Review

*Larrea tridentata* (Creosote bush), an abundant plant of Mexican and US-American deserts and its metabolite nordihydroguaiaretic acid

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**Abstract**

Although controversial, Creosote bush, *Larrea tridentata* (Sesse and Moc. ex DC) Coville, is used to treat a variety of illnesses including infertility, rheumatism, arthritis, diabetes, gallbladder and kidney stones, pain and inflammation. Recently, it has been used as a nutritional supplement. The primary product extracted from this common plant of the arid regions of northern Mexico and Southwestern United States is the potent antioxidant nordihydroguaiaretic acid (NDGA). It was widely used during the 1950s as a food preservative and to preserve naturals fibers. Later it was banned after reports of toxicity during the early 1960s. Renal and hepatotoxicity are also reported for chronic use of creosote bush and NDGA. This article reviews traditional and contemporary uses and pharmacology, including toxicology of this plant widely used in Mexican traditional medicine.

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**Keywords:** *Larrea tridentata*; Gobernadora; Creosote bush; Nordihydroguaiaretic acid; Herbal medicine; Traditional medicine; Hepatotoxicity; Renal toxicity

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1. Introduction

Creosote bush, *Larrea tridentata* (Sesse and Moc. ex DC, Zygophyllaceae) Coville is a common shrub of North American warm deserts. Its dominance has increased within 19 million ha of lands previously considered desert grasslands in response to disturbances such as grazing (Van Aften, 2000; Whorf and Gould, 2001). While often viewed as an indicator of desertified conditions and the focus of extensive control efforts (Herbel and Gould, 1995), it is also an important plant with a long history of medicinal use (Timmermann, 1979). Among the proposed medicinal properties of creosote bush, the most prominent is its antioxidant effects (Sheikh et al., 1977). The family Zygophyllaceae includes more than 30 genera and approximately 250 species (Jones, 1987). *L. tridentata* is used in a variety of forms. Traditionally leaves and twigs are used to prepare a tea, but it is also used in capsules and tablets, prepared for oral consumption. In Mexico the tea is used traditionally as a treatment of kidney and gall-bladder stones (Diaz, 1976). Current use is limited by reports of toxic hepatitis (Brent, 1999), and a case of cystic renal disease (Smith, 1994) associated with its chronic use. Hepatic impairment resulting from the use of conventional drugs is widely acknowledged, but there is less awareness of the potential hepatotoxicity of herbal preparations and other botanicals, many of which are believed to be harmless and are commonly used for self-medication without supervision (Stickel et al., 2000). Creosote bush is also known as charnara and greasewood in the United States, and gobernadora (governor) and hediondilla (little smelly one) in Mexico. This review initially presents a short botanical description of the plant and an overall view of its phytochemical diversity. Then the traditional and contemporary uses reported are briefly summarised, together with in vitro studies dealing with some medicinal uses. It should be mentioned that most of the medicinal uses of *Larrea tridentata* are not supported by experimental or clinical studies. In the section on pharmacology, in vivo studies on the beneficial, which propose possible therapeutic additional uses, and toxic properties of both the plant and its main metabolite, nordihydroguaiaretic acid, are reviewed, as well as their hepatic metabolism.

2. Botanical description and distribution

*Larrea tridentata* is an evergreen shrub 1–3 m high, branched and knotty. Leaves are opposite with two asymmetrical leaflets measuring ca. 1 cm in length. Leaves are glossy with a thick resinous coating secreted by a glandular epidermis of the stipules, located on the knots; the stem is woody, knotty and inerm. Flowers are complete and borne solitary in the axils, with five yellow clawed petals. The fruit is a roundish capsule, covered with a dense concentration of white hairs (De la Cerda, 1967; Nelies, 1997). The plant discharges a penetrant odor and has a bitter flavor. It is perennial, retaining most of its leaves across the drought and low temperatures within its range of distribution, without undergoing irreparable damage. However, periodic extreme freezes may contribute to limiting the present distribution of *L. tridentata* since freezing induces xylem embolism and cavitation (Martinez-Vilalta and Pockman, 2002). Creosote bush is unpalatable to livestock and most wildlife, is usually toxic, sometimes causing death (Gay and Dwyer, 1998). Sheep, especially pregnant ewes, have been reported to die after eating the leaves (Utah State University, 2005). Creosote bush is abundant in the desert areas of the Mexican states of San Luis Potosí, Coahuila, Chihuahua, Durango, Sonora, Zacatecas, Baja California Norte and Sur, and in the Southwest of the United States in Arizona, California, Nevada, Texas and New Mexico (Rzedowski and Huerta, 1994). Similar species are found in arid zones of South America, mostly in Argentina and Bolivia. It has been established that the plant is of South American origin, with a disjunct distribution (Lia et al., 2001).

3. Phytochemistry

*Larrea tridentata* is a notable source of natural products with approximately 50% of the leaves dry weight as extractable matter. The resin that covers the leaves yielded 19 flavonoid aglycones, as well as several lignans, notably including the antioxidant NDGA (Fig. 1; Konno et al., 1990). Some glycosylated flavonoids, sapogenins, essential oils, halogenated alkaloids (Argueta, 1994) and waxes were isolated from creosote bush (Romero de Vivar, 1985). *Larrea tridentata* contains about 0.1% of dry weight as volatile oils. Within the volatile fraction 67 compounds have been identified constituting more than 90% of the known creosote bush oils; the remaining 10% is a mixture of more than 300 constituents, mainly monoterpenoids and aromatic sesquiterpenoids (Mabry and Bohnstedt, 1981; Xue et al., 1988). Products from the mevalonic, shikimic and fatty acid pathways are predominant. Besides a great number of substances, the vinyl and methyl ketones contribute significantly to the creosote bush characteristic odor. Three common sterols have also been identified: campesterol, stigmasterol and sitosterol, as well as sapogenins of the C20-ursolic type that represent less than 1% of this species dry weight (Mabry and Bohnstedt, 1981). Alkaloids have been isolated from the bark and roots, but not from the leaves and flowers (Lara and Martinez, 1996). In terms of natural products chemistry creosote bush is best known by the large amount of the lignan NDGA, which is deposited on the

![Fig. 1. Nordihydroguaiaretic acid structure.](image-url)
through vaginal baths with infusions of the leaves and tea from the creosote bush (Tabernaemontana divaricata) for the treatment of diabetes (Winkelman, 1989). This plant was also used as a contraceptive (Moser, 1970; Argueta, 1994; Brent, 1999). Centaury (Centaurium erythraea) has been reported to be effective against menstrual pains and post-parturient inflammation (Estudillo and Hinojosa, 1988; Argueta, 1994; Brent, 1999). Whereas an infusion of the root is also used as a contraceptive (Mosser, 1970; Argueta, 1994).

4. Ethnobotany and in vitro pharmacology related to traditional uses

4.1. Reported medicinal uses

Creosote bush has been used in traditional medicine to treat more than 50 illnesses (Table 1). Most common uses are associated to diseases of renal and gynacologic origins. The plant is used as aqueous or alcoholic liquid extract of leaves and twigs; in addition, it is available in capsules and tablets for oral use, while leaves and branches can be used for poultice and fomentation. Historically, aqueous extracts of the creosote bush have been used by native healers of the Southwest region of North America, and is commonly referred to as chaparral tea. In México it is reported that an infusion of the leaves dissolves gallbladder and kidney stones when the tea is consumed throughout the day (“Agua de uso”; Martínez, 1969; Díaz, 1976; Lara and Márquez, 1996). It is used in the treatment of diseases of the liver, and as a liver tonic (Sheikh et al., 1997; Brent, 1999). Creosote bush has also been employed for kidney pain and cystitis (Martínez, 1969) dysuria (Martínez, 1969; Lara and Márquez, 1996), as a diuretic (Mabry et al., 1979b; Tyler and Foster, 1999), to treat infections of the urinary tract and venereal diseases (Timmermann, 1977; Mabry et al., 1979b; Brent, 1999). Oral decoctions and extracts of leaves and twigs have been used by the Pima Indians in the Southwest of Unitst States and in Mexico for the treatment of diabetes (Winkelman, 1989).

Likewise, creosote bush is employed against sterility through vaginal baths with infusions of the leaves and tea from the creosote bush (Tabernaemontana divaricata) for the treatment of diabetes (Winkelman, 1989). This plant was also used as a contraceptive (Moser, 1970; Argueta, 1994). Externally Larrea tridentata has medicinal uses as tincture and salves, as antiseptic and poultice to excoriations, wounds (Timmermann, 1977; Mabry et al., 1979b; Argueta, 1994; Kay, 1996; Lara and Márquez, 1996), acne, psoriasis and dandruff (Estudillo and Hinojosa, 1988; Brent, 1999). It has also been used as salves against burns (Sheikh et al., 1997; Brent, 1999), bruises and hemorphoids, for cicatrization (Argueta, 1994), chicken pox (Timmermann, 1977; Sheikh et al., 1997), snakebite pain, chronic cutaneous disorders (Nellessen, 1997; Sheikh et al., 1997; Brent, 1999) and allergic problems (Brintner, 1993).

Several antibiotic (Mabry et al., 1979a; Argueta, 1994), antifungal (Mabry et al., 1979a; Barragán et al., 1994; Brent, 1999) and antiviral properties (Brent, 1999) have been attributed to creosote bush. Dried chaparral is described as one of the best herbal antibiotics, being useful against bacteria, viruses and parasites, both internally and externally (Smith, 1994). The alcoholic extract of creosote bush has antifungal activity against tested species of Aspergillus, Penicillium and Fusarium (Tequida et al., 2002). Similarly, the ethanolic extract showed good antimicrobial activity against growth of yeast and some molds and bacteria (Verastegui et al., 1996). Among the multiples medicinal properties of chaparral, it has been mentioned as antiamoeban at low doses (Brent, 1999). Ethanolic and chloroformic extracts of creosote bush and NDGA showed a marked growth inhibition of Entamoeba histolytica and Entamoeba invadens in culture (Segura, 1978; Mabry et al., 1979a; Calzado-Flores et al., 1995). It is said to possess analgesic and anti-inflammatory properties, when applied as a poultice of powdered leaves (Timmermann, 1977; Argueta, 1994; Kay, 1996; Tyler and Foster, 1999), is helpful in the treatment of neuritis and sciatica (Timmermann, 1977; Mabry et al., 1979b) and has been employed as a tea to help with cramps (Brintner, 1993), toothache (Brent, 1999) and headache (Argueta, 1994). Chaparral and nordihydroguaiaretic acid (NDGA) have potent anti-inflammatory activity, possibly due to their ability to inhibit the enzyme lipoxygenase in vitro (Bokoch and Reed, 1981; Salari et al., 1984; Safayhi et al., 1992).

Other potential medicinal uses are against arthritis and rheumatism. The branches are macerated in alcohol and rubbed onto the affected area (Mabry et al., 1979b; Brintner, 1993; Argueta, 1994; Lara and Márquez, 1996; Tyler and Foster, 1999). A tea of the branches is consumed for bowel cramps and inflammation (Mabry et al., 1979b; Tyler and Foster, 1999), stomach pain, diarrhea (Argueta, 1994; Sheikh et al., 1997), ulcer and indigestion (Argueta, 1994), as an emetic (Mabry et al., 1979b; Tyler and Foster, 1999), weight-loss (Sheikh et al., 1997).

Chaparral tea has also been used in the treatment of respiratory diseases (Mabry et al., 1979b; Brent, 1999), such as cold, cough and influenza (Argueta, 1994; Brent, 1999; Tyler and Foster, 1999), bronchitis (Timmermann, 1977; Sheikh et al., 1997) and tuberculosis (Timmermann, 1977; Tyler and Foster, 1999). A large great variety of illnesses have been treated with creosote bush, such as anemia (Argueta,
Table 1

Main ethnobotanical uses of the leaves and twigs of Creosote bush

<table>
<thead>
<tr>
<th>Uses</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acne, psoriasis and dandruff</td>
<td>Estudillo and Hinojosa (1988) and Brent (1999)</td>
</tr>
<tr>
<td>Allergic problems</td>
<td>Brinker (1993)</td>
</tr>
<tr>
<td>Altered blood pressure</td>
<td>Argueta (1994) and Sheikh et al. (1997)</td>
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<tr>
<td>Anemia</td>
<td>Argueta (1994)</td>
</tr>
<tr>
<td>Antiarthritic</td>
<td>Brent (1999) and Segura (1976)</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Mabry et al. (1976a) and Arpeta (1994)</td>
</tr>
<tr>
<td>Antifungal</td>
<td>Mabry et al. (1976a), Buresan et al. (1994) and Brent (1999)</td>
</tr>
<tr>
<td>Antineoplasic</td>
<td>Sheikh et al. (1997) and Tyler and Foster (1999)</td>
</tr>
<tr>
<td>Antiviral</td>
<td>Brent (1999)</td>
</tr>
<tr>
<td>Blood purifier</td>
<td>Sheikh (1997)</td>
</tr>
<tr>
<td>Bowel cramps and inflammation</td>
<td>Mabry et al. (1976b) and Tyler and Foster (1999)</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>Timmermann (1977) and Sheikh et al. (1997)</td>
</tr>
<tr>
<td>Burns</td>
<td>Sheikh et al. (1997) and Brent (1999)</td>
</tr>
<tr>
<td>Chicken pox</td>
<td>Timmermann (1977) and Sheikh et al. (1997)</td>
</tr>
<tr>
<td>Cicatrization, bruises and hemorrhoids</td>
<td>Argueta (1994)</td>
</tr>
<tr>
<td>Cold, cough and influenza</td>
<td>Argueta (1994), Brent (1999) and Tyler and Foster (1999)</td>
</tr>
<tr>
<td>Contraceptive agent (roots of the plant)</td>
<td>Moser (1976b) and Arpeta (1994)</td>
</tr>
<tr>
<td>Cramping</td>
<td>Brinker (1993)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Winkelman (1989) and Argueta (1994)</td>
</tr>
<tr>
<td>Diseases of the liver, and as a liver tonic</td>
<td>Sheikh et al. (1997) and Brent (1999)</td>
</tr>
<tr>
<td>Dysuria</td>
<td>Martinez (1990) and Lara and Marquez (1996)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>Mabry et al. (1976b) and Tyler and Foster (1999)</td>
</tr>
<tr>
<td>Dermatitis</td>
<td>Mabry et al. (1976b) and Tyler and Foster (1999)</td>
</tr>
<tr>
<td>Headache</td>
<td>Argueta (1994)</td>
</tr>
<tr>
<td>Kidney and gallbladder stones</td>
<td>Diaz (1976)</td>
</tr>
<tr>
<td>Kidney pain and cystitis</td>
<td>Martinez (1990)</td>
</tr>
<tr>
<td>Menstrual pains and inflammation after delivery</td>
<td>Estudillo and Hinojosa (1988), Argueta (1994) and Brent (1999)</td>
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<tr>
<td>Neuritis and sciatica</td>
<td>Timmermann (1977) and Mabry et al. (1976b)</td>
</tr>
<tr>
<td>Parasites</td>
<td>Mabry et al. (1976b) and Brinker (1993)</td>
</tr>
<tr>
<td>Snakelike pain, chronic cutaneous disorders</td>
<td>Nollesn (1997), Sheikh et al. (1997) and Brent (1999)</td>
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<tr>
<td>Splenitis</td>
<td>Estudillo and Hinojosa (1988) and Arpeta (1994)</td>
</tr>
<tr>
<td>Stomach pains and diarrhea</td>
<td>Argueta (1994) and Sheikh et al. (1997)</td>
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<tr>
<td>Toothache</td>
<td>Brent (1999)</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>Timmermann (1977) and Tyler and Foster (1999)</td>
</tr>
<tr>
<td>Ulcer and indigestion</td>
<td>Argueta (1994)</td>
</tr>
<tr>
<td>Urinary tract infections and venereal diseases</td>
<td>Timmermann (1977), Mabry et al. (1976b) and Brent (1999)</td>
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<tr>
<td>Weight-loss</td>
<td>Sheikh et al. (1997)</td>
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1994), altered blood pressure (Argueta, 1994; Sheikh et al., 1997) and diabetes (Argueta, 1994), as well as blood purifier (Sheikh et al., 1997) and antineoplasic (Sheikh et al., 1997; Tyler and Foster, 1999).

The beneficial effect in most of these treatments has not been demonstrated using appropriate in vivo models or clinical studies. However, two possible properties of creosote bush (and/or NDGA) for which some in vitro evidence exists seem to be of relevance for many different diseases: antibiotic and anti-inflammatory activities. In other cases this does not seem to be so, such as in diabetes and gallstones, for which, however, some experimental evidence exist (Luo et al., 1998, Arteaga, 1997). More recently, creosote bush has been introduced as a dietary supplement, mainly due to its antioxidant activity. This could explain its use and abuse in other situations, as well as the increase in toxicity reports.

4.2. Other reported uses

The essential oil has been employed in soaps and creams, as well as a shoe polish. Due to its tannin content it has been employed in tanning. The extraction, crystallization and use of NDGA from creosote bush as a food antioxidant was approved by the Meat Inspection Division of the US War Food Administration in 1943. However, in 1970 this use was banned by the US Food and Drug Administration (Timmermann, 1981). Currently, NDGA is employed as an antioxidant in the storage of natural and synthetic rubber. The resin is used as a thermofixed polymer adhesive for wood and cardboard, due to its strong antimicrobial properties, which prevent the rotting of natural fibers. It has also been used to cover capsules, tablets and pills. The whole plant has been used for house roofing and firewood (Mabry and Bohnstedt,
5.2 Side effects and toxicity

Chaparral products, mainly tablets and capsules of powdered leaves and twigs, have been marketed as dietary supplements, due to their antioxidant properties. However, non-recommended uses of these products have led to hepatic damage (Stickel et al., 2000). Several cases of chaparral-associated hepatitis have been reported to the U.S. Food and Drug Administration (FDA) between 1992 and 1994 (Obermeyer et al., 1995). In one report, there was evidence of hepatotoxicity in 13 of 18 patients; the predominant pattern of liver injury was characterized as toxic or drug-induced cholestatic hepatitis, jaundice and marked increase in serum liver chemistry values; in four individuals progression to cirrhosis was observed and in two individuals there was acute fulminant liver failure requiring liver transplant (Sheikh et al., 1997). In another case, a patient developed similar symptoms after taking chaparral tablets, 160 mg/day, for 2 months, documented severe cholestasis and hepato cellular injury. The serum enzyme levels were markedly elevated and severely narrowed biliary ducts were observed, without sclerosing cholangitis, distal obstruction, tumor, or stenosis (Alderman et al., 1994). Another patient developed hepatitis 2–3 months after beginning daily consumption of creosote bush leaf (proven by biopsy). The patient recovered after ceasing creosote bush intake (Batchelor et al., 1995). Yet another study reported hepatic and renal failure attributed to prolonged consumption of creosote bush products. The author of this report noted that when taken in capsule or tablet form creosote bush can cause subacute hepatitis (Gordon et al., 1995). Six patients exhibited clinical, biochemical and histological evidence of severe hepatitis after taking herbal remedies, among them chaparral. The symptoms were jaundice, fatigue, pruritus and high liver enzymes, indicating hepato cellular damage (Stickel et al., 2000). Several cases of chaparral-associated hepatitis have been reported to the U.S. Food and Drug Administration (FDA) between 1992 and 1994 (Obermeyer et al., 1995). In one report, there was evidence of hepatotoxicity in 13 of 18 patients; the predominant pattern of liver injury was characterized as toxic or drug-induced cholestatic hepatitis, jaundice and marked increase in serum liver chemistry values; in four individuals progression to cirrhosis was observed and in two individuals there was acute fulminant liver failure requiring liver transplant (Sheikh et al., 1997). In another case, a patient developed similar symptoms after taking chaparral tablets, 160 mg/day, for 2 months, documented severe cholestasis and hepato cellular injury. The serum enzyme levels were markedly elevated and severely narrowed biliary ducts were observed, without sclerosing cholangitis, distal obstruction, tumor, or stenosis (Alderman et al., 1994). Another patient developed hepatitis 2–3 months after beginning daily consumption of creosote bush leaf (proven by biopsy). The patient recovered after ceasing creosote bush intake (Batchelor et al., 1995). Yet another study reported hepatic and renal failure attributed to prolonged consumption of creosote bush products. The author of this report noted that when taken in capsule or tablet form creosote bush can cause subacute hepatitis (Gordon et al., 1995). Six patients exhibited clinical, biochemical and histological evidence of severe hepatitis after taking herbal remedies, among them chaparral. The symptoms were jaundice, fatigue, pruritus and high liver enzymes, indicating hepato cellular damage, in all biopsies portal and lobular hepatitis 5.2 Side effects and toxicity

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5.2 Side effects and toxicity

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Creosote bush has been reported as an agent producing the biliary duct vanishing syndrome, observed in cholestasis induced by drugs (Chitturi and Farrel, 2001). Herbal preparations are marketed as natural and safer alternatives to conventional medicines for the prevention and treatment of a variety of ailments, however, consumers may not be fully aware of their potential side effects.

5.3. Experimental studies on possible additional uses of NDGA

This lignan possesses several beneficial properties. It has been used in the treatment of the Sjögren-Larsson syndrome, a severe neurocutaneous disorder due to fatty aldehyde dehydrogenase involved in the degradation of leukotriene B4 (Willesen, 2000). This is its only clinical use reported. It modulates the expression of endothelial nitric oxide synthetase in vitro, which has implications in the treatment of cardiopathies (Ramasamy et al., 1999) and it also reduces blood pressure in rats with hypertension induced by fructose (Gwon et al., 1999). NDGA is converted by the gut microflora to estrogenic compounds, which have estrogenic activity in vitro as well as in vivo (Fujimoto et al., 2004).

Several studies suggest that NDGA could have a role in cancer therapy. NDGA and a leaf extract of a South American subspecies of creosote bush were found to exert an antitumor effect in rats (Birkenfeld et al., 1987). NDGA, at 2 mg/day p.o. for 1 week, is a potent inhibitor of hepatic toxicity and renal tumor promotion mediated by ferric-nitrilotriacetate in mice (Assar et al., 1999). It was shown to be a possible chemoprotective agent in patients at risk or with lung cancer (Soriano et al., 1999). Moreover, non-small and small-cell lung cancer cell lines are inhibited by NDGA with an IC50 of 5–7 μM (Moody et al., 1998). It also reduced the frequency of micronuclei induced by methyl methanosulfonate in vivo (Diaz et al., 1999). Similar to other lipoxigenase inhibitors, NDGA induced a more differentiated state and apoptosis in several human pancreatic cancer cell lines (Ding et al., 1999), suppress breast cancer cell growth (Earashi et al., 1999), and also shows an additive or synergistic effect with retinoic acid on the inhibition of mammary tumor cell transformation and proliferation (Kubow et al., 2000). NDGA significantly inhibited UVB-induced signaling pathways in the human keratinocyte cell line HaCaT, which suggests it to be a potential agent in the prevention of skin cancer (Gonzales and Bowden, 2002). These reports suggest two different modes of action for NDGA in cancer. The first one as an antioxidant, prevent the harmful effect of reactive oxygen species, the other as an agent that affects genetic expression and differentiation, probably through its effect on leukotriene synthesis.

Several lignans derivatives from Larrea tridentata show anti-HIV activities (Gnabre et al., 1996), and several methylated NDGA were produced in the laboratory which exhibited similar or even higher anti-HIV activities than the natural compounds (Hwa et al., 1998). Of these lignans the synthetic derivative tetramethyl-O-NDGA, which shows the highest anti-HIV activity, inhibited (IC50 = 43.5 μM) the replication of herpes simplex virus in Vero cells (Chen et al., 1998) and human papilloma virus (Craigie et al., 2000).

NDGA has been used in research as an antioxidant to test the participation of lipid peroxidation in some processes. Creosote bush scavenges the superoxide anion radical O2− in a dose-dependent manner (Zang et al., 1999). NDGA inhibited the alterations of airway epithelial barrier and active ion transport properties of guinea pig tracheobronchial monolayers induced by nitrogen dioxide (Robinson and Kwang-Jun, 1996), the apoptotic cell death of the trophoblast layer of chorion tissues during development (Ohyama et al., 2001), and protected cultured rat hippocampal neurons against the toxicity of amyloid β-peptide, interrupting a neurodegenerative pathway relevant to the pathophysiology of Alzheimer’s disease (Goodman et al., 1994).

5.4. Studies on NDGA toxicity

Besides being a potent lipoxigenase and cyclooxygenase inhibitor (Safayhi et al., 1992), NDGA is also an inhibitor of intracellular vesicular transport at concentrations between 50 and 100 μM (Drecktrath et al., 1998; Ramoner et al., 1998): it not only interrupts the secretory vesicular route, but also the endocytic pathway in human dendritic cells. Protein recycling between endoplasmic reticulum and Golgi is reversibly blocked by NDGA at 30 μM, disrupting the Golgi apparatus. However, at 100 μM it inhibits protein synthesis and alters the Golgi irreversibly (Fujisawa et al., 1999a). Likewise, it has been found that the ethanolic extract of creosote bush and NDGA have a reversible cholestatic effect in hepatocyte couplets at concentrations between 2 and 12 μg/ml (Cárdenas et al., 2000), which could be related to its inhibition of intracellular movement of transporters. NDGA reduces cellular ATP through inhibition of electron flux in the respiratory chain (Fujisawa et al., 1998b); it also inhibits the regulation of cellular volume by swelling, through an inhibition of taunine channels at 50–150 μM (Ballatori and Wang, 1997). Increases in intracellular Ca2+ in a concentration-dependent manner are induced by NDGA between 10 and 100 μM. This action is modulated by phospholipase A2 (Jan and Tseng, 2000). NDGA caused a dose-dependent reduction of the ovariatory rate in the isolated perfused rat ovary, with a reduction of ovarian prostaglandin and leukotriene concentrations (Mikuni et al., 1998).

The US Federal Drugs Administration prohibited the use of NDGA as food additive since it was shown to inhibit several enzymes such as peroxidase, catalase and alcohol dehydrogenase, as well as NADH-dehydrogenase and succinate dehydrogenase (Timmermann, 1981). NDGA also inhibits phospholipase A2 (Jacobson and Schier, 1993), cytochrome P-450 (Agarwal et al., 1991) and carboxylesterases (Sato and Hosokawa, 1998).

Reported acute NDGA LD50 for rodents range between 800 and 5500 mg/kg b.w. orally (Oliveto, 1972). Rats fed diets with 3% NDGA developed cortical and medullary cysts...
in the kidney (Timmermann, 1977), and one case of human cystic renal disease and cystic adenocarcinoma associated to chaparral tea consumption has been reported (Smith, 1994).

5.5. Hepatic metabolism of creosote bush and NDGA

Little is known on the metabolism of creosote bush components. However, it has been reported on the hepatic processing of NDGA. The intravenous injection of 50 mg/kg of NDGA to mice resulted in a peak plasma concentration of 14.7 mg/ml, with a half-life of 135.0 min, and a clearance of 201.9 ml/min/kg (Lambert et al., 2001). The high clearance indicates that NDGA may be cleared by non-renal mechanisms. Mono and diglucuronides of NDGA have been identified in bile from mice injected intraperitoneally with 120 mg/kg of NDGA (Lambert et al., 2002). In this latter study, an LD₅₀ of 75 mg/kg was established 5 days after a single i.p. dose, and an increase in alanine amino transferase was found with 50 mg/kg.

Similarly, woodrats fed with alfalfa pellets containing increasing levels of the phenolic resin from creosote bush showed increased glucuronide and sulfide conjugates level with increasing resin intake, and it seems that woodrats from the Mojave Desert tolerate more resin because they have a greater capacity for glucuronide excretion (Mangione et al., 2001).

6. Conclusions

Creosote bush and its main metabolite NDGA have shown to be useful in traditional medicine, industry and research. Although several medicinal properties of creosote bush have support in experimental studies, with some exceptions none has been tested at the clinical level. One of the uses reported in traditional medicine is the treatment of kidney stones. However, there has not been any experimental assay dealing with this issue.

On the other hand, the toxicity of creosote bush has been demonstrated. However, as far as we can conclude from these data the reported toxic doses in humans and experimental animals always exceeded the traditional use of the plant and are often confounded with use of other herbs and potentially with lifestyle choices. Overall, prescribed appropriately there is a wide margin of safety for many popular remedies (Elvin-Lewis, 2001). Further studies are needed to establish the therapeutic and toxic doses of creosote bush in humans and experimental animals. Creosote bush is readily available plant that potentially has many beneficial applications. However, due to some reports of toxicity care must be taken when the plant is used in traditional preparation.

Acknowledgements

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Anti-diarrhoeal activity of *Butea monosperma* in experimental animals

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Abstract

The anti-diarrhoeal potential of the ethanolic extract of stem bark of *Butea monosperma* (Lam) Kuntz has been evaluated using several experimental models in Wistar albino rats. The extract inhibited castor oil induced diarrhoea and PGE2 induced enteropooling in rats; it also reduced gastrointestinal motility after charcoal meal administration. The results obtained establish the efficacy and substantiate the use of this herbal remedy as a non-specific treatment for diarrhoea in folk medicine.

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Keywords: *Butea monosperma*; Fabaceae; Anti-diarrhoeal; Intestinal secretion; Gastrointestinal motility

1. Introduction

In developing countries, a quarter of infant and child-

Abstract

hood mortality is related to diarrhoea (Jousilahti et al., 1977). Many plants conveniently available in India are used in tra-

ditional folklore medicine for the treatment of diarrhoea and dysentery (Chopra et al., 1956). The plant *Butea monosperma* (Lam) Kuntz (Fabaceae), also known as Bastard Teak, is a medium sized tree native of the mountainous regions of In-
dia and Burma (Anonymous, 1988). The stem bark of this plant is used in indigenous medicine for the treatment of dyspepsia, diarrhoea, dysentery, diabetes, ulcers, sore throat and snake bites (Jayaweera, 1981; Varier, 1993). Although many chemical constituents such as, kino-tannic acid, gal-
lic acid and proanthocyanidins have been isolated from the stem bark and gum (Seshadri and Trikha, 1972; Nadkarani and Nadkarani, 1976), together with (−) medicarpin from the stem bark (Bandara et al., 1989), and pterocarps, phenols and lipids from the stem (Mishra et al., 2000; Shukla et al., 2002), we wish to report therein on the anti-diarrhoeal effect of the ethanolic extract of the stem bark of the plant.

2. Materials and methods

2.1. Plant material

Stem bark of *Butea monosperma* was collected from the Valasa Malai in the Dharmapuri district of Tamilnadu, India in November 2002. It was identified by Professor P. Jayaraman, Taxonomist, Plant Anatomy Research Centre, Chennai. A voucher specimen (GPT/19) has been deposited in the Herbarium Section of the Department of Pharmacognosy, K.P. College of Pharmacy, Thiruvananthapuram, Tamilnadu, for future reference. The stem bark (1.5 kg) was air dried and pulverized using a mechanical grinder.

2.2. Preparation of extract

The powdered plant material (750 g) was subjected to maceration process with 95% ethanol at room temperature. After exhaustive extraction, the ethanolic extract (EBM) was
concentrated under reduced pressure at 50–55 °C. A reddish-brown coloured residue was obtained (93 g; yield 12.4%, w/w) and stored in desiccator. For pharmacological studies, a weighed amount of the dried extract was suspended in a 2% (w/v) aqueous acacia solution.

2.3. Animals used
Swiss albino mice (30–40 g) and Albino (Wistar) rats (210–235 g) of either sex were maintained at uniform laboratory conditions in standard polypropylene cages and provided with food and water ad libitum. The animals were acclimatized for a period of 14 days prior to performing the experiments.

2.4. Acute toxicity study
Swiss albino mice were divided into eight groups of six individuals. The extract was administered orally at doses ranging from 0.1 to 5 g/kg following a standard method (Turner, 1965). A group of animals treated with 2% (w/v) aqueous acacia suspension (vehicle control). The animals were continuously observed for 2 h to detect changes in the autonomic or behavioural responses. Mortality in each group was observed for 7 days. The doses of 200, 400 and 800 mg/kg were selected based on the results of preliminary toxicity testing.

2.5. Castor oil induced diarrhoea in rats
The method reported by Awouters et al. (1978), with modifications, has been used in the present study. Rats of either sex (210–235 g) were fasted for 18 h; they were then divided into five groups of five individuals. The EBM extract was administered orally at doses of 200, 400 and 800 mg/kg by gavage as suspension to the first three groups of animals. The fourth group received loperamide (3 mg/kg) orally as suspension to the first three groups of animals. The fifth group received atropine (0.1 mg/kg, i.p.), the standard drug for comparison and the fifth group was treated with aqueous acacia suspension (vehicle control). Thirty minutes later, each animal was sacrificed and the intestinal distance moved by the charcoal meal from the pylorus was cut, measured, and expressed as a percentage of the distance from the pylorus to caecum for each animal (Mukherjee et al., 1998).

2.7. PGE2-induced enteropooling
In this method, rats were deprived of food and water for 18 h and placed in five cages, with five animals per cage. The first three groups were treated with 200, 400 and 800 mg/kg doses of EBM. The fourth group was treated with 1 ml of a 5% (v/v) ethanol in normal saline (i.p.) and then it was treated with aqueous acacia suspension, which served as vehicle control. Immediately after the extract administration PGE2 (Astra Zeneca, India) was administered orally to each rat (100 µg/kg) in the first three groups. The fifth group was treated with PGE2 (100 µg/kg) as well as with aqueous acacia suspension and served as the PGE2 control group. After 30 min following administration of PGE2, each rat was sacrificed and the whole length of the intestine from the pylorus to the caecum was dissected out, its content collected in a test tube, and the volume measured (Mukherjee et al., 1998).

2.8. Statistical analysis
All data was expressed as the mean ± S.E.M. Statistical significance testing was performed by Student’s t-test; *P* < 0.05 imply significance.

3. Results
The presence of steroids, flavonoids, phenolic compounds, tannins and glycosides were detected on preliminary phytochemical screening of the dried extract. The EBM extract, when orally administered in the dose range 0.1–5.0 g/kg to mice did not produce any significant changes in the autonomic or behavioural response during the observation period. No mortality was observed up to 7 days of monitoring. Hence, the extract seems to be safe for administration up to 5.0 g/kg.

The EBM extract significantly inhibited the frequency of defaecation, when compared to untreated control rats (Table 1); the activity was similar to that of loperamide, the standard anti-diarrhoeal agent. The extract also reduced the wetness of faecal droppings. The EBM extract at doses of 400 and 800 mg/kg decreased the propulsion of charcoal meal through the gastrointestinal tract, as compared with the control group. Atropine (0.1 mg/kg) reduced the motility of the intestine to a greater extent (*P* < 0.001) (Table 2). The extract (EBM) significantly inhibited PGE2 induced enteropooling in rats in higher dose levels (Table 3). PGE2 induced a significant increase in the fluid volume of the rat intestine when compared with control animals, received ethanol in normal saline.
The anti-enteropooling effect of EBM extract in rats

Table 3
Effect of EBM extract on small intestinal transit

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean volume of intestinal fluid (ml)</th>
<th>Mean number of wet faeces in 4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (atropine 0.1 mg/kg)</td>
<td>29 ± 0.17 b</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>Standard (lopamidine 3 mg/kg)</td>
<td>0.80 ± 0.58</td>
<td>0.00</td>
</tr>
<tr>
<td>EBM (400 mg/kg)</td>
<td>1.8 ± 0.42</td>
<td>0.80 ± 0.52 b</td>
</tr>
<tr>
<td>EBM (800 mg/kg)</td>
<td>1.80 ± 0.48</td>
<td>0.40 ± 0.24</td>
</tr>
</tbody>
</table>

Results are mean ± S.E.M., n = 5. Statistical significance test with control was done by Student’s t-test.

a P < 0.05.
b P < 0.01.
c P < 0.001.

Table 4
Effect of EBM extract on castor oil induced diarrhoea in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean defecations in 4 h</th>
<th>Mean number of wet faeces in 4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (acacia suspension 5 ml/kg)</td>
<td>3.60 ± 0.50</td>
<td>3.00 ± 0.50</td>
</tr>
<tr>
<td>Standard (atropine 0.1 mg/kg)</td>
<td>29 ± 0.17 b</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>EBM (200 mg/kg)</td>
<td>1.42 ± 0.05</td>
<td>1.20 ± 0.05 b</td>
</tr>
<tr>
<td>EBM (400 mg/kg)</td>
<td>1.80 ± 0.48</td>
<td>0.40 ± 0.24</td>
</tr>
<tr>
<td>EBM (800 mg/kg)</td>
<td>1.00 ± 0.48</td>
<td>0.40 ± 0.24</td>
</tr>
</tbody>
</table>

Results are mean ± S.E.M., n = 5. Statistical significance test with control was done by Student’s t-test.

a With respect to EBM extract.
b With respect to PGE2 treatment.

4. Discussion

In the present study, the extract of Butea monosperma exhibited significant anti-diarrhoeal activity against castor oil induced diarrhoea in rats. The extract had a similar activity as lopamidine, when tested at 400 and 800 mg/kg and significantly inhibited the frequency of defecation and the wetness of the faecal droppings when compared to control rats. Castor oil releases ricinoleic acid which induces changes in mucosal fluid and electrolyte transport that results in a hypersecretory response and diarrhoea (Ammon et al., 1974; Gagninella et al., 1975). The experimental studies in rats demonstrated a significant increase in the portal venous PGE2 concentration following oral administration of castor oil (Luderer et al., 1980). Ricinoleic acid markedly increased the PGE2 content in the gut lumen and also caused on increase of the net secretion of the water and electrolytes into the small intestine (Beubler and Juan, 1979). Inhibitors of prostaglandin biosynthesis delayed castor oil induced diarrhoea (Awouters et al., 1978).

The extract appears to act on all parts of the intestine. Thus, it reduced the intestinal propulsive movement in the charcoal meal treated model; at 800 mg/kg EBM showed activity similar to that of atropine. The extract (EBM) at different dose levels 400 and 800 mg/kg significantly inhibited the PGE2 induced intestinal fluid accumulation (enteropooling). These observations tend to suggest that the EBM extract at doses of 400 and 800 mg/kg reduced diarrhoea by inhibiting gastrointestinal motility and PGE2 induced enteropooling.

The present results indicate that the ethanolic extract of Butea monosperma possesses significant anti-diarrhoeal activity due to its inhibitory effect both on gastrointestinal propulsion and fluid secretion. The inhibitory effect of the extract justifies the use of the plant as a non-specific anti-diarrhoeal agent in folk medicine.

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References


Effects of wild ginseng (Panax ginseng C.A. Meyer) leaves on lipid peroxidation levels and antioxidant enzyme activities in streptozotocin diabetic rats

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Abstract

The aim of this study was to examine the possible antioxidant activities of wild Panax ginseng leaf extract intake in streptozotocin (STZ)-induced diabetic rats (WGLE). Initial blood glucose levels increased abruptly after streptozotocin injection. After 4 weeks of WGLE supplementation, blood glucose levels were lower in animals fed 40 mg/kg (266 mg/dL) and 200 mg/kg (239 mg/dL) than those in no-WGLE fed diabetic rats (464 mg/dL). The concentration of blood TBARS, which are considered the main products of glucose oxidation in blood, was also lowered by WGLE supplementation. These results indicate that WGLE supplementation is involved in suppressing a sudden increase in blood glucose levels and a consequent decrease in TBARS levels in diabetic rats. TBARS levels in the liver, kidney and spleen of WGLE-fed diabetic groups were also significantly lower than in the control diabetic group indicating that oral administration of WGLE effectively suppresses lipid peroxidation that occurs in the organs of diabetic rats. Antioxidant activities of WGLE supplementation further extend in suppressing activities of antioxidant related enzymes, such as glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD), in organs of diabetic rats. These results confirm the effectiveness of WGLE supplementation in detoxifying free radicals that are produced excessively in diabetic-induced complications.

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Keywords: Wild ginseng; Panax ginseng leaves; Streptozotocin-induced diabetic rats; Thiobarbituric acid reactive substances (TBARS); Antioxidant activities

1. Introduction

Diabetes mellitus (DM), characterized by hyperglycemia and long-term complications affecting the eyes, kidneys, nerves and blood vessels, is the most common endocrine disorder. Although the underlying mechanism of diabetic complications remains unclear, much attention has been focused on the role of oxidative stress. It has been suggested that oxidative stress may contribute to the pathogenesis of different diabetic complications (Ceriello, 2000). Diabetic experimental animal models have shown that oxidative stress causes persistent and chronic hyperglycemia, thereby depleting activities of the antioxidant defense system and otherwise promoting free radicals generation (Kakkar et al., 1997; Bhor et al., 2004).

Panax ginseng C.A. Meyer (Araliaceae) is a valuable herb in East Asia that has also gained popularity in the West because of its pharmacological properties (Zhang et al., 1996; Mahady et al., 2000). Panax ginseng is categorized as either cultivated or wild according to different nurturing methods. Cultivated ginseng is systematically farmed on open land and harvested after a 5–6 year cultivation period. On the
other hand, wild ginseng is planted through seeding in a deep mountain at an altitude between 800 and 1500 m. Wild ginseng is slower in growth and more sensitive to environmental changes than cultivated ginseng, showing a preference for areas with fluctuating daily temperatures and less exposure to direct sunlight. These differences may result in a variation of active compounds between cultivated and wild ginseng. It is widely accepted in both Korea and China that wild ginseng is more active than cultivated ginseng. However, only a few studies have been conducted to compare the pharmacological activities of wild and cultivated ginseng. Traditionally, Panax ginseng leaves have been consumed mostly in the form of tea (KFDA, 2002). Ginseng leaves have long been used as folk medicine in the treatment of diabetes in Korea. Because most studies on the use of ginseng as a treatment for diabetes focused on roots rather than leaves, little has been reported on the antidiabetic effectiveness and compounds found in ginseng leaves. Xie et al. (2004) have researched hypoglycemic activities in diabetic rat models and concluded that these activities come primarily from ginsenosides (Xie et al., 2004).

Ginseng leaves are known to have six major ginsenosides, Rb1, Rb2, Rc, Rd, Re and Rg1 (Li et al., 1996). It is generally accepted in both Korea and China that wild ginseng leaves exhibit very potent antioxidant activities (Park et al., 1999). In addition, the phenolic acids and flavonoids in ginseng leaves exhibit very potent antioxidant activities (Park et al., 1990).

2. Materials and methods

2.1. Chemicals

Standard ginsenoside Rg1, Rc, Rb1, Re and Rd were obtained from Wako (Osaka, Japan). Ginsenoside Rb2 and Rf were purchased from Extrasynthese (Genay, France). Streptozotocin and all other chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Plant material

The wild Panax ginseng C.A. Meyer leaves were collected from ginseng plants that were grown for more than 12 years at a mountain in Inje-gun, Gangwon-do, Korea. Voucher specimens have been deposited in the laboratory of KT&G Center Research Institute, in Gyunggi-do, Korea.

2.3. Preparation of ginseng leaf extract

The wild ginseng leaves were freeze-dried and ground into a fine powder. The powder was extracted twice with water under reflux in a water bath at 90°C for 1 h. The extract was then filtered and freeze-dried. The extract yield was 46% (w/w, dry basis). Finally, the extract was dissolved in distilled water for oral administration to the diabetic rats.

2.4. Determination of ginsenosides, phenolics and flavonoids

The freeze-dried extract was dissolved in 20% (v/v) acetonitrile/water and filtrated through a 0.45 μm membrane filter to analyze ginsenosides by HPLC (Li et al., 1996). Total phenolic contents in WGLE were determined using Folin-Ciocalteu’s reagent according to the method of Singleton and Lamuela-Raventos (1999). Total flavonoid contents in WGLE were determined by the method of Kumazawa et al. (2004).

2.5. Animals and treatment

Male Sprague–Dawley rats (250 ± 10 g) were housed in steel cages. They were allowed free access to drinking water and normal diet during the experiment. The rats were acclimatized for 7 days and randomly allotted into five groups. All animal experiments were performed under the guidelines of the Laboratory Animal Experiment Committee of Korea Food Research Institute. Body weight was recorded at 2-day intervals in every group. Amount of food consumption was measured by subtracting leftovers from the diet provided to the rats at 2-day intervals.

2.6. Experimental design

In the normal groups, rats were allotted into two subgroups: normal rats (N) and normal rats fed with WGLE (200 mg/kg, N+W200). Diabetic animals were distributed into control diabetic (DM) and WGLE-fed diabetic group. WGLE-fed diabetic animals were allotted into DM + W40 (40 mg/kg) and DM+W200 (200 mg/kg) according to the amounts of WGLE supplementation. Diabetes was induced by intravenous injection of streptozotocin into the tail vein at a dose of 45 mg/kg body weight in a 0.1 M fresh cold citrate buffer (pH 4.3). Six days after the STZ injection, glucose in the orbital vein plexus was measured in all test rats. STZ-treated rats having a blood glucose level of 280–380 mg/dL were included in the study. After 4 weeks, all animals were sacrificed and the liver, kidney and spleen were isolated from each rat, washed in 0.9% saline solution, blotted dry and weighed.
2.7. Determination of glucose and thiobarbituric acid reactive substances (TBARS) in blood

Blood was obtained from the orbital vein plexus of all rats. Blood glucose concentrations were measured by a glucometer kit (Abbott Laboratories, Illinois, USA). TBARS in the serum were estimated by Satoh (1978) method based on thiobarbituric acid (TBA) reactivity. 1.0 mL of 20% trichloroacetic acid (TCA) was added to the 0.5 mL of serum and the tube was left to stand for 10 min at room temperature. After centrifugation at 4000 × g for 10 min, 0.5 mL of supernatant and 0.8% TBA were added. The coupling of lipid peroxide with TBA was carried out by heating in a boiling water bath for 30 min. After cooling in cold water, the resulting chromogen was extracted with 4.0 mL of n-butanol by vigorous shaking. Separating of the organic phase was facilitated by centrifugation at 4000 × g for 10 min and its absorbance was determined at a wavelength of 532 nm. The standard for TBARS in this study was malondialdehyde (MDA). MDA was prepared from 1,1,3,3-tetramethoxypropane. The MDA concentration in tissue homogenates was normalized with protein concentration, which was measured by the Lowry method (Lowry et al., 1951).

2.8. Tissue samples

Tissue was chopped into small pieces on ice. A 10% (w/v) homogenate was prepared in 10 mM phosphate buffer (pH 7.4) and centrifuged at 13,000 × g for 10 min and its absorbance was determined at a wavelength of 532 nm. Tissue samples were normalized with protein concentration, which was measured by the Lowry method (Lowry et al., 1951).

2.9. Determination of thiobarbituric acid reactive substances in tissues

TBARS in tissue was estimated by the method of Ohkawa et al. (1979). Some 0.2 mL of the tissue sample was added to 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid solution (pH 3.5) and 1.5 mL of 0.8% TBA. The mixture was made up to 4.0 mL with distilled water and heated in a water bath at 90°C for 60 min. After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of n-butanol were added and shaken vigorously and centrifuged at 4000 × g for 10 min. The upper butanol layer was taken and its absorbance at 532 nm was read.

2.10. Antioxidant enzyme activities

Activity of glutathione peroxidase (GSH-Px) was determined according to the method of Lawrence and Burk (1976). The assay mixture consisted of 2.0 mL of 75 mM phosphate buffer (pH 7.0), 50 μL of 60 mM glutathione, 0.1 mL of 30 units/mL glutathione reductase, 0.1 mL of 15 mM EDTA, 0.1 mL of 3 mM NADPH and the appropriate amount of tissue supernatant to a final volume of 3.0 mL. The reaction was started by the addition of 0.1 mL of 7.5 mM H₂O₂. The rate of change of absorbance during the conversion of NADPH to NADP⁺ was recorded spectrophotometrically at 340 nm for 3 min. GSH-Px activity for tissues was expressed as μmoles of NADPH oxidized to NADP⁺ min⁻¹ mg⁻¹ protein.

Catalase (CAT) activity was measured according to the Aebi (1983) method. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 μmol of H₂O₂ in 1 min. In a cuvette containing 1.95 mL of a 50 mM phosphate buffer (pH 7.0), 0.05 mL of tissue supernatant was added. The reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically at 240 nm for 1 min. Using the reaction time (Δt) of the absorbance (A₁ and A₂), the following equation was generated to calculate the rate constant (k): k = (2.31/Δt)log(A₂/A₁). The enzyme activity was expressed as k mg⁻¹ protein.

SOD activity was measured by the inhibition of pyrogallol autoxidation at 420 nm for 10 min according to Marklund and Marklund (1974) method. The enzyme activity was expressed as U/mg protein, where 1 U is the amount of enzyme required to bring about 50% inhibition of the autoxidation of pyrogallol. The assay mixture consisted of 1.8 mL of 50 mM Tris–HCl buffer (containing 10 mM EDTA), 0.1 mL of 6.0 mM pyrogallol and the diluted tissue supernatant to a final volume of 2.0 mL. The reaction was stopped by adding 0.05 mL of 1N HCl.

2.11. Statistical analysis

All experimental data were analyzed for variance (ANOVA) and significant difference among the means from triplicate analysis was set at P < 0.05. This was determined by Duncan’s multiple range test using the Statistical Analysis System (SAS).

3. Results

3.1. Effects of WGLE supplementation on blood glucose and TBARS in diabetic rats

As shown in Table 1, there were significant differences in body weight gain between normal (N) and diabetic animals (DM). Unlike the normal group, which gained weight during the experimental period, the diabetic group lost weight (−53.26 ± 10.38 g) after 4 weeks. Increased food consumption and decreased body weight observed in diabetic rats in comparison to normal rats indicates polyphagic condition and weight loss due to the excessive breakdown of tissue proteins (Chatterjea and Shinde, 2002). Weight loss of WGLE fed diabetic groups (DM + W40 and DM + W200), however, were less severe than that of the DM group.

The result of WGLE effect on lowering blood glucose and TBARS in diabetic rats is also presented in Table 1. There was no significant difference in blood glucose levels after 4 weeks of WGLE supplementation on normal groups.
indicating that feeding WGLE to normal rats does not cause any changes in blood glucose levels. In diabetic groups, however, the effect of WGLE supplementation on lowering blood glucose was clearly observed. DM groups with no WGLE supplementation showed an abrupt increase in blood glucose from 338.6 to 464.6 mg/dL after 4 weeks. Although the initial blood glucose level in WGLE fed DM groups (DM + W40 and DM + W200) was around 330 mg/dL, it was significantly reduced to 266.0 mg/dL in DM + W40 and 239.6 mg/dL in DM + W200 after 4 weeks of WGLE feeding. As in the case of blood glucose levels, TBARS levels in all DM groups were initially higher than those in normal groups. All WGLE fed diabetic rats maintained constant blood glucose levels even though control diabetic rats had increased TBARS levels after 4 weeks.

### 3.2. Effects of WGLE supplementation on antioxidant enzyme activities and TBARS in organs of diabetic rats

Table 2 shows antioxidant related enzyme activities in the livers, kidneys and spleens of normal and diabetic groups. In general, normal groups maintained higher enzyme activities than diabetic groups. Although daily administrations of 40 and 200 mg/kg for 4 weeks were not effective in fully recovering enzyme activities as detected in normal groups, WGLE supplementation seemed to play a certain role in recovering them in diabetic groups. By STZ-induction, TBARS in each organ were increased by 25–40% (Table 2). In most of the organs in the diabetic groups, TBARS were effectively reduced by the administration of WGLE in a dose dependent manner.

### Table 2

**Activities of glutathione peroxidase, catalase, superoxide dismutase in liver, kidney and spleen from normal and diabetic rats treated with WGLE**

<table>
<thead>
<tr>
<th>Group</th>
<th>N (n=8)</th>
<th>N + W200 (n=8)</th>
<th>DM (n=7)</th>
<th>DM + W40 (n=8)</th>
<th>DM + W200 (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBARS</td>
<td>1.07 ± 0.13 a</td>
<td>1.10 ± 0.16 a</td>
<td>1.71 ± 0.10 c</td>
<td>1.45 ± 0.05 b</td>
<td>1.41 ± 0.06 b</td>
</tr>
<tr>
<td>GSHPx</td>
<td>460.64 ± 23.39 a</td>
<td>455.80 ± 20.23 a</td>
<td>301.67 ± 12.94 c</td>
<td>321.67 ± 10.67 c</td>
<td>360.25 ± 17.31 c</td>
</tr>
<tr>
<td>CAT</td>
<td>0.84 ± 0.04 a</td>
<td>0.87 ± 0.04 a</td>
<td>0.46 ± 0.03 d</td>
<td>0.70 ± 0.04 c</td>
<td>0.79 ± 0.02 b</td>
</tr>
<tr>
<td>SOD</td>
<td>6.15 ± 0.58 a</td>
<td>6.15 ± 0.55 a</td>
<td>3.44 ± 0.34 a</td>
<td>4.46 ± 0.36 b</td>
<td>4.77 ± 0.31 b</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBARS</td>
<td>0.77 ± 0.07 a</td>
<td>0.81 ± 0.03 a</td>
<td>1.05 ± 0.10 c</td>
<td>0.94 ± 0.06 b</td>
<td>0.90 ± 0.05 b</td>
</tr>
<tr>
<td>GSHPx</td>
<td>146.75 ± 11.56 a</td>
<td>144.82 ± 11.52 a</td>
<td>97.93 ± 9.71 c</td>
<td>107.30 ± 10.96 c</td>
<td>129.95 ± 10.97 c</td>
</tr>
<tr>
<td>CAT</td>
<td>0.61 ± 0.02 a</td>
<td>0.60 ± 0.05 a</td>
<td>0.25 ± 0.02 c</td>
<td>0.27 ± 0.01 b</td>
<td>0.30 ± 0.01 b</td>
</tr>
<tr>
<td>SOD</td>
<td>4.57 ± 0.34 a</td>
<td>4.58 ± 0.34 a</td>
<td>6.11 ± 0.46 c</td>
<td>5.38 ± 0.33 b</td>
<td>5.12 ± 0.29 b</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBARS</td>
<td>0.27 ± 0.04 a</td>
<td>0.28 ± 0.03 a</td>
<td>0.38 ± 0.05 b</td>
<td>0.35 ± 0.03 a</td>
<td>0.28 ± 0.04 a</td>
</tr>
<tr>
<td>GSHPx</td>
<td>177.55 ± 12.56 a</td>
<td>181.45 ± 10.10 a</td>
<td>138.20 ± 5.96 c</td>
<td>137.23 ± 11.41 c</td>
<td>165.85 ± 5.12 c</td>
</tr>
<tr>
<td>CAT</td>
<td>0.21 ± 0.01 a</td>
<td>0.22 ± 0.02 a</td>
<td>0.14 ± 0.01 b</td>
<td>0.14 ± 0.01 b</td>
<td>0.14 ± 0.02 b</td>
</tr>
<tr>
<td>SOD</td>
<td>4.43 ± 0.50 a</td>
<td>4.39 ± 0.22 a</td>
<td>2.65 ± 0.33 c</td>
<td>3.08 ± 0.54 b</td>
<td>3.67 ± 0.20 b</td>
</tr>
</tbody>
</table>

N: normal group, no streptozotocin injected; N + W200: normal group orally fed with WGLE(200 mg/kg day); DM: diabetic control group; DM + W40: diabetic group orally fed with WGLE(40 mg/kg day); DM + W200: diabetic group orally fed with WGLE(200 mg/kg day). All values are mean ± S.E.M. Mean values with different letters are significantly different (P<0.05) by Duncan’s multiple-range test.
4. Discussion

STZ is a commonly employed compound for the induction of type-1 diabetes in rats (Tomlinson et al., 1992). STZ causes diabetes by the rapid depletion of β-cells, which leads to a reduction in the insulin release. An insufficient release of insulin causes high blood glucose, namely hyperglycemia, which results in oxidative damage by the generation of reactive oxygen species (ROS) (Mohamed et al., 1999) and the development of diabetic complications (Dominini et al., 1996). Furthermore, STZ diabetic animals may exhibit most other diabetic complications such as myocardial, cardiovascular, gastrointestinal, nervous and urinary bladder dysfunctions (Ozturk et al., 1996).

In this study, we examined oxidative stress pathway markers in rats with STZ-induced diabetes. As shown in our results, diabetic rats had much higher blood glucose levels than normal rats during the experimental period. By feeding WGLE to diabetic rats, however, blood glucose levels were controlled to a certain degree. Although the mechanism involved with suppressing blood glucose levels by WGLE supplementation was not clearly demonstrated in this study, at least three possibilities can be suggested: (1) modulation of glucose transport (Yamasaki et al., 1993), (2) glucose disposal (Yokozawa et al., 1984), or (3) insulin secretion (Waki et al., 1992). Some components such as phenolics and flavonoids that compose 1.67 and 0.95% of dry ginseng leaves (data not shown) are known to be responsible for hypoglycemic activity (Ragunathan and Sulochna, 1994).

Since high blood glucose is susceptible to oxidation, hyperglycemia causes high ROS production and, in turn, leads to high TBARS in tissues (Wolf and Dean, 1987; Das et al., 2000). As in the case of hypoglycemic activity by WGLE feeding, it was assumed that TBARS levels in blood were also lowered by WGLE feeding in diabetic groups. In addition, direct radical scavenging activities by phenolic compounds in ginseng leaves may result in lowered blood TBARS levels in WGLE supplemented groups (Park et al., 1990).

As shown in the results (Table 2), activities of antioxidant related enzymes were deteriorated by STZ induction. This may be due to a direct attack of reactive oxygen species on these enzymes. GSH-Px plays an important role in the reduction of H$_2$O$_2$ in the presence of reduced glutathione (GSH). This detoxifying action of GSH-Px against H$_2$O$_2$ protects cell membrane against oxidative damage (Jacob, 1995). WGLE supplementation in diabetic rats protect, to a certain degree, further deterioration of GSH-Px and CAT in all organs. WGLE supplementation also increases SOD activity in the livers and spleens in diabetic groups whereas it significantly decreases in kidneys.

As in the case of blood analysis, it was found that STZ induction caused a significant increase in TBARS in organs. This increase in TBARS could be suppressed more or less by WGLE feeding. The present study indicates that WGLE exerts a protective effect against lipid peroxidation by scavenging reactive oxygen species and (or) elevating the activity of antioxidant enzymes (Shirwaikar et al., 2004). Another important component in ginseng leaves, ginsenosides, produces powerful antioxidant activities other than radical scavenging activities by simulating gene expression of antioxidant enzymes and enhancing their activities (Wee et al., 1989; Kim et al., 1996a). These enhanced activities of antioxidant enzymes contribute to further scavenging of ROS or other free radicals formed through lipid peroxidation (Fan et al., 1993; Kim et al., 1996b). In our results, ginsenosides in WGLE composed 16.99% (data not shown). Conclusively, WGLE supplementation causes both reactivation of antioxidant enzymes and scavenging of free radicals, which, in turn, results in decreased TBARS synthesis and its accumulation.

Acknowledgments

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References


Reevaluation of risks with the use of *Ficus insipida* latex as a traditional anthelmintic remedy in the Amazon

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Abstract

The anthelmintic remedy ojé, prepared latex of *Ficus insipida*, is still used by indigenous and local people in the Amazonian regions. However, overdosage leading to toxic reactions occurs despite the broadcasting of a clinically accepted dosage that is effective and safe. The intoxication of a 10-year-old girl in Pucallpa, who had received ojé in a dose close to the recommended one, led us to study retrospectively the records of all hospitalized patients with toxic reactions to ojé over a 12-year-period. The use of ojé in and around Pucallpa was estimated. Most cases with toxic reactions, out of a total of 39 for the 12-year-period, were probably due to an overdose, defined as more than 1.5 cm³/kg; the recommended dose being 1 cm³/kg. In only five cases did toxic reactions occur at doses up to 1.5 cm³/kg, which were interpreted as idiosyncratic reactions; all of them occurred in children, and in two cases it was a severe reaction. One fatal outcome was noted among the 37 hospitalized patients. Two other fatal outcomes were observed in the 12-year-period but they occurred outside the hospital. The mortality rate is estimated to have been 0.01–0.015% among patients supposedly treated with ojé in the area. Severe intoxication led to symptoms of cerebral edema. The main treatment was osmotic diuresis with mannitol which started in 1996. Although hypersensitivity reactions have been observed with other *Ficus* spp., there was apparently no such reaction in our cases. Recommendations are given so as to avoid toxic reactions from an expected continued use of ojé.

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Keywords: Ojé; *Ficus insipida*; Anthelmintic; Toxic reactions; Safety; Cerebral edema

1. Introduction

The white latex of *Ficus insipida* Willdenow (Moraceae) has been used for centuries among indigenous people and settlers in the neotropics, particularly in the Amazon region, for intestinal helminthiasis (Hansson et al., 1986; Phillips, 1990; Casner et al., 1998). In Peru this majestic fig tree is known as ojé. Synonymous scientific names are *Ficus glabrata* H.B.K. and *Ficus anthelmintica* Martius. The pharmacologically active component is thought to be the proteolytic enzyme ficin, in fact, a complex of sulfhydryl endopeptidases, although other components such as the terpenoids eloxanthine and moretenolactone could also be involved (Gaughran, 1976; Ayala, 1984; Hansson et al., 1986; Lopes et al., 1993; De Amorin et al., 1999). A clinical trial was carried out in 181 persons to find a dosage that was clinically effective for common intestinal helminths in reducing the worm burden without disturbing adverse effects (Hansson et al., 1986). The popular dosage had varied tremendously and occasional serious adverse effects had been described. The overall prevalence of intestinal helminthiasis has in many studies been reported to be up to more than 90% in Amazonian villages (Hansson et al., 1986). With a dose of 1 cm³/kg (or a simplified dosage with spoons according to age) of prepared latex taken for three consecutive mornings a reasonably effective and safe treatment was established and recommended for mass campaigns or individual treatments (Hansson et al., 1986; Caldecott,
As this is a retrospective study, informed consent could not be given by the patients. The anonymity of the patients is protected in the publication of this study. Furthermore, there was no experimental intervention as a result of this investigation. Consequently, there was no violation of international rules on bioethics.

2.2. Plant material

The ojé tree is supposedly recognizable by all collectors of the crude latex and also by a high proportion of the rural population in Ucayali. The tree has been botanically identified in published studies; it is Ficus insipida Willdenow, sometimes identified with a synonymous name (see, e.g. Tournon et al., 1986). It is most improbable that collectors make a mistake when choosing ojé trees for latex tapping. There are other Ficus spp. in the area but they are generally very different with regard to visible signs, e.g. a strangler or an epiphyte instead of a tree, small leaves instead of large ones, equipped with spines instead of having none, or with regard to the taste, smell and colour of the latex. In none cases of toxic reactions were the preparations of ojé consumed available. The preparations of ojé in the markets appeared to be authentic as judged by one of the authors who had much experience in this field of work. Preparations of ojé from a previous investigation (Hansson et al., 1986). In that study it was also demonstrated that ojé preparations from a market were all genuine. The white latex is nearly always mixed with sugarcane brandy, “aguardiente”, with an ethanol content of about 40%. This is done soon after tapping in the approximate proportion 4:1. With this preparation the mixture can be stored for several months without decomposition or fungal growth (Hansson et al., 1986). If the latex is not mixed with alcohol it easily ferments, changing in colour and smell and is then regarded as unfit for consumption. Based on the above-mentioned facts, our assumption is that the preparations of ojé taken by the hospitalized patients were actually based on latex from Ficus insipida although no voucher specimen is available.

2.3. Statistical analysis

Doses, times elapsed before toxic symptoms, and hospitalization time are expressed with mean and median values and are furthermore grouped together according to “normal” dose, moderate overdose, and heavy overdose. Median values were calculated to get a more stable measure as there were 1–2 very extreme values for some parameters. Correlation coefficients have been calculated where possible and their significance has been tested (Clarke, 1980). Toxic reactions and anatomical systems involved are expressed in factual numbers and percentages.

2.4. Market data

All six main markets in Pucallpa were visited once or twice in May 2003 to find where sales of ojé took place. Apparently,
no sales of ojé took place in shops specializing in herbal medicines at this time. Vendors selling ojé were interviewed about the quantity sold, its preparation, recommended doses, and their knowledge of adverse effects.

3. Results

3.1. Hospital data

In the hospital files (from both the Regional Hospital and the Hospital Amazonico) 37 patients with a diagnosis of “intoxicación por ojé” (ojé poisoning) were found for the 12 years from 1992 to 2003; 31 from the Regional Hospital and 6 from the Hospital Amazonico. The cases were distributed throughout the years without any tendency to decrease or increase; 17 cases in the first 6 years and 20 cases in the second. There were 20 cases of children (less than 15 years old) and 17 of adults. The youngest child was only 1-year-old; furthermore there was one 4-year-old child and three 5-year-old children. There were, in total, three cases with fatal outcomes, two of which were not registered in the case records of the hospital as they were dead on arrival. More or less complete case records could be found for 29 patients: 17 children and 12 adults.

It was stated to be an overdose when the single dose was 1.5 cm³/kg or larger, and a heavy overdose when it was 4 cm³/kg or larger. This definition was made having in mind the safe dosage of 1 cm³/kg or less observed in many clinical studies. In 19 out of 24 records, where the dosage was stated, an overdose had been administered; in eight of these cases it was a heavy overdose (Table 1). The maximum dose was 7.3 cm³/kg, noted for an adult. The highest dose for a child was 5.0 cm³/kg for a 12-year-old girl. Mean dose was 3.3 cm³/kg as was the median. In the five cases in which the dose was close to the recommended one the doses were 0.5 cm³/kg (9-year-old boy), 0.6 cm³/kg (11-year-old girl), 1.1 cm³/kg (6-year-old boy), 1.2 cm³/kg (1-year-old girl) and 1.4 cm³/kg (10-year-old girl).

The time between ingestion and the appearance of adverse or toxic reactions varied between 20 min and 2 days in 23 cases where information was available. Mean value was 8.2 h and median value was 6.0 h. The correlation coefficient between dose and time was 0.58, p < 0.01. This positive correlation is surprising as it could be expected that a higher dose would lead to toxic reactions appearing more rapidly. However, when time was grouped according to dose interval another picture could be seen (Table 1). At doses lower than 1.5 cm³/kg median time was 5.0 h, at a moderate overdose 7.0 h and at a heavy overdose 4.0 h. For the five non-overdose patients information on time was only available in three cases. However, for these patients a relatively short time passed before symptoms appeared, which might indicate some kind of idiosyncratic reaction not directly related to the dose.

The number of days the patients were hospitalized varied between 1 and 12 days; records were available in 28 cases. Mean time was 3.9 days and median time 3.5 days. Admission time was longer in cases of a heavy overdose (Table 1). The five children with about the recommended doses were on average admitted after 4.0 days, median time 2.0 days. The correlation coefficient between dose and hospitalization time was 0.68, p < 0.001. This appears logical, and it concurs with the results when the median values are compared.

Among the 29 patients (with available data) admitted for ojé poisoning CNS symptoms and gastrointestinal symptoms dominated in both children and adults (Table 2). There were slightly more musculoskeletal reactions among children, and slightly more cardiovascular and respiratory reactions among adults. The most common adverse or toxic reactions were vomiting/nausea and irritability/psychomotor excitation (Table 3). Cerebral edema was apparent in many cases. Eleven patients were unconscious and four had fainting fits. There was no clear relation between dose and different reactions and no obvious hypersensitivity reactions.

<table>
<thead>
<tr>
<th>Ojé dose (cm³/kg)</th>
<th>≤1.5</th>
<th>1.5–4.0</th>
<th>&gt;4.0</th>
<th>Unknown</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution of patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>5</td>
<td>6</td>
<td>2</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>Adults</td>
<td>0</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>11</td>
<td>8</td>
<td>13</td>
<td>37</td>
</tr>
<tr>
<td>Time elapsed before symptoms appeared</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median time (h)</td>
<td>5.0 (n=3)</td>
<td>7.0 (n=11)</td>
<td>4.0 (n=6)</td>
<td>4.0 (n=3)</td>
<td>6.0 (n=23)</td>
</tr>
<tr>
<td>Time in hospital</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median time (days)</td>
<td>2.0 (n=5)</td>
<td>3.0 (n=11)</td>
<td>4.0 (n=8)</td>
<td>3.0 (n=4)</td>
<td>3.5 (n=28)</td>
</tr>
</tbody>
</table>
Mydriasis 5 (17)
Abdominal discomfort 7 (24)
Headache 7 (24)
Diarrhea 8 (28)
Convulsions 10 (35)
Unconsciousness 11 (38)
Irritability/psychomotor excitation 14 (48)
Vomiting/nausea 14 (48)

Most common toxic symptoms or signs

Table 3

<table>
<thead>
<tr>
<th>Symptom/sign</th>
<th>Number of patients (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vomiting/nausea</td>
<td>14 (48)</td>
</tr>
<tr>
<td>Irritability/psychomotor excitation</td>
<td>14 (48)</td>
</tr>
<tr>
<td>Unconsciousness</td>
<td>11 (38)</td>
</tr>
<tr>
<td>Convulsions</td>
<td>10 (35)</td>
</tr>
<tr>
<td>Diaphora</td>
<td>8 (28)</td>
</tr>
<tr>
<td>Headache</td>
<td>7 (24)</td>
</tr>
<tr>
<td>Abdominal discomfort</td>
<td>7 (24)</td>
</tr>
<tr>
<td>Somnolence</td>
<td>5 (17)</td>
</tr>
<tr>
<td>Mydriasis</td>
<td>5 (17)</td>
</tr>
</tbody>
</table>

Of the 11 patients who suffered unconsciousness 6 were children, 3 boys and 3 girls aged between 4 and 12 years. Doses for four of these children (with data available) were 0.5, 1.4, 1.6 and 2.7 cm³/kg. Doses for four out of five adults were 2, 4.7, 6.0 and 7.3 cm³/kg. The case with 0.5 cm³/kg is the most alarming one. It involved a 9-year-old boy who also showed symptoms of vomiting, hypotonia, mydriasis, abdominal distension and psychomotor agitation.

Treatment was according to symptoms and usually included intravenous rehydration, registered in 17 of 29 cases. When cerebral edema was diagnosed an infusion of mannitol was given, sometimes supported with furosemide as a diuretic. However, the use of the mannitol as an osmotic diuretic for cerebral edema was not prescribed in any cases before 1996. The adequate dose of mannitol was found to be 1–2 g/kg every sixth hour. Diazepam (or other benzodiazepine) was given in 17 cases, mannitol in 15 cases, histamin-2-blockers (ranitidine/famotidine) in 15 cases, antibiotics (for initial fear of sepsis) in 13 cases, dexamethasone or other steroid in 10 cases, anticonvulsants (phenobarbital/fenytoin) in 8 cases, antipyretics/analgesics in 8 cases, furosemide in 7 cases and antihypertensives in 4 cases. Other occasional treatments included neuroleptics, antacids, pyridoxin, oxygen, atropin and dimethylurea. Gastric lavage had been performed in two cases.

The cases with fatal outcomes occurred in the years 1994 and 1995 (two cases). Unfortunately, no specific data are available in the last two cases when the persons were dead on arrival at the hospital; they were aged 14 and 8 years. In the third case, an 11-year-old boy died in the hospital of a cerebral edema after 1 day; dose of ojé unknown; symptoms appeared 4 h after intake. At this time, 1994, mannitol was not given as a treatment for cerebral edema.

3.2 Market data

Vendors of ojé were found in two of the main markets in Pucallpa, Mercado no. 2 and Bellavista. In four other markets there was no sale of ojé; despite questioning, it appeared as if sales never occurred there and that sales of ojé were concentrated in the two markets mentioned above. In a total of two visits to these markets 12 vendors were located and 7 of them were interviewed. The approximate sales of ojé for these 12 vendors were calculated to be 22 bottles (each bottle = 0.5 L) a month. In all cases the latex had been mixed with “aguar-diente” to prevent fermentation. There might be a few more vendors and some people might get ojé directly from ojé collectors or in some other way; we have supposed five bottles a month. It was thus estimated that a total of 27 bottles were administered in most months in the Pucallpa area. According to the vendors information sales were up 50% in January to March before schools started after the holidays and when more ojé was available, as latex is more abundant in the trees in the rainy season. According to these estimates about 365 bottles (27 × 9 + 40.5 × 3) were sold per year around 2003. It is likely that the quantity has had a tendency to decrease because of greater access to commercial drugs. On the other hand, the rapid population growth in Pucallpa has probably had an opposite effect. We therefore assume that this quantity is representative for the whole period from 1992. Based on the interviews and previous experience in the riverine villages we assume that the number of administrations of ojé is roughly the same among children as adults.

The vendors generally recommend a single dose of a quarter (of a bottle) for an adult and half the dose or less for a child but rarely for children under the age of 5 years. This means a dose that is around 2 cm³/kg, but it is not clear whether these recommendations are followed. The dose could also be divided and given for 3 days. The dose should be taken in the morning on an empty stomach and a traditional diet observed during the day, avoiding, e.g., pork, chilies and salt. The dose should be repeated once or twice a year. For administration the ojé preparation can be mixed with orange or sugarcane juice, honey, or banana gruel. The vendors say they have sold ojé for many years and they know the collectors of the latex well. Harvesting is mostly done from trees at the riversides or close to the river.

The vendors interviewed did not know of any toxic reactions on the part of their respective clients. Asked about serious adverse reactions they, like many other people, as-severe that it is due to (1) using latex from another tree, or mixing one latex with another; (2) non-compliance with diet or close to the river.

3.3 Estimate of incidence of toxic reactions

A fatal outcome occurred in three cases over 12 years, giving an incidence of between 0.01 and 0.015% among patients supposedly treated with ojé. Unconsciousness was observed in at least 11 patients, giving an incidence of at least 0.06% among ojé-ingesting persons. Toxic reactions leading to hospitalization occurred in 39 cases (two of which were dead on arrival), meaning an incidence of between 0.13 and 0.2%.
4. Discussion and conclusions

The therapeutic use of prepared latex of *Ficus insipida*, ojé, still occurs to some extent in the city of Pucallpa including its surroundings. In distant, rural areas among indigenous peoples its use is much more frequent, in some villages reaching up to 25% of the population, based on sporadic interviews. Cases of toxic reactions can occur as has previously been reported anecdotically (Hansson et al., 1986). In this study, the incidence of hospitalization due to intoxication was estimated at 0.13–0.2% of all ojé consumers, between a half and third of these cases showing such serious reactions as unconsciousness. A fatal outcome is estimated to have an incidence rate of 0.01–0.015% during the 12-year-period. In most cases of toxic reactions an overdose of ojé, more than 1.5 cm³/kg, had been administered, although in five children the dose was close to the recommended one; in these cases the reactions might be characterized as being atypical or idiosyncratic.

In the tropical regions of Peru many clinical trials of ojé were carried out in recent decades to establish an effective and safe anthelmintic dosage of this traditional remedy to be integrated into primary health programs in remote regions as an alternative to commercial drugs. The study reports or articles available to us were put together in a standardized form (Table 4). Most patients belong to different indigenous peoples. The dose was generally about 1 cm³/kg, although in one minor study the dose was about 6 cm³/kg. In total, 395 patients received 1–7 doses of ojé, generally in preparations with 70–85% of pure latex. There were 39 reports of adverse reactions (Table 5). Gastrointestinal symptoms dominated. A miscarriage (at about 4 months of pregnancy and 5 days after a dose of 0.25 cm³/kg) occurred in an 18-year-old girl who had not admitted being pregnant at her pretreatment examination; ojé is contraindicated in pregnant women. In one of the studies placebo was used as a control (Chávez, 1986). Gastrointestinal reactions occurred in 10 cases with placebo (n = 70) versus 25 cases with ojé (n = 78) (Table 4). Atypical reactions were noted in three cases; allergic itching in one case and edema of the lower extremities in two cases (Table 5). These atypical or idiosyncratic reactions, thus had an incidence of 0.75%.

In one of the studies it was clearly stated that there was no blood, microscopically or macroscopically, after treatment in the stool samples of the 181 patients examined for helminthic egg (larva) count (Hansson et al., 1986). In another of the studies there was an additional anecdotal report, involving cases outside the anthelmintic trial, with unknown doses of ojé where the persons had suffered generalized edema with renal insufficiency in three cases, and hematuria with severe anemia in one case (Giove, 1996).

In this context a preclinical study in mice infected with two oxiurid species and one cestod species should be mentioned (De Amorin et al., 1999). Prepared ojé latex was administered intragastrically, 4 cm³/kg/day for 3 days. As anthelmintic activity was moderate and hemorrhagic enteritis was observed at necropsy, continued clinical use of ojé latex...
in traditional medicine was not recommended, apparently a conclusion without due regard to all the clinical trials performed. In Pucallpa and its surroundings anthelminthic mass campaigns with single-dosed albendazol are now carried out frequently. This is a beneficial and welcome measure in an area where intestinal helminthiasis is prevalent. The most common adverse effects with albendazol are non-serious and include: abdominal pain, nausea, vomiting, alopecia, increased serum aminotransferase and neutropenia (Liu and Weller, 1976). The use will probably recede in the urban area and prior sensitization to rubber latex could, perhaps, be a possible explanation of the rare hypersensitivity reactions observed with ojé in other studies (Table 5). However, specific studies are needed to clarify such possible connections. Ficin can be sensitizing but only after inhalation of dry powder. Sensitization by the oral and topical routes have not been noted (Gaughran, 1976). The importance of the traditional diet generally followed together with an ojé intake must also be taken into account to explain idiosyncratic or hypersensitivity reactions.

With a continued therapeutic use of the latex of Ficus insipida the following precautions should be followed and broadcast through the health authorities in both urban and rural areas and through indigenous organizations. High dosages of ojé should be avoided as the risk of toxic reactions increases. Ojé should not be used with children under the age of 3 years and, as mentioned previously, not with pregnant women. Prepared ojé should only be purchased from known vendors or prepared by knowledgeable people so as not to use the potentially toxic latex of other tree species, which might be used by unscrupulous people. Should symptoms of poisoning occur owing to an overdose or a rare idiopathic reaction, the patient should be transferred to a hospital or a health centre. In severe cases with cerebral edema the use of mannitol is recommended to save the patient.

### Acknowledgements

We are grateful to Philip O’Brien, MA (Oxon), of the British Centre, Pucallpa for proof-reading the manuscript for any infelicities in our use of the English language. We are also grateful to Beatriz Luján and Christian Felipa for assistance in getting data from the Archive Departments of the two hospitals involved.

### References


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Table 5

<table>
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<tr>
<th>Adverse effect</th>
<th>Number of patients (%)</th>
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<td>Abdominal pain</td>
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<tr>
<td>Abdominal distension</td>
<td>4 (1.0)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>4 (1.0)</td>
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<tr>
<td>Edema of leg</td>
<td>2 (0.5)</td>
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<tr>
<td>Nausea</td>
<td>1 (0.25)</td>
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<tr>
<td>Miscarriage</td>
<td>1 (0.25)</td>
</tr>
<tr>
<td>Allergic pneumonia</td>
<td>1 (0.25)</td>
</tr>
</tbody>
</table>


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Landírez, E., 1984b. Tratamiento y resultados de enteroparasitosis con resina de Ficus anthelmintica (oje, potoc) al 70%. Archives of Save the Children, Lima, pp. 1–4.


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Effects of *Bambusae concretio* Salicae (Chunchukhwang) on amyloid β-induced cell toxicity and antioxidative enzymes in cultured rat neuronal astrocytes

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Abstract

*Bambusae concretio* Salicae (BCS; plant family name: *Phyllostachys bambusoides* Siebold et Zuccarinii) is a medicinal plant used in Korea for the treatment of various symptoms accompanying hypertension and cerebrovascular disorders. Previously, it was shown that BCS is an effective protectant against oxidative glutamate toxicity in the murine neuroblastoma cells and human neuroblastoma cells. Treatment with BCS increased the secretion of the non-amyloidogenic amyloid precursor protein fragment, and decreased the secretion of amyloid-β (Aβ) peptides from neuronal cells [Jeong, J.C., Seo, Y.J., Kim, H.M., Lee, Y.C., Kim, C.H., 2003. Inhibitory effects of *Bambusae concretio* Salicae on neuronal secretion of Alzheimer’s Aβ-amyloid peptides, a neuro-degenerative peptide. Neurochemical Research 28, 1785–1792.]. To further examine the pharmacological activity of BCS, we studied the protective effect of the water extracts on Aβ25-35 peptide-induced neuronal death by microscopic observation and lactate dehydrogenase (LDH) assay, and action on antioxidative enzymes using cultured astrocyte cells. Ten μM Aβ25-35-induced cell death was protected by the application of water extract of BCS in a dose-dependent manner, and concentrations of 1–10 μg/ml had a significant effect compared to exposure to Aβ25-35 only. When antioxidative enzyme activities such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were assayed after Aβ25-35 treatment, the enzymes were decreased in a similar fashion. However, those activities were enhanced by BCS treatment and this may have resulted from the potentiation of antioxidative ability by BCS. The ability of BCS to reduce cellular cytotoxicity induced by 10 μM Aβ25-35 suggests that BCS may be a protective agent for free radical generating compounds such as Aβ25-35, and that Aβ25-35 is not only a potent lipid peroxide inducer, but also causes changes in antioxidative enzymes. From the results, it was concluded that BCS has a protective effect on Aβ-induced neuronal death in cultured astrocyte cells through the inhibition of lipid peroxidation and protection of antioxidative enzymes. © 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: Astrocyte; Alzheimer’s disease; Amyloid-β; *Bambusae concretio* Salicae (Chunchukhwang); Antioxidative enzyme

Abbreviations: BCS, *Bambusae concretio* Salicae water extract; Aβ, amyloid-β; LDH, lactate dehydrogenase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; AD, Alzheimer’s disease; APP, amyloid precursor protein; FBS, fetal bovine serum; DMEM, Dulbecco’s modified eagle’s medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; TBA, 2-thiobarbituric acid; TEPA, 1,1,3,3-tetraethoxypropane; EDTA, ethylenediamine tetraacetic acid; SOD, superoxide dismutase; XOD, xanthine, xanthine oxidase; NBT, nitro blue tetrazolium; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; GSH, reduced glutathione; GSSG-reductase, oxidized glutathione-reductase; NADPH, nicotinamide adenine dinucleotide phosphate; CDNB, 1-chloro-2,4-dinitrobenzene; NaN3, sodium azide; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive decline resulting from selective neuronal dysfunction, synaptic loss, and neuronal cell death. The well-studied neuropathological features of AD show compacted deposits of amyloid β (Aβ) aggregates (Selkoe, 1991; Terry, 1996). Aβ is 39–43 amino acids long and proteolytically derived from an integral membrane protein termed amyloid precursor protein (APP) (Kang et al., 1987; Kim and Suh, 1996), although the mechanism for APP processing is still unknown. There are many in vitro studies demonstrating that Aβ is directly neurotoxic and increases neuronal susceptibility to other toxic agents (Cotman et al., 1996; Kim and Suh, 1996; Yankner, 1996). The toxic effect of Aβ is correlated with its ability to form aggregates (Pike et al., 1995). Both oxygen species (Goodman et al., 1994) and excessive Ca2+ influx (Mattson et al., 1993) are also implicated in the mechanism of Aβ neurotoxicity. In contrast, it was also reported that Aβ promotes neurite outgrowth under certain culture conditions instead of having a toxic action (Kee et al., 1995).

On the other hand, AD could be induced by surrounding free radicals and also be protected by enzymes such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) (Seifert et al., 1988, Halliwell, 1994). Antioxidants scavenge and minimize the formation of oxygen-derived species and inhibit oxidative damage induced by free radicals. They also recover the level of intracellular antioxidants (vitamins, methionine, glutathione and glutathione-related minerals) (Ip et al., 1991; Schrauzer, 1992). Hence, these antioxidants may be particularly important in diminishing cumulative oxidative damage.

Recently, several reports have suggested that natural dietary plants may play an antioxidative role in the prevention of aging and carcinogenesis and may offer effective protection from lipid peroxidative damage in vitro and in vivo (Ruch et al., 1989; Tsuda et al., 1994). Therefore, much attention has been focused on natural antioxidants (Wang et al., 1988a,b).

In particular, it was reported that the extract of_Bombusae concretio Salicae (Chunghuhwagw in Korean; _Phyllostachys bambusoides_ Siebold et Zuccarinii in plant family name; BCS) is specifically effective for cerebrovascular lesions and aphasia during the treatment of wind-heat syndrome and heat-phlegm in oriental medicine (Lee, 1986). BCS has been long being used in Traditional Korean Medicine for clinical treatment of degenerative neuronal disorders (Lee, 1986; Kourwon et al., 1994). Previously, the pharmacological mechanism for BCS was attributed to anti-aging and sexual-reinforcing activities in experimental in vitro and in vivo systems (Chen et al., 1992; Lee and Chung, 1998). Recently, to delineate the mechanisms of action of BCS in experimental systems of oxidative neuronal cell death, we have investigated the neuroprotective effects exhibited by in different concentrations of BCS (Jeong et al., 2003). The idea of a novel and neuroprotective function of BCS had arisen because BCS showed comparable neuroprotection (Jeong et al., 2003). This study was carried out to investigate the effect of BCS on cultured astrocytes, lipid peroxidation and antioxidative enzyme activities following Aβ25-35 treatment.

2. Materials and methods

2.1. Materials

The Aβ25-35 peptide was synthesized by Applied Biosystem’s Protein Synthesizer Model 470A (Peptron Co. Ltd., Taejon, Korea). Fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Gibco-BRL (Grand Island, New York, USA). Dulbecco’s Modified Eagle’s Medium (DME), glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), ethylenediamine tetracetic acid (EDTA), superoxide dismutase (SOD: from bovine liver), xanthine, xanthine oxidase (XOD), nitro blue tetrazolium (NBT), catalase (from bovine liver), diethylene triamine pentaacetic acid (DETPAC), reduced glutathione (GSH), oxidized glutathione-reductase (GSSG-reductase), and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chem. Co. (St. Louis, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) and sodium azide (NaN₃) were obtain from Aldrich Chem. Co. (Milwaukee, WI).

2.2. Bombusae concretio Salicae and the extraction

Three hundred grams of BCS (_Phyllostachys bambusoides_ Siebold et Zuccarinii in plant family name) (OHC-B-3 in herbarium record) was obtained from Oriental Herbal Center (OHC), Oriental Medical Hospital, Dongguk University College of Oriental Medicine, and extracted with 500 ml of boiling water for 3 h. After the extract was centrifuged at 7500 rpm for 30 min, the supernatant was lyophilized. For direct use, the extract solution was stored at 4°C in aliquots.

2.3. Cell culture and treatment of BCS

Cortical astrocyte cultures were prepared from neonatal rat (1–2 day old) pups by the method of Levison and McCarthy (Levison and McCarthy, 1991). Cerebral cortex was dissected from neonatal day 1–2 Sprague–Dawley rats and dissociated by gentle trituration. Cells were plated in six-well culture plates (0.2 mg/ml in sodium borate buffer, pH 8.3) at a density of 40,000 cells per well. After overnight incubation in DMEM supplemented with 20% fetal bovine serum, the medium was changed to serum-free defined medium for neurons (DMEM supplemented with 2 mM glutamine, 1 mM pyruvate, penicillin-streptomycin–amphotericin B mixture, 5 mM HEPES, 0.5% glucose, 10 µg/ml insulin, 30 mM sodium selenite, 20 mM progesterone,
100 μM putrescine, and 20 μg/ml transferrin. The cultures were incubated at 37 °C in an atmosphere of 5% CO2/95% room air, and the medium was replaced every other day. Experiments were performed on 6–7-day-old cultures.

Depending upon the experimental group, 1–10 μg/ml BCS was added (at 2 vol.% in culture medium) to or omitted from flasks. After 16–18 h, cells were washed twice with warm phosphate-buffered saline (PBS) and serum-free medium added to the flask. Then the cells were treated with 10 μM Aβ25-35 for 2 h and enzyme activities measured. Aβ25-35 was diluted in serum-free medium and added to the cultures.

2.4. Determination of cell viability and toxicity assay

Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the coloured formazan product by mitochondrial enzymes in viable cells (Sladowski et al., 1993). Cells were cultured in 24- or 96-well culture plates at a density of 10,000 cells per well for lactate dehydrogenase (LDH) assay or 40,000 cells per well for MTT reduction assay. LDH activities in the medium were measured by a Cytotox 96 non-radioactive cytotoxicity assay kit (Promega) according to the manufacturer’s instructions. The results were expressed as percentages of peak LDH release on complete cell lysis (control).

The MTT reduction was measured essentially as described previously (Kim and Suh, 1996) with a slight modification. In brief, after incubating cells for 48 h with various samples, Aβ25-35, MTT (Sigma) solution in PBS was added to a final concentration of 0.5 mg/ml, and the cells were further incubated for 4 h at 37 °C. After incubation, the plates were centrifuged at 900 × g for 10 min to obtain the resulting insoluble formazan precipitates. To dissolve the crystal precipitates, 150 or 600 μl of a 1:1 mix of ethanol and DMSO were added to each well. Each plate was gently shaken for approximately 20 min before reading on the enzyme-linked immunosorbent assay (ELISA) reader (measurement 570 nm, reference 620 nm). Absorbance of converted dye was measured. Assay values obtained on addition of vehicle were taken as 100%, and complete inhibition of MTT reduction (0%) was defined as the value obtained following addition of 0.9% Triton X-100.

To examine whether BCS could attenuate the cytotoxicity of Aβ peptide, cultures were pre-treated with indicated concentrations of 10 μg/ml of BCS for 4 h. Thereafter 10 μM Aβ25-35 was added to cultures and incubated for 48 h. LDH activity in the culture medium was determined as described above. To investigate the effect of pretreatment with Aβ peptide on the cytotoxicity induced by hydrogen peroxide or glutamate, cells were pretreated with 10 μM Aβ25-35 peptide for 48 h, and then 100 μM hydrogen peroxide or 100 μM glutamate was added to cultures and incubated further for 4 or 1 h, respectively.

For all findings, each condition represents five separate wells per experiment and is repeated in two or five independent experiments.

2.5. Treatment of astrocytes with Aβ peptide

Confluent astrocytes were trypsinized and plated into 75 tissue culture flasks at a density of 5 × 10^6 cells/m (for MTT reduction assays). After 24 h, cells were washed with PBS to remove serum, and cultures were incubated in DMEM free FBS for an additional 12 h before addition of 0.1, 0.5, 1.0, 5.0, 10, 25, 50 and 100 μM Aβ25-35 peptides or control buffer.

2.6. Examination of astrocyte morphology

Cell morphology was examined under a Nikon TMS (H-III) inverted microscope equipped with a Nikon FDX-35 photo camera.

2.7. Assay of catalase activity

Catalase activity was measured as the decrease in hydrogen peroxide absorbance at 240 nm on a Gilford Response spectrophotometer using 30 mM hydrogen peroxide according to Aebi (1974). Briefly, 100 μl of cellular extract was placed on ice bath for 30 min and then for another 30 min at room temperature. Ten microliter Triton X-100 was added to the each tube. In a cuvette containing 200 μl phosphate buffer and 50 μl of cellular extract, was added 250 μl of 0.006 M H2O2 (in phosphate buffer) and decrease in optical density was measured at 240 nm for 60 s. The molar extinction coefficient of 43.6 M cm⁻¹ was used to determine catalase activity. One unit of activity is equal to the moles of H2O2 degraded/min/mg protein.

2.8. Assay of superoxide dismutase activity

SOD was assayed by recording the inhibition of ferricytochrome c reduction with xanthine and xanthine oxidase, EDTA being replaced by DETAPAC (1 mM) (Oberley and Spitz, 1984; Oyanagui, 1984). Briefly, 2.8 ml of reactant mixture (xanthine 9.13 mg/200 ml distilled water, EDTA 25 mg/100 ml, sodium carbonate, 2.54 g/60 ml, bovine albumin 30 mg/30 ml) is added to 0.1 ml sample and 50 μl xanthine oxidase (10 μl in 2 M ammonium sulphate), incubated at 25 °C for 20 min and mixed with 0.1 ml copper chloride (108 mg/100 ml). The colour reaction is detected at 560 nm.

One unit of SOD activity was defined as the amount of enzyme needed to obtain 50% inhibition of cytochrome c reduction at pH 7.8 (25 °C) in a 3.0 ml reaction volume at 560 nm using a Gilford Response spectrophotometer.
2.9. Assay of glutathione peroxidase activity

GPx activity was determined by the modified coupled assay developed by Paglia and Lawrence (Paglia and Valentine, 1967; Lawrence and Burk, 1976). The reaction mixture consisted of 500 μl phosphate buffer and 100 μl glutathione reductase (0.24 units). One hundred microliters of cellular extract was added to the reaction mixture and incubated at 37 °C for 10 min. Fifty microliters of 12 mM t-butyldihydroperoxide was added to 450 μl of the cellular reaction mixture and measured at 340 nm for 180 s. The molar extinction coefficient of 6.22 × 10^3 cm⁻¹ was used to determine the activity. The reaction was started by addition of 2.2 mM hydrogen peroxide as substrate. The change in absorbance at 340 nm was measured for 1 min on a Gilford Response spectrophotometer. The activity was expressed as μM of NADPH oxidized/min/mg protein.

2.10. Assay of glutathione-S-transferase activity

GST activity was assayed with CDNB as substrate and enzyme activity of GST towards the glutathione conjugation of CDNB (Habig et al., 1974). Briefly, prepared TCA supernatant was added to 1 ml of a solution containing 0.1 M phosphate buffer (pH 6.5), 1 mM GSH and 1 mM CDNB. The formation of the CDNB-conjugate was followed at 340 nm (25 °C) with a Gilford Response spectrophotometer.

2.11. Protein determination

Protein was determined on each sample by the method of Smith et al. (1985) (using bicinchoninic acid), using bovine serum albumin as the standard.

2.12. Statistical analysis

Standard procedures were used to calculate means and standard deviation of the mean. Mean values were compared using Duncan’s Multiple Range Test with an SAS program (SAS Institute, Cary, NC); P < 0.05 was considered significant.

3. Results

3.1. Effect of Aβ25-35 peptide on cell cytotoxicity in cultured astrocyte cells

The toxicity of Aβ25-35 on astrocytes was assessed by LDH assay. The results were expressed as a percentage of peak LDH release obtained on complete lysis. The Aβ25-35 peptide increased LDH release by 52.6% of the maximal value at 100 μM concentration at 4h incubation. Aβ25-35 induced LDH release of 12.3% and 32.5%, respectively, at 25 and 50 μM (Table 1). However, a non-toxic fragment of Aβ had little effect on LDH release up to 100 μM. For examination of effects of BCS treatment, cells were treated with Aβ25-35 for 2 h and further treated with the indicated concentration (10 μg/ml) of BCS, and then LDH activities in the culture medium of cultured rat cortical astrocytes were assayed 48 h after treatment with BCS. LDH releases were severely decreased in the cells, indicating that BCS treatment reduced the cell injury and protected the cells against Aβ25-35-induced cytotoxicity (Table 1).

3.2. Aβ25-35 induces morphological activation of astrocytes

Cells, which were certificated to be approximately 98% astrocytes, as assessed by positive staining for astrocyte intermediate filament protein, GFAP (Cotman et al., 1996) were grown in serum-rich media for 24 h. They were flat and polygonal-shaped and growing in a monolayer, a typical morphology in culture (Fig. 1(A)). Aβ25-35 exposure induced neuronal degeneration appeared as swelling of the soma and bright pycnotic mass of nucleus (Fig. 1(B)). This indicates that cortical astrocyte cultures treated with Aβ25-35 exhibited a marked morphological changes, which show the activated morphology such as stellate-shaped a more spherical and phase-bright cell soma compared to the flat, polygonal control cells (100×).
Table 1

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>LDH activity of maximal release</th>
<th>MTT reduction of control</th>
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<tr>
<td></td>
<td>[Aβ25-35]</td>
<td>[Aβ25-35 + BCS]</td>
</tr>
<tr>
<td>0</td>
<td>1.2 ± 0.1</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
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The results are expressed as percentage of maximal LDH release that was obtained on complete cell lysis. For effects of BCS treatment (10 µg/ml at 2 vol.% in culture medium), cells were treated with [Aβ25-35] for 2 h and further treated with indicated concentration of BCS. MTT assay values obtained on addition of vehicle were taken as 100%, and complete inhibition of MTT reduction (0%) was defined as the value obtained following addition of 0.9% Triton X-100.

Data are mean ± S.E.M. values obtained from five culture wells per experiment.

* P < 0.05.
** P < 0.01.
*** P < 0.001.

death were prevented by BCS (1–10 µg/ml). The representative effect of BCS on the neuronal death at concentration of 10 µg/ml was shown in Fig. 1(C), suggesting that BCS protected cells from this [Aβ25-35]-induced neuronal damage.

3.3. Effect of BCS on antioxidative enzyme activities in [Aβ25-35]-treated astrocyte cultures

The effect of BCS on catalase activity by [Aβ25-35] treatment is shown in Fig. 2. The [Aβ25-35] treatment group resulted in a decrease of catalase activity when compared with normal group. In contrast, BCS pretreatment (10 µg/ml) markedly increased compared to those of untreated normal groups. However, BCS + [Aβ25-35] group was significantly increased compared to [Aβ25-35] treatment group, although this increase was lower than that of BCS treatment group.

3.4. Superoxide dismutase activity

Fig. 3 shows that [Aβ25-35] treatment for 2 h decreased about 2.0-fold of the normal group of the SOD activity in the cultured cell. BCS pretreatment at concentration of 10 µg/ml had a slightly enhanced effect on SOD activity compared to normal group. The SOD activity by BCS itself (10 µg/ml) was significantly increased by 1.5-fold compared with normal group. Also, the enzyme activity of BCS + [Aβ25-35] group
3.5. Glutathione peroxidase activity

As shown in Fig. 4, GPx activity in Aβ25-35 treatment group was 1.7-fold higher than that of normal group, while the GPx activity was not changed by BCS itself (10 μg/ml). The GPx activity was significantly decreased in the BCS + Aβ25-35 group (P < 0.01) when compared with Aβ25-35 treatment group.

3.6. Glutathione-S-transferase activity

The effect of GST activity by Aβ25-35 treatment and BCS pretreatment (10 μg/ml) is documented in Fig. 5. The GST activity by Aβ25-35 itself was significantly increased by 4.0-fold as compared to that of normal group. In addition, BCS pretreatment have significantly enhanced GST activity compared to the normal group (2.5-fold) (P < 0.001). In the case of the BCS + Aβ25-35 group, the GST activity was also significantly increased when compared to the Aβ25-35 treatment group (P < 0.01).

4. Discussion

The present study was carried out to investigate the effects of BCS on cultured astrocyte cell system, Aβ25-35-induced cytotoxicity and antioxidative enzyme activities in Aβ25-35 treated conditions. Cellular cytotoxicity was significantly enhanced by addition of increasing concentrations of Aβ25-35. Pretreatment of BCS (10 μg/ml) attenuated cell killing enhanced by increasing concentrations of Aβ25-35. In our previous paper (Lee and Chung, 1998), MDA level induced by Aβ25-35 treatment was significantly increased and the level was slightly reduced by BCS. These results of cellular cytotoxicity and MDA level by Aβ25-35 treatment are in good agreement with that of Glascott et al. (1992, 1995).

Lipid peroxidation was prevented or greatly reduced by addition of antioxidants (Vit E, Vit C, DPPD or deferoxamine) (Sharma and Buettner, 1993). For example, addition of antioxidants in cell culture medium significantly reduced cell killing and content of intracellular antioxidants. Recently, it was reported that repeated oral administration of deer antler extract showed inhibitory effect on monoamine oxidase activity, one of the senescence marker enzymes, reduced MDA level and increased SOD activity in the liver and brain tissues of aged mice (Wang et al., 1988a,b). For plant resources, a large number of exogenous compounds such as quercetin, catechin, stilbens and resveratrol have also been shown to exhibit antioxidative properties (Fauconneau et al., 1997; Murkies et al., 1998; Bravo, 1998; Ashby et al., 2000). They have been suggested to exert beneficial pharmacological effects on neurological disorders on the basis of in vitro observations (Oyama et al., 1994; Skaper et al., 1997). Several novel antioxidant and prooxidant phenolic compounds such as 3-O-(3′-methylcaffeoyl)quinic acid, 5-O-caffeoyl-4-methylquinic acid and 3-O-cafeoyl-1-methylquinic acid have been isolated from BCS (Hu et al., 2000; Kweon et al., 2001). Therefore, it was suggested that changes in MDA level are related to the alterations of antioxidative enzyme activity and these biochemical changes were recovered to normal levels by the addition of antioxidants (Halliwell and Gutteridge, 1990).

In the present study, we have examined several enzymes to evaluate the effects of Aβ25-35 treatment and pretreatment of BCS. Particular importance was placed on catalase, SOD,
GP and GST for well-known parameters as the antioxidative enzymes. The results showed a decrease in the activity of catalase in cultured cell by Aj25-35 treatment. However, catalase activity by pretreatment of BCS was increased compared to control groups. These results suggest that the reduced catalase activity by Aj25-35 treatment possibly is due to a direct toxic effect of Aj25-35 or its metabolites. On the other hand, SOD activity by Aj25-35 treatment was significantly decreased when compared with the normal group, and the decreased activity was significantly increased by BCS pretreatment (BCS + Aj25-35 group, P < 0.001). Furthermore, the BCS pretreatment group showed a significantly increased effect on SOD activity when compared to the normal group (P < 0.05). In GPx, our results showed elevation in the activity of GPx in Aj25-35 treated cultured cell. It has been shown that exposed to oxidant drug or materials resulted in the inactivation of GPx and these were recovered by addition of antioxidant. GST activity by Aj25-35 treatment was markedly increased when compared to the control group. Also, BCS pretreatment slightly enhanced GST activity above that of the normal group.

In the present study, antioxidative enzyme activities by Aj25-35 treatment were decreased in a similar fashion. These results possibly due to adaptive phenomenon by the direct toxic effect of Aj25-35 or its metabolites may cause these effects. Furthermore, enhanced activities by BCS pretreatment may be resulted from the potentiation of antioxidative ability. In summary, the ability of BCS to reduce cellular cytotoxicity and MDA level induced by Aj25-35 suggests that BCS may be a protective agent for free radical generating compounds such as Aj25-35, although we cannot in detail explain to effects of BCS at present. We are now in progress to isolate the active molecules and investigate the pharmacological mechanism.

Acknowledgments

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Anti-inflammatory, analgesic and antipyretic effects of methanol extract from *Bauhinia racemosa* stem bark in animal models

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Abstract

In this study, the anti-inflammatory, analgesic, and antipyretic effects of 50, 100 and 200 mg/kg body weight of methanol extract obtained from *Bauhinia racemosa* stem bark, the so-called MEBR, were investigated. The effects of MEBR on the acute and chronic phases of inflammation were studied in carrageenan, dextran and mediators (histamine and serotonin)-induced paw oedema and cotton pellet-induced granuloma, respectively. Analgesic effect of MEBR was evaluated in acetic acid-induced writhing and hotplate tests. Antipyretic activity of MEBR was evaluated by yeast-induced hyperpyrexia in rats. The anti-oedema effect of MEBR was compared with 10 mg/kg of indomethacin orally. In acute phase of inflammation, a maximum inhibition of 44.9, 43.2, 44.8 and 45.9% (*P* < 0.001) was noted at the dose of 200 mg/kg b.w. after 3 h of treatment with MEBR in carrageenan, dextran, histamine and serotonin-induced paw oedema, respectively. Administration of MEBR (200 mg/kg b.w.) and indomethacin (10 mg/kg b.w.) significantly (*P* < 0.05) decreased the formation of granuloma tissue induced by cotton pellet method at a rate of 50.4 and 56.2%, respectively. The extract also inhibited peritoneal leukocyte migration in mice. The MEBR also produced significant (*P* < 0.01) analgesic activity in both models. Further, the MEBR potentiated the morphine- and aspirin-induced analgesic in mice. Treatment with MEBR showed a significant (*P* < 0.01) dose-dependent reduction in pyrexia in rats. The results suggest that MEBR possess potent anti-inflammatory, analgesic and antipyretic activity.

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Keywords: *Bauhinia racemosa*; Anti-inflammatory; Analgesic; Antipyretic

1. Introduction

*Bauhinia racemosa* L. (*Caesalpiniaceae*), a small crooked tree with dark scabrous bark, is widely distributed throughout India, Ceylon, China and Timor. The bark and leaves of this plant are reported to be medicinally important in the traditional system of medicine and are used extensively for the treatment of inflammation, headache, fever, tumors, skin infection, disease of the blood, dysentery, and diarrhoea (Kirtikar and Basu, 1975; Wealth of India, 1952). The ethanol extract of leaves of this plant was evaluated for analgesic, anti-inflammatory, antipyretic and antispasmodic activity and was reported to be active (El-Khatib and Khaleel, 1995). The fresh flower buds of this plant were screened for antiulcer activity (Akhtar and Ahmad, 1995). Dried entire plant showed antimicrobial activity (Ali et al., 1999). The cytotoxic, hypotensive and hypothermic activities of seeds of *Bauhinia racemosa* have also been reported (Dhar et al., 1995).

Several chemical constituents of *Bauhinia racemosa* have been identified mainly as flavonols, coumarins, triterpenoids, stilbenes, steroids and tannins (El-Hossary et al., 2000; Prakash and Khosa, 1976; Anjaneyulu et al., 1984, 1986; Balasooriya et al., 1982). Previous results from this laboratory have also demonstrated the antioxidant and hepatoprotective effects in rats (Gupta et al., 2004a), and antitumor and antioxidant activity of *Bauhinia racemosa* against Ehrlich ascites carcinoma in Swiss albino mice (Gupta et al., 2004b). The present study was focussed on anti-inflammatory, analgesic and antipyretic...
effects of methanol extract of Bauhinia racemosa (MEBR) stem bark in animal models.

2. Materials and methods

2.1. Plant material

The stem bark of the plant Bauhinia racemosa L. (Family—Caesalpinaceae) was collected from Kolli Hills of TamilNadu, India. The plant material was taxonomically identified by the Botanical Survey of India, Kolkata. A voucher specimen (No GMS-1) has been preserved in our laboratory. The stem barks were dried under shade and then powdered with a mechanical grinder and stored in airtight container. The dried powder material of the bark was extracted with methanol (yield 9.25%), in a soxhlet apparatus. Phytochemical screening of the extracts revealed the presence of flavonoids, triterpenoids, coumarins, tannins and steroids.

2.2. Chemicals and drugs

Carrageenan (S.D. Fine Chemicals Limited, Bombay), 5-hydroxytryptamine hydrochloride (serotonin), histamine (Sigma, USA) and dextran (Sigma, USA) were used in the study, and indomethacin (Recon Bangalore), aspirin (USV Bombay), paracetamol (IPCA, Bombay) and morphine (M.M. Pharma, New Delhi) were used as the standard drugs.

2.3. Animals

Studies were carried out using male Wistar albino rats weighing 180–200 g and male Swiss albino mice weighing 18–22 g. They were obtained from the animal house, Indian Institute of Chemical Biology (IICB), Kolkata, India. The animals were grouped and housed in polycrylic cages (38 cm × 23 cm × 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C with dark and light cycle (14/10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water ad libitum. The rats were acclimatized to laboratory condition for 10 days before commencement of experiment. All procedures described were reviewed and approved by the University animal ethical committee.

2.4. Toxicity study

The LD₅₀ was determined using the graphical method of Litchfield and Wilcoxon (1949), in mice. Briefly, geometric doses of the extract (100–1750 mg/kg) were administered i.p. to 10 groups of mice. Control group received normal saline (5 ml/kg i.p.). Signs of toxicity and mortality within 24–72 h were noted. Confirmatory test was carried out and the LD₃₀ was calculated from the graph of percent mortality against probit log dose of the extract.

2.5. Anti-inflammatory activity

2.5.1. Carrageenan-induced rat paw oedema

The animals were divided into five groups (n = 6). The different groups were treated with MEBR (50, 100 and 200 mg/kg b.w., p.o.), indomethacin (10 mg/kg, p.o.) and vehicle control (10% propylene p.o. and the paw volume was measured at 0 h and 3 h after carrageenan injection using plethysmometer (Winter and Porter, 1957). The animals were pretreated with the extract 1 h before the administration of carrageenan. Acute inflammation was produced by the subplantar administration of 0.1 ml of 1% carrageenan in normal saline in the right paw of the rats. The anti-inflammatory effect of MEBR was calculated by the following equation: Anti-inflammatory activity (%) = (1 − DC/D) × 100, where D represents the percentage difference in paw volume after MEBR was administered to the rats and C represents the percentage difference of volume in the control groups (Suleyman et al., 1991).

2.5.2. Dextran-induced paw oedema

The animals were treated in a manner similar to that of carrageenan-induced paw oedema models; dextran (0.1 ml, 1% w/v in normal saline) was used in the place of carrageenan (Winter and Porter, 1957).

2.5.3. Histamine- and serotonin-induced inflammation

The anti-inflammatory activity of the MEBR was measured with phlogistic agents (viz. histamine, 5-HT) which act as mediator of inflammation. The paw oedema was induced in rats by subplantar injection of freshly prepared histamine (1 mg/kg b.w.) and serotonin (1 mg/kg b.w.) solutions respectively, and the paw oedema was measured as mentioned earlier (Winter et al., 1962).

2.5.4. Cotton pellets-induced granuloma

The rats were divided into five groups (n = 6). After shaving the fur, the rats were anaesthetized and 10 mg of sterile cotton pellets were inserted, one in each axilla. The MEBR (50, 100 and 200 mg/kg b.w., p.o.) and indomethacin (10 mg/kg b.w., p.o.) and control vehicle were administered orally for seven consecutive days from the day of cotton pellet implantation. The animals were anaesthetized on the eighth day and cotton pellets were removed surgically and made free from extraneous tissues. The moist pellets were weighed and then dried at 60 °C for 24 h, after that dried pellets were weighed again. Increment in the dry weight of the pellets was taken as measure of granuloma formation. The antiproliferative effect of MEBR was compared with the control.

2.6. Mouse carrageenan peritonitis

The mice were divided into five groups (n = 6). Inflammation was induced by modification of the technique as
previously described (Griswold et al., 1987). The extract was administered orally at doses of 50, 100 and 200 mg/kg and indomethacin at a dose of 10 mg/kg p.o., and carrageenan (0.25 ml, 0.75% in saline) was injected intraperitoneally 1 h later and after 4 h the animals were sacrificed by cervical dislocation for further investigation. Ca²⁺ and Mg²⁺ free phosphate buffered saline was used during the collection of peritoneal fluids. The total leucocyte count was determined in a Neubauer chamber and the differential cell count was determined (Wintrobe et al., 1961; D’Amour et al., 1965). The percentage of the leucocyte inhibition = (1 – T/C) × 100, where T represents the treated groups leucocyte counts and C represents the control groups leucocyte counts.

Neutrophils changes were calculated by the following equation: Neutrophils changes = Neutrophils counts of treated groups/Neutrophils counts of control groups × 100.

2.7. Analgesic activity

This was investigated by monitoring test animals exposed to chemical and thermal stimuli.

2.7.1. Acetic acid-induced writhing response in mice

Eight groups of mice were selected for the present study. Group one received acetic acid solution (300 mg/kg) (control) and group two received standard drug aspirin (100 mg/kg). Remaining six different groups of mice received 50, 100 and 200 mg/kg b.w. of MEBR (i.p.) and the combination of these doses of extract with the standard drug (aspirin 100 mg/kg b.w.). Acetic acid solution (15 mg/ml) at the dose of 300 mg/kg b.w. was injected i.p. and the number of writhes by drug treatments as compared to vehicle control animals was considered as a positive analgesic response. The percentage inhibition of writhing was then calculated.

2.7.2. Hot plate reaction time in mice

Eight groups of mice were selected for the present study. Group one received normal saline (5 ml/kg b.w.) (Control) and group two received standard drug morphine (5 mg/kg). Remaining six different groups of mice received 50, 100 and 200 mg/kg b.w. of MEBR (i.p.) and the combination of these doses of extract with the standard drug (aspirin 100 mg/kg b.w.). Mice were screened by placing them on a hot plate maintained at 55 ± 1°C and recorded the reaction time in seconds for licking of hind paw or jumping (Turner, 1965). The mice which reacted within 15 s and which did not show large variation when tested on four separated occasions were selected for studies.

2.8. Yeast-induced hyperpyrexia in mice

Hyperpyrexia was induced in rat by subcutaneous injection of 10 ml/kg b.w. of a 15% aqueous suspension of brewer’s yeast in the back below the nape of the rat (Al-Ghamdi, 2001). The animals were then fasted for the duration of the experiment, water ad libitum. Control temperatures were taken 24 h after the yeast injection to determine the pyretic response of yeast. Temperatures taken 1 h prior to drug administration in fevered animal served as the pre-drug control. Extract (50, 100 and 200 mg/kg b.w.) and paracetamol (150 mg/kg b.w.) served as the reference drug given orally 24 h after the yeast injection. The temperatures were recorded at 1–4 h after the drug treatment.

2.9. Statistical analysis

Values were expressed as mean ± S.E.M. Statistical significance was determined by ANOVA, followed by Student’s t-test; values with P < 0.05 were considered as statistically significant.

3. Results

3.1. Toxicity study

The LD₅₀ value of MEBR was estimated to be 955.04 mg/kg (915.80–984.75 mg/kg) body weight i.p. in mice.

3.2. Anti-inflammatory studies

The anti-inflammatory activity of MEBR was measured at the dose of 50, 100 and 200 mg/kg b.w. against acute paw oedema induced by carrageenan, dextran and mediators (histamine and serotonin), and is summarized in Fig. 1. The MEBR produced significant (P < 0.001) anti-inflammatory activity and the results were comparable to that of indomethacin as a standard anti-inflammatory drug. MEBR at the doses 50, 100 and 200 mg/kg showed an inhibition (28.1, 39.3 and 44.9%), (26.1, 37.5 and 43.2%), (27.6, 39.1 and 44.8%) and (28.2, 40.0, 45.9%) against acute paw oedema-induced by carrageenan, dextran, histamine and serotonin, respectively.

The effects of MEBR and indomethacin on the proliferative phase of inflammation are summarized in Table 1. It was seen that MEBR was responsible for anti-inflammatory effect, which would be calculated depending on the moist and dry weight of cotton pellets. According to these results, the antiproliferative effects of MEBR (200 mg/kg b.w.) and indomethacin (10 mg/kg b.w.) were calculated as 44.8 and 51.5% (P < 0.05), respectively. After they were dried, the antiproliferative effects were calculated on the basis of dry weight pellets; the inhibition of inflammation by MEBR and indomethacin were established as 50.4 and 56.2% (P < 0.05), respectively.
Fig. 1. Effect of methanol extract of Bauhinia racemosa (MEBR) stem bark on carrageenan-, dextran-, histamine- and serotonin-induced pedal edema in rats. Difference of mean of edema volume (ml) between control and treatment values at different doses ±S.E.M. Variation compared to the control animals. ANOVA followed by Student’s t-test, *P < 0.001.

3.3. Mouse carrageenan peritonitis

The MEBR also inhibited peritoneal leukocyte migration at the rate of 36.13, 58.14 and 78.51% at the doses of 50, 100 and 200 mg/kg, respectively, whereas the inhibition produced by indomethacin (10 mg/kg) 60.81% was found to be in carrageenan-induced peritonitis model as shown in Table 1.

3.4. Acetic acid-induced writhing in mice

Analgesic effects induced by different doses of MEBR on the writhing test in mice are shown in Table 2. MEBR at the dose of 50, 100 and 200 mg/kg b.w. and aspirin 100 mg/kg b.w. exhibited significant (P < 0.01) inhibition of the control writhes at the rate of 20.58, 36.71, 53.41 and 66.72%, respectively in the acetic acid-induced writhing. In addition, MEBR at the different doses also potentiated (71.36, 77.04 and 84.02%) the aspirin-induced analgesia.

3.5. Hot plate reaction time in mice

As shown in Table 3, the MEBR produced significant (P < 0.01) analgesic activity at all the tested doses. Additionally, MEBR at different doses potentiated the analgesic activity of morphine (5 mg/kg b.w.).

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Weight of cotton pellet (mg) (moist)</th>
<th>Percentage of inhibition</th>
<th>Weight of cotton pellet (mg) (Dried)</th>
<th>Percentage of inhibition</th>
<th>Leukocytes (10^5 mL^-1)</th>
<th>Leukocytes inhibition</th>
<th>Neutrophils 10^6 mL^-1</th>
<th>Neutrophils changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>203.51 ± 15.9</td>
<td>46.82 ± 1.9</td>
<td>4.18 ± 0.34</td>
<td>2.50 ± 0.41</td>
<td>33.63</td>
<td>10</td>
<td>33.63</td>
<td>8.43</td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>98.42 ± 8.7</td>
<td>51.46</td>
<td>56.23</td>
<td>20.54 ± 0.7</td>
<td>33.63</td>
<td>10</td>
<td>33.63</td>
<td>8.43</td>
<td></td>
</tr>
<tr>
<td>MEBR 50</td>
<td>152.32 ± 12.1</td>
<td>25.15</td>
<td>28.64</td>
<td>26.67 ± 0.11</td>
<td>36.13</td>
<td>10</td>
<td>36.13</td>
<td>11.64</td>
<td></td>
</tr>
<tr>
<td>MEBR 100</td>
<td>126.71 ± 10.0</td>
<td>37.73</td>
<td>17.5 ± 0.14</td>
<td>36.13</td>
<td>10</td>
<td>36.13</td>
<td>11.64</td>
<td>8.43</td>
<td></td>
</tr>
<tr>
<td>MEBR 200</td>
<td>112.33 ± 11.8</td>
<td>44.81</td>
<td>23.21 ± 0.6</td>
<td>30.41</td>
<td>0.00 ± 0.08</td>
<td>78.51</td>
<td>21.0 ± 0.08</td>
<td>8.43</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M (n=6).

* Experimental groups were compared with control (P < 0.01).

** Experimental groups were compared with control (P < 0.05).
Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Number of writhes (per 30 min)</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid control</td>
<td>300</td>
<td>33.42 ± 2.75</td>
<td>–</td>
</tr>
<tr>
<td>Aspirin</td>
<td>100</td>
<td>11.12 ± 1.19</td>
<td>66.72</td>
</tr>
<tr>
<td>MEBR</td>
<td>50</td>
<td>26.54 ± 2.13</td>
<td>20.58</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>21.15 ± 1.12</td>
<td>36.71</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>15.57 ± 1.17</td>
<td>53.41</td>
</tr>
<tr>
<td>MEBR + Aspirin</td>
<td>50 + 100</td>
<td>9.57 ± 0.79</td>
<td>71.36</td>
</tr>
<tr>
<td></td>
<td>100 + 100</td>
<td>7.67 ± 0.71</td>
<td>77.04</td>
</tr>
<tr>
<td></td>
<td>200 + 100</td>
<td>5.34 ± 0.49</td>
<td>84.02</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6).

* Experimental groups were compared with control (P < 0.01).

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Mean latent time</th>
<th>Initial After 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline 5 ml/kg</td>
<td></td>
<td>9.43 ± 0.83</td>
<td>9.36 ± 0.74</td>
</tr>
<tr>
<td>Morphine</td>
<td>5</td>
<td>9.03 ± 0.72</td>
<td>19.78 ± 1.12*</td>
</tr>
<tr>
<td>MEBR</td>
<td>50</td>
<td>9.05 ± 0.74</td>
<td>14.13 ± 1.32*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>9.37 ± 0.84</td>
<td>15.27 ± 1.21*</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>9.21 ± 0.65</td>
<td>16.57 ± 1.34</td>
</tr>
<tr>
<td>MEBR + Morphine</td>
<td>50 + 5</td>
<td>8.74 ± 0.65</td>
<td>22.15 ± 1.94*</td>
</tr>
<tr>
<td></td>
<td>100 + 5</td>
<td>8.93 ± 0.71</td>
<td>24.74 ± 1.75*</td>
</tr>
<tr>
<td></td>
<td>200 + 5</td>
<td>8.64 ± 0.69</td>
<td>27.96 ± 1.87*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6).

* Experimental groups were compared with control (P < 0.01).

Fig. 2. Effect of methanol extract of Bauhinia racemosa (MEBR) stem bark on yeast-induced pyrexia in rats. The results are given are mean ± S.E.M.; number of animals used (n = 6). *P < 0.01 compared to control by ANOVA.
This effect may be due to the cellular migration to injured tors in the immediate response of inflammation in rats. The greater effect of the MEBR on the inflammation media tors and it is a potent vasodilator substance and increases the vascular permeability (Linardi et al., 2002; Cuman et al., 2001). The result obtained from both the standard and MEBR treated rats were compared with that of control and a significant reduction in the yeast-induced elevated rectal temperature was observed (Fig. 2).

4. Discussion

4.1. Anti-inflammatory activity

The MEBR showed a dose-dependent anti-oedematogenic effects on paw oedema induced by carrageenan at 3 h. Dextran-induced paw oedema is known to be mediated both by histamine and serotonin. The MEBR also exhibited significant \( P < 0.001 \) anti-inflammatory in dextran-induced paw oedema. Histamine is one of the important inflammation media tors and it is a potent vasodilator substance and increases the vascular permeability (Linardi et al., 2002; Cuman et al., 2001). This study showed that all the doses of MEBR effectively suppressed the oedema produced by histamine, so it may be suggested that its anti-inflammatory activity is possibly backed by its antihistaminic activity. The MEBR also effectively suppressed the inflammation produced by serotonin induced by hind paw edema, which indicates that the MEBR may exhibit its anti-inflammatory action by means of either inhibiting the synthesis, release or action of inflammatory media tors viz. histamine, serotonin and prostaglandins that might be involved in inflammation. From the above results it is suggested that the anti-oedematogenic effects of MEBR on carrageenan, dextran and mediators-induced paw oedema may be related to inhibition of inflammation mediator formation.

The cotton pellet granuloma method is widely used to evaluate the transudative and proliferative components of the chronic inflammation. The moist weight of the cotton pellet correlates with the transudate; the dry weight of the pellet correlates with the amount of the granulomatous tissue (Olajide et al., 1999, 2000). Administration of MEBR (50, 100 and 200 mg/kg b.w.) and indomethacin (10 mg/kg b.w.) appear to be effective in inhibiting the moist weight of cotton pellet. On the other hand, the MEBR effect on dry weight of the cotton pellet was almost near to that of indomethacin. These data support the hypothesis of the greater effect of the MEBR on the inflammation media tors in the immediate response of inflammation in rats. This effect may be due to the cellular migration to injured sites and accumulation of collagen and mucopolysaccharide.

4.2. Mouse carrageenan peritonitis

Leukocyte aggregation at the site of inflammation is a fundamental event in the inflammatory process. Cell migration occurs as a result of much different process including adhesion and cell mobility (Meade et al., 1986). In the present investigation we compared the effect of the extract at the doses of 50, 100 and 200 mg/kg b.w. and indomethacin on the cell migration. The MEBR also inhibited the carrageenan-induced leukocyte migration in peritonitis model in mice. The MEBR was found to inhibit leukocyte migration more potent than indomethacin. The extract (in peritonitis model) drastically reduced the migration of neutrophils.

4.3. Analgesic activity

In acetic acid-induced abdominal writhing which is the visceral pain model, the processor releases arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis plays a role in the nociceptive mechanism (Franzotii et al., 2002). Results of the present study show that all the doses of the MEBR produced significant analgesic effect and this effect may be due to inhibition of the synthesis of the arachidonic acid metabolite. In addition, MEBR potentiates the analgesic activity of aspirin. The hot plate test has been found to be suitable for evaluation of centrally acting analgesics. The validity of this test has been shown even in the presence of substantial impairment of motor performance (Plummer et al., 1996). The present study findings indicate that the MEBR may be centrally acting.

4.4. Antipyretic

Fever may be a result of infection or one of the sequelae of tissue damage, inflammation, graft rejection, or other disease states. Antipyretic are drugs, which reduce the elevated body temperature. Regulation of body temperature requires a delicate balance between production and loss of heat, and the hypothalamus regulates the set point at which body temperature is maintained. In fever this set point elevates and a drug like paracetamol does not influence body temperature when it is elevated by the factors such as exercise or increase in ambient temperature (Goodman and Gilman, 1996). The MEBR possesses a significant antipyretic effect in yeast-induced elevation of body temperature in rats and this may be due to anti-inflammatory effect. Based on the results of the present study it can be concluded that MEBR has potential anti-inflammatory activity against acute and chronic phases of inflammation. The MEBR produce analgesic activity, which is both central and peripher analgesia, and also significant antipyretic action.
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CNS pharmacological effects of the hydroalcoholic extract of *Sida cordifolia* L. leaves

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**Abstract**

*Sida cordifolia* L. (Malvaceae), known as “malva branca”, is a plant used in the popular medicine for the treatment stomatitis, of asthma and nasal congestion. This work researched the acute toxicity of *Sida cordifolia* and its action on the central nervous system (CNS) because no data in the literature have been found about the pharmacological activity of this plant in the CNS. The hydroalcoholic extract of *Sida cordifolia* leaves (HESc) was used and the psychopharmacology approach began with the determination of LD50, where a low toxicity was observed in mice. Depressive activity on CNS was demonstrated by several alterations in mice’s behavior in the pharmacological screening. In the motility test, the HESc showed significant reduction of spontaneous activity at a dose of 1000 mg/kg (i.p.) at 30 and 60 min. The same form the HESc also decreased the ambulation and rearing in open-field test at 30, 60 and 120 min at a dose of 1000 mg/kg (i.p.).

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**Keywords:** *Sida cordifolia*; Malvaceae; Medicinal plants; CNS depressant activity

1. **Introduction**

In Brazil *Sida cordifolia* is popularly known as “malva branca” or “malva branca sedosa” and found throughout the country with considerable distribution in the northeast region. It is used in the popular medicine for the treatment stomatitis, of asthma and nasal congestion (Balbach, 1978).

This plant contains mainly alkaloids, oils, steroids, resin acids, mucin and potassium nitrate (Diwan and Kanth, 1999). Studies showed that the roots possess diuretic and tonic properties and administered for nervous disorders such as hemiplegia and facial paralysis (Rastogi and Malhotra, 1985).

Pharmacological investigation carried out with an aqueous extract of this plant’s leaves demonstrated an anti-inflammatory and analgesic activity (Antoniolli et al., 2000). Although preliminary pharmacological studies with *Sida cordifolia* have been undertaken, there are no data about the pharmacological effects of this species on behavior and CNS. The aim of this study was to carry out a pharmacological behavioral screening, determine the acute toxicity and to evaluate the effects of HESc in psychopharmacological animal’s models.

2. **Material and methods**

2.1. **Plant material and preparation of extract**

*Sida cordifolia* was collected in the botanical garden of Universidade Federal de Sergipe (UFS) (Brazil) in January 1999. The plant was identified by Dr. C. Dias Silva Jr. and a voucher specimen (no. 30171) is deposited in the Herbarium of the Biology Department at the same institution. *Sida cordifolia*
leaves were dried at 40 ± 1 °C and ground into a granulated powder. The extract was obtained using 494 g of this powder with EtOH 70% at 50 °C for 72 h in Södert following by filtration. The filtrate was concentrated in rotavaporator at 50 ± 5 °C for 48 h, lyophilized for 8 h and stored at 5 °C, yielding 88 g of lyophilized active material. The extract was freshly prepared with 0.9% saline and cremophor (vehicle) for pharmacological experiments.

2.2. Animals

Male Swiss mice (weighing 25–35 g, 90 days old) were obtained from our research animal house and were maintained at controlled room temperature (21 ± 2 °C) on a 12 h light/dark cycle (lights on at 06:00–18:00 a.m.) with free access to food and water. All experiments were conducted between 8:00 and 13:00 h. Procedures were approved by the Laboratório de Tecnologia Farmacêutica Animal Care and Use Committee.

2.3. Drugs

Sodium pentobarbital and cremophor were purchased from Sigma (USA). All drugs and the HESc were immediately prepared before each assay and administered in a volume of 0.1 ml/10 g body weight (mice). At the time of use, the extract was suspended in vehicle (saline 0.9% and one drop of cremophor) at the desired concentrations and the sodium pentobarbital was diluted in a saline 0.9% solution.

2.4. Acute toxicity

Different doses of HESc were administered intraperitoneally (i.p.) (500–3000 mg/kg) and orally (p.o.) (500–5000 mg/kg), while the control group received only the vehicle. The groups were observed for 48 h and at the end of this period mortality was recorded for each group (Dietrich, 1983).

2.5. Pharmacological behavioral screening

Groups of 10 mice were treated with HESc at the dose of 1000 mg/kg, i.p. or p.o. (experimental) or vehicle (control) while behavioral effects were observed and quantified as described by Almeida et al. (1999). After the treatment, the mice were observed from 30 min up to 4 h for studying behavioral changes.

2.6. Spontaneous locomotion test

This experimental model was described by Carlini (1973) to evaluate the interference of a substance in the motor activity of the animals. Groups of 10 mice were treated with HESc of dose of 1000 mg/kg (i.p. or p.o.) or vehicle. The animals were placed in the activity cage (with a square area of 48 cm, 30 cm in height and demarcation squares of 12 cm × 12 cm).

After 30, 60 and 120 min of treatment, the number of squares invaded within a period of 3 min were counted (De Lima et al., 1993; Almeida et al., 2001). The invasion criterion adopted was the presence of all paws of the animal within the square (Vásquez-Freire et al., 1994).

2.7. Open-field

This method is used to evaluate exploratory activity and emotionality of animals (Carlini et al., 1986). The open-field consisted of a white painted arena measuring 55 cm in diameter with a 100 W lamp. The floor of the arena was divided into several units by black painted lines. Groups of 10 mice were treated with HESc at dose of 1000 mg/kg (i.p. or p.o.) or vehicle. After 30, 60 and 120 min of administration, each mouse was placed in the center of the arena and defecation, ambulation, rearing and grooming were recorded for 5 min (Arletti et al., 2000).

2.8. Rotarod test

This method was described by Dunham and Miyata (1957). Mice were placed on a rotating rod (2.5 cm diameter, rotating at 7 rpm) for a pre-selection and those able to remain on the rod for 3 or more minutes in two successive trials were selected for testing (Morais et al., 1998). After 24 h of pre-selection, groups of ten mice were treated with HESc at dose of 1000 mg/kg (i.p. or p.o.) or vehicle. After 30, 60 and 120 min of treatments the animals were placed on a rotative bar of the rotarod apparatus for 5 min and the time spent by each animal on the rotarod was recorded (Carlini and Burgos, 1979; Morais et al., 1998).

2.9. Pentobarbital-induced sleep time

This methodology evaluate the depressive action of a given drug in CNS that possess sedative activity and characteristics of a hypnotic drug (Carlini et al., 1986). Groups of 10 mice were treated with HESc at a dose of 1000 mg/kg (i.p. or p.o.) or vehicle. After 60 min of the pre-treatment the animals were treated with sodium pentobarbital (50 mg/kg, i.p.) (Pal et al., 1996; Perez et al., 1998; Morais et al., 1998). The time between loss and recovery of the righting reflex, taken as sleeping time, was recorded for the saline and the drug pre-treated animals (Speroni and Minghetti, 1988).

2.10. Statistical analysis

Calculation of the LD₅₀ values with 95% confidence limits and comparisons of the results were performed using computerized linear regression analysis, in GraphPad Prism, version 3.02, a registered trademark of GraphPad Software Inc. The statistical analysis of data was made by analysis of variance (ANOVA) followed by Bonferroni test. In all cases differences were considered significant if p < 0.05.
3. Results

3.1. Acute toxicity of HESc

The hydroalcoholic extract of *S. cordifolia* was toxic at high doses administered (i.p.). The LD$_{50}$ values were 2639 mg/kg with 95% confidence limits of 2068–3367 mg/kg for i.p. administration. Deaths were not observed among orally treated animals.

3.2. Pharmacological behavioral screen of HESc

The HESc at a dose of 1000 mg/kg (i.p. and p.o.) produced sedation, decrease of the ambulation, reduction of answer to the touch, analgesia and decrease of urination. The effects of HESc were more pronounced in animals treated intraperitoneally.

3.3. Effect of HESc on spontaneous motor activity in mice

The mice treated with HESc at a dose of 1000 mg/kg (i.p.) caused significant reduction (p < 0.001) of the spontaneous locomotor activity in comparison with the control group at 30 and 60 min. The animals treated p.o. showed a decrease (p < 0.05) of ambulation at 60 min (Fig. 1).

3.4. Effect of HESc in open-field

The HESc at a dose of 1000 mg/kg (i.p.) significantly (p < 0.05) reduced the ambulation of mice at 30, 60 and 120 min. On the other hand, the animals treated p.o. presented a decrease (p < 0.05) of ambulation at 60 and 120 min. Another important data observed was the reduction of rearing in animals treated at 30, 60 and 120 min, both i.p. or p.o. (Table 1).

3.5. Effect of HESc on rotarod test

The HESc at a dose of 1000 mg/kg (i.p. or p.o.) did not cause a significant difference in the motor coordination of the treated animals in comparison with the control group (Fig. 2).

3.6. Effect of HESc on pentobarbital-induced sleep time

The HESc at a dose of 1000 mg/kg (i.p. or p.o.) did not produce a significant alteration of the latency and the time of sleep of the treated animals in comparison with those from the control group (Fig. 3a and b).

### Table 1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Groups</th>
<th>Dose/route (mg/kg)</th>
<th>Ambulation</th>
<th>Rearing</th>
<th>Grooming</th>
<th>Defecation</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Control</td>
<td>–</td>
<td>54.2 ± 2.8</td>
<td>23.4 ± 3.9</td>
<td>1.0 ± 0.2</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>HESc 1000i.p.</td>
<td>31.7 ± 7.5&quot;</td>
<td>5.5 ± 2.8&quot;</td>
<td>1.0 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HESc 1000p.o.</td>
<td>54.9 ± 4.9</td>
<td>8.6 ± 2.8&quot;</td>
<td>0.6 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>Control</td>
<td>–</td>
<td>41.6 ± 2.4</td>
<td>18.0 ± 2.7</td>
<td>1.8 ± 0.4</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>HESc 1000i.p.</td>
<td>28.4 ± 4.4&quot;</td>
<td>1.8 ± 0.4&quot;</td>
<td>1.7 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HESc 1000p.o.</td>
<td>29.4 ± 5.2&quot;</td>
<td>6.5 ± 2.6&quot;</td>
<td>1.7 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>Control</td>
<td>–</td>
<td>33.6 ± 3.8</td>
<td>10.1 ± 1.7</td>
<td>1.2 ± 0.4</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>HESc 1000i.p.</td>
<td>12.9 ± 3.9&quot;</td>
<td>1.5 ± 1.0&quot;</td>
<td>1.5 ± 0.4</td>
<td>0.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HESc 1000p.o.</td>
<td>14.6 ± 3.7&quot;</td>
<td>3.8 ± 2.1&quot;</td>
<td>1.2 ± 0.4</td>
<td>0.2 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

n = 10, values represent mean ± S.E.M.

* p < 0.05 significantly different from control.

** p < 0.01 significantly different from control.

*** p < 0.001 significantly different from control.
Fig. 3. Effect of HESc on: (a) the latency of pentobarbital-induced sleep and (b) the time of sleep induced pentobarbital. The values represent mean ± S.E.M. (n = 10).

4. Discussion and conclusion

In this work, the effects of the hydroalcoholic extract of *Sida cordifolia* leaves was studied in several behavioral animal models for the evaluation of central activity: pharmacological behavioral screening, spontaneous locomotion, open-field, pentobarbital-induced sleeping time and rotarod test. These are classical animal models of preliminary pharmacological tests of activities on CNS, which provide information about action upon psychomotor performance, motor behavior and neurotoxicity. Increasing doses of the hydroalcoholic extract of *Sida cordifolia* up to 5 g/kg administered to mice p.o. were not lethal, this is an indication of the low toxicity of the extract (Dietrich, 1983).

In pharmacological behavioral screening, the animals treated with HESc showed decrease of response to the touch and reduction of motor activity. These data are indicative of depressive activity of the CNS according to Almeida et al. (1999). It is important to note that the i.p. dose chosen make with basis in preliminary tests that showed more effective.

The general depressive activity was confirmed in the spontaneous locomotion test where the HESc significantly reduced spontaneous motor activity. The decrease in motor activity gives an indication of the level of excitability of the CNS (Masur et al., 1971) and this decrease may be related to sedation resulting from depression of CNS (Ozturk et al., 1996). This behavioral effect is similar to those obtained in previous studies of depressive drugs of CNS in agreement with Moore and Kenyon (1994, pp. 114–123), cited in Almeida et al. (2001). Radhakrishnan et al. (2001) affirmed that the reduction of motor activity can be due to the inhibitory effects of the extract in SNC or the muscular relaxing activity.

Another methodology carried out to assess the depressant effect of HESc in CNS was the pentobarbital-induced sleep test. The results showed that the animals treated with HESc did not present any alteration in latency and sleep time. According to Lovell (1986), the animals' response in this model can be affected by environmental (diet, temperature and bedding material) and genetic factors. However, the depressive activity of HESc in mice was better evidenced by the decrease of ambulation in spontaneous locomotion and open-field tests. Therefore the absence of effects in motor coordination performance and in pentobarbital-induced sleep time suggests a possible absence of neurotoxicity.

On the basis of the present study, we may suggest that the HESc has depressant effect on CNS without interfering with motor coordination with a low toxicity, thus justifying its extensive use by the northeast Brazilian population. It is necessary to determine the major evidence indicating depressant activity as well as the possible action mechanisms.

Acknowledgements

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References


In vitro antiplasmodial activity and cytotoxicity of 33 West African plants used for treatment of malaria

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Abstract

Thirty-three plants commonly used in West tropical Africa by traditional healers for the treatment of malaria were collected and ethanolic extracts were obtained by decoction. The antiplasmodial activity of extracts was evaluated in vitro against the chloroquine-resistant FcB1 strain of Plasmodium falciparum. Cytotoxicity was determined on the human MRC-5 and the rat L-6 cell lines. Of the 33 plant extracts, eight (24.5%) showed significant antimalarial activity (IC 50 values ranging from 2.3 to 13.7 μg/ml), 14 (42.5%) weak activity (IC 50 values ranging from 15 to 50 μg/ml) and 11 (33%) appeared inactive (IC 50 values >50 μg/ml). Five plants were of particular interest, associating good antiplasmodial activity and weak cytotoxicity. These five included Nauclea latifolia with known antiplasmodial activity and four, Fagara macrophylla, Funtumia elastica, Phyllanthus muellerianus and Rauvolfia vomitoria, for which the description of antiplasmodial activity is entirely novel.

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Keywords: Plasmodium falciparum; Antiplasmodial activity; Medicinal plants; Fagara macrophylla; Rauvolfia vomitoria; Phyllanthus muellerianus; Funtumia elastica

1. Introduction

Malaria is the major tropical disease due to parasites, responsible for significant morbidity and mortality in the world. A dramatic recrudescence of malaria is ongoing due to the increasing resistance of vectors to insecticides and the progressive resistance of the parasite, mainly Plasmodium falciparum, to drugs. These developments and the difficulty of creating efficient vaccines underline the urgent need for new antimalarial drugs.

In endemic countries, accessible treatments against malaria are mainly based on the use of traditional herbal remedies. Indeed, indigenous plants play an important role in the treatment of many diseases (Phillipson and Wright, 1991) and 80% of people world wide are estimated to use herbal remedies. However, few data are available on their efficiency and safety, despite the fact that validation of traditional practices could lead to innovative strategies in malaria control. What is more, natural products from plants or other organisms, represent a virtually inexhaustible reservoir of molecules, most of which are hardly explored and can constitute lead molecules for new antimalarial drugs, such as artemisinin, initially isolated from Artemisia annua (Kayser et al., 2003).

During an ethnobotanical survey in the central west Ivory Coast (Zirihi, 1991), 33 plants were selected on their common use by traditional healers for the treatment of malaria...
and fever. The present study investigates the in vitro antiplasmodial activity of ethanolic extracts of these plants against *Plasmodium falciparum* and their toxicity as assessed in two mammalian cell lines.

### 2. Materials and methods

#### 2.1. Plant extracts

Plants were selected during an ethnobotanical survey with the guidance of traditional healers (ACCT, 1986; Zirihi, 1991) and collected in Issia (central west Ivory Coast).

### Table 1

<table>
<thead>
<tr>
<th>Family</th>
<th>Plant species (specimen no.)</th>
<th>Aspect</th>
<th>Part used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amaranthaceae</td>
<td>Alternanthera repens (Linn.) Link (L. Ake Assi 14199)</td>
<td>Herb (0.5–1 m)</td>
<td>Whole plant</td>
</tr>
<tr>
<td>Anacardiaceae</td>
<td>Altinia boosei De Wild. (L. Ake Assi 13872)</td>
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</tr>
<tr>
<td>Apocynaceae</td>
<td>Foutumana elegans (Perr.) Steup. (L. Ake Assi 15378)</td>
<td>Tree (10–20 m)</td>
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</tr>
<tr>
<td></td>
<td>Rauvolfia vomitoria Aizel. (E. Ake Assi 155)</td>
<td>Tree (4–8 m)</td>
<td>Root bark</td>
</tr>
<tr>
<td>Asclepiadaceae</td>
<td>Parquetina nigrescens (Aizel.) Bullock. (L. Ake Assi 15031)</td>
<td>Liana</td>
<td>Leaf</td>
</tr>
<tr>
<td>Asteraceae</td>
<td>Cassia alata Linn. (L. Ake Assi 15915)</td>
<td>Shrub (0.5–1 m)</td>
<td>Leaf</td>
</tr>
<tr>
<td></td>
<td>Euphorbia cordifolia (Schumach. and Thonn.) Müll. Arg. (Adjanohoun and Ake Assi 234)</td>
<td>Shrub (5–12 m)</td>
<td>Leaf</td>
</tr>
<tr>
<td></td>
<td>Euphorbia hirta Linn. (E. Ake Assi 132)</td>
<td>Herb (0–2.0–1.3 m)</td>
<td>Whole plant</td>
</tr>
<tr>
<td></td>
<td>Mucuna pruriens (Benth.) Müll. Arg. (Adjanohoun and Ake Assi 337)</td>
<td>Tree (8–12 m)</td>
<td>Stem bark</td>
</tr>
<tr>
<td></td>
<td>Phyllanthus muellerianus (Kuntze) Exell. (E. Ake Assi 156)</td>
<td>Shrub (3–4 m)</td>
<td>Leaf</td>
</tr>
<tr>
<td>Euphorbiaceae</td>
<td>Alchornea cordifolia (Schumach. and Thonn.) Müll. Arg. (Adjanohoun and Ake Assi 234)</td>
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<td>Leaf</td>
</tr>
<tr>
<td></td>
<td>Euphorbia hirta Linn. (E. Ake Assi 132)</td>
<td>Herb (0–2.0–1.3 m)</td>
<td>Whole plant</td>
</tr>
<tr>
<td></td>
<td>Mucuna pruriens (Benth.) Müll. Arg. (Adjanohoun and Ake Assi 337)</td>
<td>Tree (8–12 m)</td>
<td>Stem bark</td>
</tr>
<tr>
<td>Fabaceae</td>
<td>Antholca incana (Ach.) Chevalier (L. Ake Assi 10513)</td>
<td>Tree (10–15 m)</td>
<td>Stem bark</td>
</tr>
<tr>
<td></td>
<td>Anthocleista djalonensis A. Chevalier (L. Ake Assi 10513)</td>
<td>Tree (10–15 m)</td>
<td>Stem bark</td>
</tr>
<tr>
<td></td>
<td>Bersama abyssinica Fresen. (Adjanohoun and Ake Assi 215)</td>
<td>Tree (10–15 m)</td>
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</tr>
<tr>
<td></td>
<td>Bethea azythina Miers (Adjanohoun and Ake Assi 15927)</td>
<td>Tree (10–15 m)</td>
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</tr>
<tr>
<td></td>
<td>Kigelia africana Miers (Adjanohoun and Ake Assi 15927)</td>
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<td></td>
<td>Physalis angulata Linn. (L. Ake Assi 15767)</td>
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<td>Whole plant</td>
</tr>
<tr>
<td></td>
<td>Solanum indicum (Thom.) Bitter (E. Ake Assi 101)</td>
<td>Herb (0–3–0.5 m)</td>
<td>Fruit</td>
</tr>
<tr>
<td></td>
<td>Solanum nigrum Linn. (E. Ake Assi 100)</td>
<td>Herb (0–3–0.5 m)</td>
<td>Fruit</td>
</tr>
</tbody>
</table>

### 2.2. In vitro antiplasmodial activity

Extracts were tested against the chloroquine-resistant FcB1/Colombia strain of *Plasmodium falciparum* (IC50) value for chloroquine of 0.1 μM (Frappier et al., 1996). The antiplasmodial activity was determined according to Desjardins et al. (1979). Extracts were prepared in DMSO at a concentration of 10 mg/ml and serially diluted with culture medium before to be added to asynchronous parasite cul-
In vitro antiplasmodial activity of ethanol extracts of plants on Plasmodium falciparum

Table 2

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Plasmodium falciparum IC50 (µg/ml)</th>
<th>L-A. IC50 (µg/ml)</th>
<th>SL L6/Pf IC50 (µg/ml)</th>
<th>MRC-5 IC50 (µg/ml)</th>
<th>SI MRC-5/Pf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacia tortilis</td>
<td>15.7 ± 1.8</td>
<td>22.7 ± 2.4</td>
<td>1.6</td>
<td>10.4 ± 1.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Aloe vera</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Aloe vera</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Aloe vera</td>
<td>&gt;50</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>Alternanthera dioica</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Alternanthera dioica</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Annona squamosa</td>
<td>2.5</td>
<td>&gt;50</td>
<td>&gt;5.6</td>
<td>&gt;50</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Berrera abyssinica</td>
<td>23.9 ± 5.7</td>
<td>ND</td>
<td>ND</td>
<td>5.3 ± 0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Cassia alata</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cassia alata</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Capsicum officinale</td>
<td>36.9 ± 5.6</td>
<td>&gt;50</td>
<td>&gt;1</td>
<td>32.8 ± 3.4</td>
<td>1</td>
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<tr>
<td>Erythroxylum fordii</td>
<td>36.9 ± 5.8</td>
<td>15.2 ± 1.1</td>
<td>0.5</td>
<td>&gt;50</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Ervatamia hispida</td>
<td>44.7 ± 14.7</td>
<td>&gt;50</td>
<td>&gt;1</td>
<td>&gt;50</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Forsimia capensis</td>
<td>23.1 ± 3.3</td>
<td>28.5 ± 0.5</td>
<td>12.4</td>
<td>20.8 ± 3.2</td>
<td>9</td>
</tr>
<tr>
<td>Ficus carica</td>
<td>45.3 ± 5.1</td>
<td>&gt;50</td>
<td>&gt;1</td>
<td>&gt;50</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Ficus carica</td>
<td>45.3 ± 5.1</td>
<td>&gt;50</td>
<td>&gt;1</td>
<td>&gt;50</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Irvingia gabonensis</td>
<td>21.6 ± 7.4</td>
<td>37.4 ± 1.5</td>
<td>1.7</td>
<td>46.6 ± 2.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Muntingia indica</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Myrsina congesta</td>
<td>27.6 ± 1.9</td>
<td>146.4 ± 0.2</td>
<td>0.5</td>
<td>7.0 ± 0.2</td>
<td>0.25</td>
</tr>
<tr>
<td>Metassonera scuderi</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Microdendron krysanum</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Microlepis peruviana</td>
<td>33.1 ± 4.1</td>
<td>&gt;50</td>
<td>&gt;1</td>
<td>&gt;50</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Muehlenbeckia cyanea</td>
<td>18.1 ± 2.6</td>
<td>32.3 ± 4.1</td>
<td>2</td>
<td>34.4 ± 3.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Nectandra mirtoides</td>
<td>11.6 ± 3.7</td>
<td>42.2 ± 3.2</td>
<td>3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neolania latifolia</td>
<td>8.9 ± 2.5</td>
<td>&gt;50</td>
<td>&gt;5.6</td>
<td>&gt;50</td>
<td>&gt;5.6</td>
</tr>
<tr>
<td>Parkia horrida</td>
<td>21.2 ± 3.0</td>
<td>23.3 ± 0.8</td>
<td>1</td>
<td>27.5 ± 4.8</td>
<td>1</td>
</tr>
<tr>
<td>Physalis paniculata</td>
<td>9.4 ± 2.9</td>
<td>&gt;50</td>
<td>&gt;5.3</td>
<td>&gt;50</td>
<td>&gt;5.3</td>
</tr>
<tr>
<td>Physalis spinyata</td>
<td>7.9 ± 0.7</td>
<td>27.4 ± 1.5</td>
<td>3.5</td>
<td>27.9 ± 1.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Pseudospondias argentea</td>
<td>18.2 ± 2.7</td>
<td>47.9 ± 2.6</td>
<td>2.6</td>
<td>&gt;50</td>
<td>&gt;2.7</td>
</tr>
<tr>
<td>Rauwolfia vomitoria</td>
<td>2.5 ± 1.0</td>
<td>22.5 ± 1.5</td>
<td>10</td>
<td>22.6 ± 1.4</td>
<td>9</td>
</tr>
<tr>
<td>Bixa orellana munitifera</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Solanum indicum</td>
<td>41.3 ± 7.0</td>
<td>&gt;50</td>
<td>&gt;1</td>
<td>&gt;50</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Solanum nigrum</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Solanum pseudocapsicum</td>
<td>21.8 ± 6.7</td>
<td>42.7 ± 6.2</td>
<td>1.9</td>
<td>&gt;50</td>
<td>&gt;2.3</td>
</tr>
</tbody>
</table>

IC50 values are the mean ± the standard deviations from three independent experiments; SI: selectivity index, defined as the ratio of the IC50 value determined on the mammalian cell line on the IC50 value determined on Plasmodium falciparum; ND: not determined.
in the study have previously been described as having antiplasmodial activity. This was the case for *Acanthospermum hispidum* (Sanon et al., 2003); *Morinda morindoides* (Tona et al., 2001), *Psychanthus angolensis* (do Ceu de Madureira et al., 2002), and *Physalis angulata* (Ankrah et al., 2003). With the exception of *Mureya micrantha*, *Bersama abyssinica*, *Eugenia floribunda* and *Acanthospermum hispidum*, plant extracts presented no or a weak cytotoxicity on the mammalian cells (IC₅₀ values > 20 μg/ml).

Several extracts are of particular interest, associating a good antimalarial activity with a low cytotoxicity: the root bark extract of *Rauwolfia vomitoria* (IC₅₀ = 2.5 μg/ml, SI = 9–10), the stem bark extract of *Funtumia elastica* (IC₅₀ = 3.3 μg/ml, SI > 15) and the stem bark extract of *Fagara macrophylla* (IC₅₀ = 2.3 μg/ml, SI = 9–12).

Although Apocynaceae are used in traditional medicine in Africa (Omino and Kokwaro, 1993), the in vitro antimalarial activity of *Rauwolfia vomitoria* and *Funtumia elastica* have not previously been reported. Stem latex of *Funtumia elastica* is used for the washing of wounds and the leaves are widely used in traditional medicine for the treatment of haemorrhoids. *Rauwolfia vomitoria* is an important medicinal plant used in many ills such as venereal diseases, neuropsychiatric disorders, jaundice, gastro-intestinal, sexual complaint, measles (Iwu and Court, 1977). *Rauwolfia vomitoria* also belongs with *Alstonia boonei* and *Elais guineensis*, to a Ghanaian herbal preparation with anti-inflammatory activity known to be used in the management of rheumatoid arthritis (Kweefo-Okaa, 1991).

*Fagara macrophylla* possesses a potent antifeedant activity against larvae of both *Spodoptera frugiperda* and *Spodoptera littoralis* (Tringali et al., 2001) and these substances might also be involved in the antimalarial activity. This hypothesis is supported by the fact that several acridone derivatives have been described as having potent activity against *Plasmodium falciparum* (Busco et al., 1994). Benzoylphenanthridine alkaloids with antitumor activity have also been isolated from *Fagara macrophylla* (Wall et al., 1987). They are DNA ligands and active as topoisomerase I-targeting agents (Li et al., 2003), the nitidine alkaloid present in *Fagara macrophylla* being the best known. Nitidine has also been isolated from *Todalia asiatica*, a plant used by the Pokot tribe of Kenya to treat fevers and showed a potent antimalarial activity against *Plasmodium falciparum* with IC₅₀ values in the low nanomolar range, and a lack of cross-resistance between chloroquine and nitidine (Gakunju et al., 1995). Benzoylphenanthridine alkaloids might thus be involved in the antimalarial activity of the *Fagara macrophylla* extract by inhibiting DNA replication.

The liana bark extract of *Nauclea latifolia* and the leaf extract of *Phyllanthus muellerianus* showed a weaker antimalarial activity (IC₅₀ values of 8.9 and 9.4 μg/ml, respectively) but they also have significant parasitic selectivity (SI > 5). These observations also confirm the previously described potent antimalarial activity of *Nauclea latifolia* (Benoit-Vical et al., 1998), although the active components against *Plasmodium* have yet to be identified. Also, several *Phyllanthus* species showed antimalarial activity (Omulokoli et al., 1997), whereas, no activity has previously been reported for *Phyllanthus muellerianus*.

Investigations to identify the active antimalarial compounds of *Fagara macrophylla*, *Funtumia elastica*, *Phyllanthus muellerianus* and *Rauwolfia vomitoria* by bioassay-guided fractionation are now in progress.

Acknowledgements

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References


Effect of *Commiphora opobalsamum* (L.) Engl. (Balessan) on experimental gastric ulcers and secretion in rats

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Abstract

The ulcer protective potential of an ethanol extract of *Commiphora opobalsamum* (L.) Engl. (Burseraceae) ‘Balessan’ was assessed against different acute gastric ulcer models in rats induced by necrotizing agents (80% ethanol, 0.2 M NaOH and 25% NaCl), hypothermic restraint stress, pyloric ligation (Shay) and indomethacin. Balessan, 250 and 500 mg/kg administered orally (intraperitoneally in Shay rat model) showed a dose-dependent ulcer protective effects in all the above ulcer models. Besides, the extract offered protection against ethanol-induced depletion of stomach wall mucus and reduction in nonprotein sulfhydryl (NP-SH) concentration. Ethanol treatment also caused histopathological lesions of the stomach wall. Pretreatment with Balessan extract provided a complete protection of gastric mucosa through supporting both the offensive and defensive factors. Balessan extract was also showed a large margin of safety without any apparent adverse effects in rats.

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Keywords: *Commiphora opobalsamum*; Burseraceae; Gastric anti-ulcer; Biochemical and histopathological changes in rats

1. Introduction

During field surveys in various parts of the Kingdom of Saudi Arabia, we found that the aerial parts of *Commiphora opobalsamum* (L.) Engl. (Burseraceae) ‘Balessan’ are used by local people for the treatment of various diseases including dyspepsia, colic, joint pain and to promote urine output or to expel renal calculi. This information was gathered from the local inhabitants of Farasan Island (an island in the Red Sea, Southwest of Saudi Arabia). In Unani or Greeco–Arab medicine, this plant is one of the important ingredients of a polyherbal formulation and in the form of decoction is used to improve digestion and to treat gastrointestinal disorders (Dymock et al., 1890; Anonymous, 1967; Chopra et al., 1956). Hartwell (1982) reported its potential use in stomach problems and cancer. In an earlier investigation, Abdul and Amin (1997) have reported hypotensive effect of an aqueous extract of *Commiphora opobalsamum* in rats. Since, no studies of anti-ulcer activity with this plant are available, the present study was carried to investigate the influence of this plant on different experimental models of gastric ulcers, which operate via distinct mechanisms of ulcerogenesis (Desai and Parmar, 1994).

2. Materials and methods

2.1. Plant material and preparation of crude extract

The aerial parts of Balessan used in this study were collected from Farasan Island of Red Sea (Saudi Arabia) in the
month of March 2002, and identified by Dr. M.A. Rahman, a voucher specimen (14312) was deposited in the herbarium of Medicinal, Aromatic and Poisonous Plants Research Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia for future reference. Each 500 g coarse powder of aerial parts of the plant was macerated in 3 L of 96% ethanol for 72 h using percolation method. The solvent was then removed at 40 °C under reduced pressure in a rotary evaporator. The yield was 11.02% of (w/w) in terms of the dried starting material. The extract was kept in a refrigerator maintained at a temperature of 2–4 °C for biological testing. The extract was suspended in distilled water using sodium carboxymethylcellulose (0.25%) just before the necrotizing agents. The stomach of each of the animals was excised and opened along the greater curvature. After washing with normal saline the gastric lesions were quantified using a binocular magnifier. The ulcers were scored according to the method reported earlier (Sedlak and Lindsay, 1968). The animals in the test groups were given 1 mL of necrotic agents, either 80% ethanol, 0.2 M NaOH or 25% NaCl, which are known to produce gastric lesions (Robert et al., 1979). Hypertonic saline and NaOH (0.2 M) were used only in cytoprotection studies. Balessan extract was given 30 min before the necrotizing agents. The animals were killed under anesthesia, using diethyl ether 1 h after treatment with the necrotic agents. The stomach of each of the animals was excised and opened along the greater curvature. After washing with normal saline the gastric lesions were quantified using a binocular magnifier. The ulcers were scored according to the method reported earlier (Sedlak and Lindsay, 1968). The modified procedure of Corne et al. (1974) was used to determine gastric-wall mucus. The glandular segments from the stomachs of control and treated rats were removed and weighed. Each segment was transferred immediately to 1% Alcian blue solution (in sucrose solution, buffered with sodium acetate, pH 5), and the excess dye was removed by rinsing with sucrose solution. The dye complexed with the gastric wall mucus was extracted with magnesium chloride. A 4-μL sample of blue extract was then shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged and the absorbance of the aqueous layer was measured at 580 nm. The quantity of Alcian blue extracted per gram (net) of glandular tissue was then calculated.

2.6. Pylorus ligated (Shay) rats

The animals were fasted for 36 h with access to water ad libitum before the pylorus was ligated under ether anesthesia, care being taken not to cause bleeding or to occlude blood vessels (Shay et al., 1945). Balessan extract (250 and 500 mg/kg body weight) was administered immediately after pylorus ligation by intraperitoneal injection. The animals were sacrificed 6 h after the pylorus ligation, stomachs were removed, and contents were collected, measured, centrifuged, and subjected to analysis for titratable acidity against 0.01N NaOH to pH 7. Each stomach was examined for lesions as described above.

2.7. Gastric wall mucus determination

The modified procedure of Corne et al. (1974) was used to determine gastric-wall mucus. The glandular segments from the stomachs of control and treated rats were removed and weighed. Each segment was transferred immediately to 1% Alcian blue solution (in sucrose solution, buffered with sodium acetate, pH 5), and the excess dye was removed by rinsing with sucrose solution. The dye complexed with the gastric wall mucus was extracted with magnesium chloride. A 4-μL sample of blue extract was then shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged and the absorbance of the aqueous layer was measured at 580 nm. The quantity of Alcian blue extracted per gram (net) of glandular tissue was then calculated.

2.8. Indomethacin-induced gastric ulcers

Indomethacin was suspended in 1% carboxymethylcellulose in water (6 mg/mL) and administered to the fasted rats in a dose of 30 mg/kg (0.5 mL/100 g). Rats were treated with Balessan extract (250 and 500 mg/kg, orally) 30 min before indomethacin. Control rats were treated similarly with an equivalent amount of vehicle (Bharagava et al., 1973). The stomachs of the animals were removed, rinsed with normal saline and studied according to the standard procedure (Szabo et al., 1985).

2.9. Estimation of nonprotein sulfhydryl groups (NP-SH)

Gastric mucusal NP-SH was measured according to the method reported earlier (Sedlak and Lindsay, 1966). The glandular stomachs of control and treated rats were removed and homogenized in ice-cold 0.02 M ethylenediaminetetraacetic acid. The homogenate was mixed with distilled water and then centrifuged at 10,000 × g for 15 min. The supernatant was used for determining NP-SH, according to the following arbitrary scale: 0, no blood detectable; 1, thin blood follows the rugae; 2, thick blood follows the rugae with blood clots in certain areas; and 4, thick blood (Chiu et al., 1984). After wiping the blood off, the total area of lesions in each stomach was scored as described above.

2.2. Animal stock

Wistar albino rats of either sex (home bred) aged 7–8 weeks and weighing 150–200 g, were obtained from the Experimental Animal Care Centre, King Saud University, Riyadh, Saudi Arabia. The animals were fed on Purina chow diet and water ad libitum and were maintained under standard conditions of humidity (55 ± 5%), temperature (22 ± 2 °C) and light (12-h light/12-h dark cycle). The rats were randomly assigned to different control and treatment groups.

2.3. Chemicals

Ethanol (BDH, England), indomethacin (Sigma), sodium hydroxide and sodium chloride (Merck) were used.

2.4. Gastric lesions induced by necrotizing agents

The stomachs of the animals were removed, rinsed with normal saline and studied according to the standard procedure (Szabo et al., 1985). The animals in the test groups were given 1 mL of necrotic agents, either 80% ethanol, 0.2 M NaOH or 25% NaCl, which are known to produce gastric lesions (Robert et al., 1979). Hypertonic saline and NaOH (0.2 M) were used only in cytoprotection studies. Balessan extract was given 30 min before the necrotizing agents. The animals were killed under anesthesia, using diethyl ether 1 h after treatment with the necrotic agents. The stomachs of control and treated rats were removed, measured, centrifuged, and studied according to the standard procedure (Szabo et al., 1985). The stomachs of the animals were removed, rinsed with normal saline and studied according to the standard procedure (Szabo et al., 1985).

2.5. Hypothermic restraint stress-induced ulcers

The method of Levine (1971) was followed with slight modification. The animals were fasted for 36 h with access to water ad libitum. One hour after receiving oral Balessan extract (250 and 500 mg/kg body weight), the rats were immobilized in restraint cages and placed inside a ventilated refrigerator maintained at a temperature of 2–4 °C. After 3 h, they were taken out and sacrificed. The stomachs were excised and examined for the severity of intraluminal bleeding according to the following arbitrary scale: 0, no blood detectable; 1, thin blood follows the rugae; 2, thick blood follows the rugae with blood clots in certain areas; and 4, thick blood (Chiu et al., 1984). After wiping the blood off, the total area of lesions in each stomach was scored as described above.

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Table 1

Effect of Balessan extract on the induction of gastric ulcers by various necrotic agents in rats

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment and dose (mg/kg body weight)</th>
<th>Ulcer index (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>80% EtOH</td>
</tr>
<tr>
<td>1.</td>
<td>Control</td>
<td>6.66 ± 0.72</td>
</tr>
<tr>
<td>2.</td>
<td>Balessan extract (250)</td>
<td>4.33 ± 0.55** (34.98)</td>
</tr>
<tr>
<td>3.</td>
<td>Balessan extract (500)</td>
<td>1.66 ± 0.33*** (75.05)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percent inhibition. Groups 2 and 3 were statistically compared with group 1. Six animals were used in each group.

∗P < 0.05 (Student’s t-test).
∗∗P < 0.01 (Student’s t-test).
∗∗∗P < 0.001 (Student’s t-test).

2.10. Determination of LD50 in rats

Wistar albino rats were divided into various groups and each group was orally treated with Balessan extract in the dose range up to 10 g/kg. Following treatments, the animals were observed for six continuous hours and thereafter at intervals of 2–12 h for up to 72 h. All behavioral changes and death during the observation period were recorded. The percentage of death in each group was then calculated. The LD50 was then determined using the methods outlined by Ghosh (1984).

2.11. Phytochemical screening

A preliminary phytochemical screening of aerial parts of Balessan was conducted to determine the presence or absence of alkaloids, cardiac glycosides, flavonoids, tannins, coumarins, anthaquinones, saponins, volatile oil, volatile bases, cyanogenic glycosides, glucosinolates, sugars, sterols and/or triterpenes according to the methods described by Farnsworth (1966).

2.12. Statistical analysis

The differences between control and treated groups were compared using the ANOVA and Student’s t-test as appropriate and were considered significant if P was < 0.05.

2.13. Histopathological studies

The gastric tissue was fixed in 10% ethanol buffered formalin and processed through graded ethanol, xylene and impregnated with paraffin wax; sections were made by microtome. After staining with haemotoxylin and eosin stain (Culling, 1974), the sections were examined under a research microscope by a person who was not aware of experimental protocols. The different histopathological indices screened were: congestion, hemorrhage, edema, necrosis, inflammatory and dysplastic changes erosions and ulcerations.

3. Results and discussion

The results of the present study are summarized in Tables 1–6. Balessan treatment was found to offer the gastric mucosa, a statistically significant and dose-dependent protection against ulcerations caused by 80% ethanol, 0.2 M NaOH and 25% NaCl (Table 1). The results were substantiated by histopathological findings. The histopathological studies showed that ethanol treatment caused haemorrhagic necrosis to the gastric mucosa in rats (Figs. 1 and 2). Pretreatment with Balessan in doses of (250 and 500 mg/kg) was found to preserve the functional cytoarchitecture of the entire gastric mucosa (Figs. 3 and 4). These findings confirm the cytoprotective nature of Balessan (Rafatullah et al., 1994). Ethanol-induced gastric lesions are thought to arise as a result of direct damage of gastric mucosal cells, resulting in the development of free radicals and hyperoxidation of lipid (Puurunen et al., 1980). The cytoprotective action of some anti-ulcer drugs are mediated by the action of endogenous prostaglandins known to play an important role in maintaining mucosal integrity (Miller, 1983) and to protect the gas-
Table 3
Balessan extract on the basal gastric secretion, acidity and lesion in 6h pylorus ligated Shay rats

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment and dose (mg/kg body weight)</th>
<th>Volume of basal gastric secretion (mean ± S.E.)</th>
<th>Titratable acidity (mEq/L) (mean ± S.E.)</th>
<th>Ulcer index (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>8.66 ± 0.66</td>
<td>163.27 ± 3.94</td>
<td>1.33 ± 0.33</td>
</tr>
<tr>
<td>2.</td>
<td>Balessan extract (250)</td>
<td>6.06 ± 0.65</td>
<td>140.35 ± 9.06</td>
<td>0.00**</td>
</tr>
<tr>
<td>3.</td>
<td>Balessan extract (500)</td>
<td>6.16 ± 0.98</td>
<td>81.10 ± 6.18</td>
<td>0.00**</td>
</tr>
</tbody>
</table>

Group 2 and 3 were statistically compared with group 1. Six animals were used in each group.

∗ P < 0.05 (Student’s t-test).

∗∗ P < 0.001 (Student’s t-test).

Table 4
Effect of Balessan extract on the induction of changes in gastric wall mucus by 80% ethanol

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment and dose (mg/kg body weight)</th>
<th>Number of rats</th>
<th>Gastric-wall mucus (Alcian blue H9262 g/g wet glandular tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control (vehicle 1 mL/rat)</td>
<td>6</td>
<td>338.57 ± 8.06</td>
</tr>
<tr>
<td>2.</td>
<td>80% Ethanol (1 mL/rat)</td>
<td>6</td>
<td>184.22 ± 6.16</td>
</tr>
<tr>
<td>3.</td>
<td>Balessan extract (250) + 80% ethanol</td>
<td>6</td>
<td>209.31 ± 12.00</td>
</tr>
<tr>
<td>4.</td>
<td>Balessan extract (500) + 80% ethanol</td>
<td>6</td>
<td>270.76 ± 5.71</td>
</tr>
</tbody>
</table>

Table 5
Effect of Balessan extract on indomethacin-induced gastric mucosal damage in rats

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment and dose (mg/kg body weight)</th>
<th>Lesions score (mean ± S.E.)</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Indomethacin (30) + (distilled water)</td>
<td>33.50 ± 3.24</td>
<td>56.41</td>
</tr>
<tr>
<td>2.</td>
<td>Indomethacin (30) + Balessan extract (250)</td>
<td>14.60 ± 2.92**</td>
<td>71.16</td>
</tr>
<tr>
<td>3.</td>
<td>Indomethacin (30) + Balessan extract (500)</td>
<td>9.66 ± 2.23**</td>
<td></td>
</tr>
</tbody>
</table>

Group 2 and 3 were statistically compared with group 1. Six animals were used in each group.

∗∗ P < 0.01 and <0.001 (Student’s t-test).

Table 6
Effect of Balessan extract on NP-SH concentrations in gastric ulcers induced by 80% ethanol

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment and dose (mg/kg body weight)</th>
<th>Number of animals</th>
<th>NP-SH concentration (μmol/g tissue, mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control (vehicle)</td>
<td>6</td>
<td>1.66 ± 0.08</td>
</tr>
<tr>
<td>2.</td>
<td>80% Ethanol (1 mL/rat)</td>
<td>6</td>
<td>0.96 ± 0.04</td>
</tr>
<tr>
<td>3.</td>
<td>Balessan extract (250) + 80% ethanol</td>
<td>6</td>
<td>1.11 ± 0.04</td>
</tr>
<tr>
<td>4.</td>
<td>Balessan extract (500) + 80% ethanol</td>
<td>6</td>
<td>1.37 ± 0.05</td>
</tr>
</tbody>
</table>

Group 2 was statistically compared with group 1. Groups 3 and 4 were statistically compared with group 2.

∗ P < 0.05 (Student’s t-test).

∗∗ P < 0.001 (Student’s t-test).
factor, gastric acid secretion. The anti-ulcerogenic effect of the extract may be related to its anti-secretory action since acid is a major factor in the development of peptic ulcer (Glavin and Szabo, 1992). However, certain anti-ulcer drugs increase the amount of gastric mucus secretion in gastric mucosa (Bolton et al., 1976; Robert et al., 1984). This mucus consists of mucin-type glycoproteins, which can be detected by amounts of alcian blue binding (Bolton et al., 1978).
Balessan extract increased the alcian blue binding to mucosa. Alcian blue dye is able to bind negatively charged materials. The increase in bound alcian blue suggested protective effect of orally administered Balessan extract. This may be via the formation of protecting complexes between Balessan extract and mucus, which can act as a barrier against several agents introduced in the stomach (Clamp et al., 1978; Sun et al., 1991). In the present study, the extract of Balessan significantly replenished the depleted level of gastric mucus by ethanol challenge (Table 4), indicating the probable local increase in prostaglandin synthesis. Our results also showed (Table 6) a significant reduction in nonprotein sulfhydrals content of gastric mucosa after 80% ethanol administration. Pretreatment of rats with Balessan extract significantly prevented NP-SH depletion. Decreased NP-SH level by ethanol is in agreement with other reports which have demonstrated the important role of NP-SH in gastric mucosal damage by ethanol (Miller et al., 1985). An increased NP-SH level is thought to protect gastric damage against various noxious chemicals (Szabo et al., 1981). These findings clearly showed the possible involvement of NP-SH in the cytoprotective and antioxidant activities of Balessan extract. Al-Harbi et al. (1994, 1997) have reported similar finding for another Commiphora spec. (Commiphora molmol) of Commiphora opobalsamum.

The preliminary phytochemical screening of Balessan revealed the presence of flavonoids, saponins, volatile oil, sterol and/or triterpenes. Though, we have not studied the active principles responsible for the anti-ulcer activity of Balessan, it is likely that the presence of flavonoids in this plant may be involved in the ulcer preventing action as flavonoids possess significant anti-ulcer activity in various experimental models of gastric and duodenal ulceration (Parmar and Parmar, 1998; Deshpande et al., 2003). Additionally, saponins and volatile oil of some plants are known to affect the integrity of mucus membranes and shown their ability to prevent the formation of ulceration of the gastric mucosa in different models of experimentally induced gastric ulcers in rats and mice (Hiruma-Lima et al., 1999; Guaraldo et al., 2001; Al-Rehaily et al., 2002). LD50 studies on acute toxicity determination showed no deleterious symptoms and a large margin of safety in rats was recorded. The LD50 value was found to be 4.75 g.

In the results of histopathological investigation (Figs. 1–4), the gastric mucosa of rats revealed that the pretreatment of Balessan extract absolutely inhibited the ethanol-induced haemorrhagic necrosis of rat stomach. Our results are in corroboration with the antigastric ulcer activity of the extract observed under the studies on pharmacological and biochemical evaluation.

In conclusion, the oral administration of an ethanol extract of Commiphora opobalsamum displayed a significant antiulcer activity without any apparent toxicological effects, which supports the use of Balessan in herbal medicine of Saudi Arabia. Further experiments and detailed phytochemical analyses are underway to determine the phytoconstituent(s) responsible for as well as the anti-ulcer mechanisms involved.

Acknowledgements

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In vitro estrogenic activities of Chinese medicinal plants traditionally used for the management of menopausal symptoms

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Abstract

The estrogenic activity of 70% EtOH extracts of 32 traditional Chinese medicinal plants, selected according to their reported efficacy for the treatment of menopausal symptoms, was assessed using a recombinant yeast system with both a human estrogen receptor expression plasmid and a reporter plasmid. Among them, 11 (34%) species proved to be active. Polygonum cuspidatum had the highest estrogenic relative potency (RP) (3.28 × 10^{-3}), followed by Rheum palmatum (3.85 × 10^{-4}), Cassia obtusifolia (3.49 × 10^{-4}), Polygonum multiflorum (2.87 × 10^{-4}), Epimedium brevicornum (2.30 × 10^{-4}), Psoralea corylifolia (1.90 × 10^{-4}), Cynomorium songaricum (1.78 × 10^{-4}), Scutellaria baicalensis (8.77 × 10^{-5}), Astragalus membranaceus (8.47 × 10^{-5}) and Pueraria lobata (6.17 × 10^{-5}). The EC50 value of 17β-hydroxyestradiol used as the positive control was 0.205 ± 0.025 ng/ml (RP = 100). This study gave support to the reported efficacy of Chinese medicines used for hormone replacement therapy.

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Keywords: Phytoestrogens; Hormone replacement therapy; Chinese medicine; Saccharomyces cerevisiae

1. Introduction

Hormones such as estrogen and progesterone play a very important role in human growth. It is responsible in regulating the complex cellular events associated with differentiation, function and growth of female reproductive tissues. Women in the menopause have always had to suffer bone density reduction, sweating and anxieties because of a lack of hormones (Harlow and Signorello, 2000). Hormone replacement therapy (HRT) was introduced to improve the menopausal symptoms 20 years ago (Nichols et al., 1984), which quickly took effect but increased the risk of breast cancer (Beral et al., 1999). It was found that natural compounds from certain plants called phytoestrogens could be used for management of menopausal symptoms and have few side effects (Thompson, 1993; Glazier and Bowman, 2001).

Traditional Chinese medicine has been used to heal many diseases for thousands of years and is now well known as natural medicine throughout the world. Many herbal medicines such as Angelica sinensis (Oliv.) Diels, Panax notoginseng (Burk.) F. H. Chen and so on are effective for improving female function according to the oldest traditional Chinese medical book, Sheng-nong Ben-cao Jing. It has been proven that some plant extracts have estrogenic components possessing a potential human use in dietary supplements and treatment of menopausal symptoms (Liu et al., 2001).

In vivo and in vitro assays have been developed to test estrogenic substances. Although in vivo assays are widely used,
they are unsuitable for large-scale screening and their utility is further limited due to the cost and relatively poor sensitivity. In vitro assays, however, are based on well-elucidated mechanisms of action and utilize more definitive end points than in vivo assays (Zacharewski, 1997). Among them, the assay system based on the binding of a ligand to estrogen receptor is the simplest. Yeast cells carrying the human estrogen receptor (hER) gene, estrogen response elements (ERE) and Escherichia coli β-galactosidase gene (lacZ) are very suitable for large-scale screening and sensitive analysis of estrogenic compounds. It is useful for the assay and discovery of novel estrogenic substances in natural specimens (Breithofer et al., 1998; Routledge and Sumpter, 1996).

In this study, a recombinant yeast with both a human estrogen receptor expression plasmid and a reporter plasmid was employed to search for phytoestrogens in selected Chinese medicinal plants, which have been used for hormone replacement therapy. A total of 32 Chinese medicinal plants (Table 1) were selected according to their reported efficacy for the treatment of menopausal symptoms and the estrogenic activities of their crude 70% EtOH extracts was assessed in order to give support to their reported activity and find crude drugs containing phytoestrogens in high concentration or highly active.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (E2) and o-nitrophenol-β-D-pyrogalactoside (oNPG) were purchased from Sigma. Yeast nitrogen base without amino acids was purchased from Fluka. All other reagents used in the study were of analytical grade.

2.2. Plant materials and extraction

A total of 32 Chinese medicinal plants were purchased from Darentang drugstore in Dalian, China, originating from different regions in China. The plants were identified by Dr. H. Sun, College of Pharmacy, Hei Longjiang University. Voucher specimens were preserved in College of Bio and Food Technology, Dalian Institute of Light Industry, China. The voucher numbers are shown in Table 1.

The minced plants (100 g) were extracted with 70% EtOH (800 ml) at room temperature for 24 h with shaking (Chen et al., 1987). The extracts were taken to dryness under reduced pressure at a temperature of 40–50 °C to yield gummy solids. The extraction yields (w/w) ranged from 0.6 to 12.1% (Table 1). The extracts were kept in a refrigerator for further activity assay.

2.3. Yeast strain and growth

For all transactivation assays Saccharomyces cerevisiae strain BJ3505 originally developed by Glaxo was used, which was kindly provided by Prof. W.Z. Wu, Institute of Hydrobiology, Chinese Academy of Sciences. This strain carried the hER expression plasmid YEPE10 and the estrogen responsive reporter plasmid YRPE2 (Santiso-Mere et al., 1991). The reporter gene (β-galactosidase) was controlled by ERE. The activity of β-galactosidase resulted in a color reaction, which was measured absorbance at 420 nm. The absorbance at 600 nm was selected to measure cell density and viability. The yeast strain was grown at 30 °C, 180 rpm, in selective medium with 50 μM CuSO4 but without tryptophane and uracil (Wu et al., 2002a).

2.4. Preparation of test samples

The plant extracts were dissolved in dimethylsulfoxide (DMSO) and used as samples for screening tests. 17β-Estradiol was dissolved in DMSO and used as positive control.

2.5. Design of the experiments

The experiments were designed according to Wang et al. (2003) with some modification. For all experiments, overnight cultures were diluted to OD600 = 1.0 prior to the induction of hER expression and addition of 17β-estradiol (positive control), test samples or DMSO (negative control). All the samples at concentrations of 0.1–1000 μg/ml (dried extract/ml) and 17β-estradiol at concentrations of 0.001–10 ng/ml were prepared in DMSO. The final concentration of DMSO in the assays was less than 1.0%. In this test, 5 μl of samples, 17β-estradiol or DMSO were added to 985 μl of yeast culture containing 50 μM CuSO4, which induced the expression of estrogen receptor (ER). After incubation at 30 °C for 2 h with shaking (150–180 rpm), the yeast cells were used for β-galactosidase assays.

2.6. β-Galactosidase assays

For the β-galactosidase assays, 100 μl cell suspensions were added to the wells of a 96-well microplate. The cells were permeabilized by addition of 100 μl assay buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 2 mg/ml oNPG, 38 mM β-mercaptoethanol, 0.01% Triton X-100, 15U/ml lyticase). The microplate was incubated at 30 °C until the color became yellow, which resulted from β-galactosidase cleavage of oNPG. Then, 100 μl of 1 M Na2CO3 was added to stop the reaction. In our test system, for 17β-estradiol, the incubation time was 50 min, and for the samples, it was less than 90 min. The resulting absorption was measured at 420 nm with a plate reader (TECAN, Austria). Each test sample and E2 was assayed in triplicate. E2 as a positive control and DMSO as a negative control for activity were performed in each test run.
<table>
<thead>
<tr>
<th>Plant family and species</th>
<th>Chinese name</th>
<th>Plant part</th>
<th>Collection place and voucher number</th>
<th>Extraction yield (w/w)</th>
<th>EC_{50} (µg/ml)</th>
<th>RIE</th>
<th>RP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amaranthaceae</strong></td>
<td><em>Amaranthus caudatus</em></td>
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<td>6.2</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
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<td><em>Amaryllis belladonna</em></td>
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<td>–</td>
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</tr>
<tr>
<td><strong>Anaphylactis</strong></td>
<td><em>Anaphylactis officinalis</em></td>
<td>Rhizome</td>
<td>Sichuan, DL-A AB318</td>
<td>6.2</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Artemisia</strong></td>
<td><em>Artemisia argyi</em></td>
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<tr>
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<td><em>Berberis aristata</em></td>
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<tr>
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<td>–</td>
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</tr>
<tr>
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<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>–</td>
<td>–</td>
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<tr>
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<td><em>Veronica officinalis</em></td>
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<td><em>Zea mays</em></td>
<td>Stem</td>
<td>Sichuan, DL-A AB318</td>
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</tr>
</tbody>
</table>
2.7. Calculation of β-galactosidase activity

The β-galactosidase activity is dependent on the binding of the ligand to the estrogen receptor and was measured according to Miller (1972), using the following formula (Wang et al., 2003):

\[
u = 100 \times \left( \frac{A_{600}(\text{sample}) - A_{600}(\text{blank})}{A_{600}(\text{blank})} \right) \times \frac{S}{t}
\]

where \( u \) is the β-galactosidase activity, \( t \) the incubation time (min) of enzyme reaction, \( A_{600} \) the absorbance of enzyme reaction at 420 nm and \( A_{600}(\text{blank}) \) the absorbance of the cells of the sample at 600 nm.

2.8. Curve fitting and EC50

Data derived from transactivation assays were fitted using four parameters logistic model based on the Marquardt-Levenberg algorithm (SigmaPlot 4.0, SPSS Inc., Chicago, IL, USA) (Rehmann et al., 1999):

\[ Y = \frac{A - D}{1 + \left( \frac{X}{C} \right)^2} + D \]

where \( Y \) is the response value (β-galactosidase activity), \( X \) the sample concentration, \( A \) the maximum induction of β-galactosidase activity, \( B \) the relative slope of the middle region, \( C \) the sample concentration at half-maximal response and \( D \) the detection limit. EC50 value is the value of C in the equation.

2.9. Definition of estrogenic relative potency and relative inductive efficiency (RIE)

In order to compare each assay directly, the relative potency and the relative inductive efficiency are employed. The estrogenic relative potency of samples is computed by dividing the EC50 of E2 by the EC50 of the test samples and then multiplying these values by 100 (the RP value of E2 is 100 in the definition). But it is not enough to evaluate estrogenic activity using RP alone (Coldham et al., 1997). The relative inductive efficiency of β-galactosidase activity could be used for further evaluating estrogenic activity. The RIE is the ratio of maximal β-galactosidase activity of the samples to that of E2 and then multiplying these values by 100 (the RIE value of E2 is 100 in the definition).

3. Results

3.1. Standard dose–response in yeast

To the induced culture 17β-estradiol was added to reach a final hormone concentration between 0.001 and 10 ng/ml and incubated for 2 h, then the β-galactosidase activity was assayed. The limit of detection was 0.04, the maximum β-galactosidase activity was 3.80 u and the EC50 was 0.205 ± 0.025 ng/ml.

3.2. Estrogenic activities of the selected Chinese medicinal plants

The EtOH extracts of 32 Chinese medicinal plants used to treat menopausal symptoms were assayed for estrogenic activities by a recombinant yeast system. The test samples in DMSO were added to the culture reaching final concentrations between 0.1 and 1000 μg/ml and incubated for 2 h, and then the β-galactosidase activity was assayed.

Table 1 shows that 11 extracts activated the transcription of lacZ. Polygonum cuspidatum, Rheum palmatum, Cassia obtusifolia and Polygonum multiflorum had a higher estrogenic relative potency with RP ranging from 3.28 × 10^-3 to 2.87 × 10^-4. Among them, Polygonum cuspidatum had the highest estrogenic potency and was about 100,000 times less estrogenic than 17β-estradiol (RP of E2 was 100). On the other hand, Belamcanda chinensis, Pueraria corystifolia and Polygonum multiflorum had a higher estrogenic relative inductive efficiency with RIE ranging from 83.7 to 52.1 (RIE of E2 was 100). The results indicated their potential efficacy for the treatment of menopausal symptoms.

4. Discussion

The recombinant yeast cells, MCF-7 human breast cancer cells and a prepubertal mouse uterotrophic bioassay have been used for phytoestrogen screening and environmental estrogen assays. The recombinant yeast cell bioassay is approximately two and five orders of magnitude more sensitive to E2 than MCF-7 cells and the uterotrophic assay, respectively (Coldham et al., 1997). So it is thought to be a good method for screening potential estrogens because of its exquisite sensitivity, absence of test compound biotransformation, ease of
results were very stable and the EC50 of 17β-estradiol was 0.205 ± 0.025 ng/ml. Normally, estrogenic activity is judged by the RP value (Wu et al., 2002a). According to this, Polygonum cuspidatum is the highest estrogen, followed by Rheum palmatum, Cassia obtusifolia, Polygonum multiflorum, Epimedium brevicornum, Psoralea corylifolia, Cynomorium songaricum, Belamcanda chinensis, Scutellaria baicalensis and Astragalus membranaceus. Estrogenic activity is also judged as fully estrogenic if the RIE is >75%, partially estrogenic if the RIE is 25–75%, weakly estrogenic if the RIE is 10–25% and negative if the RIE is below 10% (Andersen et al., 1999). According to this, Belamcanda chinensis is fully estrogenic, Cynomorium songaricum, Epimedium brevicornum, Polygonum cuspidatum, Polygonum multiflorum, Psoralea corylifolia, Pueraria lobata and Rheum palmatum are partially estrogenic, Cassia obtusifolia and Astragalus membranaceus are weakly estrogenic, and other species are negative. These data suggest a potential use of certain Chinese crude drugs against hormone-dependent breast and prostate cancers, and cardiovascular diseases, and as estrogen replacement therapy for postmenopausal women.

The physiological role of bioactive compounds present in plants has attracted more attention over the last decade, especially the phytoestrogens. Traditional Chinese medicines have been used in the treatment of menopausal symptoms for several thousands of years in Asian countries, but many mysteries remain about the relationship between the active component and their function owing to the complexity of the mixtures. It has been proven that phytoestrogens of genistein and daidzein, the main components of soy, make a contribute to the inhibition of the growth of breast cell lines and reduce bone turnover (Vissac-Sabatier et al., 2003; Setchell et al., 2001). According to their chemical structure, phytoestrogens can be classified into four main groups, i.e., isoflavonoids, flavonoids, stilbenes and lignans (Cos et al., 2003). It is known that isoflavonoids, flavonoids, anthraquinones, triterpenes and saponins are the main components in the 11 active herbs, but it is unknown whether or not a single component or the additive effect of certain components contributes to estrogenic activity. Further studies will focus on the isolation and identification of active compounds in the active species.

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Effect of propolis, some isolated compounds and its source plant on antibody production

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Abstract

Propolis is a beehive product with a very complex chemical composition, widely used in folk medicine because of its several therapeutic activities. Its biological properties and chemical composition may vary according to the geographic location and to the different plant sources. The possible mechanism of action of propolis as well as of its active compounds has been the subject of researchers in recent years. In this work, first we reported the results of our study on the seasonal effect of the immunomodulatory action of propolis on antibody production in bovine serum albumin (BSA)-immunized rats. Then, we compared the effect of Brazilian and Bulgarian propolis, some isolated compounds and Baccharis extract on anti-BSA antibody levels. Based on the results, we conclude that propolis stimulates antibody production, independently of the season and geographic origin. Caffeic acid, quercetin and Baccharis extract had no effect on antibody production, although the importance of isolated compounds is well reported in other biological assays. Propolis action is a consequence of plant-derived products with synergic effects, while isolated compounds or extracts from its plant sources had no effect in this assay.

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Keywords: Propolis; Antibody; Caffeic acid; Quercetin; Baccharis

1. Introduction

Propolis is a resinous hive product, built by honeybees from various plant sources, consisting of a complex mixture of constituents (Burdock, 1998; Bankova et al., 2000). This natural product has been used in folk medicine and its various biological activities have been reported, including antibacterial (Sforcin et al., 2000), antiviral (Vynograd et al., 2000), antiinflammatory (Khayyal et al., 1993), anticarcinogenic (Bazo et al., 2002) and immunomodulatory (Sforcin et al., 2002a; Sá-Nunes et al., 2003) activities.

Its biological properties and chemical composition may vary according to the geographic location and to the different plant sources (Markham et al., 1996). In temperate zone, propolis is collected from the bud exudate of poplar trees, such as Populus species (Bankova et al., 1999b). There are no poplars in tropical regions and the main propolis source in Botucatu, São Paulo State, Brazil, is Baccharis dracunculifolia DC. (Compositae), followed by Eucalyptus citriodora Hook. (Myrtaceae) and Araucaria angustifolia (Bert.) O. Kuntze (Araucariaceae) (Bankova et al., 1999). Moreover, in the temperate zone of the Northern Hemisphere, bees collect propolis only in the summer (including late spring and early autumn), while in Brazil, propolis collection proceeds throughout the year, but seasonal variations are possible.

With respect to the immune system, in recent studies in our laboratory, we have shown that propolis increases the natural killer activity against tumor cells (Sforcin et al., 2002a), modulates both in vitro and in vivo nitric oxide and hydrogen peroxide production by peritoneal macrophages (Orsi et al., 2000), and increases the fungicidal activity of these cells (Murad et al., 2002).

The possible mechanism of action of propolis, as well as its active compounds, has been the subject of research in recent years (Banskota et al., 2001).
In this work, we wish to report the results of our study on the effect of seasonal variations over propolis immunomodu- latory action, specifically on antibody production, by BSA- immunized rats. We compared the effect of propolis from Brazil against propolis from Bulgaria, and some active com- pounds on anti-BSA antibody levels. The effect of *Baccharis* extract, one of the source plants in Brazil, was also analyzed, in order to compare the plant source with propolis activity.

2. Methodology

2.1. Propolis samples

Propolis was produced by Africanized honeybees (*Apis mellifera* L.) in the Beekeeping Section of the Lageado Farm (UNESP, Botucatu). Samples were collected throughout the year from plastic nets. At the end of each month, nets were taken and frozen to facilitate propolis removal. Samples were pooled by season and were ground and extracted (30 g of propolis, completing the volume to 100 ml with 70% ethanol), protecting it from bright light, with moderate shaking, at room temperature. After a week, extracts were filtered and final concentrations were calculated, thus obtaining the dry weight of the extracts (120 mg/ml) (Sforcin et al., 1995).

Bulgarian propolis was kindly given by Dr. Bankova and was prepared in the same way (dry weight: 170 mg/ml).

2.2. Caffeic acid and quercetin administration

Caffeic acid and quercetin were isolated at the Institute of Organic Chemistry in Bulgaria by Dr. Bankova and were dissolved in hot distilled water before administration to rats (100 mg/kg body weight), giving 0.4 ml p.o.

2.3. Baccharis extract

Extracts of *Baccharis dracunculifolia* were prepared in the same way, obtaining 10% *Baccharis* extract (1 ml extract + 9 ml distilled water) (Lopes et al., 2003). The plant was identified in the Herbário Botu, Department of Botany of Biosciences Institute, UNESP, where a voucher specimen (BOTO 09867–18.03.98) has been deposited.

2.4. Animals, treatments and immunization procedure

Male rats (*Rattus norvegicus*) weighing 200 g were used.

2.4.1. First assay

In order to investigate the seasonal effect on Brazilian propolis activity, 42 rats were divided into 6 groups (G1, G2, G3, G4, G5 and G6) of 7 rats each.

G1, G2, G3 and G4 received 10% propolis extracts (1 ml of propolis + 9 ml distilled water) from spring, summer, autumn and winter samples, respectively. G5 received 10% hydroalcoholic solution (Labsynth), in order to observe a possible ethanol effect as propolis solvent, while G6, control, received physiological salt solution (0.9% NaCl) only. Animals received 0.4 ml of the respective solutions by gavage, twice a day, for 3 days (Sforcin et al., 2002a). After 24 h of the last treatment, rats were immunized through sc injection with bovine serum albumin (Sigma: 4 mg/ml) with complete Freund adjuvant, in 0.5 ml saline. After 15 and 30 days, rats were similarly immunized (sc) with BSA (4 mg/ml) with incomplete Freund adjuvant and only BSA (2 mg/ml), respectively.

2.4.2. Second assay

The effect of Brazilian and Bulgarian propolis, as well as the effect of caffeic acid, quercetin and *Baccharis* extract, were analysed for antibody production in order to observe a possible involvement of active compounds as well as the main vegetal source of propolis in this assay.

Animals were divided into seven groups of seven rats each.

G1 and G2 were treated with Brazilian and Bulgarian 10% propolis extracts, respectively. G3 and G4 received caffeic acid and quercetin (100 mg/kg), respectively. G5 received 10% *Baccharis* extract. G6 received 10% hydroalcoholic solution (propolis vehicle) and G7 was considered as control (0.9% NaCl).

Animals were treated and immunized as previously de- scribed.

2.5. Serum

Blood samples were collected 15 and 30 days after the last immunization by retro-orbital puncture, in order to determine the anti-BSA antibodies. Blood samples were centrifuged, serum was taken and aliquots were frozen at −20 °C.

2.6. Humoral immune response

Enzyme-linked immunosorbent assay (ELISA) was used to detect antibodies (anti-BSA IgG) (Ivanovska et al., 1997). Plates were coated by overnight incubation with BSA (20 µg/ml) in carbonate–bicarbonate buffer 0.2 M, pH 9.6, then washed with phosphate-buffered saline (PBS)/0.05% Tween 20 (0.1 ml) and quenched with PBS/5% low-fat milk (0.12 ml). After 30 min, appropriately diluted test serum in PBS/5% low-fat milk was added for 1 h at 37 °C.

Plates were washed and 80 µl goat peroxidase-labelled antirat IgG was added. Within 45 min, 80 µl of 0.1 M phosphate citrate buffer, pH 5.5, containing 0.8 µl/ml hydrogen peroxide, was added to each well. After 30 min, 0.05 ml of 4 M H₂SO₄ was added and the absorbance (OD 492 nm) was measured using ELISA reader (Labsystems).

2.7. Statistical analysis

Antibody titers were determined as −log₂ of the last dilution to statistical analysis. Non-parametric
3. Results

Brazilian propolis administration to rats increased antibody production after 15 days of immunization (Fig. 1A; Table 1) \( (p < 0.05) \). No significant differences were seen after 30 days of the last immunization (Fig. 1B; Table 1).

No differences were seen between the samples from each season.

Brazilian and Bulgarian propolis stimulated antibody production, in the same magnitude, after 15 days of immunization (Fig. 1C; Table 2) \( (p < 0.05) \). Although the effect of Brazilian propolis was a little higher than the control and the Bulgarian propolis after 30 days, no significant differences were detected (Fig. 1D; Table 2).

Hydroalcoholic solution-treated groups showed a pattern of antibody production similar to control, in all assays.

Caffeic acid and quercetin had no effects on antibody production.

\textit{Baccharis dracunculifolia} extract increased antibody production not significantly after 15 days of immunization when compared to control, but efficiently when compared to propolis-treated rats. No effects were seen after 30 days (Fig. 1C and D).

Table 1

<table>
<thead>
<tr>
<th>Control</th>
<th>Alcohol</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 days*</td>
<td>1.8192</td>
<td>1.16384</td>
<td>1.65536</td>
<td>1.131072</td>
<td>1.65536</td>
</tr>
<tr>
<td>30 days</td>
<td>1.65536</td>
<td>1.131072</td>
<td>1.65536</td>
<td>1.131072</td>
<td>1.65536</td>
</tr>
</tbody>
</table>

Anti-BSA antibody titer of control groups and groups treated with propolis from each season, 15 and 30 days after immunization.

* Control significantly different from four seasons \( (p < 0.05) \).
4. Discussion and conclusion

Many scientific articles have been published in the last years related to the biological activities of propolis. Banskota et al. (2001) compiled important findings on pharmacological properties of propolis and its possible mechanism of action, as well as its active constituents.

Propolis administration to rats increased antibody production. Propolis ability to modulate antibody synthesis is a part of its adjuvant activity, since it has been shown recently that propolis has a potent effect on different cells of innate immune response (Orsi et al., 2000; Murad et al., 2002; Sforcin et al., 2002a).

Scheller et al. (1988) observed that propolis stimulates antibody production in mice immunized by sheep red blood cells (SRBC) using a different methodology (plaque-forming cells). These authors concluded that propolis acts on a short-term basis on the immune system. Ivanovska et al. (1993) observed that SRBC administered simultaneously with cinnamic acid lysine derivative (CN.Ly), strongly elevated the antibody titters of mice, through haemagglutination determination. Increasing concentrations of CN.Ly lowered the antibody levels. Some propolis constituents augment host-defence response influencing lymphocyte proliferation and induction of cytokines release (Ivanovska et al., 1995).

With respect to the effects of seasons on propolis activity, no differences were seen between samples from each season. These data are in accordance with those previously obtained in our laboratory (Sforcin et al., 1999a, 2002a, 2002b).

The chemical analysis of our propolis samples by gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) and thin-layer chromatography (TLC) revealed that seasonal variations in its composition are not significant and are predominantly quantitative (Boudourova-Krasteva et al., 1997; Bankova et al., 1999a,b).

In the second part of this investigation, we compared Brazilian and Bulgarian propolis, since their biological activities and chemical compositions may show some differences because of the local flora. One may see that both samples stimulated antibody production, in the same magnitude, after 15 days of immunization. Although Brazilian propolis showed a little higher activity than control and the Bulgarian propolis after 30 days, no significant differences were detected. These results are in conformity with our recent work, in which we observed that both Brazilian and Bulgarian propolis had a modulatory action on macrophages, without significant differences between them (Murad et al., 2002).

From our data, it is clear that caffeic acid and quercetin have no effects on antibody production. Caffeic acid phenethyl ester and quercetin are propolis constituents responsible for several biological properties, such as antimicrobial effect (Mirzoeva et al., 1997). Caffeic acid esters possess significant cytotoxicity towards various tumor cells and antitumor activity (Lee et al., 2000), although other phenolic compounds and diterpenoids isolated from propolis also have antitumor properties (Banskota et al., 2001). Besides the effect of individual constituents, there may be synergistic effects of several compounds, thus conferring propolis different pharmacological activities.

Kuznetsova et al. (1999) suggested that general pharmacological properties of propolis are due to a natural mixture of its components and a single propolis constituent does not have an activity greater than that of the total extract.

Baccharis dracunculifolia extract, the main source of propolis in our region, did not increase antibody production significantly after 15 days of immunization when compared to control, but efficiently when compared to propolis-treated rats. No effects were seen after 30 days.

We evaluated the action of three vegetal sources of propolis (Araucaria angustifolia, Baccharis dracunculifolia and Eucalyptus citriodora) on macrophages activation, through oxygen (H₂O₂) and nitrogen (NO) metabolites determination, comparing these results with those previously obtained with propolis, in the same assays, in our laboratory (Orsi et al., 2000). Propolis and plant secretions were investigated using TLC and GC–MS analysis (Bankova et al., 1999). The data suggest that no effects are attributed to such extracts on metabolic production, while propolis induces an elevation in H₂O₂ release and inhibits NO generation, depending on the concentration (Lopes et al., 2003). Propolis action is a consequence of plant-derived products and isolated extracts of its vegetal sources, which did not have the same effect in this assay.

Raised antibody titters are important for microbial killing by antibody-mediated mechanisms. Based on the results of the present work, we may conclude that propolis stimulates antibody production, independently of the season and geographic origin, while caffeic acid, quercetin and Baccharis extract had no effect on antibody production.

### Table 2

<table>
<thead>
<tr>
<th>Geographic origin, isolated compounds of Brazilian and Bulgarian propolis and Baccharis effect on antibody production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>---------</td>
</tr>
</tbody>
</table>

<sup>a</sup> Control significantly different from Brazilian and Bulgarian propolis (p < 0.05).

<sup>b</sup> Brazilian propolis significantly different from Baccharis, caffeic acid and quercetin (p < 0.05).
New assays are being carried out in our laboratory, in order to investigate propolis effect on the immune system and to permit a better understanding of its mechanism of action.

References


Medicinal plant wealth of local communities in some villages in Shimoga District of Karnataka, India

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Abstract

An ethnomedicinal survey (1998–2000) was conducted in three villages of Shimoga district of Karnataka, India, using a questionnaire designed by Sinha (1996) [Sinha, R.K., 1996. Ethnobotany—The Renaissance of Traditional Herbal Medicine. Ina Shree Publishers, Jaipur, India, 242 pp.]. The herbal practitioners in the study area were interviewed and information on medicinal plants, their local names, habitat and their seasonal availability was collected. The survey revealed the utilization of 47 species of plants belonging to 46 genera in 28 families used to treat 9 infectious and 16 non-infectious diseases. Twelve new claims on ethnomedical knowledge were reported and there were formulations that were similar to that described already in the literature.

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Keywords: Ethnomedicine; Local community; Infectious diseases; Non-infectious diseases; Western Ghats; Karnataka

1. Introduction

Traditional knowledge of herbal remedy to treat human diseases is fast declining in many parts of the world, including India. Even today, tribal and certain local communities in India still practice herbal medicine to cure a variety of diseases and disorders. They collect and preserve locally available, wild and cultivated plant species. Compared to the large number of floristic surveys in southern peninsular India (Saldanha and Nicolson, 1976; Yogararasimhan et al., 1981; Gamble, 1995), there are few surveys that reveal the practice of herbal medicine by either tribals or indigenous communities (Bhandary et al., 1995, 1996; Harsha et al., 2002, 2003; Parinitha et al., 2004). It is apparent from these surveys that tribal and communities residing in remote places followed different practices. However, even certain local communities residing near towns and cities do follow traditional healing systems. A preliminary survey of villages around Shimoga town of Karnataka, revealed that local communities residing in three villages are still practicing herbal medicine extensively in their primary health care. These villages are located next to Bhadra wild life sanctuary. There are no previous records on ethnomedical knowledge from the study area. Hence, an attempt has been made to document plant species, medicinal formulations and treatment of particular diseases by various communities residing in this area.

2. Methodology

The study covered three villages: Nellisara, Malenahalli and Shankaraghatta located about 30 km away from Shimoga town, at an elevation of 620 m above sea level, 13° 43′ latitude and 75° 38′ longitude (Fig. 1). The study area is situated in the Western Ghats of Karnataka, which is one of the biodiversity hotspots in India. The population of the study area is about 2500, comprising various communities and castes whose major occupation is agriculture, while some are labourers. About 60% of households in these villages use locally available, wild and cultivated medicinal plants to treat common diseases. In each village, medicinal plants are used by
healers in different formulations to treat ailments, including skin diseases, stomach and kidney ailments, asthma, cough, diabetes, leprosy, jaundice and wounds.

Extensive surveys were undertaken for the period of 1998–1999 and 1999–2000 in the study area for the purpose of documenting plants used by the local communities. They were convinced of the academic significance and bona fide intention of the study through repeated contacts, explanations and interviews. They consented orally to document and publish the results of the study in the interest of the society. A previously prepared questionnaire designed by Sinha (1996) was used to collect ethnobotanical information from the herbal practitioners and knowledgeable elders in the study area, and information on the plant species and their parts used for the formulation of medicine. Information on the habitat of the plants, their local names and seasonal availability was also collected. Plants were identified with the help of published regional flora (Saldanha and Nicolson, 1976; Yogararssinhan et al., 1981; Gamble, 1995) and by comparing voucher specimens with identified herbarium collections. A set of voucher specimens has been deposited at the Department of Applied Botany, Kuvempu University. The information recorded was further ascertained or cross-checked by consulting the beneficiaries, villagers and other medicine men. The conservation status of each medicinal plant species collected was assessed using the IUCN Red list and its criteria (Nayar and Sastri, 1990; Gowda et al., 1997; Seetharam et al., 1998; Ravikumar and Ved, 2000).

3. Results and discussion

Twenty informants of Nayaka, Chelvadi, Lambani, Tamil and Muslim communities, in the age group of 30–80 years, who practiced herbal medicine in the villages of the study area, were interviewed. They had knowledge of 47 species of medicinal plants used in therapy, belonging to 46 genera in 28 families, to treat 25 diseases and disorders. Of the 47 species, 28 were collected from the wild, 11 cultivated, and 8 both collected from the wild and cultivated (25 trees, 10 shrubs, 9 herbs and 3 climbers). For the sake of convenience, 25 diseases and disorders were grouped into infectious and non-infectious diseases (Tables 1 and 2). The information gathered is arranged alphabetically by disease together with the botanical name of the plants, their families, local and common names and information on part used, method of preparation, dosage, duration, ingredients and other recommendations (Tables 1 and 2).

The present study reveals that the local medicine men of the study area have good knowledge of the medicinal property of a variety of plant species that grow around their locality. They use 20 plant species to treat nine infectious diseases and 30 species to treat 16 non-infectious diseases. Twelve species of plants belonging to 19 genera and 14 families have not been previously cited in the literature for the treatment of human diseases. Among the species of plants listed in Tables 1 and 2, five are endangered in the wild, three are vulnerable and two are of lower risk category based on the Red Book category (Nayar and Sastri, 1990; Gowda et al., 1997; Seetharam et al., 1998; Ravikumar and Ved, 2000). Most of the species used in the preparation of herbal medicine are collected fresh; very rarely, stored materials are used. Among the various plant parts used for the herbal formulations, leaves, followed by stem bark and root, were preferred over other plant parts. Upon interviews with the beneficiaries, elders and residents of the study area and neighbouring villages, they unanimously agree to the efficacy of the herbal formulation.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Plant species used to treat infectious diseases</th>
<th>Part and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholera</td>
<td><em>Oldenlandia auricularia</em> K. Schum. a (Rubiaceae) (KUSGV/055)</td>
<td>Paste of whole plant mixed with cow’s milk or water and taken orally with sugar, for 10–12 days.</td>
</tr>
<tr>
<td></td>
<td><em>Terminalia paniculata</em> Rothm. a (Combretaceae) (KUSGV/056)</td>
<td>Flowers mixed with leaves of <em>Cocculus volubilis</em> DC a (Vasanthika) (KUSGV/MH 322) plant, made into a paste and taken orally.</td>
</tr>
<tr>
<td>Chronic dysentery</td>
<td><em>Phyllanthus amarus</em> Schum. et Thomm. a (Euphorbiaceae) (KUSGV/057)</td>
<td>Leaves ground with <em>Aerva sanguinolenta</em> (Santakhdaha) (KUSGV/SG/N 327) leaves, added sugar and given orally, or tender leaves ground with cow’s milk card green orally, for 2–5 days, before food.</td>
</tr>
<tr>
<td></td>
<td><em>Mangifera indica</em> L. b (Anacardiaceae) (KUSGV/058)</td>
<td>Fruits and seeds ground and mixed with cow’s milk, taken orally for 2–3 days before food.</td>
</tr>
<tr>
<td></td>
<td><em>Jatropha curcas</em> L. c (Euphorbiaceae) (KUSGV/059)</td>
<td>Fruits and seeds ground and mixed with cow’s milk, taken orally for 2–3 days before food.</td>
</tr>
<tr>
<td></td>
<td><em>Syzygium cumini</em> Lam. c (Myrtaceae) (KUSGV/060)</td>
<td>Fruits and seeds ground and mixed with cow’s milk, taken orally for 2–3 days before food.</td>
</tr>
<tr>
<td>Cough</td>
<td><em>Ocimum sanctum</em> L. b (Lamiaceae) (KUSGV/061)</td>
<td>Leaf paste made with cow’s milk or water taken orally, two to three times a day.</td>
</tr>
<tr>
<td></td>
<td><em>Butea frondosa</em> Koenig ex Roxb. a (Papilionaceae) (KUSGV/062)</td>
<td>Gum of tree with cow’s milk taken orally, for 2–3 days.</td>
</tr>
<tr>
<td></td>
<td><em>Areca catechu</em> L. b (Arecaceae) (KUSGV/MH 063)</td>
<td>Nut paste mixed with three to four drops of honey taken orally, for 4 days.</td>
</tr>
<tr>
<td>Herpes simplex</td>
<td><em>Tamarindus indica</em> L. b (Leguminosae) (KUSGV/064)</td>
<td>Fruit paste with coconut oil and <em>Asystoma mexicana</em> leaves ground and applied locally throughout the affected part.</td>
</tr>
<tr>
<td></td>
<td><em>Holoptelea integrifolia</em> Planch. a [Vu] (Ulmaceae) (KUSGV/065)</td>
<td>Fruits and seeds ground and mixed with cow’s milk, taken orally for 2–3 days before food.</td>
</tr>
<tr>
<td>Iitching</td>
<td><em>Tapasi</em> (Holy basil)</td>
<td>Leaf paste made with cow’s milk or water taken orally, two to three times a day.</td>
</tr>
<tr>
<td></td>
<td><em>Butea frondosa</em> Koenig ex Roxb. a (Papilionaceae) (KUSGV/066)</td>
<td>Gum of tree with cow’s milk taken orally, for 2–3 days.</td>
</tr>
<tr>
<td></td>
<td><em>Leucas aspera</em> Spreng. a (Lamiaceae) (KUSGV/067)</td>
<td>Fruits and seeds ground and mixed with cow’s milk, taken orally for 2–3 days before food.</td>
</tr>
<tr>
<td></td>
<td><em>Momordica charantia</em> L. b (Cucurbitaceae) (KUSGV/068)</td>
<td>Fruits and seeds ground and mixed with cow’s milk, taken orally for 2–3 days before food.</td>
</tr>
<tr>
<td></td>
<td><em>Butea frondosa</em> Koenig ex Roxb. (Leguminosae) (KUSGV/069)</td>
<td>Fruits and seeds ground and mixed with cow’s milk, taken orally for 2–3 days before food.</td>
</tr>
<tr>
<td>Skin diseases, dishi rich and ringsworm</td>
<td><em>Leucas aspera</em> Spreng. a (Lamiaceae) (KUSGV/070)</td>
<td>Leaf paste applied over the skin, for 5 days.</td>
</tr>
<tr>
<td></td>
<td><em>Momordica charantia</em> L. b (Cucurbitaceae) (KUSGV/071)</td>
<td>Leaf paste applied over the skin, for 5 days.</td>
</tr>
<tr>
<td></td>
<td><em>Butea frondosa</em> Koenig ex Roxb. (Leguminosae) (KUSGV/072)</td>
<td>Leaf paste applied over the skin, for 5 days.</td>
</tr>
</tbody>
</table>

[EW]: Endangered in wild; [Vu]: Vulnerable.

 a Wild
 b Cultivated
 c Wild as well as cultivated
Table 2

<table>
<thead>
<tr>
<th>Disease</th>
<th>Botanical name and family</th>
<th>Local (common) name</th>
<th>Part and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>Terminalia bellerica Roxb. a [Vu] (Combretaceae) (KU/BS/KH 042)</td>
<td>Tara (Beleric myrobalans)</td>
<td>Macerated fruit taken orally with honey, for 2–3 days.</td>
</tr>
<tr>
<td></td>
<td>Tylophora asthmatica Wight et Arn. a (Verbenaceae) (KU/BS/BS 012)</td>
<td>Pitta mat (Indian ipecacuanha)</td>
<td>Leaf decoction mixed with two to three drops of honey taken orally, for 5 days in the morning. Root decoction is taken orally with garlic and cow’s milk, two to four times a day.</td>
</tr>
<tr>
<td></td>
<td>Hilsorina tonomyrtalisDonal. a [EW] (Solanaceae) (KU/SG/NS 071)</td>
<td>Ashwagandha (Winter cherry)</td>
<td>Dried fruits pulverized and taken with water.</td>
</tr>
<tr>
<td></td>
<td>Ficus religiosa L. a (Moraceae) (KU/SG/SV 072)</td>
<td>Arali (Sacred fig)</td>
<td></td>
</tr>
<tr>
<td>Boils, bums and sores</td>
<td>Diospyros montana Roxb. a [LR] (Ebenaceae) (KU/SG/NS 073)</td>
<td>Jagalaganti (Mountain persimmon)</td>
<td>Fruit made into a powder and applied on burnt parts.</td>
</tr>
<tr>
<td></td>
<td>Bombax malabaricum DC. a (Bombacaceae) (KU/SG/NS 074)</td>
<td>Booruga (Silk cotton tree)</td>
<td></td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Plumbago zeylanica L. (Plumbaginaceae) (KU/BS/MA 023)</td>
<td>Chitramula (White lead wort)</td>
<td>Roots ground with lime juice and applied over the part with symptom, three to five times a day.</td>
</tr>
<tr>
<td>Cataract</td>
<td>Breynia rhamnoides Moul.-Arg. a (Euphorbiaceae) (KU/BS/V 075)</td>
<td>Hullkadi (Tiktaar)</td>
<td>Root exudate collected in the morning and dropped carefully into eyes, two to three times a day. Medicine men need to take care not to touch the exudate.</td>
</tr>
<tr>
<td>Dog bite</td>
<td>Acalypa indica L. a (Euphorbiaceae) (KU/SG/NS 076)</td>
<td>Kuppi (Indian acalypha)</td>
<td>Leaf paste with a little lime applied to bitten area two times a day, for 3–4 days.</td>
</tr>
<tr>
<td></td>
<td>Ricinus communis L. a (Euphorbiaceae) (KU/SG/NS 076)</td>
<td>Harala (Castor)</td>
<td>Leaf paste applied over bitten area for 5 days and a small quantity of paste taken orally with food.</td>
</tr>
<tr>
<td></td>
<td>Acerula indica A. Juss. (Meliaceae) (KU/SG/NS 077)</td>
<td>Buevo (Neem)</td>
<td>Leaf paste of this and of Butea monosperma DC. a (Bombacaceae) (KU/BS/V 072) applied over the bitten area, for 3–4 days. Advised not to use cow’s milk or curd.</td>
</tr>
<tr>
<td>Fever</td>
<td>Ruta graveolens L. a (Rutaceae) (KU/BS/MA 078)</td>
<td>Naagdali (Garden rue)</td>
<td>Leaf paste with honey or cow’s milk taken orally, two times a day.</td>
</tr>
<tr>
<td></td>
<td>Adhatoda vasica Nees. c (Acanthaceae) (KU/SG/JS 223)</td>
<td>Chritamula (White lead wort)</td>
<td>Leaf paste mixed with black pepper powder (Piper nigrum L.) (KU/BS/SM 053) made into pills taken orally, two to three times daily.</td>
</tr>
<tr>
<td>Hair fall (severe)</td>
<td>Vitex trifolia L. a (Verbenaceae) (KU/SG/NS 079)</td>
<td>Nira lakki (Indian wild pepper)</td>
<td>Leaf paste blended with coconut oil and applied to hair and scalp.</td>
</tr>
<tr>
<td>Infertility</td>
<td>Careya arborea Roxb. a (Lecythidaceae) (KU/BS/SM 036)</td>
<td>Kavalu (Kumbhi)</td>
<td>Flower paste prepared with fruits of Emblica officinalis (KU/SG/NS 223), Terminalia chebula Retz. a [Vu], and macerated with ghee, taken orally in empty stomach for 4 days. Advised not to use salt.</td>
</tr>
<tr>
<td>Jaundice</td>
<td>Telopatea arbutifolia L. a (Verbenaceae) (KU/BS/SU 042)</td>
<td>Pitta maa (Indian ipecacuanha)</td>
<td>Roots with black pepper (Piper nigrum L.) (KU/BS/SM 053), garlic and fruits of Butea monosperma DC. a (Bombacaceae) (KU/BS/V 072), Terminalia chebula Retz. a (Verbenaceae) (KU/BS/NS 223), Terminalia chebula Retz. a (Verbenaceae) (KU/BS/NS 223).</td>
</tr>
</tbody>
</table>
suggested by the local herbalists. They also point out that allopathic medicines, which are available in the nearby towns, are expensive and have side effects in comparison to the herbal medicine. This might indicate the reason for the dependence of local residents on herbal medicine rather than allopathic medicine.

The findings of the present study are in conformity with study published by Nadakarni (1976) in the treatment of certain diseases with specific medicinal plants. For example, plant species recommended for the treatment of asthma, cholera, cough, dysentery, jaundice, leprosy and snakebite are essentially the same species, although the plant parts differed. However, there are certain examples of other plant species, which are used exclusively for the treatment of specific diseases in the study area and represent the first time these are used for such diseases. For example, Breynia rhamnoides is used to treat cataract, leaf paste of Ricinus communis to treat dog bite, flower of Careya arborea to treat infertility, and the bark of Xylica xylocarpa for leprosy. It is interesting to note that decoction of various parts of Holoptelea integrifolia, Jatropha curcas, Clerodendrum inerme and Azadirachta indica are used to treat malaria.

The present investigation points out that often, more than one plant belonging to different taxonomic groups are being used to treat a specific disease or disorder. A proper scientific understanding of the pharmacological effect of herbal drugs is necessary for effective therapy of diseases.

Several cases of indirect evidence on the pharmacological effects of certain medicinal herbs (Aristolochia trifolata, Artemisia absinthium, Centella asiatica, Lignea aspera and Plumbago cyanus) have been documented in the literature (Samy and Ignacimuthu, 2000; Quinlan et al., 2002; Somchit et al., 2003 and Nessa et al., 2004). Rao (2000) provides a list of herbs that have various medicinal properties in his book on database of medicinal plants. Certain herbal drugs listed in the ayurveda and other traditional medicine systems are not only time-tested but have also been screened for their pharmacological properties (Dev, 1997, 1999). For example, guggul, the gum resin from Commiphora wightii, has been used to treat rheumatoid arthritis and lipid disorders in addition to other diseases. It has been shown that two antihyperlipoproteinemic compounds, Z-guggulsterone and E-guggulsterone extracted from the gum resin, have hypolipidemic activity similar to that of the synthetic drug, clofibrate (Dev, 1999). The presence of resveratrol and pterostilbene in darakchasava, an ayurvedic medicine whose principal component being grapes, has been shown to reduce cardiac disease rate and carcinogenesis (Paul et al., 1999).

Certain Chinese traditional herbal drugs used to retard ageing and to treat several other diseases have been found to

Table 2 (Continued)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plant Parts</th>
<th>Medicinal Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parkinson's</td>
<td>Pudina (Peppermint)</td>
<td>Plant parts of M. arvensis and seeds of Trachyspermum ammi (L.) Sprague. * (Carum) in equal proportions along with rock salt taken with coffee, three to four times a day.</td>
</tr>
<tr>
<td>Piles</td>
<td>Careya arborea Roxb. (Lecythidaceae) (KUB/SV/LV 086)</td>
<td>Kavalu (Kumbri)</td>
</tr>
<tr>
<td>Snakebite</td>
<td>Acacia indica L. (Euphorbiaceae) (KU/SV/GH 075)</td>
<td>Koppu (Indian acalypha)</td>
</tr>
<tr>
<td></td>
<td>Tephrosia urvilleana Wright et Arn. (Asclepiadaceae) (KU/SV/KH 042)</td>
<td>Pita maa (Indian souchambuli)</td>
</tr>
<tr>
<td></td>
<td>Careya arborea Roxb. ex Kar. * [LR] (Lecythidaceae) (KU/BS/LV 036)</td>
<td>Kaurac (Kurta)</td>
</tr>
<tr>
<td></td>
<td>Careya arborea Roxb. ex Kar. * [LR] (Lecythidaceae) (KU/BS/LV 036)</td>
<td>Ekkuk (Safed Ak)</td>
</tr>
<tr>
<td>Scorpion sting</td>
<td>Careya arborea Roxb. (Lecythidaceae) (KUB/SV/LV 036)</td>
<td>Kavalu (Kumbri)</td>
</tr>
<tr>
<td>Stomach ache</td>
<td>Azadirachta indica A. Juss. (Meliaceae) (KU/SV/MO 067)</td>
<td>Baerv (Neem)</td>
</tr>
<tr>
<td></td>
<td>Homodendron indicum R. Br. * [LR] (Asclepiadaceae) (KUB/SV/HR 001)</td>
<td>Squajana bera (Indian souchambuli)</td>
</tr>
<tr>
<td>Mouth ulcer</td>
<td>Anogeissus shootii Correa ex Roxb. * [Vul] (Rutaceae) (KU/SV/GV 085)</td>
<td>Billa patte (Bael tree)</td>
</tr>
<tr>
<td></td>
<td>Wrightia tinctoria R. Br. * (Apocynaceae) (KUB/SV/GV 086)</td>
<td>Belpalle (Sweet indraj)</td>
</tr>
</tbody>
</table>


* Wild.

b Cultivated.
c Wild as well as cultivated.
contain considerably high amounts of melatonin, which is related to the scavenging of free radicals (Chen et al., 2003).

The present results also suggest that different groups of people practice herbal medicine differently, and their formulations might not resemble that of herbal medicine of people residing either in the far-off places or nearby places. For example, ‘Siddis’ and ‘Gowils’ of Uttar Kannada in Karnataka used entirely different types of plant species for the treatment of a variety of human diseases. Bhandary et al. (1995) reported that ‘Siddis’ used bank of Careya arborea to treat dysentery and ear pain. On the other hand, local communities of the study area used flowers of the above plant for treating infertility, piles and scorpion sting. ‘Gowils’ of Uttar Kannada used Rauvolfia serpentina to treat herpes infection (Bhandary et al., 1996), while people of the study area for revealing their traditional medico-botanical knowledge to a wider audience for the benefit of everyone.

Acknowledgements

The authors express thanks to the herbal doctors in the study area for revealing their traditional medicos-botanical knowledge and for their permission to communicate their knowledge to a wider audience for the benefit of everyone. Thanks are also expressed to Prof. M. Krishnappa, Department of Post Graduate Studies and Research in Applied Botany, Kuvempu University for the co-operation extended during the study.

References


The ameliorative effect of dates (Phoenix dactylifera L.) on ethanol-induced gastric ulcer in rats


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Abstract

The present work aimed at testing, in a rat model of ethanol-induced gastric ulceration, a local folk medicinal claim that dates are beneficial in gastric ulcers in humans. Aqueous and ethanolic undialyzed and dialyzed extracts from date fruit and pits were given orally to rats at a dose of 4 ml/kg for 14 consecutive days. On the last day of treatment, rats were fasted for 24 h, and were then given ethanol, 80% (1 ml/rat) by gastric intubation to induce gastric ulcer. Rats were killed after 1 h of ethanol exposure, and the incidence and severity of the ulceration were estimated, as well as the concentrations of gastrin in plasma, and histamine and mucus in the gastric mucosa. A single group of rats that were fasted for 24 h, was administered orally with lansoprazole (30 mg/kg), and was given 80% ethanol as above, 8 h thereafter, served as a positive control.

The results indicated that the aqueous and ethanolic extracts of the date fruit and, to a lesser extent, date pits, were effective in ameliorating the severity of gastric ulceration and mitigating the ethanol-induced increase in histamine and gastrin concentrations, and the decrease in mucus gastric levels. The ethanolic undialyzed extract was more effective than the rest of the other extracts used. It is postulated that the basis of the gastroprotective action of date extracts may be multi-factorial, and may include an anti-oxidant action.

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Keywords: Dates; Ethanol-induced ulcer; Gastric histamine; Gastrin; Lansoprazole; Mucin; Phoenix dactylifera L.; Palmae

1. Introduction

Date palms (Phoenix dactylifera L., Palmae) have been cultivated in the Middle East over at least 6000 years ago (Copley et al., 2001). For the natives in this region, dates are considered a staple carbohydrate food (Al-Shahib and Marshall, 2003). Date fruits are also used in the production of local beverages and spirits. In local medicinal practices dates are considered a “tonic” and “aphrodisiac”, and in some communities they are thought to be useful against ulcer (Rasheed, personal communication). In fact, Muslims believe that “He who eats seven dates every morning will not be affected by poison or magic on the day he eats them” (cited by Miller et al., 2003).

The pollen grains of date palm have been used in Egyptian local practices to improve fertility in women, and in some locations in Arabia date pits are roasted and used in lieu of coffee as a hot beverage.

Relatively few pharmacological studies have been conducted on dates. For example, it has been shown that, depending on the type of extract used, date fruit and pit extracts significantly increase or decrease gastrointestinal transit (GIT) in mice (Al-Qarawi et al., 2003), and that date fruit extract has strong antioxidant and antimutagenic properties (Vayali, 2002). Date palm kernels have been shown to exhibit antiaging properties and significant reduction in skin wrinkles in women (Bauza, 2002), and natural fats from date palm has been reported to prevent irritant contact dermatitis (Schliemann-Willers et al., 2002). In animals, the pits have been included in the diet of chickens, sheep, fish and rats, and have been shown to enhance...
growth in these species (see Ali et al., 1999 and references therein).

In view of the wide consumption of dates in our region, the fact that dates are anecdotally reputed to be useful against peptic ulcers, and the fact that Muslims customarily consume more of the dates during the fasting month of Ramadan, possibly to protect the gastric mucosa from the damaging effect of gastric acid, and because of the scarcity of information on the pharmacological properties of date fruits and pits, we considered undertaking this study to assess the influence of date extracts on the incidence and severity of ethanol-induced gastric ulceration. In addition, the effect of date extracts on the gastric concentrations of histamine and mucin, and the plasma concentration of the hormone gastrin has also been investigated.

2. Materials and methods

2.1. Animals

Fifty-four adult male Wistar rats weighing between 200 and 250 g were used in this work. They were obtained for the Animal House of King Saud University in Riyadh, and were divided into eight equal groups. The animals were kept at a controlled temperature of 23±2 °C, relative humidity of 65–80% and a light regime of 12 h light:12 h dark (lights on at 6:00). Except otherwise mentioned, pelleted Purina chow and water were provided to the rats ad libitum.

2.2. Plant material

Fresh fruits of Sukari dates (P. dactylifera L.) were obtained from a local date manufacturing factory. Samples of these dates were kept frozen for future reference.

2.3. Plant preparation and administration

The date fruits were manually separated from the pits and the latter were washed clear of any fruit, dried at room temperature and ground into powder using a stainless-steel blender. The water extract of the date fruit was made by adding distilled water to coarsely pounded date fruit (3:1), and leaving for 48 h in a refrigerator (4 °C) with continuous stirring. The aqueous extract was then used daily for 14 consecutive days. To remove sugars from the extract, the aqueous extract was dialyzed. Dialysis was carried out under running tap water for 24 h. The dialyzed water extract was kept refrigerated and used daily for 14 consecutive days.

A soxhlet apparatus was used to obtain an ethanol extract. The ethanol was then evaporated and the residue diluted with water to give the required concentration. The powdered date fruit or pits were added (1:3) to either ethanol or distilled water. Extraction was carried out at 4 °C with continuous stirring. The ethanol extracts were then concentrated to dryness and the residue dissolved in distilled water to the appropriate doses just prior to use.

Dialysis to remove sugars was performed using cellulose tubing (Spectra/Por, width 32 mm, diameter 20.4 mm, volume/length 303 ml, from Spectrum Medical Instruments, Inc., USA).

2.4. Experimental design

Rats were randomly assigned to the following experimental groups:

- **Group 1**: Rats were given distilled water (4 ml/kg) orally for 14 consecutive days. On the last day rats were given normal saline (1 ml) 1 h before killing.
- **Group 2**: Rats were given distilled water (4 ml/kg) orally for 14 consecutive days. On the last day rats were given 80% ethanol (1 ml) 1 h before killing.
- **Group 3**: Rats were given the aqueous date fruit extract (4 ml/kg) for 14 consecutive days. On the last day rats were given 80% ethanol (1 ml) 1 h before killing.
- **Group 4**: Rats were given the dialyzed aqueous date fruit extract (4 ml/kg) for 14 consecutive days. On the last day rats were given 80% ethanol (1 ml) 1 h before killing.
- **Group 5**: Rats were given the ethanolic date fruit extract (4 ml/kg) for 14 consecutive days. On the last day rats were given 80% ethanol (1 ml) 1 h before killing.
- **Group 6**: Rats were given the aqueous date pit extract (4 ml/kg) for 14 consecutive days. On the last day rats were given 80% ethanol (1 ml) 1 h before killing.
- **Group 7**: Rats were given the dialyzed date pit extract (4 ml/kg) for 14 consecutive days. On the last day rats were given 80% ethanol (1 ml) 1 h before killing.
- **Group 8**: Rats were given the ethanolic date pit extract (4 ml/kg) for 14 consecutive days.
- **Group 9**: Rats were given a single oral dose of lansoprazole (30 mg/kg), and 8 h later was given 80% ethanol as above, 1 h before killing.

2.5. Ethanol-induced gastric lesion

Rats were deprived from food (but not water) on day 14 of the experiment. On the last day of experiment (day 15) rats were given 80% ethanol (1 ml) by gastric intubation 1 h before killing, except for rats in group 1 which were given normal saline (1 ml/rat), and group 9 (positive control) which consisted of six rats that were fasted for 24 h, administered orally with lansoprazole (Sigma, MO, USA) (30 mg/kg), and was given 80% ethanol as above, 8 h thereafter.

The animals were anesthetized with ether, and rapidly decapitated 1 h after ethanol treatment. Blood was collected in heparinized tubes and centrifuged at 900 × g for 15 min at 5 °C. The plasma obtained was stored at −20 °C pending gastrin assay. The stomach of each animal was excised and opened along the greater curvature. After washing with
normal saline and removal of blood, the gastric lesion was quantified using a stereo-microscope. Gross mucosal damage was assessed in a “blinded” manner by calculation of a lesion index based on the number and severity factor of lesions as described previously (Agrawal et al., 2000). The stomachs were inflated with 10 ml of 1% formalin for 10 min to fix the inner walls. The average number of ulcers per stomach was recorded. The lesion index was calculated as the total number of lesions added to their respective severity factor.

2.6. Measurement of the mucin content in the gastric wall

Gastric mucus was quantitatively measured as described by Corne et al. (1974). The stomachs were removed and were soaked in 0.1% Alcian blue solution for 2 h. The uncomplexed dye was removed by two successive washes at 15 and 45 min in 0.25 M aqueous sucrose solution. Dye complexes with gastric wall mucous were extracted by immersion in 10 ml of 0.5 M MgCl$_2$ for 2 h. The resulting blue solution was shaken with equal volumes of diethyl ether and the optical density of the aqueous phase was measured at 605 nm by a UV–visible spectrophotometer (B&L 2000). The quantity of mucin was expressed as μg of Alcian blue extracted per weight (g) of stomach.

2.7. Gastrin measurement

The gastrin hormone was assayed in the collected plasma samples using a radioimmunoassay (RIA) technique according to the procedure described by the manufacturer (IBL- Hamburg, Germany, cat. No: MI 131 01).

2.8. Histamine estimation

Histamine in the gastric mucosa was separated by thin layer chromatography according to the modified method of Shalaby (1994). Histamine dihydrochloride (Sigma, MO, USA) was used as standard for identification of the spots in the plates and for spectrophotometric determination (Spectronic 2000, Bausch and Lomb at 570 nm) of samples after elution from the plates. Values were expressed as μg/g of stomach weight.

2.9. Histopathological examination

Gastric tissue samples were fixed in 10% neutral formalin and processed for routine paraffin blocking and H&E staining. These were “blindly” examined under the microscope for histopathological change such as congestion, oedema, erosions, ulcerations and necrosis. The severity of histopathological changes was expressed according to an arbitrary scale (between − to +++ + ).

2.10. Statistical analysis

Values reported are expressed as mean ± S.E.M. The significance of differences between the control and ethanol and/or date-treated groups was tested by least squares analysis of variance using the general linear models (GLM) procedures of the statistical analysis system (SAS, 1996).

3. Results

The results of this work are shown in Tables 1–3. Table 1 shows the effect of treatment with various date extracts on the number, severity and ulcer index. The ethanolic and, to a lesser extent, the aqueous date fruit extract ameliorated the severity of gastric ulceration. The effect of the date pit extract was less evident. The positive control lansoprazole significantly reduced the lesion index.

Table 2 summarizes the histological changes seen after treatment with ethanol and date extracts. Ethanol treatment induced severe necrosis, haemorrhage, congestion and oedema in stomach sections. These actions were markedly ameliorated by lansoprazole, and to a lesser extent by pretreatment with date fruits and pits. The ethanolic undialyzed date fruit extract was more active in this regard than the other extracts tested.

The concentrations of gastrin in the plasma, and histamine and mucin in the gastric mucosa in control and treated rats with date extracts and lansoprazole are shown in Table 3. Ethanol treatment induced a significant increase in the concentrations of gastrin and histamine, and significantly decreased that of mucin (P < 0.05). These effects were significantly antagonized by the pre-treatment of rats with lansoprazole and by date fruit aqueous and ethanolic extracts (P < 0.05). Date pits extracts were not significantly effective in antagonizing these effects.

4. Discussion

Rat gastric mucosal damage induced by high concentrations of ethanol has widely been used to investigate gastroprotective effect of medicinal plants (e.g. Zhu et al., 1997). The present results suggest that pretreatment with date fruit (and to lesser extent pit) ethanol and aqueous extracts for 14 days markedly ameliorated the ulcer index, and some histological and biochemical indices of ethanol-induced gastric ulceration in rats. This lends support to the local folk medicinal claim that dates may be useful to humans with ulcers.

In this work we selected lansoprazole as a reference anti-ulcer drug (rather than a histamine antagonist) because it has been shown that prostaglandins provide a much better anti-ulcer effect on ethanol-induced gastric damage (Cho and Ogle, 1992). Treatment with ethanol induced the expected actions in the stomach of rats, and those included severe histological damages (necrosis, haemorrhages), and a signi-
Effect of date fruit and pits extracts on ethanol-induced gastric ulcers in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of ulcers/stomach (x)</th>
<th>Severity per stomach (y)</th>
<th>Lesion index (x + y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>distilled water + saline</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 2</td>
<td>distilled water + ethanol</td>
<td>$8.1 \pm 0.9^d$</td>
<td>$8.0 \pm 0.9^d$</td>
</tr>
<tr>
<td>Group 3</td>
<td>date fruit aqueous extract + ethanol</td>
<td>$4.2 \pm 0.5^b$</td>
<td>$5.4 \pm 0.4^d$</td>
</tr>
<tr>
<td>Group 4</td>
<td>dialyzed date fruit aqueous extract + ethanol</td>
<td>$6.1 \pm 0.5^a$</td>
<td>$6.1 \pm 0.5^a$</td>
</tr>
<tr>
<td>Group 5</td>
<td>date fruit ethanolic extract + ethanol</td>
<td>$3.1 \pm 0.3^c$</td>
<td>$4.2 \pm 0.4^c$</td>
</tr>
<tr>
<td>Group 6</td>
<td>date pits aqueous extract + ethanol</td>
<td>$7.0 \pm 0.4^d$</td>
<td>$6.1 \pm 0.4^d$</td>
</tr>
<tr>
<td>Group 7</td>
<td>dialyzed date pits aqueous extract + ethanol</td>
<td>$6.6 \pm 0.5^c$</td>
<td>$5.4 \pm 0.3^c$</td>
</tr>
<tr>
<td>Group 8</td>
<td>date pits ethanolic extract + ethanol</td>
<td>$6.0 \pm 0.5^d$</td>
<td>$4.9 \pm 0.5^d$</td>
</tr>
<tr>
<td>Group 9</td>
<td>lansoprazole + ethanol</td>
<td>$1.6 \pm 0.3^d$</td>
<td>$0.9 \pm 0.1^d$</td>
</tr>
</tbody>
</table>

Values (means ± S.E.M. from six rats), with different superscripts are significantly different ($P < 0.05$) from the values in the same column. Distilled water (4 ml/kg) or various date extracts were given daily as an oral dose of 4 ml/kg orally for 14 consecutive days; 24 h after the last day of treatment, rats were given 80% ethanol (1 ml) per os 1 h before killing. A single group of rats that were fasted for 24 h, was administered orally with lansoprazole (50 mg/kg), and was given 80% ethanol as above, 8 h thereafter, served as a positive control.

Table 2: Histopathological evaluations of the effects of date aqueous and ethanolic extracts on the induction of gastric lesions in the rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Necrosis</th>
<th>Haemorrhage</th>
<th>Congestion</th>
<th>Oedema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>distilled water + saline</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 2</td>
<td>distilled water + ethanol</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Group 3</td>
<td>date fruit aqueous extract + ethanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 4</td>
<td>dialyzed date fruit aqueous extract + ethanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 5</td>
<td>date fruit ethanolic extract + ethanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 6</td>
<td>date pits aqueous extract + ethanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 7</td>
<td>dialyzed date pits aqueous extract + ethanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Group 8</td>
<td>date pits ethanolic extract + ethanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Group 9</td>
<td>lansoprazole + ethanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Values (means ± S.E.M. from six rats), with different superscripts are significantly different ($P < 0.05$) from the values in the same column. Distilled water (4 ml/kg) or various date extracts were given daily as an oral dose of 4 ml/kg orally for 14 consecutive days; 24 h after the last day of treatment, rats were given 80% ethanol (1 ml) per os 1 h before killing. A single group of rats that were fasted for 24 h, was administered orally with lansoprazole (50 mg/kg), and was given 80% ethanol as above, 8 h thereafter, served as a positive control.

Table 3: Effect of treatment of rats with date fruit and pits extracts on plasma gastrin, gastric histamine and gastric juice mucin activity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Gastrin (pg/ml plasma)</th>
<th>Histamine mg/g stomach weight</th>
<th>Mucin mg/g stomach weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>distilled water + saline</td>
<td>$15.2 \pm 1.9^c$</td>
<td>$22.0 \pm 1.5^c$</td>
</tr>
<tr>
<td>Group 2</td>
<td>distilled water + ethanol</td>
<td>$125.2 \pm 2.6^d$</td>
<td>$298.3 \pm 18.2^d$</td>
</tr>
<tr>
<td>Group 3</td>
<td>date fruit aqueous extract + ethanol</td>
<td>$107.7 \pm 12.3^d$</td>
<td>$217.3 \pm 19.6^d$</td>
</tr>
<tr>
<td>Group 4</td>
<td>dialyzed date fruit aqueous extract + ethanol</td>
<td>$118.5 \pm 13.2^d$</td>
<td>$230.2 \pm 24.9^d$</td>
</tr>
<tr>
<td>Group 5</td>
<td>date fruit ethanolic extract + ethanol</td>
<td>$98.5 \pm 10.1^d$</td>
<td>$211.2 \pm 3.1^d$</td>
</tr>
<tr>
<td>Group 6</td>
<td>date pits aqueous extract + ethanol</td>
<td>$165.6 \pm 12.3^d$</td>
<td>$228.4 \pm 19.9^d$</td>
</tr>
<tr>
<td>Group 7</td>
<td>dialyzed date pits aqueous extract + ethanol</td>
<td>$113.8 \pm 13.1^d$</td>
<td>$225.8 \pm 17.4^d$</td>
</tr>
<tr>
<td>Group 8</td>
<td>date pits ethanolic extract + ethanol</td>
<td>$107.7 \pm 11.9^d$</td>
<td>$205.5 \pm 20.2^d$</td>
</tr>
<tr>
<td>Group 9</td>
<td>lansoprazole + ethanol</td>
<td>$34.3 \pm 4.3^d$</td>
<td>$205.5 \pm 20.2^d$</td>
</tr>
</tbody>
</table>

Values (means ± S.E.M. from six rats), with different superscripts are significantly different ($P < 0.05$) from the values in the same column. Distilled water (4 ml/kg) or various date extracts were given daily as an oral dose of 4 ml/kg orally for 14 consecutive days; 24 h after the last day of treatment, rats were given 80% ethanol (1 ml) 1 h before killing. A single group of rats that were fasted for 24 h, was administered orally with lansoprazole (50 mg/kg), and was given 80% ethanol as above, 8 h thereafter, served as a positive control.

significant increase in plasma concentrations of the gastric hormone gastrin, reduction in mucin and an increase in the histamine concentrations in the gastric mucosa. These parameters were selected for study because of their relevance to the pathogenesis of gastric ulceration. For example, gastrin is a gastrointestinal hormone that, among other various functions, regulates gastric acid secretion, releases histamine, and regulates gastric endocrine cell proliferation (Walsh, 1993). Stimulation of the oxyntic cells by histamine is the final common pathway by which neural and endocrine mechanisms act in inducing acid secretion. Histamine is involved in a cycle of events leading to the production of arteriolar vasodilation in injured tissues (Black, 1993). Gastric mucus (mucin) is an important protective factor for the gastric mucosa and consists of a viscous, elastic, adherent and transparent gel formed by 95% water and 5% glycoproteins that cover the entire gastrointestinal mucosa. Moreover, mucus is capable of acting as an antioxidant, and thus can reduce mucosal damage mediated by oxygen free radicals (Repetto and Llesuy, 2002).
effects on GIT transit (Al-Qarawi et al., 2003). In the present work, non-dialyzed extracts seemed to be more active as gastroprotectants than dialyzed extracts.

Ethanol-induced gastric ulceration is known to be related to an anti-oxidant action, increased lipid peroxidation and generation of free-radicals (Terano et al., 1989). Recently Vayallil (2002) discovered that date extracts possess significant anti-inflammatory action in vitro. This may, at least partially, be one of the possible mechanisms by which date extracts have ameliorated the ethanol-induced gastric ulceration. Recently we have found that dates contain relatively high concentrations of the anti-oxidants melatonin and vitamin E (Al-Qarawi et al., unpublished data). It has been reported that treatment with melatonin prevents gastric ulcerogenesis and decreases ulcer index (Bandypadhyay et al., 2001; Bubnesh, 2002). Vitamin E in palm oil has also been shown to reduce ethanol-induced gastric ulcer (Jarrin et al., 1999). Taken together, these results corroborate our present finding of an ameliorative action of dates on ethanol-induced gastric ulceration, possibly due to its relatively high content of anti-oxidant substances.

In conclusion, the present work has suggested that date extracts can ameliorate ethanol-induced gastric ulcers, and that, in view of the fact that ethanol induces ulceration by an anti-inflammatory action, and that dates contain relatively high amounts of anti-oxidant substances, it is possible that the mechanism of the gastroprotective action is via an anti-inflammatory action, however, other mechanisms cannot be excluded. Pending further pharmacological and toxicological studies to delineate the mechanism(s) of action of dates as gastro-protective agent, and their toxic effects, compounds from dates may potentially be useful in ulcers.

Acknowledgements

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References


Tanshinone inhibits intimal hyperplasia in the ligated carotid artery in mice

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Abstract

Vascular smooth muscle cell (VSMC) proliferation is considered to play a central role in the development of intimal hyperplasia with pathological artery healing. Danshen, the Salvia miltiorrhiza Bge., has long been regarded as an effective traditional Chinese medicine for cardiovascular diseases. In this paper, the effects of tanshinone (TA), the lipid-soluble pharmacological constituents of danshen, on the intima hyperplasia and proliferating state of VSMC were described in a mouse carotid artery injured by complete cessation of blood flow. This study showed that oral administration of TA could significantly decrease the intimal thickening of injured vessels and proliferating cell nuclear antigen (PCNA)-positive VSMC in intimal area. These results suggested that the suppressive effects of TA on intimal hyperplasia might partly result from its inhibitory effect against VSMC proliferation.

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Keywords: Tanshinone; Restenosis; Proliferation of smooth muscle cell; Intimal hyperplasia

Percutaneous transluminal coronary angioplasty (PTCA) has revolutionized the treatment of atherosclerosis coronary arterial stenosis, but the long-term success of angioplasty remains limited by the high occurrence of restenosis. From a number of clinical and experimental studies that used intracoronary ultrasound and/or histological evaluation of tissues, it is clear that there are three major mechanisms responsible for restenosis: intimal hyperplasia, elastic recoil, and vessel wall remodeling (Safian et al., 1990; Post et al., 1994; Mintz et al., 1996). Vessel wall remodeling and elastic recoil can be effectively counteracted by permanently fixing the vessel size by the placement of an intracoronary stent (Gordon et al., 1993; Painter et al., 1995). However, restenosis due to intimal hyperplasia and production of extracellular matrix material within the neointima still occurs in a significant percentage of patients, more importantly after intracoronary stenting (Gordon et al., 1993; Painter et al., 1995). Intimal hyperplasia is a process of vascular smooth muscle cell (VSMC) proliferation, migration, and transition to a secretary phenotype that is induced by multiple growth stimuli released during the arterial injury (Cassells et al., 1994). Therapeutic interventions that inhibit the proliferation of VSMC may therefore be beneficial in the prevention of restenosis.

Danshen is a well-known traditional Chinese medicine used for the treatment of cardiovascular diseases. Recent studies have showed that danshen significantly inhibited intimal hyperplasia and attenuated restenosis (Zhang et al., 1998; Chen et al., 2001). Tanshinone (TA) is the major lipid-soluble pharmacological constituent of danshen. Our previous study demonstrated that TA-IAA, one of the main components of TA, had dose-dependent inhibition on the basic fibroblast growth factor (bFGF)-induced human Smooth muscle cell (SMC) proliferation in vitro (Du, 1999). The purpose of this paper is to investigate the effect of TA on intimal hyperplasia in vivo. Therefore, we used a mouse model with carotid artery ligation, in which the lumen narrowing occurred by the formation of an extensive SMC-rich neointima and a re-
duction in vessel diameter (Kumar and Lindner, 1997). The inhibition of TA on intimal hyperplasia and proliferating state of SMC was observed.

1. Materials and methods

1.1. Mouse intimal hyperplasia model

All animal studies were approved by the Institutional Animal Care and Use Committee in China. Thirty-six female KM mice (7–8 weeks old, West China animal center of Sichuan University) weighing 20–22 g were used in all experiments. The animals were randomly divided into three groups: model control, TA 0.3 and 0.6 g/kg BW/d groups. Each group had 12 mice. The controlateral carotid artery of ligated vessel of model control group was regarded as normal control group. The mouse intimal hyperplasia model was set up as described (Kumar and Lindner, 1997). In brief, mice were anesthetized by intraperitoneal injection of a solution of xylazine (5 mg/kg body weight) and ketamine (80 mg/kg body weight). The left carotid arteries of the mice were dissected and ligated near the carotid bifurcation, leading to intimal hyperplasia and thus establishing restenosis model. All animals recovered and showed no symptoms of a stroke. 90.5% purity of TA (w/w) (Hang Zhou Tian Nong Material Extract Company, Hang Zhou, China) was dispersed in 0.5% sodium carboxymethyl cellulose, and orally administered 2 days before ligation of vessel and continued until the mice were killed 4 weeks after operation. The control animals were administrated the equal volume of the solvent.

1.2. Histopathological staining and morphometry

After a 4-week treatment, the mice were anesthetized again and fixed for 3 min by perfusion with 4% p-formaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.3). After excision of the left and right carotid arteries, all specimens were immersion fixed in 10% formalin and then embedded in paraffin. Serial sections (5 μm thick) were cut for hematoxylin–eosin staining. The morphometric analysis of vessels was carried out by the observation under light microscope, and the digitized images were analyzed with graphic analysis software (Nikon & Spot, USA). The circumferences (lengths) of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were determined by tracing along the luminal surface, IEL, and EEL. Under the assumption that the structures were circular, these measurements were used to calculate intimal area, medial area, intima-to-media ratio and relative lumen area. The medial area was calculated by subtracting the area defined by the IEL from the area defined by the EEL, and intimal area determined by subtracting the lumen area from the area defined by the IEL. Intima-to-media ratio was the ratio of vessel intimal area to medial area. Relative lumen area is the percentage of lumen area to the sum of lumen and vessel wall area.

1.3. Immunohistochemical staining

PCNA-positive VSMC was identified by staining with a mouse monoclonal antibody (Mab, DAKO, Carpinteria, CA). The avidin–biotin–peroxidase complex method was used for the immunostaining of PCNA as previously reported (Lehr et al., 1995). In brief, after dewaxing, inactivating endogenous peroxidase activity and blocking cross-reactivity with normal serum (Vectastain Elite Kit; Vector, Burlingame, CA), the sections were incubated overnight at 4 °C with a diluted solution of the primary antibodies (1:200 for PCNA). Location of the primary antibodies was achieved by subsequent application of a biotinylated anti-primary antibody, an avidin–biotin complex conjugated to horseradish peroxidase, and diaminobenzidine (Vectorstain Elite Kit, Vector, Burlingame, CA). The slides were counter-stained by hematoxylin. Negative controls were established by replacing the primary antibody with PBS. Known immunostaining-positive slides were used as positive controls. The numbers of total and stained nuclei were counted separately for the intima with image analysis software (Nikon & Spot, USA) and PCNA-positive indices [(stained nuclei/total nuclei) × 100] were calculated.

1.4. Statistical analysis

Data were expressed as mean ± S.E.M. Unpaired Student’s t-tests were used to compare the values between normal vessels and ligated vessels. The effect of TA treatment was compared by repeated-measures ANOVA. Means were considered significantly different if P < 0.05.

2. Results

2.1. Histopathological changes

The entire length of the vessels was obtained from the mice 4 weeks after ligation of the common carotid artery. Sections from the middle portion of specimens were analyzed. Among the normal control vessel, the luminal surface is smooth, endothelial cell tightly lined with IEL, vascular wall only composed of few layers of cell and lumen area enough for the blood flow (Fig. 1a). As for the model control vessel, intimal hyperplasia is significant. Extensive VSMC was present in subendothelial space, lumen area was reduced and vascular wall increased in thickness (Fig. 1b). Treatment with TA 0.3 or 0.6 g/kg/d could efficiently improve the above histopathological changes and prevent the extent of neointima (Fig. 1c and d).

2.2. Morphometric analysis

Morphometric analysis of the changes in vascular wall geometry was carried out by the software. All histological parameters of neointima formation in ligated vessels were sig-
Fig. 1. TA inhibits intimal thickening in ligated mouse carotid arteries. HE staining (original magnification ×200). (a) cross-section of a normal vessel; (b) cross-section of a 4-week ligated vessel showing an intimal hyperplasia and narrowed lumen; (c) and (d) cross-sections of 4-week treated ligated vessels with TA 0.6 or 0.3 g/kg BW/d showing the inhibition of intimal hyperplasia and improvement of narrowed lumen.

As compared with those of the normal group, the intimal area, medial area and intima-to-media ratio of ligated artery of the model control group increased and the relative lumen area decreased obviously ($P<0.01$). TA could significantly improve all the changes in these morphological parameters ($P<0.01$). No significant difference was observed between TA 0.3 and 0.6 g/kg/d groups (Table 1).

2.3. PCNA expression

The dark brown labeled PCNA-positive nuclei were observed within the vascular neointima. PCNA is significantly less expressed in the TA-treated groups as compared to the model control group. The PCNA-positive indice in model control, TA 0.3 and 0.6 g/kg BW/d groups were $32.52 \pm 2.46$, $5.67 \pm 1.42$ and $5.21 \pm 0.95\%$, respectively (Fig. 2). Thus, TA significantly reduced the PCNA positive indice in neointima, consistent with histopathological changes in neointima ($P<0.01$).

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Intimal area ($\mu m^2$)</th>
<th>Medial area ($\mu m^2$)</th>
<th>Relative lumen area (%)</th>
<th>Ratio of intima/media (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>410 ± 141</td>
<td>56637 ± 33224</td>
<td>329.7 ± 1.7</td>
<td>1.2 ± 1.1</td>
</tr>
<tr>
<td>Model control</td>
<td>37778 ± 22314</td>
<td>125311 ± 62483</td>
<td>32.2 ± 14.9*</td>
<td>31.8 ± 11.2**</td>
</tr>
<tr>
<td>TA 0.3 g/kg/d × 4 weeks</td>
<td>5334 ± 2716**</td>
<td>58746 ± 30753**</td>
<td>43.4 ± 6.9*</td>
<td>10.0 ± 4.9**</td>
</tr>
<tr>
<td>TA 0.6 g/kg/d × 4 weeks</td>
<td>4787 ± 2679**</td>
<td>44984 ± 23428**</td>
<td>46.8 ± 9.0*</td>
<td>10.9 ± 5.8**</td>
</tr>
</tbody>
</table>

** $P<0.01$, compared with normal control group.
* $P<0.05$, compared with model control group.
** $P<0.01$, compared with model control group.

3. Discussion

The main pathological changes of restenosis after PTCA are the abnormal hyperplasia of vascular intima and narrowed lumen. Proliferation and immigration of VSMC from media to intima have long been regarded as the primary mechanism of restenosis. Among which models of animal restenosis (Kumar and Lindner, 1997; Leidenfrost et al., 2003; Sirsjo et al., 2003; Uwatoku et al., 2003), the carotid-ligated mouse is regarded as a reproducible one since complete cessation of blood flow can induce rapid proliferation of medial VSMC, thus leading to extensive neointima formation and also the other conspicuous advantages such as easy manipulation and low cost. In the present experiment, the ligated vessels showed marked intimal hyperplasia as compared with the normal ones. TA could efficiently prevent the neointima formation. By orally administrated 0.3 or 0.6 g/kg/d, TA could significantly improve the histopathological changes in neointima: reducing the intimal area, medial area and the ratio of intima to media, and increasing the relative lumen area ($P<0.01$). TA also inhibited PCNA expression in intima. PCNA is a special marker for cellular proliferation and found most cell phases during proliferation (Yue et al., 2000). The number of PCNA-positive cells was used as an index of VSMC proliferation in injured arteries. In this paper, a significant difference in PCNA-positive indices in neointima was observed between model control and TA-treated groups ($P<0.01$). Thus, the suppressive effect of TA on intimal hyperplasia may, at least in part, result from the inhibition of the proliferation of VSMC. The inhibitory effects of TA on VSMC proliferation in the present in vivo experiment were comparable with those found previously in in vitro experiment (Du, 1999).
It is known that restenosis is a complex vascular repair process in response to luminal injury that is induced by multiple growth stimuli released during the arterial injury. These growth stimuli include local factors and blood-borne growth factors, especially associated with lipoprotein oxidation (Steinberg et al., 1989) and activation of platelets (Li et al., 1989). On the basis of the ‘oxidation theory,’ the oxidation of LDL in the artery wall is an early initiating event and contributes to atherogenesis. Previous studies have shown that TA was an effective antioxidant against LDL oxidation (Zhou et al., 1999; Niu et al., 2000). TA cannot only inhibit lipid peroxidation but also increase some endogenous antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase. Therefore, TA may interfere with restenosis process via its antioxidant activity.

Taken together, intimal hyperplasia remains a significant problem of restenosis because effective clinical therapy to reduce intimal hyperplasia is still unavailable. Here, our novel in vivo finding shows that TA inhibits the development of intimal thickening in an animal model of arterial ligation, which, as expected, is associated with the inhibition of VSMC proliferation. Since TA may affect many vascular processes responsible for restenosis, more experiments are needed to clarify the exact mechanisms underlying its protective effect on restenosis. The efficacy of TA in preventing neointimal proliferation in injured vessels and the other vascular protective effects of TA support a potential clinical use of TA to reduce restenosis in humans.

References


Antiviral activity in vitro of *Urtica dioica* L., *Parietaria diffusa* M. et K. and *Sambucus nigra* L.

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Abstract

*Parietaria diffusa* M. et K., *Urtica dioica* L. (Urticaceae) and *Sambucus nigra* L. (Caprifoliaceae) are plants usually used in popular medicine of central Italy for treating numerous diseases, first of all *Herpes zoster*. Several plant products have been described as potential antiviral agents, with special attention being devoted to those having retroviruses as etiological agents, including acquired immunodeficiency syndrome (AIDS), in which a retrovirus, the designated human immunodeficiency virus HIV, has been clearly identified as the primary cause of this disease. The present study proposes a preliminary screening of the antiviral activity of *Parietaria diffusa*, *Sambucus nigra* and *Urtica dioica* preparation against the feline immunodeficiency virus (FIV) infection. The feline immunodeficiency virus is a widespread lentivirus of domestic cats sharing numerous biological and pathogenic features with the human immunodeficiency virus (HIV). FIV infection in cats has therefore been proposed as an animal model for AIDS studies with respect to pathogenesis, chemotherapy, and vaccine development [Pedersen, N.C., 1993. Feline immunodeficiency virus infection. In: Levy, J.A. (Ed.), The Retroviridae. Plenum Press, New York; Bendinelli, M., Pistello, M., Pirotta, M., Lombardi, S., Poli, A., Garzelli, C., Matteucci, D., Cecherini-Nelli, L., Malvaldi, G., Tozzi, F., 1995. Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. Clinical Microbiology Revue 8, 87–112; North, T.W., LaCasse, R.A., 1995. Testing anti-HIV drugs in the FIV model. Nature Medicine 1, 410–411; Matteucci, D., Pistello, M., Mazzetti, P., Giannecchini, S., Isola, P., Mirex, A., Zaccaro, L., Rizzati, A., Bendinelli, M., 2000. AIDS vaccination studies using feline immunodeficiency virus as a model: immunisation with inactivated whole virus suppresses viraemia levels following intravaginal challenge with infected cells but non-following intravenous challenge with cell-free virus. Vaccine 18, 119–130]. Early studies showed that some of them presented antiviral activity against infection of FIV as assayed by syncytia formation using feline kidney Crandell cells (CPK).

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Keywords: Ethnopharmacobotany, Tuscany, Italy

I. Introduction

Also in fully-developed countries – such as central Italy – numerous plants are used in popular medicine and very interesting traditions are still maintained [Martini, 1983; Leporatti et al., 1985; Leporatti and Parrechi, 1990; Uncini Manganelli and Tomei, 1999a]. In Tuscany ethno-pharmacobotanical studies have been in progress for over ten years with the presence of more than 500 plant species; some of these are used in handicrafts, in liqueurs, as ornaments, etc. [Uncini Manganelli et al., 2002], some belong to popular medicine (Uncini Manganelli and Tomei, 1999a; Camangi et al., 2001; Camangi and Uncini Manganelli, 2002). An anthropological and botanical in loco research was done first, which was then followed by pharmacological and phytotoxicological research, to verify the value of some original uses [Calderone et al., 1998; Uncini Manganelli et al., 2000; Testai et al., 2002; Cheritoni et al., 2003]. Among the plants used as antivirals we began the analysis of *Parietaria diffusa* M. et K. and *Urtica dioica* L. (Urticaceae) and of *Sambucus nigra* L. (Caprifoliaceae) *Parietaria diffusa* M. et K., is also used for treating *Herpes zoster* and *Herpes labialis*; for this the freshly crushed plant...
is applied as a poultice which is changed every 5–6 h (Uncini Manganelli and Tomei, 1999b).

In inland areas – for example, the Appennino – *Sambucus nigra* L. is used; the bark is cooked in olive oil and bees-wax is added; this cream is applied often during the day (Uncini Manganelli and Tomei, 1996).

The *Urtica dioica* decoction is applied with a compress and is changed every 5–6 h.

2. Methodology

2.1. Plant material and extraction

All the plant material was collected in their respective areas of usage, and a voucher specimen was deposited at the Botanic Garden of Lucca (LU) (*Parietaria diffusa* M. et K.: LUCCA 5 229; *Sambucus nigra* L.: LUCCA 73 361; *Urtica dioica* L.: LUCCA 5 223).

The fresh bark of *Sambucus nigra* was boiled for 20 min about (1 g/10 ml); the extract was filtered and then lyophilized. The lyophilized extract was melted in bidistilled water at protocol percentage. The same process was done with the root of *Urtica dioica*.

*Parietaria officinalis* was instead centrifuged, filtered and then lyophilized.

2.2. Cells

Crandell feline kidney cells (CrFK, ATCC clone CCL94) were grown in Eagle’s minimal essential medium containing 0.5% fetal calf serum (FBS), 2 nM l-glutamine, penicillin (50 IU/ml) and streptomycin (50 μg/ml), a variety of growth factors and other supplements (Tozzini and Bandecchi, 1985).

2.3. Virus

Feline immunodeficiency viruses, the California isolate Petaluma strain (FIV-Pet) kindly provided by Yamasato et al. (1988, 1991) has been used. This virus induces formation of syncytia in the CrFK cells. The titration of the viral stocks was performed as described by Tozzini et al. (1992). The viral titres were expressed as syncytium forming units virus (SFU/ml).

2.4. Experimental protocol

The extract plants were screened for the capability to inhibit FIV replication using an assay based on FIV-induced syncytia formation (Tozzini et al., 1992). Briefly, different concentrations of plant extracts were added to CrFK cells grown in 24-well plates (2 × 10⁶ cells per well) in 0.8 ml of culture medium containing 0.5% FBS. After 1 h at 37 °C approximately 50–100 syncytia-forming units (SFU) of FIV-Pet were added in 0.2 ml volume. Six days later, the cultures were stained by crystal violet (0.5%) in methanol 30% and the number of syncytia was counted under the microscope. Only the syncytia composed of at least seven nuclei were counted. The cytotoxicity of the extracts of the three plants in CrFK cells was determined by microscopic examination of cell death and integrity after an incubation time of 72 h (data not shown). The positive check/test was carried out using a compound whose antiviral activity and action mechanism in vitro are well known, that is to say destrane (Tanabe-Tochikura et al., 1992) (Fig. 1).

2.5. Statistical analysis

Data are expressed as means ± S.D. The significance of differences between groups was determined by single-factor ANOVA, when compared to control cultures in the absence of plant extract. A value of *p* lower than 0.05 was considered as significant.

3. Results

Acute infection and cell-to-cell transmission of FIV were assayed by the syncytia formation assay. This assay, based on the interaction between fusigenic FIV-infected cells, has been used with other natural products (Calderone et al., 1998; Nicoletti et al., 1999). After an incubation of six days all extracts showed an antiviral activity, with a pattern similar to that of destrane (Fig. 1).

The aqueous extract of *Urtica dioica* indicated a good inhibition on the development of syncytia (Fig. 2) with low doses (0.5–1 μg/ml) and increased when the concentration rose until it reached an inhibition level of 84% which, however, began to show cytotoxic effects.

The inhibition trend on the syncytia formation based on *Parietaria diffusa* is reported in Fig. 3. The inhibiting activity of the syncytia reached good levels in a short time, but then it remained stable at insufficient values and obtained complete inhibition reaching the maximum value of 88.5% regarding the sub-toxic concentration of 32 μg/ml.

![Fig. 1. Inhibition of FIV-induced syncytium formation by destrane.](image-url)
The assay of syncytia inhibition for *Sambucus nigra* showed a noticeable anti-FIV activity, even if the inhibition curve is not regular (Fig. 4).

At the beginning, the decrease in number of syncytia happens progressively and reaches values which are over 43% for concentrations of 1 μg/ml and to values over 85% for concentrations of 31 μg/ml; then there is a digression of about 60–70% for concentrations between 62 and 125 μg/ml continuing up to 100% inhibition at 500 μg/ml.

4. Discussion and conclusion

The therapeutic properties of plants have been attributed to crude extracts or isolated compounds, often reflecting their role in traditional medicine.

In the study of natural products that have antiviral activity, previous studies have shown that all three plants have a good antiviral activity; the cytotoxicity stopped the attainment of total inhibition for *Urtica dioica* and *Parietaria diffusa*.

*Parietaria diffusa* is a well-known and studied plant due to its allergenic properties, its pollen being mainly responsible for numerous spring allergies.

From a therapeutic point of view, in popular medicine the infusion of its aerial components, taken orally, is considered to be helpful in treating the urinary tracts and to increase diuresis; the fresh plant is applied locally to soothe the skin bitten by insects or touched by stinging nettles.

Not many studies on the therapeutical uses of this plant can be found in literature, however, its diuretic and uricosuric properties are well known (Giachetti et al., 1986).

No information is available concerning its possible antiviral use.

The nettle, in popular medicine, is used very much in various minor pathologies, besides the above-mentioned use for treating *Herpes zoster* the infusion of its aerial components can be drunk to favor diuresis, to soothe rheumatism, and to lower high blood pressure; externally it can be rubbed on the scalp to stimulate hair growth. Recent studies have confirmed diuretic and hypertensive properties regarding *Urtica dioica* (Tahri et al., 2000); interesting results have been obtained from the discovery that the aqueous extract has antihyperglycemic properties (Bnouham et al., 2003), and it is also a good antioxidant (Pieroni et al., 2002).

The data we have obtained using the entire extract of *Urtica dioica* and the syncytium formation test are similar to those obtained using the active principles derived from the same plant. The N-acetylglucosamine-specific lectin from *Urtica dioica* was a strong inhibitor of syncytium formation between HUT-78 cells and CD4+ Molt/4 cells permanently infected by HIV-1 and HIV-2 (Balzarini et al., 1992).
The most important differences regard the effective dosage (lecitin dosage is lower than the raw extract) and the amount of inhibition, which towards HIV is total, towards FIV is partial, even if high.

The reason for this is likely to be found in the different composition of the substances which have been tested: the extract used in the inhibition test is a raw extract, which is, qualitatively and quantitatively, not well defined, while the results reported in literature were obtained assaying a pure compound having a well-defined biological activity.

It is necessary to continue these studies to determine the chemical identification of all constituents.

_Sambucus nigra_ is a very well-known plant in official phytotherapy, whose flowers stimulate bronchial secretion and have a diaphoretic activity (Longo, 1996); recent studies have confirmed the presence of non-toxic ribosome-inactivating protein (RIP) in the bark, leading to the inhibition of protein synthesis. Conjugation of RIP to monoclonal antibodies is a promising tool for cancer therapy (Girbes et al., 2003).

In folk medicine many parts of this tree are used: the fruit as a laxative; the leaves are applied to wounds and bruises to help soothe and heal, the infusion of the flowers is applied to sore red eyes or is drunk for gastro-intestinal problems. Concerning the antiviral activity of elder, the analysis of its main components are now in progress.

Further studies to make a sub-division of the extract and the analysis of its main components are now in progress.

**Acknowledgement**

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In vitro anti-*Helicobacter pylori* action of 30 Chinese herbal medicines used to treat ulcer diseases

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**Abstract**

Infection by *Helicobacter pylori* has been ascertained to be an important etiologic impetus leading usually to chronic active gastritis and gastric ulcer with growing incidences worldwide. Utilizing as the test pathogen a standard and five clinic strains of *Helicobacter pylori*, the antibacterial action was assessed in vitro with ethanol extracts of 30 Chinese herbal medicines which have been frequently prescribed since ancient times for treating gastritis-like disorders. Among the 30 tested materials, the ethanol extracts of *Abrus cantoniensis* (Fabaceae), *Saussurea lappa* (Asteraceae) and *Eugenia caryophyllata* (Myrtaceae) were strongly inhibitory to all test strains (MICs: \(\sim 40\) μg/ml), and *Hippophae rhamnoides* (Elaeagnaceae), *Fritillaria thunbergii* (Liliaceae), *Magnolia officinalis* and *Schisandra chinensis* (Magnoliaceae), *Corydalis yanhusuo* (Papaveraceae), *Citrus reticulata* (Rutaceae), *Bupleurum chinense* and *Ligusticum chuanxiong* (Apiaceae) substantially active with MICs close to 60.0 μg/ml. As to antibacterial actions of the aqueous extracts of the same drugs, those derived from *Cassia obtusifolia* (Fabaceae), *Fritillaria thunbergii* and *Eugenia caryophyllata* were remarkably inhibitory against all the six *Helicobacter pylori* strains (MICs: \(\sim 60\) μg/ml). The work compared almost quantitatively the magnitude of the anti-*Helicobacter pylori* actions of the 30 most prescribed gastritis-treating Chinese herbal drugs, and located as well some source plants where potent anti-*Helicobacter pylori* phytochemicals could be characterized.

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**Keywords:** Traditional Chinese medicines; *Helicobacter pylori*; Gastric ulcer; Antibacterial

### 1. Introduction

The human bacterial pathogen *Helicobacter pylori* has been recognized as a major causative factor in peptic ulcer diseases (Graham, 1989; Blaser, 1992; Axon, 1993). As to the therapy of those illnesses, high cure-rates are sometime afforded by sophisticated therapeutic approaches based on an on-demand empirical combination of histamine-2- (or H\(_2\)-) receptor antagonist(s), proton pump inhibitor(s) and bismuth compound(s) (De Korwin and Lozniewski, 1996). However, the infection of *Helicobacter pylori* is still as a whole under a poor management since the eradication failure rate remains as high as 5–20% along with frequent relapses of gastric ulcers even after the discerned 'complete healing' (Bazukli et al., 1994; Bell et al., 1995; Bayerdörffer et al., 1995). This situation could be attributed to a couple of reasons: (1) Some strains of *Helicobacter pylori* harboring deeply inside gut tissues could not be entirely killed even after a long-time administration of pertinent antibiotics (Korman et al., 1997); (2) The drug resistance of the bacterium could be developed during repeated applications of anti-*Helicobacter pylori* agents (Adamek et al., 1998; Marshall, 1991); and (3) as a result of patient compliance and/or ulcerogenic side-effects of some drugs co-administered imperatively for the management of other accompanying diseases. Accordingly, there is a growing need for finding new anti-*Helicobacter pylori* agents that can hopefully eradicate the invasion and presence of the survived strain of *Helicobacter pylori* to avoid relapses of the gastric ulcer it causes.

In China, most traditional medicines have experienced for dozens of centuries continuous clinical practices with opti-
mized combinations. Among those well-proven herbal drugs, some have been documented to have significant efficacy for treating gastric ulcers. Thus, a thorough literature search was conducted to select a total of 30 traditional Chinese herbal medicines (belonging to 19 families), which have been most frequently prescribed for that therapeutic purpose. A comparative investigation was subsequently performed concerning the anti-\textit{Helicobacter pylori} actions of the ethanol and aqueous extracts of the selected herbs.

2. Materials and methods

2.1. General

Columbia agar was purchased from Biomérieux sa (France). Horse serum was obtained from Yuanheng Shenna (Beijing, China). Ampicillin was from the National Institute for the Control of Pharmaceutical and Biological Products (China). All other chemicals used in the study are of chemical grade.

2.2. Plant material

The plant materials were collected or purchased from different parts of China (Table 1). Each voucher specimen under a given registration number, authenticated by Associate Prof. L.X. Zhang, was preserved in the Herbarium of Nanjing University, Nanjing, People’s Republic of China.

2.3. Preparation of extracts

Two sets (40 g each) of roughly ground air-dried plant materials of every selected species were extracted twice separately with 95% ethanol and water (400 ml for each extraction) by refluxing for 4 h on a water bath at 90 °C. In vacuo evaporation of solvents from the filtered ethanol and water extracts gave residues, which were subjected to an immediate lyophilization into dry powders with the yields given in Table 1.

2.4. \textit{Helicobacter pylori} strains

Used in this study were a standard strain (ATCC 43504) and five clinical isolates of \textit{Helicobacter pylori} obtained from antral biopsies of childish and adult patients hospitalized at Jiangsu People’s Hospital in Nanjing. They were inoculated into Columbia agar base plates supplemented with 7% horse serum and cultured for 3 days at 37 °C under microaerophilic conditions with high humidity as detailed elsewhere (Fabry \textit{et al.}, 1996).

2.5. MIC measurements

The minimum inhibitory concentration (MIC) was detected with the agar dilution method described earlier (Megraud \textit{et al.}, 1999). Briefly, 1 ml of each stock solutions at given concentrations of every plant extract in sterile water (possibly less amounts of DMSO were used for an acceptable intermiscibility) was separately added into petri dishes containing 8 ml of unsolidified Columbia agar base supplemented with 1 ml of horse serum. Final concentrations of each plant extract in the medium were set to be 200.0, 100.0, 60.0, 40.0, 20.0, 10.0 and 5.0 μg/ml with DMSO concentration lower than 1%. Different \textit{Helicobacter pylori} strains pre-activated in seed cultures were immediately diluted with Brucella broth with bacterial cells at approximately \textit{5 × 10^7} CFU/ml in resultant liquors. Subsequently, 0.1 ml of each thus-prepared liquor was inoculated onto the surface of the sample-supplemented agar plates, followed by being kept at 37 °C for 72 h in anaerobic jar (containing: 85% N_2, 10% CO_2 and 5% O_2). The MICs were defined as the lowest concentration at which no microbial growth could be detectable. Ampicillin was co-assayed as a positive reference at concentrations of 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg/ml.

3. Results and discussion

Ethanol and aqueous extracts of 30 herbal medicines used traditionally for the treatments of gastric ulcers were screened in vitro for the anti-\textit{Helicobacter pylori} actions. As summarized in Table 1, the strongest growth inhibitions (MICs: ~40.0 μg/ml) against all the six test strains of \textit{Helicobacter pylori} were discerned with the ethanol extracts from the aerial parts of \textit{Abrus cantoniensis}, the stems of \textit{Saussurea lappa} and the flowers of \textit{Eugenia caryophyllata}. Also significant anti-\textit{Helicobacter pylori} actions with MICs around 60.0 μg/ml were exhibited by 11 additional ethanol extracts of \textit{Hippophae rhamnoides}, \textit{Fritillaria thunbergii}, \textit{Magnolia officinalis} and \textit{Schisandra chinensis}, \textit{Corydalis yanhusuo}, \textit{Citrus reticulata}, \textit{Bupleurum chinense} and \textit{Ligusticum chuanxiong}. As to the anti-\textit{Helicobacter pylori} action of aqueous extracts, only three derived from the leaves of \textit{Cassia obtusifolia}, the stems of \textit{Fritillaria thunbergii} and the flowers of \textit{Eugenia caryophyllata} exhibited strong inhibitions against all the test strains with the MICs of ~60 μg/ml. As far as the ethanol and aqueous extracts of a single herb were compared, the former seemed more active than the latter (Table 1). Furthermore, the MICs of those active extracts being usually complex mixtures consisting of numerous phytochemicals are quite acceptable although they are higher than that (2.0 μg/ml) of ampicillin co-assayed as a positive reference in the study.

A reference survey on the chemical composition of the three most anti-\textit{Helicobacter pylori} medicines have been performed. The previous phytochemical attention to \textit{Eugenia caryophyllata} had led to the characterization of volatile oil (mainly contains eugenol and caryophyllen), a chromosome C-glucoside, elagitanins, phytosterols (Yu and Hung, 1981; Xiao \textit{et al.}, 2002). Among these plant constituents, eugenol was disclosed to have prominently antibacterial properties (Yu and Hung, 1981; Pattanak \textit{et al.}, 1997). Furthermore, the
### Table 1: Minimum inhibitory concentrations (MICs: μg/ml) of ethanol (EE) and aqueous (AE) extracts of 30 medicinal plants against *Helicobacter pylori* strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Parts</th>
<th>MIC (μg/ml)</th>
<th>Collection</th>
<th>Voucher number</th>
</tr>
</thead>
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<tr>
<td><em>Apocynaceae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bupleurum chinense</em> DC.</td>
<td>AP</td>
<td>60</td>
<td>JS</td>
<td>April 2001</td>
</tr>
<tr>
<td><em>Eriastrum vulgare</em> Mill.</td>
<td>FT</td>
<td>&gt;100</td>
<td>HLI</td>
<td>October 2000</td>
</tr>
<tr>
<td><em>Ligusticum chuanxiong</em></td>
<td>RT</td>
<td>60</td>
<td>SC</td>
<td>July 1999</td>
</tr>
<tr>
<td><em>Aristolochiaceae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aristolochia debilis</em> Sieb. et Zucc</td>
<td>RT</td>
<td>&gt;100</td>
<td>JS</td>
<td>May 1999</td>
</tr>
<tr>
<td><em>Aristolochia mallissima</em> Hance</td>
<td>LF</td>
<td>&gt;100 &gt;100</td>
<td>JS</td>
<td>October 1998</td>
</tr>
<tr>
<td><em>Asteraceae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Saussurea lappa</em> Clarke.</td>
<td>RT</td>
<td>40</td>
<td>YN</td>
<td>December 2001</td>
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<tr>
<td><em>Xanthium sibiricum</em> Patr.</td>
<td>FT</td>
<td>0 &gt;100</td>
<td>JL</td>
<td>September 1999</td>
</tr>
<tr>
<td><em>Elaeagnaceae</em></td>
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</tr>
<tr>
<td><em>Hippophae rhamnoides</em> L.</td>
<td>LF</td>
<td>60</td>
<td>JS</td>
<td>November 2000</td>
</tr>
<tr>
<td><em>Fabaceae</em></td>
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<td></td>
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</tr>
<tr>
<td><em>Abrus cantoniensis</em> Bge.</td>
<td>AP</td>
<td>40</td>
<td>GD</td>
<td>September 1999</td>
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<tr>
<td><em>Astragalus membranaceus</em> Bge.</td>
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<td>&gt;100</td>
<td>GS</td>
<td>September 2001</td>
</tr>
<tr>
<td><em>Cassia obtusifolia</em> Vahl.</td>
<td>LF</td>
<td>&gt;100 60</td>
<td>JS</td>
<td>August 2000</td>
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<tr>
<td><em>Glycyrrhiza uralensis</em> Fisch.</td>
<td>RH</td>
<td>&gt;100 100</td>
<td>HB</td>
<td>October 2001</td>
</tr>
<tr>
<td><em>Sophora flavescens</em> Ait.</td>
<td>RT</td>
<td>&gt;100 &gt;100</td>
<td>JS</td>
<td>April 2001</td>
</tr>
<tr>
<td><em>Trigonella foenum-graecum</em> L.</td>
<td>FT</td>
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<td>JL</td>
<td>September 1999</td>
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<td><em>Lauraceae</em></td>
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<tr>
<td><em>Lindera strychnifolia</em> Vill.</td>
<td>ST</td>
<td>&gt;100</td>
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<td>March 2001</td>
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<td><em>Lamiaceae</em></td>
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<tr>
<td><em>Mentha haplocalyx</em> Briq.</td>
<td>AP</td>
<td>&gt;100</td>
<td>JS</td>
<td>October 2001</td>
</tr>
<tr>
<td><em>Scutellaria barbata</em> D.Don</td>
<td>WP</td>
<td>&gt;100 100</td>
<td>SX</td>
<td>August 2000</td>
</tr>
<tr>
<td><em>Liliaceae</em></td>
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<tr>
<td><em>Fritillaria thunbergii</em> Miq.</td>
<td>ST</td>
<td>60 60</td>
<td>ZJ</td>
<td>July 1998</td>
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<td><em>Magnololaeae</em></td>
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<tr>
<td><em>Magnolia officinalis</em> Rehd. et Wils.</td>
<td>BK</td>
<td>60 &gt;100</td>
<td>HB</td>
<td>March 2000</td>
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<tr>
<td><em>Schizandra chinensis</em> Baill.</td>
<td>FT</td>
<td>60 0</td>
<td>LN</td>
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<td><em>Meliae</em></td>
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<td></td>
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<tr>
<td><em>Melia toosendan</em> Sieb. et Zucc.</td>
<td>FT</td>
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<td>GS</td>
<td>December 2001</td>
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<td><em>Menispermacaeae</em></td>
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<tr>
<td><em>Tinospora sagittata</em> Gagnep.</td>
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<td>&gt;100 100</td>
<td>HL</td>
<td>September 1999</td>
</tr>
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<tr>
<td><em>Eugenia carophyllate</em> Thunb</td>
<td>FL</td>
<td>40 60</td>
<td>HS</td>
<td>October 2000</td>
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<td><em>Ochnaceae</em></td>
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<tr>
<td><em>Bretilla strata</em> Reischl F.</td>
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<td>&gt;100</td>
<td>AH</td>
<td>October 2001</td>
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<td><em>Papaveraceae</em></td>
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<td><em>Corydalis yanhusuo</em> W.T. Wang</td>
<td>BT</td>
<td>60 100</td>
<td>SD</td>
<td>June 2001</td>
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<td><em>Piperaceae</em></td>
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<tr>
<td><em>Piper longum</em> L.</td>
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<td>100 &gt;100</td>
<td>YN</td>
<td>November 1998</td>
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<tr>
<td><em>Poria cocos</em> Wolf</td>
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<td>AH</td>
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<td><em>Germaphus chinensis</em> Hance</td>
<td>WP</td>
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<td>FI</td>
<td>April 2000</td>
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<td><em>Rutaceae</em></td>
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<td><em>Citrus reticulata</em> Blanco</td>
<td>FS</td>
<td>60 100</td>
<td>ZJ</td>
<td>October 2000</td>
</tr>
</tbody>
</table>

**Species:** Collection Site Period Collection Yield (%) Voucher number

**Parts:** AP, aerial part; BK, bark; FL, flower; FT, fruit; FS, fruit shell; LF, leaf; RH, rhizome; RT, root; SD, seed; SP, spike; ST, stem; WP, whole plant.

**Collection Sites:** AH, Anhui; FJ, Fujian; GD, Guangdong; GS, Gansu; HB, Hunan; HLJ, Heilongjiang; HK, Henan; JS, Jiangsu; JL, Jilin; KS, Jiangsu; LN, Liaoning; SC, Sichuan; SD, Shandong; SX, Shanxi; YN, Yunnan; ZJ, Zhejiang.
preliminary study also showed that this plant has an anti-
Helicobacter pylori activity, which was consistent with our
results (Bae et al., 1998). Accordingly, anti-Helicobacter pylori
action of the ethanol extract of Eugenia carophyllata
in this study might be related to the presence of eugenol al-
though none of the reported principles has been shown to be
anti-Helicobacter pylori.
The ethanol extract of Abrus cantoniensis displayed as
well the most significant inhibition to the growth of Heli-
cobacter pylori. Phytochemically, this plant has been as-
certained to be a common source of triterpenic saponins, an-
thaquinones, alkaloids, flavonoids, and some other con-
stituents such as abrin, abrinic acid, uric acid, glycolbro-
lide (Xiao et al., 2002). Flavonoids and isoflavonoids present
in the plant could be the anti-Helicobacter pylori sub-
stances since some structurally related analogs such as
flavonoids ponciretin and hesperitin as well as isoflavonoids
i.e. cabrevin, irisinolide, genistin and licorisoflavone were
revealed to possess potent anti-Helicobacter pylori activities
(Bae et al., 1999; Obasaki et al., 1999; Fukui et al., 2002).
The third most anti-Helicobacter pylori traditional medic-
inal plant is Saussurea lappa, which has been demonstrated
to contain sesquiterpenes, monoterpenes, triterpenes, aro-
matic compounds, sterols, alkaloid (Yang et al., 1997; Mat-
suda et al., 2003, 2000). Concerning the antibacterial action,
volatile oils from the plant were demonstrated to be antibac-
terial to Staphylococcus aureus and Streptococcus pneumoniae
(Pattnaik et al., 1997; Wang, 1997). Therefore it is suggested
that the volatile oils may be the constituents responsible for
the anti-Helicobacter pylori activity.
Some anti-Helicobacter pylori natural products have been
reviewed earlier (Cowan, 1999). Also reported elsewhere to
be anti-Helicobacter pylori oil-macerated garlic con-
stituents (Ohba et al., 1999), fatty acids and terpenes in Acis-
tolochia paucnervis (Gadhi et al., 1999), costunolides in
Magnolia sieboldii (Park et al., 1997), and some acidic and
alkaline constituents in Coptis japonica, Eugenia carophyl-
lata, Magnolia officinalis and Rhus javanica (Bae et al.,
1999). The present results, along with the observation, sug-
gested that a wider range of phytochemicals could be anti-
Helicobacter pylori, rationalizing the utility and efficacy of
the traditional Chinese medicines in the treatment of gastric ulcers.

Acknowledgements
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Science Foundation of China (No. 30171040) and from the
Ministry of Education (No. 104195).

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troenterology 96, 613-625.
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a Brazilian medicinal plant, Myroxylon peruiferum and the activity of
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Effect of an avocado oil-rich diet over an angiotensin II-induced blood pressure response

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Abstract

We studied the effect of an avocado oil-rich diet on (1) the blood pressure response to angiotensin II (AngII) and (2) the fatty acid composition of cardiac and renal membranes on male Wistar rats. The avocado oil-rich diet induced a slightly higher AngII-induced blood pressure response in the rats as compared to the control rats. In cardiac microsomes, avocado oil induced an increase in oleic acid content (13.18 ± 0.33% versus 15.46 ± 0.59%), while in renal microsomes, the oil decreased α-linolenic acid content (0.34 ± 0.02% versus 0.16 ± 0.12%), but increased the arachidonic acid proportion (24.02 ± 0.54% versus 26.25 ± 0.54%), compared to control. In conclusion, avocado oil-rich diet modifies the fatty acid content in cardiac and renal membranes in a tissue-specific manner. The rise in renal arachidonic acid suggests that diet content can be a key factor in vascular responses.

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Keywords: Avocado oil; Blood pressure; Membrane fatty acid composition; Arachidonic acid; Angiotensin II

1. Introduction

Persea americana Miller (Lauraceae) or avocado, native to Mexico, is valued due to its nutritional and therapeutic qualities. Avocado fruit and leaves have been used in Mexican folk medicine to treat a wide variety of diseases. Francisco Hernández reported as early as the XVI century, that oil obtained from pressing the seed was useful in the treatment of rashes and scars, had an astringent effect, and could also be used to treat dysentery (Argueta-Villamar et al., 1994). Hot water infusion from the leaves can be taken as an emmenagogue, diuretic, to treat coughs and colds, and diarrhea. An important use of avocado leaves is to treat hypertension, which is not used exclusively by Mexican populations, but people in countries like Brazil, Indonesia, Jamaica, Nigeria and Panama also use it for the same therapeutic benefits (Ross, 1999; Adeboye et al., 1999).

Recent studies carried out among Mexican populations that consume avocados have shown that the avocado decreases serum total cholesterol, LDL-cholesterol and triglycerides, and increases HDL-cholesterol levels compared to the control diet (Alvizouri-Munoz et al., 1992; Lopez-Ledesma et al., 1996).

The consumption of oil from different sources exerts different effects over the lipid composition of the cellular membranes and their function. Therefore, we studied the effect of a diet rich in avocado oil and a control diet, on the blood pressure response to angiotensin II (AngII). In addition, we evaluated the effect of an avocado oil diet on fatty acid composition of cardiac and renal membranes in order to correlate biochemical changes and physiological responses.
2. Materials and methods

2.1. Experimental animals

Male Wistar rats (220–250 g), bred and raised in our facilities, were placed into metabolic cages 3 days prior to the beginning of the protocol, offered tap water and lab chow ad libitum, and maintained on a 12-h light:12-h dark cycle in a temperature-controlled room. Animals were randomly divided into two experimental groups of five rats each. The control group received lab chow, while the treated group received a 10% (w/w) avocado oil-rich diet for a 2-week period. At the end of the treatment, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and were either prepared for blood pressure measurement, or the heart and kidneys were removed. All procedures were conducted in accordance with Institutional ethical guidelines.

2.2. Diet preparation

The avocado-enriched diet was prepared in bulk in our laboratory by mixing ground lab chow with avocado oil obtained from fresh fruit (10%, w/w). Both the control diet (ground lab chow) and avocado oil-rich diet were partitioned into daily rations packaged in plastic bags, and flushed with nitrogen to minimize oxidation and stored at 4°C.

2.3. Fatty acid methyl esters analysis

Cardiac and renal microsomes were obtained as described by Garg and co-workers (1988) in a homogenizing buffer containing: 250 mM sucrose, 0.1 mM etilenediamino tetraacetic acid (EDTA), 62 mM potassium phosphate, 150 mM potassium chloride, 5 mM magnesium chloride, and 1 mM dithiothreitol (DTT), at pH 7.4. The microsomes, containing butylated hydroxy toluene (BHT, 0.02%), were stored at −70°C until processed. The lipids were extracted as described by Folch et al. (1957). The lipid extracts were trans-esterified to their fatty acid methyl esters as described by Christie (1989), separated and identified by gas–liquid chromatography in a Carlo Erba Fratovap 2300 chromatograph, fitted with a 25 m × 0.25 mm i.d. fused-silica capillary column, coated with CP-Sil 88 (film thickness, 0.25 μm). The analysis was carried out at an isothermal temperature of 195°C, using helium gas as a carrier at a flow rate of 1 ml/min.

2.4. Angiotensin II-induced increase of blood pressure

At the end of the 2-week treatment, both control rats and rats fed with avocado oil-rich diet were anesthetized and we performed intra-artery measurement of the blood pressure as described by Adeboye et al. (1999). AngII (100, 300, and 1000 ng/kg, i.v.) was administered and the basal- and AngII-induced blood pressure changes were measured with a Blood Pressure monitor (BP Monitor, WPI, USA).

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. Statistical evaluation of the data was performed using Student’s t-test for unpaired comparisons; p < 0.05 was considered statistically significant.

3. Results

3.1. Effect on body weight

Two weeks of an avocado oil-rich diet had no significant influence on the rat’s body weight. Body weight of control rats was 247.5 ± 1.4 g, while in the avocado oil-rich diet was 249.1 ± 1.7 g.

3.2. Effect on AngII-induced change in blood pressure

Basal systolic blood pressure for control and avocado oil-rich diet rats was: 95 ± 3.1 and 97 ± 2.6 mmHg, respectively. The administration of AngII (100, 300, and 1000 ng/kg, i.v.) induced an increase in blood pressure in both control rats and rats fed with avocado oil-rich diet; however, this increment was slightly higher in the rats fed with avocado oil-rich diet, as compared to the control rats (Fig. 1).

3.3. Effect on fatty acid composition

Avocado oil-rich diet induced an increase in oleic acid proportion in cardiac microsomes (15.46 ± 0.5% of total) compared to those from control rats (13.18 ± 0.3%, p < 0.05). In renal microsomes, avocado oil-rich diet elicited a decrease in n-linolenic acid (0.34 ± 0.02 and 0.16 ± 0.12% of total, for control and avocado oil-treated rats, respectively, p < 0.05); as well as an increase in arachidonic acid proportion: for control,
The data represent the mean ± S.E. of five different experiments.

4. Discussion and conclusions

In this present study, we found that an avocado oil-rich diet administered for 2 weeks to Wistar rat, induced a higher AngII-induced blood pressure response, and modified the fatty acid composition of cardiac and renal microsomes.

The fatty acid composition of cardiac membranes is sensitive to modification through dietary factors (Pepe and McLennan, 2002). It has been reported that the level and nature of polyunsaturated fatty acids (PUFA) incorporated in cardiac membranes is related to the dietary n-3 PUFA. In their data, Sergiel et al. (1998) reported that rats given a diet rich in docosahexaenoic acid (DHA) had a decrease in the arachidonic acid content of the cardiac membranes, and that DHA constituted almost the only membrane n-3 PUFA, suggesting a very low level of retroconversion. Our data show that avocado oil induces an increase in the proportion of oleic acid in the heart microsomes and thus can modify the structure and function of the membrane, including the biosynthesis of prostaglandins involved in the regulation of the vascular function. Indeed, oleic acid has been shown to exert a pressor effect in rats, when administered intravenously (Grekin et al., 1995). However, this pattern is different in renal microsomes, where we found not only a lack of differences in oleic acid content, but a decrease in α-linolenic acid and an increase in arachidonic acid content. The tendency to a higher increase in Ang II-induced blood pressure may be partially explained by these renal modifications. It has been reported that enhanced dietary intake of α-linolenic acid decreased blood pressure in spontaneously hypertensive rats and increased prostaglandin formation (Rupp et al., 1996).

Upon AngII-stimulation, AA is released from the membrane phospholipids and metabolized in a cell/tissue-specific manner (Croft et al., 2000). Since the avocado oil-rich diet increased the renal content of AA, and we observed a higher increase in AngII-induced blood pressure, it is possible to postulate that the production of metabolites with vasconstrictor properties is increased.

The use of avocado (fresh fruit and leaves) in folk medicine has shown to be effective. However, our results show that the favorable effects for the cardiovascular system cannot be attributed to the components of the oil and suggests that other active substances present in fresh fruit and leaves may be responsible. Further studies are required to establish the ratio of hypcholesterolemic/cardiovascular benefits, when avocado oil is consumed as well as the component(s) responsible. Further studies are required to establish the ratio of hypcholesterolemic/cardiovascular benefits, when avocado oil is consumed as well as the component(s) responsible. Further studies are required to establish the ratio of hypcholesterolemic/cardiovascular benefits, when avocado oil is consumed as well as the component(s) responsible. Further studies are required to establish the ratio of hypcholesterolemic/cardiovascular benefits, when avocado oil is consumed as well as the component(s) responsible. Further studies are required to establish the ratio of hypcholesterolemic/cardiovascular benefits, when avocado oil is consumed as well as the component(s) responsible.

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References


Inhibitory effect of jaceosidin isolated from Artemisia argyi on the function of E6 and E7 oncoproteins of HPV 16

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Abstract

Jaceosidin (4′,5,7-trihydroxy-3′,6-dimethoxyflavone) was isolated from Artemisia argyi as a putative oncogene inhibitor. Jaceosidin inhibited binding between oncoprotein E6 of the human papillomavirus and the p53 tumor suppressor protein. In addition, jaceosidin inhibited binding between the E7 oncoprotein and the Rb tumor suppressor protein, and also inhibited the function of HPV-16 harboring cervical cancer cells, including SiHa and CaSki. Collectively, jaceosidin inhibited the functions of the E6 and E7 oncoproteins of the human papillomavirus, suggesting that this compound might be used as a potential drug for the treatment of cervical cancers associated with the human papillomavirus.

Keywords: Cervical cancer; HPV; E6; E7; Artemisia argyi; Jaceosidin

1. Introduction

The genus Artemisia consists of more than 350 species, and the aerial portions of the plant have been used in traditional medicine for the treatment of prickly heat and jaundice, as well as for treating uterine metrorrhagia and metritis in Korea. Human papillomaviruses (HPVs) are small epitheliotropic DNA viruses, which are involved, in several human malignancies. HPVs are major risk factors associated with the development of cervical carcinomas (Wright and Sun, 1996). The HPV E6 and E7 oncoproteins and the E6AP/E6-associated protein gene are uniformly retained and regularly expressed in tumors, underlining their importance in the development or maintenance of neoplastic phenotypes (Woodworth et al., 1992). The E6 and E7 proteins have been shown to interact specifically with the p53 and pRb tumor suppressor proteins, respectively (Scheffner et al., 1991). Both pRb and p53 negatively regulate the cell cycle, and also appear to inhibit G0–G1 and G1–S phase transitions. These interactions apparently play important roles in the induction of cell immortality. The importance of p53-mediated apoptosis has been recognized in terms of the maintenance of homeostasis, as well as the prevention of neoplastic transformation. Above all, p53 is a target of several oncoproteins, which are encoded by DNA tumor viruses (Kessis et al., 1993). E6 forms a ternary complex with p53 and E6AP, resulting in the degradation of p53 via the ubiquitination pathway (Hubbert et al., 1992). pRb is normally activated when it is hypophosphorylated, and thus, negatively regulates G1–S cell cycle phase transitions, resulting...
2.3. Isolation of jaceosidin from Artemisia argyi

Plant Diversity Research Center, KRIBB. by Dr. Hyeong-Kyu Lee, a manager at the Plant Extract Bank, our laboratory. The authenticity of the plant was confirmed in October 2001. A voucher specimen (No. 143) was deposited in the medicine cooperative association of Daejeon, Korea, in October. J’sphere ODS-H80, 150 mm x 20 mm, S-4 µm, 80 Å, detection at 20 mm i.d., S-4 µm, 80 Å, detection at 254 nm using 75% MeOH to yield jaceosidin (40 mg).

2. Materials and methods

2.1. General

The following were purchased: organic solvents (Duksan Chemical Co., Daejeon, Korea); cisplatin (Sigma, Deisenhofen, Germany); the analytical and preparative HPLC column (J’sphere ODS-H80, 150 mm x 20 mm, S-4 µm, 80 Å, YMC Inc., Kyoto, Japan); HPLC solvents (Fisher Scientific, Pittsburgh, PA); TLC: pre-coated silica gel plate (Silica gel 60F254, 0.5 mm) and pre-coated RP plate (RP: 18F354, 25DC-platten, Merck Co., Darmstadt, Germany); Maxisorb 96-well plates (Nunc, Netherlands); NGL-NTA column (Peptron Inc., Daejeon, Korea) and glutathione sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

2.2. Plant materials

Artemisia argyi leaves were purchased from the herbal medicine cooperative association of Daejeon, Korea, in October 2001. A voucher specimen (No. 143) was deposited in our laboratory. The authenticity of the plant was confirmed by Dr. Hyoong-Kyu Lee, a manager at the Plant Extract Bank, Plant Diversity Research Center, KRIBB.

2.3. Isolation of jaceosidin from Artemisia argyi

Dried Artemisia argyi (2 kg) was extracted twice with MeOH at room temperature. The evaporated MeOH extract (250 g) was then dissolved in water, and partitioned with EtOAc. After concentration, the EtOAc-soluble extract was subjected to silica gel chromatography (Silica gel, Kieselgel 60, 0.063–0.2 mm, MERCK Co.) via elution with a mixture of hexane, ethylacetate, chloroform, and MeOH. Fractions of similar composition, as determined by TLC analysis, were pooled. The active fraction was further chromatographed on ODS-18 resin (ODS-1A 120A, S-150 µm, YMC Inc.), eluting with a stepwise gradient from 40 to 100% MeOH, in H2O. The fraction eluted with 70% MeOH exhibited inhibitory activity on ELISA systems, based on binding between oncoproteins and tumor suppressors, as described below. Finally, the active fraction was purified by preparative HPLC (J’sphere ODS-H80, 150 mm x 20 mm i.d., S-4 µm, 80 Å, detection at 254 nm) using 75% MeOH to yield jaceosidin (40 mg).

2.4. Overexpression and purification of E6, E6AP, E7 and pRb

Fresh E6, E6AP, and E7 were prepared with pGEX vector (Amersham Pharmacia Biotech AB) and pET vector (Novagen, Madison, USA), as described in our previous reports (Cho et al., 2000, 2001). pRb tumor suppressor protein was expressed in pET3 vector, and was constructed to improve solubility in a supernatant fraction, resulting in the production of a truncated protein composed of an N-terminal and a pocket domain (aa 373–928), as described in previous reports (Cho et al., 2001). To express recombinant proteins, pET or pGEX recombinant vectors were transformed into DH5α. The other vector, pET28a, was expressed in BL21 (DE3). These recombinant proteins were induced and purified for use, as described in previous reports (Cho et al., 2000, 2001).

2.5. Effects of jaceosidin on binding between oncoproteins and tumor suppressors

The purified E6, E6AP, and E7 proteins were each coated onto Maxisorb 96-well plates (Nunc, Netherlands), at a final concentration of 4 µg/ml. These plates were blocked with PBS containing 3% skimmed milk for 2 h at room temperature, in order to preclude non-specific binding. Jaceosidin and p53 lysate were added to plates, which had been pre-coated with E6 or E6AP. Jaceosidin and p53 lysate were added to E7 pre-coated plates as described (Cho et al., 2001). After 1 h of incubation, the respective plates were washed three times with PBS containing 0.05% Tween-20 (PBST). E6 or E6AP coated plates were treated with p53 antibody (Onco-gene, Cambridge, MA), and E7-coated plates were treated with pRb antibody (Ab-6) (Oncogene) for 1 h. Horseradish peroxidase-conjugated secondary antibody was then added to the plates, after an additional washing with PBST. After 30 min of further incubation, 100 µl of substrates (4 mg, o-phenylenediamine 5 µl, 37% H2O2/10 ml of 0.1 M citrate buffer, pH 5.1) were added. The enzyme reaction was stopped with 50 µl of 2N H2SO4, and the plate was measured at 490 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA).
2.6. Cytotoxic effect of jaceosidin on HPV (+) cervical carcinomas and HPV (−) control cell lines

Based on the cleavage of tetrazolium salt WST-1 (Boehringer Mannheim, Germany) by mitochondrial dehydrogenases in viable cells, a colorimetric assay for cell viability was employed. CaSki, SiHa, HeLa, C33A, HaCaT, and C3 were used as cell lines in the viability inhibition test. HPV 16-positive cervical carcinoma cell lines, SiHa, and CaSki, were purchased from the American Type Culture Collection (Rockville, MD). C33A is an HPV (−) cervical carcinoma cell line. It has been established that the SiHa and CaSki cell lines contain 1.2 copies and 60–600 copies of the HPV 16 genome, respectively (Baker et al., 1987; Meissner, 1999). These cell lines were grown in DMEM containing 100 U/ml penicillin-streptomycin, 25 ng/ml amphotericin B, and 10% FBS. A humidified incubator was maintained at 37°C and 5% CO₂. These cell lines were then seeded in 96-well plates, at a concentration of 1 x 10⁵/100 μl/well. After an overnight incubation, the culture medium on the plates was exchanged for fresh medium. Various concentrations of jaceosidin, or the known anti-cancer agent, cisplatin, were subsequently added. After another 20h of incubation, 10 μl of cell proliferation reagent WST-1 was added to the plates. After more incubation for 0.5 to 4h, the plates were shaken thoroughly for 1 min with a shaker. The absorbance was measured with an ELISA reader (Molecular Devices) at 490 nm.

2.7. Statistical analysis

All data were expressed as the means ± S.E.M. These data were analyzed by one-way ANOVA using SPSS/PC(+). Post hoc comparisons were assessed using Tukey’s method.

3. Results and discussion

HPV E6 and E7 oncogenes play critical roles in carcinogenesis of the cervix. Thus, the functions of E6 and E7 should be inhibited in order to prevent HPV infection.

In order to isolate the HPV oncogene inhibitor from Artemisia argyi, the MeOH extract, which exhibited inhibitory activity on ELISA systems based on binding between oncoproteins and tumor suppressors, was further investigated. The ELISA assay guided fractionation, accomplished by a variety of chromatographic techniques, led to the isolation of the active component. This active component was identified as jaceosidin (Fig. 1), by comparing NMR and MS data results with those in the literature (Tomas-Barberan et al., 1987; Yadava and Saurabh, 1998; Nakasugi et al., 2000).

In order to evaluate the inhibitory effects of jaceosidin on the function of the E6 and E7 oncoproteins of HPV, we evaluated the effects of jaceosidin on the binding between viral oncoproteins (E6 and E7) and tumor suppressor proteins (p53, Rb). In the ELISA-based binding assay, jaceosidin exhibited inhibitory effects on the binding between E6 and p53, as well as the binding between E6AP and p53 (Fig. 2). Jaceosidin also inhibited binding between E7 and Rb. Cisplatin, a potent inducer of growth arrest and/or apoptosis in most cell types, is among the most effective and widely used chemotherapeutic agents employed in the treatment of human cancers (Wang et
Fig. 3. The cytotoxic effects of jaceosidin on HPV-containing or non-HPV-containing cervical carcinoma cell lines, and HPV-negative keratinocytes. Cells were seeded in 96-well plates at a concentration of 1 × 10⁴/100 μl/well. After 1 day incubation, jaceosidin or the anti-cancer agent, cisplatin, were added, followed by 20 h of incubation, at which time, 10 μl of WST-1 was dispensed into these plates, and the absorbance was measured with an ELISA reader. Vehicle 70% MeOH was used as a control solvent. Cytotoxicity is represented as the mean value (mean ± S.E.M) of at least three independent experiments. Data were represented from three independent experiments performed at least in duplicate; *p < 0.05 vs. untreated control (ANOVA followed by Tukey test).

al., 2000). Cisplatin effectively inhibited binding between E7 and Rb, and also inhibited binding between E6 (or E6AP) and the p53 tumor suppressor (Fig. 2). Collectively, jaceosidin proved able to attenuate the binding activity between oncoproteins and tumor suppressors in a dose-dependent manner. Therefore, jaceosidin may be considered a putative oncogene inhibitor.

The HPV E6 and E7 oncogenes are required for the continuous growth of tumor cell lines containing HPV. Jaceosidin, as a putative inhibitor of the E6 and E7 oncoproteins, may not only interfere with the HPV life cycle, but also arrest HPV 16-associated malignancies. As jaceosidin had clearly exhibited inhibitory effects on the interactions between E6 and p53, and between E7 and pRb, we attempted to determine whether it would also have an effect on cervical cancer cell lines. Therefore, we administered jaceosidin to the HPV 16-containing cervical type cell lines, SiHa and CaSki. HaCaT cells were also used as an immortalized human skin epithelial cell line, which did not contain HPV. In addition, we used the HPV 18 type cell line, HeLa. C33A is a cervical carcinoma cell, which contain no HPV. Jaceosidin’s inhibitory effects on cell viability were evaluated by its addition to the HPV-containing cervical carcinomas and non-HPV-containing cell lines (Fig. 3). In this set of assays, jaceosidin was found to specifically inhibit HPV 16-positive cervical carcinoma cell lines SiHa and CaSki, in a dose-responsive manner (Fig. 3). Little or no inhibition, however, was observed in the HPV-negative HaCaT and HPV 18-positive HeLa cells, suggesting that jaceosidin was specific to the E6 and E7 oncogenes of HPV type 16. Cisplatin was also added into cervical cancer cells, as a control anti-cancer agent. The viability of cervical cancer cells was also inhibited (Fig. 3). Jaceosidin effectively inhibited the viability of CaSki cells, which harbor a larger amount of HPV 16 genomes (60–600 copies) than do other cervical cells. Our results suggest that jaceosidin abrogated the function of HPV 16 types, via the functional inhibition of the E6 and E7 oncoproteins. In summary, jaceosidin inhibited interactions between oncoproteins (E6 and E7) and tumor suppressors (p53, pRb) (Fig. 2), and decreased the growth of immortalized cell lines containing HPV 16 (Fig. 3). The inhibition of these interactions between oncogenes and tumor suppressors appears to result in the recovery of the functions of the p53 and pRb tumor suppressors.

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References


Hypolipidemic activity of aqueous extract of \textit{Capparis spinosa} L. in normal and diabetic rats

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Abstract

The purpose of this study was to examine the effect of single and repeated oral administrations of the aqueous extract of \textit{Capparis spinosa} L. (CS) at a dose of 20 mg/kg on lipid metabolism in normal and streptozotocin-induced diabetic rats. In normal rats, the aqueous extract of CS induced a significant decrease on plasma triglycerides concentrations 1 week ($p<0.05$) and 2 weeks ($p<0.01$) after once daily repeated oral administration. A significant decrease of plasma cholesterol levels was also observed 4 days ($p<0.05$) and 1 week ($p<0.05$) after repeated oral administration. In diabetic rats, CS treatment caused a significant decrease of plasma triglycerides levels after repeated oral administration. Four days after repeated oral administration of aqueous CS extract, the plasma cholesterol levels were significantly decreased ($p<0.05$) and still dropped after 2 weeks ($p<0.01$). On the other hand, the repeated oral administration of CS aqueous extract caused a significant decrease of body weight 4 days after repeated oral treatment in diabetic rats ($p<0.05$).

We conclude that the aqueous extract of CS (20 mg/kg) exhibits a potent lipid lowering activity in both normal and severe hyperglycemic rats after repeated oral administration of CS aqueous extract.

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Keywords: \textit{Capparis spinosa} L.; Hypolipidemic; Cholesterol; Triglycerides; Body weight; Aqueous extract; Oral administration

1. Introduction

Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas and/or by the ineffectiveness of the insulin produced. Such a deficiency results in increased plasma glucose, which in turn damages many of the body’s systems in particular the blood vessels and nerves. Besides hyperglycemia, the levels of plasma lipids are usually raised in diabetes mellitus causing a risk factor for coronary heart disease (Kannel and McGee, 1979). Hypertriglyceridemia is also related to insulin resistance and glucose intolerance (Ginsberg, 1994).

Despite the remarkable progress in the management of diabetes mellitus by synthetic drugs, there has been a renewed interest in medicinal plants attributed with therapeutic virtues. Morocco has a rich heritage of medicinal plants of wide diversity, which are used by the local population and the traditional healers for the treatment of several diseases including diabetes (Ziyyat et al., 1997; Eddouks et al., 2002). For cultural and economic reasons, the local pharmacopoeia continues to be considered as an important source of remedies for primary healthcare. \textit{Capparis spinosa} L. (CS) (Capparidaceae), locally known as “Kebbar” is a native shrub widely distributed throughout the south-eastern region of Morocco (Tafilalet). This plant is traditionally used in diabetes control and treatment according to our previous ethnopharmacological surveys in two great areas of Morocco, Tafilalet and Fez-Boulemane regions (Jouad et al., 2001; Eddouks et al., 2002). CS is used in phytomedicine around the world as anti-oxidative (Germano et al., 2002), antifungal (Ali-...
Shtayeh and Abu Ghdeib, 1999), anthepatotoxic (Gadgoli and Mishra, 1999), anti-inflammatory (Al-Said et al., 1988) and anti-diabetic (Yaniv et al., 1987; Ziyyat et al., 1997). Several medicinal plants have been reported in the scientific literature to possess significant hypolipidemic activities in animal models of diabetes (Ahmed et al., 2001; Sharma et al., 2003). In diabetes phytotherapy, the effect of aqueous CS extract on lipid metabolism has never been demonstrated experimentally. In the south-eastern region of Morocco (Tafilalet), CS fruits are recognized as potent hypoglycaemic agents by several traditional healers (Eddouks et al., 2002).

The present study was conducted in order to evaluate the beneficial effects of the oral administration of the aqueous extract of CS fruits, which are the most commonly found in the Moroccan kitchen and used as medicinal plant for the treatment of diabetes mellitus, on plasma lipid parameters in normal and streptozotocin (STZ) diabetic rats.

2. Material and methods

2.1. Plant material

Specimens of CS (Capparidaceae) were collected from the Tafilalet region (semi-arid area) of Morocco in May–June 2001, and air-dried at 40°C. The plant was previously identified and authenticated by Pr. M. Rejdali (Agronomy and Veterinary Institute, Rabat) and a voucher specimen (ME 60) (Eddouks et al., 2002) was deposited at the herbarium of the Faculty of Sciences and Techniques Errachidia.

2.2. Preparation of the aqueous extract

Plant material was prepared according to the traditional method used in Morocco (decoction): 1 g of powdered fruits mixed with 100 ml distilled water were boiled for 10 min and then cooled for 15 min. Thereafter, the aqueous extract was filtered using a Millipore filter (Millipore 0.2 mm, St Quentin en Yvelines, France) to remove particulate matter. The filtrate was then freeze-dried and the desired dose (mg of lyophilized aqueous extract of CS fruits per kg body weight) was then prepared and reconstituted in 1.5 ml of distilled water. The aqueous extracts were prepared daily, just before administration. The extracts obtained were then given orally to different groups of rats at a dose of 20 mg/kg body weight. The dose of 20 mg/kg was used according to the Moroccan traditional phytotherapy. The extract was green coloured with a percent yield of 13%, its average osmolality was 34 mOsm, pH 6.5, and with a very low viscosity.

Fig. 1. Plasma triglycerides levels over 6 h after single oral administration of an aqueous CS extract (20 mg/kg) in normal (panel a) and diabetic rats (panel b). Data are expressed as means ± S.E.M., n = 6 rats per group. **p < 0.001, when compared to baseline values. (□) Control; (△) CS; (●) Vanadate.

Fig. 2. Plasma cholesterol levels over 6 h after single oral administration of an aqueous CS extract (20 mg/kg) in normal (panel a) and diabetic rats (panel b). Data are expressed as means ± S.E.M., n = 6 rats per group. **p < 0.01, when compared to baseline values. (□) Control; (△) CS; (●) Vanadate.
2.3. Animals used

Experiments were performed in adult male Wistar rats weighing from 200 to 230 g. The animals were housed under standard environmental conditions (23 ± 1 °C, with 55 ± 5% humidity and a 12 h light/dark cycle) and maintained with free access to water and ad libitum standard laboratory diet (70% carbohydrates, 25% proteins, 5% lipids).

2.4. Induction of diabetes

Streptozotocin (Sigma, St Louis, MO, USA) was dissolved in 0.1 M fresh cold citrate buffer at pH 4.5 before use, and injected intravenously into the tail vein at a dose of 65 mg/kg. After 18 h, the rats with stable fasting blood glucose levels greater than 16 mmol/l were considered as diabetic and used in the present study. The percentage of response to streptozotocin injection was 90%.

2.5. Single oral administration

Normal and diabetic rats were randomly assigned to three different groups containing six rats each. One control group received distilled water, a second treated group received the aqueous extract of CS fruits at a dose of 20 mg/kg (20 mg of lyophilized aqueous extract of CS per kg body weight) and the third group received a reference drug (Vanadate (Na\(^{+}\)VO\(_3\)) at a dose of 0.8 mg/kg). For single oral administration, distilled water (control), Vanadate (0.8 mg/kg) or the aqueous extract (20 mg/kg) were administered and plasma cholesterol and triglycerides levels were measured before and 6h after CS treatment.

2.6. Repeated oral administration

For repeated oral administration, rats were treated once daily at a dose of 20 mg/kg for 2 weeks and plasma cholesterol and triglycerides levels were followed during this period. The rats (n = 6 in each group) were treated once daily. Blood samples were collected from the tail vein and plasma triglycerides and cholesterol levels are determined enzymatically by colorimetric specific kits (Randox, UK), respectively. The kits used in this study for substrates analysis were specified for both human and rat blood samples at the same percentage.

2.7. Statistical analysis

Data were expressed as mean ± S.E.M. The statistical analysis was performed by the Student’s t-test. The values were considered significantly different when the P-value was
less than 0.05 in comparison to baseline values (starting values).

3. Results

3.1. Single oral administration

In normal and STZ diabetic rats, no significant changes of both plasma cholesterol and triglycerides concentrations 6 h were noted after a single administration of CS (20 mg/kg) (Figs. 1 and 2). In normal rats, Vanadate (0.8 mg/kg) did not affect significantly the plasma cholesterol and triglycerides concentrations after a single oral dose (Figs. 1a and 2a). However, in STZ rats, vanadate reduced both the plasma cholesterol ($p < 0.01$) and triglycerides levels ($p < 0.01$) 6 h after CS administration (Figs. 1b and 2b).

3.2. Repeated oral administration

In normal rats, a significant reduction in plasma triglycerides was observed in CS-treated group from the 7th day ($p < 0.05$) to the 50th day of CS treatment ($p < 0.01$) (Fig. 3a). Plasma cholesterol levels dropped significantly, in normal rats, from the 4th day ($p < 0.05$) to the 7th day ($p < 0.01$) after CS administration. Vanadate caused a significant decrease of plasma triglycerides ($p < 0.05$) (Fig. 3a) and cholesterol levels ($p < 0.001$) after a single administration of CS (20 mg/kg) (Fig. 4a).

In STZ diabetic rats, CS extract decreased significantly the plasma triglycerides levels from the first week ($p < 0.01$) to the 2nd ($p < 0.01$) (Fig. 3b). The plasma cholesterol levels were decreased from the 4th day ($p < 0.05$) to the 50th day ($p < 0.01$) after CS treatment (Fig. 4b). Daily vanadate administration (0.8 mg/kg) for 2 weeks produced a statistically significant decrease in both plasma cholesterol ($p < 0.001$) and triglycerides ($p < 0.001$) concentrations (Figs. 3b and 4b).

3.3. Body weight loss

A significant decrease on body weight was observed in distilled water treated group from the second day ($p < 0.05$) of STZ injection. CS aqueous extract (20 mg/kg) caused a significant weight loss 2 weeks after once daily repeated oral treatment only in STZ rats ($p < 0.01$) (Fig. 5b) but not in normal rats (Fig. 5a). Vanadate caused also a significant decrease on body weight in both normal ($p < 0.01$) and STZ ($p < 0.001$) rats after repeated oral administration (Fig. 5).

4. Discussion

This study was carried out in order to elucidate the influence of daily oral administration for 15 days of the aqueous extract of CS fruits on plasma triglycerides, plasma cholesterol and body weight in normal and STZ diabetic rats. Vanadate, a potent inhibitor of tyrosine phosphatases (Tsiani et al., 1998), was used as a drug reference. Vanadate mimics several insulin actions in vivo: the stimulation of hexose uptake, the stimulation of lipogenesis and the inhibition of lipolysis. Administration of this compound to STZ diabetic rats normalises plasma levels of glucose, lipids, creatinine and thyroid hormones (Gupta et al., 1999).

The levels of plasma lipids are usually raised in diabetes mellitus. Such elevation represents a risk factor for coronary heart diseases (Kannel and McGee, 1979). The abnormally high concentration of plasma lipids is mainly due to the increase in the mobilization of free fatty acids from the peripheral depots (Ahmed et al., 2001). The results demonstrated that the aqueous extracts of CS fruits produced a significant decrease in plasma triglycerides and cholesterol levels for the repeated oral administration (long-term treatment) in both normal and STZ diabetic rats. However, CS treatment did not affect lipid profile after single oral administration (short-term treatment) in both normal and STZ diabetic rats. The underlying mechanism by which CS exerts its cholesterol lowering effect seems to be a decrease in cholesterol absorption from the intestine, by binding with bile acids within the intestine and increasing bile acids excretion (Kritchevsky, 1978; Kelly and Tsai, 1978). CS can also act by decreasing the cholesterol biosynthesis especially by decreasing the
3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) activity, a key enzyme of cholesterol biosynthesis (Kedar and Chakrabarti, 1982; Sharma et al., 2003) and/or by reducing the NADPH required for fatty acids and cholesterol synthesis (Chi, 1982).

In addition, CS fruits may improve hypercholesterolemia by modifying lipoprotein metabolism: enhanced uptake of LDL by increasing LDL receptors (Slater et al., 1980) and/or by increasing the lecithin:cholesterol acyl transferase (LCAT) activity (Khanna et al., 2002) which may contribute to the regulation of blood lipids. LCAT plays a key role in incorporating free cholesterol into HDL and transferring it back to VLDL or IDL, which is taken back by the liver cells (Rajlakshmi and Sharma, 2004). Caper may facilitate rapid catabolism of LDL. On the other hand, repeated oral administration of aqueous extract of CS fruits for 15 days produced a significant decrease in plasma triglycerides in both normal and STZ diabetic rats. The observed hypotriglyceridemic effect may be due to a decrease of fatty acids synthesis (Bopanna et al., 1997), enhanced catabolism of LDL, activation of LCAT and/or inhibition of acetyl-CoA carboxylase (Mc Carty, 2001) and production of triglycerides precursors such as acetyl-CoA and glycerol phosphate.

The marked hyperlipidemia that characterizes the STZ diabetic rats (Riyad et al., 1988; Choi et al., 1991) seems to be a consequence of un inhibited action of lipolytic hormones on adipose tissue. Since insulin inhibits adipose tissue hormone-sensitive lipase and reduces lipolysis, CS may mimic insulin action. No significant change in plasma insulin concentrations was noted in both normal and STZ diabetic rats after CS treatment (Eddouks et al., 2004). It seems that CS reduced plasma cholesterol and triglycerides levels without stimulating insulin secretion. It is well known that the level of glycemic control is the major determinant of plasma VLDL and triglycerides levels. Previously, we have reported that CS produced a potenti antihyperglycemic activity in STZ diabetic rats (Eddouks et al., 2004), so the strong hypolipidemic effect of CS fruits could also be mediated by the improvement of glycemia.

Chemical studies on CS have reported the presence of alkaloids, flavonoids and glucosinolates as main chemicals constituents (Brevard and Brambilla, 1992; Sharaf et al., 2000; Calis et al., 2002). One or more of these chemical compounds of the plant is/are also likely to have contributed to the observed hypolipidemic activity of aqueous extract of CS fruit. Flavonoids are plant polyphenols found frequently in fruits. A myriad of nutritional benefits has been attributed to these phytochemicals. It has been reported that administration of these compounds to hypercholesterolemic and hypertriglyceremic rats evokes a significant lipid lowering activity and improves dyslipidemia (Kenyi et al., 2001; Anila and Vijayalakshmi, 2002). The intervention of other phytochemical constituents as bioactive hypolipidemic agents is not excluded.

In conclusion, our study demonstrated that repeated oral administration of CS fruits for 15 days evokes a beneficial effect on the hyperlipidemia associated with hyperglycaemia in. This finding supports its use by the Moroccan population for the treatment and management of diabetes mellitus and cardiovascular diseases. This implies that CS fruits consumption can prevent or be helpful in reducing the complications of dyslipidemia associated with diabetes. Finally, the precise mechanism(s) and site(s) of this activity and the active constituent(s) of CS fruits involved are still to be determined in addition to toxicological studies.

Acknowledgements

We would like to extend our thanks to the “Comité Inter Universitaire Maroco-Français, Action intégrée N° MA/ 03/83” and Moroccan Government for supporting this work.

References


Antimalarial remedies in French Guiana: A knowledge attitudes and practices study

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Abstract

A “knowledge attitudes and practices” study about malaria treatments was undertaken in French Guiana, along with an ethnopharmacological study. One hundred and seventeen people from five different groups and nationalities (Créole, Palikur, Galibi, Brazilian, and European) answered the questionnaire. The results were analysed using univariate and multivariate statistical analysis.

First, we evaluated the overall knowledge about malaria from the interviewed people. According to bio-medical concepts, we noticed that they have a good knowledge of this illness. Secondly, we studied the treatment used by sick people during their last malaria attack. We demonstrated that, although bio-medical treatment is available in this area, people use both modern drugs and traditional remedies. Finally, preventive attitudes have been examined. One-third of the interviewed people drink regularly some herbal remedy to prevent febrile illnesses and malaria, thus displaying a strong concern about this disease. The ethnopharmacological study highlighted the frequent use of traditional remedies, along with their mode of preparation and administration. A total of 34 different species (both from flora and fauna) have been registered as antimalarial. Twenty-seven are used for curative purposes, 20 as preventive and 13 of them are used for both purposes. *Quassia amara* (Simaroubaceae) whose antimalarial activity has already been demonstrated was the species most frequently used as antimalarial for curative and preventive purposes.

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Keywords: French Guiana; Malaria; Treatment; Medicinal plant; Traditional medicine; KAP

1. Introduction

French Guiana is a 91,000 km² overseas department of France, located northeast of the South American continent between 2° and 6° North and 52°–54° West. Its frontiers with Brazil are formed by the River Oiapoque (east) and the Tumuc-Humac mountains (south), while the River Maroni delimits the western border with Suriname. The average temperature ranges from 26 to 28 °C all year round, with two marked seasons: the dry one occurring from July to November and the rainy one from December to June. Humidity varies from 50 (dry season) up to 100% during the wet season. Mean annual precipitation on the coastline is 3000 mm (Groussin, 2001). Floristically, French Guiana belongs to the Guianas floristic province, delimited east by the River Amazon and west by the River Orenoque. More than 90% of French Guiana territory is covered by equatorial forest, and other types of vegetation include savannahs, swamps, and mangroves. Actually, approximately 5200 phanerogamic species have been registered (De Granville, 2001).

Due to its history and geographic location, this French department is a mosaic of ethnic groups. The overall population is estimated 170,000 (Grenand and Grenand, 2001). Amerindians constitute the first inhabitants and belong to three distinct linguistic groups: the Tups-Guarani (Wayampi and Emerillon), the Arawak (Palikur and Arawak) and the Karib (Galibi and Wayana). During the 17th Century, an-
other community the “noirs marrons” (including the Ndjuka, Aliku, Saramaka, Paramaka ethnic groups) settled down in French Guiana and today they number around 15,000 people (Price and Price, 2003). Asian people and European people are also important communities. The Créoles (from French Guiana or originating from other part of the Caribbean zone) represent the highest part of the population. Finally, Haitian, Brazilian, and people from Suriname represent almost one-third of the overall population (Charrier, 2002).

This department is severely affected by endemic malaria. It is estimated to be one of the South American countries where the highest endemic prevalence exists, together with Guiana and Suriname. Every year, between 3500 and 5000 malaria cases are recorded (Carme and Venturin, 1999). Case repartition depends upon the place, most of them occurring along the Oiapoque (higher prevalence of *Plasmodium vivax*) and Maroni (higher prevalence of *Plasmodium falciparum*) boarding river (Huguet and Clausse, 2003) (see Fig. 1). Although epidemiological factors are unfavourable (in-land difficult access, constant and unpredictable migration of the population, high prevalence of *Plasmodium falciparum*, high-chemoresistance level), the malaria mortality rate is very low (no more than five cases a year). It is assumed that good health coverage, free access to diagnostic and treatment, and specific vectors control program together with educational program strongly help in reducing the impact on the population (Anonymous, 2002).

Within our research program aiming to detect new natural substances with antimalarial activities (Bourdy et al., 2004; Deharo et al., 2001, 2002), we became interested in the biological evaluation of indigenous phytotherapeutic preparations, rather than solvent-based plant extracts. This re-orientation of our work is in accordance with new trends in this field, as displayed by recent publications (Willcox et al., 2004) and WHO directives (WHO, 1998, 2002). The fact that malaria affects the world’s poorest, and that many treat themselves with herbal medicine because it is more available and affordable, proves the urgency of antimalarial programs that would detect local remedies with the greatest efficacy. These remedies should be investigated thoroughly, and could be promoted in other areas where the relevant plant exist or could be cultivated (Willcox and Bodeker, 2004; WHO, 1997). In a long-term program, questions to be addressed here are: which plant species are used, how widely they are used, and which one should be prioritized for further research (Willcox et al., 2004)? In order to answer these questions, we started a KAP (knowledge attitudes and practices) survey in French Guiana, applied to 117 people, mainly directed to assess disease perception and knowledge, treatment seeking...
attitude, use of therapeutic and prophylactic treatments. This survey was analysed using correspondence analysis procedure, a specific statistical analysis routinely used in epidemiological studies (Murgue et al., 1999). The following reveals the results of this survey with emphasis on the treatment followed and the traditional remedies used, both preventives and curatives. Other issues, such as validation of activity, will be dealt in following papers.

2. Methodology

The study encompassed two different sections: the first one was strictly a KAP questionnaire; the second part was based on the compilation of traditional remedies used by informants.

2.1. The KAP questionnaire

The KAP questionnaire was previously tested in a pre-survey including 20 people. This pre-survey allowed us to check if the questions were well understood, and all together pertinent to our project. Minor corrections were made accordingly, so that the questionnaire is in a definitive form.

The last version included open and closed questions, and was divided in seven parts (available from GB).

They were as follows:

1. Survey data.
2. Sociological data.
3. Knowledge of malaria. This part aimed to define what the word malaria meant to the interviewed people. Questions were oriented towards numerous symptoms encountered when a person was suffering from this disease. People were also invited to speak freely on this topic. This preliminary approach ensured us to reduce possible misunderstanding between local conception of the disease and its bio-medical definition (Willcox and Bodeker, 2004; Espino et al., 1997; Gessler et al., 1995).
4. Characteristics of the last malaria crisis (if any) and diagnosis/treatment seeking attitudes.
5. Treatment used during the last malarial crisis.
6. Estimation and comparison of the different treatments available, definition of the ideal treatment (everyone was questioned).
7. Context of use of the preventives remedies. In this part, we focused on self-medication practices used in order to avoid malaria or fever. Generally epidemiological studies try to quantify some risk-related attitudes (Quinones et al., 1998; Snow et al., 1996) but preventive measures, such as regular ingestion of some plant remedies, are rarely documented nor taken into account except, maybe from an ethnopharmacological point of view (Brandao et al., 1992). Within this survey, it seemed pertinent to focus on these practices, keeping in mind that these prophylactic preparations might have an impact on the malaria incidence. Other preventive attitudes (use of bed nets, etc.) were not recorded.

The questionnaire was usually given at the house of the interviewed people, sometimes outside, in an open place. During interviews with people who did not speak French, an interpreter was used.

2.2. Ethnopharmacological study

Preventive and curative recipes were recorded with as many details as possible. Four parts of herbarium vouchers for each species mentioned were collected. Vouchers were deposited in French Guiana Herbarium (Huguet and Clause, 2003). The villages of Saul, Ouanary, Saint-Georges and Kamuyene were selected as places of study (see Fig. 1).

2.4. Choice of the interviewed population

Places of study were selected according to their facility of access, and also their epidemiological status in order to have a representation of different transmission zones, sporadic and medium-high, as classified in French Guiana (Huguet and Clause, 2003). The villages of Saul, Ouanary, Saint-Georges and Kamuyene were selected as places of study (see Fig. 1).

2.5. Statistical analysis

Data have been recorded using Excel® software.

We made univariate and multivariate statistical analysis. The goal of the univariate analysis is to analyse all answers of each question in the questionnaire. Mean, median and variance are calculated for assessing the distribution of each quantitative variable. Proportions are calculated for expressing the results of each category of answer of the questions in the questionnaire.

The multivariate statistical analysis is a correspondence analysis procedure (CAP), which allows assessing the particular link of several variables to each other. Correspondence analysis procedure is a multivariate technique that converts tables of qualitative data into graphical displays in which rows (individual cases) and columns (variables categories) are depicted as labelled points on a graph (Deparis et al., 1999; Murgue et al., 1999). Instead of trying to compare them using proportions in several analysis, we broke down the measures of association between variables into a number of components, or “factors” represented by axes. The coordinates of
each individual’s case and each variable’s category on these axes are calculated and computed, so that each successive axis or factor accounts for a decreasing portion of the total association between variables, as represented by Pearson X² statistics or, in other words, a decreasing portion of the total.

Due to the mechanism of this analysis, the first calculated factor (F1) contains more information than the second (F2), the second more than the third (F3), and so on. Therefore, the first two first factors are the most informative because they contain the largest portion of the information resulting from the analysis of the questionnaires. This permits most of the information included in the data to be graphically presented at the same time. In the plots, (i) the greater the distance from the origin indicates the greater importance of the categories of the variable in the analysis, (ii) the proximity of points representing categories from two distinct variables is interpreted as an association between these categories; e.g. the proximity of the points “take preventive remedies” and “age > 60 years” suggests an association which can be interpreted as people >60 years old often drink more preventive remedies than other people, (iii) the proximity of the category points from the same variable is interpreted as similarity in the mean presentation between the groups of people expressed in these categories; e.g. the proximity of the points “male” and “female” indicates similarity of the mean presentation independently of genders.

3. Results and discussion

3.1. Epidemiological study: analysis of the KAP questionnaire

3.1.1. Sociological data

A total of 117 people were interviewed. The sex ratio of the interviewed population was nearly 1:1 (61 men and 56 women). Median and average ages were both close to 40 years of age. The interviewed population included Galibis (14 people), Créoles (46), Palikur (35), Hmong (1), European (7) and Brazilian (14).

3.1.2. Knowledge of malaria

A great homogeneity was recorded in the answers to questions dealing with symptoms, transmission mode, and estimation of malaria severity. A large majority of informants responded in accordance with bio-medical definitions demonstrating strong knowledge of malaria. For the majority, the fever symptom is always associated with malaria, but chills, strong headache; diarrhoea, vomits and loss of appetite are also identified as possible accompanying symptoms.

A very interesting fact is that 80% of the interviewed people declared that malaria was different from fever: malaria is recognized as a disease of its own and consequently, people said that malaria treatment is different from fever treatment.

The mode of transmission is also well known, 96% of the people quoting the mosquito as the agent responsible for malaria. The perceived severity of this disease is underlined by the fact that 115 people said that malaria could be fatal, both for children and adults.

3.1.3. Characteristics of the last malaria crisis, and diagnosis/treatment seeking attitudes

Seventy-two people stated that they did suffer a malaria crisis, at least once in their life. The Galibis, Brazilian and Palikurs, along with some non-native people of the area where the interview took place, appeared to have a higher risk of catching malaria. They represent the mobile population as opposed to the rest (Créoles, Europeans) who travel few inland, if not at all. Risk of catching malaria is the same for both sexes, and sick people can suffer many crises, between two and five.

Almost everyone suspecting a malaria crisis went directly to the nearest health centre for a diagnosis set. Everyone, but two informants, mentioned that the diagnostic method used to confirm a malaria crisis was a smear test, which is routinely made in French Guiana. Only four people did not go to the health centre because they quickly made their own prompt diagnostic based upon the occurring symptoms. More than half (51%) of the sick people remembered what type of parasite they got infected with during their last crisis, Plasmodium falciparum or Plasmodium vivax. The compilation of both data confirms that informants have a good knowledge of malaria, not only because they live in endemic area, but also because of educational and preventive measures undertaken in French Guiana.

3.1.4. Treatment used during the last malaria crisis

Seventy-two informants said that they had been sick at least once. Twenty-seven declared that they had treated themselves with modern drugs alone and three had used traditional remedy alone. The other 42 had used a combination of medicinal plants and modern drugs. When traditional remedies were used in combination with modern drugs, we recorded different patterns of association: (i) traditional remedies are administered at the same time as modern drugs. Sometimes, the administration of traditional remedies overpasses the time of drugs, and the length of this type of treatment is of 15 days approximately, (ii) treatment starts with modern drugs alone. Then, traditional remedies are administered, for approximately 7 days after the last tablet, (iii) same as above but there are a few days (from 1 week up to one month) of break between both treatments, (iv) treatment starts with traditional remedies alone (two cases). In both cases of traditional remedy treatment, it was followed by a modern drug treatment, because in one case the Geissospermum sp. bark decoction was too bitter to be drunk, and in the other case, the thinning effect of Phyllanthus amarus did not last. After one week of the Phyllanthus treatment, fever rose up again, and prompted the patient to change his treatment for modern drugs.
Throughout the correspondence analysis procedure, we tried to better define who were the people who relied on traditional remedies to cure their last malaria crisis. We found out that the choice of traditional remedies was not dependent upon sociological factors (i.e. ethnic group, place of residence, sex, age, education level, marital status) but was dependent upon the fact of having suffered a previous episode of malaria before the last crisis; in other words, the occurrence of a first malaria crisis shapes the next treatment and orients patients towards the use of traditional remedies.

3.1.6. Context of use of preventive remedies

For a high majority of informants, modern drugs are perceived as very efficient, acting very fast. The efficiency of modern drugs is, therefore, considered essential, but complementary. "Modern drugs cure, but plants heal". The role of modern drugs along with plants and it was often quoted that it was easy to find, free, and impair relapses. In fact, the ideal treatment method is very much appreciated and is a conclusive proof of activity for everyone. Nevertheless, modern drugs are perceived as potentially toxic, and are perceived to have strong damaging incidence on hepatic functions, thus enhancing the toxic effect of malaria itself on the liver.

On the other hand, traditional remedies are said to be efficient, but for the majority of the interviewed people they are perceived more as "liver and body purifier" and "cleansing body impurities". It was mentioned that both, plants and modern drugs are easy to find, free, and impair relapses. In fact, the ideal treatment as defined by all our informants, is a combination of modern drugs along with plants and it was often quoted that "Modern drugs cure, but plants heal". The role of modern drugs is, therefore, considered essential, but complementary to plants, warrant of true healing.

3.1.5. Estimation and comparison of the different treatments available, definition of the ideal treatment

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3.1.6. Context of use of preventive remedies

In French Guiana and in the French West Indies, especially within the Créole ethnic group exists the tradition of the bitter ("amer" in French), initially an alcoholic drink (Grenand et al., 1987; Vilayleck, 2002). A bitter is basically a preparation made out of ingredients selected upon their organoleptic characteristic of strong bitterness. Many therapeutic virtues are attributed to these ingredients, i.e., stomachic, digestive, cholagogue, choleric, liver protective, appetite enhancer, or vermifuge properties. This way of selecting medicinal plants upon organoleptic characteristics has also been recorded in many places, and theoretically demonstrated by Leonti in Mexico (Leonti et al., 2001).

In French Guiana, a traditional bitter is made by alcholic maceration (in rum, locally called taffia, or in another alcoholic beverage) of selected ingredients for a few weeks. When ready, a small liquor glass is drunk every now and then and sometimes, many times a week. Species used to prepare bitters are selected depending upon their availabiliy and personal taste. Generally, the bitter is prepared and drunk at home because it is different from its aromatic version, which is drunk at the bar, called décottage, richer in fragrance and less bitter species. For people who do not wish to drink alcohol, a bitter can be prepared simply by boiling plants parts. Finally a bitter, among the Créoles is also a three-step sequence of administration of different plants preparations (namely rafraîchis, lok, purge), especially to children in order to get rid of worms and parasites. In that very case, plants are totally different from the previous ones (Grenand et al., 1987, and our observations). Underlying this practice is the idea that a bitter body (skin and blood, outside and inside) is much stronger and is well protected against any attack; this idea has been expressed many times by our informants, whatever their ethnic origin.

During our interviews, we discovered that repeated administration of preventive remedies was also performed within other ethnic groups, especially among Palikur and Galibi. In the Palikur group, the word tisvine (which is used to name all things considered as unpalatable or bitter) was used for these preparations, perceived as a special class of remedies, characterised also by their bitterness. In fact, in French Guiana and among Galibi, Palikur, and Créole people, bitter seems to be a generic term, passed over people, used for any plant-based preparation administered preventively.

From our questionnaire, the main reason alleged to drink bitters were in order of importance: stay healthy, prevent worms attack, and prevent malaria and fever. One-third of the interviewed people (38) declared expressly that they did drink bitters regularly for that last purpose.

For those who drank preventive traditional remedies against malaria and fever, the frequency of administration of these preparations could vary strongly, but on average, a traditional preventive remedy is taken every 28 days. Anyhow, for some people, these prophylactic preparations are considered efficient only if they drank some every day, this being especially necessary if there is a malaria epidemic running around. Their action was compared to be like “a vaccine against fever and malaria“. Unlike, others consider it is not good to take these preparations daily because when a strong crisis arises, “their body will not respond” because it is too accustomed. They say that the “strong”, the “bitter” must be used sparingly otherwise it loses its power.

Also, though many informants mentioned the fact that drugs delivered in the pharmacy/health centre can also be used for prophylaxis, none of them use them for such purposes; preventive remedies are always local recipes.

Using the correspondence analysis procedure, we discovered that bitter self-administration against fever and malaria was not dependent upon sociological factors, but was dependent upon the occurrence of a malaria crisis in the informant’s life, and also, was correlated with the administration of bitter during childhood as a family habit. It’s as if the informants that already got malaria, had a protection that was recognized necessary and actively looked for. This protection-seeking attitude is part of a tradition and perpetuates in adult life.
<table>
<thead>
<tr>
<th>Species</th>
<th>Part used</th>
<th>In curative treatment, used alone or in combination&lt;sup&gt;a&lt;/sup&gt;</th>
<th>In preventive treatment, used alone or in combination&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aristolochia stahelii</em> O.C. Schmidt</td>
<td>Stem</td>
<td>–</td>
<td>Used alone</td>
</tr>
<tr>
<td><em>Aristolochia trilobata</em> L.</td>
<td>Leaf</td>
<td>Always in combination and preferably with <em>Geissospermum</em> spp.</td>
<td>Always in combination and preferably with <em>Geissospermum</em> spp., <em>Quassia amara</em>, <em>Tinospora crispa</em>, <em>Eryngium foetidum</em>, <em>Aristolochia trilobata</em>, <em>Curcuma longa</em></td>
</tr>
<tr>
<td><em>Artemisia spp.</em> A.</td>
<td>Leaf</td>
<td>Always in combination and preferably with <em>Geissospermum</em> spp., <em>Quassia amara</em>, <em>Tinospora crispa</em>, <em>Eryngium foetidum</em>, <em>Aristolochia trilobata</em>, <em>Curcuma longa</em></td>
<td>Always in combination and preferably with <em>Geissospermum</em> spp., <em>Quassia amara</em>, <em>Tinospora crispa</em>, <em>Eryngium foetidum</em>, <em>Aristolochia trilobata</em>, <em>Curcuma longa</em></td>
</tr>
<tr>
<td><em>Acapana triplinervia</em> (Vahl.) R. King and H. Robinson</td>
<td>Aerial part</td>
<td>Always in combination</td>
<td>–</td>
</tr>
<tr>
<td><em>Borbonia guianensis</em> Aubl.</td>
<td>Leaf</td>
<td>Always in combination</td>
<td>–</td>
</tr>
<tr>
<td><em>Campomanesia aromatica</em> (Aubl.) Griesch or <em>Campomanesia grandiflora</em> (Aubl.) Sagot.</td>
<td>Leaf</td>
<td>Always in combination and preferably with <em>Siparuna</em> spp.</td>
<td>–</td>
</tr>
<tr>
<td><em>Carica papaya</em> L.</td>
<td>Root, leaf</td>
<td>Always in combination and preferably with <em>Quassia amara</em>, <em>Citrus</em> sp. (lemon), <em>Euterpe oleracea</em></td>
<td>Always in combination and preferably with <em>Quassia amara</em>, <em>Carica papaya</em>, <em>Euterpe oleracea</em></td>
</tr>
<tr>
<td><em>Cirtus</em> sp. (orange)</td>
<td>Root, leaf</td>
<td>Always in combination and preferably with <em>Quassia amara</em>, <em>Carica papaya</em>, <em>Euterpe oleracea</em></td>
<td>–</td>
</tr>
<tr>
<td><em>Cypripedium citratus</em> Stapf.</td>
<td>Leaf</td>
<td>Always in combination</td>
<td>–</td>
</tr>
<tr>
<td><em>Eryngium foetidum</em></td>
<td>Whole plant</td>
<td>Always in combination</td>
<td>–</td>
</tr>
<tr>
<td><em>Euterpe oleracea</em> Mart.</td>
<td>Inner bark</td>
<td>Always in combination and preferably with <em>Siparuna</em> spp.</td>
<td>–</td>
</tr>
<tr>
<td><em>Eutrema longa</em> L.</td>
<td>Rhizome</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Euphorbia toxiferum</em> A. Chev.</td>
<td>Leaf</td>
<td>Always in combination</td>
<td>–</td>
</tr>
<tr>
<td><em>Hyptis pectinata</em> (L.) Poit.</td>
<td>Leaf</td>
<td>Always in combination</td>
<td>–</td>
</tr>
<tr>
<td><em>Lantana camara</em> L.</td>
<td>Leaf</td>
<td>Always in combination</td>
<td>–</td>
</tr>
<tr>
<td><em>Leonotis nepetifolia</em> (L.) R. Br.</td>
<td>Leaf</td>
<td>Always in combination</td>
<td>–</td>
</tr>
<tr>
<td><em>Lippia alba</em> (Mill.) N. E. Brown</td>
<td>Leaf</td>
<td>Always in combination</td>
<td>–</td>
</tr>
<tr>
<td><em>Mikania guaco</em> Humb. and Bompl. or <em>Mikania micrantha</em> Kunth.</td>
<td>Leaf</td>
<td>Always in combination and preferably with <em>Quassia amara</em>, <em>Gymnanthemum amygdalinum</em></td>
<td>Always in combination and preferably with <em>Quassia amara</em></td>
</tr>
<tr>
<td><em>Ocimum basilicum</em> L.</td>
<td>Whole animal</td>
<td>–</td>
<td>Always in combination</td>
</tr>
<tr>
<td><em>Petiveria alliacea</em> L.</td>
<td>Leaf</td>
<td>Always in combination</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> In curative treatment, used alone or in combination with other species; In preventive treatment, used alone or in combination with other species.
Unfortunately, the correspondence analysis procedure revealed that drinking bitters did not have any influence on the occurrence of malaria crisis. The study reveals that people who declared that they drank bitters regularly in order to avoid malaria are just as prone as the others to get it.

3.2. Ethnopharmacological study

3.2.1. Curatives remedies

Forty-five informants declared that they used a traditional recipe to cure their last malarial crisis. Thirty-nine species were mentioned to be used alone; they are Coutoubea spicata, Geissospermum spp., Mikania spp., Phyllanthus spp., Quassia amara, Senna spp. and Solanum leucocarpon.

In multi-ingredients recipes, we noticed that they were species more likely to be found together in combination; this is the case for Cymbopogon citratus, Lippia alba, Ocimum campechianum, and Citrus spp. (orange). Another combination frequently encountered is from Carica papaya, Euterpe oleracea and lemon roots.

Most frequently used species, such as Coutoubea spicata and Quassia amara, are used alone or they appear in combinations with almost any listed species; they can be considered as the most versatile species, being the stable element of the recipes. This is maybe due to their effective antimalarial activity (Moretti, 1986) though this still needs to be confirmed for Coutoubea spicata.

All recipes (but one, a concentrated juice of Mikania spp. soft leaves) are long-lasting decoction; generally, all ingredients are put in a large aluminium pot, cold water is poured over and is left to boil for a while. A dose is then taken from the aluminium pot, generally without removing plant material. The dose to be drunk varies from one to four cups (150 ml) daily, all day round, as water substitute. For preparations containing Picrolemma pseudocoffea, recognised by all informants as very toxic, and Plectranthus barbatus (designated as extremely bitter), the dose...
is a small coffee cup (50 ml) three times a day for a few days.

The warm decoction is also poured all over the patient's body, and this is called a “bath”. Some people, enhancing the symbolic aspect of this cure, explained that the preparation had to be passed all over the skin with the help of an old patient’s cloth previously soaked in the decoction, then thrown away. Warm “baths” must be administered at least twice a day.

Reasons behind administering warm remedies is that they cause profuse sweating which “makes the fever go out the body”, “helps eliminate malaria completely”, “drives malaria out of the body”, and “eliminates all impurities”.

Some of the species used as curative in this KAP study have already been retained for biological and chemical analysis, and a few of them displayed in vitro or in vivo activities. This is the case for Quassia amara (Ajayebba et al., 1999, Moretti, 1986). Geissospermum spp. (Muñoz et al., 2000), Tinospora crispa (Pachaly and Adnan, 1992), Picrodendron pseudocoffea (Moretti, 1986), Gymnanthemum amygdalinum (Huffman et al., 1997), Siparuna spp. (Jenett Siems et al., 1999), Zanthoxylum rhoifolium (Georges, 2002, DeFilipps et al., 2004). In regards to the other species, antimalarial activity still needs to be proved.

3.2.2 Remedies used for prevention

We retained for analysis only the last preventive remedies that have been taken by informants against malaria and/or fever. Thirty-eight different preventive recipes were recorded based on the use of 20 different species (listed in Table 1).

Eight species have been mentioned to be used as prophylactics alone, but preventive remedies are mainly multi-components recipes, made out of plants and one animal (a caterpillar). Two modes of preparation were recorded: alcoholic maceration or decoction. When prepared in alcoholic maceration, 

bitters have a significantly higher number of ingredients than when they are made out of water. We also revealed through the CAP that the ingredients combination within a recipe is related to the ethnic group. This confirms the previous statement: 

bitters are part of tradition, so 
bitters recipes are passed over generation within the family.

Most species used in alcoholic bitters are: Geissospermum spp. bark, Tinospora crispa stem, Aristolochia trilobata leaves, Quassia amara (bark or leaves), Picrodendron pseudocoffea (stem, leaves or roots), and Parides sp., a butterfly-caterpillar feeding on Aristolochia trilobata leaves. Sometimes, in these alcoholic bitters macerations other flavoured species might also be used, an aromatic additive such as Curcuma longa (rhizom) or Artemisia spp. (leaves). The latter species are never used in water.

Preventive remedies are administered orally and also, are rubbed all over the body (as “bath” in case of watery preparations and in friction for the alcoholic ones). An interesting fact is that, in opposition with curative remedies that have to be administered warm, preventive remedies are drunk cold, even at fridge temperature.

3.2.3 Preventive remedies versus curative remedies

Even though, it has been expressed frequently by our informants that “the plants which cure malaria are the same ones that can prevent it”, in fact, less than one-third (13 over 34) of the species are used for both purposes. This might be due to the fact that those were retained for analysis “malaria and/or fever preventive remedies”, thus increasing and widening the range of preventive species.

Digging deeper, we discovered that, among the 13 plants species indiscriminately as preventive and curative, their citation frequency in remedies varies greatly according to their use. In other words, even if a species is quoted for both uses, it has a strong tendency to be much more used for one or the other purpose. For example it was quite a surprise to see that one of the uttermost renowned antimalarial Amazonian species, Geissospermum spp. (Mülliken, 1997; Grenand et al., 1987; Brandao et al., 1992; Muñoz et al., 2000; and many others) was so poorly employed as curative, but on the contrary, used much more in favour as preventive. Same thing for Mikania spp., Tinospora crispa, all of them being species having a strong antimalarial reputation, but in fact, in the frame of our study, poorly used as curative. From our point of view, this reflects the difference between theoretical knowledge and real practice.

4. Conclusion

Previous ethnopharmacological studies have been undertaken in French Guiana in order to document related traditional knowledge, and also to detect plants with promising biological activity (Andreu-Verin, 1998, Berton, 1997; Grenand et al., 1987; Hay, 1998; Lecat, 2002; Fleury, 1991; Leduc, 2002), and especially plants with antimalarial potential (Tandeur et al., 1985; Moretti, 1986; Sauvain, 1989).

From some already published ethnopharmacological data, it appears that there are broadly around 150 species used against malaria and/or fever in French Guiana (Grenand et al., 1987). In Latin America, another study (Mülliken, 1997) reported 956 species with antimalarial or antipyretic reputation. Oliveira et al. (2002) reviewed the Brazilian ethnomedical literature and reported that, in this country, 197 species are used for the treatment of malaria and fever.

In our study, only thirty-four species have been used as antimalarial, and this number is more in accordance with the survey undertaken in northern Brazil by Brandao et al. (1992), in which 4689 people declared they used 40 plants as malaria curative (and one as preventive). This discrepancy between
the numbers of plants displayed in different studies might be
due to the fact that the literature compiled plants used against
malaria and plants used against fever, thus increasing greatly
the number of species. In our study, it was clear that because
malaria is perceived differently than fever, treatments used to
treat malaria are very specific and more likely to prove some
efficiency. This is of importance when it comes to selecting
accurately plants for future antimalarial study.

As far as we know, no study was fully dedicated to the
analysis of antimalarial treatment really used by the popu-
lation, nor in Guiana, nor elsewhere. Also, very few studies
are centred on the preventive plants-based methods used by
the population, though some studies try to correlate the vari-
ations in the diet with the incidence of malaria cases (Etkin
and Ross, 1983). We are clearly aware that this study should
be considered as preliminary, and should be extent to a much
larger part of the population, including more people from dif-
ferent places, especially people living along the Maroni river
considered as a zone of permanent high transmission with a
predominance of Plasmodium falciparum cases (Huguet and
Clauzou, 2003).

Further studies should be oriented to the biological evalua-
tion of some remedies in the form they are prepared locally by
the population (mainly water extract). The evaluation should
be done also on some multi-components remedies, in order to
evaluate the possible synergism of action of the constituents
of the preparation. Preventive activities of remedies should be
looked for. The study of the interaction between remedy
and modern antimalarial drugs should also be undertaken.

This last analysis would be a good indicator in regard to the
estimation of possible benefits gained by the local population
when using both treatments in combination.

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We would like to express our thanks to people who were
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Ethnopharmacological communication

Inhibitory effects of Okbyungpoong-Gamhmi on anaphylactic responses

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Abstract

We investigated the effect of a herbal formulation Okbyungpoong-Gamhmi (OG) on mast cell-dependent anaphylactic reactions by intra-rectal administration. OG concentration dependently inhibited compound 48/80-induced anaphylaxis-like response and ear swelling response with doses of 0.01–1 g/kg. OG also inhibited the passive cutaneous anaphylaxis at the same concentrations. The histamine release induced by compound 48/80 or IgE from the rat peritoneal mast cells was reduced by 64.2 and 63.6%, respectively, at 1 g/l. These results provide evidence that intra-rectal therapy of OG may be beneficial in the treatment of anaphylactic response.

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Keywords: Intra-rectal administration; Ear swelling response; Passive cutaneous anaphylaxis; Systemic allergic reaction; Atractylodis japonica

1. Introduction

1.1. Plant

The traditional herbal formulation Okbyungpoong-Gamhmi (OG) consists of Atractylodis Rhizoma Alba (Atractylodis japonica Koidzumi, Compositae), Astragalii Radix (Astragalus membranaceus Bunge, Leguminosae), Ledebouriellae Radix (Ledebouriella seseloides Wolf, Umbelliferae), Cinnamomi Ramulus (Cinnamomum cassia Blume, Lauraceae), Sutellariae Radix (Sutellaria baikalensis Georgi, Labiatae), Perillae herba (Perilla frutescens Britton var. acuta Kudo, Labiatae), Moutan Cortex Radicis (Paeonia moutan Sims, Paeoniaceae), Adenophorae Radix (Adenophora trypylla var. japonica Hara, Campanulaceae), Altheae Cortex (Althea japonica Durazz, Leguminosae), and Magnoliae Flos (Magnolia denudata Desrousseaux, Magnoliaceae). Each of the dried individual herbs were purchased from Keum-dang yakupsa, a herbal drug company, and authenticated by Professor H. J. Song, Wonkwang University.

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Inhibitory effects of Okbyungpoong-Gamhmi on anaphylactic responses
Atractylosid Rhizoma Alba, Cinnamomi Ramulus, Perillae Herba, and Magnoliae Flos (Studeninkova and Khaletskii, 1966; Xu et al., 2001; Wilson et al., 1977; Nagasawa et al., 1969), saponins from Astragali Radix (Yu, 1986), coumarins from Ledebouriellae Radix (Jin et al., 1992), flavonoids from Scutellariae Radix (Takido et al., 1975), phenolic compounds from Moutan Cortex Radicis (Harada and Yamashita, 1969), triterpenoids from Alizziae Cortex, alkaloids from Scutellariae Radix (Takido et al., 1975), phenolic compounds from Ledebouriellae Radix (Kinjo et al., 1992), and alkaloids from Adenophorae Radix (Asano et al., 2000) have been previously reported.

2. Material and methods

2.1. Reagents

Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), o-phthalaldehyde (OPA), Evans blue, fetal bovine serum, o-minimum essential medium, and metrizamide (density = 1.120 g/ml) were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Animals

The original stock of male ICR mice (4-week age) and male Sprague-Dawley rats (7-week age) were purchased from the Dae-Han Experimental Animal Center (Taejon, Korea) and the animals were maintained at the College of Pharmacy, Wonkwang University. The animals were housed from 5 to 10 per cage in a laminar air-flow room, maintained at a temperature of 22 ± 2 °C and relative humidity of 55 ± 10% throughout the study.

2.3. Preparation of OG

Extract of OG was prepared by decocting the dried prescription of herbs with boiling distilled water. The extraction decocted for approximately 3 h has been filtered, lyophilized, and kept at 4 °C. Yield of lyophilized powder was 16% (w/w). OG was administered to mice in liquid state dissolved in saline. OG formula contains 11.8% of Atractylosid Rhizoma Alba, 5.9% of Astragali Radix, 7.1% of Cinnamomi Ramulus, 7.1% of Scutellariae Radix, 10.5% of Perillae Herba, 10.5% of Moutan Cortex Radicis, 17.6% of Adenophorae Radix, 11.8% of Magnoliae Flos. Components of OG were analyzed by HPLC. The chromatographic system consisted of a pump (Waters 2690XE HPLC pump), and a UV-detector (Waters M996 PDA detector). A Nova-pak C18 (3.9 × 150) column (waters, U.S.) was used. Acetomrilite-H2O-acetic acid (100:900:10) was used as the mobile phase. Detection of the peaks at 254 nm and the sensitivity was set of 0.50 AUFS. The injection volume was 30 μl and flow rate was 1.0 ml/min. Standard solution was prepared by dissolving in distilled water (10 mg/100 ml). The solution was filtered through 0.45 μm membrane filter and applied to HPLC. Well known major chemical constituents of individual herbs include atractylon (Atractylosid Rhizoma Alba), astragaloside (Astragal Radix), psoralen (Ledebouriellae Radix), cinnamaldehyde (Cinnamomi Ramulus), baicalin (Scutellariae Radix), perillaldehyde (Perillae Herba), paconol (Moutan Cortex Radicis), and cineole (Magnoliae Flos).

2.4. Compound 48/80-induced systemic anaphylactic reaction

Mice were given an intraperitoneal (i.p.) injection of 7 mg/kg compound 48/80 to evoke systemic anaphylactic reaction. The OG was dissolved in saline and administered rectally 1 h before the injection of compound 48/80. Mortality was monitored for 1 h after induction of the fatal response.

2.5. Ear swelling response

Ear swelling response was investigated by the method previously used (Hong et al., 2001). Compound 48/80 was freshly dissolved in saline (5 g/l) and injected intradermally into the dorsal aspect of a mouse ear (100 μg/site, 20 μl) using a microsyringe with a 28-gauge hypodermic needle. Ear thickness was measured with a digital micrometer (Murutoyo, Japan) under mild anesthesia induced by i.p. injection of 1:1 mixture (50 μl) of ketamin (1 mg/ml) and xylazine hydrochloride (23.32 mg/ml). Mice were kept in immobility state during the measurement. Ear swelling response represented an increment in thickness above baseline control values. Ear swelling response was determined 40 min after compound 48/80 or vehicle injection.

OG was administered rectally 40 min before the compound 48/80-injection (100 μg/site). The values obtained would appear to represent the effect of compound 48/80 rather than the effect of the vehicle injection, since the ear swelling response evoked by physiologic saline returned to almost baseline thickness within 40 min.

2.6. Preparation of rat peritoneal mast cells (RPMC)

RPMC were isolated as previously described (Jippo et al., 2000). Mast cells were separated from the major components of rat peritoneal cells (i.e., macrophages and small lymphocytes) according to the method described earlier (Yurt et al., 1977). More than 97% of the cells were viable as judged by Trypan blue uptake.

2.7. Histamine release

Histamine release was assayed as previously described (Kim et al., 2003). The histamine content was measured by a reported method of the OPA spectrofluorometric procedure (Shore et al., 1959). The fluorescence intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer.
inhibition percentage of histamine release can be calculated using the following equation:

\[ \% \text{Inhibition} = \left( \frac{\text{histamine release with OG} - \text{histamine release with OG}}{\text{histamine release without OG}} \right) \times 100 \]

2.8. PCA reaction

The effect of PCA was investigated as another way to evaluate anti-anaphylactic property of OG (Hong et al., 2001). OG dissolved in saline was administered intra-rectally 1 h prior to the challenge with antigen. The DNP-HSA was diluted in phosphate-buffered saline (PBS). The mice was injected intradermally with 250 ng/site of anti-DNP IgE into each of the three dorsal skin sites that had been shaved 48 h earlier. DNP-HSA was applied to the mice together with Evans blue as a mixture. The pigment sites on the dorsal skin were punched out. Dye was extracted from the skin with 1 ml of 1.0 N KOH and 9 ml of a mixture of acetone and phosphoric acid (with the ratio of 5:13) (Katayama et al., 1978). The cutaneous reaction can be quantified by measuring the concentration of leaked dye. Quantified dye amount measured from the OG-administered mouse was compared with that from the vehicle-administered mouse. The absorbance at 620 nm of the extract was measured in spectrophotometer and the amount of dye was calculated with the standard line of Evans blue.

2.9. Statistical analysis

The results were expressed as mean ± S.E. for the number of experiments. Statistical significance was compared between each treated and control group by the Student’s t-test. Results with \( P < 0.05 \) were considered statistically significant.

3. Results

The in vivo model of systemic anaphylactic shock reaction was designed to assess the contribution of OG in anaphylactic reactions. As shown in Table 1, an oral administration of 200 mg/kg as a control (i.e. compound 48/80 injection without using OG) proved a 100% fatal shock. When the OG was administered through rectum (at doses ranging from 0.01 to 1 g/kg) 1 h before compound 48/80 injection, the mortality was reduced dose-dependently. OG-inhibited compound 48/80 induced mortality by 42.7 and 75% at doses of 0.1 and 1 g/kg, respectively. It has been reported that the compound 48/80 induced mortality by 42.7 and 75% at doses of 0.1 and 1 g/kg, respectively. OG-inhibited compound 48/80 was reduced in a dose-dependent manner. OG inhibited compound 48/80-induced ear-oedema response by 26 and 32.3% at the doses of 0.1 and 1 g/kg, respectively.

4. Discussion and conclusion

Stimulation of mast cell with compound 48/80 initiates the activation of signal-transduction pathway that leads to histamine release. Some studies have shown that compound 48/80 and other polybasic compounds are able to activate G proteins (Moulin et al., 1990a, 1990b). The membrane permeability increase may be an essential factor for the release of the mediators from mast cells. We assume that OG might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80 and regulate the degranulation of the mast cells in mouse skin by stabilizing membrane fluidity.

The OG-administered mice were protected from IgE-mediated local allergic reaction. This was supported by the effect of OG on PCA. OG also showed protection activity of
Table 1

<table>
<thead>
<tr>
<th>Dose of OG (mg/kg)</th>
<th>Systemic allergic reaction$^a$</th>
<th>Ear swelling response$^c$</th>
<th>PCA$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Compound 48/80 (mg/kg) Mortality (%)</td>
<td>Inhibition (%)</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>None (D.W.)</td>
<td>+ 100 + 2.093 ± 1.143 – 0 ± 3.5</td>
<td>– 0.9 ± 3.8</td>
<td>– 0.9 ± 3.8</td>
</tr>
<tr>
<td>10</td>
<td>+ 100 + 2.012 ± 1.04 4 5.7 ± 3.5</td>
<td>– 0.9 ± 3.8</td>
<td>– 0.9 ± 3.8</td>
</tr>
<tr>
<td>100</td>
<td>+ 75.0 + 1.550 ± 1.18 26$^*$</td>
<td>– 0.9 ± 3.8</td>
<td>– 0.9 ± 3.8</td>
</tr>
<tr>
<td>1000</td>
<td>+ 42.7 + 1.417 ± 1.13 32.3$^*$</td>
<td>– 32.8 ± 5.6$^*$</td>
<td>– 32.8 ± 5.6$^*$</td>
</tr>
</tbody>
</table>

Each table shows effect of OG on compound 48/80-induced systemic allergic reaction and on the PCA in mice, respectively.

$^a$ 200 μl distilled water (D.W.) or OG was intra-recetrally given at various doses 1 h before (n=6/group) the compound 48/80 injection. The compound 48/80 solutions were intraperitoneally given to mice. Mortality (%) within 1 h following the compound 48/80 injection is presented as the number of dead mice/total number of experimental mice.

$^b$ 50 μl D.W. or OG was intra-recetrally given at various doses 40 min before (n=6/group) the compound 48/80 injection. 20 μl of compound 48/80 (100 μg/site) were applied topically to the ears of mice. Each value represents the mean ± S.E.M. of three independent experiments. *P<0.05; **P<0.01, significantly different from the D.W. value.

$^c$ OG was administered intra-recetrally 1 h (n=6/group) prior to the challenge with antigen. Each value is presented as the mean ± S.E.M. of four independent experiments. *P<0.05; **P<0.01, significantly different from the D.W. value.

IgE-induced histamine release from RPMCs. Antigen stimulation of mast cells via IgE receptor elicits release of numerous mediators including histamine in minutes and cytokines in hours. Consequently, these mediators induce immediate or late hypersensitivity reaction. The mechanism of histamine release in mast cells is mainly for further studies.

In conclusion, our results obtained in this study proved that OG-inhibited both compound 48/80-induced anaphylaxis-like response and IgE-mediated anaphylactic response in vivo and in vitro in murine model. To our knowledge, this is the first report of mast cell-mediated immediate-type allergic reaction by OG.

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Ethnopharmacological communication

Alkaloids from *Boophane disticha* with affinity to the serotonin transporter in rat brain

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Abstract

Bulbs and leaves of *Boophane disticha* are used in South African traditional medicine in the treatment of anxiety. Crude extracts of the leaves have shown affinity to the SSRI site on the serotonin transporter in a radioligand binding assay. In this study, two compounds, buphanidrine and buphanamine, were isolated by bioassay-guided fractionation on VLC and preparative TLC. The structures of the compounds were determined by \textsuperscript{1}H and \textsuperscript{13}C NMR. Fractions were tested for affinity to the serotonin transporter in a binding assay using \textsuperscript{3}H-citalopram as ligand. The \textit{K}_i values of buphanidrine and buphanamine were 274 \mu M (\textit{K}_i = 132 \mu M) and 1799 \mu M (\textit{K}_i = 868 \mu M), respectively. The two alkaloids were also tested for affinity to the 5HT\textsubscript{1A} receptor, but only showed slight affinity.

Keywords: *Boophane disticha*; Buphanamine; Buphanidrine; \textsuperscript{3}H-Citalopram; Depression; Radioligand binding assay; Serotonin transporter; 5HT\textsubscript{1A}

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Plant leaves of *Boophane disticha* (L.f.) Herb (Amaryllidaceae) were collected in the Botanical Gardens, Pietermaritzburg, South Africa in March. A voucher specimen (Stafford 53 NU) is deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg.

Uses in traditional medicine scales from the bulb of *Boophane disticha* is traditionally used for numerous purposes, e.g., treatment of hysteria in young women (Van Wyk et al., 1997). The bulb has narcotic properties and leads to visual hallucinations in toxic doses (Du Plooy et al., 2001). In a screening of plants used for anxiety and depression for affinity to the serotonin transporter in rat brain, leaf extracts of *Boophane disticha* had high affinity for the SSRI site (Nielsen et al., 2004).

Abbreviations: DPA T, dipropylaminotetralin; SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitor

1. Materials and methods

1.1. Isolation

Hundred g fresh leaves of *Boophane disticha* were extracted with 70% ethanol (3 × 500 ml). The extract was fractionated on 150 g of Merck silica gel 60 in a vacuum column. Five hundred millilitres of each of following solvents were used as eluents: hexane; hexane:ethyl acetate 50:50; 25:75; ethyl acetate; ethyl acetate:methanol 90:10; 80:20; 70:30; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90; 2 × methanol; water. Active fractions from VLC (ethyl acetate:methanol 90:10 and 80:20) where dissolved in 100 ml 70% ethanol, adjusted
to pH 3 with 4% acetic acid and partitioned against di-
ethylether (3 × 100 ml). The pH was adjusted to 10 with
NaOH and the aqueous phase was partitioned against di-
ethylether (3 × 100 ml), ethylacetate (3 × 100 ml) and but-
anol (4 × 70 ml). The active fraction (diethylether from basic
partition) was separated on preparative TLC using ethyl ac-
etate:methanol:water (90:20:10) as mobile phase. The TLC
plate was detected under UV 254/365 nm and a small part of
the TLC plate was sprayed with Dragendorff reagent to detect
alkaloids. Five bands were scraped off the TLC, eluted with
methanol and tested for activity. The isolated compounds
were dissolved in CDCl3 and the structures elucidated by
1H NMR and 13C NMR.

1.2. Tissue preparation

All procedures were carried out at 0–4 °C. Whole rat
brains, except cerebellum, were homogenised with an Ul-
terra Turrax homogenizer in 1:10 (w/v) buffer (5 mM TRIS
base, 150 mM NaCl and 20 mM EDTA, pH 7.5). The ho-

<table>
<thead>
<tr>
<th>Position</th>
<th>1H</th>
<th>13C</th>
<th>COSY (H → H)</th>
<th>NOESY (H → H)</th>
<th>HMBC (C → H)</th>
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<td>1</td>
<td>4.30 d (1J1,2 = 5.6)</td>
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<td>2</td>
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<td>2</td>
<td>6.05 dddd (1J2,3 = 10.0, 1J2,1 = 5.6, 1J2,4 = 2.8, 1J2,6 = 1.9)</td>
<td>125.4</td>
<td>1, 3, 4β</td>
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<td>5.86 dddd (1J3,4 = 10.0, 1J3,2 = 4.6, 1J3,6 = 2.9)</td>
<td>127.0</td>
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<td>4</td>
<td>2.83 dddd (1J4,5 = 19.6, 1J4,6 = 5.6, 1J4,2 = 4.6, 1J4,3 = 1.9)</td>
<td>26.1</td>
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<td>3, 4β, 4a</td>
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<td>55.4</td>
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<td>9</td>
<td>1.99 dddd (1J9,10 = 12.3, 1J9,10endo = 8.7, 1J9,10exo = 12.3, 1J9,10endo = 1.3)</td>
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<td>2.12 ddd (1J10,11 = 12.3, 1J10,11endo = 10.9, 1J10,11exo = 8.2)</td>
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</table>

Table 1

NMR data for buphanamine (100 MHz for 1H, 600 MHz for 13C, CDCl3)
and 5 mM KCl, pH 7.5) and then centrifuged at 16,000 × g for 10 min. The supernatant was discarded and the pellet finally suspended in 120 ml buffer (50 mM TRIS base, 120 mM NaCl and 5 mM KCl, pH 7.5). This homogenate was kept at −70 °C until use.

1.3. [3H]-Citalopram binding assay

The method described by Plenge et al. (1990) was used with modifications (Nielsen et al., 2004). Two hundred microlitres of test solution were mixed with 50 μl of [3H]-citalopram (4 nM) and 50 μl of tissue suspension, respectively. Paroxetine (1.5 μM) was used for the determination of unspecific binding. The total binding of [3H]-citalopram was determined with a buffer blank. All samples were incubated for 2 h at 25 °C and then filtered under vacuum using glass fibre filters. After 24 h the radioactivity on the filters was determined by liquid scintillation. Specific binding was calculated as total binding minus unspecific binding. All experiments were done in duplicate.

1.4. 8-Hydroxy[3H]DPAT binding assay

Two hundred microlitres of test solution were mixed with 50 μl of 8-hydroxy[3H]DPAT and 50 μl of tissue suspension. Buspirone (1 μM) was used to determine unspecific binding. Subsequent procedures were as for the [3H]-citalopram binding assay.

1.5. Estimation of IC50 values and Ki values

The IC50 values were calculated using GraFit 5 from Erithacus Software utilising a full four-parameter equation. The Ki values, which is independent of ligand concentration and thus more useful for inter-lab comparison, was calculated by $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the ligand concentration (here 0.75 nM) and $K_d$ is 0.7 nM for citalopram.

2. Results

Two compounds, buphanidrine (7.6 mg) and buphanamine (5.6 mg) (Fig. 1), with activity on the SERT were isolated. Buphanidrine was identified by comparison with NMR data previously reported (Viladomat et al., 1995). NMR data for buphanamine are given in Table 1.

Structurally, buphanamine and buphanadrine have the benzo-1,3-dioxole moiety in common with the clinically used SSRI paroxetine, which could explain their affinity to the SERT. The hallucinogenic effects obtained after accidental or purposeful overdosing with Boophane disticha extracts (De Smet, 1996; van Wyk et al., 2002) indicate that the alkaloids reach the CNS.

In the SERT assay the IC50 values of buphanidrine and buphanamine were 274 and 1799 μM, respectively (Fig. 2.). The Ki values of the two compounds were calculated to be 132 μM for buphanidrine and 686 μM for buphanamine. For citalopram the IC50 value was 1.3 nM, giving a Ki value of 0.6 nM. Both buphanidrine and buphanamine bound to the 5HT1A receptor with low affinity, the IC50 values were 1203 and 2975 μM, respectively (Fig. 2.). For the SSRIs, and most other types of antidepressant drugs, it takes 2–4 weeks for the therapeutic action to develop. A leading hypothesis of this delayed pharmacological action is desensitisation of somatodendritic serotonin 1A autoreceptors (5HT1A) in the midbrain raphe, which act by reducing neuronal firing (Stahl, 1998; Holmes et al., 2002). A major goal of antidepressant development is to improve preceding drug classes with more rapid onset of antidepressant action. Therapeutic agents acting both by inhibition of serotonin reuptake and by inhibiting the action of 5HT1A autoreceptors might result in a more rapid onset of antidepressant action. Buphanidrine and buphanamine had only slight affinity to 5HT1A, eliminating the possibility of a dual-action system. Although the activities of buphanidrine and buphanamine on the SERT were lower than the activity of the clinically used SSRI citalopram, the activity supports the traditional use of Boophane disticha as a remedy for depression and anxiety.
Acknowledgements

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References