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Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

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Full Length Research Paper

Evaluation of biologic effects of an *Ilex paraguariensis* aqueous extract on the labeling of blood constituents with technetium-99m and on the morphology of red blood cells

Ana Cristina S. Braga¹, Maria Luisa Gomes², Josefina da Silva Santos¹, Joelma Fonseca de Oliveira¹, Lúcia de Fátima Amorim³, Gláucio Diré Feliciano⁴, Sebastião David Santos-Filho^{1*} and Mario Bernardo-Filho¹

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Ilex paraguariensis (mate-tea) has been used to treat type-2 diabetes and emphysema. This tea presents antioxidant activity and promotes satiety, body weight lowering and improves bone mineral density. Blood constituents have been labeled with technetium-99m (^{99m}Tc) and this labeling depends on the presence of a reducing agent. Stannous chloride has been widely utilized with this purpose. The influence of natural and synthetic drugs in this procedure has been reported. It was evaluated with effect of an aqueous mate-tea extract on the labeling of blood constituents with ^{99m}Tc and on the morphology of erythrocyte. Blood samples of Wistar rats were incubated with mate-tea extract, stannous chloride and ^{99m}Tc-sodium pertechnetate. Blood cells (BC) and plasma (P) were isolated. BC and P were also precipitated and soluble (SF) and insoluble (IF) fractions separated. The radioactivity was counted and percentage of incorporated radioactivity (%ATI) determined. Blood smears were performed for morphological evaluation. The data show a significant ($p < 0.05$) alteration of %ATI in BC, IF-C and IF-P as well as on the erythrocyte morphology. These findings could be related to the redox properties of the substances of the mate-tea extract, as well as due to the interference in the transport of stannous and/pertechnetate into the BC due to the morphological alterations of the erythrocytes.

Key words: *Ilex paraguariensis*, blood constituents, radiolabeling, morphology, technetium-99m, stannous ion.

INTRODUCTION

Ilex paraguariensis (mate-tea) is an herb whose leaves are utilized to prepare infusion. This infusion is a popular

drink known as mate-tea. It has been consumed under different forms. Various properties of this plant in the

form of tea have been already described and it has been popularly used to treat type-2 diabetes (Hussein et al., 2011) and emphysema (Lanzetti et al., 2011). In addition, data have demonstrated that this tea possesses antioxidant activity (Coentão et al., 2011) and promotes satiety, body weight lowering (Hussein et al., 2011) and improves bone mineral density (Confort et al., 2012). Consumption of mate powder would significantly contribute to antioxidant and stimulant intake, providing high amounts of phenolic acids, tannins and methylxanthines with biological effects potentially beneficial for human health (Vieira et al., 2010).

Blood constituents have been labeled with technetium-99m (^{99m}Tc) and used in several procedures in nuclear medicine. These radiopharmaceuticals can be used for important applications, including imaging of cardiovascular system (Niemeyer et al, 1995; Crandall et al, 2008; Javadi et al, 2011), peripheral arterial blood flow (Blond and Madsen, 2000; Harel et al, 2005; Hsieh et al, 2009), evaluation of gastrointestinal bleeding (Zaman et al, 2004; Wong et al, 2004; Olds et al, 2005; Schillaci et al, 2009; Dolezal et al, 2011), measurement of red cell volume (Hladik III et al, 1987), hepatic hemangiomas (Artiko et al, 2004; Verdu et al, 2005; Yarlagaadda et al, 2008), renal carcinoma (Cortes et al, 2003), splenic reticuloendothelial system (Jin et al, 2004; Slart et al, 2004) and imaging infection (Stoeckli et al, 1996)..

The labeling process of red blood cells (RBC) is based on the transmembrane transport of the reducing agent (Sn^{+2}) and pertechnetate ($^{99m}\text{TcO}_4^-$) ions into the RBC, reduction of $^{99m}\text{TcO}_4^-$ by Sn^{+2} , and subsequent binding of the reduced ^{99m}Tc to internal structures (Early and Soddee, 1995; Dewanjee et al., 1982). The band-3 anion transport system and calcium channels may be involved in transport of $^{99m}\text{TcO}_4^-$ and Sn^{+2} , respectively (Callahan and Rabito, 1990; Gutfilen et al., 1992; Sampson, 1996). The fixation of ^{99m}Tc on plasma proteins also depends on the presence of a reducing agent (Saha, 2010).

Data have demonstrated the effects of synthetic and natural drugs on this radiolabeling process (Mousinho et al., 2008; Holanda et al., 2009; Bustani et al., 2009; Rocha et al., 2009; De et al., 2009; Cekic et al., 2011; Souza et al., 2011). In consequence, the labeling of blood constituents with ^{99m}Tc has been used as an *in vitro* assay to screening effects of synthetic or natural products that could have actions related to the band-3 and calcium channels or antioxidant/oxidant properties. Moreover, qualitative and quantitative morphological analysis of RBC has been used as a method to evaluate if the effects of drugs on this radiolabeling process could be related to changes on shape of RBC (Rocha et al., 2009; De et al., 2009).

The aim of this study was to evaluate biologic effects of an aqueous extract of mate-tea on the labeling of the blood constituents with ^{99m}Tc and on the morphology of

RBC.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (3 to 4 months of age, body weight 250 to 350 g) were maintained in a controlled environment. The animals had free access to water and food and ambient temperature was kept at $25 \pm 2^\circ\text{C}$. Experiments were conducted in accordance with the Institutional Committee of Animal Care of the Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro that has approved the protocols used with the number CEA/213/2007.

Preparation of mate-tea extract

As the *I. paraguayensis* extract can be used in different forms of beverage, it is difficult to determine a specific concentration to be studied (Bastos et al., 2007; Miranda et al., 2008; Burris et al., 2012). In our investigation, to prepare the extracts, 10 g of dry leaves of commercial *I. paraguayensis* (Shapira, Brasil) were vortexed in 50 ml NaCl 0.9%. The crude extract was filtered, centrifuged (clinical centrifuge, 1500 rpm, 5 min) to obtain the final extract. The supernatant was considered to be in the concentration of 200 mg/ml.

In vitro labeling of blood constituents assay

Heparinized blood (500 μl), was withdrawn from Wistar rats and incubated with 100 μl of mate-tea extract at different concentrations (12.5, 25, 50, 100 and 200 mg/ml) or with a saline solution alone, as control, for 1 h (room temperature). Afterwards, 500 μl of stannous chloride (1.20 $\mu\text{g/ml}$) was added and the incubation continued for further 1 h. After this period of time, 100 μl of ^{99m}Tc (3.7 MBq) as sodium pertechnetate ($\text{Na}^{99m}\text{TcO}_4$), recently milked from a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear, São Paulo, Brasil) were added and the incubation was continued for 10 min. These samples were centrifuged in a clinical centrifuge (1500 rpm, 5 min) and aliquots of 20 μl of plasma (P) and blood cells (BC) were isolated. Another aliquots of 20 μl of P and BC were separated and precipitated in 1.0 ml of trichloroacetic acid (5%) and centrifuged (1500 rpm, 5 min) to isolate soluble (SF) and insoluble fractions (IF). The radioactivity in P, BC, SF-P, IF-P, SF-BC and IF-BC were determined in a well counter (Packard, model C5002, Illinois, USA) and the percentage of incorporated radioactivity (%ATI) was calculated as described elsewhere (Oliveira et al., 2003).

Morphological evaluation of red blood cells

Histological preparations were carried out with blood samples *in vitro* treated with mate-tea extract at different concentrations during 60 min at room temperature, or with saline solution as control group. Blood smears were prepared, dried, fixed and stained by May-Grünwald-Giemsa method (Bancroft and Gamble, 2007). After that, the images of the red blood cells were acquired (Optronics, USA) from blood smears to qualitative morphology analysis under optical microscopy ($\times 1000$, Olympus, BX model, Japan).

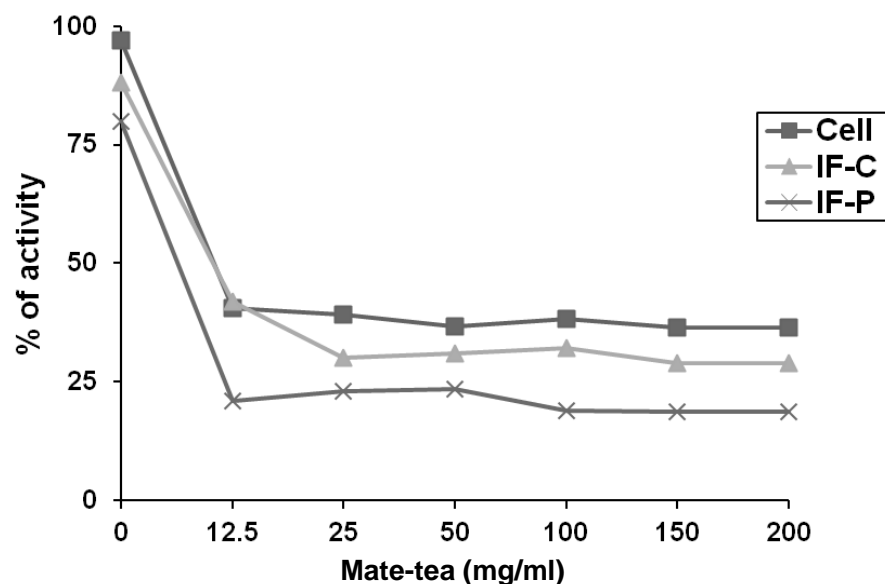


Figure 1. Effect of mate-tea extract on the uptake of the ^{99m}Tc in the blood cells (Cell), insoluble (IF-C) fractions of blood cells and insoluble (IF-P) fractions of plasma, in the radiolabeling procedure of blood constituents. Heparinized blood samples of *wistar* rats were incubated with different concentrations of mate-tea extract (1 h) and after with SnCl_2 (1.20 $\mu\text{g/ml}$, 1 h) and in sequence with $\text{Na}^{99m}\text{TcO}_4$ (3.7 MBq, 10 min). Insoluble and soluble fractions of plasma were obtained by precipitation with trichloroacetic acid (5%) and centrifugation (1500 rpm, 5 min). the radioactivity in these fractions was counted and the %ATI were calculated.

Statistical analysis

Data are reported as (means \pm SD) of %ATI. The comparison of the obtained data of the treated ($n = 10$ for each extract concentration) and control groups ($n = 10$) by one way analysis of variance – (ANOVA), followed by Tukey post test, with a $p < 0.05$ as significant level was performed. InStat Graphpad software was used in the statistical analysis (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California, USA).

RESULTS

Figure 1 shows the distribution of the radioactivity in the cells (cellular compartment) when the blood was treated with different concentrations of the mate-tea extract (12.5, 25.5, 100 and 200 mg/ml). The results show an inhibition in the process of labeling of the cells where the efficiency of labeling was reduced significantly ($p < 0.05$) for all the concentrations of the mate-tea extract tested.

In addition, Figure 1 also shows the fixation of the radioactivity in the insoluble fractions of the plasma (IF-P) and the insoluble fractions of the cells (IF-C) when treated with different concentrations of the mate-tea extract (12.5, 25.5, 100 and 200 mg/ml). The results obtained after the treatment of the blood with the different concentrations of the mate-tea extract, show that it produced

significant reduction ($p < 0.05$) in the efficiency of labeling of the IF-C, from 88.0 ± 3.7 to 41.8 ± 3.7 , until the concentration of 12.5 mg/ml with this reduction more accentuated for the concentration of 200 mg/ml when the %ATI was reduced from 88.0 ± 3.7 to 29.0 ± 1.3 . An important consideration is that these effects are not related to a dose-response relationship. Moreover, with a strong dilution of the extract, its effect was observed.

Figures 2, 3, 4 and 5 represented photomicrographs of the blood smears from samples of whole blood treated with saline solution (control) or with an aqueous mate-tea extract with different concentrations (12.5, 50 and 200 mg/ml), respectively. The qualitative morphological analysis by the comparison between these figures suggests the treatment with mate-tea extract could induce important changes on shape of red blood cells observed under optical microscopy. Furthermore, these qualitative morphological effects seem to be independent on the dose of the extract and they appear with strong dilutions of the extract.

DISCUSSION

The knowledge about properties and effects of a natural product is worthwhile due to the consumption of these

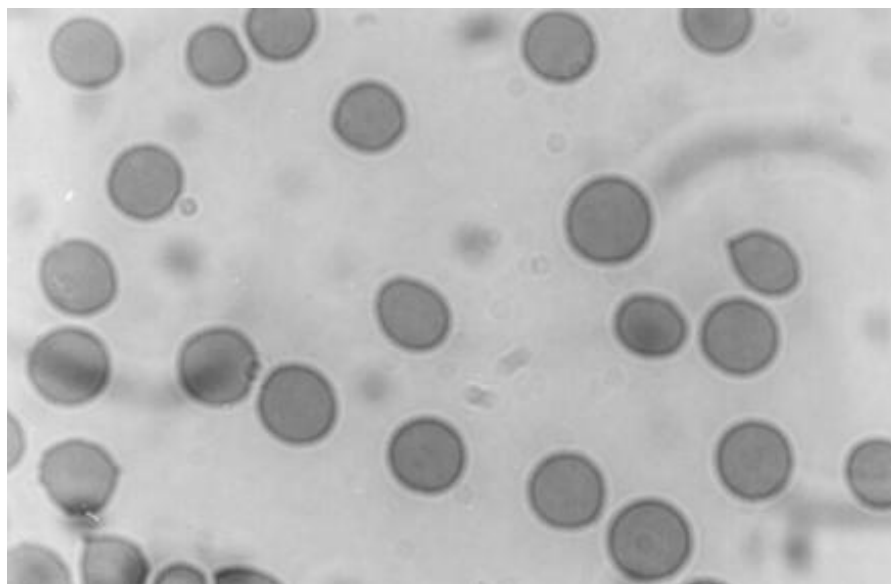


Figure 2. Photomicrography of blood smears from blood samples treated with NaCl 0.9% solution (control). Samples of whole blood from Wistar rats were treated with NaCl 0.9% solution during 60 minutes. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy after image capture.

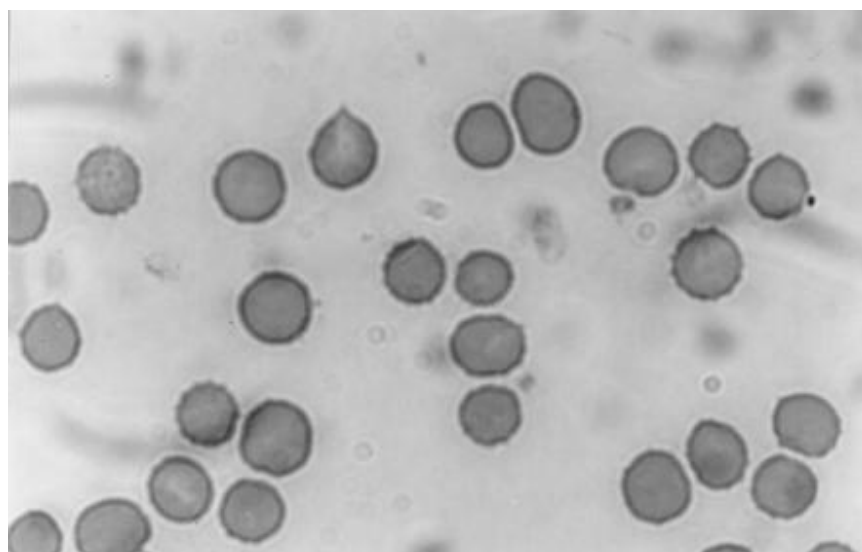


Figure 3. Photomicrography of blood smears from blood samples treated with mate-tea extract. Samples of whole blood from Wistar rats were treated with aqueous mate-tea extract (12.5 mg/ml) during 60 min. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy ($\times 1000$) after image capture. The arrows indicate the alterations on the erythrocyte membrane.

products which are increasing in the entire world (Rotblatt and Ziment, 2002). *I. paraguariensis*, as tea, was capable

in altering the distribution of ^{99m}Tc on plasma and cellular compartments, as well as the fixation of this radionuclide

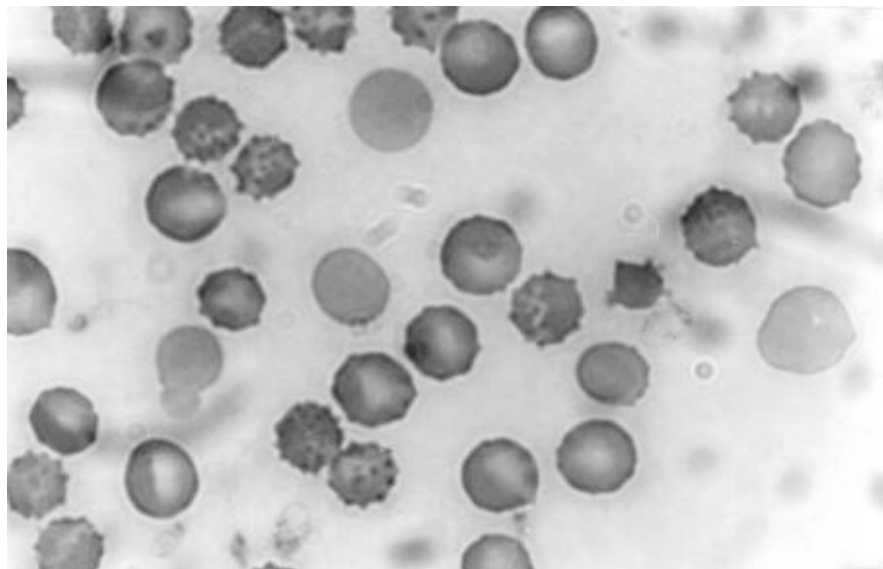


Figure 4. Photomicrography of blood smears from blood samples treated with mate-tea extract. Samples of whole blood from Wistar rats were treated with aqueous mate-tea extract (50 mg/ml) during 60 min. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy ($\times 1000$) after image capture. The arrows indicate the alterations on the erythrocyte membrane.

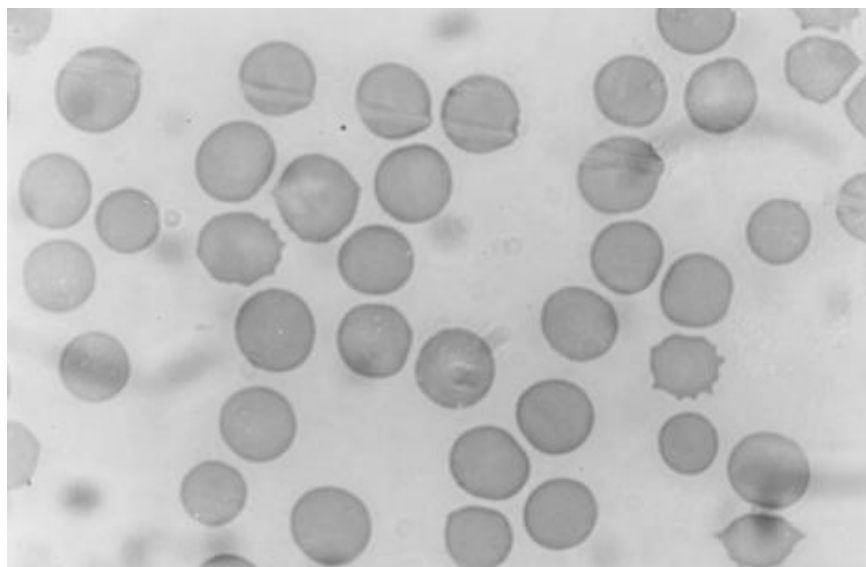


Figure 5. Photomicrography of blood smears from blood samples treated with mate-tea extract. Samples of whole blood from Wistar rats were treated with aqueous mate-tea extract (200 mg/ml) during 60 min. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy ($\times 1000$) after image capture. The arrows indicate the alterations on the erythrocyte membrane.

in the insoluble fractions of plasma and blood cells (Figure 1). It is reported that mate-tea extracts contain

some compounds that have redoxi properties such as phenolic acids, and its properties are attributed to

methylxanthines, such as caffeine referred in folk medicine (Vieira et al., 2010). As the labeling of blood constituents depends on a reducing agent, the redox properties of the substances present in the *I. paraguariensis* could be responsible by the reduction of the labeling of blood constituents with ^{99m}Tc .

In addition, in the labeling of blood cells with ^{99m}Tc , the stannous and pertechnetate ions must reach the cell compartment by channels of erythrocyte membrane. It was observed (Figures 3, 4, 5) that independently on the dose, the extract could cause qualitative and important morphological modifications on the membrane of the red blood cells and this fact can contribute to justify the alteration on the labeling of these cells with ^{99m}Tc . Our results of morphology of RBC could be related to modifications on membrane structures involved in ions transport that could alter the transport of the stannous and pertechnetate ions into cell justifying the decrease of the labeling of blood cells with ^{99m}Tc (Figure 1).

Conclusion

Our findings could be related to the redox properties of the substances of the mate-tea extract, as well as due to the interference in the transport of ions (stannous and/pertechnetate) into the blood cells due to morphological modifications of the erythrocyte membrane. Although, our experiments were performed in controlled conditions and with blood withdrawn from rats, we suggest precaution in the interpretation of the examinations done in a Nuclear Medicine Department in patients that are undertaken mate-tea.

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Full Length Research Paper

Evaluation of the reversibility and possible mechanisms of antifertility of *Catha edulis* F. (khat) extract following subacute administration in rodents

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The study of antifertility mechanisms of khat can be used to counteract the possible toxicities during pregnancy as abuse of khat is common in all segments of female population. The present study was initiated to assess the reversibility of antifertility effect and to evaluate the possible mechanisms of antifertility of the chloroform diethyl ether extract of *Catha edulis*. K300 exhibited significant decrease in the mean number of litters and mean birth weight of live litters compared to K100 and control group (CON) ($p < 0.01$ in both cases). The anti-implantation and antifertility effects were reversible after a 21 day washout period. Post-implantation losses for K200 and K400 were 27% ($p < 0.01$) and 30% ($p < 0.01$), respectively. Maternal mortality in pregnant mice and morphological changes like massive leukocyte infiltration, necrosis and haemorrhage in rat uterus were observed in K400. No morphological changes were shown in ovary at all doses of the extract. Khat has antifertility effect with possibility of complete sterility in higher dose treated groups. The possible mechanisms include decreased serum estradiol concentration and massive leukocyte infiltration in uterus stroma cells. Khat use should be discouraged in female population especially during pregnancy.

Key words: *Catha edulis*, anti-implantation, reversibility, mechanism, rodents.

INTRODUCTION

Catha edulis Forsk (Celastraceae), commonly called 'khat', is an evergreen perennial plant and mostly refers to the leaves and young shoots (Figure 1). The plant is widely distributed throughout the horn of Africa and Arabian Peninsula and chewed fresh daily by millions of people as a recreational drug. Once harvested, khat is a perishable commodity. Hence, its distribution was limited to these areas (Lemessa, 2001). Owing to the possibility of air transport, however, khat has now made its appearance in Europe and North America (Nezar et al., 2005).

Khat buds and leaves contain alkaloids; cathinone,

cathine, and norephedrine, and are chewed by high proportion of the adult population for the pleasant mild stimulant effect (Hassan et al., 2007). Khat also has flavonoids, tannins, oils, vitamins, amino acids and minerals. Moreover, khat consists of terpenoids, alkane hydrocarbons, β -sitosterol (Al-Motarreb et al., 2002; Chappell and Lee, 2010). Cathinone is transformed mainly to cathine in khat leaves and to norephedrine by human metabolism (Rizk et al., 1989).

Recent studies demonstrated that cathinone persists in dried khat for a time frame of several years and simple drying techniques might be used as an effective means to



Figure 1. Photograph of *Catha edulis* leaves purchased from 'Belechie' market.

preserve seized khat evidence for long term storage (Chappell and Lee, 2010).

Even though khat use is a common practice in men, nowadays it is also common in women, even during pregnancy. Fresh khat is thought to increase sexual motivation or libido which is more frequently observed in females than males (Aziz et al., 2009).

Methanolic khat extract showed dose dependent anti-implantation, abortifacient and anti-estrogenic property (Tariq et al., 1987). Other studies indicated that khat possesses both dose dependent embryotoxic as well as teratogenic properties (Islam et al., 1994). Reports also show that 41.6% of mice did not give any litters upon administration of khat extract (Bedada and Engidawork, 2010). Khat alkaloid (+) norpseudoephedrine causes vasoconstriction in the utero-placental vascular bed which may in turn impair fetal growth through reduction of placental blood flow, which is one of the proposed mechanisms for anti-fertility effect of khat (Jansson et al., 1988).

Previous studies showed that the methanolic extract of khat extract had antifertility effect in experimental animals. However, no study had been done whether the antifertility effect of khat extract is reversible or not. In addition to Khat alkaloid (+) norpseudoephedrine induced vasoconstriction mechanism, other possible mechanisms for antifertility and anti-implantation effect of khat extract need to be understood. This work also tries to address the extent of anti-implantation and antifertility effect of khat extract using different animal models.

Intrauterine growth retardation, low fetal birth weight and infant mortality are some of the most important reproductive health problems affecting most developing countries. In addition to this, khat chewing during pregnancy is on the increase among women of reproductive age, and questions have been raised on the potential effects of khat on maternal fertility and fetal development. Therefore, this study tries to address anti-implantation and antifertility effect, the possible mechanisms and

reversibility of these effects of khat extract.

MATERIALS AND METHODS

Chemicals and solvents

Diethyl ether and chloroform were obtained from Fischer scientific, United Kingdom through a locally available distributor. Buffered formalin and phosphate buffer saline (PBS) were prepared in the laboratory.

Experimental animals

Wistar female rats aged 3 to 4 months and Swiss albino mice of 2 to 3 months were maintained at laboratory conditions of controlled environment provided with a 12:12 h light and dark cycle at approximate temperature of 25°C and relative humidity of 50%. Each animal was fed with laboratory diet and water *ad libitum*. Animals with regular estrous cycle were used throughout the experimental period (Marcondes et al., 2002). The animals were cared and treated humanely according to the principles of laboratory animal care (ILAR, 1996) and the protocol was approved by the School of Pharmacy, Addis Ababa University ethics committee.

Plant collection and extraction

Bundles of fresh *C. edulis* shoots and small branches (about 3000 g) were purchased in late December 2010 at local market in 'Beleche', one of the common natural habitats, 290 km South of Addis Ababa, Ethiopia. The fresh bundles were packed in plastic bags and transported in an ice box to the laboratory. The fresh leaves were immediately kept at a deep freezer. The plant was identified by a taxonomist and a voucher specimen was deposited in the National Herbarium, Addis Ababa University (specimen number AS 001). The chopped leaves and buds (1800 g) of *C. edulis* were prepared and extracted as described elsewhere (Banjaw and Schmidt, 2004). The yield was 1.83% and the solid residue was kept in an amber bottle covered with aluminium foil and kept refrigerated for the whole experiment period.

Grouping and dosing of animals

The experimental animals were randomly assigned to four groups. Group I served as control (CON) group and received vehicle (2% V/V Tween 80 in water). Group II were treated with 100 mg/kg khat extract (K100); Group III with 200 mg/kg khat extract (K200) and Group IV with 400 mg/kg khat extract (K400). The doses used were selected based on previous studies (Bedada and Engidawork, 2010; Admassie and Engidawork, 2011).

Reversibility study

Female mice (7 to 8) at stage of proestrus and estrus were left overnight with mature male mice of proven fertility at a ratio of 2:1. The vaginal smear was examined for presence of spermatozoa and/or vaginal plug in the next morning. The day on which the spermatozoa were found in the smear was considered as day 1 of pregnancy. The pregnant mice were then randomly divided into four groups. However, mice in group IV which received K400 showed signs of toxicity and died. So the dose was reduced to 300 mg/kg (K300). The mice received treatments throughout the term and the number and weight of litters delivered (if any) and the gestation

Table 1. Reproductive effects of *Catha edulis* extract administration throughout gestation period and after a 21 day washout period.

Dose	Fertility index (%)		Fetal mortality index (%)		Mean number of live litters		Mean birth weight of litters	
	Before	After	Before	After	Before	After	Before	After
CON	100.0	100.0	0.00	0.00	8.50 ± 0.72	8.88 ± 1.06	1.49 ± 0.05	1.53 ± 0.04
K100	87.5	100.0	0.00	0.00	7.00 ± 1.55	8.88 ± 0.85	1.47 ± 0.92	1.52 ± 0.05
K200	42.85	100.0	11.11	0.00	3.86 ± 1.55 ^{a*}	8.00 ± 0.85	1.32 ± 0.01	1.60 ± 0.03
K300	25.0	75.0	27.27	0.00	1.00 ± 0.7 ^{a,b***}	7.37 ± 1.71	1.10 ± 0.14 ^{a,b***}	1.51 ± 0.08

Data are mean ± SEM, *p<0.05, **p<0.01; n=7-8 mice per group, a as compared to CON, b as compared to K100; CON: Control; K100: 100 mg/kg; K200: 200 mg/kg; K300: 300 mg/kg.

period of dams were recorded. The reversibility of antifertility effect was studied based on previous studies with slight modification (Salhab et al., 1997). After 21 days of extract withdrawal, animals were allowed to mate with male mice in the ratio of 4:1. Pregnant mouse was taken from the group and kept in a separate cage to prevent cannibalism during delivery. The number of litters and birth weight were determined at the end of the term.

Anti-implantation and antifertility study

The anti-implantation and antifertility activities of *C. edulis* in 6 rats per group were determined as described elsewhere with slight modification (Costa-Silva et al., 2007). Pregnancy date was determined as described earlier in reversibility study. The animals were then treated with vehicle or extract for two weeks: pre-implantation period (1st to 6th day) and organogenesis period (7 to 14th day). On the 20th day of pregnancy, the rats were euthanized by cervical dislocation, laparatomized and the uterine horns removed. At day of laparotomy, the number of implants, resorption, live and dead fetuses was recorded. The ovaries were fixed in 10% phosphate-buffered formalin immediately after weighing, then processed for routine hematoxylin and eosin (H&E) staining and the numbers of corpora lutea were counted under light microscope (Armanda-Dias et al., 2001).

Hormonal levels and lipid profile determination

Cycling non-pregnant Wistar rats of 5 per group were treated with either vehicle or extract for 15 days. On day 16th, the animals were anesthetised using diethyl ether and blood was drawn from the orbital sinus, and the ovaries and uteri were dissected out. The blood was then allowed to coagulate for an hour. The serum was separated by centrifuging the coagulated blood at 3000 rpm for 20 min and stored at -20°C. Progesterone, estrogen and cortisol levels were analysed by electro chemiluminescence immunoassay (ECLIA) method using Elecsys 2010 Immunoassay kit (Roche diagnostics, Indianapolis, USA). One ovary from each animal was homogenized with phosphate buffered saline (PBS) (pH 7.4). The serum and homogenized ovary tissue were used for measurement of cholesterol, high density lipoprotein (HDL) (using HDL spin reactor), low density lipoprotein (LDL) and triglyceride contents by enzymatic colorimetric methods.

Histopathological studies of ovary and uterus

The remaining ovary and contralateral uterus were immediately fixed in 10% phosphate buffered formalin, and then routine H&E staining was done (Shivalingappa et al., 2002). The morphologic features including the presence of inflammation, edema, hemorrhage and necrosis were assessed.

Statistical analysis

All data are expressed as mean ± standard error of the mean. Mean from different groups were compared using one way analysis of variance (ANOVA) followed by post hoc tukey test. paired T-test was also used in some parameters. The data were analysed using SPSS version 19.01 and significance was set at p< 0.05.

RESULTS

Reversibility study

Administration of K400 resulted in death of 85.7% of mice within a week after showing signs of toxicity like decreased mobility, decreased food intake, seizure, erected hair, and decrease in body weight. Because of these adverse reactions, the dose was decreased to 300 mg/kg.

Although dams exposed to khat extract during pregnancy exhibited decrease in fertility index and increase in mortality of litters (Table 1); dams which gave birth were within the usual expected period of gestation (20 to 22 days). The fertility index of K100 and K200 after washout period was 100% which was comparable to CON. About 25% of K300 were unable to give birth despite efforts of long period of stay (around 2 months) by keeping them with males of proven fertility. The K100, K200 and K300 showed an increase in fertility index by 12.5, 57.2 and 66.7%, respectively, after 21 days of washout period. In case of fetal mortality index, no dead litter was found both in CON and K100. However, K200 and K300 showed 11 and 27% fetal mortality, respectively, which was decreased by 100% after washout period.

Female mice in khat extract treated groups delivered decreased number of litters per dam as compared to CON. K300 decreased mean number of litters compared to K100 and CON (p<0.01 in both cases). The K200 also decreased mean number of litters as compared to CON (p<0.05). Withdrawal of treatment for 21 days increased mean number of live litters in K100 (p<0.05), K200 (p<0.01) and K300 (p<0.01) by 21.1, 51.8 and 86.4%, respectively compared to treatment values. No apparent difference was, however, noted between controls in pre- and post-withdrawal values (Figure 2A).

K300 decreased mean birth weight of live litters as

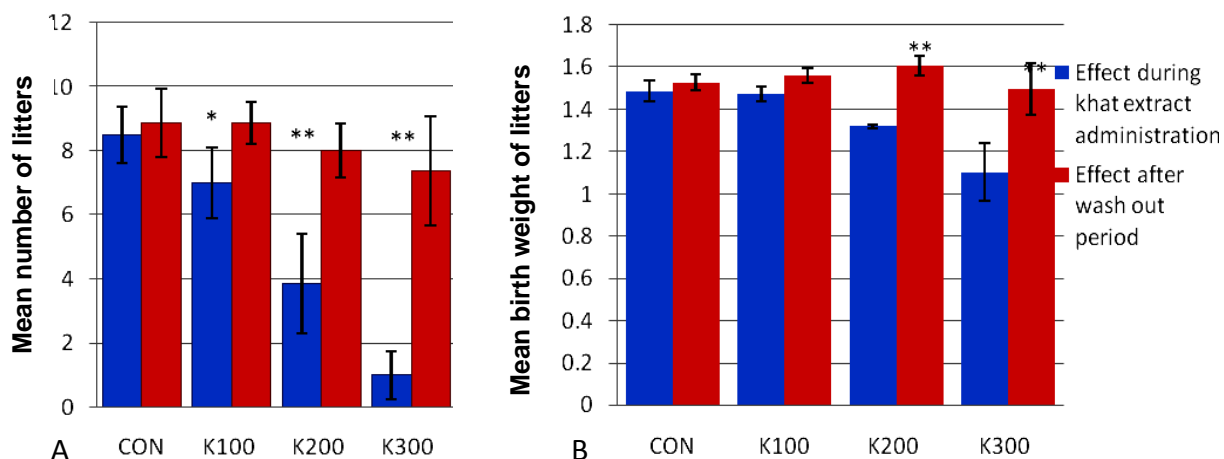


Figure 2. Comparison of mean number of litters (A) and mean birth weight of litters (B) during khat extract administration and after washout period. Data are mean \pm SEM, n=7-8 mice per group, * p<0.05, **p<0.01.

Table 2. Reproductive parameters of female rats treated with *Catha edulis* crude extract from 1st to 14th day (pre-implantation and organogenesis period) of pregnancy.

Reproductive parameter	CON	Khat extract	
		K200	K400
Number of corpora lutea	9.67 \pm 0.80	9.83 \pm 0.60	12.50 \pm 1.80
Number of implantation sites	9.50 \pm 0.76	8.33 \pm 0.42	6.00 \pm 1.97
Number of live fetuses	9.33 \pm 0.88	6.17 \pm 0.75	4.17 \pm 1.35 ^{a**}
Number of resorptions	0.17 \pm 0.17	1.67 \pm 0.33 ^{a**}	1.75 \pm 0.25 ^{a**}
Implantation index	98.48 \pm 1.52	85.89 \pm 5.46	61.25 \pm 19.45
Resorption index	2.38 \pm 2.38	20.31 \pm 4.03 ^{a**}	19.23 \pm 1.90 ^{a**}
Fetal wastage index	2.38 \pm 2.38	24.24 \pm 6.00 ^{a**}	30.12 \pm 3.08 ^{a**}
Ovary per body weight/100g	0.091 \pm 0.011	0.057 \pm 0.002 ^{a**}	0.057 \pm 0.003 ^{a**}
Mean fetal weight (g)	5.27 \pm 0.19	5.21 \pm 0.26	4.10 \pm 0.34 ^{a,b*}
Pre-implantation loss (%)	1.52 \pm 1.52	15.44 \pm 4.90	38.75 \pm 19.45
Post-implantation loss (%)	2.38 \pm 2.38	26.63 \pm 6.77 ^{a**}	30.12 \pm 3.08 ^{a**}
Anti-implantation loss (%)	3.89 \pm 2.55	37.89 \pm 5.23 ^{a*}	57.31 \pm 13.57 ^{a**}

Data are mean \pm SEM, n=6 rats per group, *p<0.05, **p<0.01; ^a as compared to CON, ^b as compared to K200; CON: Control; K200: khat extract 200

compared to CON and K100 (p<0.01 for both cases). Although K200 group displayed a decrease in mean birth weight of live litters compared to CON, it failed to reach statistical significance. Litters of extract treated groups regained the mean birth weight after a 21 day washout period. The increase in weight was significant with K200 (17.7%, p<0.01) and K300 (26.9%, p<0.01) groups. Although there was an increase in weight gain in CON and K100 groups by 2.8 and 2.9%, respectively, the difference was not statistically significant (Figure 2B).

Anti-implantation and antifertility study

During the treatment period with *C. edulis*, a death of

16.7% pregnant rat treated with K400 was registered and some clinical signs of maternal toxicity were observed before death. Only two doses of khat extract were used as K100 had no significant antifertility effect during reversibility study in mice. There was no difference in the number of corpora lutea and implantation sites between khat extract treated groups and CON. Likewise, no difference was noted in percentage of implantation in K200 and K400.

As shown in Table 2, K400 decreased the number of live fetuses per dam by 55.3% as compared to CON (p<0.01). Though K200 was able to reduce the number of live fetuses per dam by 33.9%, the difference failed to reach statistical significance. However, K200 and K400 increased fetal wastage by 24 and 30% and percentage

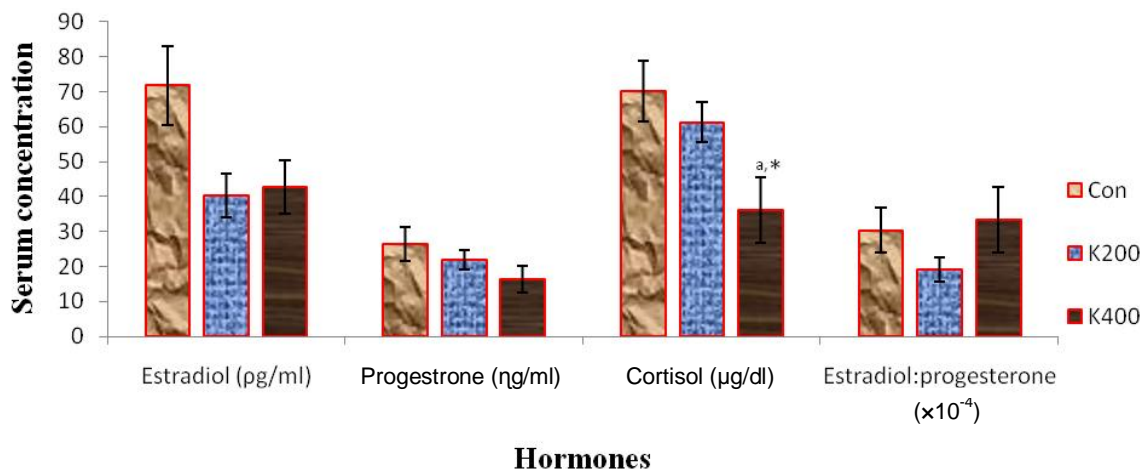


Figure 3. The effects of subacute administration of *Catha edulis* crude extract on serum concentration of hormones in non pregnant rats. Data are mean \pm SEM, n=5 rats per group, *p<0.05, ^aas compared to control, CON: control; K200: 200 mg/kg; K400:400 mg/kg of khat extract.

Table 3. The effects of subacute administration of *Catha edulis* crude extract on lipid profile of non pregnant rats.

Parameter	CON	<i>Catha edulis</i>	
		K200	K400
Total cholesterol(mg/dl)	100.64 \pm 8.34	88.48 \pm 2.88	63.05 \pm 2.84 ^a
Triglyceride(mg/dl)	271.36 \pm 152.72	82.79 \pm 5.48	70.87 \pm 9.86
LDL (mg/dl)	8.27 \pm 0.57	6.51 \pm 0.76	5.38 \pm 0.69 ^a
HDL (mg/dl)	37.20 \pm 1.77	38.91 \pm 8.68	47.20 \pm 6.39
Ovary cholesterol (mg/dl)	22.60 \pm 6.46	57.20 \pm 13.65	47.03 \pm 19.95
Ovary triglyceride (mg/dl)	529.82 \pm 102.16	820.37 \pm 140.18	710.75 \pm 87.34
Ovary weight (mg) /100g	0.048 \pm 0.0047	0.048 \pm 0.0029	0.043 \pm 0.0031
Uterus weight (mg) /100g	0.242 \pm 0.052	0.158 \pm 0.014	0.194 \pm 0.039

Data are expressed mean \pm SEM; n= 5 rats per group; ^a against CON, *p<0.05, CON: Control; K200: 200 mg/kg; K400: 400 mg/kg.

of resorption by 20.3 and 19.2%, respectively, compared to CON (2.4%). The mean fetal weight was decreased in K400 (p<0.05) compared to K200 and CON. The percentage of pre-implantation loss in K200 and K400 were 15.4 and 38.75%, respectively. The percentage of post-implantation loss for K200 and K400 were 26.6 and 30.1%, respectively. Thus, K200 and K400 showed significant increase in post-implantation loss rate (p<0.05) than pre-implantation loss rate. K400 (p<0.01) and K200 (p<0.05) group had significant anti-implantation loss as compared to CON.

Hormonal and lipid profile

As shown in Figure 3, K200 and K400 decreased serum estradiol levels by 43.7 and 40.6%, respectively, the difference, however, did not reach statistical significance. Similarly, K200 and K400 tended to decrease serum

progesterone levels, though once again the difference was not statistically significant. No detectable change was noted in serum cortisol with K200 though levels were decreased by 12.5%. Cortisol levels, however, significantly decreased (49%, p<0.05) with K400 compared to CON.

As shown in Table 3, no apparent difference was observed between CON and K200 in serum cholesterol. By contrast, K400 demonstrated a significant reduction (37%, p<0.05) in total serum cholesterol. Similarly, no detectable change was observed in serum LDL cholesterol between CON and K200, though K200 tended to decrease levels by 21%. K400, however, was capable of significantly reducing LDL levels (35%, p<0.05) compared to CON. Khat failed to increase serum HDL and decrease serum triglyceride levels significantly, although K400 tended to increase HDL by 21.2% and decrease triglyceride levels by 73.9%. Obviously, ovary cholesterol and triglyceride levels were increased by khat treatment.

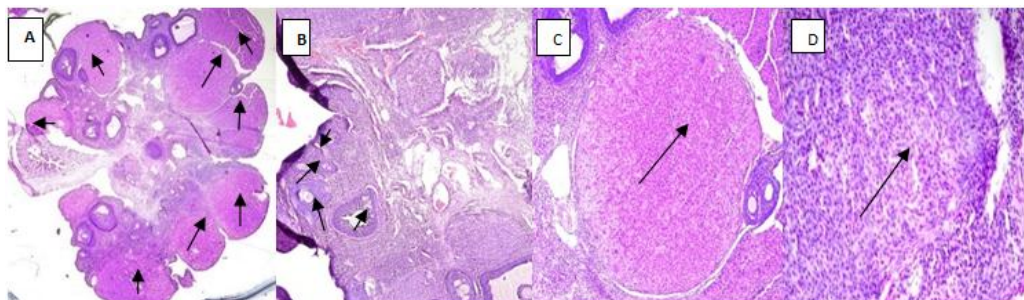


Figure 4. Photomicrograph of hematoxylin and eosin stained ovarian sections for control and *Catha edulis* treated rats. CON showed normal ovary morphology with corpora lutea, 2.5x (A); K100, 10x showed ovary components with primary, secondary and tertiary follicles (B); normal structure of ovary with corpora lutea in K200 group, 10x(C) and normal structure of stromal cells in corpora lutea of K400 group, 40x(D).

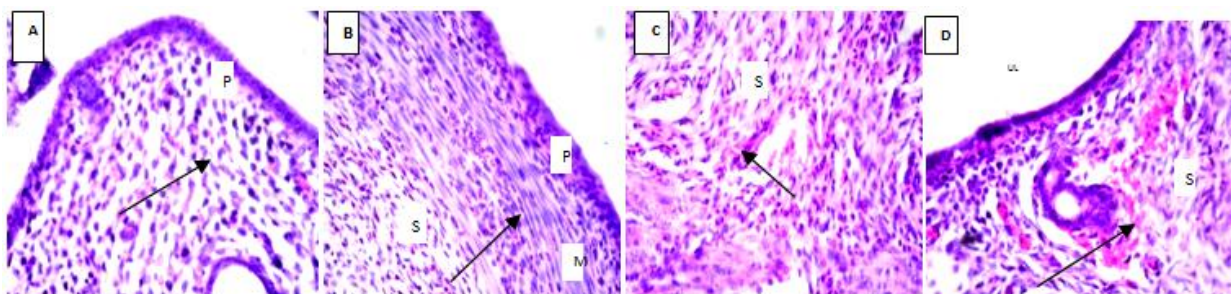


Figure 5. Photomicrograph of hematoxylin and eosin stained uterine sections for control and *Catha edulis* treated rats: CON showed normal stroma cells, 40x (A); K200 rats show evidence of inflammation that migrate towards myometrium, 40x (B); those treated with K400 show inflammatory infiltration, 40x (C) and haemorrhage, 40x (D). M-Myometrium; S-Endometrial Stroma; P-Perimetrium; UL-Uterine Lumen.

Morphometric analysis

The weights of ovary and uterus were decreased in both khat extract treated groups but failed to reach statistical significance. The histological sections of CON, K200 and K400 treated animals showed normal ovarian morphology and no morphological change was observed between the control and khat treated groups (Figure 4). However, extensive inflammation was observed in myometrium in K200 group. K400 also showed extensive inflammatory infiltrates within the endometrial stroma cells with edema, hemorrhage and necrosis (Figure 5).

DISCUSSION

The present study was designed to further evaluate the antifertility effects of *C. edulis*, which is popularly used for its euphoric effects. All the necessary precautions were taken to prevent degradation of cathinone throughout the extraction procedure. The reduction in fertility index, mean number of live litters and increment in fetal mortality index in cyclic rodents after subacute treatment with khat extract reinforces the notion that khat is

endowed with antifertility activities in rodents. Methanolic extract of khat at a dose of 500 mg/kg is reported to have around 40% anti-implantation activity (Tariq et al., 1987), which is lower than the present finding (57%) at a dose of 400 mg/kg. The decrease in anti-implantation effect in the previous work might be due to the following reasons: (1) use of dried leaves instead of fresh leaves; (2) safe extraction procedure employed in the present study that used easily volatile solvents and lyophilizer, which enabled to maintain an increasing yield of the highly potent and easily degradable component, cathinone (Banjaw and Schmidt, 2004). It has been demonstrated that drying the green khat leaves with heat decreases the cathinone content by chemical reduction (Chappell and Lee, 2010).

The decrease in the number of live litters per dam at higher dose might be due to increase in the number of resorptions and dead fetuses as *in utero* treatment of khat extract has been reported to increase resorptions during pregnancy (Islam et al., 1994).

The observed decrease in mean birth weight of litters in rodents was also reported in humans (Al Harazi, 2009), and was a contributing risk factor for both fetal and litter mortality. When testing possible fetal toxicity of a specific

substance, it is necessary to establish if these effects are due to direct action on the fetus or an indirect action through the maternal toxicity that could secondarily interfere with the fetus. Clinical signs of maternal toxicity that were observed in rodents indicated that indirect action is also implicated in fetal toxicity. From the evidence that severe toxicity was observed in gavage administration of mice, and it is also possible to conclude that pregnant mice are more sensitive to gavage administration of *C. edulis* than non-pregnant rats like berberine (Jahnke et al., 2006). Fetal mortality might also be due to placental insufficiency that resulted from vasoconstriction caused by khat extract (Jansson et al., 1988). In addition, anorexia is a well known side effect of khat (Zelger and Carlini, 1980) and the possible role of anorexia for intrauterine growth retardation cannot be excluded.

The present finding is perhaps the first report on the reversibility of anti-fertility activity of khat in mice at lower doses (100 and 200 mg/kg) with possibility of sterility in large dose (400 mg/kg) treated mice. The observation that the absence of significant change in the number of litters between control and the post-extract groups and recovery of all induced antifertility effects after withdrawal for 3 weeks shows that the anti-fertility and anti-implantation effects were transient and reversible. The present observations are in agreement with previous studies on the reversible antifertility effect of *Ricinus communis* (castor beans) in female rabbits (Salhab et al., 1997).

The decreased implantation in the present investigation might be due to massive leukocyte infiltration in stroma cells that resulted in degeneration of the early embryos and post-implantation embryonic resorptions in the uteri. This massive leukocyte infiltration was observed in K200 and K400 group. Such infiltration was also reported in khat extract treated hepatic portal tract and the infiltration was reported to increase with dose (Al-Habori et al., 2002). The lack of antifertility effect in K100 could be explained by the pharmacokinetic characteristics of khat components. The main alkaloid, cathinone, has a rate of absorption similar to the rate of inactivation, which limits the cathinone blood levels attainable by gavage administration. This characteristic is important for absence of many adverse effects observed due to cathinone accumulation. However, with increasing dose treatment results in cathinone accumulation and thereby results in anti-implantation/anti-fertility effects (Cox and Rampes, 2003).

Any imbalance in levels of estrogen and progesterone is implicated in infertility. As a result, one possible mechanism of antifertility effect of khat may be hormonal imbalance, as demonstrated by a decrease in serum estrogen and alteration of the estrogen: progesterone ratio. The decreased serum estradiol along with massive leukocyte infiltration might affect the estrogen responsive epidermal growth factor receptor (EGFR) localization in the stroma cells. Overall uterine and vaginal organ

growth, in response to estrogen, require EGFR signaling for DNA synthesis in the fibro-muscular stroma and a decrease in estradiol might results in degeneration of the early embryos and post-implantation embryonic resorptions in the uteri (Juneja et al., 1996; Hom et al., 1998). Another hormone which might be implicated for antifertility effect of khat was cortisol. The decrease in cortisol level after khat extract treatment suggests that cortisol is unlikely to play a role in the antifertility mechanisms of khat.

The decreased LDL cholesterol could also be considered as another mechanism for antifertility effect of *C. edulis*, as LDL and HDL cholesterol are the major transporters of fertility factors. But this is not true in rodents as the main transporter for cholesterol to ovarian steroidogenesis is HDL unlike that of other mammals and humans which uses LDL as major transporter (Cedars, 2007). The lack of change in HDL thus excludes the role of lipids as a possible mechanism for anti-fertility effects of khat.

Conclusions

The antifertility and anti-implantation activity of fresh khat extract with possibility of complete sterility was demonstrated. The possible mechanisms include decreased serum estradiol concentration, altered estrogen to progesterone ratio, and massive leukocyte infiltration in stromal cells that prevent the sequential effects of estrogen in uterus by different components of khat. Further researches are recommended on how all these mechanisms work together.

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Full Length Research Paper

Gastroprotective activity of mirtazapine, escitalopram and venlafaxine in depressed rats

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The study investigated the gastro-protective effects of certain antidepressants in relation to ranitidine on indomethacin-induced ulcer in depressed rats. Animals were divided into 6 groups (n = 8). Induction of depression was done by clonidine (0.8 mg/kg; i.p.) for 10 days in all groups except the 1st one (normal control). Depressive-like behavior was confirmed by increased immobility time in forced swimming test. Groups 1 and 2 received saline (normal and depressed controls, respectively). Groups 3 to 6 received p.o. mirtazapine (10 mg/kg), escitalopram (10 mg/kg), venlafaxine (20 mg/kg) and ranitidine (50 mg/kg), respectively for 30 days. After the last treatment, gastric ulcer was induced using indomethacin (25 mg/kg; p.o.) in groups 2 to 6. An additional group received indomethacin alone (control indomethacin). Animals were sacrificed and ulcer scores were determined. Part of the stomach was preserved for histopathologic studies while the other part was used for determination of reduced glutathione (GSH), malondialdehyde (MDA), nitric oxide (NO), tumor necrosis factor-alpha (TNF- α) and interleukin-10 (IL-10) contents. Pretreatment with all antidepressants used ameliorated indomethacin-induced changes in rat stomach. Biochemical findings were supported by histologic studies. In conclusion, the observed gastroprotective effects of mirtazapine, escitalopram and venlafaxine are possibly mediated by modulation of inflammatory cytokines and antioxidant effects.

Key words: Mirtazapine, escitalopram, venlafaxine, depression, indomethacin.

INTRODUCTION

Gastric ulcer is a multifactorial etiological disease involving depression, stress and production of reactive oxygen species (ROS) as major factors (Kwiecien et al., 2012; Goodwin et al., 2013). An increased vulnerability to depression (Pare, 1989) in experimental animals is paralleled with ulcer development and the same occurs in humans (Sjodin et al., 1985). Interestingly, certain antidepressants, such as duloxetine, amitriptyline, fluoxetine, were shown to reduce stress ulcer formation, perhaps to a greater extent than that seen with traditional therapies

such as cimetidine and antacids (Dajani et al., 2003; Olden, 2005; Bilici et al., 2009).

Mirtazapine, escitalopram and venlafaxine are extensively used antidepressants in clinical practice. Mirtazapine, a norepinephrine and specific serotonin antidepressant, enhances both serotonin (5-HT) and norepinephrine (NE) neurotransmission (Haddjeri et al., 1996). Escitalopram is the S-enantiomer of the racemic selective serotonin reuptake inhibitor compound citalopram with reported marked antidepressant and

anxiolytic activities (Sanchez et al., 2003). Venlafaxine inhibits the reuptake of 5-HT, NE and dopamine. Although its mechanism of action is similar to the tricyclic antidepressants (TCAs) drugs; it acts more specifically at those receptors and does not bind to the receptors responsible for the side effects of TCAs (Adelman et al., 2000).

Chronic inflammation and oxidative stress have been implicated in the pathophysiology of major depressive disorder (MDD) (Berk et al., 2011). MDD is accompanied by activation of inflammatory pathways, reflected by an increased levels of inflammatory cytokines, such as interleukin-1beta, interleukin-2, interleukin-6, interferon-gamma and tumor necrosis factor-alpha (TNF- α) (Maes, 1999).

The current study aimed to examine the possible anti-ulcer effect of mirtazapine, escitalopram and venlafaxine in indomethacin induced ulcer in depressed rats and to compare their effects with that of ranitidine, a well-known H₂ blocker. Indomethacin-induced gastric ulcer model has been chosen as it produces higher gastric damage in rats when compared to other non-steroidal anti-inflammatory drugs (NSAIDs) (Takeuchi et al., 2005). It seemed also important to investigate the effects of the chosen agents on certain factors known to be involved in ulcer and depression pathophysiology as oxidative stress biomarkers as well as production of pro-inflammatory and anti-inflammatory cytokines.

MATERIALS AND METHODS

Animals

Adult male albino Wistar rats weighing 130 to 160 g between 10 to 12 weeks age were obtained from the animal house colony of the National Research Center. The animals were housed under standard light, temperature, and room humidity conditions during the study. Animal experiments were performed in accordance with guidelines for the use and care of laboratory animals of the Ethics Committee of Faculty of Pharmacy, Cairo University.

Chemicals

All chemicals for laboratory experimentation were purchased from Sigma Chemical (Germany). Indomethacin, mirtazapine, escitalopram, venlafaxine and ranitidine were obtained from Khahira (Egypt), Novartis (Egypt), Lundbeck (Denmark), Wyeth (USA) and Pharco (Egypt) companies, respectively. Drugs were prepared as suspensions in 1% tween 80 and orally administered.

Experimental design

Rats were divided into 6 groups (n = 8). Induction of depression was done by i.p. injection with clonidine (0.8 mg/kg) (Enginar and Eroglu, 1990) once daily for 10 days in all groups except the 1st one which received saline i.p. (normal control). From 10th day

onwards, after the confirmation of depression by forced swimming test (FST), groups 3 to 6 were orally treated with mirtazapine (10 mg/kg) (Ji et al., 2012), escitalopram (10 mg/kg) (Saglam et al., 2008), venlafaxine (20 mg/kg) (Abdel-Wahab and Salama, 2011) and ranitidine (50 mg/kg) (Cayci and Dayioglu, 2009), respectively for 30 days. The 1st two groups received 1% tween 80 (p.o.) and served as normal and depressed control groups, respectively. Rats were subjected again to FST after the last dose of antidepressants. Thereafter, groups 2 to 6 in addition to a 7th group (indomethacin control) received 25 mg/kg indomethacin p.o. (Dengiz et al., 2013). Six hours later, all rats were sacrificed using a high dose of thiopental sodium (50 mg/kg, i.p.), the stomachs were excised and gastric erosions on the surfaces of the stomachs were examined macroscopically for mucosal necrotic lesions, red streaks and red erosions (Mozsik et al., 1982). Total lesions number was counted and the severity of lesions was determined based on the following scores:

- 0 = No ulcer;
- 1 = Lesion size < 1 to 2 mm;
- 2 = Lesion of size 1 to 2 mm;
- 4 = Lesion of size 3 to 4 mm.

Part of each stomach was preserved in 10% formalin for histopathologic examination and the remaining part was used to prepare 10% homogenate in phosphate buffer. The prepared homogenates were used for estimation of stomach contents of lipid peroxides, reduced glutathione (GSH), nitric oxide (NO), TNF- α and interleukin-10 (IL-10).

Forced swimming test

Depression-like behavior in FST was assessed as previously described (Cryan et al., 2001). Rats were individually placed in a transparent plexi-glass cylinder. The latency to immobility was recorded. Immobility was defined as the total absence of movement except slight motions to maintain the head above the water (Cryan et al., 2002). At the end of the experiment, rats were dried and placed back into their home cage with littermates.

Biochemical and histologic analysis

Lipid peroxides were determined as thiobarbituric acid reactive substances (TBARS) using malondialdehyde (MDA) as a reference according to the method described by Mihara and Uchiyama (1978) and expressed as nmol/g wet tissue. GSH content was determined using Ellman's reagent according to the method of Beutler et al. (1963) and expressed as mg/g wet tissue. NO content was determined as total nitrate/nitrite (NO_x) using Griess reagent according to the method described by Miranda et al. (2001) and expressed as μ M/g wet tissue. Stomach contents of TNF- α and IL-10 were estimated using rat specific immunoassay kit (Biosource, USA) and expressed as pg/g wet tissue. Tissue samples preserved for histopathologic examination were fixed in 10% formalin and used to prepare paraffin blocks. Sections of 5 μ m were obtained and stained with Hematoxylin and Eosin (H & E). Images were captured and processed using Adobe Photoshop (version 8).

Statistical analysis

Values were expressed as means \pm standard error (SE). Results of ulcer number and severity were analyzed using Kruskal-Wallis non-

Table 1. Effects of 30 days administration of mirtazapine, escitalopram, venlafaxine and ranitidine on immobility time of depressed rats in forced swimming test (FST).

Group	Immobility time	
	Day 0	Day 30
Normal	1.42 [®] ±0.28	1.61±0.24
Control	2.73*±0.09	3.13*±0.15
Depressed rats		
Mirtazapine (10 mg/kg)	2.58*±0.20	2.05 [®] ±0.16
Escitalopram (10 mg/kg)	2.86*±0.14	1.67 [®] ±0.10
Venlafaxine (20 mg/kg)	2.88*±0.10	1.05 [®] ±0.15
Ranitidine (50 mg/kg)	2.68*±0.16	2.88*±0.10

Induction of depression was done by i.p. administration of clonidine (0.8 mg/kg) for 10 successive days. FST was performed after the last dose of clonidine (day 0) and after the last dose of test agents (day 30). Values are means ± SE of 8 rats. Statistical analysis was done by one way ANOVA followed by Tukey Kramer multiple comparisons test. *Significantly different from normal control group at *p* < 0.05. [®]Significantly different from control depressed group at *p* < 0.05.

parametric one way analysis of variance (ANOVA) followed by Mann Whitney multiple comparisons test. The results of the remaining experiments were analyzed using one way ANOVA followed by Tukey Kramer multiple comparisons test. *P* < 0.05 was accepted as being significant in all types of statistical tests. Graph Pad Software InStat (version 2) was used to carry out these statistical tests.

RESULTS

Effects of mirtazapine, escitalopram, venlafaxine and ranitidine on immobility time of depressed rats in forced swimming test

The results depicted in Table 1 show that clonidine-induced depression increased immobility time of rats in FST when compared to the normal control group. Administration of mirtazapine (10 mg/kg), escitalopram (10 mg/kg) and venlafaxine (20 mg/kg) for 30 days decreased the immobility time of depressed rats by 34.50, 46.64 and 66.45%, respectively when compared to control group. On the other hand, administration of ranitidine (50 mg/kg) did not significantly change immobility time when compared to control depressed group (Table 1).

Effects of mirtazapine, escitalopram, venlafaxine and ranitidine on gastric ulcer number and severity induced by indomethacin in depressed rats

Macroscopic lesions with evident borderlines in various forms and sizes were dispersed irregularly on all stomach

Table 2. Effects of 30 days administration of mirtazapine, escitalopram, venlafaxine and ranitidine on gastric ulcer number and severity induced by indomethacin in depressed rats.

Groups	Score	
	Ulcer number	Ulcer severity
Normal	-	-
Indomethacin	12.50*±0.89	18.80*±1.51
Depressed + indomethacin		
Control	13.00*±1.53	20.30*±2.22
Mirtazapine (10 mg/kg)	3.50 [®] ±0.43	6.17* [®] ±1.66
Escitalopram (10mg/kg)	5.00* [®] ±1.29	5.83* [®] ±0.62
Venlafaxine (20 mg/kg)	3.17 [®] ±0.91	3.33 [®] ±0.21
Ranitidine (50 mg/kg)	2.17 [®] ±0.28	2.33 [®] ±0.21

Induction of depression was done by i.p. administration of clonidine (0.8 mg/kg) for 10 successive days. Treatment with test agents started following induction of depression. Gastric ulcer was induced by oral administration of indomethacin (20 mg/kg) after the last dose of test agents. Values are means ± SE of 8 rats. Statistical analysis was done by Kruskal-Wallis non-parametric one way ANOVA followed by Mann Whitney multiple comparisons test. *Significantly different from normal control group at *p* < 0.05. [®]Significantly different from control indomethacin group at *p* < 0.05.

surfaces of untreated rats receiving indomethacin (control indomethacin and control depressed). Similarly, hyperemia was more evident in either groups when compared to the groups treated with ranitidine or any of the antidepressants used (Table 2). The severity of hyperemia paralleled with an increase in ulcer numbers. As seen in Table 2, treatment with mirtazapine (10 mg/kg), escitalopram (10 mg/kg), venlafaxine (20 mg/kg) and ranitidine (50 mg/kg) decreased ulcer number by 72, 60, 74.64, and 82.64%, respectively as compared to control indomethacin group.

Effects of mirtazapine, escitalopram, venlafaxine and ranitidine on stomach contents of malondialdehyde, reduced glutathione and total nitrate/nitrite in depressed rats with indomethacin-induced gastric ulcer

Indomethacin administration significantly decreased stomach contents of GSH and NO_x by 30.2 and 84.25%, respectively parallel to an increase in MDA content by 65.65% when compared to the normal group. Similarly, administration of indomethacin in depressed rats decreased stomach contents of GSH and NO_x by 31.5 and 68%, respectively parallel to an increase in MDA content by 62.51%. Treatment of depressed rats with mirtazapine, venlafaxine, escitalopram and ranitidine protected against indomethacin-induced depletion of

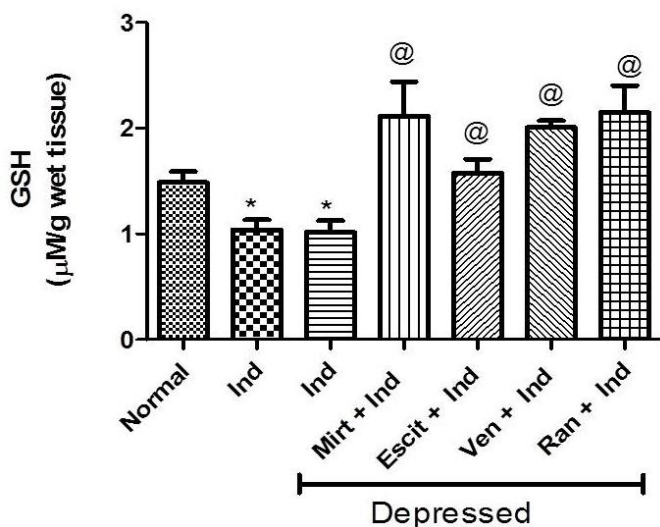


Figure 1. Effects of 30 days administration of mirtazapine (10 mg/kg), escitalopram (10 mg/kg), venlafaxine (20 mg/kg) and ranitidine (50 mg/kg) on stomach reduced glutathione (GSH) content of depressed rats with indomethacin-induced ulcer. Induction of depression was done by i.p. administration of clonidine (0.8 mg/kg) for 10 successive days. Treatment with test agents started following induction of depression. Gastric ulcer was induced by oral administration of indomethacin (20 mg/kg) after the last dose of test agents. Ind: indomethacin; Mirt: mirtazapine; Escit: escitalopram; Ven: Venlafaxine; Ran: ranitidine. Values are means \pm SE of 8 rats. Statistical analysis was done by one way ANOVA followed by Tukey Kramer multiple comparisons test. *Significantly different from normal control group at $p < 0.05$. @Significantly different from control indomethacin group at $p < 0.05$.

stomach GSH content (Figure 1). Similarly, treatment with venlafaxine and ranitidine prevented the decrease in NO_x stomach content induced by indomethacin (Figure 2). In addition, both mirtazapine and ranitidine prevented indomethacin-induced lipid peroxidation in stomach tissues (Figure 3).

Effects of mirtazapine, escitalopram, venlafaxine and ranitidine on stomach contents of tumor necrosis factor- α and interleukin-10 in depressed rats with indomethacin-induced gastric ulcer

Indomethacin administration in normal and depressed rats significantly increased stomach TNF- α contents by 44.45 and 42.51%, respectively when compared to the normal control group (Figures 4 and 5). Meanwhile, IL-10 content decreased in both groups by 28.11 and 36%, respectively when compared to the normal group. Treatment of depressed rats with any of the test agents prevented indomethacin-induced increase in stomach TNF- α content. Similarly, all treatments except escitalo-

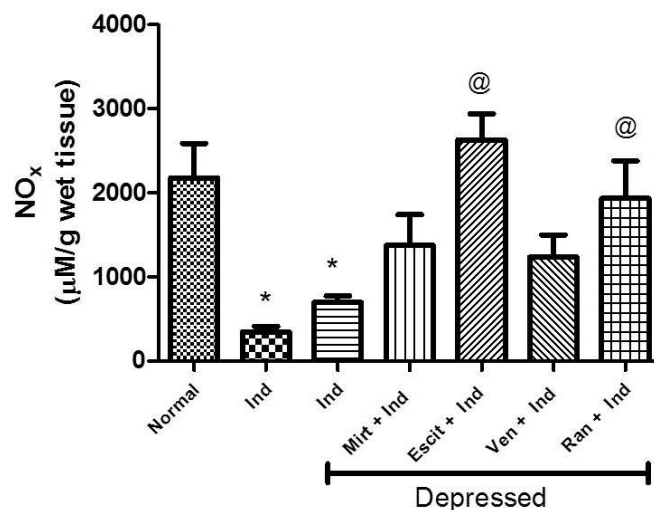


Figure 2. Effects of 30 days administration of mirtazapine (10 mg/kg), escitalopram (10 mg/kg), venlafaxine (20 mg/kg) and ranitidine (50 mg/kg) on stomach total nitrate/nitrite (NO_x) content of depressed rats with indomethacin-induced ulcer. Induction of depression was done by i.p. administration of clonidine (0.8 mg/kg) for 10 successive days. Treatment with test agents started following induction of depression. Gastric ulcer was induced by oral administration of indomethacin (20 mg/kg) after the last dose of test agents. Ind: indomethacin; Mirt: mirtazapine; Escit: escitalopram; Ven: Venlafaxine; Ran: ranitidine. Values are means \pm SE of 8 rats. Statistical analysis was done by one way ANOVA followed by Tukey Kramer multiple comparisons test. *Significantly different from normal control group at $p < 0.05$. @Significantly different from control indomethacin group at $p < 0.05$.

pram prevented the decrease in IL-10 content induced by indomethacin.

Effects of mirtazapine, escitalopram, venlafaxine and ranitidine on histopathologic changes in stomach of depressed rats with indomethacin-induced gastric ulcer

Administration of indomethacin in normal or depressed untreated rats caused marked necrosis and hemorrhage in mucosal layer coupled with edema of sub-mucosal layer. Treatment of depressed rats with ranitidine or any of the used antidepressants ameliorated indomethacin-induced histologic changes (Figure 6).

DISCUSSION

FST remains one of the most used tools for assessing pharmacological antidepressant activity (Petit-Demouliere et al., 2005). Antidepressant treatments reduce immobility time of rats in this test. Indeed in the current

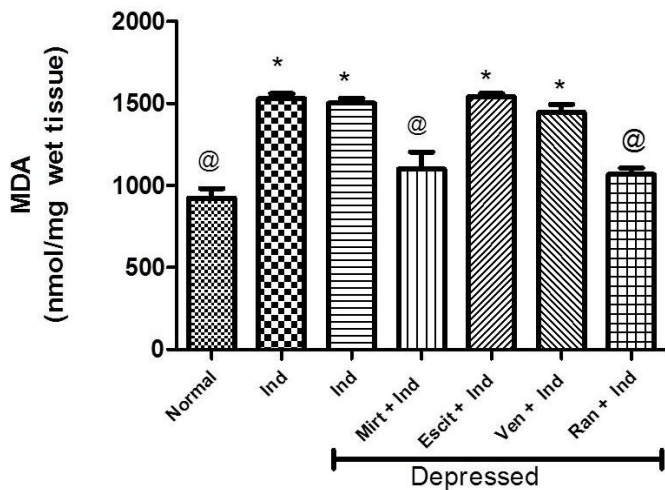


Figure 3. Effects of 30 days administration of mirtazapine (10 mg/kg), escitalopram (10 mg/kg), venlafaxine (20 mg/kg) and ranitidine (50 mg/kg) on stomach malondialdehyde (MDA) content of depressed rats with indomethacin-induced ulcer. Induction of depression was done by i.p. administration of clonidine (0.8 mg/kg) for 10 successive days. Treatment with test agents started following induction of depression. Gastric ulcer was induced by oral administration of indomethacin (20 mg/kg) after the last dose of test agents. Ind: indomethacin; Mirt: mirtazapine; Escit: escitalopram; Ven: Venlafaxine; Ran: ranitidine. Values are means \pm SE of 8 rats. Statistical analysis was done by one way ANOVA followed by Tukey Kramer multiple comparisons test. *Significantly different from normal control group at $p < 0.05$. @Significantly different from control indomethacin group at $p < 0.05$.

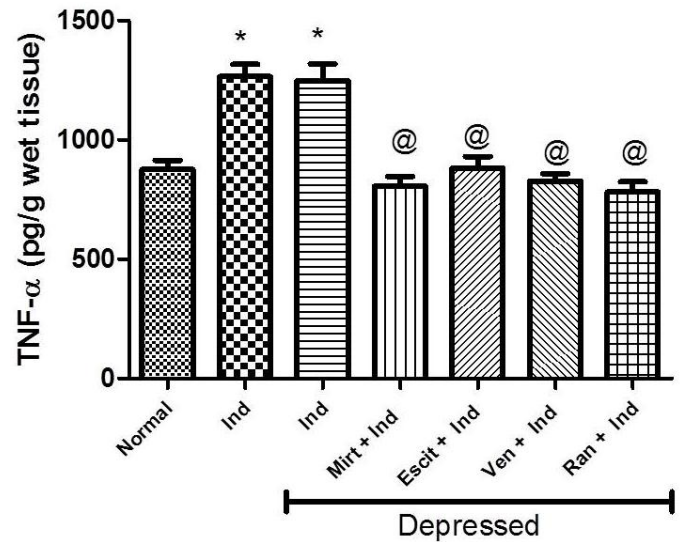


Figure 4. Effects of 30 days administration of mirtazapine (10 mg/kg), escitalopram (10 mg/kg), venlafaxine (20 mg/kg) and ranitidine (50 mg/kg) on stomach tumor necrosis factor-alpha (TNF- α) content of depressed rats with indomethacin-induced ulcer. Induction of depression was done by i.p. administration of clonidine (0.8 mg/kg) for 10 successive days. Treatment with test agents started following induction of depression. Gastric ulcer was induced by oral administration of indomethacin (20 mg/kg) after the last dose of test agents. Ind: indomethacin; Mirt: mirtazapine; Escit: escitalopram; Ven: Venlafaxine; Ran: ranitidine. Values are means \pm SE of 8 rats. Statistical analysis was done by one way ANOVA followed by Tukey Kramer multiple comparisons test. *Significantly different from normal control group at $p < 0.05$. @Significantly different from control indomethacin group at $p < 0.05$.

experiments, treatment of depressed rats for 30 days with mirtazapine, escitalopram or venlafaxine reduced immobility time of rats in FST. The present results are in accordance with other studies that revealed the effectiveness of escitalopram and venlafaxine in FST (Zomkowski et al., 2010).

The present results demonstrated that mirtazapine, escitalopram and venlafaxine displayed a significant reduction of the mucosal damage induced by indomethacin. The three antidepressants reduced ulcer numbers and severity as well as indomethacin-induced histopathologic injuries and their results were comparable to ranitidine, a well-known H₂ blocker used in treatment of gastric ulcers. Similar results were reported by Bilici et al. (2009), using mirtazapine in doses 15, 30 and 60 mg/kg. Similarly, Ji et al. (2012) reported anti-ulcer effects of mirtazapine (10 and 20 mg/kg) in gastric ulcer models induced by water-immersion plus restraint stress or reserpine. The gastroprotective effect of citalopram, an enantiomer of escitalopram, was also reported in stress and pyloric ligation mediated ulcers (Saxena and Singh, 2011).

Antidepressant drugs have been shown to produce antiulcer effects by reducing histamine secretion from

mast cells, inhibiting gastric acid secretion, and blocking leukotriene receptors (Hano et al., 1978). Moreover, Saxena and Singh (2011) mentioned that the protective effects of citalopram was related to increasing -SH groups, NO production and PGs.

In the current experiments, indomethacin administration resulted in increased lipid peroxidation manifested by increased stomach content of MDA, an end product of the process of lipid peroxidation parallel to reduced GSH content. The important primary factor in indomethacin-induced gastric damage is ROS mediated lipid peroxidation (Naito et al., 1998) coupled with reduced antioxidant defenses (Hassan et al., 1998). We investigated effects of mirtazapine (10 mg/kg), escitalopram (10 mg/kg) and venlafaxine (20 mg/kg) on stomach contents of GSH, MDA, and NO in indomethacin-induced ulcer in rats. Similarly, the effects of the chosen antidepressants on stomach content of TNF- α as a pro-inflammatory cytokine and IL-10, as an anti-inflammatory cytokine were assessed as an approach to investigate the mechanism behind their antiulcer effects. Treatment of depressed rats with mirtazapine or ranitidine prevented indomethacin-

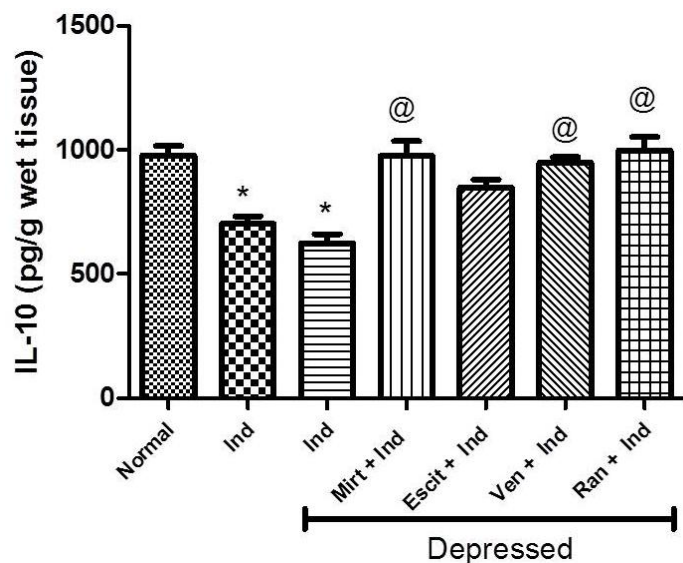


Figure 5. Effects of 30 days administration of mirtazapine (10 mg/kg), escitalopram (10 mg/kg), venlafaxine (20 mg/kg) and ranitidine (50 mg/kg) on stomach interleukin-10 (IL-10) content of depressed rats with indomethacin-induced ulcer. Induction of depression was done by i.p. administration of clonidine (0.8 mg/kg) for 10 successive days. Treatment with test agents started following induction of depression. Gastric ulcer was induced by oral administration of indomethacin (20 mg/kg) after the last dose of test agents. Ind: indomethacin; Mirt: mirtazapine; Escit: escitalopram; Ven: Venlafaxine; Ran: ranitidine. Values are means \pm SE of 8 rats. Statistical analysis was done by one way ANOVA followed by Tukey Kramer multiple comparisons test. *Significantly different from normal control group at $p < 0.05$. @Significantly different from control indomethacin group at $p < 0.05$.

induced lipid peroxidation and depletion in stomach GSH stores. Similarly, venlafaxine and escitalopram protected against indomethacin-induced GSH depletion, although stomach MDA contents were still elevated in both groups. In a study performed by Bilici et al. (2009), using mirtazapine was associated with similar protection against indomethacin-induced lipid peroxidation. Mirtazapine restored stomach content of GSH and activities of superoxide dismutase and catalase. Similarly, venlafaxine in doses of 5, 10 and 20 mg/kg/day for 21 days protected against stress-induced DNA oxidative damage in hippocampus of mice and reduced associated lipid peroxidation while increasing brain GSH and total antioxidant stores (Abdel-Wahab and Salama, 2011). Moreover, the protective effects of escitalopram were reported against depression-induced oxidative stress in rat brains (Eren et al., 2007).

The present findings indicate the important relationship between gastric GSH contents and ulcer severity. GSH detoxifies hydrogen peroxide and/or organic acids chemically; thus hydrogen peroxide accumulates in the

absence of GSH and in the presence of transition metals such as Fe and Cu, it reacts with superoxide anion to form hydroxyl radical, the most reactive and cytotoxic form of ROS (Dalle-Donne et al., 2003).

In the current study, gastric NO content was reduced by indomethacin administration. The present findings confirm the positive correlation between the decrease in NO content and the severity of gastric damage as previously reported (Lanas et al., 2000; Cadirci et al., 2007). NO, as a vasodilator, maintains gastric epithelium integrity by accelerating ulcer healing (Li et al., 2000) and modulating acid levels as well as gastric mucus secretion (Martin et al., 2001). Moreover, NO has also been reported to prevent membrane lipid peroxidation (Hogg and Kalyanaraman, 1999). Treatment with escitalopram or ranitidine, in the current study, increased gastric NO levels significantly when compared to control indomethacin group. The ability of ranitidine to increase stomach NO content in indomethacin-induced ulcer was previously reported and explained by its ability to increase constitutive nitric oxide synthase (Bayir et al., 2006). The noted effect of escitalopram on stomach NO content could be one of the factors responsible for its observed decrease of ulcer-index and ulcer severity induced by indomethacin. The effects of escitalopram on NO content could be mediated via increased synthesis of NO through nitric oxide synthases (NOS). Indeed, Saglam et al. (2008) reported increased NOS activity in brain of rats treated with escitalopram in doses of 2.5, 5 and 10 mg/kg.

Induction of indomethacin ulcer in normal or depressed rats was associated by increased stomach contents of TNF- α , a pro-inflammatory cytokine, parallel to decreased IL-10, an anti-inflammatory cytokine. This observation is consistent with previous observations showing the importance of TNF- α in the process of ulcer healing (Konturek et al., 2000). Indeed, TNF- α was shown to delay ulcer healing by inhibition of cell proliferation, angiogenesis and gastric microcirculation (Shimizu et al., 2000). Treatment with any of the three used antidepressants or ranitidine attenuated indomethacin-induced changes in stomach TNF- α and IL-10 contents except for escitalopram which did not significantly affect IL-10 content when compared to indomethacin control group.

The present findings find support in previous published literature. Ranitidine inhibited the production of TNF- α in monocytes stimulated with lipopolysaccharide *in vitro* (Okajima et al., 2002) and in stomach of rats subjected to pyloric ligation (Sood and Muthuraman, 2009). Mirtazapine was shown to reduce TNF- α expression in tumor-bearing mice (Fang et al., 2012) and to reduce TNF- α in brain of rats parallel to IL-10 up-regulation (Zhu et al., 2008). Venlafaxine was also shown to suppress TNF- α up-regulation and up-regulate IL-10 level in patients and experimental animals (Kubera et al., 2001;

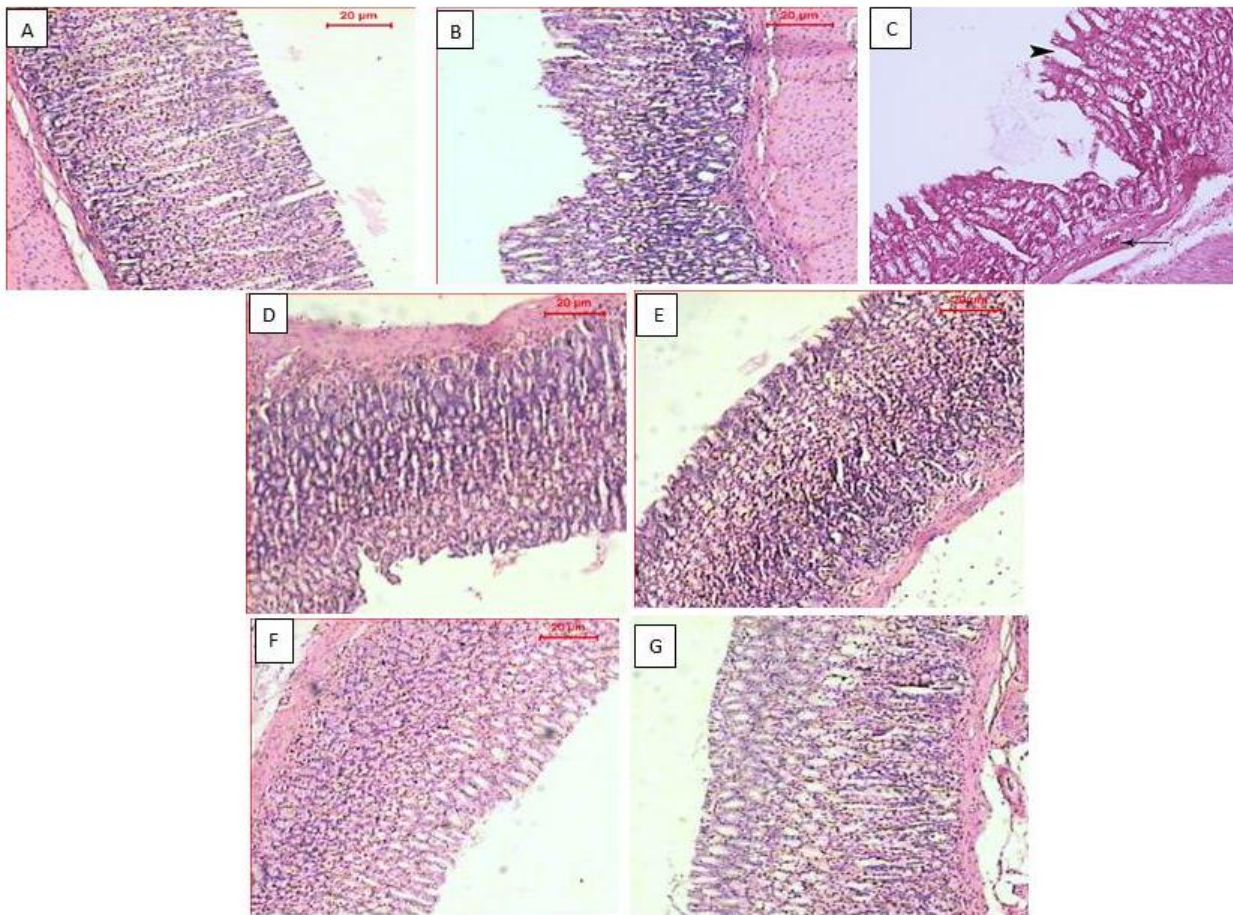


Figure 6. Effects of 30 days administration of mirtazapine (10 mg/kg), escitalopram (10 mg/kg), venlafaxine (20 mg/kg) and ranitidine (50 mg/kg) on stomach histopathologic changes of depressed rats with indomethacin-induced ulcer. (A): Stomach of normal control rat showing normal structure of stomach tissue; (B): Stomach of rat treated with indomethacin showing necrosis of the surface mucous cells and fundus gland, hemorrhage in mucosal layer and edema of submucosal layer; (C): Stomach of control depressed rat treated with indomethacin showing major injuries in mucosal layer, as well as necrosis of the surface mucous cells; (D): Stomach of depressed rats treated with indomethacin and mirtazapine showing necrosis of the surface mucous cells and fundus gland, hemorrhage in mucosal layer; (E): Stomach of depressed rat treated with indomethacin and escitalopram showing no obvious injuries in mucosal layer; (F): Stomach of depressed rats treated with indomethacin and venlafaxine showing no obvious injuries in the most of the mucosal layer. (G): Stomach of depressed rats treated with ranitidine showing regular and intact structure. (H & E; $\times 100$).

Li et al., 2013). Moreover, suppression of TNF- α was recognized as one of the antidepressant mechanisms of escitalopram (Bah et al., 2011; Powell et al., 2012). The current data indicate the important role played by TNF- α and IL-10 in the observed gastroprotective effects of mirtazapine, escitalopram and venlafaxine.

Conclusion

We report that mirtazapine, escitalopram and venlafaxine have antiulcer effects in depressed rats receiving indomethacin. The chosen antidepressants appear to

exert their antiulcer effects by activation of antioxidant mechanisms, inhibition of toxic oxidant mechanisms in stomach tissues in addition to their anti-inflammatory effects revealed by reducing TNF- α and increasing IL-10 contents. Histologic examination of stomach tissues confirmed the present biochemical findings.

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Full Length Research Paper

Antioxidant studies of various solvent fractions and chemical constituents of *Potentilla evestita* Th. Wolf

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In the present research work acacetin (1), chrysin (2) and umbelliferone (3) were isolated for the first time from the chloroform fraction of *Potentilla evestita*. The isolated compounds as well as various solvent fractions of the crude ethanolic extract were assessed for their antioxidant potentials. A significant DPPH free radical scavenging effect was observed in the methanolic and chloroform fractions. The acacetic and chrysin demonstrated significant free radical scavenging activity. Likewise compound 3 showed moderate activity as comparative. It was concluded that the therapeutic potential of this plant is due to its antioxidant potential and the antioxidant effect of *P. evestita* is due to the presence of these three isolated antioxidant compounds.

Key words: *Potentilla evestita*, acacetin, chrysin, and umbelliferone, antioxidant activity.

INTRODUCTION

Antioxidant property of any natural products or chemical constituent provides protection to human body against free radicals by inhibiting various oxidizing chain reactions. When these constituents are present at low concentration in the body they stop the oxidation of an oxidizable substrate (Chun et al., 2005). These antioxidants play important roles in delaying the development of prolonged diseases such as cardiovascular diseases, cancer, atherosclerosis and inflammatory diseases (Velioglu et al., 1998). Plants are the best natural antioxidant products (Muhammad et al., 2011).

Potentilla evestita belongs to the family Rosaceae (Tomczyk et al., 2009). It is distributed in the Eastern Himalayan range from Indus to Kumaon (Anonymous., 1998), in the Arctic, Alpine and temperate regions of the Northern hemisphere. In Pakistan, it is well distributed in

Gilgit. Several medicinal uses of *P. evestita* has been reported such as antimicrobial, anti-inflammatory, anti-diarrheal, anti-diabetic, hepatoprotective, anticancer, anti-spasmodic and ulcerative colitis (Tomczyk et al., 2009).

Phytochemically, approximately 43 compounds have been isolated from *P. evestita*. The rhizomes of *P. evestita* contains rich amount of tannins that is 3.5% hydrolysable tannins and 15-20% condensed tannins, pregnane derivative, 2,6 beta,7beta-trihydroxy-4-methyl-19-norpregna-1,3,5(10)-trien-17-one, and pregnane derivative, 11alpha,17alpha,21-trihydroxypregna-4,16(22)-diene-3,20-dione, have also been isolated from *P. evestita* (Khan et al., 2011). In continuation of our research work on medicinal plants (Uddin et al., 2011a; 2012b; Rauf et al., 2012a) herein we are reporting the isolation of three compounds using chromatographic

methods and characterization by spectroscopic techniques followed by their test for antioxidant activities.

MATERIALS AND METHODS

Materials

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical and vitamin C were obtained from Sigma-Aldrich. All other chemicals and reagents used were of analytical grade.

The whole plant of *P. evestita* (15 kg) was collected from Gilgit, Pakistan. The plant was identified by the Taxonomist Department of Botany, University of Karachi. Voucher specimen (voucher No. 707) has been deposited at the herbarium (Herbarium No. 71212) of the Department of Botany, University of Karachi, Pakistan.

Extraction and Isolation

Shade dried whole plant (15 kg) of *P. evestita* was ground into fine powder and soaked in 25 L ethanol for 10 days at room temperature. The resulting extract was filtered and the filtrate evaporated under reduced pressure at 45°C to yield 300 g dark brown residue. The residue was suspended in water and subsequently extracted with solvents of increasing polarity, namely *n*-hexane (3×10 L), chloroform (3×14 L), ethyl acetate (3×12 L) and methanol (1×3 L). Each extract evaporated under reduced pressure to afford *n*-hexane fraction (70 g), chloroform (75 g) EtOAc extract (8 g), and methanol (40 g). The chloroform fraction (60 g) was subjected to column chromatography on silica gel (Merck Silica gel 60 (0.063 - 0.200 mm), 5 × 60 cm). The column was first eluted with hexane-ethyl acetate (100:0 → 0:100) as solvent system. A total of 100 fractions, RF-1 to RF-100 were obtained based on TLC profiles. Elution of the chromatogram with hexane-ethyl acetate (100:0 → 100:0) gave the isolation of three known compounds, acacetin **1** (80 mg), chrysin **2** (99 mg) and umbelliferone **3** (60 mg). The structures (Figure 1) of compounds were confirmed by comparing their NMR and physical data with the reported data in literature (Dawson et al., 2006; Hemila et al., 1992; Battelli et al., 2001).

DPPH radical scavenging assay

The antioxidant activity was performed by DPPH radical scavenging assay according to standard protocol as earlier discussed (Rauf et al., 2012b; Uddin et al., 2012c; 2012d; 2012e). The positive control used was vitamin C. The hydrogen atom or electron donation abilities of the corresponding extracts/fractions and standards were measured from the bleaching of the purple-colored methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH.) Experiments were carried out in triplicate. Briefly, a 1 mM solution of DPPH radical solution in methanol was prepared and 1 ml of this solution was mixed with 3 ml of the sample (extracts/fractions) solutions in methanol (containing 10 – 100 ug) for various fraction while (containing 5 – 100 ug) for pure compounds and control (without sample). The solution was allowed to stand for 30 min, in dark the absorbance was measured at 517 nm. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. Scavenging of free radicals by DPPH as percent radical scavenging activities (%RSA) was calculated as follows:

$$\% \text{ DPPH} = (\text{OD control} - \text{OD sample}) \times 100 / \text{OD control}$$

where OD control is the absorbance of the blank sample, and OD sample is the absorbance of samples or standard sample.

Statistical analysis

Data were presented as mean and standard errors of mean. The statistical analysis was performed using Prism Graphed. ANOVA followed by Dunnet test for multiple comparison of groups were employed for assessing the statistical significance of the differences observed among the groups.

RESULTS AND DISCUSSION

The results of antioxidant potential of crude ethanolic extract and its various solvent fractions are presented in Table 1. The maximum free radical scavenging effect was demonstrated by methanolic fraction having 96.88, 92.54, 87, 84.40, 78.43 and 55.20% at the tested dose of 100, 80, 60, 40, 20 and 10 ppm, respectively. When the sub fraction (chloroform) was tested for antioxidant effect at accumulative concentration, a dose dependant manner was observed. The percent antioxidant effect at 100 ppm was 91.22% and at lowest concentration the effect was 30.22%. The effect of crude ethanolic extract was lower than methanolic and chloroform fraction but was significant and exhibited 18.55, 28.52, 35.33, 60.11, 70.99 and 78.54% at tested concentrations of 10, 20, 40, 60, 80 and 100 ppm. Ethyl acetate and *n*-hexane fractions were proved comparatively to be weak antioxidants.

The antioxidant activity of isolated compounds is presented in Table 2. When these isolated bioactive constituents were tested at cumulative concentrations (5, 10, 15, 20, 40, 60, 80 and 100 ppm) a dose dependant antioxidant effect was observed. The maximum free radical scavenging effect was observed with compound **2**. The percent protection of DPPH free radical by compound **2** was 90.51, 81.31, 72.23, 50.21, 35.51, 18.31, 10.33, and 6.58% at the tested concentrations of 100, 80, 60, 40, 20, 10 and 5 ppm respectively. The effect of compound **1** was somewhat lesser than compound **2**. The percent activity of compound **1** was 70.55, 66.52, 54.21, 40.83, 30.89, 20.26, 10.22 and 4.22% at the same tested concentrations. When compound **3** was tested against DPPH induced free radicals, a weak antioxidant effect was observed.

The multiple use of this valuable plant in different diseases is directly attributed to the antioxidant effect of various solvent fractions and the isolated chemical constituents are responsible for this radical scavenging effect. It is the antioxidant effect which protects the human body from different diseases.

The discovery or synthesis of new, effective and easily available antioxidant is a big challenge to the researcher in the modern era. It is common perception among the public that natural products are safe due to their minimal

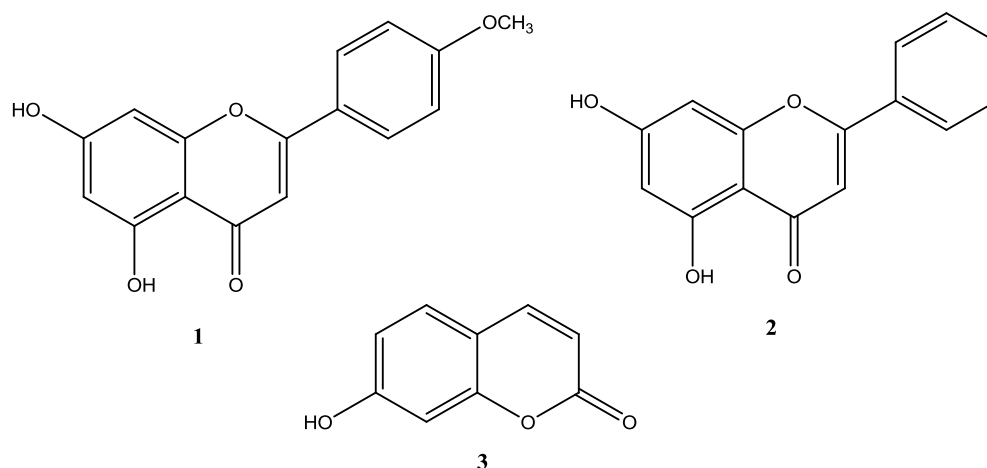


Figure 1. Chemical structures of compound 1, 2 and 3.

Table 1. Antioxidant effect of various solvent fractions.

Sample	Various tested concentration (ppm)					
	10	20	40	60	80	100
Ethanol	18.55±1.77	28.51±1.44	35.33±1.11	60.11±1.23	70.99±1.03	78.54±1.22
Methanol	55.20±1.00	78.43±1.76	84.40±3.09	87.00±1.20	92.54±1.88	96.88±1.03
Ethyl acetate	15.44±1.33	20.22±1.34	30.21±2.76	40.62±1.03	52.66±1.87	57.10±1.70
Chloroform	30.22±1.13	58.31±1.03	60.01±1.03	72.42±1.89	80.21±1.03	91.22±1.76
n-hexane	4.00±1.23	8.11±1.88	16.90±1.03	20.10±1.22	30.91±1.55	40.11±1.63

Table 2. Antioxidant effect of isolated compounds.

Compound	Tested concentrations (ppm)						
	5	10	20	40	60	80	100
1	4.22±1.55	10.0±2.11	20.21±1.22	30.81±1.89	40.80±1.89	54.21±1.890	70.50±1.03
2	6.58±1.23	10.31±2.98	18.30±1.44	35.50±1.88	50.210±1.89	72.20±1.89	90.510±1.03
3	2.21±1.03	6.48±3.00	14.50±1.73	20.28±1.46	28.40±1.89	34.30±1.89	44.10±1.03

side effects. With the hope of finding natural, safe and effective antioxidants we scrutinized the crude ethanolic extract and the various solvent fractions of *P. evestita* and further the three compounds were isolated from the chloroform fraction of this valuable medicinal plant. Antioxidants protect the human body from many ailments. The production of various types of free radicals is believed to be the cause of a large number of disease conditions. Several medicinal uses of *P. evestita* has been reported such as antimicrobial, anti-inflammatory, anti-diarrheal, anti-diabetic, hepatoprotective, anticancer, antispasmodic and as remedy for ulcerative colitis (Tomczyk et al., 2009). The use of this plant in these diseases is directly attributed to its antioxidant potential.

The evaluations of various solvent fractions for various bioassays are due to the changes in the chemical composition of these solvent fractions (polar, non-polar or of intermediate polarity) (Muhammad et al., 2012; 2013). In the current study the maximum antioxidant effect was observed with the methanolic fraction followed by the chloroform extract. This deference in antioxidant effect is due to the different chemical composition. Therefore it is concluded that in the methanolic fraction, maximum antioxidant chemical moieties are accumulated as compared to the other solvent fractions.

For any compound showing antioxidant effect, the reactive site like hydroxyl groups or unsaturated bonds are very necessary. Compounds having maximum

number of unsaturated bonds or hydroxyl groups are probably exhibit more antioxidant activity. These unsaturated sites are more reactive than saturated sites and responsible for the scavenging effect of free radicals. In the present study, the maximum effect was demonstrated with compound **2**, followed by compounds **1** and **3**. Compounds **1** and **2** are structural analog and the only difference between them is the lack of the methoxy group in compound **1**. This methoxy group causes a shielding hindrance which makes the reaction weaker to complete. The presence of methoxy group might reduce the antioxidant potential of compound **1**. It is therefore recommended to medicinal chemists to synthesize various derivatives of these isolated natural compounds which may be proved to be good antioxidants.

Conclusion

It is concluded that *P. evestita* is a rich source of antioxidant molecules and its methanolic extract is a significant antioxidant. This research work provides strong scientific background to the folkloric uses of this plant in various ailments. The therapeutic potential of *P. evestita* is therefore due to its antioxidant effect which is due to the presence of these three isolated compounds.

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