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Full Length Research Paper

Subchronic toxicity evaluation of the hydroethanolic extract from *Endopleura uchi* (Huber) Cuatrec in Wistar rats

Beatriz M. Sá[1,2], Clarissa S. Lima[1,2], Uriel Davi A. Silva[1], Helison O. Carvalho[1], Caio P. Fernandes[1], Rafael L. Resque[1], Tania T. de Oliveira[3] and José Carlos T. Carvalho[1,2*]

[1]Laboratório de Pesquisa em Fármacos,Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá, Amapá, Brasil.
[2]Programa de Pós-Graduação em Biodiversidade Tropical, Universidade Federal do Amapá, Amapá, Brasil.
[3]Laboratório de Biofarmacos, Departamento de Bioquimica e Biologia Molecar, Universidade Federal de Viçosa, Minas Gerais, Brasil.

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***Endopleura uchi* (Huber) Cuatrec. (Humiriaceae) is a species from Brazilian Amazon rainforest, popularly used against menstrual disorders and uterine inflammation. This study aimed to evaluate the subchronic toxicity of hydroethanolic extract from *E. uchi* (EHEEu) in Wistar rats based on biochemical and hematological parameters. Rats were treated with daily doses of EHEEu (500 mg/kg - gavage), and then hematological and biochemical parameters were observed. The results shows that the treatment performed produced no signs of toxicity or death, as well as no changes in weight gain or daily intake of water and food. Biochemical and hematological parameters were not modified by EHEEu administration, with the exception of erythrocyte index of rats (males) in the treated group, however, it was not assigned clinical relevance once it remained within the reference range for the species. Thus, subchronic administration of EHEEu produced no toxic effects in Wistar rats.**

Key words: *Endopleura uchi*, subchronic treatment, hematology, biochemistry.

INTRODUCTION

The traditional use of medicinal plants based on popular knowledge, along with the belief that being natural does not cause adverse reactions, made only a few medicinal plants were evaluated through pre-clinical and clinical studies to prove its effectiveness and security (Turolla and Nascimento, 2006). However, over time it was realized that certain plants have potentially dangerous substances and therefore should be used with care,

*Corresponding author. E-mail: farmacos@unifap.br.

respecting their toxicological risks (Veiga-Junior and Pinto, 2005).*Endopleura uchi* (Huber) Cuatrec. (Humiriaceae) is a botanical species typical from the Brazilian Amazon rain-forest This plant is found in not too flooded ground forest, which is widely spread in the Amazon Basin.It is popularly known in the region as uchi, uxi, axuá, uchi-pucu, uxi-amarelo, uxi-liso e uxi-ordinário (Cuatrecasas, 1961; Schultes, 1979). The stem bark of *E. uchi* is used as tea for menstrual disorders and uterine inflammation (Revilla, 2001). Other indications are also known and cited as antimicrobial activity (Politi, 2009), high anti-oxidant activity and absence of cytotoxicity (Politi et al., 2011). These results justify the increasingly growing interest in the study of this plant.

According to Politi (2009), the bark of *E. uchi* consists mainly of three types of secondary metabolites: tannins, coumarins and saponins. Another phytochemical study from crude ethanol extract of bark led to the isolation of bergenin isocoumarins and 8, 10 dimetoxibergenina, pentacyclic triterpenoids, maslinic acid and its methyl ester maslininate (Luna et al., 2000).

The bergenin extracted from the bark of *E. uchi* have been identified as mainly responsible against the biological activities such as anti-inflammatory (Nunomura et al., 2009), anti-microbial (Silva et al., 2009), neuroprotective (Takahashi et al., 2003) and antinociceptive (Oliveira et al., 2011). Until this moment, toxicological studies do not support the safe use of *E. uchi*. However, Politi et al. (2010) evaluated the acute oral toxicity in male rats with an extract of powdered bark of *E. uchi*. Thus, the present study aimed to evaluate the subchronic toxicity of crude hydroethanolic extract of the stem bark of *E. uchi* in Wistar rats of both sexes.

MATERIALS AND METHODS

Collection and authentication

The stem barks of *E. uchi* were collected in April, 2005 in the city of Ananideua, Pará State, Brazil. The voucher specimen of the plant (number 180611) was deposited in the Herbarium of the Brazilian Agricultural Research Corporation - EMBRAPA, Belém, Pará, Brazil.

Extract preparation

The stem bark of *E. uchi* were dried in an air circulating oven at 40°C for 72 h and after drying, it was triturated in a knives mill, obtaining the powder of the plant (464 g). Subsequently, this material was macerated in a 75% hydroalcoholic solution in a ratio of 1:5 at room temperature for 7 days, under stirring. Then, the mash was filtered and concentrated on a rotaevaporator (Quimis Model Q 218.2) at 45°C until complete evaporation of the solvent. The concentrated filtrate was subjected to lyophilization providing 10.65% yield.

Analysis of the content of total polyphenols and total tannins

The method of Brazilian Pharmacopeia, 5th edition (2010) described for Rhatany species (*Ratanhiae radix*) was adapted and subjected to linear regression curve prepared with pyrogallic acid, all analyses were performed in triplicate. The polyphenols contents were calculated from the equation of the line obtained by standard curve of acid pyrogallic in concentrations from 0.01 to 0.05 mg/ml submitted to reaction with phosphomolybdic tungstic acid in alkaline medium following the method described by Carvalho et al. (2013).

Animals

Rats (*Rattus norvegicus albinus*), Wistar strain (males and females), weighing around 170 to 215 g from the vivarium of the Center for Reproductive Biology, Federal University of Juiz de Fora were used. These went through a period of adjustment, kept under controlled lighting conditions (cycle 12 h light/dark), temperature (23 ± 2°C) and received water and food *ad libitum*. The experimental protocol was approved by the Ethics Committee of the Federal University of Amapá (Case No. 001A/2012).

Treatment of animals and evaluation of biochemical and hematological parameters

Twenty rats corresponding to two groups n = 10/group (5 males and 5 females) were randomized into five subgroups and treated for 22 consecutive days orally with EHEEu at a dose of 500 mg/kg (treated group) and distilled water (control group). Parameters indicative of toxicity and blood-biochemical analyzes were performed according to the method described by Silva et al. (2005), with some adaptations. Parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, HDL cholesterol, triglycerides, alkaline phosphatase (ALP), albumin, glucose, creatinine, total and differential blood count were determined using the multiparametric equipment for biochemical determination (Alizé) from Biomérieux and automatic hematologic cells analyzer HumaCount Plus.

Statistical analysis

The results obtained in different analyzes were expressed as average ± standard error of the average (average ± SEA) for each experimental group. For biochemical and hematological analysis, we used Mann - Whitney, and to compare the data of weight gain, water and food intake the student "t" test (unpaired) was used. Test was performed using GraphPad Prism software® (version 5.03). Results with $p < 0.05$ were considered statistically significant.

RESULTS

The lyophilized extract showed total polyphenols equal to 7.01% corresponding to an average of 0.021 ± 0.008 mg/ml (n = 3). The percentage of total tannins was 1.5% with 0.0045 ± 0.0011 mg/ml (n = 3) representing 21.4% of all polyphenols in the extract (Figure 1). The oral administration for 22 days with EHEEu at a dose of 500 mg/kg in rats, males and females, did not affect the weight gain

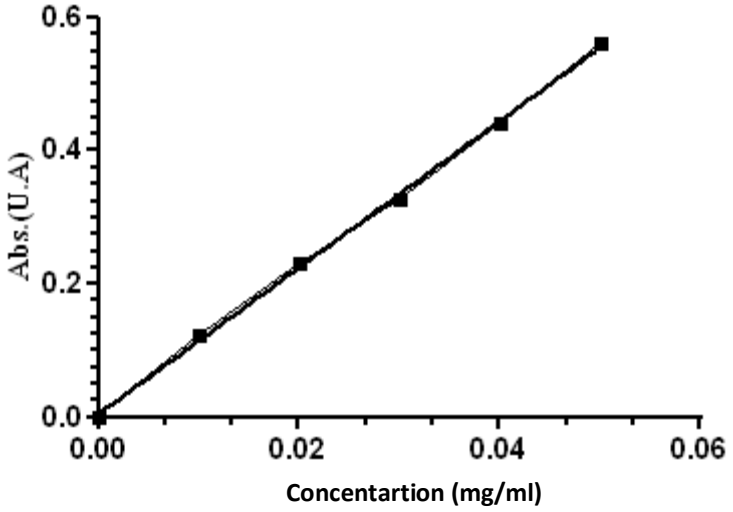

Figure 1. Standard curve of pyrogallic acid for spectrophotometrically (λ = 760 nm), concentrations from 0.01 to 0.05 mg/mL subjected to reaction with phosphomolybdictungstic acid in alkaline medium with read-after 2 minutes of reaction.
Linear regression of the results obtained r^2 = 0.9987 correlation coefficient with the equation of the line y = 10.450x 0.0118.

Table 1. Effect of the treatment (po) of EHEEu (500 mg/kg) and distilled water (control) for 22 consecutive days, on the biochemical parameters of Wistar rats (males and females).

Parameter	Female		Male	
	Control	EHEEu	Control	EHEEu
AST (U/L)	241.6 ± 50.0	245.0 ± 39.9	193.2 ± 27.6	228.0 ± 37.1
ALT (U/L)	83.6 ± 22.9	79.0 ± 17.8	53.0 ± 4.8	54.5 ± 5.8
Total cholesterol (mg/dl)	66.5 ± 1.8	73.8 ± 4.6	61.6 ± 3.3	65.6 ± 4.2
HDL cholesterol (mg/dl)	31.6 ± 1.2	30.7 ± 1.4	31.2 ± 1.1	30.4 ± 0.8
Triglycerides (mg/dl)	82.0 ± 4.5	76.8 ± 6.3	55.5± 2.3	74.4 ± 1.6
ALP (U/L)	23.6 ± 2.4	26.2 ± 6.2	90.8 ± 8.9	90.0 ± 17.9
Albumin (g/dl)	3.5 ± 0,0	3.6 ± 0,1	3.7 ± 0.0	3.6 ± 0.0
Glucose (mg/dl)	111.7 ± 20.9	123.3 ±17.3	154.5 ± 6.75	146.4 ± 13.5
Creatinine (mg/dl)	0.5 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.5 ± 0.0

The values represent mean ± S.E.M. (n = 5/group). *p < 0.05 compared to the control group (Mann-Whitney test). AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; FA: Alkaline phosphatase.

gain and daily food and water intake of these animals, since significant changes were not observed compared to the control group (Figures 2, 3 and 4). The level of serum AST, both in males and females (control and treated) and ALT in males (control and treated) were above the reference values (Table 1). The treatment of animals with EHEEu did not alter the levels of triglycerides and total cholesterol, however, the levels of HDL cholesterol in males and females (control and treated group) animals remained below the reference values (Table 1). Oral treatment of animals with EHEEu did not interfere in hematologic rates, except for the values of mean corpuscular hemoglobin concentration (MCHC) of animals (males), which showed a significant increase compared to the control group. The differential count of lymphocytes, monocytes, neutrophils and eosinophils showed similar values, with no statistically significant differences between the control group and treated with EHEEu (Table 2).

DISCUSSION

Polyphenols vary from simple molecules to complex

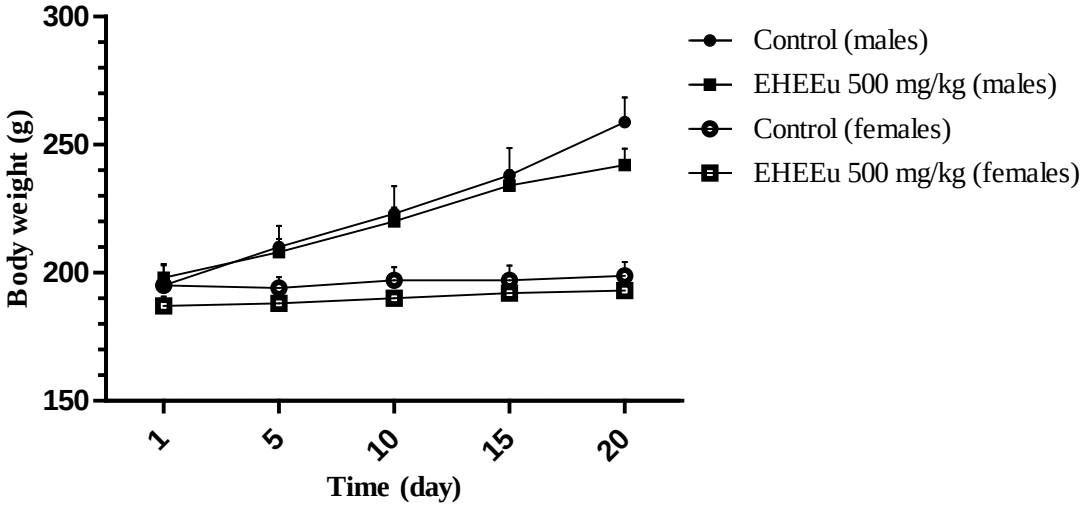

Figure 2. Effects of the administration (po) of EHEEu (500 mg/kg) and distilled water (control) on the body weight of Wistar rats (males and females), by 22 consecutive days. The values represent mean ± SEM. (n = 5/group). *p < 0.05 compared to the control group.

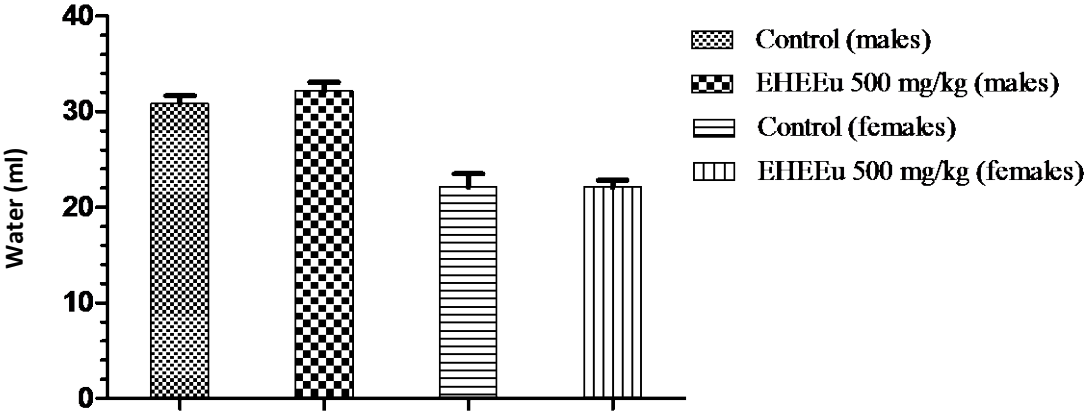

Figure 3. Effects of the administration (po) of EHEEu (500 mg/kg) and distilled water (control) on the daily water consumption of Wistar rats (males and females), for 22 consecutive days. The values represent mean ± S.E.M. (n = 5/group). *p < 0.05 compared to the control group.

molecules with a high degree of polymerization and are classified into several classes of secondary metabolites such as flavonoids, phenolic acids, simple phenols, coumarins, tannins, lignins, and tocopherols among others and are easily oxidizable by metals light, heat and enzymatic processes and are associated the antioxidant actions (Shahidi and Naczk 1995; Simões et al., 2004). In this study, the EHEEu showed concentration significant for tannins.

One of the indicators of adverse effects of drugs and chemicals is the change in animal body weight (Tofovic and Jackson, 1999; Raza et al., 2002; Teo et al., 2002). Systemic toxicity can also be diagnosed by decreased water intake, diet, behavioral changes such as apathy and prostration, and by the appearance of rough hair coat (Melo, 2001). In this study, it was observed that the gain in body weight in rats (males) from control and EHEEu treated groups was higher than those rats (females) from both groups. However, it was found that oral administration for 22 days with EHEEu in rats, males and females did not affect the weight gain and daily food and water intake of these animals (Figures 2, 3 and 4).

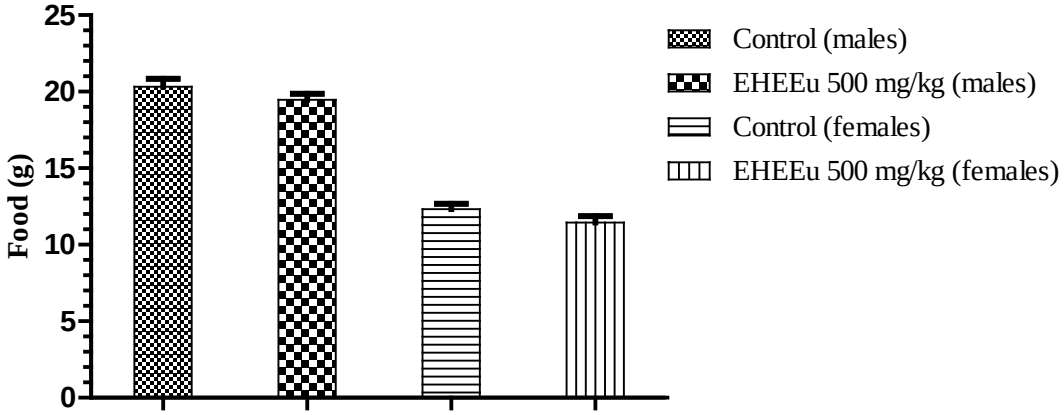

Figure 4. Effects of the administration (po) of EHEEu (500 mg/kg) and distilled water (control) on the daily feed intake of Wistar rats (males and females), for 22 consecutive days. The values represent mean ± S.E.M. (n = 5/group). *p < 0.05 compared to the control group.

Table 2. Effect of the treatment (po) of EHEEu (500 mg/kg) and distilled water (control) for 22 consecutive days, on hematological parameters of Wistar rats (males and females).

Parameter	Females		Males	
	Control	EHEEu	Control	EHEEu
RBC (x10^6/mm^3)	7.9 ± 0.1	7.9 ± 0.3	9.2 ± 0.0	9.1 ± 0.2
HGB (g/dl)	14.8 ± 0.1	15.1 ± 0.5	15.7 ± 0.1	15.9 ± 0.2
HCT (%)	41.3 ± 0.4	40.8 ± 1.7	48.0 ± 0.3	47.0 ± 1.7
MCV (fl)	52.2 ± 0.6	51.5 ± 0.6	52.0 ± 0.3	51.2 ± 0.5
MCH (pg)	18.6 ± 0.1	19.1 ± 0.4	17.0 ± 0.1	17.4 ± 0.2
MCCH (g/dl)	35.7 ± 0.3	37.1 ± 0.5	32.7 ± 0.1	34.7 ± 0.4*
WBC (×10^3/mm^3)	3.7 ± 0.6	3.4 ± 0.4	3.6 ± 0.6	3.2 ± 0.7
Lymphocytes (%)	56.2 ± 3.8	52.2 ± 3.6	63.8 ± 2.1	61.8 ± 1.3
Monocytes (%)	2.4 ± 0.6	3.0 ± 0.3	1.6 ± 0.4	2.0 ± 0.5
Segmented (%)	40.0 ± 3.5	43.0 ± 3.6	32.6 ± 2.2	34.6 ± 1.2
Eosinophils (%)	1.4 ± 0.2	1.8 ± 0.3	2.0 ± 0.8	1.6 ± 0.5
Platelets (×10^3/mm^3)	1069.0 ± 35.5	996.25 ± 13.9	1026.3 ± 159.0	1007± 63.1

The values represent mean ± S.E.M. (n = 5/group). *p < 0,05 compared to the control group (Mann-Whitney test). RBC: Red Blood Cells; HGB: Hemoglobin; HCT: Hematocrit; MVC: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCCH: Mean corpuscular hemoglobin concentration.

Although the food intake of the treated group was lower than in the control group (Figure 4), the fact is not associated with EHEEu toxic effects because there was no interference with the body mass gain of these animals during treatment. During the experimental period, the animals of different groups showed no clinical signs of toxicity and no death was registered. The decrease in glomerular filtration, in general, leads to increased plasma creatinine concentrations. In rats, plasma levels of creatinine change can be a reliable indicator for the presence of renal injury, because its serum level is not influenced by diet, age or sex (Alves, 2007). The administration EHEEu does not change serum creatinine levels in animals, which indicates that renal function was not affected (Table 1).

According to Alves (2007), some enzymes can be used as indicators of hepatic injury, such as ALT, AST and ALP. ALT is found mainly in the cytoplasm of hepatocytes, while 80% of AST is present in mitochondria. In light hepatocellular damage the serum is predominantly cytoplasmic, while in severe injuries the mitochondrial enzyme is released (Motta, 2003). The ALP is present

mainly at bone tissue, at the hepatobiliary system and the gastrointestinal mucosa, it is indicative of cholestasis, which may lead to increased serum levels up to 10 times (Scheffer and Gonzalez, 2003). In the present study, these enzymes were not altered after administration of EHEEu, however the level of serum AST, both in males and females (control and treated) and ALT in males (control and treated) were above the reference values (Table 1) (Clifford and Giknis, 2008).

The importance of lipid levels is fundamental because elevated levels of total cholesterol are closely related to risk of ischemic coronary disease (Araújo et al., 2011). Treatment of animals with EHEEu did not alter the levels of triglycerides and total cholesterol, probably indicating that there was no change in the lipid metabolism of these animals, however, the levels of HDL cholesterol in males and females (control and treated group) animals remained below the reference values (Table 1) (Dantas et al., 2006).

Despite the existence of own mechanisms to control the physiological parameters values, it is known that in certain groups of animals, such as rats and mice, these parameters can vary, mainly related to gender, strain, genotype and may be influenced by age, diet, handling, environment, among other factors (Pinheiro et al., 2003). Although some biochemical parameters in animals treated with EHEEu are below or above the reference values, cannot be assigned clinical importance because the same results were found in the control group, with no statistically significant difference between them (Table 1). However, it is necessary for the histopathological study of the liver and kidneys of these animals.

According to Silva et al. (2012), hematological parameters are important for the toxicity study, due to the hematopoietic system that is highly sensitive to the toxic agents' activities, such as those with cytotoxic or mutagenic potential. These toxicants may result in many changes such as qualitative or quantitative, transient or permanent and may also limit the use of drugs. In oral treatment of animals with EHEEu, in hematologic rates, only the values of mean corpuscular hemoglobin concentration (MCHC) of animals (males) was changed, which showed a significant increase compared to the control group (Table 2). According to Alves (2007), analysis of red blood cell (RBC) indices are important indicators in determining the morphological type of anemia. The increased erythrocyte MCHC index of male animals had no clinical relevance because they have values within the range recommended by the study of Charles River (Giknis and Clifford, 2008).

Conclusion

With the obtained results, we can suggest that oral treatment with EHEEu at a dose of 500 mg/kg for 22 days produced no signs of systemic toxicity in Wistar rats males and females when compared to the control group. So the EHEEu showed high degree of safety at dose and period in which the animals were exposed.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Full Length Research Paper

Evaluation of anti-inflammatory activity of the bark of *Eysenhardtia polystachya* in experimental animal models

Rosa Martha Perez Gutierrez

Laboratory of Research on Natural Products, School of Chemical Engineering and Extractive Industries del IPN Unidad Profesional Adolfo Lopez Mateos, Zacatenco, del IPN. Av. Instituto Politécnico Nacional S/N. CP.07508, México.

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The bark of *Eysenhardtia polystachya*, has been used in Mexican folk medicine for treatment of anti-inflammatory diseases. This study aimed to investigate the anti-inflammatory activity, on various animal models using carrageenan-induced oedema, cotton pellets-induced granuloma, induction of acute inflammation by histamine, croton oil induced ear oedema, activity of myeloperoxidase, adjuvant-induced arthritis, quantification of tumor necrosis factors alpha (TNFα), interleukin-1 beta (IL-1β), prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4) in arthritic rat. Methanol extract (PAM) was also performed by assessing the activities of lipoxygenase and xanthine-oxidase. Our data indicate that PAM exhibited significant anti-inflammatory activity in all the trials of paw and ear oedema induced exhibiting also anti-arthritic activity. PAM could also markedly inhibit production of proinflammatory cytokines, especially TNFα, IL-1β, PGE_2 and LTB_4. These effects resulted in an attenuation of the inflammatory cytokines and ultimately suppression of the oedema. The extract also inhibited lipoxygenase and xanthine-oxidase. It was seen that PAM is effective on chronic inflammation and acute inflammation.

Key words: *Eysenhardtia polystachya,* anti-inflammatory, cytokines, inflammation.

INTRODUCTION

The tree *Eysenhardtia polystachya*, (Ortega) Sarg, belonging to the Leguminosae family, is known as “palo azul” and has widely been used for the treatment of nephrolithiasis, as a blood depurative, diuretic and anti-rheumatic and bladder disorders developed with diabetes (Perez et al., 1998). Phytochemical studies indicate that *E. polystachya* contains polyphenols (Burns et al., 1984). In another study, isoflavans displayed moderate cytotoxic activity against KB cell lines (Alvarez et al., 1999). The methanolic extract of branches displayed hypoglycaemic activity (Alvarez and Delgado, 1999a). In another report, methanolic bark extract was further separated by column chromatography, yielding four known substances: (-)-epicatechin, (+)-afzelechin, eriodictyol, (+)-quercetin-3-O-*p*-D-galactopyranoside, all of which showed scavenging properties against free

E-mail: rmpg@prodigy.net.mx

radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Narvaez-Mastache et al., 2008). In previous studies, the hypoglycemic, antioxidant potential and advanced glycation endproducts (AGEs) inhibition capacity of the methanol-water extract from the bark of *E. polystachya in vitro* assays, and also using diabetes-induced oxidative damage in the liver, kidney and pancreas was evaluated (Gutierrez and Baez, 2014). This study was undertaken to evaluate the anti-inflammatory potential of plant extracts using various animal models.

MATERIALS AND METHODS

Plant

Bark of *E. polystacya* was collected in Ixmiquilpan, Hidalgo, México in June 2011. Voucher specimens (4532) were deposited in the Herbario de la ENEP-Iztacala UNAM for further reference.

Animals

Male Wistar rats weighing 150 to 200 g were obtained from the Laboratory Animal Services Center, Escuela Nacional de Ciencias Biologicas-IPN, Mexico. All animals were acclimated for 1 week under 12 h light and 12 h dark cycle at room temperature of 22±1°C. Chow diet and water were provided *ad libitum.* Experiments reported in this study were carried out following the guidelines stated in Principles of Laboratory Animal Care (National Institute of Health Publication (NIH) 85-23, revised 1985 and the Mexican Official Normativity (NOM-062-Z00-1999)). All animals' procedures were performed in accordance with the recommendations for the care and use of laboratory animals (756/lab/ENCB).

Preparation of plant extracts

A total of 1000 g of the bark of *E. polystachya* were dried and powdered in a mechanical grinder. The ground material was extracted with 5 L of hexane (PAH), chloroform (PAC) and methanol (PAM) consecutively using a soxhlet apparatus. These extracts were filtered and concentrated by a rotary vacuum evaporator and kept in a vacuum desiccator for complete solvent removal.

Carrageenan-induced rat paw oedema

The rats were divided into 5 groups (n=6). Acute inflammation was produced by the subplantar administration of 0.1 ml of 1% carrageenan in normal saline in the right paw of the rats. The different groups were treated with PAH, PAC and PAM (50, 100, and 200 mg/kg, p.o.), indomethacin (10 mg/kg, p.o.) and control vehicle were administered orally. The paw volume was measured at 0 and 3 h after carrageenan injection using plethysmometer. The animals were pretreated with the extracts 1 h before the administration of carrageenan. The extracts and the standard used for this study were prepared in the same manner as mentioned earlier. The ratio of the anti-inflammatory effect of the extracts was calculated by the following equation: anti-inflammatory activity (%) = (1- D/C) × 100, where D represents the percentage difference in paw volume after extract was administered to the rats, and C represents the percentage difference of volume in the control groups (Washiyama et al., 2009).

Cotton pellets-induced granuloma

The rats were divided into four groups (n=6). After shaving, the rats were anaesthetized and 10 mg of sterile cotton pellets were inserted, one in each axilla. The extracts (50, 100, 200 mg/kg, p.o.) and indomethacin (10 mg/kg, p.o.) and control vehicle were administered orally for 7 consecutive days from the day of cotton pellet implantation. The animals were anaesthetized on the eighth day and cotton pellets were removed surgically and made free from extraneous tissues. The pellets were incubated at 37°C for 24 h and dried at 60°C to constant weight. Increment in the dry weight of the pellets was taken as measure of granuloma formation (Gupta et al., 2003).

Induction of acute inflammation in rat hind paws by histamine

The anti-inflammatory activity of the extract was measured with phlogistic agents (namely, histamine, 5-HT) which act as mediator of inflammation. Acute inflammation in the hind paws was induced by the subcutaneous injection of 0.05 ml of the prepared solutions of histamine (1%) into the right hind paws of the rats. The left hind paws without injection were used as control. The volumes (ml) of both hind paws of rat were measured using a plethysmometer (Plethysmometer 7150, UGO Basile, Italy) at 1 h before induction and 0.5, 1, 2, 3, 4, 6 h after induction of the inflammation. The increased volumes (paw edema) of the right hind paws of rats were calculated by the following equation: the increased rate (%) = (B-A)/A × 100, where A and B represent the paw volumes before induction of inflammation and at different time points after the induction, respectively. Extracts (100, 50, 25, 12.5 mg/kg) or vehicle were intraperitoneally administrated 10 min prior to histamine injection. The reference drug, indomethacin (10 mg/kg), was orally administered 1 h prior to histamine injection (Jing-Rong et al., 2008).

Croton oil induced ear edema in mouse

The edema was induced by application of 20 µl of croton oil (200 µg) diluted in a solution of acetone/water (7:3) to the inner surface of the mouse's left ear. The right ear received only the vehicle (20 µl). Immediately after injecting the phlogistic agent, in the groups of treated animals we applied 20 µl of the total extract (1.25, 2.5, 5.0, 7.5 mg) to the left ear. In the control group, 20 µl of the vehicle was applied to the left ear. After 6 h, the animals were killed, and the ears were sectioned in discs 6.0 mm in diameter and weighed (mg) in an analytical balance. The percentage of inhibition of edema was determined (Van Arman, 1974).

Activity of myeloperoxidase (MPO)

The MPO activity was evaluated in the supernatant of homogenates of the ear sections (controls and those treated with crude extract, 5.0 mg; or indomethacin, 1.0 mg), according to the technique described by Bradley eI al. (1982). The ear tissue was placed in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadeciltrimethylammonium bromide (Sigma, 1 ml/50 mg of tissue) in a Potter homogenizer. The homogenate was vortex-mixed and centrifuged for 5.0 min at 2500 rpm. Ten microliters of the supernatant thus obtained was added to a 96-well microplate, in triplicate, followed by addition of 200 µl of a buffer solution containing O-dianisidine dihydrochloride (Sigma, 16.7 mg), bidistilled water (90 ml), potassium phosphate buffer (10 ml) and 1% H_2O_2 (50 µl). The enzyme activity was determined by measuring the absorbancy (460 nm).

Table 1. Effect of the PAM extract on carrageenan induced paw oedema and on cotton-pellet induced granuloma in rats.

Treatment	Dose mg/kg	Paw edema (Percentage inhibition) at different time intervals			
		1	2	3	4
Carrageenan control	0	0	0	0	0
PAM	50	12.44 ± 2.22[b]	19.41 ± 6.12[b]	23.07 ± 6.43[b]	34.61 ± 7.38[b]
PAM	100	28.31 ± 4.52[a]	36.26 ± 8.58[a]	48.45 ± 4.67[a]	61.53 ± 8.10[a]
PAM	200	37.62 ± 5.31[a]	48.73± 7.52[a]	60.32 ± 7.28[a]	73.26 ± 7.31[a]
Indomethacin	10	52.23 ± 6.23[b]	57.62 ± 3.49[b]	73.21 ± 5.52[a]	78.43 ± 6.28[a]
STD	10	21.47 ± 7.21[a]	39.46 ± 4.72[a]	55.43 ± 6.78[a]	68.12 ± 4.21[a]

Values represent mean ± SEM (n=8). One way ANOVA followed by Dunnet's multiple comparison test. [a]p<0.05, [b]p<0.01, statistically significant relative to control. Diclofenac sodium (STD).

Adjuvant-induced developing arthritis in rat

Adjuvant arthritis was induced in rat by the subplantar injection of 0.02 ml freshly prepared suspension (5.0 mg/ml) of steam killed *Mycobacterium tuberculosis* (Difco, USA) prepared in liquid paraffin in the left hind foot pad. The volume of the injected (primary inflanmmatory response) (Arrigoni-Martelli and Bramm, 1975) and uninjected (secondary immune mediated response) paws were quantitated on day 13 after the adjuvant injection. The volume of the injected as well as uninjected paws was measured by a volume differential meter model 7101, Ugo Basile, Italy and the effect determined on day 13.

Quantification of tumor necrosis factors alpha (TNFα), interleukin-1 beta (IL-1β), prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4) in serum samples of arthritic rat

Samples of the serum obtained on day 14 from different groups of animals were prepared for the analysis of cytokine mediators. TNFα, IL-1β, PGE_2 and LTB_4 were estimated using commercially available kits based on sandwich and competitive enzyme-linked immunosorbent assay (ELISA) technique (R&D Systems, MN, USA) according to the manufacturer's instructions. All cytokine concentrations were carried out by means of colorimetric measurement at 450 nm on an ELISA plate reader (Multiskan, Thermo Electron Corporation, MA, USA) by interpolation from a standard curve.

Enzyme inhibition assay

Xanthine oxidase (XO) inhibition assay

The xanthine oxidase inhibition activity was assayed on a spectrophometer according to a method previously described by Owen and Timothy (1999). The assay mixture consisted of 150 µl of phosphate buffer (0.066 M; pH 7.5), 50 µl of extract solution (1 mg/ml in phosphate buffer), and 50 µl of enzyme solution (0.28 U/ml). After pre-ineubation at room temperature (25°C) for 3 min, the reaction was initiated by addition of 250 µl of substrate solution (Xanthine, 0.15 M in the same buffer). A blank without enzyme solution was also prepared. The reaction was monitored for 3 min at 295 nm and velocity (Vo) was recorded. Phosphate buffer was used as negative control (activity of the enzyme without extract solution). Allopurinol was used as positive control. The percentage of xanthine oxidase inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = [(Vo_{control} - Vo_{sample}) \times 100]/Vo_{control}$$

$Vo_{control}$ is the activity of enzyme without macerate/fraction and Vo_{sample} is the nzyme activity in the presence of extract or allopurinol

Lipoxygenase (LOX) inhibition assay

The lipoxygenase inhibiting activity was assayed spectrophotometrically as described by Lyckander and Malterud (1992). Briefly 100 µl of the enzyme solution (at the final concentration of 200 U/ml) was prepared in boric acid buffer (0.2 M; pH 9) and mixed with 25 µl of extract solution (1 mg/ml in boric acid buffer) and then incubated at room temperature for 3 min. Reaction was subsequently initiated by the addition of substrate solution (linoleic acid, 250 µM), and the velocity was recorded for 2 min at 234 nm. Negative control was prepared and contained 1% methanol solution without extract solution. Quercetin was used as positive control. The percentage of lipoxygenase inhibition was calculated according to the following equation:

$$\text{Inhibition (\%)} = [(Vo_{control} - Vo_{simple}) \times 100]/Vo_{control}$$

$Vo_{control}$ is the activity of enzyme in absence of extract solution and Vo_{simple} is the activity of the enzyme in the presence of extract or quercetin.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). The level of statistical significance was determined by analysis of variance followed by Duncan's new multiple range test.

RESULTS

The hexane, chloroform and methanol extracts of the bark of *E. polystachya* was evaluated for anti-inflammatory activity in experimental animal models. The methanol extract (PAM) exhibited significant (p<0.05) anti-inflammatory activity in all assays, instead hexane and chloroform extracts showed no anti-inflammatory activity (the results are not presented here).

As shown in Table 1, the methanol extract showed maximum inhibition of 73.26% at the dose of 200 mg/kg after 4 h of drug treatment in carrageenan induced paw oedema, whereas the standard drug indomethacin and

Table 2. Effect of the PAM extract on cotton-pellet induced granuloma in rats.

Treatment	Dose (mg/kg)	Weight of cotton pellet	Percentage of inhibition
Cotton-Pellet control	0	48.4 ± 2.8	-
PAM	50	37.1 ± 6.0	23.3
PAM	100	30.5 ± 24.7	37.0[b]
PAM	200	22.7 ± 23.9	53.19[a]
Indomethacin	10	21.3 ± 4.7	56.0[a]

Values represent mean ± SEM (n=8). One way ANOVA followed by Dunnet's multiple comparison test. [a]p<0.05, [b]p<0.01, statistically significant relative to control.

Table 3. Inhibition of histamine-induced paw edema of rats by treatment of methanol Extract (PAM).

Doses mg/kg		Time (h) after histamine injection				
		0	1	2	3	4
Control	0	36.8 ± 7.9	27.5 ±6 .9	22.2 ± 7.6	17.5 ± 5.6	13.7 ± 4.4
12.5	0	27.2 ± 3.5 (26)[a]	18.1 ± 2.8 (34)[b]	16.1 ± 4.5 (27)[a]	14.2 ± 1.9 (19)[a]	5.4 ± 1.32 (60)[b]
25	0	25.0 ± 6.1 (32)[a]	17.1 ± 2.7 (38)[b]	15.2 ± 3.8 (31)[b]	10.3 ± 4.7 (41)[a]	4.8 ± 0.78 (64)[a]
50	0	21.3 ± 2.9 (42)[b]	16.4 ±2.9 (40)[a]	13.4 ± 2.9 (39)[a]	8.1 ± 3.7 (53)[a]	4.3 ± 1.23 (68)[a]
100	0	19.3 ± 4.7 (47)[b]	15.3 ± 5.2 (44)[a]	12.1 ± 4.6 (45)[b]	5.8 ± 2.4 (66)[a]	3.9 ± 0.76 (71)[a]
In 10	0	14.2 ±1.8 (61)[a]	14.6 ± 3.6 (46)[a]	11.8 ± 6.2 (47)[a]	5.0 ± 1.0 (71)[b]	3.1 ± 0.98 (77)[a]

PAM and the vehicle were i.p. injected 10 min prior to the induction. Reference drug was orally administered 1 h in advance. Values are the mean ± SEM (n=8). ap<0.05, bp<0.01. Increase of paw volume (%). Indomethacin (In).

Table 4. Effect of the PAM extract on edema of the ear and the activity of MPO**.**

Group (mg)	Ear edema (mg)	Group (mg)	MPO activity (UMPO/mg)
Basal	7.3 ± 1.4	Basal	0.001 ± 0.0004
CO	14.6 ± 4.3	CO	0.98 ± 0.05
CO + 1.25	13.3 ± 4.2	CO + 5.0	0.41 ± 0.07[ab]
CO + 2.5	12.4 ± 3.7	CO + Indo	0.26 ± 0.05[ab]
CO + 5.0	11.1 ±3.8[a]	-	-
CO + 7.5	10.0 ± 1.4[a]	-	-
CO + Indo	8.1 ± 2.6[ab]	-	-

Indomethacin (Indo), 1 mg which was administered topically, was used as the reference anti-inflammatory (positive control). Ears which received only an application of the vehicle (basal). Each column represents the mean weight of the ears ± SEM, 6 h after application of the croton oil (CO) and the MPO-mediated activity± SEM, [a]p<0.01, compared to the control group (CO), [b]p<0.05, compared to the Indo group (ANOVA, Tukey's test).

diclofenac sodium showed 78.43 and 68.12% of inhibition, respectively.

In the chronic model (cotton pellets-induced granuloma) PAM at dose of 200 mg/kg and standard drug (indomethacin) showed decreased formation of granuloma tissue of 53.19 and 56.0%, respectively (Table 2).

In histamine-induced rat paw edema, measurements were conducted at 0.5, 1, 2, 3, 4 and 6 h after subcutaneous injection of histamine. As shown in Table 3, the hind paw edema of rats rapidly decreased from 1 h onward after the injection. The left hind paws that were used as control showed no increase of paw.

Application of croton oil to the left ear of the mice induced a very evident inflammatory response by 6 h. The weight of the ear doubled as compared to the right ear (basal, with no croton oil applied). PAM failed to inhibit the intensity of edema at a dose of 1.25 mg; however, at doses of 2.5, 5.0, and 7.5 mg, this extract significantly ($p<0.01$) reduced the intensity of edema (Table 4).

MPO is an enzyme present in the intracellular granules of neutrophils, and can be used as a marker for the influx of polymorphonuclear leukocytes into inflamed tissues. Application of croton oil induced an increase in MPO activity on the order of 20-fold, at 6 h after application of

Table 5. Effect of the PAM extract on antiarthritic activity in *Micobecterium tuberculosis* induced arthritis in rat.

Doses (mg/kg)	Oedema (mean ± mm)
Control	1.03 ± 0.02
1	0.90 ± 0.05
2	0.74 ± 0.08[ab]
4	0.60 ± 0.06[ab]
Prednisolone 5	0.52 ± 0.04[a]

[a]p<0.01, compared to the control group , [b]p<0.05, compared to the Prednisolone group (ANOVA, Tukey's test).

Table 6. Effect of the PAM extract on TNFα, IL-1β, PGE_2 and LTB_4 in arthritis induced rat.

Doses (mg/kg)	TNFα (pg/ml)	IL-1β (pg/ml)	PGE_2(pg/ml)	LTB_4 (pg/ml)
Control	4500	1980	701	470
1	3100[a]	1488[a]	531[b]	402
2	2200[a]	975[b]	419[a]	358
4	2000[b]	769[b]	263[a]	268[a]
Prednisolone 5	1007[b]	526[b]	143[b]	161[b]

Table 7. Inhibitory activities PAM against lipoxygenase (LOX) and xanthine oxidase (XO).

Treatment	LOX	XO
PAM	46.1 ± 3.29	67.17 ± 4.19
Allopurinol	ND	75.32 ± 5.84
Quercetin	54.21 ± 6.43	71.50 ± 5.16

Values are the mean ± SEM (n=6). [a]p<0.01, [b]p<0.05, Student's t-test. Showing significant inhibition of TNFα, IL-1β, PGE_2 parameters at higher dose levels of 2 and 4 mg/kg. Expression of LTB_4 was also inhibited but was not very significant statistically compared with prednisolone.

the stimulus. PAM extract (5.0 mg) and indomethacin (1.0 mg) significantly inhibited the activity of the enzyme. These results are as shown in Table 3. PAM showed statistically significant dose related inhibition of oedema in the injected paw with a maximum effect at dose levels of 2 and 4 mg/kg orally (Table 5). The PAM administered groups also did not show significant swelling in the uninjected paw (secondary response) of the experimental animals when compared with the vehicle control group.

Antiarthritic activity of PAM in *M. tuberculosis* induced inflammatory arthritis in mice (injected paw) is as shown in Table 6. At dose of 4 mg/kg (p.o.), PAM significantly decreased TNFα, IL-1β, PGE2 and LTB4 levels.

The activity of the extracts from *E. polystachya* against XO and LOX, were evaluated and the results are summarized as shown in Table 7. The data indicated that PAM possesses strong activity in XO and LOX enzyme inhibition compared to that produced by allopurinol and quercetin.

DISCUSSION

In this investigation, PAM has been demonstrated to have anti-inflammatory effect at the dosages of 50, 100 and 200 mg/kg on the carrageenan-induced paw edema in rats. Carrageenan induced oedema is commonly used as an experimental animal model for acute inflammation and the time course of edema development in carrageenan induced paw edema in rats is generally represented by a biphasic curve (Gepdiremen et al., 2004), of which the first phase occurs within 1 h of injection and it is mediated by the release of histamine and 5-HT induced hind paw edema. Prostaglandins play a major role in the development of the second phase which is measured at 3 h time, which indicates that the methanolic extract exhibits its anti-inflammatory action by means of either inhibiting the synthesis, release or action of inflammatory mediators, namely, histamine, serotonin and prostaglandins might be involved in inflammation in the

later phase.

In the current study, the anti-inflammatory effect of PAM was further evaluated in the histamine-induced paw edema in rats. Histamine is a potent mediator to act in acute inflammation; it is produced in the early phase of acute inflammation to increase vascular permeability. Since the action of histamine is transient, the inflammation decreases very quickly after induction. Hence, the early inflammatory response appears to be mediated mainly by histamine. In our study, PAM showed strong and dose-dependent inhibition on the paw edema in the early phase of the inflammation, implying that PAM exert the anti-inflammatory effect by acting on the early phase of the inflammation.

Chronic inflammation is a reaction arising when the acute response is insufficient to eliminate proinflammatory agents. Chronic inflammation includes a proliferation of fibroblasts and the infiltration of neutrophils and exudation (Dunne, 1990). Chronic inflammation occurs by means of the development of proliferative cells. These cells can be either spread or in granuloma form. PAM showed significant anti-inflammatory activity in cotton-pellet induced granuloma and thus found to be effective in chronic inflammatory conditions, which reflected its efficacy in inhibiting the increase in the number of fibroblasts and synthesis of collagen and mucopoly-saccharides during granuloma tissue formation (Recio et al., 1995).

PAM showed significant ($p<0.05$) activity on edema of the ear induced by local application of croton oil which involves the activation of phospholipase A_2 and, consequently, biosynthesis of prostaglandins and leucotriens (Rotelli et al., 2003).

PAM possess significantly dose-related antiarthritic activity in *M. tuberculosis*-induced adjuvant arthritis test in rat, which is considered the close to simulating human rheumatoid arthritis. The appearance of secondary lesions (uninjected paw swelling) is the manifestation of cell mediated immunity (T cell response) and the suppression of this response by PAM suggests it to have immunosuppressive activity (Luster et al., 1982).

PAM significantly inhibited the expression of TNF-α, LTB_4 and IL-1β 1evels which are potent triggers involved in leukocyte migration (Crofford el al., 1997) in arthritic animal and this was also evident in the inhibition of cell migration and the exudate volume by decreasing the influx of leucocytes. TNF-α, a key proinflammatory mediator of this activated immune network, connects other ancillary cells in the process of activation with a detrimental outcome (Aggarwal and Gutterman, 1992). PGE_2 is another polypeptide mediator, activates lymphocytes and induces several components of the host's acute-phase response to infection and injury (Schrader and Thompson, 1994).

LOX and XO representing the key enzymes in the biosynthesis of leukotrienes that have been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases. Leukotrienes function as initiators of inflammation and their inhibition is considered to be partly responsible for the anti-inflammatory activity (Ammon et al., 1992). In the present study, PAM showed good anti-LOX and anti-XO activities. The inhibition percentage by PAM on XO is comparable to that allopurinol, is a therapeutic drug used to treat gout, which also suffers from many side effects such as hypersensitivity syndrome. Thus, there is a need to develop compounds with XO activity which is devoid of the undesirable side effects of allopurinol. Flavonoids and polyphenolic crude extracts have been reported to possess xanthine oxidase and lipoxygenase inhibitory activities (Halliwill, 1996). The high antioxidant capacity of PAM may be due to the presence of flavonoids or polyphenols in the extract (Perez and Baez, 2014).

The results of this study demonstrated that methanol extract of the bark of *E. polystachya* acts as an anti-inflammatory agent. It also can be a good source of effective crude inhibitors for XO and LOX. The finding presented in this study are encouraging and substanciate the search for newer pharmacophores in palo azul behind the anti-inflammatory effect.

Conflict of interest

Authors declare that there are no conflicts of interest.

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Review

Review: The potential of chalcones as a source of drugs

Rosa Martha Perez Gutierrez*, Alethia Muñiz-Ramirez and Jahel Valdes Sauceda

Laboratorio de Investigacion de Productos Naturales, Escuela Superior de Ingeniería Química e Industrias Extractivas, IPN. Av. Instituto Politecnico Nacional S/N Unidad Profesional Adolfo Lopez Mateos, Col. Zacatenco, CP 07758, Mexico D.F., Mexico.

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Chalcone and dihydrochalcones are intermediates in the biosynthesis of flavonoids and isoflavonoids in plants. These compounds are widely investigated for their anticancer, anti-inflammatory, antimicrobial, antiprotozoal, antifilarial, larvicidal, anticonvulsant, anti-rheumatoid and antioxidant activities and their use as food additives. Chalcones are considered to be an active ingredient in a large number of medicinal herbs. Further chemical investigation of these plants has now resulted in the isolation of chalcone and biologically active derivatives. Chalcone and their derivatives are an attractive molecular scaffold for the search of new biologically active molecules. This review provides a comprehensive analysis of the source plants, chemistry, structure-activity, pharmacological reports of chalcone and derivatives isolated and identified from plants. In recent years a considerable number of investigations conducted on the biological activities of these compounds suggested a wide range of clinical applications.

Key words: Chalcones, dihydrochalcones, derivatives, bioactives, flavonoids, phytochemistry.

INTRODUCTION

Chalcones structure differs considerably from the other members of the flavonoid family. Approximately 201 aglycone structures with varied patterns of hydroxylation, and in some cases, methylation and prenylation, are known. Although many chalcones occur as glycosides, the majority are found as free aglycones. Chalcones are isomerized to flavanones in plants by the enzyme chalcone isomerase, but are readily isomerized *in vitro* in the presence of acid (Seigler, 2002). The biological effect of chalcones was found to be dependent on the presence, the number and position of functional groups such as methoxy, glycosides, hydroxyl, halogens, etc. in both A and B rings (Dhar, 2003).

Chalcones are abundant in edible plants fruits, vegetables, spices, tea and have also been shown to display pharmacologicall varied effects (Chimenti et al.,

*Corresponding author. E-mail: rmpg@prodigy.net.mx.

2009). They present a broad spectrum of biological activities such as anticancer, anti-inflammatory, antimalarial, antifungal, antilipidemic, antiprotozoal (antileishmanial and antitrypanosomal), antibacterial, antifilarial, larvicidal, antioxidant, anticonvulsant antimicrobial and antiviral (Rahman, 2011). There has been a tremendous interest in these compounds (Appendix 1) as evidenced by the voluminous work. Therefore, we aimed to compile an up to date and comprehensive review of chalcones that covers their traditional and folk medicine uses, phytochemistry and pharmacology.

BIOLOGICAL ACTIVITY

Scientific investigations of the medicinal properties of chalcones dates back to the 1980s. A summary of the findings of these studies performed is presented below.

Antiinflammatory activity

Recent reports indicate the importance of chalcones as anti-inflammatory agents involved in the inhibition of cell migration and the inhibition of TNF-α production in mouse model. Chalcone derivatives have been extensively reported to inhibit NO synthesis, iNOS and cycloxygenase 2 protein expression in lipopolysaccharide (LPS) stimulated cells. The structure-activity analysis demonstrated that chalcones with substituents that reduce the electronic density in the B ring, such as chlorine atoms or nitro groups, show better biological activity and selectivity in the inhibition of nitrite production, and position 2 in B ring seems to be more important (Wu et al., 2011). Six chalcones were isolated from *Angelica keiskei* 2',4',4-trihydroxy-3'-[2-hydroxy-7-methyl-3-methylene-6-octaenyl]chalcone] (1), 2',4',4-trihydroxy-3'-geranylchalcone (2), 2',4',4-trihydroxy-3'-[6-hydroxy-3,7-dimethyl-2,7-octadienyl]chalcone (3), 2',4-dihydroxy-4'-methoxy-3'-[2-hydroperoxy-3-methyl-3-butenyl] chalcone (4), 2',4-dihydroxy-4'-methoxy-3'-geranylchalcone (5), and 2',4-dihydroxy-4'-methoxy-3'-[3-methyl-3-butenyl]chalcone (6). Among them, compounds 1 to 3 showed potent inhibitory activity of IL-6 production in TNF-α-stimulated MG-63 cell, while compounds 4 to 6 did not. The inhibitory activity of IL-6 production in TNF-α-stimulated MG-63 cell is likely to be affected by the presence of C-4' hydroxyl group in the chalcone moiety (Shin et al., 2011).

The chalcone derivatives isolated from the fruits of *Malotus philippinensis* called mallotophilippens C (7), D (8) and E (9) xanthohumol (10), and dihydroxanthohumol (11) inhibited the production of NO induced by LPS and IFN-y in murine macrophage-like cell line, RAW 264.7. Furthermore, mallotophilippens inhibited inducible iNOS, COX-2, IL-6 and IL-113 mRNA gene expression (Nowakowska, 2007). Daikonya and co-workers hypothesized that the main inhibitory mechanism of these compounds may be the inactivation of the nuclear factor KB (NF-KB) (Daikonya et al., 2004).

Antimicrobial effect

Licochalcone A (12) is a retrochalcone isolated from the roots and rhizomes of *Glycyrrhiza inflata.* It is active against a wide range of Gram positive organisms but not against Gram negative bacteria and eukaryotes. Licochalcone A structure-activity relationship study showed that, of the two phenolic hydroxyl (OH) groups attached to rings A and B of licochalcone A, the OH on ring A was more important for antibacterial activity. The prenyl side chain on ring B contributed to lipophilicity, and could be replaced by groups with comparable lipophilíc character, like n-hexyl, without loss of antibacterial activity. Licochalcone A has been used as a lead compound for the design of more potent antibacterial agents based on the chalcone template (Liu et al., 2008).

Drewes and van Vuuren (2008) isolated from flowers of *Helichrysum gymnocomum* the chalcones 4',6', 8',trihydroxychalcone (14) and 2-hydroxy-4',6'-dibenzyloxychalcone (13) which had minimum inhibitory concentration (MIC) value below 64 µg against of pathogens including *Staphylococcus aureus* and the *S. aureus* methicillin and gentamycin resistant strain. The existence of the benzyloxy group, as well as the presence of the unsubstituted B-ring in chalcone play a role in influencing the antimicrobial activity. Other studies show that *Artocarpus nobilis* (Moraceaes) yielded 2',4',4-trihydroxy-3'-geranylchalcone (15), 2',4',4-trihydroxy-3'-[6-hydroxy-3,7-dimethyl-'2(E),7-oetadienyl] chalcone (16), 2',4',4-trihydroxy-3'-['2-hydroxy-7-methyl-3-methylene-6-oetaenyl]chalcone (17), 2',3,4,4'-tetrahydroxy-3'-geranylchalcone (18) and 2'3,4,4'-tetrahydroxy-3'-[6-hydroxy-3,7-dimethyl-2(E),7-octadienyl] chalcone (19). All the compounds showed fungicidal activity at 5 µg/spot against *Cladosporium cladosporioides*. Furthermore, four chalcones, were isolated from an ethanol extract of the leaves of *Maclura tinctoria* (L.) Gaud. Compounds 2',4',4,2''-tetrahydroxy-3'-[3''-methylbutyl-3'''enyl]chalcone] (20), isovachalcone (21), bakuchalcone (22), and bavachromanol. Isovabachalcone was active against *Candida albicans* (IC_{50} of 3 µg.ml^{-1}) and *Cryptococcus neoformans* (IC_{50} of 7 µg ml^{-1}) (Jayasinghe et al., 2004). Other studies show that the methanolic extract of *Zuccagnia punctata* consisting of 2',4'-dihydroxy-3'-methoxychalcone (23) and 2',4'-dihydroxy chalcone (24) displayed very good activities (MIC = 6.25 and 3.12 µg ml^{-1}) against *Phomopsis*

longicolla Hobbs CE117, and (MIC = 6.25 µg ml^{-1}) against *Colletotrichum truncatum* CE175 (Svetaz et al., 2004). 2',4'-Dihidroxy-3',5'-dimethyl-6' methoxychalcone (25) (Belofsky et al., 2004) isolated from *Dalea versicolor* exhibited individually and in synergy with known antibiotics (berberin, erythromycin and tetracycline) the activity towards the human pathogen *S. aureus* and the opportunistic pathogen *B. cereus*. This compound in the presence of berberine effected a dramatic 30-fold increase in activity against *B. cereus.*

Antiosteoporosis effect

Dimeric dihydrochalcone cycloaltilisin 6 (26) and AC-5-1 (27) were isolated of the bud covers of *Artocarpus altilis*. All the compounds shown to be potent inhibitors of cathepsin K (is a cysteine protease that has been implicated in osteoporosis). Cycloaltilisin 6 was found to be the most potent inhibitor with an IC_{50} of 98 nM followed by AC-5-1 with an IC_{50} of 170 nM and cycloaltilisin 7 (28) with an IC_{50} of 840 nM (Patil et al., 2002).

Antioxidant effect

The methanol extract of *Maclura tinctoria* stem bark led to the isolation of four chalcone glycosides 4'-O-β-D-(2''-p-coumaroyl)glucopyranosyl-4,2',3'-trihydroxychalcone (29), 4'-O-β-D-(2''-*p*-coumaroyl)-6''-acetylglucopyranosyl-4,2',3'-trihydroxychalcone (30), 3'-(3-methyl-2-butenyl)-4'O-β-D-(glucopyranosyl-4,2'-dihydroxy chalcone (31) and 4'-O-β-D-(2''-acetyl-6''-cinnamoyl)glucopyranosyl-4,2',3'-trihydroxychalcone (32). The results showed that 3'-(3-methyl-2-butenyl)-4'O-β-D-(glucopyranosyl-4,2'-dihydroxychalcone was the most active chalcone in antioxidant assays (Cioffi et al., 2003). The fruit and seeds of *Cedrelopis grevei* (Ptaeroxylaceae) yielded uvangoletin (33), flavokawin B (34), 5,7-dimethylpinocembrin (35), 2'-methoxyhelikrausichalcone (36), and the prenylated chalcones, cedrediprenone (37) and cedreprenone (38) (Koorbanally et al., 2003). The antioxidant effect of some dihydrochalcones has been reported in apple fruits (*Malus domestica*). Phloridzin (39), seboldin (40) and trilobatin (41) were isolated from the leaf of *M. domestica*. Phloridzin had a high activity in the oxygen radical antioxidant capacity (ORAC) assay, it have ability to prevent oxidative-dependent formation of AGEs the phenylephrine-induced contraction of isolated rat mesenteric arteries. Sieboldin clearly demonstrated antioxidant activity and prevented vasoeonstrietion and inhibited AGEs formation (De Bernonville et al., 2010).

Eight dihydrochalcones were isolated from the roots of *Anneslea fragrans* var. lanceolata, davidigenin-2'-O-(6''-O-4'''-hydroxybenzoyl)-β-glucoside (42), davidigenin-2'-O-(2''-O-4'''-hydroxybenzoyL)-β-glucoside (43), davidigen-2'-O-(3''-O-4'''-hydroxybenzoyl)-β-glucoside (44), davidigenin-2'-O-(6''-O-syringoyl)-β-glucopyranoside (45), 1-O-3,4-dimethoxy-5-hydroxyphenyl-6-O-(3,5-di-O-methylgalloyl)-β-gluco-pyranoside (46) davidioside (47), 4'-O-methyldavidioside (48) and davidigenin (49). Compounds 46 to 49 showed weak DPPH radical scavenging activity, whereas the other chalcones did not display any DPPH radical scavenging activity. The 2,6-dimethoxy groups of the syringoyl moiety may further stabilize the phenoxyl radicals enhancing the radical scavenging ability of compounds 45 and 46 (Huang et al., 2012).

Syzygium jambos ALston, afforded three compounds phloretin 4'-O-methyl ether (2',6'-dihydroxy-4'-methoxydihydrochalcone) (50), myrigalone G (51) and myrigalone B (52), which showed antioxidant activity higher than that of α-tocopherol by spectrophotometry method (Jayasinghe et al., 2004). Aspalathin (53) and nothofagin (54) were isolated from Rooibos (*Aspalathus linearis*). The most potent radical scavengers were aspalathin (IC_{50} = 3.33 µM) and EGCG (IC_{50} = 3.46 µM), followed by nothofagin (IC_{50} = 4.04 µM), [90].

Antiplasmodial effect

Worldwide, 300-500 million people are infected with malaria each year. Most cases occur in sub-Saharan Africa, with approximately 2 million people dying there each year. Unfortunately, the emergence of malarial parasite strains resistant to chloroquine has eroded this drug´s efficacy. Extensive programs are underway to screen natural products and synthetic derivatives for new agents to treat chloroquine-resistant malaria. The n-hexane extract of leaves of *Piper hostmannianum* var. berbicense (Miq.) (Piperaeeae) exhibited interesting activity against *Plasmodium falciparum* (IC_{50} = 8 µg ml^{-1}) (Portet et al., 2007). An activity bioassay-guided fractionation led to the isolation of dihydrochalcones hostmanin A (55), hostmanin B (56), hostmanin C (57) hostmanin D (58) and 2',6'-dihydroxy-4'-methoxydihydrochalcone (59), as well as linderatone (60), adunctin E (61) and (-)-methyllinderatin (62). All chalcones were actives in vitro against *Plasmodium falciparum*, whereas linderatone and (-)-methyllinderatin were considered to be potentially interesting.

Anticancer activities

Since apoptosis is one of the most potent defenses

against cancer development, efforts have been made to develop a chemoprevention and therapeutic strategies that selectively trigger apoptosis in malignant cancer cells. Particularly interesting are the properties of chalcones in the induction of apoptosis and their ability to change mitochondrial membrane potential (Sabzevari et al., 2004). In cancer, it has been reported that chalcones interfere in several points of the signal transduction pathways related to cellular proliferation, angiogenesis, metastasis, apoptosis and the reversal of multidrug resistance. The largenumber of research articles and patents related to chalcones is already an indication of their importance as a lead class of compounds. Chalcones with fewer hydroxyl groups on rings A and B were more effective in this regards, as compared to chalcones containing more hydroxyl groups. This difference was attributed to the acidity of the phenolic hydroxyl groups. One of the most widely cited mechanisms by which chalcones exert their cytotoxic activity is that of the interference with the mitotic phase of the cell cycle. A large number of methoxylated chalcones with antimitotic activity against HeLa cells was discovered. Other studies show that the capacity of 2'-hydroxychalcones with different methoxy subtitutions on ring B to inhibit cellular proliferation, induce apoptosis and correlate it with the chemical reactive indexes in HepG2 hepatocellular carcinoma cells (Echeverria et al., 2009).

Later, Bertl et al. (2004) studied the potential antiangiogenic effects of xanthohumol (63) and isoxanthohumol (64), chalcones isolated from *Humulus lupulus* (hopse). In *in vitro* conditions they observed a reduction of newly formed capillary growth by xanthohumol at a concentration range of 0.5 to 10 µM (IC_{50} value of 2.2 µM). The inhibitory effect of isoxanthohumol was weaker. Furthermore, xanthohumol effectively blocksed tumour angiogenesis and tumour growth *in vivo* and interferes with several steps in the angiogenic process. Xanthohumol also reduced vascular endothelial growth factor (VEGF) secretion, decreased cell invasion and metalloprotease production in acute and chronic myelogenous leukemia cell lines (DellEva et al., 2007). Moreover, licochalcone E (65), a retrochalcone isolated from the roots of *Glycyrrhiza inflata*, was found to be an inducer of apoptosis in endothelial cells by modulating NFKB and members of the Bcl-2 family (Mojzis et al., 2008).

Similarly, 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (66), extracted from the dried flower *Cleistocalyx operculatus*, blocked antiangiogenesis *in vitro* as well as *in vivo*. In *in vitro* conditions it reversibly inhibited VEGF receptor tyrosine kinase phosphorylation. It also inhibited MAPI< and AKT activation of VEGF receptor signal transduction. Systemic administration of this chalcone resulted in the inhibition of subcutaneous tumour growth of human hepatocarcinoma Bel7402 and lung cancer GLC-82 xenografts and a decrease in the tumour vessel density (Zhu et al., 2005).

TRAIL is a naturally occurring anticancer agent appearing in soluble form or expressed in immune cells. TRAIL mediates *in vitro* and *in vivo* apoptosis in cancer cells. Cytotoxic effects of chalcones and dihydrochalcone 2',6'-dihydroxy-4'-methoxychalcone (67), 2',6'-dihydroxy-4'-methoxydihydro chalcone (68) 2' 6' -dihydroxy-4,4' –dimethoxy dihydrochalcone (69) and phloretin (70) markedly augment TRAIL mediated apoptosis in LNCaP cells. Sensitization of prostate cancer cells to TRIAL-mediated apoptosis by chalcones and dihydrochalcones suggest the potential role of these compounds in anticancer immune defense in which endogenous TRAIL takes part. The TRAIL-mediated cytotoxic and apoptotic pathways may be a target of the chemopreventive agents in prostate cancer cells and the overcoming TRAIL-resistance by chalcones and dihydrochalcones may be one of the mechanisms responsible for their cancer preventive effects (Szliszka et al., 2010). The phytochemical study of chloroform extract of *Calythropsis aurea* (Myrtaceae) yielded two chalcones calythropsin (71) and dihydrocalythropsin (72). Calythropsin showed no detectable activity *in vitro* tubulin polymerization assay, however it showed weak cytotoxic activity against L1210 cells with IC_{50} of 7 µM (Beutler et al., 1993).

In another study, the chalcone derricin (73) and lonchocarpin (74) were isolated from hexanic extract from the roots of *Lonchocarpus sericeus* (Fabaceae). Both chalcones possessed cytotoxicity against CEM Leukaemia cell line, inhibiting cell growth with IC_{50} lower than 20 µg/ml. Lonchocarpin was cytotoxic against tumoral cells, but had no effect on sea urchin egg development at tested concentrations. In fact, lonchocarpin was also the least active substance against leukaemia cells presenting a maximal inhibition of 77% in higher tested concentration, while derricin almost completely stopped cell growth (Cunha et al., 2003).

Dihydrochalcones 10',6'-diacetoxy-4,4'-dimethoxydihydrochalcone (75), 4,2',6'-trihydroxy-4'-methoxy dihydrochalcone (76), 2',6'-dihydroxy-4'-methoxydihydrochalcone (77) and chalcone 2',4'-diacetoxy chalcone (78) isolated from the leaves of *Carthamus arborescens* showed cytotoxic activity on cell lines P-388, A-549 and HT-29. Of these chalcones 10',6'-diacetoxy-4,4'-dimethoxy- dihydrochalcone was the most potent against human cell line tested (Barrero et al., 1997). Litseaone A (79) and B (80) were isolated of the stem bark of *Litsea rubescens* and *Litsea pedunculata*. Both compounds exhibited moderate cytotoxic activities with IC_{50} values of 23.0 and 21.5 µg ml^{-1} against liver carcinoma (HepG-2) cell line. Chalcones displayed potent cytotoxic activities with IC_{50} values lower than 14.0 µg ml^{-1} against myeloid leukaemia (HL-60) and epidermoid carcinoma (A431) cell lines were more active than DDP.

Litseaone A exhibited cytotoxic activity against myeloid leukaemia (HL-60) with IC_{50} value 2.1-fold more sensitive to DDP. These chalcones were found to contain the rare epoxy or ethylidenedioxy group. This is the first report on the presence of chalcone in the Litsea plant genus (Li et al., 2011). *Syzygium samarangense* (Bloom) (Myrtaceae), known commonly as wax jambu, is an evergreen tree with origins in Asia. Three C-methylated chalcones, 2',4'-dihydroxy-3',5'-dimethyl-6'-methoxychalcone (81), stercurensin (82), and cardamonin (83) were isolated (Resurrección-Magno et al., 2005).

In another study, the dihydrochalcone 2',4'- dihydroxy-4,6'-dimethoxydihydrochalcone (84) was isolated from the ethyl ether extract of *Iryanthera juruensis* Warb (Myristicaceae) and it was found to be a major cytotoxic metabolite when tested against a panel of cancer cell lines (122). Panduratin A (85) is a cyclohexanyl chalcone found in *Boesenbergia rotunda* induced apoptosis on A375 cancer cells, which was mediated by prolonged ER stress at least in part via the PERK/eIF2α/ATF4/CHOP pathway revealeing that mitochondrion is the primary acting site of Panduratin A on A375 cancer cells (Lai et al., 2015). Flavokawain B (34), a kava chalcone, showed a strong *in vitro* activity against osteosarcoma cell lines. This compound inhibited cell proliferation, induced apoptosis and cell cycle arrest. Furthermore, in contrast to conventional chemotherapeutic drugs, showed less toxicity in normal bone marrow cells (Tao et al., 2013). Cardomonin (83) inhibited prostate cancer cell proliferation and decreased the expression of NFkB1. Moreover, analysis by flow cytometry showed that this compound induced DNA fragmentation, suggesting an effect on apoptosis induction in the PC-3 cell line (Pascoal et al., 2014).

Antiviral effect

Licochalcone G (84), licochalcone A (12), echinantin (86), 5-prenylbutein (87), licochalcone D (88), isoliquiritigenin (89), licoagrochalcone A (90), and kanzonol C (91) were isolated from the acetone extract of the *Glycyrrhiza inflata.* All the isolated compounds shown activity against NAs from influenza viruses. The non-prenylated chalcones echinantin and isoliquiritigenin (IC_{50} 5.80 ± 0.30 and 8.41 ± 0.39 µg ml^{-1}, respectively) exhibited higher activity than the prenylated compounds 5-prenylbutein, the C-5 hydroxy derivative of licoagrochalcone A (IC_{50} 25.87 ± 2.03 µg ml^{-1}) (Go et al., 2005). Xanthohumol (10), chalcone, isolated from *Humulus lupulus* is a selective inhibitor of HIV-1. The EC_{50}'s of xanthohumol on inhibiting HIV-1 p24 antigen and RT production were 1:28 and 1:35 µg ml^{-1}, respectively. Xanthohumol also showed activity against BVDV, HSV-2, and HSV-1, as well as additionally against cytomegalovirus (CMV) (Buckwold et al., 2004).

Licochalcones A (12) and B (92) as well as 3,3',4,4'-tetrahydroxy-2-methoxy chalcone (93) suppressed the TPA-induced HIV promoter, whereas they did not cause any apparent reduction in the Luc activity in pCMVLuc transfected cells. These chalcones had a negative effect on HIV transcription, possibly because they bind to some specific protein factors. Additionally, cardamonin exhibited an appreciable anti-HIV-I PR activity (75.1% inhibition) with an IC_{50} value of 31 µg ml^{-1} (Xu et al., 2000). Glycycoumarin, glycyrin, glycyrol and liquiritigenin isolated from *Glycyrrhiza uralensis*, as well as isoliquiritigenin, licochalcone A and glabridin, develop antivirals activity against hepatitis C virus (HCV) infection (Adianti et al., 2014).

Tyrosinase inhibitor effect

Chalcone (94), 4-hydroxychalcone (95), 4'-hydroxychalcone (96), 2'-hydroxychalcone (97), 2',4'-dihydroxychalcone (98), 2',4-dihydroxychalcone (99), 2',4',4-trihydroxychalcone (100) and 2',4',3,4-tetrahydroxychalcone (101) were tested as inhibitors of tyrosinase mono- and diphenolase activities, showing that the most important factor in their efficacy is the location of the hydroxyl groups on both aromatic rings, with a significant preference to a 4-substituted B ring, rather than a substituted A ring. Neither the number of hydroxyls nor the presence of a catechol moiety on ring B correlated with the increasing tyrosinase inhibition potency. Surprisingly, the addition of a second OH to 4-HC at position 2' (ring A) negated tyrosinase inhibition activity, as observed in 2',4-dihydroxychalcone which was practically inactive (Seo et al., 2003).

CONCLUSION

The pharmacological studies conducted on chalcones indicate the immense potential of these compounds in the treatment of conditions such as osteoporosis, cancer, influenza viruses, as inhibitor of the HIV-1, antimicrobial, tyrosinase inhibitor, plasmodial etc. Not surprisingly, chalcones also exhibits antioxidant and anti-inflammatory effects as oxidative injury underlies many of these diseases. However, the diverse pharmacological activities of the chalcones have only been assayed in *in vitro* tests using laboratory animals, and the results obtained may not necessarily be applicable situation in humans. While there are gaps in the studies conducted so far, which need to be bridged in order to exploit the full medicinal potential of chalcones, it is still very clear that there are compounds which are already widely used and also have an extraordinary potential for the future.

ABBREVIATIONS: AGEs, Advanced glycation end-prcducts; **A-549,** human non-small cell lung cancer; **AKT,** protein kinase B; **COX-2,** cycloxygenase 2; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **IFN-y;** interferon gamma; **IL-6,** interleukin 6; **IL-13,** interleukin 13; **iNOS,** NO synthetase; **HepG-2,** liver carcinoma; **HT-29,** human colon cancer; **LPS,** lipopolysaccharide; **MAPI,** multiple activation key; **NF-κB,** nuclear factor kappa-light-chain-enhancer of activated B cells; **NO,** nitrous oxide; **ORAC,** oxygen radical absorbance capacity; **P-388,** murine leukemia; **TNF-α,** tumor necrosis factor-α; **VEGF,** vascular endothelial growth factor.

Conflict of interest

Authors declare that there are no conflicts of interest.

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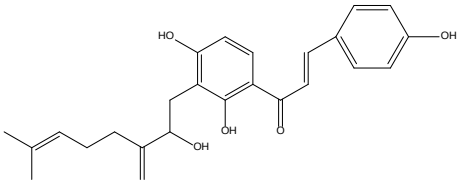
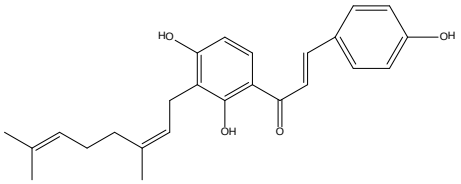
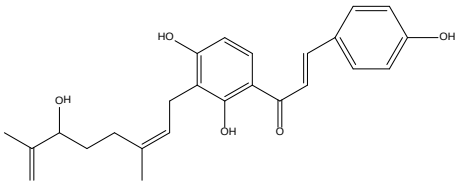
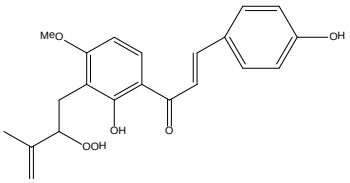
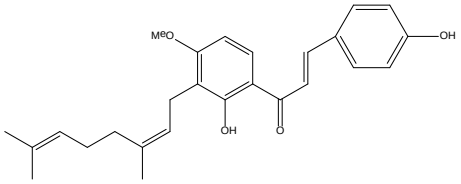
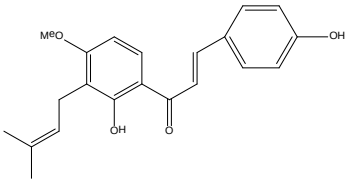
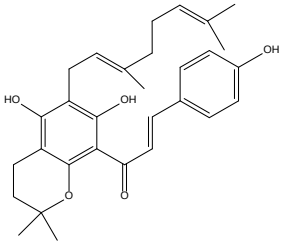
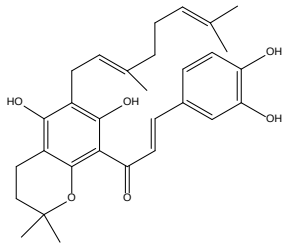
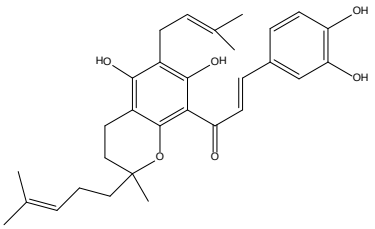
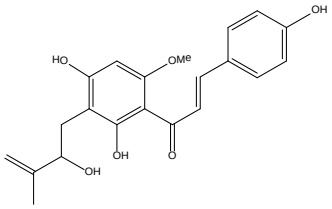
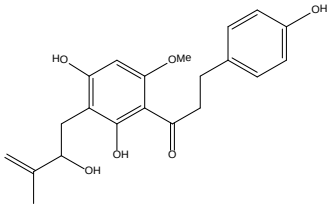
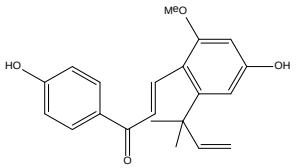
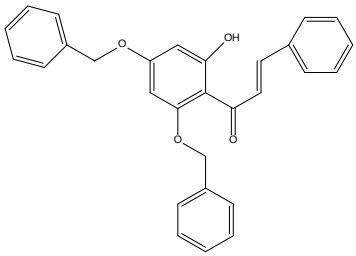
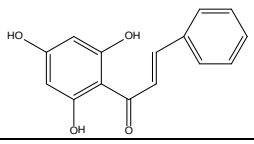
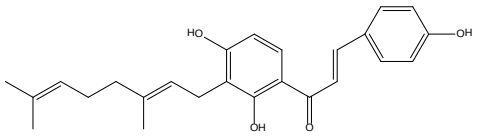
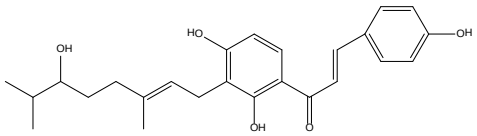
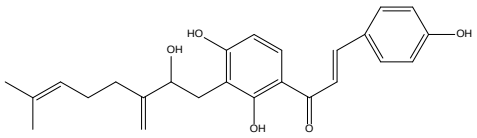
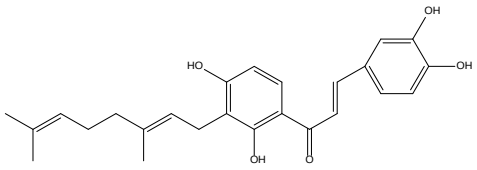
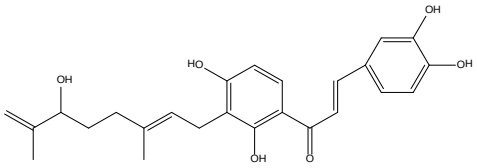
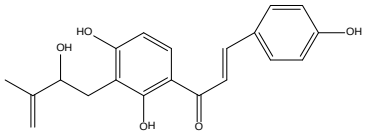
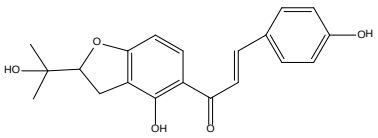
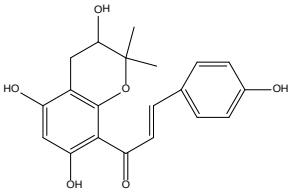
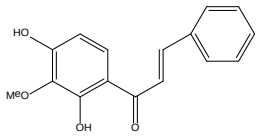
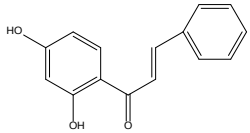
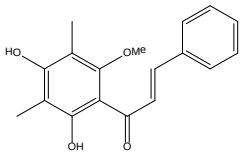
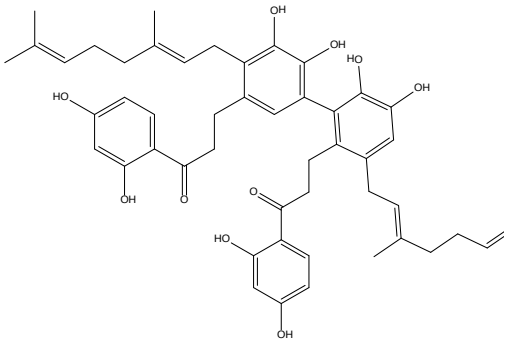
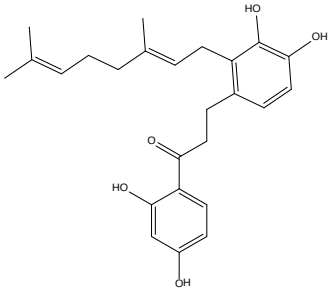
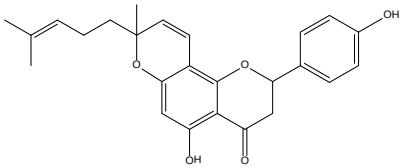
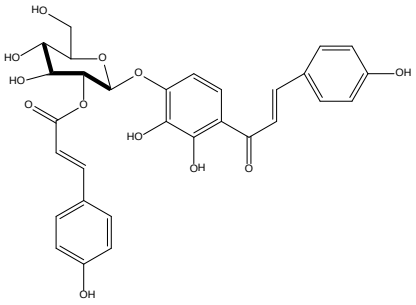
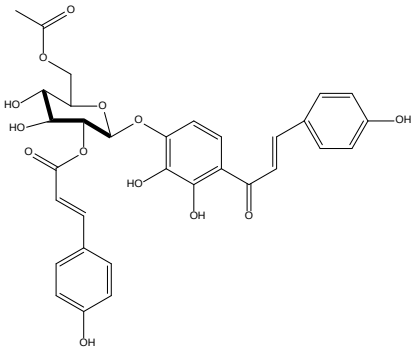
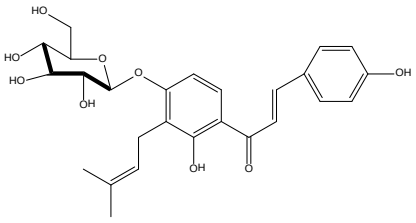
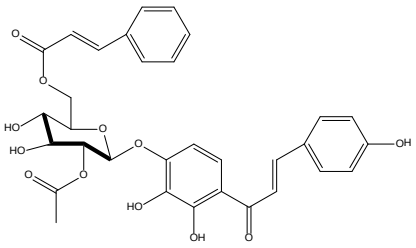
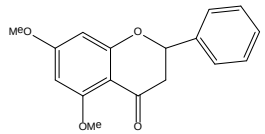
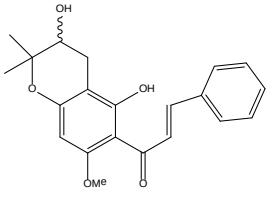
Appendix 1. Compound names and their structures.

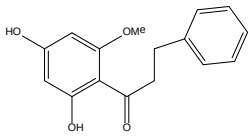
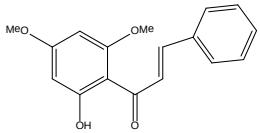
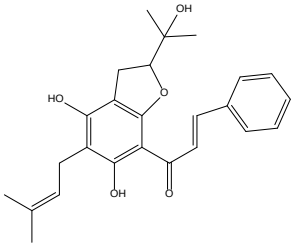
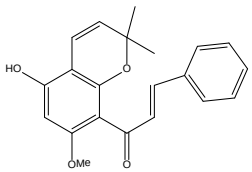
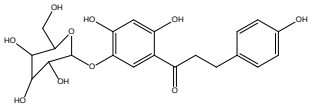
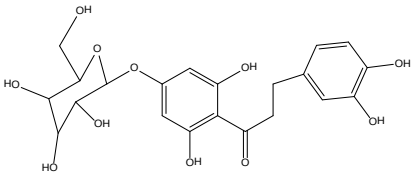
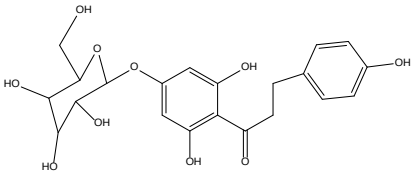
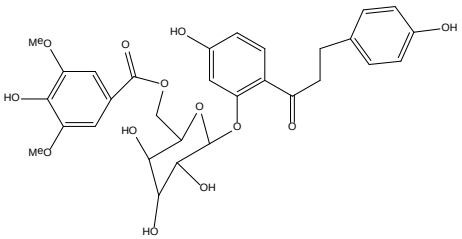
S/NO	Name of compound	Molecular structure
1	2',4',4-trihydroxy-3'-[2-hydroxy-7-methyl-3-methylene-6-octaenyl]chalcone]	
2	2',4',4-trihydroxy-3'-geranylchalcone	
3	2',4',4-trihydroxy-3'-[6-hydroxy-3,7-dimethyl-2,7-octadienyl]chalcone	
4	2',4-dihydroxy-4'-methoxy-3'-[2-hydroperoxy-3-methyl-3-butenyl] chalcone	
5	2',4-dihydroxy-4'-methoxy-3'-geranylchalcone	
6	2',4-dihydroxy-4'-methoxy-3'-[3-methyl-3-butenyl]chalcone	
7	Mallotophilippens C	

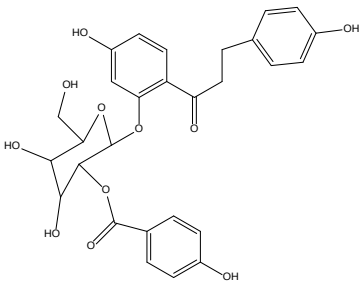
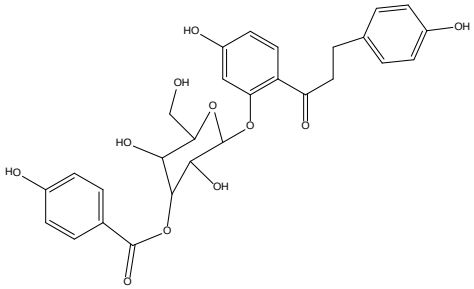
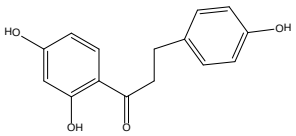
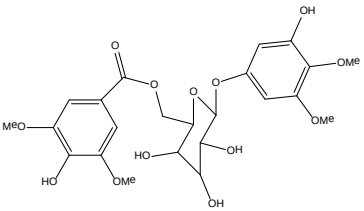
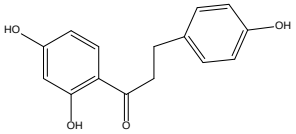
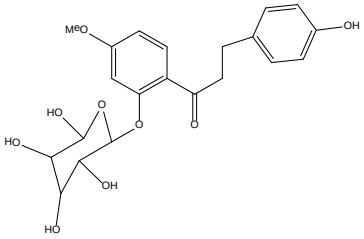
8	Mallotophilippens D	
9	Mallotophilippens E	
10	Xanthohumol	
11	Dihydroxanthohumol	
12	Licochalcone A	
13	2-Hydroxy-4',6'-dibenzyloxychalcone	
14	benzyloxy group	

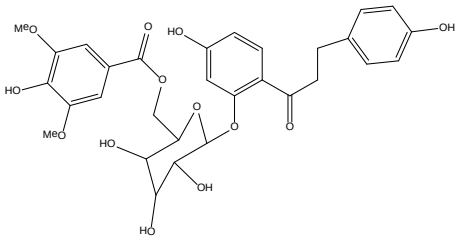
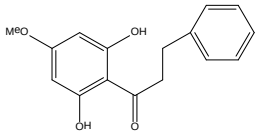
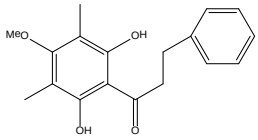
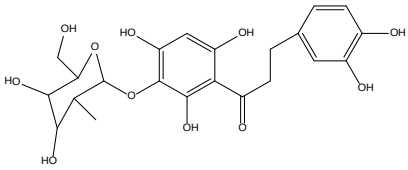
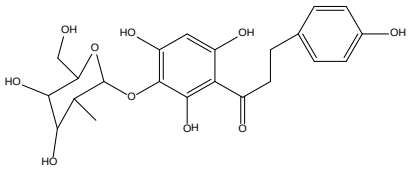
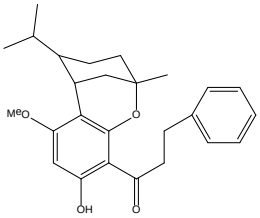
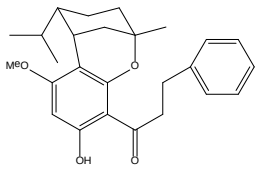
15	2',4',4-trihydroxy-3'-geranylchalcone	
16	2',4',4-trihydroxy-3'-[6-hydroxy-3,7-dimethyl-'2(E),7-oetadienyl] chalcone	
17	2',4',4-trihydroxy-3'-['2-hydroxy-7-methyl-3-methylene-6-oetaenyl)chalcone	
18	2',3,4,4'-tetrahi-droxy-3'-geranylchalcone	
19	2'3,4,4'-tetrahydroxy-3'-[6-hydroxy-3,7-dimethyl-2(E),7-octadienyl) chalcone	
20	2',4',4,2"-tetrahydroxy-3'-[3"-ethyl]chalcone	
21	Isovachalcone	
22	Bakuchalcone	

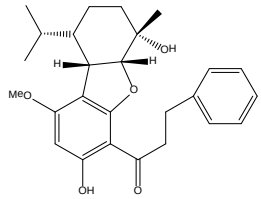
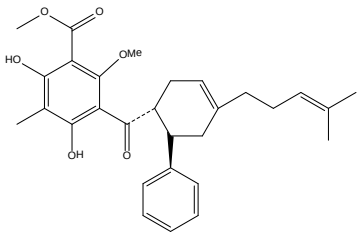
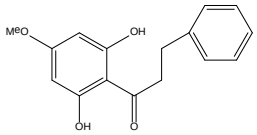
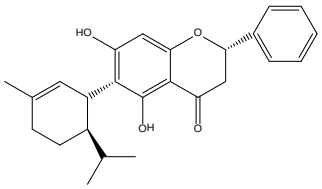
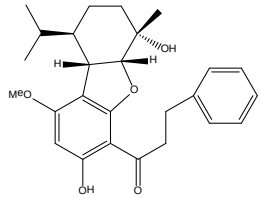
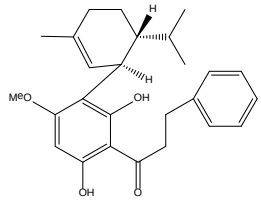
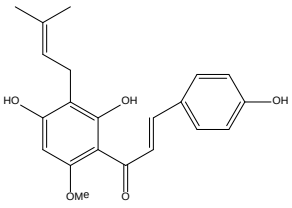
23	2',4'-dihydroxy-3'-methoxychalcone	
24	2',4'-dihydroxy chalcone	
25	2',4'-Dihidroxy-3',5'-dimethyl-6' methoxychalcone	
26	Dimeric dihydrochalcone cycloaltilisin 6	
27	AC-5-1	
28	Cycloaltilisin 7	

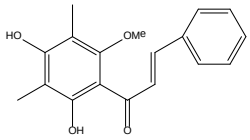
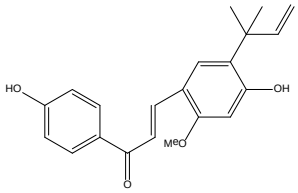
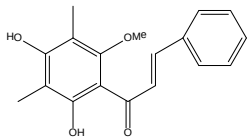
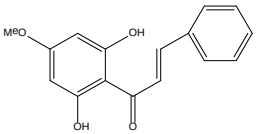
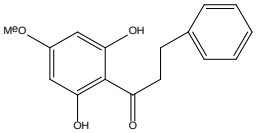
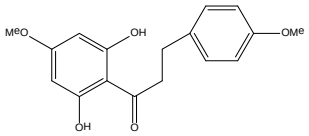
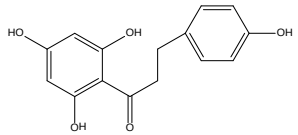
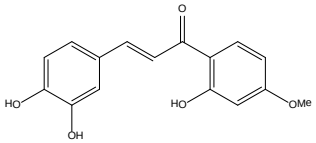
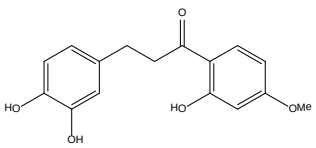
29	4'-O-β-D-(2''-p-coumaroyl)glucopyranosyl-4,2',3'-trihydroxychalcone	
30	4'-O-β-D-(2''-p-coumaroyl)-6''-acetylglucopyranosyl-4,2',3'-trihydroxychalcone	
31	3'-(3-methyl-2-butenyl)-4'O-β-D-(glucopyranosyl-4,2'-dihydroxy chalcone	
32	4'-O-β-D-(2''-acetyl-6''-cinnamoyl)glucopyranosyl-4,2',3'-trihydroxychalcone	
33	Uvangoletin	
34	Flavokawin B	

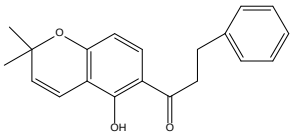
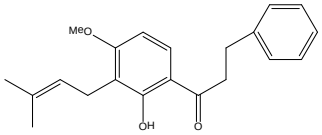
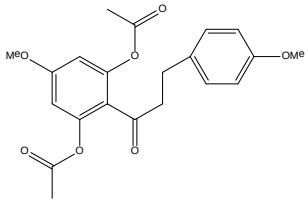
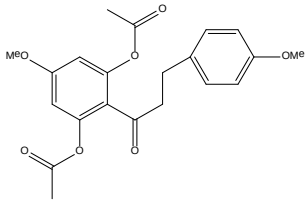
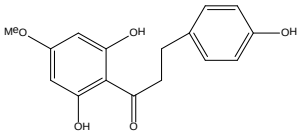
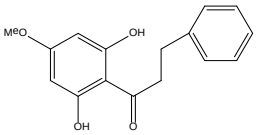
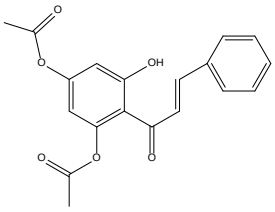
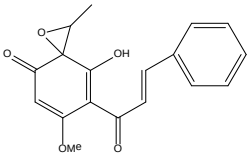
35	5,7-dimethylpinocembrin	
36	2'-methoxyhelikrausichalcone	
37	Cedrediprenone	
38	cedreprenone	
39	Phloridzin	
40	Seboldin	
41	Trilobatin	
42	Davidigenin-2'-O-(6''-O-4'''-Hydroxybenzoyl)-¡l-glucoside	

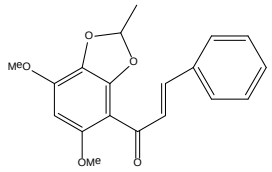
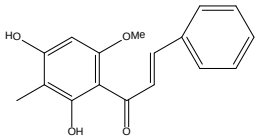
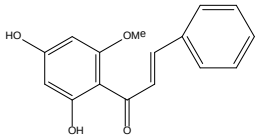
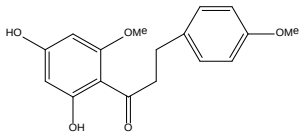
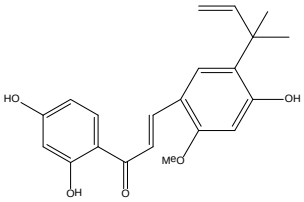
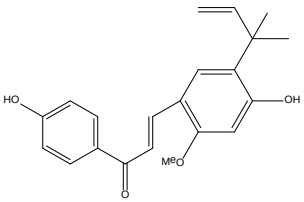
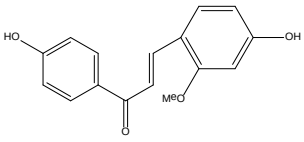
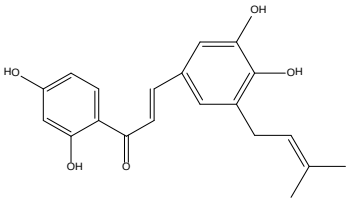
43	Davidigenin-2'-O-(2"-O-4'"-hydroxybenzoyL)-β-glucoside	
44	Davidigen-2'-O-(3"-O-4'"-hydroxybenzoyl)-β-glucoside	
45	Davidigenin-2'-O-(6"-O-syringoyl)-β-glucopyranoside	
46	1-O-3,4-dimethoxy-5-hydroxyphenyl-6-O-(3,5-di-O-methylgalloyl)-β-glucopy ranoside	
47	Davidioside	
48	4'-O-methyldavidioside	

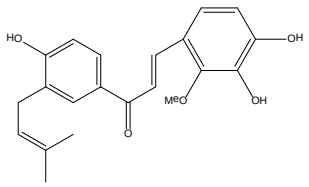
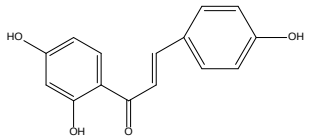
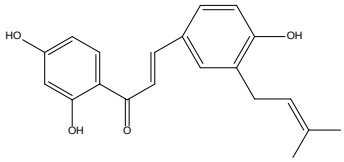
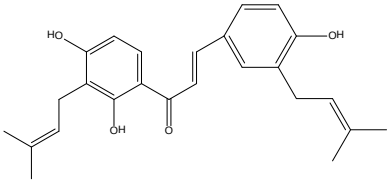
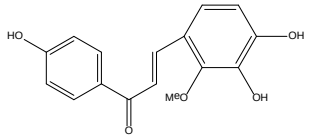
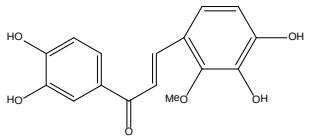
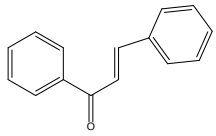
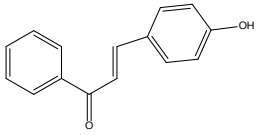
49	Davidigenin	
50	Phloretin 4'-O-methyl ether (2',6'-dihydroxy-4'-methoxydihydrochalcone)	
51	Myrigalone G	
52	Aspalathin	
53	Nothofagin	
54	Dihydrochalcones hostmanin A	
55	Hostmanin B	

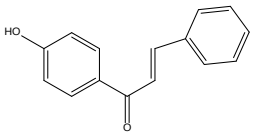
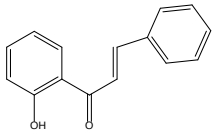
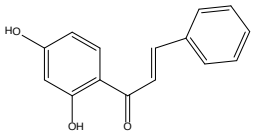
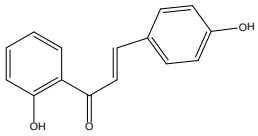
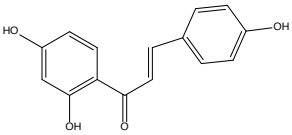
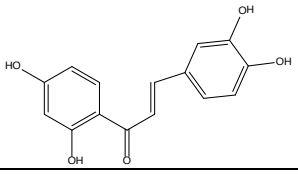
56	Hostmanin C	
57	Hostmanin D	
58	2',6'-dihydroxy-4'-methoxydihydrochalcone	
59	linderatone	
60	Adunctin E	
61	(-)-methyllinderatin	
62	Xanthohumol	

63	Isoxanthohumol	
64	licochalcone E	
65	2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone	
66	2',6'-dihydroxy-4'-methoxychalcone	
67	2',6'-dihydroxy-4'-methoxydihydro chalcone	
68	2' 6' -dihydroxy-4,4' –dimethoxy dihydrochalcone	
69	Phloretin	
70	Calythropsin	
71	Dihydrocalythropsin	

72	Chalcone derricin	
73	lonchocarpin	
74	4,10',6'-diacetoxy-4,4'-dimethoxydihydrochalcone	
75	Dihydrochalcones 10',6'-diacetoxy-4,4'-dimethoxydihydrochalcone	
76	4,2',6'-trihydroxy-4'-methoxy dihydrochalcone	
77	2',6'-dihydroxy-4'-methoxydihydrochalcone	
78	2',4'-diacetoxy chalcone	
79	Litseaone A	

80	Litseaone B	
81	Stercurensin	
82	cardamonin	
83	2',4'- dihydroxy-4,6'-dimethoxydihydrochalcone	
84	Licochalcone G	
85	licochalcone A	
86	Echinantin	
87	5-prenylbutein	

88	licochalcone D	
89	Isoliquiritigenin	
90	licoagrochalcone A	
91	kanzonol C	
92	Licochalcones A	
93	Licochalcones B	
94	Chalcone	
95	4-Hydroxychalcone	

96	4'-Hydroxychalcone	
97	2'-Hydroxychalcone	
98	2',4'-Dihydroxychalcone	
99	2',4-Dihydroxychalcone	
100	2',4',4-Trihydroxychalcone	
101	2',4',3,4-Tetrahydroxychalcone	

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Clinical duration of rocuronium becomes shorter at night: A chronopharmacological study

Menekse Ozcelik*, Ali Abbas Yılmaz, Sirali Oba, Sanem Turhan and Oya Ozatamer

Department of Anesthesiology and ICM, Ankara University School of Medicine, Ankara, Turkey

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The effects of circadian rhythms on the duration of neuromuscular blocking agents are under investigated. Therefore, the aim of this prospective, observational study was to investigate whether the dosing-time of rocuronium would cause significant differences in the neuromuscular block duration during the four different period of a day in a diurnal based study design. Forty patients were allocated into one of the following four groups according to the surgery timing during a day. Patients undergoing surgery between 07:00 am and 01:00 pm were included in Group M (Group morning, n = 10), between 01:00 pm and 07:00 pm in Group AN (Group afternoon, n = 10), between 07:00 pm and 01:00 am in Group E (Group evening, n = 10), and between 01:00 am and 07:00 am in Group N (Group night, n = 10). All patients received rocuronium in a dose of 0.6 mg/kg. The time to T1 10% and clinical duration were significantly shorter in Group N [24.4 ± 8.0, 28.5 ± 9.0 min, mean ± standard deviation (SD)] when compared with Group AN (32.1 ± 8.6, 36.7 ± 9.1 min, mean ± SD) and Group E (32.5 ± 9.9, 37.0 ± 11.4 min, mean ± SD) ($p < 0.05$). T1 10% time and clinical duration of rocuronium were significantly shorter when used at nighttime compared to afternoon and evening part of the day. The increased hepatic blood flow and metabolism of rocuronium may be the responsible factors related to this shortening at night.

Key words: Rocuronium, chronobiology, circadian rhythm, neuromuscular non-depolarizing agents, anesthesia, intravenous.

INTRODUCTION

The biologic rhythms are physiological circumstances that provide the adaptation of an organism to the external environment in the absence of environmental time determinants (Chassard and Bruguerolle, 2004). The chronobiology investigates these rhythms and their mechanisms. The most well recognized biologic rhythm is the circadian rhythm that is characterized by a biologic cycle of approximately 24 h (Chassard et al., 2005). There are a great number of studies related to drug and biologic rhythm interactions in clinical medicine (Chassard et al., 2007). However, probably due to two main reasons mentioned, the influence of chronobio-logy on the clinical practice of anesthesiology remains to be determined. The first reason is the combined use of several drugs in the course of anesthesia and therefore, the fear of underestimating or overestimating the interactions between biologic rhythms and anesthetic drugs. In addition to this, because of the concept considering that the circadian rhythms have been overcome with the homeostasis, most of the anesthe-

*Corresponding author. E-mail: ozcelikmenekse@yahoo.com

siologists have not been interested in chronobiology in their clinical practice (Cheeseman et al., 2007).In the field of anesthesia, the drug dose-response relationship represents an extremely important issue. A large number of clinical and laboratory studies have been designed to establish the factors affecting this dose-response relationship from past to present. Rocuronium is the most rapid onset and the most commonly used non-depolarizing muscle blocker currently available. Factors such as age (Baykara et al., 2002), weight (Leykin et al., 2004), temperature of the patient (Beaufort et al., 1995), as well as the anesthetic agent used for anesthesia maintenance (Maidatsi et al., 2004) affect the duration of action of rocuronium. Whether the chronobiology is another variable affecting the duration of action of rocuronium is controversial. Only one study was under-taken to evaluate the influence of time of day on the clinical duration of rocuronium in the available literature (Cheeseman et al., 2007). However, its design did not include all the time course of a day. The night part of the day denoting the resting period of human individuals had not been considered in the study design. Therefore, we have undertaken a study to in-vestigate whether the dosing-time of rocuronium would cause significant differences in the neuromuscular block duration during the whole day in a diurnal based study design.

MATERIALS AND METHODS

After local ethics committee approval, the patients undergoing elective or emergency abdominal surgery with an expected duration less than four hours between January, 2006 and July, 2006 were enrolled into the study which was conducted according to the Declaration of Helsinki and Good clinical research practice (GCRP) guidelines for pharmacodynamic studies of neuromuscular blocking agents (Fuchs-Buder et al., 2007). The inclusion criteria were 18 to 59 years of age, American Society of Anesthesiologist (ASA) status 1 or 2, actual body weight in the range of 20% of the ideal body weight, normal renal and hepatic function, no history of cardiac and neuromuscular diseases. The exclusion criteria were expected difficult airway, known allergy to one of the study drugs, history of drug usage likely to affect the neuromuscular function. The information about the general anesthesia, monitorization techniques, and the study protocol were given to all subjects and then the informed consent were requested from the patients undergoing elective and emergency surgery the day before and one hour before surgery, respectively.

The patients were allocated into one of the following four groups according to the surgery timing during a day. The patients undergoing elective surgeries between 07:00 am and 01:00 pm were included in Group M (Group morning, n = 10) and between 01:00 pm and 07:00 pm were included in Group AN (Group afternoon, n = 10). The patients undergoing emergent surgeries between 07:00 pm and 01:00 am were included in Group E (Group evening, n = 10) and between 01:00 am and 07:00 am were included in Group N (Group night, n = 10).

After arrival to the operating room, a 20-gauge intravenous (iv) catheter was inserted on the dorsum of the hand or antecubital fossa. Intravenous midazolam (0.4 mg/kg) premedication was given to all patients following routine ASA monitorization including electrocardiogram with continuous ST-T analysis, non-invasive blood pressure, body temperature, and pulse oximetry (Viridia CMS M1166A, Hewlett Packard, Germany). In addition, Bispectral Index (A-2000 XP, Aspect Medical Systems, Inc, USA), weighted sum of several electroencephalographic sub parameters was installed to monitor depth of anesthesia. Neuromuscular function was assessed quantitatively with an acceleromyograph (TOF-Watch ® SX, Organon, Dublin, Ireland) connected to a laptop computer installed with appropriate software via an interface. All patients were warmed with a blanket laid under the patients.

Anesthetic management

Total intravenous anesthesia (TIVA) with target controlled infusion (TCI) technique using propofol and remifentanil was utilized in anesthesia induction and maintenance of all patients. The demographic data such as age, actual body weight, height and sex were set on the TCI infusion pump (Orchestra ® Base Primea, Fresenius Kabi, Germany), which enables the drug concentration in the plasma (Cp) and at the effect site (Ce) such as brain, to be continuously controlled. The pharmacokinetic model described by Schnider et al. (1998) uses age as a co-variate to improve the accuracy of the model. Because this model has a smaller-volume central compartment and equilibrates more quickly with the effect site, our patients received TCI propofol driven by the Schnider model with effect-site control. Meanwhile, the infusion rate of TCI remifentanil was controlled by Minto's pharmacokinetic model incorporated into software that was previously used specifically for remifentanil (Minto et al., 1997). Syringes containing 1% propofol (10 mg/ml) and remifentanil (50 µg/ml) were simultaneously loaded onto the device and connected to the patient's intravenous catheter using a three-way stopcock.

The anesthesia induction was provided by remifentanil infusion with an effect-site concentration at 7.5 ng/ml following propofol infusion with an effect-site concentration at 2.5 µg/ml. Once the BIS value was lower than 80, the mask ventilation with 100% oxygen was started.

After reaching the target concentrations of anesthetic drugs and before administration of rocuronium, a 50 Hz tetanic stimulation for 5 s was administered followed after 1 min by immobilization of the patient’s fingers. Then the ulnar nerve at the wrist was stimulated in a train-of-four (TOF) mode every 15 s for 3 min. When the calibration process by CAL 2 mode was completed and then stable baseline of the response to TOF was reached, 0.6 mg/kg rocuronium iv was administered in 5 s. The time between rocuronium injection and 95% depression of first twitch (T1) response was noted as “onset time”. As soon as onset time was defined, all patients were intubated. All patients were mechanically ventilated with a tidal volume of 7 ml/kg and frequency of 12/min. In order to achieve the goal of maintenance of desired end-tidal carbon dioxide level (between 35 and 45 mmHg), firstly tidal volume was increased by 20% and secondly frequency of mandatory respirations was increased to 16/min.

During the maintenance period of anesthesia the remifentanil effect-site concentration was adapted by step of 2.5 ng/ml according to the hemodynamic parameters in every 5 min. In case of bradycardia and hypotension, atropine 0.5 mg and ephedrine 10 mg iv were injected, respectively. Meanwhile, propofol effect-site concentration was adjusted to intend intraoperative BIS value of 40 to 60. If BIS value was lower than 40, propofol effect-site concentration had been lowered by 0.5 µg/ml. On the contrary, propofol effect-site concentration had been increased by 0.5 µg/ml if BIS value was higher than 60. The anesthetic management was schematized in Figure 1. During the anesthesia maintenance, the time elapsing between TOF count 0 and first twitch reappearance was noted as “deep neuromuscular block duration”. Moreover, the time elapsing between administration of rocuronium and recovery to 10 and 25% of first twitch height (T1 10%, T1 25%) were recorded as “the time to T1 10% and clinical duration”, respectively. When

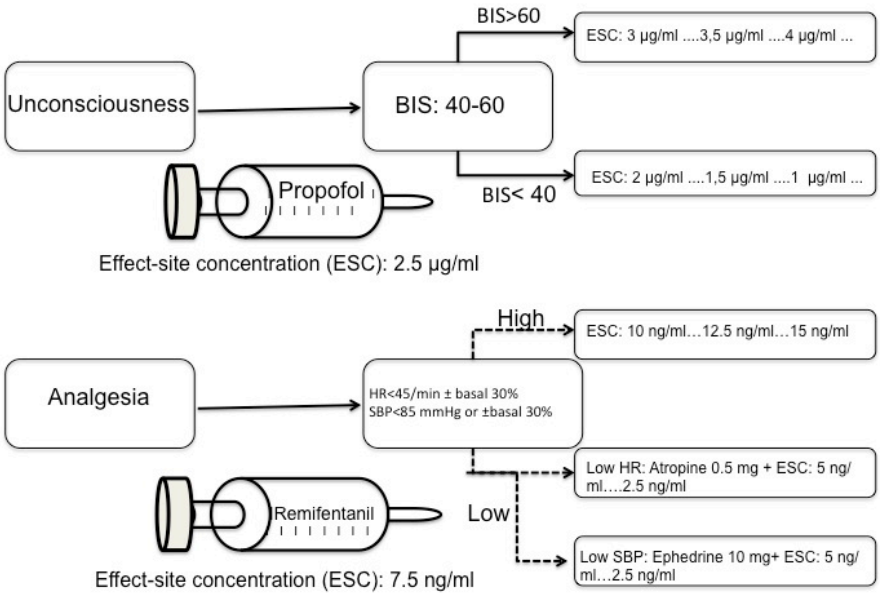

Figure 1. Anesthetic management algorithm.

Table 1. Demographic data (n=patient number).

Group	Group M (n=10)	Group AN (n=10)	Group E (n=10)	Group N (n=10)	p
Age (year)	42±8	38±12	32±13	30±11	NS
Weight (kg)	69±10	66±9	69±10	64±8	NS
Height (cm)	173±8	168±6	179±9	169±6	NS
Actual body weight (kg)	70±9	65±7	64±10	63±7	NS
ASA Classification (I/II) (n)	9/1	9/1	8/2	8/2	NS

All values are shown as mean±SD, NS, statistically not significant. Group Morning (Group M): patients undergoing surgery between 07:00 am and 01:00 pm; Group Afternoon (Group AN): patients undergoing surgery between 01:00 pm and 07:00 pm; Group Evening (Grup E): patients undergoing surgery between 07:00 pm and 01.00 am; Group Night (Group N): patients undergoing surgery between 01.00 am and 07.00 am.

"clinical duration" was achieved, if necessary, additional rocuronium in a dose of 0.15 mg/kg iv was administered and the time between the injection of rocuronium and the second recovery to 25% of first twitch height was recorded as "second clinical duration". If an additional rocuronium was not necessary due to the end of surgery, 30 µg/kg neostigmine and 10 µg/kg atropine were administered for the recovery of neuromuscular blockade. Along the recovery period, the time from 25 to 75% of first twitch height recovery was recorded as "recovery index" and the time between T1 25% and TOF ratio of 90% was also recorded as "complete recovery interval". If the TOF ratio was above 80 and the BIS level was above 70, the patients were then extubated.

All patients were followed in terms of their peripheral temperatures and BIS levels and all these parameters were recorded. In addition to the duration of anesthesia and surgery, the total and hourly propofol, remifentanil and rocuronium consumptions were also noted.

Statistical analysis

The statistical analyses were made by using SPSS 12.0 program (Statistical Program for Social Sciences, Chicago, IL, USA) for Windows with a personal computer. The numeric data were analyzed by the t-test and presented as mean ± SD. Chi-square test was used for the evaluation of categorical variables; correlation analysis was used to determine the relationship between the variables. In comparison of 3 or more groups, Kruskall-Wallis, a non-parametric test was used, in comparison of 2 groups Mann-Whitney U test was used. No power analysis was done. For statistical comparisons, $p < 0.05$ were considered as significant.

RESULTS

Forty patients were enrolled into the study. None of them were excluded due to any reason. No significant difference was found between groups in terms of patient demographics provided in Table 1. Intraoperative hemodynamic parameters were similar between groups and no hemodynamic adverse effect was found. The onset time of rocuronium did not differ between 4 groups with a mean (SD) of 2.65 (1.0) min. Similarly, there was

Table 2. Neuromuscular blockade parameters (n=patient number).

Neuromuscular parameters (min)	Group M (n=10)	Group AN (n=10)	Group E (n=10)	Group N (n=10)
Onset time	2.8 ± 1.1	2.4 ± 0.9	2.4 ± 1.0	3.0 ± 1.1
Deep neuromuscular blockade duration	19.0 ± 4.0	25.8 ± 9.3	24.6 ± 7.4	19.2 ± 8.5
The time to T1 10%	27.2 ± 6.6	32.1 ± 8.6	32.5 ± 9.9	24.4 ± 8.0*
Clinical duration	31.0 ± 7.7	36.7 ± 9.1	37.0 ± 11.4	28.5 ± 9.0*
Second clinical duration	21.4 ± 9.0	17.5 ± 4.4	16.8 ± 7.3	19.0 ± 5.0
Recovery index	5.5 ± 2.8	5.6 ± 2.8	7.6 ± 4.1	5.5 ± 3.2
Complete recovery interval	9.6 ± 2.8	10.0 ± 3.8	12.6 ± 4.9	10.8 ± 4.0

All values are shown as mean ± SD. Group Morning (Group M): patients undergoing surgery between 07:00 am and 01:00 pm; Group Afternoon (Group AN): patients undergoing surgery between 01:00 pm and 07:00 pm; Group Evening (Grup E): patients undergoing surgery between 07:00 pm and 01.00 am; Group Night (Group N): patients undergoing surgery between 01.00 am and 07.00 am. * $p < 0.05$ compared with Group AN and E.

Table 3. Intraoperative drug consumptions (n=patient number).

Parameter (mg)	Group M (n=10)	Group AN (n=10)	Group E (n=10)	Group N (n=10)
Total rocuronium consumption	84 ± 33	66 ± 22	73 ± 32	68 ± 25
Hourly rocuronium consumption	45 ± 12	41 ± 11	43 ± 14	45 ± 9
Total propofol consumption	708 ± 263	686 ± 429	671 ± 264	498 ± 216
Hourly propofol consumption	375 ± 78	388 ± 81	391 ± 101	329 ± 57
Total remifentanil consumption	2390 ± 957	2763 ± 1703	2782 ± 1268	2228 ± 775
Hourly remifentanil consumption	1265 ± 359	1521 ± 409	1592 ± 416	1526 ± 424

All values are shown as mean±SD. Group Morning (Group M): patients undergoing surgery between 07:00 am and 01:00 pm; Group Afternoon (Group AN): patients undergoing surgery between 01:00 pm and 07:00 pm; Group Evening (Grup E): patients undergoing surgery between 07:00 pm and 01.00 am; Group Night (Group N): patients undergoing surgery between 01.00 am and 07.00 am.*$p < 0.05$ compared with Group AN and E.

no difference related to the deep neuromuscular blockade duration of rocuronium among groups (Table 2). In contrast, there was a clear relationship between the administration time of rocuronium and the estimates of time to T1 10% and clinical duration of rocuronium. The time to T1 10% was significantly shorter in Group N (24.4 ± 8.0 min, mean ± SD) when compared with Group AN (32.1 ± 8.6 min, mean ± SD) and Group E (32.5 ± 9.9 min, mean ± SD) ($p < 0.05$). The shortening in T1 10% observed in Group N was 24 and 24.9% compared with Group AN and Group E, respectively.

There was no difference between Group M, Group AN and Group E in terms of time to T1 10%. The time to T1 10% did not differ between Group M and Group N. When the clinical duration of rocuronium was compared between 4 groups, it was found that clinical duration was shorter in Group N (28.5 ± 9.0 min, mean ± SD) when compared with Group AN (36.7 ± 9.1 min, mean ± SD) and Group E (37.0 ± 11.4 min, mean ± SD) ($p < 0.05$). The longest and the shortest clinical durations were noted in Group AN with a 61.4 min and in Group N with a 20.5 min, respectively. The shortening in clinical duration observed in Group N was 22.3 and 22.9% compared with Group AN and Group E, respectively. There was no difference between Group M, Group AN and Group E in terms of clinical duration. There was also no difference in clinical duration between Group M and Group N. These findings were listed in Table 2.

Although the second clinical duration was shorter in Group N compared with Group M, this was not statistically significant. The recovery index and complete recovery interval did not differ at any time throughout the day between groups (Table 2). The BIS levels and peripheral temperature measurements were similar between the groups (Figures 2 and 3). There was no statistically significant difference between four groups in terms of intraoperative total and hourly propofol, remifentanil and rocuronium consumptions (Table 3).

DISCUSSION

This is the first human study investigating the effects of the rocuronium administration time on the neuromuscular

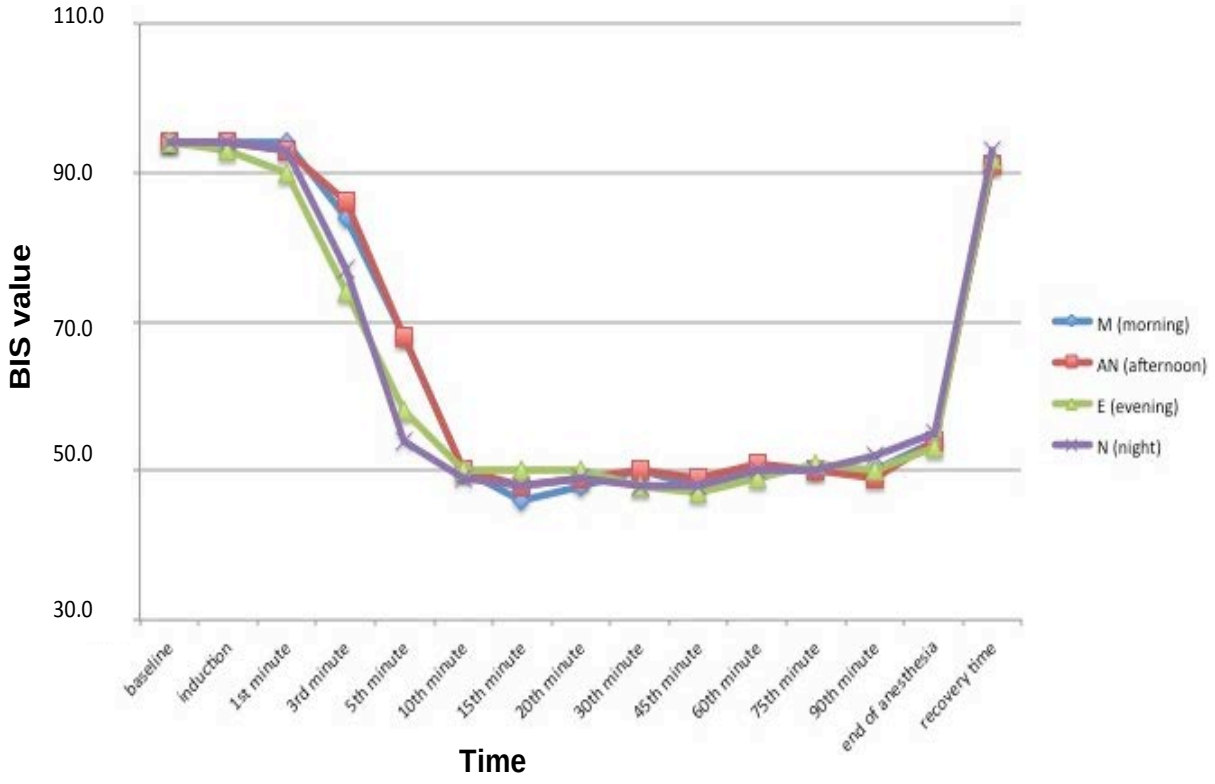

Figure 2. Intraoperative BIS values.

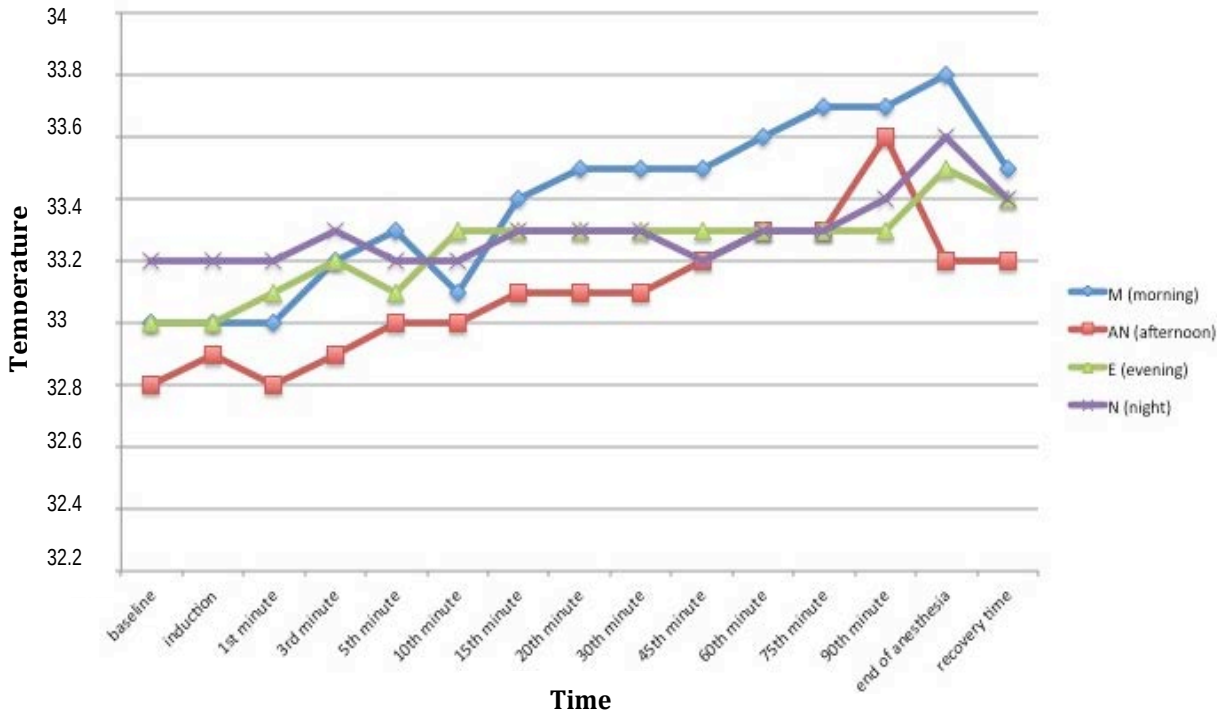

Figure 3. Intraoperative peripheral temperature measurements.

blockade characteristics during the 24 h. The primary finding of our study was that, T1 10% time and clinical duration of rocuronium were significantly shorter at night compared to afternoon and evening part of the day under total intravenous anesthesia with propofol and remifentanil. The onset time of rocuronium is approximately 1.5 to 1.7 min considering its pharmacodynamic properties (Naguib and Lien, 2009). However, the onset time was found longer in our study (mean, 2.65 min).

Na and coworkers demonstrated that the prior administration of remifentanil during TCI propofol and remifentanil anesthesia prolonged the onset time of rocuronium due to decreased cardiac output in their well-designed study (Na et al., 2012). This may also explain our prolonged onset time due to our comparable sequence of anesthesia induction. Nevertheless, there was no difference in terms of onset time or deep neuromuscular blockade of rocuronium between the 4 study groups.

There was a clear relationship between the dosing time of rocuronium and the time to T1 10%, and clinical duration. The time to T1 10% and clinical duration were shorter at night compared with afternoon and evening application. These findings were not consistent with the study data previously published.

Cheeseman et al. (2007) demonstrated that the minimum duration of action of rocuronium dosing of 0.6 mg/kg was between 14:00 and 17:00 corresponding to the timing of our Group AN. There was an important difference in study design between these two studies. Unlike from our study, there was no patient enrollment in the night period of a day due to some anticipated difficulties. Therefore, the authors could not conclude on the clinical duration of rocuronium during the resting period of participants.

The clinical duration of a neuromuscular blocker indicates that the duration of neuromuscular blocker action becomes terminated, as it has been mostly metabolized in human body. In practice, if the clinical duration of a neuromuscular blocker has been achieved, anesthesiologist should give either an additional neuromuscular blocker or a reversal agent of neuromuscular blocker according to the circumstances (that is, ongoing or ending surgery). Therefore, quantifying these parameters is very important to decide what should be done in the course of a surgical anesthesia.

The mechanism underlying this chrono-pharmacological aspect of rocuronium may be attributed to its metabolism pathway. A large portion of circulating rocuronium is taken by the liver, metabolized and then excreted into the bile. Lemmer and Nold (1991) found significant circadian differences in estimated hepatic blood flow (EHBF) measured by injection of indocyanine green during the different part of the day in healty male volunteers. In this study, the peak EHBF was reached at 04:00 am. They also showed no difference in the distribution volume. Likewise, in another study investigating the oxaliplatin pharmacokinetics and chronopharmacological aspects, the hepatic blood flow was found maximum between 02:00 am and 08:00 am, and minimum at 02:00 pm within a day (Levi et al., 2000). Therefore, we may speculate that the clinical duration of rocuronium appears to be shorter with bedtime compared with AN or E administration.

There was an absence of statistical significance in terms of second clinical duration between four groups. The additional rocuronium dosage was 0.15 mg/kg. Therefore, this finding can be explained by application error in smaller drug doses rather than the hepatic blood flow differences. There were no difference regarding recovery index and complete recovery interval between groups. There are a number of limitations in this study. Probably, the most important one is the formation of four different groups instead of two study groups. This would be more appropriate for investigations on circadian rhythms. The number of patients were not determined using a power analysis statistically. In addition to this, this study was not sufficient to establish the real mechanism related to diurnal variation of clinical duration of rocuronium.

Future clinical trials assessing the blood rocuronium levels or rocuronium and receptor interactions are needed to clarify this issue. Additionally, the effect of the diurnal changes associated with elective or emergency was another potential confounding factor in the study design. This effect was underestimated in this study.

Conclusion

This study demonstrated that the clinical duration of rocuronium became shorter at night compared with the noon and evening part of the day under propofol and remifentanil anesthesia. Although the effect of this shortening on clinical practice looks like limited, it could be more significant as enough data about the diurnal changes related with rocuronium are provided in the future.

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Conflict of interest

There is no conflict of interest as regard this study.

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Effect of grapefruit juice and sibutramine on body weight loss in obese rats

Hadir Farouk[1*], Sawsan S. Mahmoud[1], Bahia A. El-Sayeh[2], and Ola A. Sharaf[1]

[1]Pharmacology Department, National Research Center, Egypt.
[2]Pharmacology Department, Faculty of Pharmacy, Cairo University, Egypt.

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Grapefruit (Citrus Paradise, family Rutaceae) is a citrus fruit that is low in calories and rich in dietary fibers. Sibutramine (Sibutramine hydrochloride monohydrate) is an anti-obesity drug that enhances satiety. The effect of grapefruit juice and sibutramine on body weight and neurotransmitters controlling appetite was investigated in obese rats. Rats were assigned to two dietary groups for 3 weeks; control group (n=6) was fed commercial standard pellets diet and obese group (n=24) was fed cafeteria diet (hypercaloric diet consisting of highly palatable food). The effect of sibutramine and grapefruit juice was studied on obese rats. Statistical difference and interactions were evaluated through one-way analysis of variance test (one-way ANOVA) followed by Dunnett's test was used for means of different groups. For all statistical tests done, a 0.05 level of probability was used as the criterion for significance. Grapefruit juice produced its weight reduction effect after 1 week of administration and lasted till the end of the experiment and did not affect brain neurotransmitters. Sibutramine produced its weight reduction effect after 1 week of administration and lasted for only 2 weeks and produced an increase in brain noradrenaline while grapefruit juice produced its effect from the first week till the end of the study. It can be concluded that grapefruit juice is better than sibutramine since its effect lasted till the end of the experiment and also did not affect brain noradrenaline.

Key words: Obesity, sibutramine, grapefruit juice, cafeteria diet.

INTRODUCTION

Obesity represents one of the most serious global health issues that have increased to the extent that it could be considered pandemic (Abolfotouh et al., 2008).

Obesity has reached epidemic proportions globally (WHO, 2003). A recent report from the World Health Organization estimated that approximately 500 million individuals are obese while 1.5 billion are overweight worldwide (WHO, 2011). Obesity occurs through a longstanding imbalance between energy intake and energy expenditure, influenced by a complex biologic system that regulates appetite (Wilding, 2011).

During the past decade, overweight and obesity joined underweight, malnutrition, and infectious diseases as major health problems threatening the developing world (Hossain et al., 2007). At least 2.6 million people each year die as a result of being overweight or obese

*Corresponding author. E-mail: hadirfarouk@hotmail.com.

(WHO, 2010).

It seems clear that an energy-dense, high fat diet, palatable foods with high caloric density and increasingly sedentary lifestyles are particularly conducive to the development of obesity (Pi-Sunyer, 2002). Obesity is a risk factor for many chronic conditions including diabetes and cardiovascular diseases (Wang et al., 2014).

Serotonin, particularly via the $5HT_{2c}$ receptor, has also been implicated in satiety signaling. The hypothalamus is densely innervated by serotonin-containing fibres originnating in the mid-brain raphe nucleus. Serotonin is the target of a number of obesity drugs such as the re-uptake inhibitors sibutramine (Jackson et al., 1997).

In the hypothalamus, the paraventricular nucleus (PVN) (the area associated with control of food intake) (Jackson et al., 1997) is innervated by norepinephrine (NE) fibers and is a site at which infusion of exogenous NE elicits eating at low doses. Two subtypes of α-adrenergic receptors within the PVN exert antagonistic actions on eating in the rat: activation of PVN α_2-adrenoceptors increases eating, α_2-adrenoceptor agonists such as clonidine have been shown to increase food intake in rats (Jackson et al., 1997). On the other hand activation of PVN α_1-adrenoceptors suppresses eating (Wellman, 2000).

Grapefruit (Citrus Paradise, family Rutaceae) (Scora et al., 1982) is a citrus fruit that is low in calories and rich in dietary fibers (Stump et al., 2006). It is a commercial crop of great importance, mainly for the juice industry, and also as a source of essential oils and pectin (Hodgson, 1967). It had been shown that grapefruit juice decreased body weight (Fujioka et al., 2006).

Sibutramine is an anti-obesity drug that enhances satiety (suppressing the appetite) (Kim et al., 2003). It acts centrally as an inhibitor of both norepinephrine and serotonin reuptake. Due to inhibition of noradrenaline reuptake, sibutramine is expected to increase systolic and diastolic blood pressure as well as pulse rate with the result that the drug therapy being discontinued in about 5% of patients (Yanovski and Yanovski, 2002).

The aim of this work was to study the effect of grapefruit juice and sibutramine on obesity induced by cafeteria diet in rats

MATERIALS AND METHODS

Animals

Thirty female Sprague-Dawley albino rats weighing 120 to 140 g purchased from the animal house colony of the National Research Center (Dokki, Giza, Egypt) were kept in the animal house under hygienic conditions at room temperature with reversed 12 h light, dark cycle. The animals were fed on commercial standard pellets and given water *ad libitum* for an adaptation period of 1 week. Experiments were performed according to the national regulations of animal welfare and institutional animal committee (IAEC).

Animal grouping

Rats were then divided into two groups. The first group (6 rats) was fed on commercial standard pellets throughout the experiment and served as control group. The second group (24 rats) was fed high calorie highly palatable “cafeteria diet” consisting of chocolate and cookies (Hamilton and Doods, 2002) and served as obese group in addition to standard pellets for 3 weeks for the induction of obesity.

After 3 weeks, the obese group was divided into 3 subgroups: Group I received cafeteria diet for 3 weeks and served as positive control. Group II received grapefruit juice (4 ml, 3 times daily, by gavage) (Fujioka et al., 2006; Paget and Barnes, 1964) together with feeding cafeteria diet for 3 weeks. Group III received sibutramine (3 mg/kg/day, by gavage) (Brown et al., 2001) together with feeding cafeteria diet and standard pellets for 3 weeks.

Body was recorded weekly. Percent change of body weight gain was calculated as:

$$\text{Change of body weight (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

At the end of the experimental period, blood samples were collected, rats were killed by decapitation and brain tissues were isolated.

Blood samples (3 ml) were collected from retro-orbital plexus in dry centrifuge tubes and left to clot at room temperature. Samples were centrifuged at 1500 rpm for 10 min, the clear supernatant was separated and used for determination of serum glucose, triglycerides, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), creatinine and urea.

Preparation of brain samples

The brain was immediately excised on ice and weighed then homogenized in 75% high performance liquid chromatography (HPLC) methanol (1/10 weight/volume) using a homogenizer (Jake and Kunkle IKA labortechnick, Ultra-turrax T25, Germany) surrounded with an ice jacket and the homogenates were centrifuged in a cooling centrifuge (Sigma and laborzentrifugen, 2K15, Germany) for 15 min at 5000 rpm. The supernatant was used for determination of monoamines’ concentration by HPLC according to the method described by Pagel et al. (2000).

Statistical analysis

Data were expressed as mean ± standard error (SE). Statistical differences and interactions were evaluated using one-way analysis of variance test (One-way ANOVA) followed by Dunnett’s test. For all statistical tests done, a 0.05 level of probability was used as the criterion of significance.

RESULTS

Effect of feeding cafeteria diet for 3 weeks on body weight gain of rats

Feeding cafeteria diet for 3 weeks significantly increased body weight gain of rats, as compared to normal control values (50.11 ± 2.35 and 21.36 ± 1.92 g at $P<0.05$, respectively) (Figure 1).

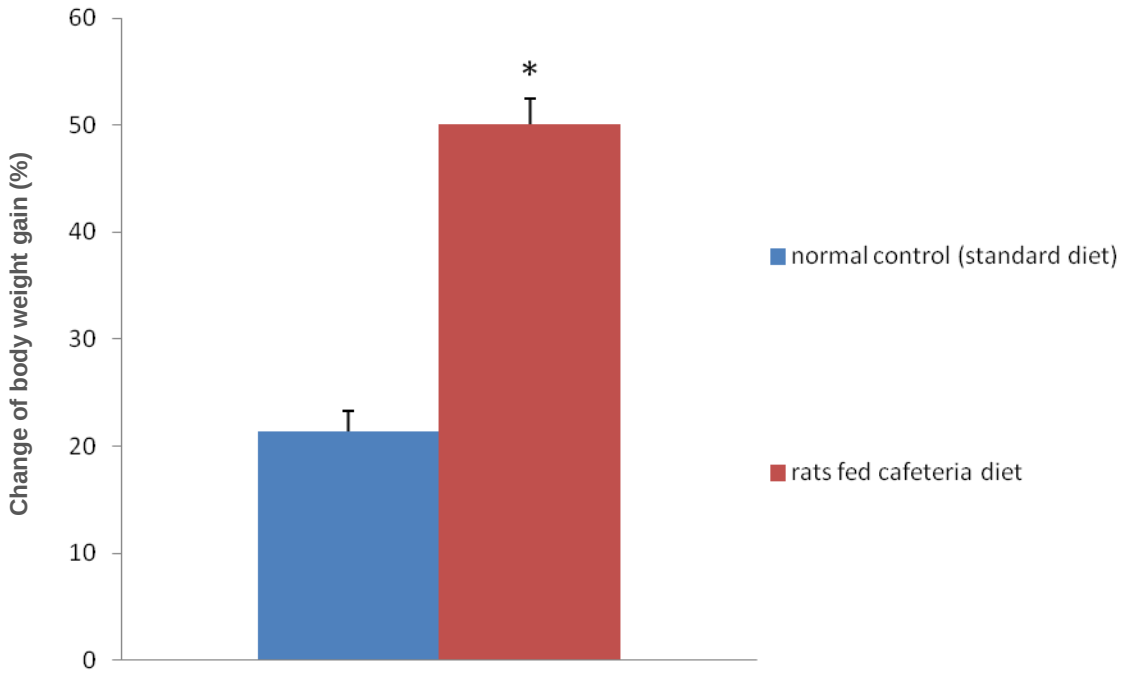

Figure 1. Effect of feeding cafeteria diet for 3 weeks on body weight gain of rats.
*Significantly different from normal control at P<0.05.

Effect of grapefruit juice and sibutramine on food consumption

Grapefruit juice significantly decreased food consumption after the second and the third weeks (323.17 ± 18.53 and 317.39 ± 29.04 Kcal at P<0.05, respectively). Sibutramine significantly decreased food consumption after the second week only (354.25 ± 13.49 Kcal at P<0.05) (Figure 2).

Effect of grapefruit juice and sibutramine on body weight

Grapefruit juice significantly decreased body weight gain after weeks 1, 2 and 3 (7.1 ±0.26, -3.58 ± 0.31 and 4.42 ± 0.45 g at P<0.05 respectively). Sibutramine significantly decreased body weight gain after week 1 and week 2 (-2 ± 0.25 and 6.2 ±0.65 g at P<0.05 respectively) (Figure 3).

Effect of grapefruit juice and sibutramine on serum glucose and triglycerides

Cafeteria diet fed rats (positive control) did not show any significant effect on serum glucose compared to normal control. All treated groups did not show any significant effect on serum glucose as compared to normal control. Cafeteria diet fed rats (positive control) showed a significant increase in serum triglycerides as compared to normal control (312.44 ± 15.24 mg/dl) at P<0.05. Grapefruit juice did not show any significant effect on serum triglycerides as compared to normal control but it showed significant decrease in serum triglycerides as compared to positive control (141.7 ± 8 mg/dl at P<0.05). Sibutramine showed significant increase in serum triglycerides compared to normal control and did not show any significant effect as compared to positive control (259.12 ± 22.7 mg/dl) (Figure 4).

Effect of grapefruit juice and sibutramine on serum GPT and GOT

Cafeteria diet fed rats (positive control) showed a significant increase in serum GPT compared to normal control (49.75 ± 2.4 U/ml) at P<0.05. All treated groups showed significant decrease in serum GPT compared to positive control and did not show any significant difference compared to normal control. All treated groups did not show any significant difference in serum GOT compared to control groups (Figure 5).

Effect of grapefruit juice and sibutramine on serum creatinine and urea

Cafeteria diet fed rats showed significant increase in serum creatinine compared to normal control (0.721 ± 0.022 mg/dl at P<0.05). All treated groups did not show any significant effect compared to positive control. Cafeteria diet fed rats showed significant decrease in

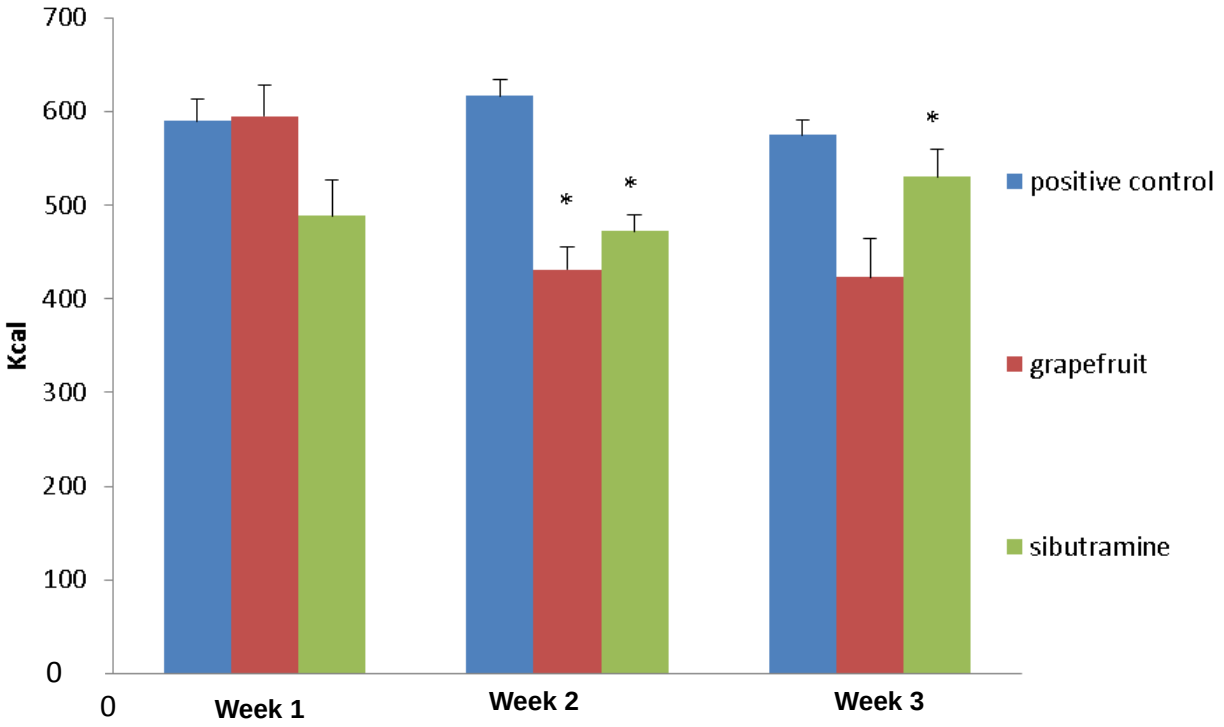

Figure 2. Effect of grapefruit juice and sibutramine on food consumption.
*Significantly different from positive control at P<0.05.

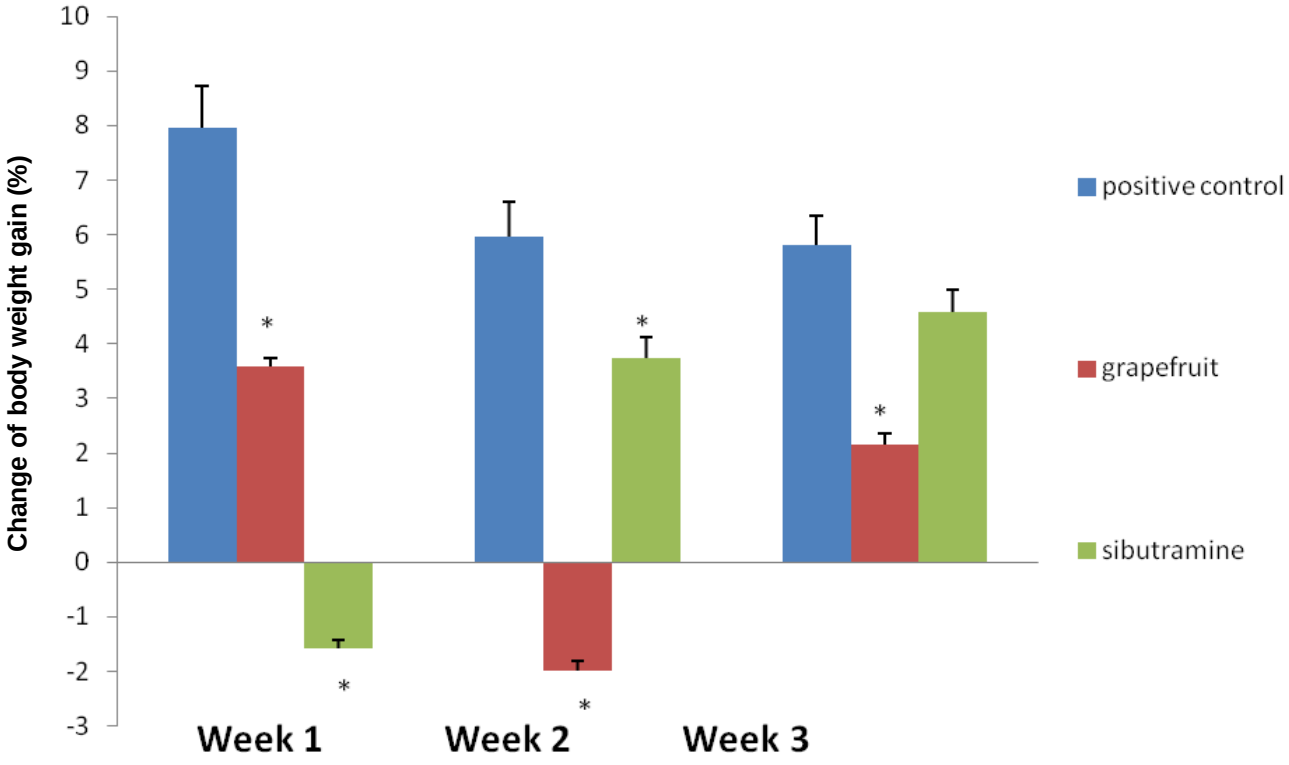

Figure 3. Effect of grapefruit and sibutramine on body weight gain in weeks 1, 2 and 3.
*Significantly different from positive control at P<0.05.

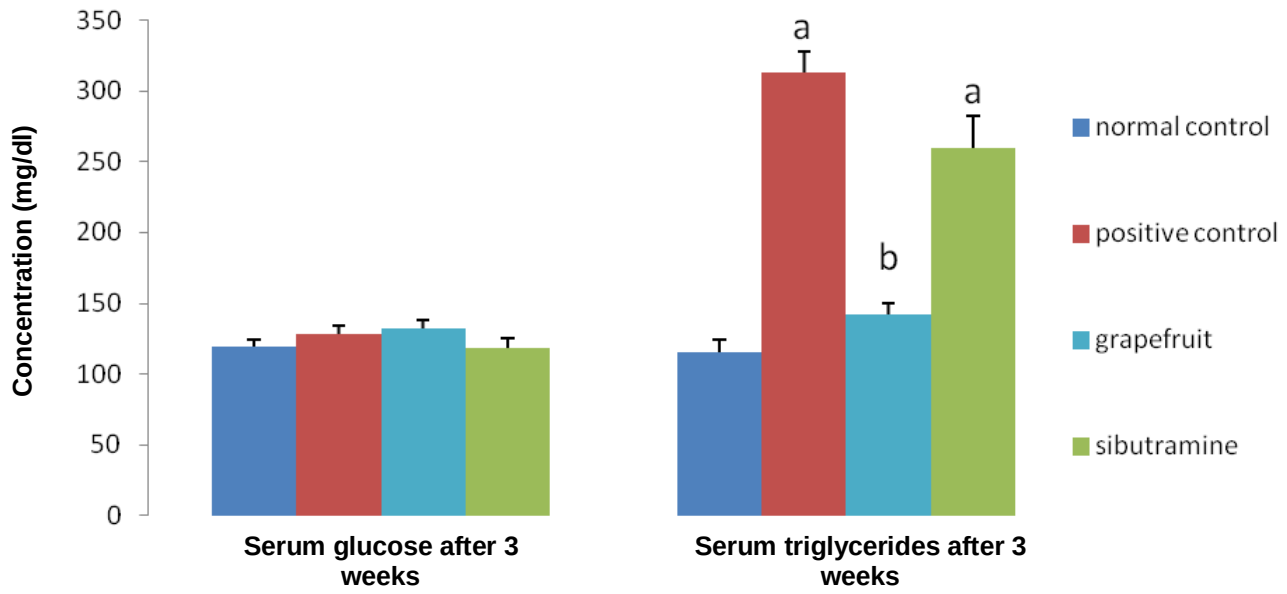

Figure 4. Effect of grapefruit and sibutramine on serum glucose and triglycerides after 3 weeks.

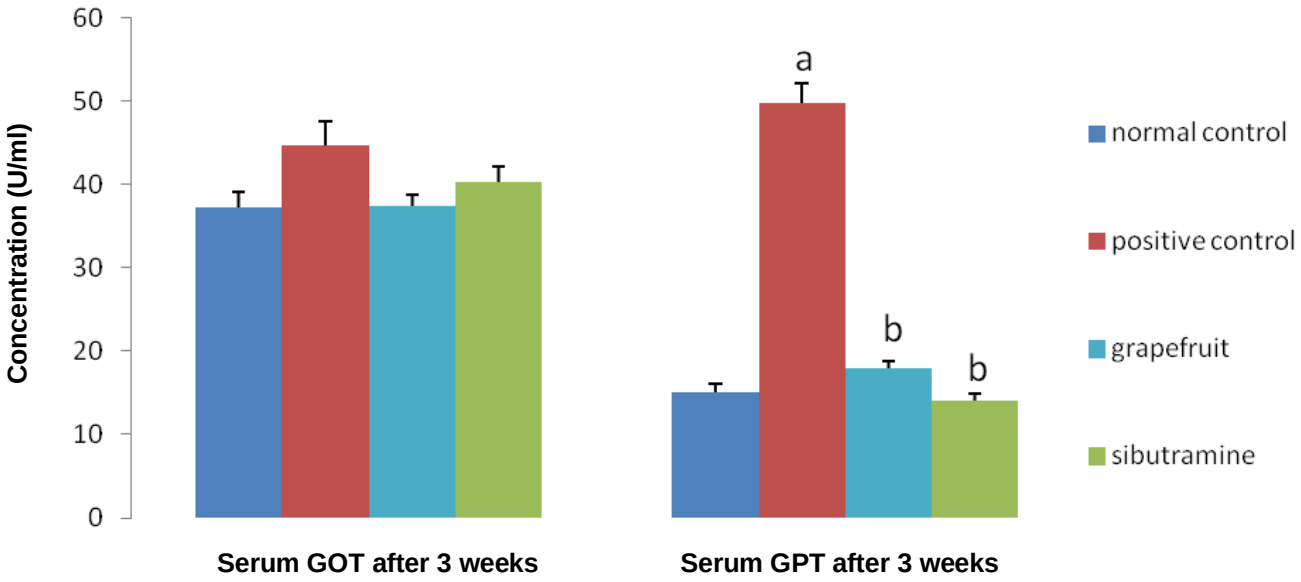

Figure 5. Effect of grapefruit and sibutramine on serum GOT and GPT after 3 weeks.

serum urea compared to normal control (23.56 ± 1.53 mg/dl at $P<0.05$). Grapefruit juice did not show any significant effect on serum urea compared to the control groups. Sibutramine showed significant increase in serum urea compared to positive control but did not show any significant effect compared to normal control (Figure 6).

Effect of grapefruit juice and sibutramine on brain serotonin and noradrenaline

Cafeteria diet did not show any significant effect on brain serotonin compared to normal control. All treated groups did not show any significant effect on serotonin compared to control groups. Sibutramine showed significant

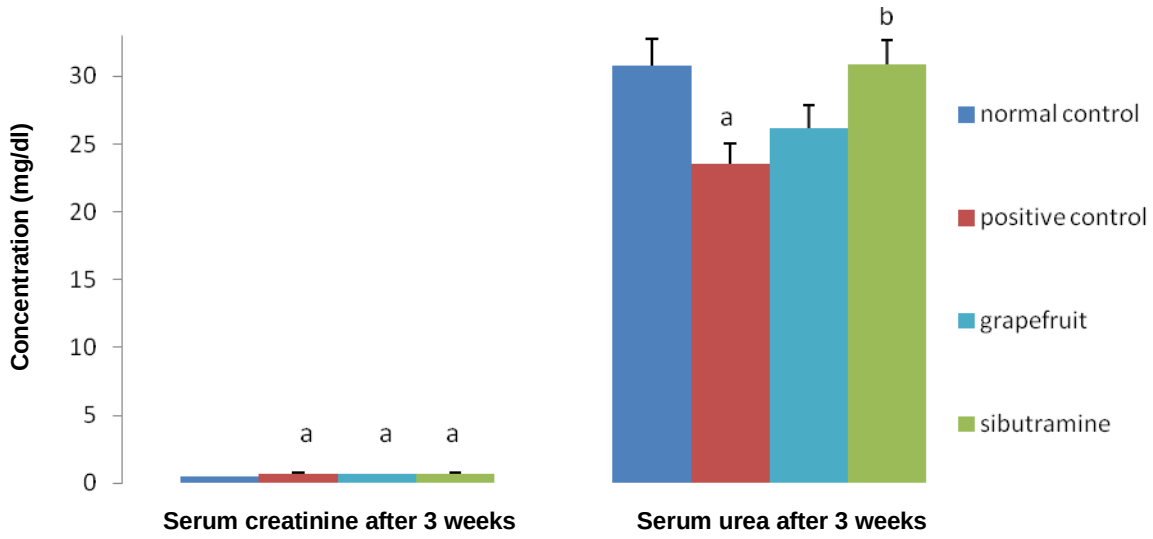

Figure 6. Effect of grapefruit and sibutramine on serum creatinine and urea after 3 weeks. [a]Significantly different from normal control at P<0.05. [b]Significantly different from positive control at P<0.05.

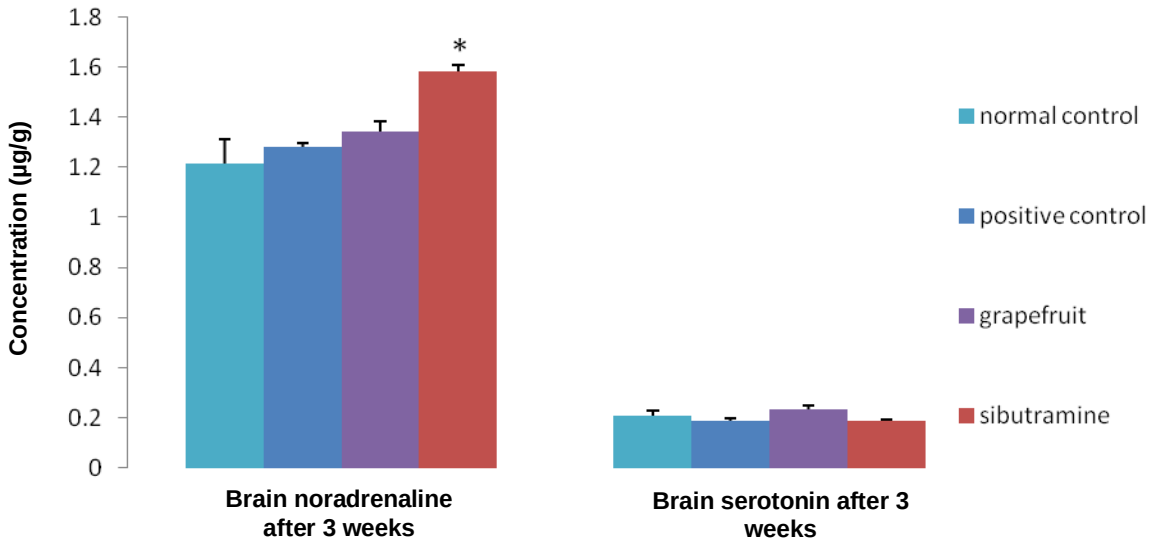

Figure 7. Effect of grapefruit and sibutramine on brain noradrenaline and serotonin after 3 weeks. *Significantly different from positive control at P<0.05.

increase in brain noradrenaline concentration compared to control groups (1.583 ± 0.03 µg/g at P<0.05) (Figure 7).

DISCUSSION

Cafeteria diet fed rats represent a useful model for human obesity studies because the cafeteria diet is a palatable hypercaloric and hyperlipidic diet that induces voluntary hyperphagia and fast body weight gain (Rodríguez et al., 2001). Exposure to a palatable diet had long-term effects on feeding patterns. Rats became overweight because they initially ate more frequently and ultimately ate more of foods with higher energy density (Martire et al., 2013).

In the present study, cafeteria (CAF) diet was used to induce obesity in rats (Hamilton and Doods, 2002). Such diet increases body weight and adipose mass in rats even after a short period of use (Rodríguez et al., 2004).

In the present study, grapefruit juice significantly decreased body weight gain after weeks 1, 2 and 3 and

after 3 weeks of administration (at the end of the treatment) compared to positive control group. The mechanism of this reduced weight may be due to delayed gastric emptying secondary to grapefruit and its relative acidity (Fujioka et al., 2006). It has been noted in other studies that a decrease in pH of gastric contents can delay gastric emptying which causes gastric distension which contribute to satiety (Chaw et al., 2001) and so decrease in body weight gain.

The decrease in body weight gain may also be attributed to nootkatone, a constituent of grapefruit, which is naturally occurring adenosine monophosphate-activated protein kinase (AMPK) activator. AMPK is a serine/threonine kinase that is implicated in the control of energy metabolism and is considered to be a molecular target for the suppression of obesity (Murase et al., 2010). It consists of three proteins (α, β, and γ subunits) that together make a functional enzyme. Each of these three subunits takes on a specific role in both the stability and activity of AMPK (Stapleton et al., 1996). The net effect of AMPK activation is stimulation of hepatic fatty acid oxidation and ketogenesis, inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis, stimulation of skeletal muscle fatty acid oxidation and muscle glucose uptake, and modulation of insulin secretion by pancreatic beta-cells (Winder and Hardie, 1999).

The energy-sensing capability of AMPK can be attributed to its ability to detect and react to fluctuations in the AMP:ATP ratio that take place during rest and exercise (muscle stimulation) (Winder and Hardie, 1996). During exercise, AMPK activity increases while the muscle cell experiences metabolic stress, and brought about by an extreme cellular demand for ATP. Upon activation, AMPK increases cellular energy levels by inhibiting anabolic energy consuming pathways (such as fatty acid synthesis and protein synthesis) and stimulating energy producing catabolic pathways (such as fatty acid oxidation and glucose transport) (Thomson et al., 2007).

Nootkatone significantly reduced high-fat and high-sucrose diet-induced body weight gain. These findings indicate that nootkatone is beneficial toward preventing obesity due to enhanced energy metabolism through AMPK activation in skeletal muscle and liver (Murase et al., 2010). The present study revealed that sibutramine (3 mg/kg) produced significant reduction of body weight gain after the first and the second weeks only, as compared to obese control values. No significant change of body weight gain was observed after the third week of drug administration, as compared to obese control values.

It has been reported that sibutramine produces significant reduction of body weight when used in conjunction with caloric restriction all over the period of its administration (Florentin et al., 2007). In the present study CAF diet did not produce a significant increase in the level of serum glucose compared to normal control, but it increased the serum triglycerides level (Kuhlmann et al., 2005). These results may be attributed to hyperinsulinemia and hyperlipidemia (Akiyamaab et al., 1996).

Studies have shown that hyperinsulinima is a major cause of obesity because insulin causes the body to store more fats (Reaven et al., 2002). Hyperinsulinemia accelerates hepatic triglyceride (TG) production which leads to elevated plasma TG concentrations (Akiyamaab et al., 1996) and this may explain why TG showed high serum level in cafeteria diet fed rats. Data of the present study showed that grapefruit juice produced a significant reduction in serum triglycerides level compared to positive control group. This is in harmony with a previous study that showed that hesperidin and naringin, bioactive compounds of citrus fruits (grapefruit), are powerful plasma lipid lowering flavonones (Shela et al., 2007). Also a previous work showed that diet supplemented with fresh grapefruit positively influences serum lipid levels of all fractions, especially serum triglycerides. The addition of fresh grapefruit to generally accepted diets could be beneficial for hyperlipidemic, especially hypertriglyceridemic (Gorinstein et al., 2006). While compared to normal control there was no significant difference which suggests that grapefruit may normalize the serum triglycerides level of obese rats as grapefruit is rich in flavonoids. Several studies have shown that flavonoids possess lipolytic activity via inhibition of cAMP-phosphodiesterase and maintain lipolysis-inducing cAMP levels. Lipolysis is a catabolic process leading to the breakdown of TG stored in fat cells (adipocytes) and the release of free fatty acids (FFA) and glycerol. And so grapefruit prevents obesity and helps to decrease body weight and body fat (Dallas et al., 2008).

Results of the current study revealed that CAF-diet-fed rats produced a significant increase in serum GPT level while non significant effect on serum GOT was shown as compared to normal control group. This may be explained by previous studies that showed that in young healthy subjects, a fast food-based diet (CAF diet) dramatically increased serum GPT (Marchesini et al., 2009), as well as that, a study done on human showed the effect of obesity was particularly important in the case of GPT than GOT (Robinson and Whitehead, 1989). Also, several studies showed that hyperlipidemia causes fatty liver disease which increases serum GPT (Kim et al., 2008).

In the present study, grapefruit juice showed an improvement in the level of serum GPT compared to positive control group. This study further shows that CAF diet produced a significant increase in serum creatinine and reduction in serum urea level compared to normal control group. These findings can be explained by the increase in nitrogen retention in CAF diet fed rats. The marked N_2 retention was due to a decrease in amino acid catabolism and in urea production by the liver (Sadhu, 2010). Therefore daily N_2 retention was greatly enhanced in these animals which is in agreement with the known protein-sparing effect of fat (excess dietary fats spare or retain the body protein) (Estornell et al., 1994).

The decrease in urea production may be due to the following facts: (i) the rate of synthesis of urea from precursors by isolated hepatocytes from cafeteria-diet-fed rats was lower than in controls, (ii) in cafeteria-diet-fed rats the activities of all the enzymes of the urea cycle are decreased, the major percentage decreases are those of carbamoylphosphate synthetase and of argininosuccinate synthetase, the enzymes involved in the regulation of the overall rate of the cycle, when rats are switched to normal chow diet, the enzyme activities return to normal values; (iii) ihe uptake of amino acids by liver of cafeteria-diet-fed rats is lower than in controls. These results contrast with those obtained previously by using other models of obesity in rat (that is genetic or hypothalamic), in which N excretion was increased (Barber et al., 1985).

Data of the present study showed that all treated groups showed a significant increase in serum creatinine level compared to normal control, but when compared with positive control they did not show any significant effect. This means that all treatments cannot normalize serum creatinine level in the presence of CAF diet. Sibutramine produced a significant increase in brain noradrenaline level compared to positive control. This is due to the reuptake inhibition effect of sibutramine on brain noradrenaline (Brown et al., 2001).

The present data showed that sibutramine did not produce a significant effect on brain serotonin level after 3 weeks of administration compared to control groups. This may be due to the low dose of sibutramine used in the present study (3 mg/kg) since previous study showed that low doses of sibutramine had no effect on serotonin concentration and only high doses (5 mg/kg) increased serotonin level (Balcioglu and Wurtaman, 2000). This may explain why the effect of sibutramine on body weight did not last till the end of the experiment since there is a synergistic interaction between serotonin and noradrenaline (Jackson et al., 1997).

Conclusion

Conclusively, grapefruit juice decreased the body weight gain from the first week till the end of the study and also decreased food consumption without affecting brain noradrenaline level. Sibutramine decreased the body weight gain and food consumption only in week 2 (that is, it did not last till the end of the treatment) but it increased the brain noradrenaline level which may lead to an increase in blood pressure.

So from the present study, it was concluded that grapefruit juice is better than sibutramine since its effect lasted till the end of the experiment and also did not affect brain noradrenaline.

Conflict of interest

The authors declare no conflict of interest.

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