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Chemical composition by HPLC-ESI-QTOF-MS/MS: Estrogenic and antioxidant effects of *Mangifera indica* L. cv. "Kent" leave extracts on ovariectomized rats

[Composición química por HPLC-ESI-QTOF-MS/MS: efectos estrogénicos y antioxidantes de extractos de *Mangifera indica* L. cv. "Kent" en ratas ovariectomizadas]

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Abstract: The chemical composition of *Mangifera indica* L. cv. "Kent" leaves was determined by HPLC-ESI-QTOF-MS/MS. Polyphenolic compounds characterized as benzophenone derivatives were the main components found in extracts (1, maclurin 3-C-(2-O-galloyl)-D-glucoside isomer; 2, maclurin 3-C-β--D-glucoside; 3, iriflophenone 3-C-β--D-glucoside; 5, maclurin 3-C-(2,3-di-O-galloyl)-β--D-glucoside; 6, iriflophenone 3-C-(2-O-galloyl)-β--D-glucoside; 7, methyl-iriflophenone 3-C-(2,6-di-O-galloyl)-β--D-glucoside) and xanthones (4, mangiferin and 8, 6-O-galloyl-mangiferin). The estrogenic and antioxidant effects of aqueous extracts from *Mangifera indica* L. cv. "Kent" leaves on ovariectomized rats were determined by uterotrophic assay and malondialdehyde (MDA) levels in erythrocytes, bone, liver, and stomach. We conclude that the polyphenolic compounds from extracts act as exogenous antioxidant agents against oxidative damage in ovariectomized rats.

Keywords: *Mangifera indica* L.; Benzophenone derivatives; Xanthones; ROS; Lipid peroxidation; Antioxidants.

Resumen: La composición química de las hojas de *Mangifera indica* L. cv. "Kent" se determinó por HPLC-ESI-QTOF-MS/MS. Compuestos polifenólicos caracterizados como derivados de benzofenona fueron los componentes principales encontrados en los extractos (1, isómero de la maclurina 3-C-(2-O-galoyil)-D-glucósido; 2, maclurina 3-C-β-D-glucósido; 3, iriflofenona 3-C-β-D-glucósido; 5, maclurina 3-C-(2,3-di-O-galloíl)-β-D-glucósido; 6, iriflofenona 3-C-(2-O-galloil)-β-D-glucósido; 7, metil-iriflofenona 3-C-(2,6-di-O-galloyl)-β-D-glucósido) y xantonas (4, mangiferina y 8, 6-O-galoyil-mangiferina). Los efectos estrogénicos y antioxidantes de los extractos acuosos de hojas de *Mangifera indica* L. cv. "Kent" en ratas ovariectomizadas se determinaron mediante ensayo uterotrófico y la medición de los niveles de malondialdehído (MDA) en eritrocitos, huesos, hígado y estómago. Concluimos que los compuestos polifenólicos de los extractos actúan como agentes antioxidantes exógenos contra el daño oxidativo en ratas ovariectomizadas.

Palabras clave: *Mangifera indica* L.; Derivados de benzofenona; Xantonas; ROS; Peroxidación lipidica; Antioxidantes.

INTRODUCTION

Mangifera indica is a fruit tree that belongs to the Anacardiaceae family, order Rutales native of Southeast Asia. It grows naturally or cultivated in tropical and subtropical regions and has been reported to be the second largest tropical fruit crop in the world (Joseph & Abolaji, 1997). Extracts of *M. indica* have been reported to possess antiviral, antibacterial, analgesic, antiinflammatory, immunomodulatory, cardiotonic, diuretic and antiamoebic activities (Tona *et al*., 1998; Scartezzini & Speroni, 2000; Makare *et al*., 2001; Prashanth *et al*., 2001; Doughari & Manzara, 2008; Ling *et al*., 2009; El-Mahmood, 2009; Benites *et al*., 2010; Agbe *et al*., 2010). In traditional medicine, the use of mango extracts as herbal drug has been used over the years. A comprehensive review describes in detail the many properties displayed by mango leave extracts (Shah *et al*., 2010).

Phenolic compounds are important role for the color and flavor of foods and beverages, and regular consumption is associated with beneficial effects for human health (Fernández-Ponce *et al*., 2012). Some phenolic compounds present in mango are antioxidants, contributing to reducing the risk of developing cardiovascular diseases, inflammation, hypertension, arthritis, and carcinogenesis (Shah *et al*., 2010; Fernández-Ponce *et al*., 2012).

Free oxygen radicals have been proposed as important causative agents of aging. Studies using rats led to observe that the differences in longevity between genders are related to the production of free radicals. Female produce less free radicals compared to males because of up-regulated estrogen-related mechanisms that influence longevity (Viña & Borrás, 2010) such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Viña *et al*., 2008). Moreover, the physiological process of aging in females is also associated with a hormonal imbalance period known as menopause (Ward & Deneris, 2018). Hormone dysregulation in menopause can cause a redox imbalance, a condition in which reactive oxygen species (ROS) and pro-oxidants overcome antioxidant capacity and cause severe cell damage (Can *et al*., 2015). Increased ROS react with polyunsaturated fatty acids to induce the release of toxic and reactive aldehyde metabolites, such as malondialdehyde (MDA), which is one of the end products of the lipid peroxidation process (Hagihara *et al*., 1984; Kurata *et al*., 1993). Lipid peroxidation

is associated with aging and a variety of chronic health diseases (Shigenaga *et al*., 1994; Bland, 1995; Marnett, 2000). Numerous studies suggest that antioxidants can prevent the oxidation of various macromolecules, such as DNA, proteins, and lipids, thus slowing the aging process and, therefore, increasing the lifespan of the organism (Taridi *et al*., 2011; Xia *et al*., 2017). In this study, we investigated the chemical composition of leaves of mango by HPLC-ESI-QTOF-MS/MS, and evaluated the potential estrogenic and antioxidant effects in ovariectomized rats.

MATERIAL AND METHODS

Plant material

The leaves of *Mangifera indica* L. cv. "Kent" were collected in November 2017 in the district of Bagua Grande, Province of Utcumba at 450 m above sea level, in the Region of Amazonas, Perú. Once collected, the specimen was identified from the "Herbarium Truxillense de la Facultad de Ciencias Biológicas de la Universidad Nacional de Trujillo". A voucher sample under accession HUT 59580 was deposited in this herbarium.

Lyophilized ethanol extract preparation

The ethanol extracts of leaves of *Mangifera indica* L. cv. "Kent" were prepared as follows: 20 g of dried leaves were weighted and added to 200 mL of ethanol, which was preheated at 50°C. The mixture was subjected to reflux for 2 hours. The extraction process was repeated two times with another 200 mL of ethanol. The extracts were mixed and filtered to remove insoluble particles and then evaporated under reduced pressure. The extract was frozen an -80°C (Arctiko) and then lyophilized with a freeze-dryer (Labconco). The lyophilized was stored at +4ºC until tested and analyzed.

Chemical identification by HPLC-ESI-QTOF-MS/MS

Samples were prepared at 5 mg/mL in methanol. The chromatographic separation was done using a HPLC Rapid Resolution (Agilent, 1200 series) composed by a binary pump, degreaser and automatic injector and using Agilent Zorbax C-18 column (4.6 x 150 mm, 1.8 μm) with a flow rate of 0.5 mL/min and 10 μL injection. The elution gradient was acetonitrile (B) and water (A) in the following ratio: 0.0 -15 min 12-20% B; 15-20 min 20% B; 20-35 min 20-12% B.

Column effluent was divided by T-valve and a fraction equivalent to 20 μL/min was introduced into the mass spectrometer. The chemical identification was performed using a Q-TOF orthogonal mass spectrometer (micrOTOF-QTM, Bruker Daltonics) equipped with electrospray ionization source (ESI). The analysis parameters were provided for the positive mode, with a mass range of 100-1000 m/z: 4500 V capillary voltage; set end plate offset -500 V; set charging voltage 2000 V; drying gas temperature 200°C; drying gas flow 10.0 mL/min; gas pressure 4 bar; collision energy (MS/MS) 35 eV; collision gas N_2. The mass data obtained were processed in Bruker Compass Data Analysis 4.2 software (Bruker Daltonics).

Animal treatments

Female Sprague-Dawley rats weighing between 150 and 200 g kept under controlled conditions (12-h dark/12-h light cycle, 23-25ºC, and 50-60% humidity) were used in this study. All experiments were conducted in accordance with international standards of animal welfare, and the experimental protocols were approved by the Ethics Committee of Universidad Nacional de Trujillo (UNT). Groups of eight animals were selected and, to reduce the influence of diurnal variation, all assays were conducted from 09:00 to 13:00 h. in a special noise-free room with controlled illumination. The animals received a standard food pellet, and before experiments, they were fasted overnight with water *ad libitum*.

The rats were randomly distributed into four experimental groups (n=6 per group), as follows: Blank, not ovariectomized (no OVX) which served as a sea level control; Control, (OVX) both were administered NaCl 0.9% orally; Problem I and II, both OVX and were treated with the lyophilized of leaves of aqueous extract of *M. indica* (EM_2) administered orally at a concentration of 0.05 g/mL at a doses of 250 mg/kg and 500 mg/kg respectively, for a period of 8 weeks.

Ovariectomy

The ovariectomy was performed during a diestrous cycle to keep the consistent lowest levels of sex hormones in rats (Zamorano *et al*., 1995). Surgery was performed under anesthesia induced by ketamine (110 mg/kg, i.p.), using standard procedures. Briefly, anesthesia was confirmed by reduced respiratory rate and absence of response to gentle pinching the footpad. A ventral incision was made through the skin on the right flank. In the OVX groups, the ovary, oviduct, and top of the fallopian tubes were clamped and removed. Skins and abdominal walls of animals of OVX groups were sutured, and animals were returned to their cages.

Vaginal cytology: collection and processing

Cells were collected from the vaginal canal with the help of a swab (Marcondes *et al*., 2001). The tip of the swab was gently inserted into the vaginal orifice at a depth of approximately 5–10 mm and then the sample is then placed evenly on the slide in a thin layer (smear). The sample was fixed with ethanol for 15 minutes and then stored at 27° C. Hematoxylin-Eosin stained (H&E) has been used to stain vaginal cytology. Then, the samples were analyzed microscopically.

Uterotrophic assay

Animals were weighed and killed by cervical dislocation twenty-four hours after the last treatment. Uteri were excised, trimmed free of fat, pierced and blotted with filter paper to remove fluid. The body of each uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries. Uterus wet weight was determined and expressed as relative weight (wet weight×100/body weight) (Müller *et al*., 2009). Body weight was measured at 0, 60 and 90 days throughout the experiment.

One uterine horn was removed and mounted in a 25 mL organ-bath chamber containing Jalón-Ringer solution (gassed with 95% O_2 and 5% CO_2 mix) during 10 minutes at 37 °C. Two experimental conditions were tested. In the first condition, baseline measurement was taken as control. In the second, contractions were evaluated in the presence of oxytocin (10 IU/mL, Sanderson Laboratory SA). Uterine horns were connected to a force transducer, and the variables of amplitude, frequency, and duration of the contraction were measured. A Power Lab 26T data acquisition system (AD instruments V100 Ma20 ML856) was used. The ion composition of Jalón-Ringer solution was (concentrations in mM): NaCl (154), KCl (563), $CaCl_2$ (0.54), $NaHCO_3$ (5.95) and Glucose (2.77) (D'ocón *et al*., 1992).

Biochemical Procedures
Malondialdehyde (MDA) measurements in erythrocytes
MDA levels were quantified using a minor modification of the method published by Gutierrez-Salinas *et al.*, 2009. Briefly, packed erythrocytes were placed in a test tube with 1.0 mL of phosphate buffered saline (PBS, pH 7.0). The mixture was incubated in a water bath for 30 min at 37°C. Then proteins were precipitated by adding equal volumes (1.5 mL) of acetic acid (5%, v/v) and of thiobarbituric acid (0.8%; w/v) and the mixture was incubated for 60 min at 90°C. Next, 1.0 mL of KCl (2%, w/v) and 3.0 mL of butanol/pyridine (1:10; v/v) were added. Then, the mixture was shaken and centrifuged at 3000 rpm for 10 min. Finally, MDA levels were measured spectrometrically at 532 nm.

Lipid peroxidation in bone
The femurs were crushed and vigorously mixed with of cold (4°C) potassium chloride (KCl, 150 mM) solution to obtain a 10% (w/v) homogenate. The homogenates were then centrifuged at $3700 \times g$ (4°C) for 15 min. Supernatants were taken for malondialdehyde analysis. In order to conduct malondialdehyde analysis, 3 mL of 1% orthophosphoric acid (H_3PO_4), 1 mL of 0.675% TBA and 0.5 mL of supernatant were mixed. The mixture was kept in a boiling water bath for 45 min. 4 mL of *n*-butanol was added after the mixture was cooled down. The values were read using spectrophotometer at 532 nm against *n*-butanol (Maniam *et al.*, 2008). Results from this method are reported as 'TBA reacting substances' (TBARS) instead of malondialdehyde in nmol/mg protein.

Lipid peroxidation in liver
Lipid peroxidation was quantified using the method proposed by Ohkawa *et al.*, 1979 with slight modifications. The liver was perfused with ice-cold 0.9% NaCl via the portal vein before homogenization. 2 g of the sample was crushed and homogenized with 50.0 mM of phosphate buffer (pH 7.4) and centrifuged at 10000 rpm for 10 min at 4°C. Then, 50 μL of phosphate buffer (50 mM, pH 7.4), 1 mL of 10 % (w/v) of trichloroacetic acid and 450 μL of supernatant were transferred into a new tube and centrifuged at 10000 rpm for 10 min at 4°C. In order to measure malondialdehyde levels, 1 mL of thiobarbituric acid (0.67%; w/v) and 1.0 mL of

supernatant were taken and mixed. The mixture was kept in a boiling water bath for 30 min. 4 mL of *n*-butanol and pyridine (15:1; v/v) was added after the mixture was cooled down. The values were read using spectrophotometer at 532 nm.

Lipid peroxidation in the stomach
Rat stomachs were promptly removed and rinsed with cold saline (4°C) for 3 times. To minimize the possibility of interference of hemoglobin with free radicals, blood adhered to the mucosa was carefully removed by rinsing the tissue with cold saline. The corpus mucosa was scraped, weighed, and homogenized in 10 mL of 100 mM KCl and 0.3 mM EDTA (10%). The homogenate (0.5 mL) was mixed with a solution containing 0.5 mL of Texapon® and 1.5 mL of trichloroacetic acid (10%; w/v). The mixture was centrifuged at 3000 rpm for 10 min, and then the supernatant was mixed with 1.5 mL of thiobarbituric acid (0.8%; w/v). The mixture was incubated at 98°C for 1 h. Upon cooling, the absorbance of the supernatant was measured at 532 nm (Albayrak *et al.*, 2015). The standard curve was obtained by using 1,1,3,3-tetramethoxypropane.

Statistical Analysis
GraphPad Prism software (San Diego, CA 92037, USA) was used, *n* represents the number of animals studied, and values are expressed as the mean ± standard error of the mean (SEM). For the statistical analysis of the groups, a one-way or two-way ANOVA was used as appropriate, followed by a Tukey post hoc test. A value $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION
The structures of secondary metabolites identified in mango leaves (*Mangifera indica* L.) are given in Figure No. 1. HPLC-ESI-QTOF-MS/MS analysis from methanol (MeOH) extract of the leaves mango showed polyphenolic compounds characterized as benzophenone derivatives and xanthones. Molecular weight and mass spectrometric data from our analysis and reported data are given in Table No. 1.

Six benzophenone derivatives were tentative identified from the MeOH extract. As shown in Figure No. 1, maclurin 3-*C*-(2-*O*-galloyl)-D-glucoside isomer **1**; maclurin 3-*C*-β-D-glucoside **2** and maclurin 3-*C*-(2,3-di-*O*-galloyl)-β-D-glucoside **5** were identified at *m/z* 577, 425, 729; and

iriflophenone glucoside derivatives were identified at m/z 409, 713 and 727 (entry **3**, **6** and **7**, Table No. 1). Two xanthones derivatives were detected in the MeOH extract of the leaves of *Mangifera indica*. The peaks at m/z 423 and 575 corresponded to [M+H]$^+$ ion and were attributed to mangiferin and 6-*O*-galloyl-mangiferin respectively.
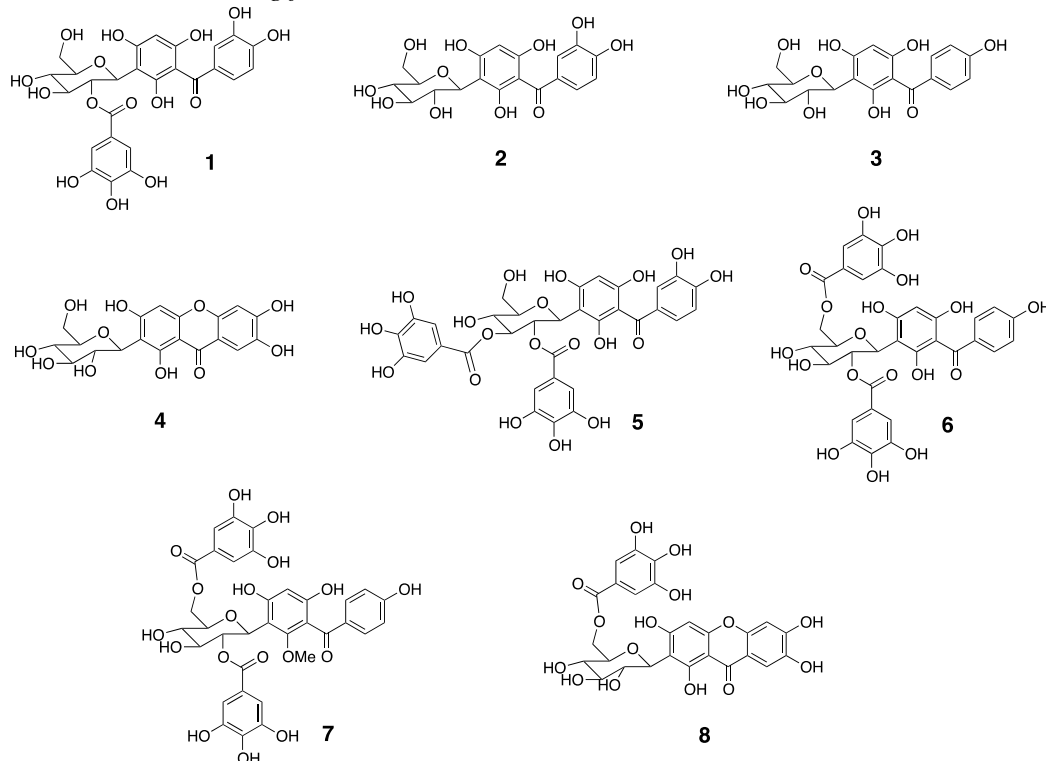

Figure No. 1

Structures of the phenolic compounds from the MeOH extract of leaves of *Mangifera indica* from Perú. Key: 1, maclurin 3-*C*-(2-*O*-galloyl)-D-glucoside isomer; 2, maclurin 3-*C*-β-D-glucoside; 3, iriflophenone 3-*C*-β-D-glucoside; 4, mangiferin; 5, maclurin 3-*C*-(2,3-di-*O*-galloyl)-β-D-glucoside; 6, iriflophenone 3-*C*-(2-*O*-galloyl)-β-D-glucoside; 7, methyl-iriflophenone 3-*C*-(2,6- di-*O*-galloyl)-β-D-glucoside; 8, 6-*O*-galloyl-mangiferin.

Table No. 1

Tentative compounds determined by HPLC-ESI-QTOF-MS/MS in the MeOH extract of leaves of *Mangifera indica* in the positive-ion mode (Rt: Retention time).

Compounds	Rt (min)	[M+H]$^+$ (m/z)	Main Fragment MS^1 (m/z)	Main Fragment MS^2 (m/z)	References
1	1.1	577	425	[425]: 137, 195, 231, 261	[Ohkawa *et al.*, 1979; Albayrak *et al.*, 2015]
2	1.4	425	425	[425]: 137, 195, 231, 261	[Ohkawa *et al.*, 1979]
3	1.8	409	409	[409]: 165, 191, 219, 261	[Ohkawa *et al.*, 1979; Barreto *et al.*, 2008]
4	2.9	423	423	[423]: 273, 303, 369	[Albayrak *et al.*, 2015]
5	4.2	729	561	[561]: 153, 195, 261, 271, 355	[Ohkawa *et al.*, 1979]
6	5.4	713	401	[401]: 204, 401	[Albayrak *et al.*, 2015, Berardini *et al.*, 2004]
7	5.8	727	465	[465]: 303; [303]: 153, 201, 229, 303	[Ohkawa *et al.*, 1979]
8	6.9	575	575	[575]: 137, 227, 253	[Albayrak *et al.*, 2015]

The fertile period in female rats is between proestrous and estrous phase (Marcondes *et al*., 2002). Environmental influences, such as diets containing phytoestrogens are known to adversely affect female reproductive cycle and fertility (Burton & Wells, 2002). The estrous cycle is functionally regulated by the pituitary-ovarian hormone; FSH, estrogen, progesterone, and Luteinizing hormone (LH) which usually peaks during the estrous phase of the cycle (Campbell *et al*., 2009). Figure No. 2 shows the vaginal smears from female Sprague-Dawley rats treated with the aqueous extract of *M. indica* (EM₂).

The study showed that EM_2 administered orally at doses of 250 mg/kg and 500 mg/kg for 8 weeks significantly disrupted the estrous cycle. Figures No. 2A and No. 2B were characterized by high numbers of small-nucleated epithelial cells found individually and in cohesive clusters. On the other hand, EM_2 treatment 250 mg/Kg and 500 mg/kg doses was characterized by the presence of predominately anucleated keratinized epithelial cells and cornified cells respectively in proestrous and estrous phases (Figure No. 2C and No. 2D).
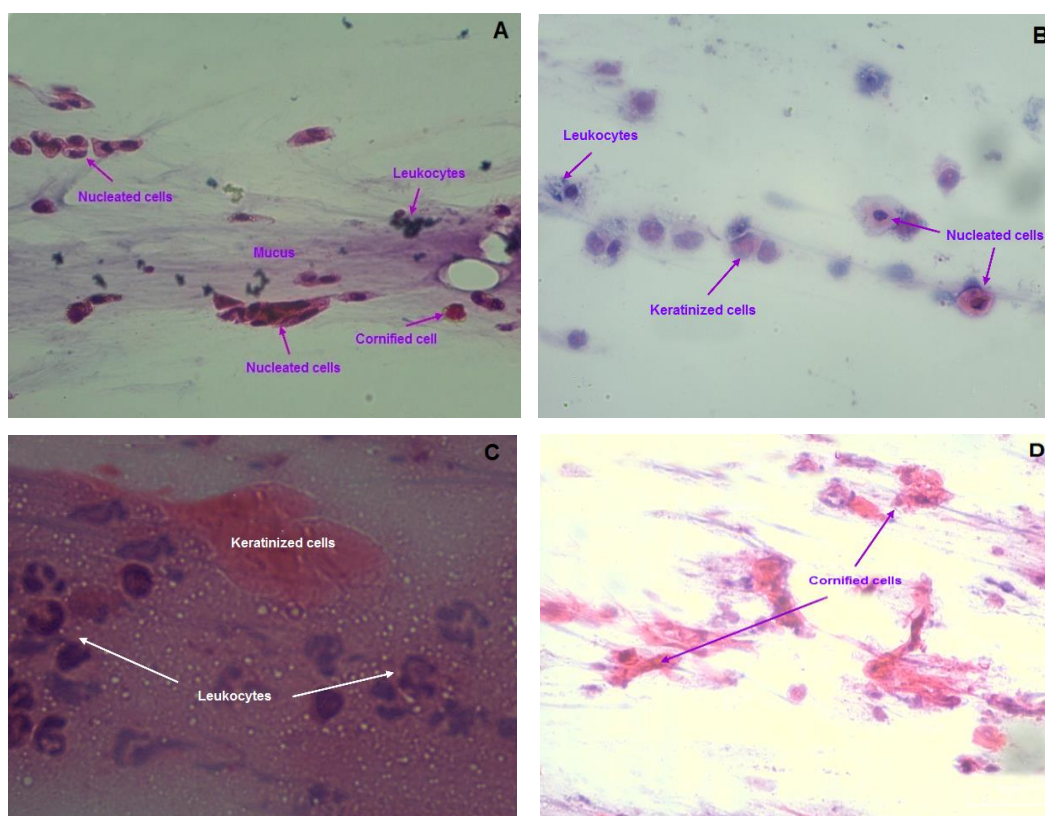

Figure No. 2
Vaginal smears from Sprague-Dawley rats and differentiation on estrous cycle, hematoxylin-eosin stain
(A-D). Original objective magnification of and 400 x (A-D). (A) Blank, estrous; (B) Control, proestrous
(C) EM₂ (250 mg/kg), proestrous; (D) EM₂ (500 mg/kg), estrous.

The relative uterine weight did not differ significantly from the groups ($p>0.05$), indicating that the EM_2 did not possess estrogenic activity in the doses tested in our study. However, the number of cornified cells of Sprague-Dawley rats that received the EM_2 (500 mg/kg) was significant higher, showing

an estrogenic effect to the extract in the dose indicated (Figure No. 3).

The ovariectomy (OVX) induced an increase in body weight when compared to blank (Figure No. 4). The body weight gain of Sprague-Dawley rats exposed to different doses (250 mg/kg and 500

mg/kg) of EM_2 did not differ significantly when compared with the control group (Figure No. 4). These results indicated that *M. indica* shows estrogenic activity in the uterotrophic assay

suggesting that chemicals present in the extract aqueous of the leaves of the mango can interact and modulate the responses of estrogenic receptors.
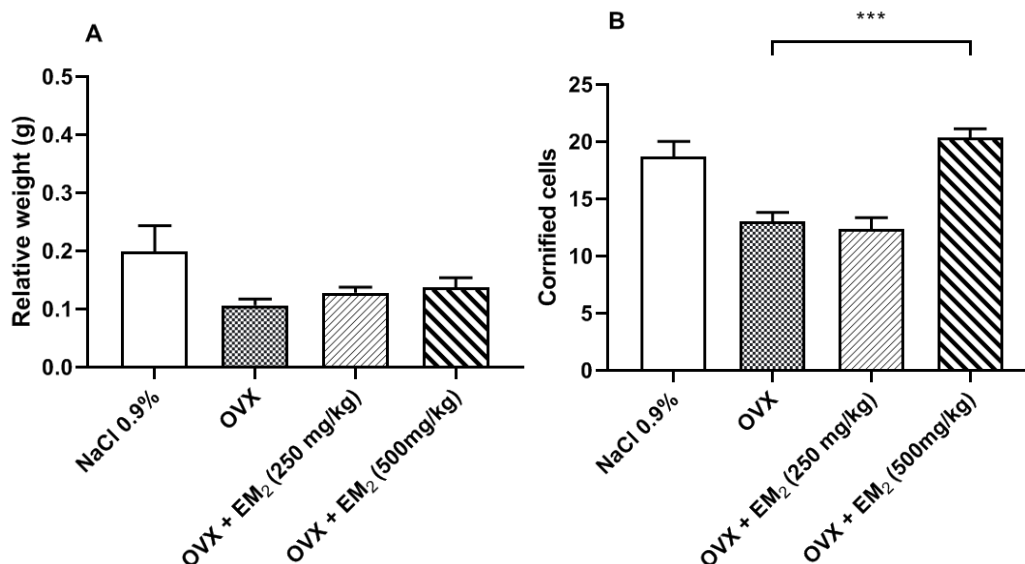

Figure No. 3
Effect of aqueous extract of *M. indica* (EM_2) on the relative uterus weight and the number of cornified cells of Sprague-Dawley rats. Significant differences are indicated with * when $p<0.001$ when compared to control group (OVX).**
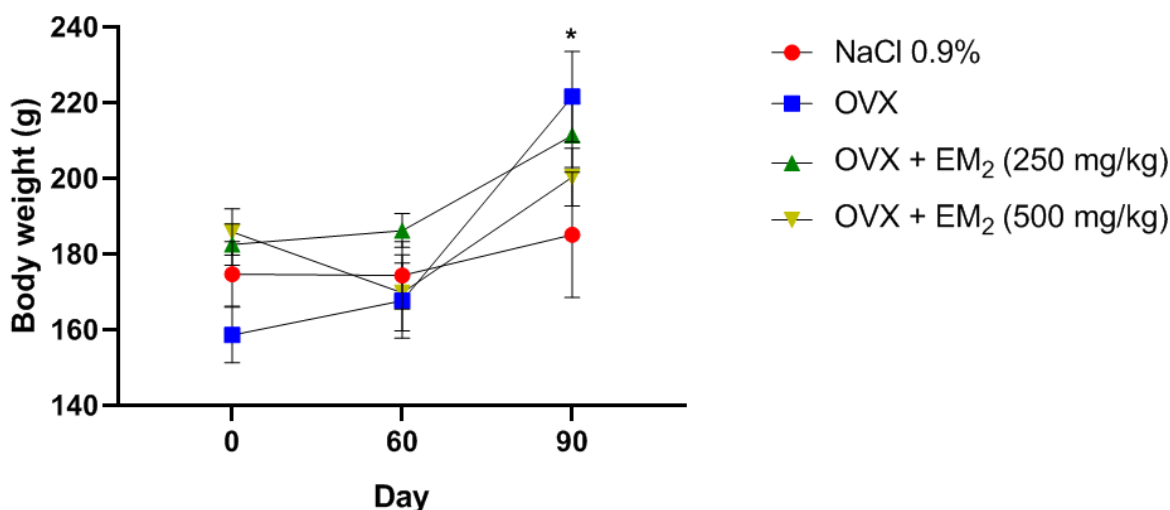

Figure No. 4
Body weight gain (g) of Sprague-Dawley rats exposed to the aqueous extract of *M. indica* (EM_2)

Figure No. 5 shows the effect of EM_2 on the isolated uterine organ of Sprague-Dawley rats

induced by oxytocin. It is noted that the administration of EM_2 (500 mg/kg) showed a

significant difference ($p<0.05$) in the duration and frequency of contractions when compared to the control group. Studies have suggested that chemicals present in the extract aqueous of the leaves of the mango as xanthones (mangiferin and 6-*O*-galloyl-mangiferin) are potential compounds to reduce oxidative stress damage in the uterus layer and increases the level of oxytocin in the blood and its receptor in the uterus (Masibo & He, 2008), simulating an estrogenic effect.
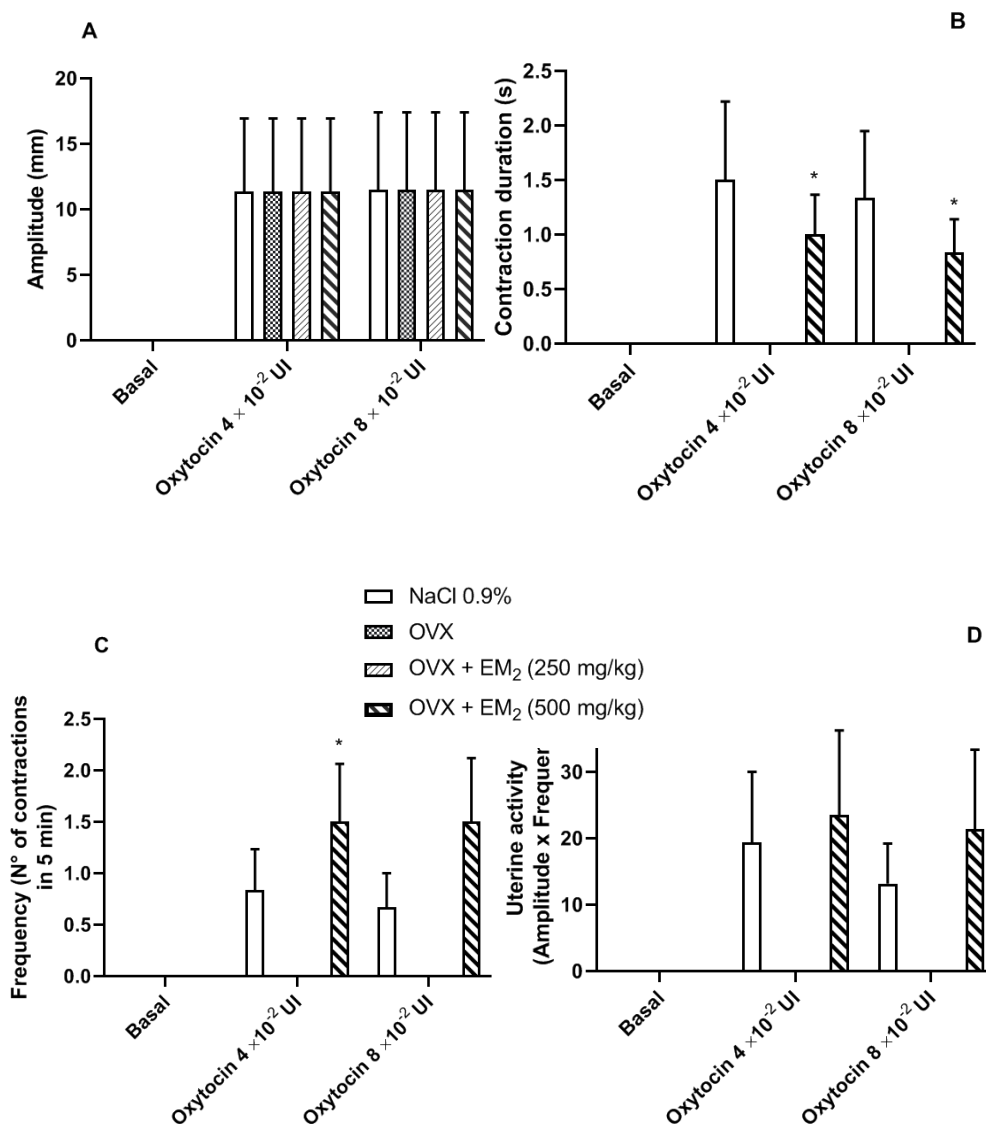

Figure No. 5
Effect of aqueous extract of *M. indica* (EM$_2$) on the isolated uterine organ of Sprague-Dawley rats induced by oxytocin. Significant differences are indicated with * when $p<0.05$ when compared to control group. (A) Amplitude of the contraction; (B) Duration of the contraction; (C) Frequency of the contraction; (D) Uterine activity.

It is well known that the increased ROS react with polyunsaturated fatty acids to induce the release of toxic and reactive aldehyde metabolites, such as MDA, which is one of the end products of the lipid peroxidation process (Hagihara *et al.*, 1984). We noted significant changes in MDA of ovariectomized (OVX) rats when compared to the blank group (Table No. 2). However, EM$_2$ treatment (250 mg/kg and 500

mg/kg) reduced lipid peroxidation when compared with the ovariectomized (OVX) rats. These results indicated that *M. indica* shows that benzophenone derivatives and xanthones present in the extract aqueous of the leaves of the mango act as exogenous antioxidants improved the endogenous antioxidant function associated with removing accumulated ROS,

preventing lipid peroxidation. The effects of EM_2 at doses of 250 mg/kg and 500 mg/kg on bone MDA are significantly ($p<0.001$ and $p<0.0001$ respectively) when compared to the control group (OVX). Moreover, mangiferin has been shown to protect osteoblast against oxidative damage via ERK/Nrf2 signaling (Xia *et al.*, 2017).

Table No. 2
Measurement of malondialdehyde (MDA) in erythrocytes, bone, liver and stomach

Experimental group	MDA levels (10^{-9} M/mL)			
	Erythrocyte	**Bone**	**Liver**	**Stomach**
Blank (NaCl 0.9%)	4.7167 ± 2.8630	0.3928 ± 0.1192	0.4893 ± 0.1119	1.4841 ± 0.6363
Control (OVX)	9.8601 ± 3.1666	1.3194 ± 0.3773	2.4616 ± 0.1279	2.1818 ± 0.7305
OVX + EM_2 (250 mg/kg)	5.7283 ± 2.8066	0.6017 ± 0.1668***	2.4366 ± 0.1218	1.8843 ± 0.6385
OVX + EM_2 (500 mg/kg)	6.1004 ± 3.4621	0.4785 ± 0.2617****	2.1087 ± 0.4081	2.6411 ± 1.2382

The values are mean ± standard deviation of (n=6) experiments. Significant differences according to Tukey test: *** $p<0.001$; **** $p<0.0001$ when compared to control group (OVX).

CONCLUSIONS
In summary, we reported here the chemical composition and the estrogenic and antioxidant effects in ovariectomized rats of the leaves of *Mangifera indica* L. cv. "Kent". Polyphenolic compounds characterized as benzophenone derivatives and xanthones exert as exogenous antioxidants against oxidative damage in ovariectomized rats.

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