

African Journal of Pharmacy and Pharmacology

Volume 9 Number 37, 8 October, 2015
ISSN 1996-0816



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Full Length Research Paper

**Antimicrobial properties of the methanol leaf extract of
Vernonia glaberrima Welw. Ex O. Hoffm (Asteraceae)****Abdullahi, M. I.^{1*}, Uba, A.¹, Alebiosu, C. O.¹, Alhassan, A. M.¹, Mode, S.¹, A. Umar¹, Nasir, I.¹,
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Received 2 August, 2015; Accepted 3 September, 2015

The emergence of multi-drug resistant microbes necessitates a continuous search for newer, effective antimicrobial agents. The crude methanol leaf extract of *Vernonia glaberrima* was screened for its antimicrobial activity against pathogenic microorganisms including Methicillin resistant *Staphylococcus aureus*, Vancomycin resistant enterococci, *Listeria monocytogenes*, *Staphylococcus aureus*, *Helicobacter pylori*, *Campylobacter fetus*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Candida tropicalis* and *Candida stellatoidea* using agar diffusion and broth dilution methods. The result of the susceptibility test showed the extract (400µg) inhibited the growth of Methicillin-resistance *S. aureus*, Vancomycin resistant enterococci, *S. aureus*, *H. pylori*, *P. fluorescens* and *C. stellatoidea* with mean zone of inhibition range of 18 to 29 mm; the most susceptible organism was *S. aureus* (29 mm) and the least was the fungus, *C. stellatoidea* (18 mm). No activity was observed against *L. monocytogenes*, *C. fetus*, *P. vulgaris* and *C. tropicalis*. Sparfloxacin (5µg/ml) the standard antibacterial drug, had inhibitory activity against all the test organisms except *H. pylori*, *P. fluorescens*, *C. tropicalis* and *C. stellatoidea* while the standard anti-fungal drug, Fluconazole (5µg/ml), showed activity only on the two fungi species, *C. tropicalis* and *C. stellatoidea*. The Minimum inhibitory concentration (MIC) and the minimum bactericidal/fungicidal concentration (MBC/MFC) ranges for the extract were 5 to 20 mg/ml and 10 to 40 mg/ml, respectively. The results of this study suggest that the methanol leaf extract of *V. glaberrima* contains bioactive principles that have good antibacterial and antifungal activity, validating the antimicrobial use of the plant in traditional medicine.

Key words: *Vernonia glaberrima*, extract, antimicrobial, MIC, MBC, evaluation.

INTRODUCTION

The World Health Organization (WHO) has long recognized antimicrobial resistance (AMR) as a growing

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global health threat, and one of the important global strategies is a continuous search for effective antimicrobial agents (WHO, 2012).

AMR is driven by both appropriate and inappropriate use of anti-infective medicines for human, animal health and food production, together with inadequate measures to control the spread of infections (Goosens et al., 2005; Mathew et al., 2007; Orzech and Nichter, 2008; WHO, 2014). More than 2 million people become infected with bacteria that are resistant to antibiotics each year in the United States out of which about 23,000 die as a direct result of these infections, while many more people die from other complications related to antibiotic resistance (Centers for Disease Control and Prevention CDC, 2013). Although, the data for developing countries such as Nigeria is not very comprehensive, the mortality figures are expectedly higher (WHO, 2014).

Theoretically, bacteria will continue to develop resistance once exposed to any antimicrobial agent, thereby imposing the need for a permanent search and development of new drugs (Silver and Bostian, 1993). Natural products have been the most significant source of drugs and drug leads in history (Newman and Cragg, 2007). The emergence of multidrug resistance in human and animal pathogenic bacteria as well as undesirable side-effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin (Ahmed and Beg, 2001). *V. glaberrima* Welw. Ex O. Hoffm (Asteraceae), Shìwaákár-ján-gágári (Hausa language - N. Nigeria) is an erect shrub, 2 m high, found on hillside grassland in Guinea to Northern Nigeria, Western Cameroon and Central Africa to Angola (Burkill, 1997). It was reported to be used against malaria, migraine, psoric and dysmenorrhoea (Burkill, 1997). The leaves decoction is also employed traditionally in Nassarawa State, Northern Nigeria for treating pain, inflammation, vertigo and microbial infections (Personal communication). Preliminary phytochemical screening, acute toxicity studies and antidiabetic property of the plant have been reported (Abdullahi et al., 2015).

The aim of this study was to evaluate the antimicrobial properties of *V. glaberrima* using agar diffusion and broth dilution methods.

MATERIALS AND METHODS

Collection and Identification of Plant material

The whole plant material of *V. glaberrima* was collected from Nasarawa State 8°32'N 8°81'E, Northern-Nigeria in June, 2012 during the rainy season. It was authenticated by Mallam U. S. Galla of the herbarium section of Biological Sciences Department, Ahmadu Bello University, Zaria. A voucher specimen (No. 899) was deposited at the herbarium for future reference.

Preparation of extract

The leaves were removed, shade dried, pulverized, labelled, and stored at room temperature in an air-tight container prior to extraction. The Powdered leaves (2500 g) were extracted with 70% methanol using maceration method for 10 days with occasional shaking. The extract was evaporated *in-vacuo* using rotary evaporator at 40°C to obtain 400 g of a gummy greenish product (16.0%w/w) subsequently referred to as the crude methanol leaf extract VGLE.

Test organisms

Clinical isolates of the test organisms were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital Zaria, Nigeria. All bacterial cultures were checked for purity and maintained in a blood agar slant while the fungi were maintained on a slant of Sabraud dextrose agar (SDA). The microbes tested include Methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin resistant *Enterococci*, *L. monocytogenes*, *H. pylori*, *C. fetus*, *S. aureus*, *P. vulgaris*, *P. fluorescens*, *C. tropicalis* and *C. stellatoidea*.

Antimicrobial evaluation

Susceptibility test

Antimicrobial activity of the methanol leaf extract of *V. glaberrima* was determined through susceptibility test using agar diffusion method (Sardari et al., 1998). The stock concentration of the extract (40 mg/ml) was prepared by dissolving 0.4 g of the extract in 10 ml dimethyl sulfoxide (DMSO). Mueller Hinton agar and the growth medium, was prepared according to manufacturer's instructions, and sterilized for 15 min at 121°C. Nutrient broth and sabraud dextrose broth were used for antibacterial and antifungal evaluations respectively. The test organisms were inoculated and incubated for 24 h for bacteria and 48 h for fungi. The solidified sterile medium contained in petri dish was seeded with 0.1 ml standard inoculum of the test microbe at 45°C. Wells were bored into the solidified inoculated nutrient agar plates using cork borer of 6 mm diameter. The wells were filled with 0.1 ml (400 µg) DMSO solution of the extract. Discs containing blank extraction solvents served as control. The reference antibacterial drug Sparfloxacin (5µg) and antifungal drug Fluconazole (5µg) were used as a positive control. The extract and standard drugs were allowed to diffuse into the agar and incubated overnight. All incubations were done at 37 and 25°C for bacteria and fungi respectively. At the end of incubation period, diameter of inhibition zone was measured using transparent ruler and recorded. The zones of inhibition of microbial growth were tested in duplicates and the mean of the results was recorded in millimeters (mm).

Minimum inhibitory concentration (MIC)

The MIC of the extract was carried out using Broth dilution method (Volekobia et al., 2001). Mueller Hinton broth was prepared of which 10 ml was dispensed into test tubes, sterilized at 121°C for 15 min, and allowed to cool; Mc-Farland's standard turbidity scale number 0.5 was prepared. Dilution of the organism suspension was done continuously using sterile normal saline until the turbidity

Table 1. Susceptibility test (Zone of Inhibition) of Methanol Leaf Extract of *V. glaberrima*.

Test organisms	Zone of Inhibition (mm)		
	VG (400 µg/ml)	Sparfloxacin (5µg/ml)	Fluconazole (5µg/ml)
<i>Staphylococcus aureus</i>	29	35	-
Methicillin Resistant <i>S. aureus</i>	20	35	-
Vancomycin Rest <i>Enterococci</i>	25	32	-
<i>Listeria monocytogenes</i>	-	37	-
<i>Helicobacter pylori</i>	24	-	-
<i>Campylobacter fetus</i>	-	38	-
<i>Proteus vulgaris</i>	-	32	-
<i>Pseudomonas fluorescens</i>	22	-	-
<i>Candida tropicalis</i>	-	-	37
<i>Candida stellatoidea</i>	18	-	35

Key: mean zone of inhibition measured in millimeter (mm), - = activity not detected, VG= *V. glaberrima* extract.

Table 2. Minimum inhibitory concentration of methanol leaf extract of *V. glaberrima* against the test organisms.

Test Organisms	Minimum Inhibitory Concentration (mg/ml)				
	40	20	10	5	2.5
<i>Staphylococcus aureus</i>	-	-	-	OA	+
Methicillin Resistant <i>S.aureus</i>	-	-	OA	+	++
Vancomycin Rest <i>Enterococci</i>	-	-	OA	+	++
<i>Listeria monocytogenes</i>	-	-	-	-	-
<i>Helicobacter pylori</i>	-	-	OA	+	++
<i>Campylobacter fetus</i>	-	-	-	-	-
<i>Proteus vulgaris</i>	-	-	-	-	-
<i>Pseudomonas fluorescens</i>	-	-	OA	+	++
<i>Candida tropicalis</i>	-	-	-	-	-
<i>Candida stellatoidea</i>	-	OA	+	++	+++

Key: - = no turbidity (no growth), OA= MIC, + = turbid (light growth), ++ = moderate turbidity, +++ = heavy growth.

matched that of Mc-Farland's scale by visual comparison. At that point, the concentration of the test microbe was about 1.5×10^8 cfu/ml. Two-fold serial dilution of the extract in the sterile broth was made to obtain the concentrations of 40, 20, 10, 5 and 2.5 mg/ml, respectively. 0.1ml of the standard inoculum of the test microbe was then inoculated into the different concentrations of the extract in the broth. The tubes were incubated at 37°C for 24 h and 25°C for 48 h for bacteria and fungi respectively after which the plates were observed for turbidity (growth). The MIC was defined as the lowest concentration of the extract inhibiting the visible growth of each micro-organism.

Minimum bactericidal concentration/Minimum Fungicidal Concentration (MBC/MFC)

The MBC/MFC was carried out to determine whether there is complete death of test microbes or just growth inhibition. Mueller

Hinton agar broth was prepared, sterilized at 121°C for 15 mins, and transferred into sterile petri dishes to cool and solidify. The contents of the MIC in the serial dilution were sub-cultured into the prepared medium and incubated at 37°C for 24 h; the plates were observed for colony growth; the MBC/MFC was the plate with lowest concentration of the extract in serial dilution without colony growth (Volekobia et al., 2001).

RESULTS AND DISCUSSION

The results of susceptibility test, MIC and MBC/MFC of the methanol leaf extract of *V. glaberrima* are shown in Tables 1, 2 and 3, respectively. The methanol leaf extract of the plant exhibited antimicrobial activity against the susceptible organisms tested.

Table 3. Minimum bactericidal/fungicidal concentration of methanol leaf extract of *V. glaberrima* against the test organisms.

Test Organisms	Minimum bactericidal/fungicidal concentration (mg/ml)				
	40	20	10	5	2.5
<i>Staphylococcus aureus</i>	-	-	OA	+	++
Methicillin Resistant <i>S.aureus</i>	OA	+	++	+++	+++
Vancomycin Rest <i>Enterococci</i>	-	OA	+	++	+++
<i>Listeria monocytogenes</i>	-	-	-	-	-
<i>Helicobacter pylori</i>	-	OA	+	++	+++
<i>Campylobacter fetus</i>	-	-	-	-	-
<i>Proteus vulgaris</i>	-	-	-	-	-
<i>Pseudomonas fluorescens</i>	OA	+	++	+++	+++
<i>Candida tropicalis</i>	-	-	-	-	-
<i>Candida stellatoidea</i>	OA	+	++	+++	+++

Key: - = no colony growth, OA= MBC/MFC, + = scanty colonies growth, ++ = moderate colonies growth, +++ = heavy colonies growth.

Susceptibility test result showed inhibition range of 18 to 29 mm against *Methicillin resistance S. aureus*, *Vancomycin resistant Enterococci*, *S. aureus*, *H. pylori*, *P. fluorescence* and the anti-fungi, *Candida stellatoidea*; The crude extract of the plant can be said to have a good broad spectrum of activity at the concentrations tested with mean zone of inhibition diameter > 18 mm (Tania et al., 2000). No activity was observed against *L. monocytogenes*, *C. fetus*, *P. vulgaris* and *Candida tropicalis*.

The standard antibacterial drug, Sparfloxacin had inhibitory activity against all the organisms except *H. pylori*, *P. flourescens*, *C. tropicalis* and *C. stellatoidea* while Fluconazole, the standard anti-fungal drug showed activity only on the two fungi species tested *C. tropicalis* and *C. stellatoidea*. *S. aureus* was the most sensitive organism (29 mm) and *C. stellatoidea* was the least (18 mm). The MIC and the Minimum Bactericidal/Fungicidal Concentration (MBC) ranges for the extract were 5 to 20 mg/ml and 10 to 40 mg/ml, respectively. The low MIC value suggests that the extract has good antimicrobial activity. The highest bactericidal activity was recorded on *S. aureus* at 10 mg/ml while *C. stellatoidea* has the least activity at 40 mg/ml.

S. aureus is the most dangerous of all the many common *staphylococcal* bacteria causing skin infections, pneumonia, breast infections, endocarditis, and other fatal infections in humans (Baorto et al., 1994). The extract also exhibited inhibitory effect on Methicillin-resistant *S. aureus* (MRSA) which is a bacterium that is resistant to many antibiotics causing life-threatening bloodstream infections, pneumonia, skin and surgical site infections (Bush, 1989; CDCP, 2015).

Helicobacter pylori has been implicated in peptic and stomach ulcer (NLM, 2014) as there is a strong association between infection with *H. pylori* and gastric ulcers (Kosunen et al., 2005; Kusters et al., 2006), and antibacterial drugs are included in the prescribed gastric and peptic ulcer treatment regimen (Sung et al., 1995). The ability of the crude extract of *V. glaberrima* to inhibit this pathogenic microbe suggests its potential relevance in the management of peptic and gastric ulcers. *P. fluorescens* is an unusual cause of disease in humans, and usually affects patients with compromised immune systems (Gershman et al., 2008)

Sparfloxacin, the standard drug is a broad spectrum antibiotic with better activity on gram positive bacteria; it exhibited high activity on the tested gram positive bacteria (MRSA, VRE, *L. monocytogenes*, and *S. aureus*) but was not active against the gram negative bacteria, *H. pylori* and *P. flourescens*. It is noteworthy that the extract inhibited the growth of *H. pylori* and *P. flourescens*, the only bacteria that were resistant to Sparfloxacin.

The ability of the extract to exert its antimicrobial effects may be attributed individually or collectively to the presence of flavonoids, alkaloids, saponins, tannins, steroids and terpenes detected in the plant (Abdullahi et al., 2015). Other *Vernonia* species such as *V. amygdalina* have been reported to possess antimicrobial activity (Ibrahim et al., 2008) though with varying degrees; the result of this work for example, showed *V. glaberrima* exhibited better inhibition of *S. aureus* (5mg/ml) than 12.5 mg/ml by *V. amygdalina* (Ibrahim et al., 2008).

These observed activities will give impetus for the isolation and characterization of bioactive constituents responsible for the observed activity, thereby aiding the

attempt to combat these pathogens which are causative agents of serious and life threatening infections.

Conclusion

The results of this study suggest that the methanol leaf extract of *V. glaberrima* contains bioactive principles that have good antibacterial and antifungal activity, validating the antimicrobial use of the plant in traditional medicine. Further work aimed at the isolation of the bioactive principles is being pursued.

Conflicts of interest

The authors have none to declare.

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Full Length Research Paper

***Camellia sinensis* extract inhibits *in vitro* pancreatic lipase and has preventive effect on obesity in female rat fed a high-fat diet**

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Received 18 March, 2015; Accepted 13 August, 2015

The aim of this study was to evaluate eight plants extract for porcine pancreatic lipase inhibition, characterizing biochemically the extract with high inhibitory activity and its effects on preventing weight gain in female rat fed a high-fat diet (HFD). *In vitro* pancreatic lipase inhibition was carried out in *p*-nitrophenyl-laurate substrates and a double-reciprocal plot was used for inhibition mechanism identification. *In vivo* experiments, female rats were fed with a standard diet or high fat diet (HFD), HFD+orlistat, HFD+22.5 mg/ml GT, and HFD+112.5 mg/ml GT. Feed intake, weight body gain, fecal lipid excretion and biochemistry parameters were analyzed. *Camellia sinensis* extract had the highest inhibitory lipase activity ($76.65 \pm 2.04\%$) with a non-competitive inhibition. *C. sinensis* administration, equivalent to 112.5 mg/ml, promoted weight loss, while 22.5 mg/ml increased fecal excretion of lipids in 31.41%. *C. sinensis* extract is certainly a promising alternative for preventive obesity treatment, since biochemical parameters analyzed showed significantly, reduction in the serum triglycerides levels and significantly decreased the LDL-cholesterol basal levels when compared with animals that did not receive a fat diet.

Key words: Enzyme inhibition, plant extracts, animal, preventive obesity.

INTRODUCTION

Lipids are important components in human nutrition, but their increased intake contributes to the development of obesity and can lead to multiple long-term complications

(Slanc et al., 2009). Life-style modifications is obviously the most appropriate approach, but therapeutic strategies such as anti-obesity agents and surgery are much sought

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by obese patients. There are limited options for medical therapy of obesity at present; in most countries, only orlistat is available as oral medication. Sibutramine (an amphetamine derivative) and rimonabant (a cannabinoid receptor blocker) have been removed from the market due to the increased cardiovascular risk associated with sibutramine and the association of depression, anxiety and suicidal ideation with rimonabant. Glucagon-like peptide (GLP)-1 receptor agonists also have potential as weight-loss agents, but so far they are only approved for the treatment of type 2 diabetes and not yet for obesity (Cameron et al., 2012). Lorcaserin, a serotonin 5-HT_{2C} receptor agonist, and phentermine plus topiramate have been approved in the USA for the treatment of obesity as an adjunct to lifestyle modifications in obese adults (body mass index [BMI] ≥ 30 kg/m²), or overweight adults (BMI ≥ 27 kg/m²) with at least one weight-related, co-morbid condition (for example, dyslipidemia, hypertension, type 2 diabetes) (Cameron et al., 2012; Gallwitz, 2013).

The potential of natural products or herbs for the treatment of obesity is still largely unexplored and might be an excellent alternative strategy for the development of safe and effective antiobesity drugs (Souza et al., 2011; Birari and Bhutani, 2007). Plant extracts, defined as raw or refined products derived from plants or parts of plants (for example, leaves, stems, buds, flowers, roots, seeds or tubers) are frequently used for the treatment of diseases (Boqué et al., 2012). Among plant extracts, grape seed extract was used as treatment to limit dietary fat absorption and accumulation of fat in adipose (Moreno et al., 2003). *Baccharis trimera* leaves is popularly used in the treatment of hepatic and digestive problems, for malaria, ulcers, diabetes, anemia, diarrhea, urinary inflammations, tonsillitis, worms, Hansen disease and weight reduction (Souza et al., 2011). *Cymbopogon citratus* leaves was used as aqueous extract for hypoglycemic and hypolipidemic study in rats (Adeneye and Agbaje, 2007). Flavonoids extracts from *Solanum melongena* showed significant hypolipidemic action in normal and cholesterol fed rats (Sudheesh et al., 1997). *Fragaria ananassa* extracts has ellagitannins that were the main active components for amylase inhibition, however, the polyphenols have been found to inhibit lipase activity *in vitro* at low levels (Boath et al., 2012). *Camellia sinensis* is an herb used for green tea and white tea. To produce green tea, the young leaves are rolled and steamed to minimize oxidation. White tea is prepared from very young tea leaves or buds covered with tiny, silvery hairs, which are harvested only once a year in the early spring (Rusak et al., 2008). Green tea is a richer source of phenolics than is white tea, but the extraction efficiency of these compounds strongly depends on the time of extraction, the solvents used and it is much slower than is the extraction of the same compounds from green tea (Rusak et al., 2008).

The main compound found in these plant extracts that have pharmacology effects on the weigh low was called

polyphenols. Polyphenols are a class of phytochemicals that are likely candidates as anti-obesity agents and several studies have suggested they can modulate the adipocyte life-cycle (Williams et al., 2013). Vegetables provide a major dietary source of polyphenols with potential anti-obesity properties. These compounds inhibit the action of pancreatic lipase by preventing the lipids from being absorbed by the enterocytes. A variety of natural products, including crude extracts and compounds isolated from plants, have been widely used traditionally in the treatment of obesity (Williams et al., 2013; Jang and Choung, 2013). The predominant constituents of *C. sinensis* (green tea), accounting for up to 35% of dry weight, are polyphenols, which include flavonols, flavones, and flavan-3-ols commonly known as catechin (Mizukami et al., 2007). Epigallocatechin-3gallate is the most abundant catechin of green tea (GT), representing 50 to 80% of the total catechin content, and other minor catechin include epicatechin3-gallate, epigallocatechin, epicatechin and catechin (Rains et al., 2011).

Experimental and clinical studies regarding the action mechanism of GT in the treatment of obesity and overweight are controversial. Diepvens et al. (2005) reported that the catechins in GT may stimulate thermogenesis and fat oxidation through an inhibition of catechol O-methyl-transferase, an enzyme that degrades noradrenaline. Juhel et al. (2000) demonstrated the *in vitro* inhibition of the two digestive lipases by green tea extract (GTE) separately. This experiment was conducted because *in vivo*, triglyceride hydrolysis is first initiated by an excess amount of gastric lipase under acidic conditions and is then completed by an excess amount of pancreatic lipase in neutral conditions. The results showed a reduced gastric and intestinal fat digestion by green tea extract mediated by direct inhibition of lipases as well as a reduction of lipid emulsification process. In studies with humans were observed significant increase of energy expenditure, lowering of body weight and good tolerance (Chantre et al., 2002). Rains et al. (2011) suggested that GT catechin may reduce glucose absorption by inhibiting gastrointestinal enzymes involved in nutrient digestion, in particular, α -amylase and α -glucosidase activity. Zhong et al. (2006) reported that GT induced carbohydrate malabsorption of 25% of the carbohydrate but did not cause triacylglycerol malabsorption or any significant increase in symptoms. Finally, Jurgens et al. (2012) related that the modest size of the reduction in weight produced by GT preparations make it then unlikely to be clinically relevant.

This study, initially, presents the screening of several methanolic plant extracts for *in vitro* inhibition of pancreatic lipase and to determine the kinetic parameters of the inhibition of green tea extract using p-nitrophenyl laurate as substrate. Furthermore, the preventive effects of green tea extract on obesity development and changing levels of lipid and lipoproteins were also

analyzed in female rats fed a high-fat diet.

MATERIALS AND METHODS

Plant

Fresh *B. trimera* and *C. citratus* were obtained from Hermínio Ometto Foundation – Uniararas' garden. Dehydrated *E. macrophyllus*, *C. sinensis* (green tea), *C. sinensis* (white tea) and *Vitis vinifera* (seed) were obtained from a compounding pharmacy, and fresh *F. ananassa* and *S. melongena* were obtained from a local market.

Methanolic extract preparation

Fresh materials were maintained in a 2.5% sodium hypochlorite solution for 30 min and washed in deionized water and picked at 1 cm². Methanolic extracts (1:10, w/v) were prepared using fresh and dehydrated materials from static maceration for 24 h, filtered, and the solvent was evaporated in bath at 45°C. Deionized water was added to residues (ratio 1:10, w/v), and then clarified at 10,000 rpm, for 20 min, at 4°C and supernatant stored at -18°C. *S. melongena* and *C. sinensis* (green tea) extracts were diluted three-times and *C. citratus*, *B. trimera* and *C. sinensis* (white tea) extracts were diluted five-times for further analysis.

Total polyphenols

Polyphenols compounds were determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965). Total polyphenols content, expressed in µg/ml, was calculated using catechin as the standard for the calibration curve. Results were expressed as catechin polyphenols (CP).

Pancreatic lipase activity

Porcine pancreatic lipase type II (Sigma-Aldrich) was dissolved in ultra-pure water at 10 mg/ml; then the supernatant was used after centrifugation at 10,000 rpm for 10 min, at 4°C. The assay buffer was 100 mM Tris-HCl buffer (pH 8.2) containing 0.5% Triton X-100, and *p*-nitrophenyl laurate (420 µM) was used as the substrate. The mixture was heated in water bath at 60°C for 15 min into dissolution of the substrate, mixed well, then cooled to room temperature (Pinsirodom and Parkin, 2001). The reaction medium was maintained in water bath at 37°C for 5 min for the reaction equilibrium. Reaction was started with the addition of porcine pancreatic lipase (2 U/ml), and maintained at 37°C for 30 min. Control was prepared without addition of enzymes. Lipase activity was determined at 410 nm (molar extinction coefficient, $\epsilon = 1.59 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). One unit of enzyme corresponded to the amount of enzymes that releases 1 µM of *p*-nitrophenol per min. All reactions were carried out in triplicate.

Porcine pancreatic lipase inhibition

Different total polyphenols concentrations were used for enzyme inhibition. Analyses were performed by adding the diluted GTE and enzyme (2 U/ml), as presented in the previous paragraph. The inhibition of the enzymes was obtained from the determination of slopes of straight lines (Abs × time) for testing of the activity of control enzymes (no sample) and enzymes + inhibitor. The slope of the results from the speed of the product formation per minute of

reaction and the presence of inhibitor cause a decrease in its slope. The inhibition percentage (I) was analyzed as follows:

$$I(\%) = \frac{(A - a) - (B - b)}{(A - a)} \times 100$$

Where: *A* absence of extract, with enzyme and substrate; *a* absence of extract and enzyme; *B* presence of extract, with enzyme and substrate; *b* absence of extract, with enzyme. All experiments were performed in quadruplicate.

Measurement of kinetic constants

In order to measure the Michaelis-Menten constant, K_m , the inhibition constant, K_i , and V_{max} , a series of substrate concentrations (5 to 650 µmol) were tested in the assay system. Each analysis was performed with and without *C. sinensis* extract. Lineweaver-Burk plots were fitted to determine the mechanism of the effect of the extract on porcine pancreatic lipase activity. The inhibition constant, K_i , was calculated from the following equation:

$$K_m \cdot \text{app} = K_m (1 + [I]K_i)$$

Where $K_m \cdot \text{app}$ and K_m represent the K_m with or without plant extract, $[I]$ represents the concentration of plant extract.

Feed composition

Conventional feed (Nuvilab[®], Sogorb Ind & Market, São Paulo, Brazil) is a balanced food for laboratory mice and rats, based on recommendations of the National Research Council and National Institute of Health - USA. The basic product composition is: calcium carbonate, corn bran, soy bran, wheat bran, dicalcium phosphate, sodium chloride, vitamins (vitamin A 12,000IU, vitamin E 30.0 mg, vitamin K 3.0 mg, vitamin B 18.2 mg, niacin 60.0 mg, pantothenic acid 20.0 mg, folic acid 1.0 mg, biotin 0.05 mg, choline 600.0 mg), amino acids (D,L-methionine 300.0 mg, lysine 100.0 mg), microelements minerals (50.00 mg iron; zinc 60.00 mg; copper 10.00 mg; iodine 2.00 mg; manganese 60.00 mg; selenium 0.05 mg; cobalt 1.50 mg), antioxidant 100.0 mg.

High-fat feed preparation

Conventional feed (Nuvilab[®]) was prepared increasing 10% (w/w) of lard (Seara Alimentos S.A., St Catarina, Brazil) in its composition. Conventional feed was triturated and lard was incorporated into the standard feed. To this preparation was added distilled water and then manually extruded to pellets formation. The pellets were dried at 60°C until constant weight. According manufacturer's information, the lard composition is (100 g): calorific value 910 kcal, carbohydrates 0%, proteins 2.0 g, total fat 89 g, saturated fat 30 g, trans unsaturated fat 0.2 g, food fiber 0 g and sodium 26 mg.

Animal and experimental conditions

Female rats (*Rattus norvegicus*), 4-months-old, Wistar strain (240 to 320 g), free of specific pathogens, were obtained from the Animal Experimentation Center (Hermínio Ometto Foundation – Uniararas, Araras, Brazil). The plain and experimental conditions complied with the Ethic and Research Committee of Uniararas (protocol n° 007/2012, May 09th, 2012). Animals were maintained in a controlled temperature room, 12:12 h artificial light-dark cycle and *ad libitum* access to the feed and water. After a random selection,

Table 1. Screening of plant extracts with *in vitro* inhibition activity of the porcine pancreatic lipase.

Plant extract	Popular name	Plant part used	Volume used	Activity Inhibition (%)
<i>Echinodorus macrophyllus</i>	Lather hat	Dried leaves and stalks	250 µl crude extract	+
<i>Camellia sinensis</i>	Green tea	Dried leaves and stalks	125 µl dilution 1:3	76.65±2.04
<i>Camellia sinensis</i>	White tea	Dried leaves and stalks	125 µl dilution 1:3	31.18±0.39
<i>Vitis vinifera</i>	Grape	Seed	750 µl dilution 1:3	+
<i>Fragaria ananassa</i>	Strawberry	Dried fruit	250 µl crude extract	14.05±1.93
<i>Solanum melongena</i>	Eggplant	Fresh fruit	750 µl dilution 1:3	+
<i>Baccharis trimera</i>	Gorse	Dried leaves	125 µl dilution 1:5	46.47±1.02
<i>Cymbopogon citratus</i>	Lemon grass	Fresh leaves	125 µl dilution 1:5	29.84±0.70

All extracts were prepared in methanol solvent. Activity inhibition is the relative activity of inhibitor trials with *versus* without inhibitor. Enzymatic assays were carried out at 37°C, 10 min, using p-nitrophenyl laurate as substrate. Dilutions were necessary due to the color of the extracts that interfere in the enzymatic analysis. Symbol: + represent the plant extracts that increased the spectrometric absorbance. Assays carried out in triplicate.

Table 2. Effects of catechin polyphenols concentrations on porcine pancreatic lipase activity.

Catechin polyphenols concentrations (µg/ml)	Relative activities (%)
14	69.5±5.2
28	70.2±2.3
42	73.0±2.1
56	76.0±6.4
70	78.0±1.2
84	80.0±4.4
98	75.0±3.2
112	52.0±3.6
126	54.0±2.9

Activity inhibition is the relative activity of inhibitor trials with *versus* without inhibitor. Enzymatic assays were carried out at 37°C, 10 min, using p-nitrophenyl laurate as substrate. Assays were carried in quadruplicate.

the rats were introduced to the standard diet (n = 10) for 15 days. The animals were weighed and randomly distributed into five groups of six subjects each. A randomized trial was carried out to assess the results. Group I received only a conventional diet (CD). Group II received only a high-fat diet (HFD). Group III received a high-fat diet and orlistat™ (1.67 mg/kg) (HFD+OR). Group IV received a high-fat diet and 22.5 mg of dry GT/ml (0.51 mg/ml CP) (HFD+CP 22.5). Group V received a high-fat diet and 112.5 mg of dry GT/ml (2.55 mg/ml CP) (HFD+CP 112.5). Intragastric administration of the extract and orlistat™ was performed with the aid of a gavage needle once a day for a period of 15 days. Every other day throughout the experiment, the feed and water intake was controlled, and individual weighing of the animals was performed at the end of treatment. Beyond the period of treatment, the animals were anesthetized with 0.3 ml of a mixture of ketamine 50 mg/ml and 2% xylazine (3:1). Blood was collected by cardiac puncture and visceral fat was removed and weighed.

Biochemical analysis

The blood samples were centrifuged at 3500 rpm for 20 min to obtain the serum. Then, the analyses of total cholesterol,

triglycerides, HDL-cholesterol and LDL-cholesterol were performed with kits for measurement of lipids and AST (aspartate aminotransferase) and ALT (alanine aminotransferase) with kits for hepatic enzyme according to the manufacturer's instructions.

Analysis of fecal fat

The fat present in the feces of animals was extracted following the modified methodology proposed by Bligh and Dyer (1959). All samples were performed in triplicate.

Statistical analysis

Statistical analyses were performed using Prism 3.0 software and the results were subjected to analysis of variance (ANOVA) and means compared by t-test, adopting a significance level of 5% (p < 0.05).

RESULTS

Plant extracts with inhibitory activity against porcine pancreatic lipase *in vitro*

Initially, eight methanolic plant extracts were analyzed for *in vitro* lipase inhibition. According to the results obtained in these experiments, *C. sinensis* extract was identified with the greater inhibitory activity of porcine pancreatic lipase (76.7 ± 2.04%), followed by *B. trimera* (46.4 ± 1.02%), *C. sinensis* (white tea) (31.18 ± 0.39%), *C. citratus* (29.84 ± 0.7%) and *F. ananassa* (14.05 ± 1.93%) (Table 1). *E. macrophyllus* and *V. vinifera* showed an activation effect of the enzyme. *S. melongena* did not inhibit the pancreatic lipase activity. In view of these results, the GTE was selected for characterizing the *in vitro* inhibitory effect on pancreatic lipase activity and to evaluate the effect on the female rats fed with a high-fat diet.

Crude GTE used for the experiments showed an equivalent of 1.70 ± 0.10 mg/ml CP. The inhibitory action of the GTE was evaluated using concentrations of 14 to 126 µg/ml CP, as shown in Table 2. The inhibitory effect

Table 3. Serum parameters in female rats fed treated with *C. sinensis* extract in female rats fed with high-fat diet.

Group	Total triglycerides (mg/dl)	Total Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)	HDL-Cholesterol (mg/dl)
Group I	170.6±23.3 ^b	98.8±15.8 ^a	64.4±8.9 ^b	28.0±1.1 ^a
Group II	227.8±21.3 ^c	129.4±13.9 ^a	98.9±7.3 ^c	36.9±1.8 ^a
Group III	149.3±14.8 ^b	123.7±14.9 ^a	68.9±7.8 ^b	34.4±1.9 ^a
Group IV	127.7±12.6 ^a	94.5±12.4 ^a	32.7±1.7 ^a	27.6±0.9 ^a
Group V	123.4±18.5 ^a	91.7±14.7 ^a	27.0±1.2 ^a	32.5±1.3 ^a

Experimental conditions: Group I received only conventional feed (control negative), Group II received only high-fat diet, Group III received high-fat diet and orlistat (1.67 mg/kg), Group IV received high-fat diet and 0.51 mg/ml of *C. sinensis* extract, Group V received high-fat diet and 2.55 mg/ml of *C. sinensis* extract. Experiments were conducted during 16 days. The values are means ± SD., $n = 10$. $p < 0.05$ compared to Group II.

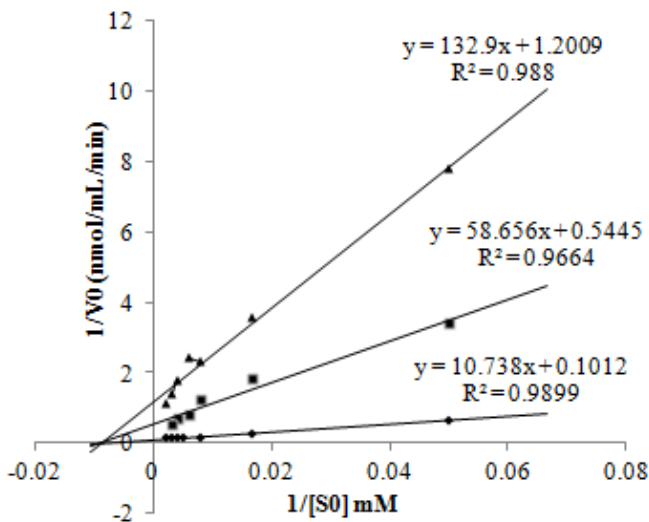


Figure 1. Lineweaver–Burk plots of the *p*-nitrophenol released from *p*-nitrophenyl laurate in the presence *C. sinensis* extract. (◆) 0 µg/ml, (■) 14 µg/ml, and (▲) 28 µg/ml. Enzymatic assays were carried out at 37°C, 10 min. Assays were carried in quadruplicate.

of a wide range of concentration of GTE (14 to 98 µg/ml CP) did not vary significantly ($p > 0.05$), remaining at approximately $72.82 \pm 3.60\%$, while values above this concentration range (112 and 126 µg/ml CP) reduced the activity of the pancreatic lipase in only $52.67 \pm 0.71\%$. Inhibition mechanism of pancreatic lipase was examined at different concentrations of the *p*-nitrophenyl laurate substrate using a extract concentration equivalent to 14 µg/ml CP (Figure 2). Kinetic data were analyzed using the double-reciprocal plot of Lineweaver-Burk and Michaelis-Menten constants K_m and V_{max} were determined (Figure 1). K_m and V_{max} values showed that methanolic GTE is a non-competitive inhibitor, as the V_{max} was reduced from 8.57 to 1.70 nmol/ml/min without changing significantly the K_m . K_i value of *C. sinensis* on the enzyme was 3.392 µg/ml CP.

Effect of the *C. sinensis* extract on the weight gain

In vivo studies, the groups that received a high-fat diet consumed less feed compared with Group CD, and Group HFD+CP 112.5 consumed significantly less feed at the final experiment (Figure 2A). The reduction of high-fat diet intake did not cause weight loss in animals in Group HFD; by contrast, the animals significantly ($p < 0.05$) increased weight gain relative to Group CD (Figure 2B). These results suggest that Group HFD had a higher energy intake while consuming the least amount of feed. Group HFD+CP 22.5 diet did not significantly prevent the weight gain compared with Group HFD+OR, but showed a clear prevention trend. Group HFD+CP 112.5 diet prevented the weight gain of the rats and promoted a significant loss of 2.80 ± 0.82 g to the final experiment. Group HFD+OR showed weight loss of 4.4 ± 1.7 g compared with Group HFD. These results show that the response of animals to the treatment with orlistat to promote weight loss is similar to that observed in humans due to its ability to inhibit the pancreatic lipase and reduce lipid absorption. Differences in weight gain of the treatments were also observed in the excretion of lipids in feces (Figure 3). Group HFD+OR and Group HFD+CP 22.5 increased the lipid excretion in feces in 54.6 and 31.4%, respectively, in final experiments. However, animals of Group HFD+CP 112.5 lost weight without increasing the fecal excretion of lipids.

Effect of *C. sinensis* extract on the changing levels of lipids and lipoproteins

The treatments in Group HFD+OR, Group HFD+CP 22.5 and Group HFD+CP 112.5 reduced triglyceride levels significantly ($p < 0.05$) during the experimental period (Table 3). The results observed in Group HFD+OR and Group HFD+CP 22.5 could be associated with the increase of lipid excretion, while in Group HFD+CP 112.5 the triglycerides reduction could be related to the feed intake reduction, increased thermogenesis, fat oxidation and energy expenditure. The total cholesterol level was

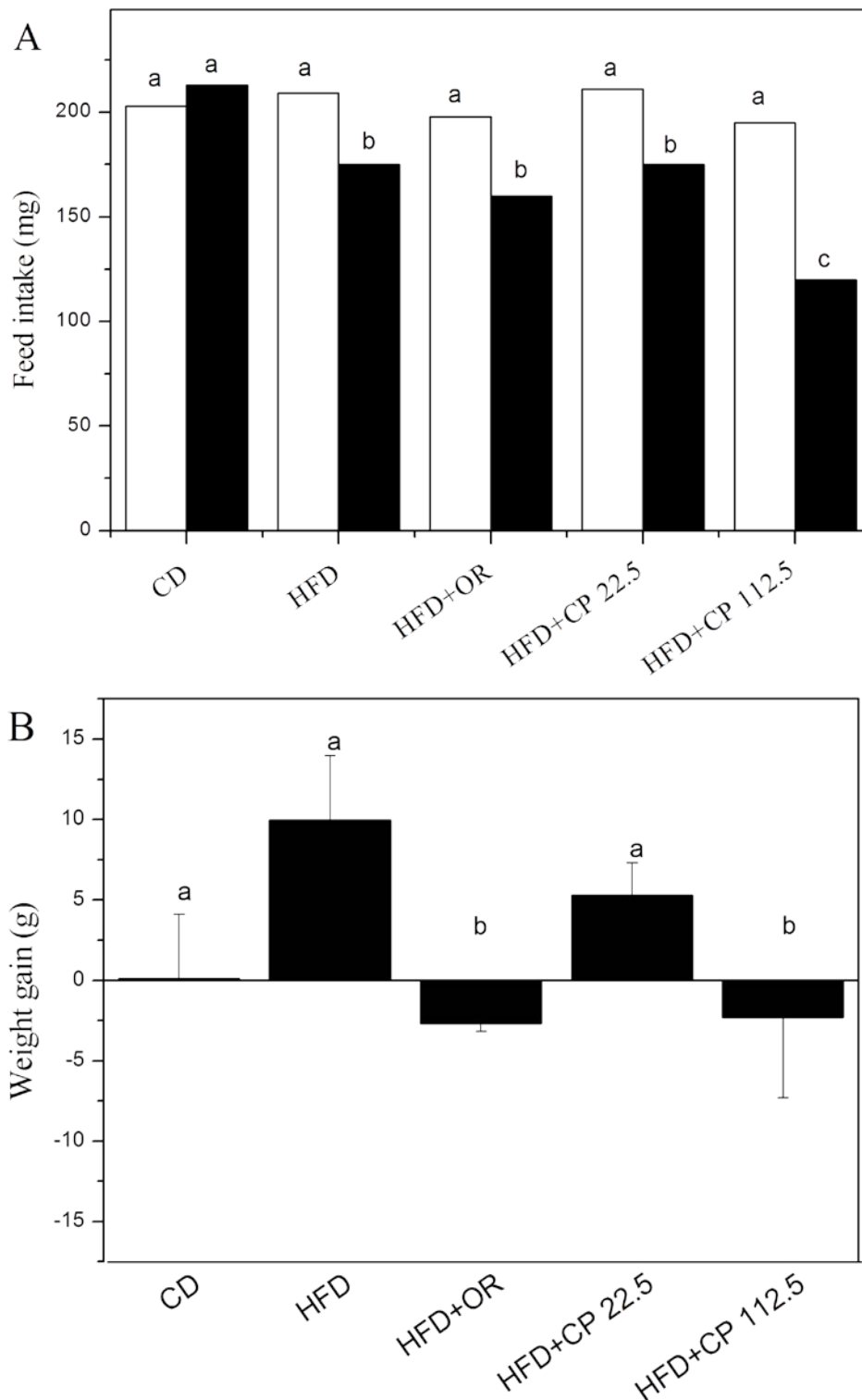


Figure 2. The effect of *C. sinensis* extract on feed intake (A) and body weight gain (B) in female rats fed the experimental diets. Group CD received only conventional feed (negative control), Group HFD received only high-fat diet (positive control), Group HFD+OR received high-fat diet and orlistat (1.67 mg/Kg), Group HFD+CP 22.5 received high-fat diet and green tea extract (22.5 mg/mL of GT), Group HFD+CP 112.5 received high-fat diet and green tea extract (112.5 mg/mL of GT). Experiments were conducted during 16 days. Legend: (□) Initial experiment, (■) Final experiment. The values are means \pm SD., $n = 10$. $p < 0.05$ compared to Group II.

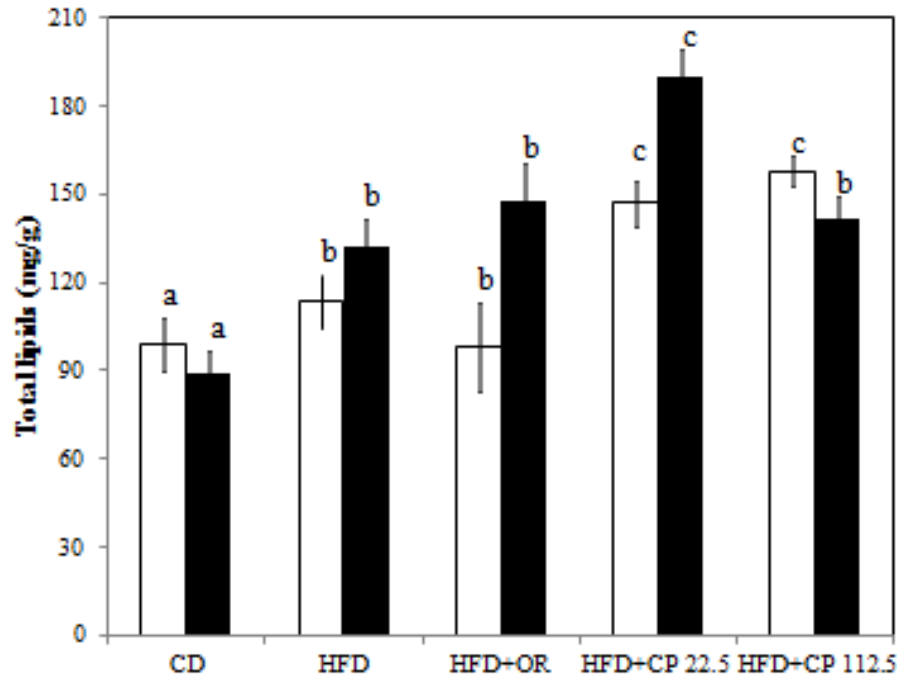


Figure 3. Total lipids of the feces in female rats fed treated with *C. sinensis* extract fed with high-fat diet. Group CD received only conventional feed (negative control), Group HFD received only high-fat diet (positive control), Group HFD+OR received high-fat diet and orlistat (1.67 mg/Kg), Group HFD+CP 22.5 received high-fat diet and green tea extract (22.5 mg/ml of GT), Group HFD+CP 112.5 received high-fat diet and green tea extract (112.5 mg/ml of GT). Experiments were conducted during 16 days. The values are means \pm SD., $n = 10$. $p < 0.05$ compared to Group II. Legend: (□) initial lipids in the feces, (■) final lipids in the feces.

not changed significantly, but tended to decrease in animals in Group HFD+CP 22.5 and Group HFD+CP 112.5. LDL-cholesterol decreased significantly ($p < 0.05$) in Group HFD+CP 22.5 and Group HFD+CP 112.5 compared with Group CD and Group HFD, while Group HFD+OR showed no change in this parameter. However, the results showed that the HDL-cholesterol and visceral fat did not differ significantly. The activities of serum hepatic aminotransferases (aspartate transaminase and alanine transaminase) of all treatments were not altered, indicating that likely the GTE did not cause a hepatotoxic effect in these animals (data not shown).

DISCUSSION

Among extracts studied, *C. sinensis* presented a strong *in vitro* inhibitory effect on lipase pancreatic. Other plant extracts, such as *Baccharis trimera*, *C. sinensis* (white tea), *Cymbopogon citratus* and *Fragaria ananassa* also had significant, but weaker inhibitory effect on the pancreatic lipase activity, whereas *Solanum melongena* were ineffective. This result confirms the inhibitory effect of GTE on the pancreatic lipase activity suggested by

Gondoin et al. (2010). The main inhibitory effect of the pancreatic lipase was attributed to catechin galatte, epigallocatechin gallate or epicatechin compounds present in the major fraction in the GT (Juhel et al., 2000; Martins et al., 2010). However, *Echinodorus macrophyllus* and *Vitis vinifera* showed activator effects on pancreatic lipase activity. These results were also observed for other plant extracts, suggesting that the lipase activation occurs due to the stabilization of the non-polar surface of the active site by the contact of the extract with a polar environment (Kato and Tosa, 1983; Nagen et al., 1995; Souza, 2009). Our results showed that using *C. sinensis* extract up to 98 $\mu\text{g/ml}$ inhibited pancreatic lipase, but above this concentration the inhibition decreased to 52.67%. These results are contradictory to those presented by Curiel (2011), which characterized the inhibitory effect of GT to be dose-dependent. Moreover, the author did not observe any reduction in inhibition of pancreatic lipase at higher concentrations of GT. Martins et al. (2010) suggested that the inhibition by polyphenols depended on how the substrate was presented to the lipases, and that the phospholipid species, especially the choline moieties, profoundly affected the lipase inhibitory activity of *Ilex*

paraguariensis. Thus, it was noted that the extract used in this study was prepared by methanol extraction, while Curiel (2011) used a *C. sinensis* infusion.

Pancreatic lipase inhibition by *C. sinensis* was first-time demonstrated to be non-competitive inhibition, and the K_i value of *C. sinensis* on the enzyme was 3.392 $\mu\text{g/ml}$ of CP. The inhibitory mechanism of polyphenols on pancreatic activity remains unclear. Won et al. (2007) showed a non-competitive inhibition of pancreatic lipase using licochalcone A, and a K_i value of 11.2 $\mu\text{g/mL}$. Martins et al. (2010) verified that the inhibition of pancreatic lipase by *I. paraguariensis* was of a competitive type, and had a K_i value of 12.9 mmol/ml (3.0 mg/ml maté tea). Gholamhoseinian et al. (2010) observed a non-competitive inhibition of pancreatic lipase for *Rosa damascene*, *Quercus infectoria* and *Eucalyptus galbie* and mixed inhibition for *Levisticum officinale*, using methanolic extracts. Chanmee et al. (2013) observed that a *Solanum stramonifolium* compound, named carpesterol, presented a competitive inhibition of pancreatic lipase.

In the *in vivo* experiments a reduction in feed intake was observed for Groups HFD+OR, HFD+CP 22.5 and HFD+CP 112.5 compared with Group HFD. These results can be associated with substances known to increase hepatic fatty acid oxidation, such as beta-adrenergic agonist, and decrease voluntary food intake in rats (Kahler et al., 1999). Kao et al. (2000) showed that rats treated with (-)-epigallocatechin-3-gallate (EGCG) by intraperitoneal injection had a reduction in food intake of 50 to 60% versus control rats. Belza et al. (2007) conducted a short-term trial in normal weight men. Subjects consumed 8% less energy at an *ad libitum* meal 4 h following the consumption of 500 mg GTE versus placebo. Given the evidence that catechin may increase hepatic fat oxidation, it is plausible that appetite may be altered by GT (Rains et al., 2011). Therefore, the reduction in feed intake by Group HFD+OR can be related to the consumption of a high-fat diet. Consumption of medium-chain fatty acids and 1,3-diacylglyceride oil, ingredients that increase hepatic fatty acid oxidation, has been shown to reduce food intake in human subjects (St-Onge and Jones, 2002).

Administration of 22.5 mg/ml of GT (Group HFD+CP 22.5) showed a clear trend of weight gain prevention compared with Group HFD+OR and increased fecal lipid excretion, while administration of 112.5 mg/ml of GT (Group HFD+CP 112.5) prevented the weight gain of the rats and promoted a significant loss of 2.80 ± 0.82 g in the final experiment. The weight gain reduction of Group HFD+CP 112.5 can be related to the feed intake, but can also be associated with other action mechanisms of GT already reported in the literature. GT catechins may stimulate thermogenesis and fat oxidation through inhibition of catechol O-methyl-transferase, resulting in increased energy expenditure that promotes weight reduction (Diepvens et al., 2005; Phung et al., 2010). In

addition to catechins, the mixture of GTE and caffeine, which has been reported *in vitro*, has thermogenic effects and can stimulate fat oxidation, in part via sympathetic activation of the central nervous system (Diepvens et al., 2005). The fact that a catechins-caffeine mixture stimulates energy expenditure cannot be completely attributed to its caffeine content because the thermogenic effect of a catechins-caffeine mixture is greater than that of an equivalent amount of caffeine (Hursel et al., 2009). In healthy men supplemented with GTE containing 270 mg EGCG and 150 mg caffeine, energy expenditure increased significantly by 4% compared with caffeine alone, and fat oxidation was 41% for GT compared with 33% for caffeine alone (McKay and Blumberg, 2007).

The *C. sinensis* extract prevented the high-fat diet-induced increases in body weight and decreased the serum triglyceride and LDL-cholesterol concentrations, but did not significantly alter the total cholesterol and HDL-cholesterol. Beside this results, the treatments did not demonstrate alteration in the hepatic enzymes (aspartate transaminase and alanine transaminase), what can indicate that the animals that received GTE did not present hepatic disorder effect. In a similar study, Chanadiri et al. (2005) investigated the effectiveness of GT catechins in the disorder of lipid metabolism, antioxidant status and excess body weight by administration of a high-fat diet in rats for 7 weeks. The results showed that the group that received the GT catechins corrected the biochemical parameters of lipid metabolism (total cholesterol, triglyceride and LDL), visceral fat and activity of antioxidant enzymes. Jang and Choung (2013) showed that rats fed with a high-fat diet initially exhibited significantly higher triglycerides, total cholesterol, LDL-cholesterol and free fatty acids, and lower HDL-cholesterol and HDL-cholesterol/total cholesterol ratio. But, with the administration of *L. japonica* extract or tea catechin, these parameters decreased to near normal levels in serum and liver, indicating that oral administration of the extract or tea catechin suppresses the accumulation of body fat in a dose-dependent manner, resulting in improved lipid profiles in serum and liver without any renal or hepatic toxicity. Ikeda et al. (2003), and Murase et al. (2002) show that GT catechins may significantly decrease body weight, visceral fat and hepatic triacylglycerol concentration, in addition to significantly increase the activity of β -oxidation of fatty acid in the liver and decrease the activity of the enzyme fatty acid synthetase, explaining the decrease in liver triacylglycerol and visceral fat deposition. Finally, our results suggest that GTE has an anti-obesity preventive function by inhibiting the hydrolysis of dietary fat in the small intestine, subsequently reducing intestinal absorption of dietary fat; however, although mechanistic studies have suggested that tea decreases lipid and carbohydrate absorption, increases lipid metabolism, inhibits de novo lipogenesis, and increases carbohydrate utilization, the relative

importance of these mechanisms to human disease remains unclear (Grove and Lambert, 2010).

Conclusion

This study demonstrated that the *in vitro* inhibition effect of *C. sinensis* extract was not dose-dependent and it was also demonstrated by the first-time that the methanolic green tea extract presented a mechanism of non-competitive inhibition on porcine pancreatic lipase. *In vivo* study, green tea extract decrease the feed intake by the animals but the weight reduction of female rats fed a high-fat diet was promoted by the polyphenols of the extract following the increase of the lipids in feces. Beside of this, green tea extract presented benefic effects on lipid metabolism that was not observed with the treatment with orlistat; and so, green tea extract can be administrated to control the biochemical parameters as total cholesterol, triglyceride and LDL, and visceral fat.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Full Length Research Paper

Anti-bacterial and anti-inflammatory effects of Tanshinone breast filler in mice

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Received 19 May, 2015; Received 27 August, 2015

The purpose of this study was to investigate the antibacterial and anti-inflammatory activities of Tanshinone breast filler (TBF) against cow mastitis. TBF on bacterial activity *in vitro* by the method of Oxford cup and the mice were treated by TBF before given the mixed bacterial by intraperitoneal injection *in vivo*. The effect of TBF against acute inflammation was studied by xylene-induced ear edema and egg white-induced paw edema in mice. The activity of TBF against chronic inflammation was assessed by the cotton pellet. TBF significantly inhibited *staphylococcus aureus*, *staphylococcus epidermidis*, *streptococcus dysgalactiae* and *escherichia coli* *in vitro* and *vivo*. TBF showed significant activity against acute inflammation on ear edema induced by xylene and paw swelling induced by egg white in mice. In chronic inflammation, TBF inhibited significant effects on cotton-induced mice granuloma. In conclusion, TBF had obvious effect against acute inflammation and chronic inflammation in mice and suppressed pathogenic bacteria *in vitro* and *vivo*.

Key words: Anti-bacterial; anti-inflammatory, TBF, cotton ball, granuloma.

INTRODUCTION

Salvia miltiorrhiza Bge (Danshen) is a perennial herbal medicine that belongs to family Lamiaceae (XW et al., 1977). The active ingredients of Danshen were Tanshinone (I, IIA and IIB), cryptotanshinone, isocryptotanshinone, miltirone, tanshinol (I and II) and salviol (Chinese Veterinary Pharmacopoeia Committees, 2010; Hao et al., 2010). Danshen has a variety of pharmacological properties including anti-oxidant, anti-bacterial, anti-inflammatory and anti-neoplastic activity. The main components of TBF contain C. tanshinone and

tanshinone IIA, and it has been reported that they have antibacterial effects on pathogen.

Anti-inflammatory mechanism of Danshen have many advantages, such as inhibiting of inflammatory mediators, improving the inflammation state and circulation, protecting vascular, inhibiting and scavenging oxygen free radicals, increasing C reaction protein decrease and antibacterial effect (Liu et al., 2015). Tanshinone IIA played an anti-inflammatory role in OVX atherosclerotic apoE (-/-) mice by activating the estrogen receptor

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through the ERK signaling pathway (Dong et al., 2009). Tanshinone IIA exerts anti-inflammatory properties by suppressing the transcription of pro-inflammatory cytokine genes that might be associated with the NF-kappabeta signaling pathway (Chang-qin et al., 2012). C. tanshinone has been reported to have anti-inflammatory activities (Li et al., 2011) and C. tanshinone was found to inhibit inflammatory cytokines production in RAW264.7 cells. Recently, it has been reported that the C. tanshinone and Tanshinone IIA has been reported to exhibit antibacterial and anti-inflammatory. In this study, we investigated that TBF could inhibit the antibacterial activities *in vitro* and *vivo*. We also used xylene-induced mice ear edema, egg white-induced mouse paw edema and cotton pellet-induced chronic inflammation to evaluate the anti-inflammatory activities of TBF *in vivo*.

MATERIALS and METHODS

Chemicals and reagents

D-D injection was purchased from the Hebei Yuanzheng Pharmaceutical Company Limited production, China. For use as the positive control, dexamethasone (DEX) were supplied by Xian Lijun Pharmaceutical Company Limited, China. Xylene was supplied from Guangfu Fine Chemical Research Insitute, China. Formaldehyde was purchased from the Xi'an Chemical Reagent Factory. Bacterial Strains used were: *S. aureus*, *S.epidermidis*, *Str. dysgalactiae*, *E. coli*, according to the standard of Veterinary Microbiology (Yi-jing, 2011) isolation and identification. The culture medium: Makanke medium was provided by Beijing aoboxing bio-tech co. Ltd, China. The nutrient broth used were: ordinary plate culture medium, 5% sheep blood agar medium, according to the practical medicine standard preparation.

Animals

Male and female mice (about 22 ± 2 g) were obtained from the Experimental Animal Center at Xi'an Jiaotong University, China. Animals were housed under standard conditions with a 12/12 h light/dark cycle. And the animals were acclimatized to their environment for a week prior to the start of experiments. All experiments were performed in accordance with the guidelines of the National Institutes of Health guidelines.

Tanshinone breast filler (TBF)

TBF was provided by the Key Laboratory of Veterinary Pharmaceutics Discovery, Ministry of Agriculture, Key Laboratory of New Animal Drug Project, Lanzhou Institute of Husbandry and Pharmaceutical Science of CAAS, China. It's the key effective content of C. tanshinone and Tanshinone IIA. The key chemical structure (Figure 1) were confirmed by nuclear magnetic resonance (NMR) spectroscopy.

Bacteriostatic test

Bacteriostatic test *in vitro*

The cultured bacteria liquid was diluted to 0.5 McIntosh turbidity standard with physiological saline and 0.5 ml bacteria liquid dilution

to 4.0 ml. Sterile Straw absorbed 0.1 ml into the culture medium and used a sterile cotton swab to bacteria liquid evenly. The culture was allowed to adhere to the plate for 3 to 4 min. After the culture was absorbed, an Oxford cup that had roasted in fire was placed on either side of the bacterial plate using sterile forceps, and labelled plates, then using the pipette, respectively, 200 μ l liquid was added to the Oxford cup and cover the dish.

After adding sample placed on the 4°C refrigerator standing for one hour, and then posted in 37°C incubator to culture 18 to 24 h (Zhu et al., 2011). Inhibition diameters were measured by vernier caliper. All experiments were repeated once. The diameter of inhibition (the distance between the edge of the disk and the edge of the bacterial colony) was measured in millimeters (Calin et al., 2009).

Bacteriostatic test *in vivo*

A total of 40 mice were randomly divided into 5 groups (n = 8 per group): the TBF (7.67, 3.84 and 1.92 g/ml), D-D and blank normal control group. The mice were infected with 0.5 ml mix bacterial suspension (final concentration of 1×10^6 to 1×10^8 CFU/ml) by intraperitoneal injection. After selecting a concentration that can make mice mortality to be up to 100%, the death rate was observed within 24 h. The mice respectively received TBF for 7 days (twice a day) according to 0.10 ml/10 g. Normal and D-D control group were given distilled water and D-D injection of the same volume. On the eighth day, the mice were infected with 0.5 ml mix bacterial suspension; determined by pre-test and by intraperitoneal injection and observed the death rate of mice in 24 h (YU et al., 2005).

Anti-inflammatory effect

Xylene-induced ear edema in mice

The mice were divided into five groups of eight. The TBF was administered orally for 6 days at the dose of 0.1 ml/10 g. In addition, dexamethasone (50 mg/kg, i.p.) was as a positive control and the negative control group received distilled water. The seventh day, 60 min after the tested mice were orally administered, Xylene (about 0.03 ml) was applied to the inner and outer surfaces of the right ear by a cotton swab and the left ear was considered as a blank control group.

Ear swellings were measured by vernier caliper (0.02 mm) at 1 h and 4 h intervals in mice, respectively. The edematous response was measured as the ear thickness difference between the right and left. The edema degree was used as the index of inflammation and the anti-inflammatory activity was evaluated by a percentage of the inhibition of edema in treated mice compared with the control mice (Hossein et al., 2000).

Egg white-induced paw swelling in mice

Edema was expressed as the difference between the control and received paws in mm. kunming mice (n = 8/group) were treated with TBF (7.67, 3.84 and 1.92 g/ml), DEX (50 mg/kg, i.p.) and saline. The TBF was administered orally for 7 days at the dose of 0.1 ml/10 g, twice a day (morning and night). After the last oral administration, the right hind paws received 0.1 ml (20%) (v/v) egg white by subcutaneous injection in mice. The thickness (mm) of the paw was measured at 2, 3, 4 and 11 h interval after the administration (Yu et al., 2013). Mean increase in paw swelling was measured:

$$\text{Paw swelling} = V_t - V_0$$

Where V_0 is the swelling before fresh egg white injection (mm);

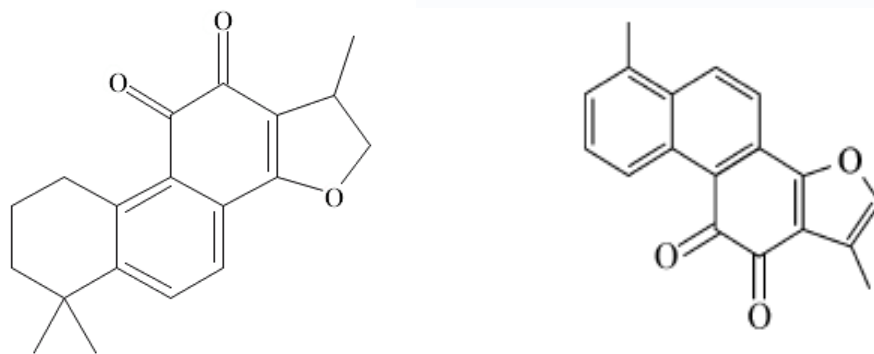


Figure 1. The chemical structure of cryptotanshinone and Tanshinone II A.

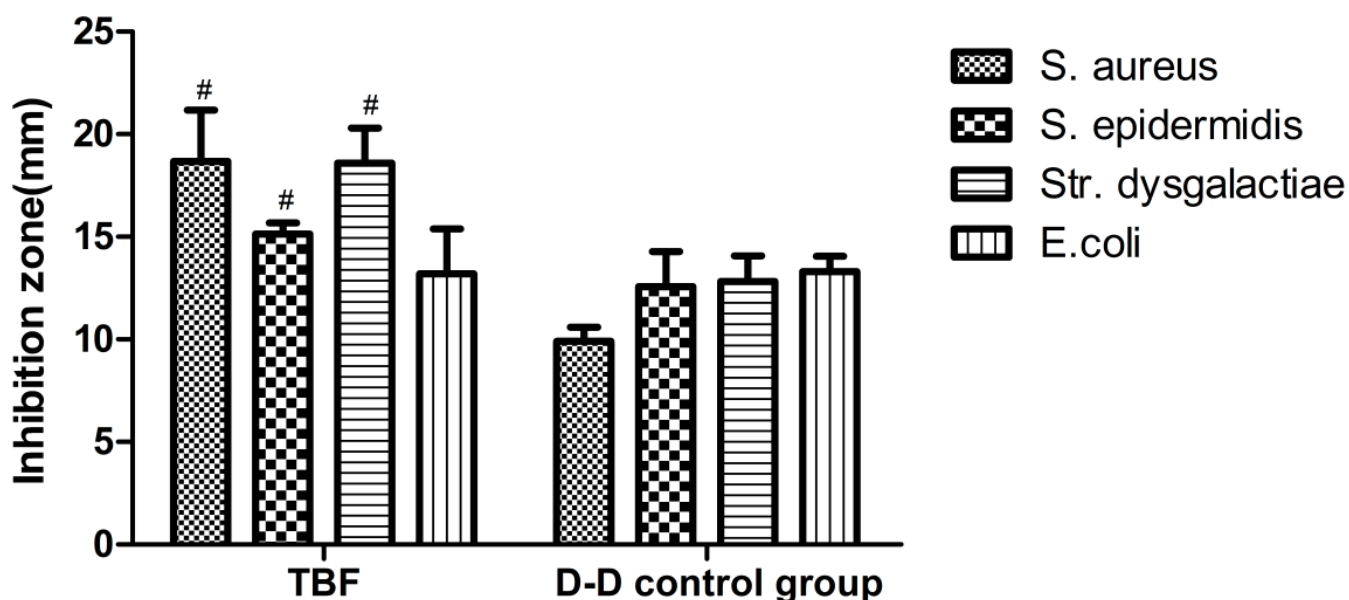


Figure 2. The effect of TBF and D-D injection on pathogenic bacteria. When compared to the D-D control group; # $P < 0.05$.

V_t is the swelling at t (h) after fresh egg white injection (mm).

Cotton pellet granuloma in mice

The mice were randomly divided into 4 groups ($n = 10$ / group): the TBF (7.67, 3.84 and 1.92 g/ml) and control group. The cotton weighing 32 mg were sterilized in vertical pressure steam sterilizer at 121°C for 21 min and impregnated with 0.6 ml of an aqueous solution penicillin and streptomycin. Under ether anesthesia, the cotton pellets were implanted subcutaneously in the back region of the mice. TBF was received twice daily according to 0.10 ml/10 g for 14 days.

Nonetheless, the control groups were given distilled water of the same volume. On day 15, the mice were killed and the pellets and surrounding granulation tissues were dried at 58°C for 24 h. Granuloma weight equal to the weight of the dry subtracted from cotton weight and the weight of granuloma was determined (Yu et al., 2012).

Statistical analysis

The data were expressed as mean values \pm SEM. and Statistical analysis was performed using a one-way analysis of variance (ANOVA) (SPSS 20.0).

RESULTS

TBF suppress pathogenic bacteria *in vitro*

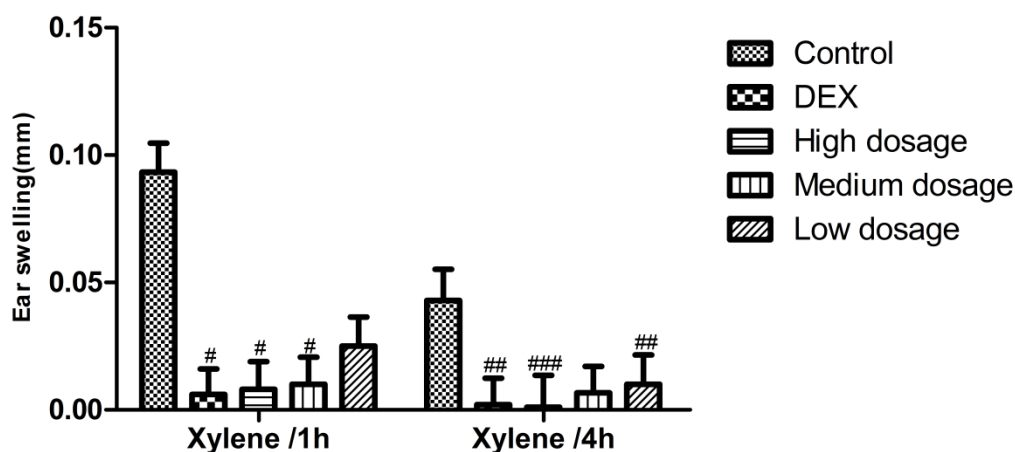
As shown in Figure 2, TBF had significant antibacterial effect on *S. aureus*, *S. epidermidis* and *Str. Dysgalactiae* ($P < 0.05$) and a certain effect on *E. coli* ($P > 0.05$) between TBF and D-D injection control groups. The results showed that TBF could inhibit pathogenic bacteria, including *S. aureus*, *S. epidermidis*, *Str.*

Table 1. Pre-experiment showed the death rate of mice (n = 5).

Groups (CFU/ml)	Dosage (ml)	Mice number	Death number	Death rate (%)
1.0×10^6	0.50	5	0	0.00
5.0×10^6	0.50	5	1	10.00
1.0×10^7	0.50	5	1	40.00
5.0×10^7	0.50	5	4	80.00
1.0×10^8	0.50	5	5	100.00

Table 2. TBF suppressed pathogenic bacteria *in vivo* (mean \pm SEM, n = 10).

Groups	Dosage (ml)	Mice number	Death number	Death rate (%)
High dosage	0.4	10	3	30.00
Medium dosage	0.4	10	4	40.00
Low dosage	0.4	10	6	60.00
D-D control	0.4	10	10	100.00
Blank control	0.4	10	10	100.00

**Figure 3.** The data were expressed as the mean \pm SD and the results were analyzed by a one-way ANOVA followed by a least significant difference (LSD) tests (*P < 0.05, **P < 0.01 compare to the control).

dysgalactiae and *E.coli*, respectively. According to the diameter of inhibition zone (Lee et al., 1999), the results showed that it was highly sensitive to *Staphylococcus* and *Str. Dysgalactiae* and sensitive to the *E. coli*.

TBF suppress pathogenic bacteria *in vivo*

As shown in Table 1, pre-experiment showed that the death rate was up to 80% after receiving mixed concentration of 5.0×10^7 CFU/ml by intraperitoneal injection in mice. From the Table 2, we can know that the mortality of the control group was up to 100%. Whereas

the death rate of TBF group was to 30, 40 and 60%. The results suggested that TBF could obviously decrease death rate in a dose-dependent manner.

The effect of TBF on xylene-induced mice ear edema

The clear effect of TBF on xylene-induced mice ear edema is shown in Figure 3. TBF inhibited significantly in a dose-dependent and time-dependent manner by xylene-induced ear edema in mice. After 1 h, we found that 7.67, 3.84, 1.92 g/ml of TBF and DEX (50 mg/kg, i.p.) treated mice reduced swelling by 91.43, 80.39, 73.20

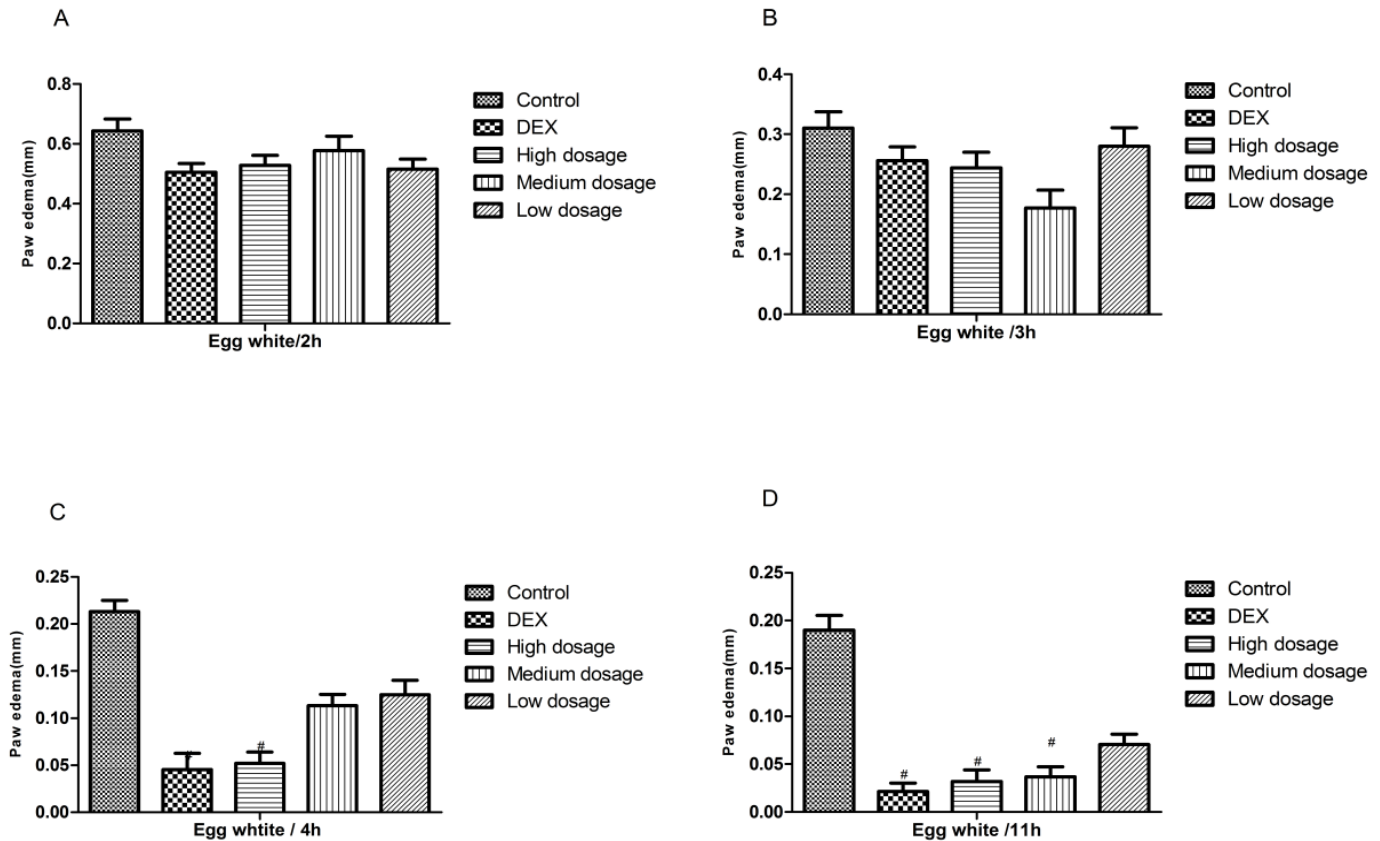


Figure 4. The data are expressed as the mean \pm SD and the results were analysed by a one-way ANOVA followed by a least significant difference (LSD) tests (* $P < 0.05$, compare with the control group).

and 93.56% ($P < 0.05$), respectively, when compared with the control group. After 4 h, the groups of TBF and DEX (50 mg/kg, i.p.) reduced swelling by 97.69, 84.38, 76.69 and 95.34 ($P < 0.01$ and $P < 0.05$), respectively, when compared with the control group. The results suggest that TBF had obviously inhibited ear edema in mice.

The effect of TBF on egg white-induced mice paw edema

As shown in Figure 4, TBF significantly inhibited paw edema in a dose-dependent and time-dependent manner by egg white-induced in mice. We used DEX as a positive control and physiological saline as a negative control group. Treatment with TBF (7.67, 3.84, 1.92 g/ml) and DEX (50 mg/kg, i.p.) significantly reduced the paw edema by subcutaneous injection. After 4 h, treatment with TBF (7.67, 3.84, 1.92 g/ml) and DEX (50 mg/kg, i.p.) obviously reduced the paw edema by 75.62% ($P < 0.05$), 46.88, 41.40 and 78.76% ($P < 0.05$), when compared with the control group, respectively. After 11 h, we found that TBF and DEX-treated mice inhibited edema by

83.16, 80.68, 60.53 and 85.36% ($P < 0.05$), when compared with the control group, respectively. The results suggest that TBF had obvious effect against paw edema in mice.

The effect of TBF on cotton pellet caused chronic inflammation

TBF inhibited the weight of granuloma by cotton-induced mice as shown in Table 3. The control group of granuloma was heavier than TBF groups. Treatment with TBF (7.67, 3.84, 1.92 g/ml) decreased the weight of granuloma by 49.86, 35.45 and 39.76% ($P < 0.01$, respectively). The weight of granuloma reduced in a dose-dependent manner by TBF treated in mice. The results suggested that TBF had obvious effect against chronic inflammation in mice.

DISCUSSION

TBF belongs to the Chinese herbal medicine, and it is natural and not only contains alkaloids, polysaccharides,

Table 3. The effect of TBF on the weight of granuloma in mice.

Groups (g/ml)	Cotton (mg)	Granuloma (mg)	Inhibition (%)
7.67	32	18.12 ± 1.36*	49.86
3.84	32	23.33 ± 0.67*	35.45
1.92	32	21.77 ± 1.32*	39.76
Normal control	32	36.14 ± 1.52	-

The effects of TBF on cotton pellet-induced mice granuloma (n = 10/group). The data with ** mean significant difference compared with control group (P < 0.01), least significant difference (LSD) tests.

saponins, volatile oil, anthracene, effective biological activity material, but also contains minerals, vitamin and nutritional factors, which has prevention, treatment and nutrition effect. Previous studies have indicated that *S. miltiorrhiza Bunge* has anti-bacterial activity against Gram positive bacteria (Li et al., 2011). The main components of TBF contain C. tanshinone and Tanshinone IIA, and it has been reported that they had antibacterial effects on pathogen.

In our study, the results showed that TBF suppressed pathogenic bacteria (*S. aureus*, *S. epidemidis*, *Str. dysgalactiae* and *E. coli*) *in vivo* and *in vitro* (Figure 2, Table 2). It was concluded that TBF had the potential protection to inhibit bacterial infection. For example, C. tanshinone demonstrated effective antibacterial activity against all 21 *S. aureus* strains tested *in vitro* (Haihua et al., 2009). Tanshinone IIA had antibacterial activity against a broad range of bacteria (Zhu and Luo, 2004). C. tanshinone and Tanshinone IIA had anti-bacterial effect (Mothana et al., 2009). C. tanshinone inhibited microbial activity against a broad range of Gram-positive and Gram-negative bacteria as well as other microorganisms (Tang et al., 2004; Honda et al., 1988).

In our present study, TBF effectively and significantly reduced cotton pellet-induced granuloma (Table 3), xylene-induced mice ear edema (Figure 3) and egg white-induced mice paw swelling (Figure 4), thereby suggested its activity in the proliferative phase of inflammation. Treatment with TBF (7.67, 3.84 and 1.92 g/ml) inhibited exudative inflammation in a dose-dependent manner, thus the data suggested that TBF possessed an anti-chronic inflammatory effect. Pre-experimental study found that *S. miltiorrhiza* has anti-inflammatory effect and not only against bacterial effect directly (Zhe et al., 2013), but can also regulate the immune function and enhance the antibacterial potential inherent in the body.

Inhibition of inflammatory mediators, improve the inflammation state and circulation, protect vascular, suppression, elimination of oxygen free radicals, the C reaction protein increased and decreased and with antibacterial function (Hao-lun et al., 2010). Pre-experimental study also found that Tanshinone IIA has anti-inflammatory (Min et al., 2015). Tanshinone IIA had a protective effect against spinal cord injury through

inhibiting the inflammatory response (Yin et al., 2012). Tanshinone IIA, one of the key components of TBF, had been reported to possess the majority of Danshen's properties with few side effects. Some studies have suggested that Tanshinone IIA inhibits the production of pro-inflammatory mediators such as TNF- α , NO, IL-1 β and IL-6 (Jang et al., 2003) by the inhibition of NF- κ B activity in RAW 264.7 cells stimulated with LPS, which were mediated by estrogen receptor activation (Fan et al., 2009). In conclusion, TBF had obvious effect against acute inflammation and chronic inflammation in mice and suppressed pathogenic bacteria *in vitro* and *vivo*.

ACKNOWLEDGMENTS

This study was supported by talent innovation and Entrepreneurship Project in Lanzhou (NO: 2014-RC-77).

Author Contributions

WCYYB FWY designed and conceived the experiments; WCYFWYLMX XHX RJZ performed the experiments; WCYJZFZ analyzed the data; WCYFWYYB LMD XHX RJZ contributed reagents/materials/analysis tools; WCYFWYYB wrote the paper.

Conflicts of interest

The authors declare that they have no competing interests.

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Full Length Research Paper

***In-vivo* antiplasmodial activity of methanol whole plant extracts of *Tapinanthus dodoneifolius* (DC) Danser in mice**

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Received 19 September, 2014; Accepted 26 June, 2015

Aqueous preparations of the whole plant of *Tapinanthus dodoneifolius* (DC) Danser growing on *Parkia biglobosa* tree are used by the nomads in Northern part of Nigeria for managing malaria, and many other ailments example, diabetes, fever, diarrhoea and wounds. To date, there are no efficacy or safety studies carried out to support its ethno-medicinal use in malaria management. This study aims to investigate the pharmacological activity of the plant relevant to the symptomatic treatment of malaria. High-performance liquid chromatography (HPLC) fractionation of methanol extract of *Tapinanthus dodoneifolius* (MCETD) produced six fractions (TDF1 –TDF6). Three concentrations of the MCETD (100, 200 and 400 mg/kg body weight); and TDF3 (25, 50 and 100 mg/kg) were evaluated for anti-plasmodial activity against *Plasmodium berghei* parasite in mice using three models: early (suppressive) infection, established (curative) infection and residual (repository, prophylactic) infection. Normal saline and Chloroquine phosphate were used as negative and positive controls respectively. All three models used showed that both the methanol whole plant extract of *T. dodoneifolius* and TDF3 fraction produced significant ($p < 0.01$) and dose-dependent chemo-suppressive effect when compared with the negative control group. They also produced a reduction in parasite count and a significant ($p < 0.01$) and dose-dependent increase in the survival times of the infected mice as compared to the negative untreated group. The phytochemical analysis revealed the presence of carbohydrate, tannins, flavonoids, anthracene, cardiac glycosides, saponin glycosides, steroid and triterpenes. The oral and intraperitoneal medium lethal doses (LD_{50}) were estimated to be greater than 5000 mg/kg and 3800 mg/kg respectively. The results suggest the presence of pharmacologically active constituents in the extract with anti-plasmodial activity against *Plasmodium berghei* that justifies its use in malaria ethno-medicine.

Key words: *Tapinanthus dodoneifolius*, *Plasmodium berghei*, anti-plasmodial activity, ethno-medicine, malaria.

INTRODUCTION

Malaria is one of the major public health concerns in more than 90 developing countries of the world today,

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affecting about 40% of the world's population. It is responsible for about 1.5 to 2.7 million deaths annually, with 75% of these deaths occurring in sub-Saharan African children. The World Health Organisation (WHO) estimated that in 2013, 3.3 billion people were at risk of malaria, of which 1.2 billion were at high risk. In high risk areas, more than one million cases occurred per 1000 population (WHO, 2014). Malaria is more serious in children, pregnant women and non-immune persons, or those suffering from sickle cell disease or Acquired Immune Deficiency Syndrome (AIDS), and can be fatal within hours or days, depending on the infecting species involved.

Malaria is endemic throughout Nigeria. The WHO mortality estimate for Nigeria is 729 cases per 100,000. Malaria is a serious worldwide health problem due to the emergence of parasites that are resistant to well established antimalarial drugs (Geib, 2007). Medicinal plants have in the past and in the last few years been the source of some of the most successful anti-malarial agents. The antimalarial potential of compounds derived from plants is proven by examples of quinine from *Cinchona* species and Artemisinin from *Artemisia annua*. Medicinal plants are commonly available in abundance, especially in the tropics. It is, therefore, of interest to screen medicinal plants for an evaluation of possible *in vitro* anti-plasmodial and *in-vivo* antimalarial activity (Adzu et al., 2007; Odugbemi, 2003).

Tapinanthus dodoneifolius belongs to the family Loranthaceae. It is a bushy parasitic plant, with stem of up to 1 metre long that grows on a wide range of trees and bushes of the wooded savannah zone. In Nigeria, it is called Etu-lonchi (Nupe), Elozie (Ibo), Kauchi (Hausa) and Afomu Igba (Yoruba). *T. dodoneifolius* plant has been used in folk medicine by the Hausa and the Fulani tribes in northern part of Nigeria (Deeni and Sadiq, 2002). It is used to treat many ailments including stomach ache, diarrhoea, dysentery, diabetes, epilepsy, hepatitis, hypertension, wounds and even cancer. In Nigeria, it is used as an antimicrobial agent in farm animals (Deeni and Sadiq 2002). It has also been reported to be used for inflammation, fever, infection, dizziness, energy loss, irritability, vertigo and headache. The plant was also shown to have a wide spectrum of antimicrobial activity against certain multiple drug resistant bacteria and fungal isolates of farm animals (Deeni and Sadiq, 2002; Cepleanu et al., 1994) revealed larvicidal and molluscicidal effects of *T. dodoneifolius*. Closely related African mistletoe specie, *Agelanthus dodoneifolius* Polh and Wiens, was shown to possess antiplasmodial activity (Builders et al., 2012).

MATERIALS AND METHODS

Plant collection and preparation

T. dodoneifolius whole plant was collected from Abuja municipal area. It was identified and authenticated at the National Institute of

Pharmaceutical Research and Development (NIPRD), Abuja. A voucher specimen (NIPRD/H/6591) was prepared and deposited at the NIPRD herbarium for future references. The plant material was cleaned, air-dried in the shade and pulverized into coarse powder using mortar and pestle. The powder was stored in a dry air-tight container until it was ready for use in the study.

Plant extraction

400 g of the coarse powdered plant was weighed and macerated in 2.5 L of 70% v/v methanol in water for 72 h with constant shaking using a GFL shaker. The resultant mixture was filtered using muslin cloth, followed by Whatman filter paper (No.1) and freeze-dried using AMSCO/FINN-AQUA GT2 Freeze dryer (Germany). The total yield of a dark green pasty substance was 39.78% w/w of crude stating material. Distilled water was used to dilute the extract before administration.

Experimental animals

Swiss albino adult mice (male and female) of 18 to 22 g body weight obtained from the Animal Facility Centre of NIPRD, Abuja were used in the study. They were kept in clean, dry cages and maintained in well-ventilated animal house maintained under standard conditions of temperature, humidity and 12 h light/darkness cycle. Water and pelleted feed were given *ad libitum* for the duration of the study, except when fasting was necessary in the course of the study. All experiments conformed to the principles for research involving animals as recommended by the Helsinki Declaration and the "National Academy of Sciences guide" (1996), on the care and use of Laboratory animals.

Malaria parasite preparation and inoculation

Chloroquine-sensitive malaria parasite, NK 65, *Plasmodium beghei* used in this study was obtained from the National Institute of Medical Research (NIMR) Lagos, and kept at the Department of Pharmacology and Toxicology National Institute of Pharmaceutical Research and Development, Abuja, Nigeria. The parasites were maintained by continuous re-infestation intraperitoneally in mice every 4 days (Adzu et al., 2007). Prior to the start of the study, one of the infected mice was kept and observed to reproduce signs and symptoms of disease similar to human malarial infection. The inoculum consisted of 1×10^7 of *P. beghei* parasitized erythrocytes per ml. Each mouse used was inoculated intraperitoneally with 0.2 ml infected blood containing 1×10^7 *P. beghei* parasitized red blood cells (Peters et al., 1993).

Phytochemical screening

The methanol whole plant extract of *T. dodoneifolius* was subjected to phytochemical screening to determine the presence of Phytochemicals such as alkaloids, carbohydrate, flavonoids, tannins, saponin, anthraquinones, steroids and triterpenoids (Trease and Evans, 1989).

Acute toxicity study

The modified method of Lorke was used. The study was carried out in two phases. In the first phase, 9 mice fasted overnight were randomly distributed into three groups of three mice each. Doses of 10, 100, 1000 mg extract/kg body weight were administered orally. The procedure was repeated using the intraperitoneal route. The

mice were observed for signs of toxicity and mortality for the first critical four hours and thereafter daily for 7 days. Symptoms of all adverse effects and death were observed and recorded. The second phase was determined by the outcome of the Phase one study. Another set of three groups of three mice were given 1600, 2900 and 5000 mg extract/kg body weight orally. The procedure was repeated using the intraperitoneal route. These mice were also observed for signs of toxicity and pattern of mortality for the first four hours, and thereafter daily for 7 days. The LD₅₀ is expressed as: LD₅₀ = geometric mean of $\sqrt{A \times B}$, where A = lowest lethal dose and B = higher non-lethal dose (0/1 and 1/1) respectively

Fractionation of extract

Methanolic extract of *T. dodoneifolius* (TD) was subjected to column chromatography using the method described by Harborne (Harborne, 1998). This consisted of Octadecylsilyl (ODS) silica gel stationary phase 100 g and 40 g of *T. dodoneifolius* whole plant extract (MCETD). Gradient elution under gravity was performed with 500 ml of each mobile phase mixture in series. The mobile phase consisted of Hexane: Ethyl acetate: Methanol, starting with Hexane (100%) and 10% increments in Ethyl acetate. This was followed by elution with Ethyl acetate (80%) and (20%) increments in Methanol. The final elution was performed with water. A total of 28 fractions were obtained. The elutes, were monitored with thin layer chromatography (TLC) using solvent system Ethyl acetate: Methanol (4:1); and elutes with similar TLC profile were pooled.

In-vivo anti-plasmodial study of methanolic extract

The anti-plasmodial activity of the whole plant extract (100, 200 and 400 mg/kg) were evaluated by determining the suppressive, curative and prophylactic *in-vivo* activity using the methods of Knight and Peters (Peters et al., 1993; Bulus et al., 2003).

Evaluation of activity on early infection (4-day test)

Thirty adult Swiss albino mice were inoculated on the first day (day 0) by intraperitoneal injection with 0.2 ml standard inoculum of *P. berghei* containing 1×10^7 infected erythrocytes. The mice were then randomly divided into five groups of 6 mice each four hours after inoculation. Mice in group I were administered 5 ml/kg normal saline, while mice in groups II, III and IV were administered 100, 200 and 400 mg/kg body weight of the extract orally daily for four days (days 0 to 3). Group V mice were given 5 mg/kg body weight Chloroquine orally daily for four days. On the fifth day (day 4) of the study, blood was collected from the tail vein of each mouse and smeared onto a microscope slide to make a film (Saidu et al., 2000). The blood films were stained with 10% Giemsa at pH 7.2 for 10 min and parasitaemia levels determined by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields of the microscope. The average percentage chemo-suppression was calculated for each dose level by comparing the parasitaemia in infected controls with those of treated mice and multiplying by 100. Average % chemo-suppression = $[(A - B)/A] \times 100$, Where A is the average parasitaemia in the negative control group, and B is the average parasitaemia in the treated group (Okonkon et al., 2006).

Evaluation of activity in established infection (Rane's test)

Thirty adult Swiss albino mice were inoculated with standard inoculum on the first day (day 0) of the study. Seventy two hours later, the mice were randomly divided into five groups of six mice each. Groups I and V mice were administered 5 ml/kg normal saline

and 5 mg/kg of Chloroquine respectively. Mice in groups II, III and IV were administered with 100 mg, 200 mg and 400 mg/kg body weight of the extract orally. The administration was for 5 days. Thin blood films were prepared from tail blood of each mouse daily for five days on microscope slide to monitor the parasitaemia level (Peters et al., 1993). The mean survival time (MST) for each group was determined arithmetically by finding the average survival time (days) of the mice (post inoculum) in each group over a period of 28 days (days 0 to 27).

$$\text{MST} = \frac{\text{Number of days survived}}{\text{Total number of days (28)}} \times 100$$

Evaluation of activity in residual (repository) infection

Thirty adult Swiss albino mice were randomly divided into five groups of six mice each. Mice in group I and V were administered normal saline 5 ml/kg and Pyrimethamine 1.2 mg/kg body weight orally respectively. Mice in groups II, III and IV received 100 mg, 200 mg and 400 mg/kg of the extract orally (days 0 to 4). All administration was for five days. On the fifth day (day 4), all the mice were inoculated with 0.2 ml of 1×10^7 *P. berghei* NK 65 infected erythrocytes infected with the parasite. Smears were then made from each mouse 72 h after treatment and examined microscopically to monitor the parasitaemia level (Okonkon et al., 2006).

Statistical analysis

All quantitative data were expressed as the mean \pm standard error of mean (SEM). Statistical analysis was carried out using one way analysis of variance (ANOVA), followed by Dunnet's post hoc test. Significant differences between means were assessed at 95% level of significance that is, p-value less than 0.05 ($p < 0.05$) was considered significant.

RESULTS AND DISCUSSION

This work sought to establish a basis for the ethno-medical use of *T. dodoneifolius* (DC) Danser (*Parkia biglobosa* mistletoe) in the treatment of malaria, and to evaluate the potential of its constituents as possible new antimalarial agent or lead to new antimalarial compound. This is to verify claim and establish a basis for its use in treatment of malaria cases in Northern part of Nigeria by traditional herbalists. This work compared the anti-plasmodial activity of *T. dodoneifolius* with the standard drug, Chloroquine, which has been used for suppressive, curative and prophylactic treatment of malaria.

The preliminary phytochemical screening of methanolic extract of *T. dodoneifolius* revealed the presence of carbohydrate, cardiac glycosides, saponin glycoside, free anthraquinones, tannins, flavonoids, unsaturated steroid and triterpenes. The presence of these diverse constituents (secondary metabolites), offer support for the various ways in which this plant is used in traditional medicine, as these compounds are known to exhibit various physiological and biological activities *in-vivo* (Tor-Anyiin and Danisa 2012; Idowu et al., 2010; Fosola and Iyama 2014; Akinmoladun et al., 2007). Anti-plasmodial

Table 1. Preliminary investigation of anti-plasmodial activity of column fractions of methanol crude extract of *T. dodoneifolius* on *P. berghei* infected mice.

Treatment	Mean parasite count \pm SEM	% Chemo suppression
N/S 5 ml/kg	11.60 \pm 0.54	-
50 mg/kg F ₁	8.67 \pm 0.54	25.26**
50 mg/kg F ₂	7.00 \pm 0.27	39.66**
50 mg/kg F ₃	1.80 \pm 0.54	84.48***
50 mg/kg F ₄	10.00 \pm 0.84	13.79*
50 mg/kg F ₅	8.01 \pm 1.54	31.03**
50 mg/kg F ₆	Insufficient quantity	-
CQ 5 mg/kg	0.50 \pm 0.02	96.00***

N/S = Normal saline; CQ = Chloroquine; F1 to F6 = Column fractions of methanol whole plant extract of *T. dodoneifolius*; *p < 0.05; **p < 0.01; ***p < 0.001; One way ANOVA; n = 6.

Table 2. The effect of methanol whole plant extract of *T. dodoneifolius* in early infection on *P. berghei* infected mice.

Treatment	Parasite count	% Suppression
N/S 5 ml/kg	15.00 \pm 1.40	-
MCETD 100 mg/kg	9.00 \pm 0.58*	40.00
MCETD 200 mg/kg	4.50 \pm 0.76**	70.00
MCETD 400 mg/kg	2.20 \pm 0.60*	85.00
CQ 5 mg/kg	1.00 \pm 0.37***	93.33

TD: *T. dodoneifolius* CQ: Chloroquine N/S: Normal saline, MCETD: Methanol crude extract of *T. dodoneifolius* *p < 0.05; **p < 0.01; ***p < 0.001; One way ANOVA; n = 6.

effects of natural plant products have been attributed to some of their active phytochemical components. Some of the Phytochemicals, such as tannins, saponins and flavonoids (detected in MCETD) have been reported to have anti-plasmodial activity (Olajide et al., 2000; Sofowora, 2008; Edeoga et al., 2005). Flavonoids, steroids, anthraquinones, alkaloids and terpenes isolated from other plant species have been found to possess anti-plasmodial activity in both *in-vitro* and *in-vivo* studies (Edeoga et al., 2005; Christensen and Kharazmi, 2001; Adebayo and Kretti, 2011; Tona et al., 2001; Karou et al., 2003).

The oral and intraperitoneal LD₅₀ of the extract was found to be greater than 5000 mg/kg and 3800 mg/kg body weight respectively. The animals presented with paw and genitalia-licking, salivation and calmness. The LD₅₀ after oral administration of 5000 mg/kg body weight of the methanol extract, and intraperitoneal administration of 3800 mg/kg within observation period of 14 days implied that the extract was practically non-toxic, even in the group administered with the highest dose (5000 mg/kg). This indicates that the doses used in the experiment (100, 200 and 400 mg/kg) were safe. Hence higher doses can be given in order to achieve better anti-

plasmodial activity. Fractionation of methanol extract produced six fractions (TDF1 to TDF6). However, the yield of fraction six (TDF6) was insignificant and hence was not used in further study. The fraction three (TDF3) gave the highest yield, and when subjected to curative anti-plasmodial tests also produced the highest activity of all the other fractions obtained (Table 1).

The results of *in-vivo* anti-plasmodial study showed that the methanol whole plant extract of TD possess significant and dose-dependent suppressive effect against early infection; curative effect against established infection and some prophylactic effect against residual infection in *P. berghei* infected mice. The extract also increased the mean survival time period of treated mice. In the early (suppressive) anti-plasmodial study, the methanol whole plant extract of *T. dodoneifolius* had a significant (p < 0.05) and dose-dependent parasite suppressive activity (40, 70 and 85%) at doses of 100, 200 and 400 mg/kg doses respectively) compared to the control group (Table 2). The chloroquine treated group showed the highest level of chemo-suppression (93.33%) when compared with all the extract doses used (p < 0.001). The TDF3 fraction showed a low to moderate level of suppressive activity ranging from 43.33, 49.33

Table 3. The effect of TDF3 fraction in early infection on *P. berghei* infected mice.

Treatment	Parasitaemia	% Chemo suppression	Mean survival time (days)
N/S 5 ml/kg	15.0±0.18	-	7.00
TDF3 25 mg/kg	8.5±0.72*	43.33	22.00
TDF3 50 mg/kg	7.6±0.16*	49.33	26.00
TDF3 100 mg/kg	5.6±0.16**	62.67	28.00
CQ 5 mg/kg	4.1±0.33***	72.67	29.0

N/S = Normal saline; CQ = Chloroquine; TDF3 = *T. dodoneifolius* column active fraction F3, *p < 0.05; **p < 0.01; ***p < 0.001; One way ANOVA; n = 6.

Table 4. The effect of methanol whole plant extract of TD in established infection in *P. berghei* infected mice.

Treatment	Mean parasite count	% Suppression	Mean survival time (days)
N/S 5 ml/kg	49.00±1.70	-	9.30±0.47
MCETD 100 mg/kg	4.80±0.53*	90.20	24.5 ±1.63*
MCETD 200 mg/kg	1.40±0.40**	97.14	28.00±0.00**
MCETD 400 mg/kg	0.60±0.40**	98.77	28.0 ±0.00**
CQ 5 mg/kg	0.20±0.20**	99.59	28.23±1.32**

TD: *T. dodoneifolius*. CQ: Chloroquine phosphate. N/S: Normal saline, MCETD: Methanol crude extract of *T. dodoneifolius* *p < 0.01; **p < 0.001; One way ANOVA, n = 6.

Table 5. The effect of TDF3 in established infection on *P. berghei* infected mice.

Treatment	Parasitaemia	% Chemo suppression	Mean survival time (days)
N/S 5 ml/kg	12.0±0.38	-	7.00
TDF3 25 mg/kg	2.50±0.28*	79.17	26.00
TDF3 50 mg/kg	2.20±0.11*	81.67	28.00
TDF3 100 mg/kg	1.80±0.21*	85.00	28.00
CQ 5 mg/kg	0.50±0.02**	96.00	29.00

N/S = Normal saline; TDF3 = *T. dodoneifolius* column active fraction F3, *P < 0.01; **p < 0.001; One way ANOVA; n = 6.

and 62.67% chemo suppression for the 25, 50 and 100 mg/kg doses respectively (Table 3). Agents with suppressive activity against *P. berghei* were known for antimalarial activity (Adebayo and Kretti, 2011; Tona et al., 2001; Karou et al., 2003).

In the established (curative) anti-plasmodial study, the methanol whole plant extract of TD at doses of 100, 200 and 400 mg/kg body weight significantly (p < 0.01) and dose-dependently reduced level of parasitaemia (90.2, 97.14 and 98.77% respectively) compared to the control group. The standard drug chloroquine produced the highest reduction in parasitaemia level (99.59%) when compared with all the extract doses used in the study, p < 0.001 (Table 4). The mean survival times for the extract doses (100, 200 and 400 mg/kg) were 24.5, 28 and 28 days respectively. Chloroquine treated group had a mean survival of 28 days, while the infected untreated group had a 9 days survival time (Table 4). The TDF3 fraction showed a high curative activity (79.1, 81.67 and 85.00% chemo suppression at doses of 25, 50 and 100 mg/kg

respectively) as compared with control untreated group (Table 5). This suggests that both the extract and fraction F3 of TD possess significant blood schizontocidal activity against *P. berghei* parasite, as seen in the high mean survival times of the treated mice (Tables 4 and 5).

In the residual (prophylactic) anti-plasmodial study, the methanol whole plant extract of *T. dodoneifolius* at doses of 100, 200 and 400 mg/kg, significantly (p < 0.05) and dose-dependently reduced the level of parasitaemia compared to the control group (Table 6). Pyrimethamine produced the highest prophylactic parasite reduction level (p < 0.001) when compared to all the extract doses used in the study. The MCETD produced a dose-dependent chemo-suppressive effect (61.5 and 76.5%) at doses of 200 and 400 mg/kg respectively. However, the 100 mg/kg extract treated group had a much lower prophylactic activity (20%) as compared to the standard drug Pyrimethamine that produced a 91.5% prophylactic effect, p < 0.001 (Table 6). The TDF3 fraction had a low level of prophylactic activity at lower dose, but the higher

Table 6. The effect of methanol whole plant extract of *TD* in residual (prophylactic) infection on *P. berghei* infected mice.

Treatment	Parasite count	% Suppression
N/S 5 ml/kg	20.00±0.42	-
MCETD 100 mg/kg	16.00±0.67*	20.00
MCETD 200 mg/kg	7.70±0.71**	61.50
MCETD 400 mg/kg	4.70±1.00***	76.50
Pyrimethamine 1.2 mg/kg	1.70±0.67***	91.50

N/S = Normal saline; MCETD = Methanol crude extract of *T. dodoneifolius*, *p < 0.05; **p < 0.01; ***p < 0.001; One way ANOVA; n = 6.

Table 7. The effect of TDF3 in residual infection on *P. berghei* infected mice.

Treatment	Parasitaemia	% Chemo suppression	Mean survival time (days)
N/S 5 ml/kg	10.0±0.14	-	9.00
TDF3 25 mg/kg	6.40±0.14*	36.00	18.00
TDF3 50 mg/kg	3.80±0.18**	62.00	24.00
TDF3 100 mg/kg	1.90±0.35**	81.00	27.00
Pyrimeth 1.2mg/kg	1.10±0.32***	89.00	29.0

N/S = Normal saline; TDF3 = *T. dodoneifolius* column active fraction F3, *p < 0.05; **p < 0.01; ***p < 0.001; One way ANOVA; n = 6.

dose levels showed moderate to high prophylactic activity of 62 and 81% for 50 and 100 mg/kg doses (Table 7).

This may be attributed to a short duration of action of the extract, perhaps limited by rapid metabolism. It might also be due to the *in-vivo* model used which lacks the insect vector, and inoculation was done in such a manner and dose that results in rapid infection of the red blood cells without the parasite going through the liver stages (Edeoga et al., 2005). Death for *P. berghei* infected untreated mice were first observed on day 8 post-infection, and most of the animals died by day 10. Parasitaemia in the control untreated group was evident on the second day, and the level increased throughout the period of the study, while those of the extract treated groups were lower but not completely eliminated. The extract treated groups had a longer survival period which was significantly higher than the maximum survival period of the untreated group (p < 0.01). The fact that all extract and TDF3 treated mice groups had a prolonged survival times when compared with infected untreated mice showed that the whole plant extract possesses intrinsic anti-plasmodial activity.

Some plant extract are known to have anti-plasmodial activity either by causing elevation of RBC oxidation (Etkin, 1997) or by inhibiting protein synthesis (Kirby et al., 1993). It is not known in this study whether the anti-plasmodial activity of the extract was due to a specific anti-plasmodial action or to general cytotoxicity. Therefore, further bioactivity guided isolation of the constituent needs to be carried out on the extract.

Conclusion

The observed anti-plasmodial activity of the methanol whole plant extract of *T. dodoneifolius* and the column active fraction F3 might be due to the presence of pharmacologically active constituents like flavonoids, triterpenes, steroids and Anthraquinones. The study, therefore provide scientific support for the ethno-medicinal claim of the use of the plant in the management of malaria

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Full Length Research Paper

Antibacterial, antifungal, insecticidal and phytotoxic activities of *Indigofera gerardiana* rootsRahman Ur Taj^{1*}, Uddin Ghias¹, Liaqat Wajiha¹ and Choudhary Iqbal Muhammad²¹Institute of Chemical Sciences, University of Peshawar, Peshawar 25120, K.P.K, Pakistan.²International Center for Chemical and Biological Sciences, H.E.J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan.

Received 31 December, 2013; Accepted 13 August, 2015

In the current study an exertion was done to perform biological investigation of *Indigofera gerardiana* to scientifically authenticate its importance. The results obtained showed that all the fractions, n-hexane, ethyl acetate, chloroform, water and residue displayed no antibacterial activities against tested bacterial strains. In case of antifungal activities, the chloroform (F3) and residue (FX3AA) fractions showed 18 and 45% inhibition against *Microsporum canis*, respectively. In case of insecticidal activities, the water fraction (F2) showed 30% activity against *Rhyzopertha dominica* and residue fraction (FX3AA) showed 40 and 16% inhibition against *Rhyzopertha dominica* and *Callosbruchus analis*. The results of phytotoxic assay indicated significant activity for all the fractions at the concentration level of 1000 µg/ml and moderate activity at 100 µg/ml concentrations. These results revealed that this plant is very important from medicinal point of view.

Key words: *I gerardiana*, antibacterial, antifungal, insecticidal and phytotoxic activities, roots.

INTRODUCTION

Indigofera. gerardiana is a deciduous shrub, which belongs to genus *Indigofera* (family leguminosae). The plant is widely found in the tropical regions of the globe. In Pakistan, it consists of about 24 species (Nasir and Ali, 1997). Earlier phytochemical investigations on the genus revealed the presence of various chemical constituents such as triterpenes, steroids, alkaloids, lignin, flavonoids, and acylphloroglucinols (Rahman et al., 2005). Other compounds isolated from the genus include saponins, quinines, tannins, garlic acid, caffeic acid, rutin, myricetin, quercitrin, myricetin and galangin (Bakasso et al., 2008).

In current phytochemical study, various fractions of *I. gerardiana* were examined for their antibacterial, antifungal, insecticidal and phytotoxic activities.

MATERIALS AND METHODS**Plant material**

The roots of medicinal plant *I. gerardiana* were collected during the month of May, 2009 from Lower Dir in Northern areas of Pakistan. Taxonomic identification of the plant was done by Dr. Samin Jan,

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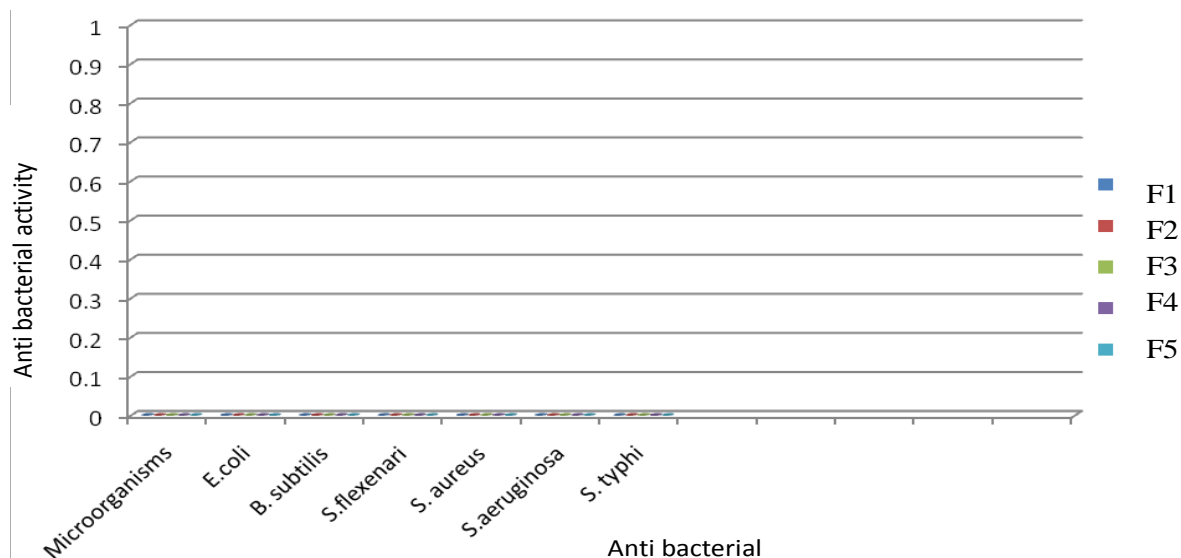


Figure 1. Antibacterial activity of the extract fractions of *M. ovalifolia* stems bark.

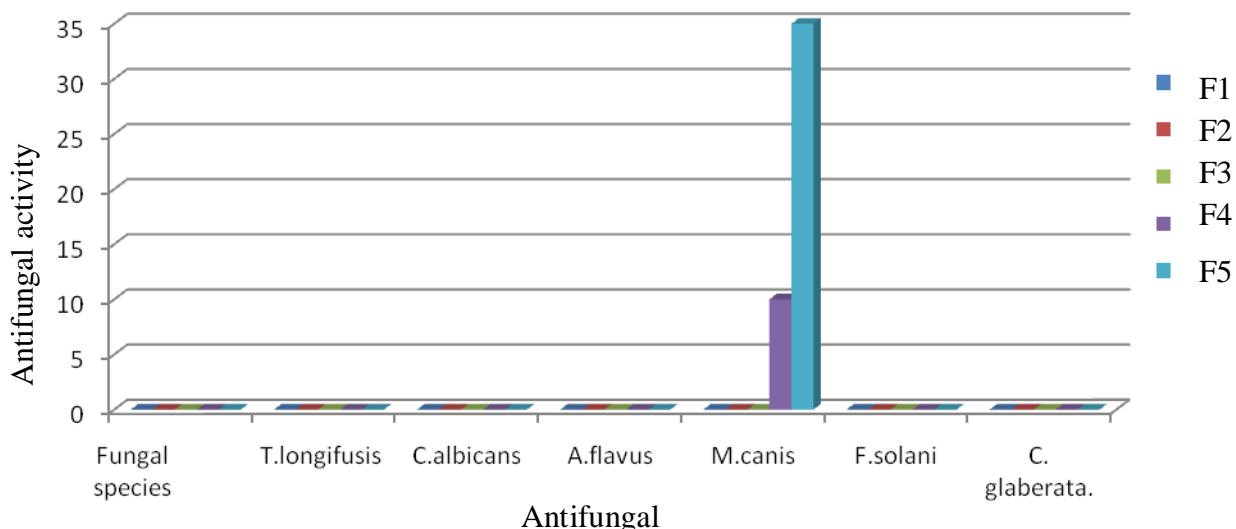


Figure 2. Antifungal activity of the extract fractions of *Indigofera gerardiana*.

Associate Professor, Department of Botany, Islamia College, Peshawar, Pakistan. The voucher specimen (SJ-42) was deposited in the herbarium of Islamia College, Peshawar, Pakistan.

Extraction

I. gerardiana roots (30 kg) were subjected to extraction (x 3) with 5% aqueous methanol for one week. The combined extract was concentrated by a rotary vacuum evaporator to obtain brownish residue of 3.40 kg (Figure 1), which was fractionated by using water

and chloroform to yield 1.20 kg (Figure 2) of water and 2.0 kg (Figures 3 and 4) of chloroform fraction. The chloroform fraction was partitioned into n-hexane and methanol fractions, which afforded FX1A (800 g) and FX1B (1.0 kg), respectively, using soxhlet extractor.

Water fraction was also partitioned with ethyl acetate (EtOAc), as a result, FX3A (600 g) of ethyl acetate fraction was obtained, which was further fractionated using ether: petroleum ether (2:1) and water to get three fractions; FX3AC (150 g), FX3AB (210 g) and residue fraction FX3AA (170 g). The fraction FX3AC was exposed

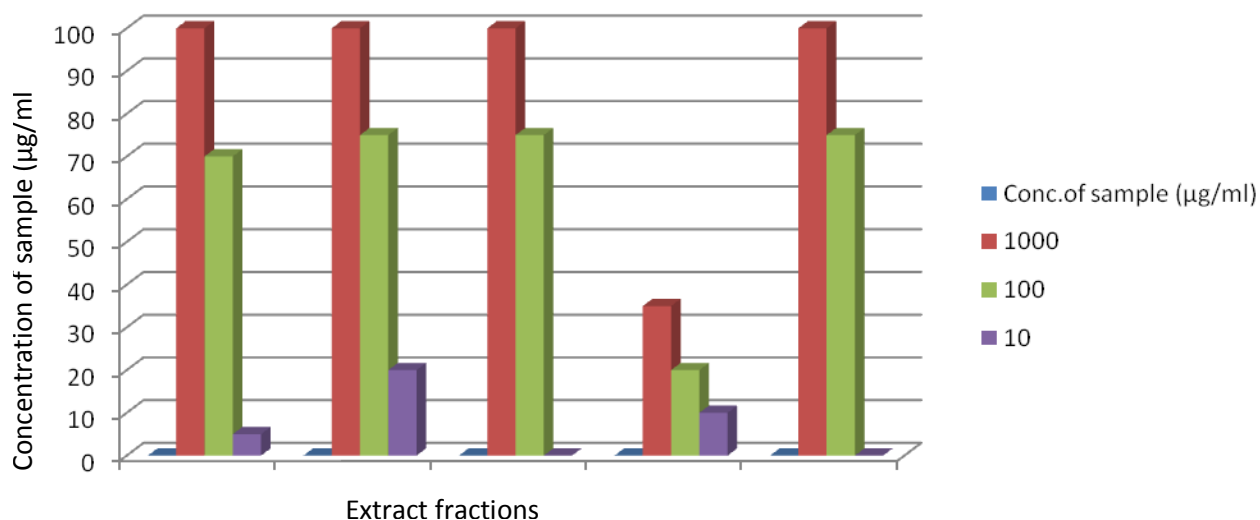


Figure 3. Phytotoxic activity of the extract fractions *M. ovalifolia* stem bark.

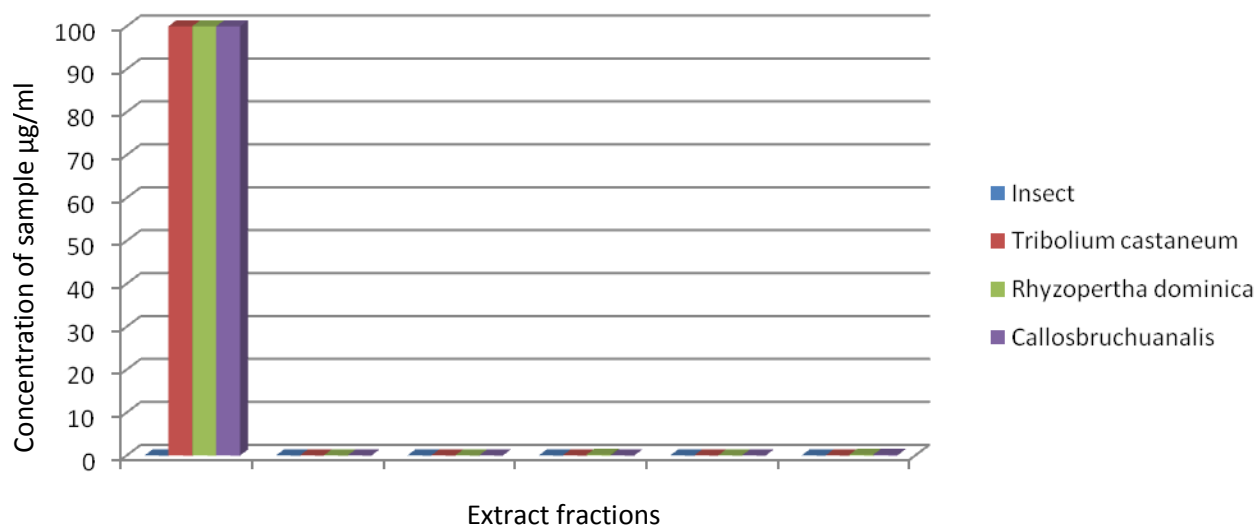


Figure 4. Insecticidal activity of the extract fractions of *M. ovalifolia* stems bark.

to column chromatography on silica gel eluted with n-hexane- ethyl acetate; in increasing polarity yielded sub fractions (A-I). The sub fractions (C-G) were combined based on TLC profile, which yielded 57 sub fractions. The sub fractions 31 to 47 were then mixed and chromatographed using n-hexane-acetone in increasing polarity to obtain various fractions.

Antibacterial activity

The microorganisms; *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC25922, *Shigella flexenari* (clinical isolate), *Staphylococcus*

aureus ATCC 25923, *Pseudomonas aeruginosa* ATCC27853 and *Salmonella typhi* ATCC19430 were used for evaluation of antibacterial activity. The microorganisms were stored on Muller Hinton agar (MHA) in refrigerator at 4°C prior to sub-culturing. Agar well diffusion method was used to study the potency of the roots extract of *I. gerardiana* for antibacterial activity. Broth media were prepared and the test organisms were transferred to the broth media from agar plate and were grown at 37°C for 24 h. After 24 h, 25 ml of MHA were poured into each petri plate and cooled in sterile condition.

The fresh culture was prepared from a day old culture, after solidification of MHA in plate, 0.6 ml of fresh culture of test

organism were poured on to MHA. Wells of 6 mm diameter were dug into the medium by using sterile borer and 60 µl of different fractions of the roots of *I. gerardiana* were used against each organism. DMSO and standard antibiotic Imipenem were used as negative and positive controls. The plates were kept in sterilized inoculation chambers for 1 hour to facilitate diffusion of the antimicrobial agent into the medium. The plates were then incubated at 37°C for 24 h and the diameters of the zone of inhibition of microbial growth were measured in millimeters (Carron et al., 1987).

Antifungal activity

The fungal strains; *Trichophyton longifusus*, *Candida albicans* ATCC2091, *Aspergillus flavus* ATCC32611, *M. canis* ATCC11622, *Fusarium solani* 11712 and *Candida glaberata* ATCC2091 were used for antifungal assay. All these strains were maintained on agar slants at 4°C. The slants were allowed to activate at 27°C for duration of 3 to 4 days on nutrient agar before any screening is made. The extract fractions were dissolved in DMSO (24 mg/ml). Sterile medium (5 ml) was placed in a test tube and inoculated with the sample solution (400 µg/ml) which was then kept in a slanting position at room temperature for overnight.

The tubes were inoculated by a piece of fungus (4 mm diameter) from seven day old culture. The samples were then incubated for 7 days at 28°C and the fungal strains started growth on the slant. The growth inhibition was observed and percentage growth inhibition was determined by calculating with reference to the positive control by applying the formula:

$$\% \text{ Inhibition} = \frac{100 - \text{linear growth and test (mm)}}{\text{Linear growth in control (mm)}} \times 100$$

Miconazole was used as standard drug for positive control (Choudhury et al., 1995; Janaki and Vijayasekaram, 1998).

Insecticidal activity

Different fractions were tested against various insects viz, *R. dominica*, *Tribolium castaneum* and *C. analis*. The samples for tests were prepared by adding 20 mg of crude fractions with 2 ml acetone which was immediately kept in Petri dish covered with the filter papers. 24 h later, ten insects were retained in every plate which was incubated at 27°C for 24 h in 50% humid environment of growth chamber. The activity was examined as percent mortality, which was calculated with reference to the negative and positive controls. Permethrin was kept as a standard drug, while acetone was used as negative controls (Rehman et al., 2001).

Phytotoxic activity

Phytotoxic activity of various fractions of the roots extract of *I. gerardiana* was tested against *Lamina minor*. The medium was prepared by mixing various constituents in 1000 ml distilled water and pH 5.5 to 6.5 was adjusted by addition of KOH solution. The medium was autoclaved at 121°C for 15 min. The extract was dissolved in ethanol (20 mg/ml) which served as stock solution. Later on, nine sterilized flasks, three for each concentration were inoculated with an amount of 1000, 100 and 10 µl of the stock solution each of 1000, 100 and 10 µg/ml, respectively.

The solvent was evaporated overnight in sterile condition. To every flask medium (20 ml) and *Lamina minor* containing a rosette of three fronds was mixed. All flasks were cotton plugged and allowed to grow in sterile condition for 7 days. The numbers of fronds per flask were counted and recorded on day seven and their growth regulation in percentage was determined by calculating with the formula given below. Parquet was used as standard growth inhibitor.

$$(\%) \text{ Growth regulation} = 100 - \frac{\text{No. of fronds in the test samples}}{\text{No. of fronds in the control samples}} \times 100$$

The result was calculated with reference to the positive and negative control. In this study, parquet was used as standard drug (McLaughlin et al., 1991).

RESULTS AND DISCUSSION

Antibacterial activity of the crude fractions

All fractions showed no antibacterial activity against selected bacterial strains (Table 1).

Antifungal activity

All fractions of roots of *I. gerardiana* were investigated for antifungal assay against selected fungal strains which include *T. longifusus*, *C. albicans*, *A. flavus*, *M. canis*, *F. solani*, *C. glaberata* (Table 2). The ethyl acetate (FX3A) and chloroform (F2) fractions showed 10 and 12% bioactivity against *M. canis*.

Insecticidal activity

All the fractions (Table 3) were evaluated for insecticidal activity against the selected insects; *T. castaneum*, *R. dominica* and *C. analis*. The results are summarized in Table 3, which clearly indicate that the water fraction has 30% activity against *R. dominica*, while residue fraction has 40 and 16% activity against *R. dominica* and *C. analis*.

Phytotoxic activity

The isolated fractions from the roots extract of *I. gerardiana* were subjected for phytotoxic assay. The results of the phytotoxic bioassay of various fractions are shown in Table 4. The n-hexane (FX1A), ethyl acetate (FX3A), chloroform (F3), water (F2) and residue fraction (FX3AA) showed significant phytotoxic activity at 1000 µg/ml and low activity at 100 µg/ml concentration. However, the most of the fractions showed no activity at

Table 1. Antibacterial activity of the extract fractions of *Indigofera gerardiana* roots.

Microorganisms	Gram	FX1A	FX3A	F2	F3	FX3AA	DMSO (-)	Imipenem 10 µg/Disc (+)
<i>Escherichia coli</i>	-	-	-	-	-	-	-	25
<i>Bacillus subtilis</i>	+	-	-	-	-	-	-	50
<i>Shigella flexenari</i>	-	-	-	-	-	-	-	28
<i>Staphylococcus aureus</i>	+	-	-	-	-	-	-	48
<i>Seudomonas aeruginosa</i>	-	-	-	-	-	-	-	23
<i>Salmonella typhi</i>	-	-	-	-	-	-	-	28

Well size 6 mm; FX1A = n-hexane; FX3A = EtOAc; F2 = water; F3 = chloroform; FX3AA = residue.

Table 2. Antifungal activity of the extract fractions of *Indigofera gerardiana* roots.

Fungal species	Miconazole (µg/ml)	FX1A	FX3A	F2	F3	FX3AA
<i>Trichophytonlongifusis</i>	70.08	-	-	-	-	-
<i>Candida albicans</i>	110.8	-	-	-	-	-
<i>Aspergillus flavus</i>	20.00	-	-	-	-	-
<i>Microsporium canis</i>	98.40	-	-	-	18	45
<i>Fusarium solani</i>	73.10	-	-	-	-	-
<i>Candida glaberata.</i>	110.80	-	-	-	-	-

FX1A = n-hexane; FX3A = EtOAc; F2 = water; F3 = chloroform; FX3AA = residue.

Table 3. Insecticidal activity of the extract fractions of *Indigofera gerardiana* roots.

Insect	NOs	FX1A	FX3A (%)	F2 (%)	F3	FX3AA (%)
<i>Tribolium castaneum</i>	100	-	-	-	-	-
<i>Rhyzopertha dominica</i>	100	-	16	30	-	40
<i>Callosbruchus analis</i>	100	-	-	-	-	40

FX1A = n-hexane; FX3A = EtOAc; F2 = water; F3 = chloroform; FX3AA = residue.

Table 4. Phytotoxic activity of the extract fractions of *Indigofera gerardiana* roots.

Concentration of sample (µg/ml)	Standard drug	FX1A	FX3A	F2	F3	FX3AA
1000	-	100	100	100	35	100
100	0.015	72	74	75	22	76
10	-	0	18	0	12	0

FX1A = n-hexane; FX3A = EtOAc; F2 = water; F3 = chloroform; FX3AA = residue.

a concentration of 10 µg/ml.

Conclusion

In the current study the roots of medicinal plant of *I. gerardiana* were investigated to explore its phytochemical importance. The results obtained showed that this plant is very important from medicinal point of view, and it needs

further phytochemical exploitation to isolate phytochemical constituents having insecticidal and phytotoxic activities.

ACKNOWLEDGMENTS

We highly acknowledge Higher Education Commission (HEC) of Pakistan for their financial support.

Conflicts of interest

Authors have none to declare

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