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Table of Contents: Volume 9 Number 43 17 November, 2015

ARTICLES

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Full Length Research Paper

Effect of *Ginkgo biloba* extract on F1 generation of male Wistar rats during fetogenesis

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Incidence of endocrine disruptors on gonadal development is the cause of many anomalies in the male reproductive system (MRS). *Ginkgo biloba* is an endocrine disruptor with affinity for β estrogen receptors, which exert an important role in the development of MRS. This study evaluated the effects of *G. biloba* on the reproductive system of F1 males born to mothers exposed to *G. biloba* during fetogenesis. Pregnant Wistar rats received, by gavage, 25, 50 or 100 mg/kg/day of *G. biloba* from the 16th to 20th day of pregnancy. The testicular descent, morphology of the glans penis, sperm concentration, organ weights, testosterone serum concentration, and the structural organization of testicular and epididymal tissues remained unchanged. However, there was an increase in the number of abnormal sperm in all *G. biloba*-treated groups. These results indicate that *G. biloba* does not promote maternal toxicity and does not interfere with the fetal and postnatal development and age of puberty onset, but induces the increase of sperm abnormalities.

Key words: *Ginkgo biloba*, sperm abnormality, fetogenesis, estrogen receptor, rat.

INTRODUCTION

Estrogen plays an important role in testicular development and spermatogenesis. Its effect is modulated via nuclear estrogen receptors (ERs), which have two isoforms: estrogen receptor α (ERα) and estrogen receptor β (ERβ) (Lucas et al., 2010; Hess et al., 2011; Chimento et al., 2014). A new ER - "G protein- coupled estrogen receptor 1" (RSPG) (Prossnitz and Arteburn, 2015) has been identified in germ and somatic cells of rat testes and was reported to mediate the action of estrogen during testicular development and spermatogenesis by triggering rapid signaling events via different pathways (Lucas et al., 2010; Chimento et al., 2014). Knockout male mice strains for estrogen receptors (ERαKO but not ERβKO) or aromatase (ArKO) were shown to exhibit

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impaired reproductive capacity, reduced sperm motility, infertility, abnormal spermatogenesis, and dysmorphic testicular structure, indicating the importance of estrogen to male reproductive functions in adults (Carreau and Levallet, 2000; Habert et al., 2006; Hamilton et al., 2014). Furthermore, during fetal development, estrogen deficiency has been reported to increase the number of germ cells and steroidogenic activity (Delbès et al., 2006).

The incidence of abnormalities in the male reproductive system is increasing and can be related to changes induced by endocrine disruptors (EDs) on fetal and gonadal development (Joensen et al., 2008; Divall, 2013). Among the EDs, there are pesticides, plastic-derived materials and natural phytoestrogens, whose presence has already been described in semen, amniotic fluid, umbilical cord and maternal milk (Safe et al., 2010; Jefferson and Williams, 2011; Jones et al., 2014). Phytoestrogens disrupt the reproductive system by mimicking the action of endogenous estrogen when they bind to ERs due to its structural similarity to estradiol (17β-estradiol or E2) (Waring et al., 2008; Patisul and Adewale, 2009; Zhao and Mu, 2011).

The extract of *Ginkgo biloba* (EGb) has been used for centuries in Asia and Europe for the prevention of several disorders (Sierpina et al., 2003; Dekosky et al., 2008), including dementia, Alzheimer's, and cognitive impairment. It has also been reported to increase cerebral blood flow (Sierpina et al., 2003; Sun et al., 2007; Weinmann, 2010).

The standard extract (EGb 761) has 24% of phytoestrogens, such as kaempferol, quercetin, and ishorhamnetin, which are the main components of the extract (Oh and Chung, 2004). EGb 716 displays estrogenic activity as well as antiestrogenic activity that depend on the concentration of E2 and EGb on the estrogen receptor and its ability to reduce E2 levels by stimulation of catabolism and synthesis inhibition (Oh and Chung, 2006).

Studies related to the reproductive toxicity and developmental toxicity of EGb 761 are scarce. Treatment of mouse blastocysts with gingkolides has been shown to induce apoptosis, decrease the number of cells, decrease post-implantation development, and increase the index of deaths of early blastocysts (Chan, 2005). In the investigation of the mouse oocyte maturation, fertilization, and subsequent embryonic development, oocytes obtained from EGb-treated females following *in vitro* cultivation and fertilization exhibited decreased oocyte maturation, fertilization and embryo development rate as well as increased embryonic resorption and reduced placental and fetal weight (Chan, 2005). The EGb embryo-fetal developmental toxicity has already been addressed in rats and mice. For instance, administration of EGb by gavage to mice during the 6th to the 15th day of pregnancy did not increase the incidence of malformations and variations or delay in the fetus development (Koch et al., 2013), but intrauterine growth retardation in rat fetuses was noted when EGb was given during the post implantation period (Pinto et al., 2007).

Moreover, the assessment of the postnatal toxicity in the offspring born to female rats treated with EGb during the lactation period did not show alterations in the offspring's physical, sensory and motor development (de Faria et al., 2008). However, no evidence of effect on the reproductive system of adult F1 male offspring born to mothers treated with EGb in critical periods of development of the male reproductive system was found in the literature.

Fetogenesis is a sensitive and critical period for the development of the male reproductive system, since it establishes the onset of gametogenesis and steroidogenesis (Delbès et al., 2006; Jones et al., 2014). The gonocytes, for instance, proliferate until the 17th post-coital day in rats, remaining quiescent until the 3rd postnatal day (Delbès et al., 2006).

Considering the possibility of using EGb during pregnancy and its effect on the male reproductive system, this study aimed to evaluate the effects of the aqueous extract of *G. biloba* on the reproductive system of male Wistar rats after maternal exposure during fetogenesis.

METHODOLOGY

Forty female Wistar rats (*Rattus norvegicus* Berkenhout, 1769) obtained from the vivarium of the Reproduction Biology Center at the Federal University of Juiz de Fora (CBR/UFJF) were used. The animals were 3 months old, nulliparous, and weighed around 175 g.

Pregnant females were housed individually in polypropylene cages (49×34×16 cm) kept in acclimatized shelves (ALESCO®) with airflow, and a controlled temperature of 22 ± 2°C and a 12 h light/dark photoperiod. They were fed on rat chow pellets NUVILAB CR1® (Nuvital Nutrients Ltda., Colombo/PR) and received filtered water *ad libitum*. The experimental protocol was approved by the Ethics Committee in Animal Experimentation of the Federal University of Juiz de Fora (protocol number 081/2012).

Extract of *Ginkgo biloba*

G. biloba extract (EGb) was provided by JR Pharma. A quality control test was carried out by Gemini Industry Pharmaceutical Drugs Ltd. and showed the presence of 24% of flavonoids, 6% ginkgolides, and 2.2% of bilobalides.

Experimental design

The guideline for toxicity studies recommends the use of at least three test groups and a control group (OECD 414, 2001). Therefore, in the present study, pregnant females were randomly distributed into four groups, containing 10 animals each, and were treated by gavage, once daily, as follows: control group (C), 1 ml of distilled water, and three groups exposed to Egb (T.Egb) at the dose levels of 25 mg/kg/day (T.Egb 25); 50 mg/kg/day (T.Egb 50)

and 100 mg/kg/day (T.Egb 100). The lowest dose was calculated using the body surface area (BSA) as a conversion factor of a daily therapeutic dose from human to rat (FDA, 2005; Reagan-Shaw et al., 2007). The two other doses - 50 and 100 mg/kg/day, correspond to twice and four times the initial recommended dose and follow the guidelines of toxicological studies (OECD, 1995).

The first day of gestation was determined by the presence of sperm in the vaginal smear and treatment was performed during fetogenesis (from the 16th to the 20th day of pregnancy), a time period in which the gonocytes remain quiescent (Delbès et al., 2006). The date of birth was considered the first postnatal day (PND 1) and 72 h after birth, the litters were reduced to four pups per mother (two males and two females) that remained under observation until weaning at PND 25. After weaning, only male pups remained under observation until the day of euthanasia at PND 65. The female pups were used in other studies.

Maternal variables

Female rats were observed daily for the occurrence of clinical signs of toxicity, such as changes in locomotor activity, weight loss, piloerection, diarrhea, stereotypies, and death (OECD, 2009).

Food intake throughout pregnancy was assessed by the difference between a pre-established amount offered to the animals and what was left 24 h later. Body weight was measured every three days from the first day until the beginning of the treatments on the 16th day of pregnancy. During this period, the body weight was measured daily until birth. After delivery, the females were weighed on PND 4, which corresponded to the day of sexing of pups to reduce the number of pups per mother.

Variables of F1 generation

Before weaning, the pups were weighed separately by sex at PND 4, 10, 15, and 20. During the experimental procedure, the occurrence of deaths or cannibalization of pups by the mother was noted. After weaning, all male pups were weighed every five days until PND 65, when the animals were then anesthetized with intraperitoneal xylazine (10 mg/kg) and ketamine (90 mg/kg) (Wolfensohn and Lloyd, 1994) for blood collection by cardiac puncture, following euthanasia by cervical dislocation (CONCEA, 2013).

Testicular descent and differentiation of the glans penis morphology

The animals were monitored daily from DPN 15 until the testicular descent was completed. From DPN 25, the morphological differentiation of the glans penis was also monitored (Yamasaki et al., 2001).

Sperm assessment

After euthanasia, sperm were collected from the epidydimal secretion of the right epididymis cauda. The number of sperm was counted using a hemocytometer with improved double Neubauer ruling in a light microscope BX41TF model (Olympus Tokyo, Japan) under 100x magnification. The total number of spermatozoa was obtained by averaging two counts, corresponding to the upper and lower field in the Neubauer chamber (Seed et al., 1996).

Smears of spermatozoa were stained according to the Shorr method (WHO, 2010) for analysis of sperm morphology in the light microscope BX41TF (Olympus Tokyo, Japan) under 100x magnification. The spermatozoa were classified as normal (intact) or abnormal when displaying: amorphous head (loss of hook shape); no head and/or tail defects (coiled or broken tails and middle piece abnormalities). The evaluation of sperm morphology was made according to the criteria used by Seed et al. (1996) and Perreault and Cancel (2001). Four hundred sperm per animal from 10 animals per group were chosen at random for analysis. The procedure was performed in duplicate per animal.

Weight of organs

Laparotomy was performed after sperm collection for the removal and weighing of the testicles, left epididymis, seminal vesicle (without secretions), kidneys, liver, spleen, and pituitary. All organs were weighed on a precision scale model AV313CP (Ohaus®, Brazil).

Testicular and epididymal histomorphometric analysis and epithelium height

The testis and epididymis were fixed in Bouin. After routine histological processing, they were sectioned at 5 µm thickness in a rotation microtome (Microm®), and were stained with hematoxylin/ eosin and Gomori's trichrome, respectively. Later, the sections were examined to determine the volumetric proportion of tubular and intertubular testicular compartments, tubular diameter and epithelial height of the seminiferous tubule, and the epitheliumheight of the epididymal duct.

Diameter and epithelial height of the seminiferous tubule: Twenty cross-sections of the seminiferous tubules (most circular ones), from five animals per group, were captured in camera AxioCam (Zeiss) attached to the microscope Primo Star (Zeiss) with 10x objective lens. The greatest and the smallest diameters of each tubule were determined and the average of the two diameters was taken as the final diameter of each measured tubule.

For the calculation of the seminiferous epithelium height, four measures per tubule were made at the same place where the lines for the diameter measurement were positioned. The final height of the seminiferous epithelium comprised the average of these four measurements.

Epithelium height of epididymal duct: Twenty cross sections of the head, body, and tail segments of the epididymis (most circular ones) per animal (n = 4 animals/group) were made as mentioned earlier. Four measures of the duct epithelium height were taken and the average of these values was considered the height of the epithelium of each tubule.

Serum concentration of testosterone

The collected blood was centrifuged at 3500 rpm for 10 min in a RC-3 - Sorval® centrifuge. The serum was aliquoted and stored at -80°C. Thereafter, the serum concentration of testosterone was determined through ELISA immunoassay, using the microplate reader apparatus ASYS HITECH GMBH, and the competitive ELISA kit (Cayman Chemicals®).

Statistical analysis

Homocedastic and normal distribution data were analyzed by one

way analysis of variance (ANOVA) followed by post hoc Dunnett test. The measurement data of the testicular diameter were transformed into log base 10 for homogenization. For heteroscedastic and non-normal distribution data, the Kruskal-Wallis-test followed by the Monte Carlo test was used. Values with $p<0.05$ were considered statistically different. For multiple comparisons, the Monte Carlo test was applied with $\alpha = 0.01$.

RESULTS

Pregnant animals showed no clinical signs of toxicity during treatment. The average food intake and weight gain of the animals exposed to EGb (T.EGb 25, T.EGb 50, T.EGb 100) and the F1 generation were not significantly different during the experimental procedure when compared with control animals (results not shown).

Testicular descent was completed around DPN 26 in all groups, coinciding with the onset of differentiation of the glans penis morphology. The end of differentiation occurred around DPN 45, which corresponds to the pubertal period, and did not vary among treated and control animals.

No significant difference in the amount of sperm found in the epididymal tail secretion was observed, however, there was an increase in the number of abnormal sperm in all treated groups in comparison with the control group (Figure 1).

Sperm abnormalities were detected on the head, middle piece, and tail, but the obtained values did not differ significantly among the groups. Broken tail and amorphous head appeared as the most common defects (Table 1).

The weight of organs of the reproductive system, the seminal vesicle, pituitary gland, and other organs analyzed were not significantly different between control and treated rats (Table 2), indicating no systemic toxicity due to treatment with *G. biloba*.

The diameter and height of the epithelium of the seminiferous tubules, and the volumetric proportion of the tubular and intertubular testicular compartments did not differ significantly between the experimental groups (Table 3).

The serum testosterone concentration was largely variable, but there was no significant difference between treated and control values (Figure 2).

DISCUSSION

Maternal toxicity is an important factor related to embryo-fetal and postnatal development (Khera, 1985). Therefore, whenever the developmental toxicity is the subject of interest in a particular study, it is necessary to eliminate confounding factors, that is, it should be determined if the toxicity in the postnatal development is due to maternal toxicity or a direct effect of the tested substance on the offspring.

Maternal toxicity is observed clinically when there are changes in body weight (Chernoff et al., 2008), reduced locomotion, diarrhea, piloerection and decreased water and food consumption and deaths (Christian, 2001). In this study, none of these clinical signs were noted.

Although it is not known yet if EGb or any of its active constituents can pass through the placenta barrier, the intrauterine growth retardation found in fetuses of rats treated during fetogenesis may be an indicator that the extract or some of its components can reach and affect the fetuses (Pinto et al., 2007).

The variables analyzed in the generation of F1 males in this study cannot be compared to others in the literature, because to the best of our knowledge, this is the first study that evaluated the influence of EGb on male offspring. The F1 generation showed no significant change in body weight or in the weight of the organs examined. This suggests absence of systemic toxicity, since such changes may indicate lesions in the organs systems that lead to physiological impairment (Sellers et al., 2007). These data are also corroborated by the similar growth observed in the litter of the treated and control groups.

In the assessment of reproductive and developmental toxicity, preputial separation and glan penis morphology are among the endpoints in the investigation of abnormal maturation caused by androgenic or antiandrogenic compounds (Stoker et al., 2000; Yamasaki et al., 2001). Yamazaki and collaborators (2001) identified three stages in the normal growth of the rat glans penis which begins with a gradual alteration on the surface of the glans penis from a protuding os penis structure to a W shape (PND 30 - 35), and a subsequent change of the W shape to a flattened surface (PND 39 – 44). Apparently, EGb did not affect the physiological processes related to puberty since both testicular descent and the three stages of differentiation of the glans penis were similar among the groups and similar to the description provided by Yamasaki et al. (2001). These parameters are regarded as the animal's puberty indicators (Neill et al., 2006) used for the study of endocrine disruptors (Yamasaki et al., 2001). Testicular descent is a prerequisite for the production of mature spermatozoa and can be affected by the action of compounds with estrogenic activity (Klonisch et al., 2004). Although evidence suggests that *in utero* exposure to such compounds may inhibit testicular descent (Klonisch et al., 2004), in this study such evidence was not observed.

The testicles weight is associated with the number of germ cells present in this organ and the spermatogenic activity (Russell et al., 1990). The weight of the testis did not differ significantly between the control and EGb-treated groups, which is suggestive of absence of changes in the morphology of the seminiferous tubules and sperm production. Similar results were found in

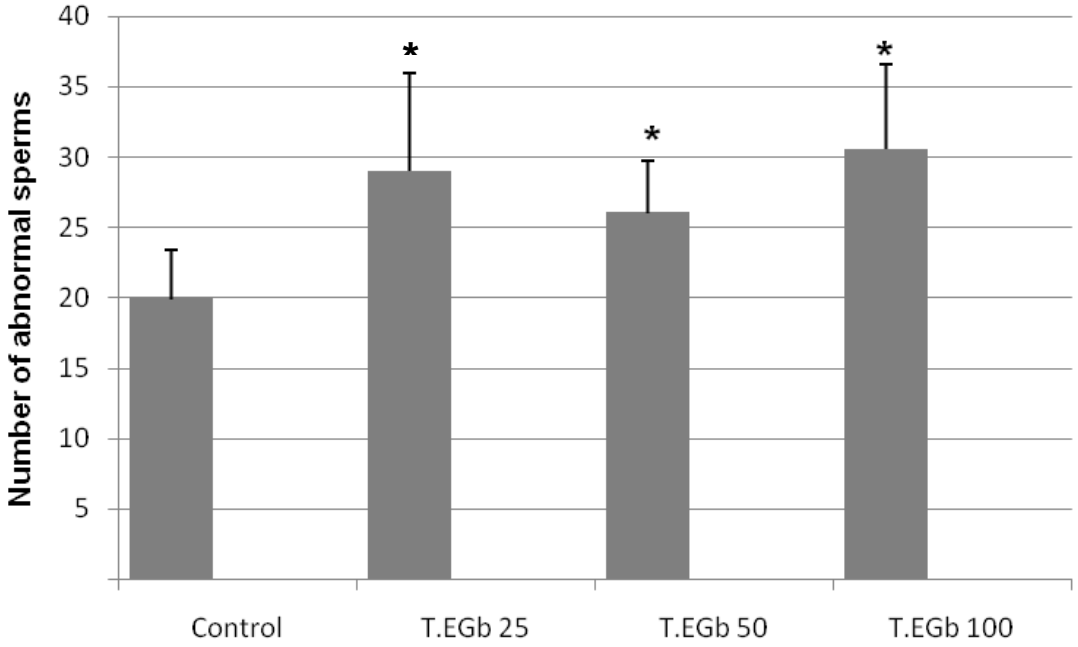

Figure 1. Abnormal sperm indexes from Wistar rats exposed *in utero* to distilled water (Control) and *Ginkgo biloba* extract (EGb) at doses of 25 (T.EGb 25), 50 (T.EGb 50) and 100 mg/kg (T.EGb 100) from the 16th to the 20th day of pregnancy. 400 sperm/animal were counted. *****p<0.05; n=10. Data analyzed by ANOVA, post hoc Dunnett t3. **p* T.EGb 25 = 0.001; *p* T.EGb 50 = 0.035; *p* T.EGb 100 = 0.001.

Table 1. Abnormal sperm from Wistar rats exposed in utero to distilled water (Control) and *Ginkgo biloba* extract (EGb) at doses of 25 (T.EGb 25), 50 (T.EGb 50) and 100 mg/kg (T.EGb 100) from the 16th to the 20th day of pregnancy. 400 sperm/animal were counted.

Abnormality	Control	T.EGb 25	T.EGb 50	T.EGb 100
Head	12.1 (24/199)	14.8 (43/290)	13.0 (34/261)	13.7 (42/306)
M. piece	20.6 (41/199)	21.4 (62/290)	24.9 (65/261)	22.2 (68/306)
Tail	67.3 (134/199)	63.8 (185/290)	62.1 (162/261)	64.1 (196/306)

Chi square p>0.05. Data expressed in percentage (absolute number of abnormals per region/absolute number of total abnormalities in the group).

Table 2. Mean weight of the organs of male Wistar rats (F1) born to mothers treated with*Ginkgo biloba* during fetogenesis (16th to 20th days of pregnancy). Control group (distilled water) and *Ginkgo biloba* extract (EGb) at doses of 25 (T.EGb 25), 50 (T.EGb 50) and 100 mg/kg (T.EGb 100).

Parameter	Control (n=20)	T.EGb 25 (n=20)	T.EGb 50 (n=20)	T.EGb 100 (n=20)	*p*
Right testis (g)	1.27 ± 0.15	1.28 ± 0.15	1.23 ± 0.06	1.26 ± 0.08	0.539
Left testis (g)	1.24 ± 0.12	1.25 ± 0.16	1.21 ± 0.10	1.26 ± 0.08	0.552
Left epididymis (g)	0.13 ± 0.01	0.13 ± 0.04	0.12 ± 0.01	0.12 ± 0.01	0.370
Seminal vesicle (g)	0.16 ± 0.02	0.16 ± 0.05	0.15 ± 0.01	0.15 ± 0.02	0.835
Spleen (g)	0.21 ± 0.01	0.22 ± 0.05	0.23 ± 0.05	0.22 ± 0.02	0.523
Left Kidney (g)	0.41 ± 0.03	0.42 ± 0.05	0.41 ± 0.05	0.42 ± 0.03	0.740
Right Kidney (g)	0.42 ± 0.03	0.43 ± 0.05	0.42 ± 0.03	0.41 ± 0.02	0.524
Liver (g)	4.49 ± 0.24	4.61 ± 0.46	4.57 ± 0.26	4.41 ± 0.30	0.160
Pituitary (g)	0.0040 ± 0.0012	0.0041 ± 0.0010	0.0038 ± 0.0018	0.0045 ± 0.0011	0.579

Data analyzed by ANOVA. *p* = p-value. Data expressed in average± standard deviation.

Table 3. Histometry of the seminiferous tubules and epididymal ductfrom Wistar rats exposed *in utero* to distilled water (Control) and *Ginkgo biloba* extract at doses of 25 (T.EGb 25), 50 (T. EGb 50) and 100 mg/kg (T.EGb 100) from the 16th to 20th days of pregnancy.

Parameter	Control (n=5)	T.EGb 25 (n=5)	T.EGb 50 (n=5)	T.EGb 100 (n=5)
TD(log) *	2.2679 ± 0.0025	2.2486 ± 0.0071	2.2635 ± 0.0027	2.2758 ± 0.0040
EH(µm)	58.88 ± 0.84	56.46 ± 0.83	59.72 ± 0.94	57.34 ± 0.71
EHH	18.45 ± 0.60	18.89 ± 1.20	16.62 ± 0.74	18.89 ± 0.62
EHB	14.65 ± 0.24	16.13 ± 0.65	15.19 ± 0.19	15.09 ± 0.74
EHT	17.33 ± 0.56	16.85 ± 0.32	18.13 ± 0.96	18.08 ± 1.16

TD: Tubular diameter (Seminiferous tubule); EH: epithelium height (Seminiferous tubule); EHH: epithelium height of the head (Epididymal duct); EHB: epithelium height of the body (Epididymal duct); EHT: epithelium height of the tail (Epididymal duct). Data analyzed by ANOVA. *p* = p-value. *post hoc Dunnett t3. p>0.05.

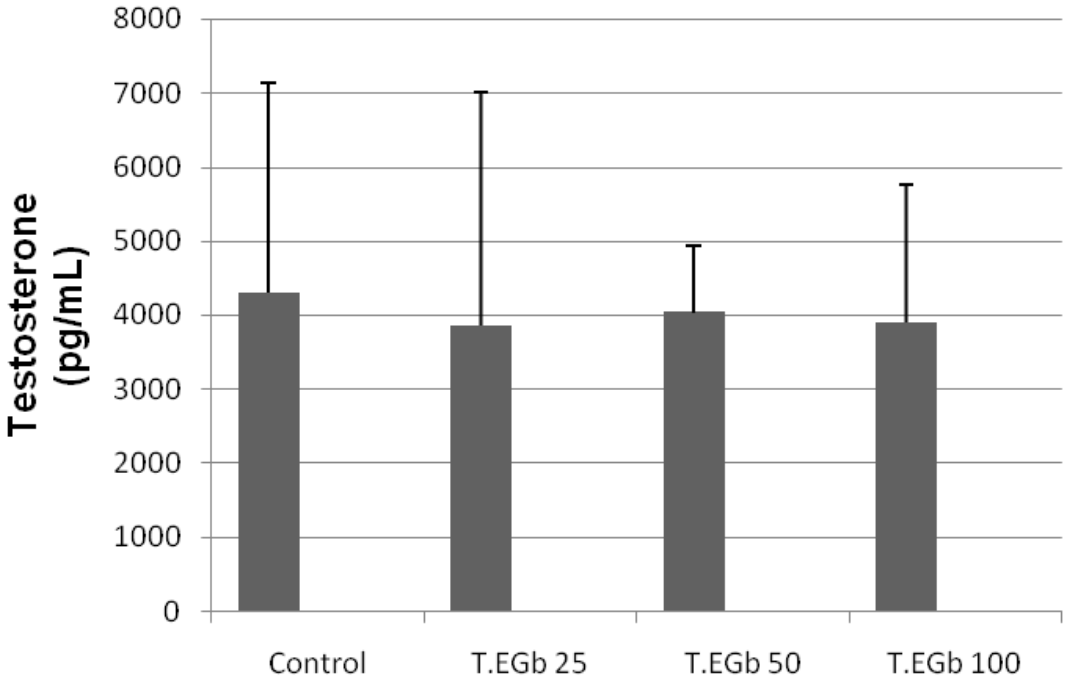

Figure 2. Total serum testosterone concentration(pg/ml) determined by ELISA assay from Wistar rats exposed *in utero* to distilled water (Control) and *Ginkgo biloba* extract at doses of 25 (T.EGb 25), 50 (50 T.EGb) and 100 mg / kg (T.EGb 100) from the 16th to 20th days of pregnancy. Data analyzed by ANOVA. *p*= 0.874.

studies with adult rats and mice, which showed no changes in the weight of the testis after oral administration of EGb (Al-Yahya et al., 2006; Yeh et al., 2008).

There is a positive correlation between the spermatogenic activity and the height of the seminiferous tubules and the seminiferous epithelium (França and Russell, 1998). Moreover, the diameter of the seminiferous tubule can also provide relevant information about possible alterations in spermatogenesis (Unsal et al., 2006). No changes in the height of the epithelium of the seminiferous tubule were observed in the EGB-treated groups, suggesting that EGb did not change the testicular histologic structure and the spermatogenesis process.

In the epididymis, the epithelium height is a parameter generally used to assess the epithelial secretory and absorptive activity (Hermo and Robaire, 2002). Each region of the epididymis has distinct functions; in particular, the tail compartment is responsible for sperm storage and phagocytosis of abnormal sperm (Robaire and Viger, 1995). This study did not identify significant changes in the height of the epididymal duct epithelium,

but showed increased number of abnormal spermatozoa in all groups treated with EGb, which may suggest that the phagocytic process was hindered by the treatment or the number of abnormal spermatozoa superior to the phagocytic capacity of the organ.

Serum testosterone levels are essential for the production of sperm as well as the development of androgen-dependent organs (Grumbach and Conte, 1992). No significant changes were observed in the serum concentration levels of testosterone in the EGB-treated groups when compared with control values. The similar serum testosterone concentrations corroborate the absence of changes in the weights of testosterone-dependent organs, such as the epididymis and seminal vesicle, and the indicators of puberty (testicular descent and morphology of the penis glans).

Although it has been shown that estrogen deficiency during fetal development increased the number of germ cells (Delbès et al., 2006), the obtained results showed that EGb did not alter the sperm concentration, but increased the number of abnormal sperm in all treated groups. The present study did not investigate the causes of these abnormalities. During fetogenesis, the testis begins the gametogenesis and steroidogenesis processes (Delbès et al., 2006; Jones et al., 2014): in the rat, the gonocytes proliferate until the 17th day of pregnancy, remaining quiescent until DPN 3 (Delbès et al., 2006), and from the 16th day of pregnancy, they begin to express the ERβ receptors (Saunders et al., 1998). Considering the administration of EGb to pregnant rats from the 16th to the 20th day of gestation and that EGb has estrogenic or antiestrogenic activity depending on the concentration of E2 and EGb on the estrogen receptor (Oh and Chung, 2006), it is possible to assume that EGB might have interfered with gene expression mechanisms of gonocytes during the period of quiescence, which may have led to the production of abnormal gametes.

In conclusion, the exposure of Wistar rats to EGb during fetogenesis, a critical period of the male reproductive system development, did not seem to cause maternal toxicity or alterations on fetal and postnatal development, and on the onset of puberty. Nevertheless, an increased number of abnormal sperm in the adult offspring was noted.

Conflict of interests

The authors have not declared any conflict of interests

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Antioxidant activity, total flavonoids and volatile constituents of *Magonia Pubescens* A.St.-Hil

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Magonia pubescens **A.St.-Hil (Sapindaceae) ('tingui') is a typical medicinal plant of the Cerrado biome. This plant is used as a larvicide and employed in poison fishing. However, little is known of its secondary metabolites. In this study, it is described for the first time as the volatile constituents of *M. pubescens*, collected via headspace. Qualitative phytochemical analyses were performed. In addition, the antioxidant activity of the ethanol extracts of flowers and leaves were evaluated, and the total flavonoids were quantified. The ethanol extracts of flowers (12.67 ± 0.05 rutin equivalent (EQ) g^{-1}) and leaves (11.81 ± 0.05 rutin EQ g^{-1}). The leaf extracts exhibited higher IC_{50} values (18.14 ± 0.02 rutin EQ g^{-1}) than did the flower extracts (31.19 ± 0.05 rutin EQ g^{-1}). Twenty volatile compounds were identified in *M. pubescens* flowers through gas chromatography coupled with mass spectrometry (GC-MS), being identified as benzilic acid (17.9%) and styrene (13.9%) as the major compounds. The antioxidant activity of *M. pubescens* could be related to the presence of flavonoids and tannins, but further studies need to be conducted to fully understand that correlation. The identified volatiles have the potential to be used in the cosmetics industry due to their socio-economic relevance, and the may also contribute to the understanding of the reproductive success of this species.**

Key words: *Magonia pubescens*, 'tingui', headspace, phytochemical screening, gas chromatography coupled with mass spectrometry (GC-MS).

INTRODUCTION

The Cerrado (Brazilian savannah) exhibits high biodiversity and includes a number of species with high bioactive potential (Rocha et al., 2008; Mendonça et al., 2008), such as *Magonia pubescens* A.St.-Hil, which is

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locally known as ‘tingui’ or‘timbó’. This plant is widely distributed in the central region of Brazil in the states of Goiás,Mato Grosso, Mato Grosso do Sul and Minas Gerais (Souza and Lorenzi, 2005). *M. pubescens* is a medium-size tree that reaches between five and nine meters in height and has paripinnate leaves, commonly used as an ornamental plant due to the lacy appearance of its foliage, and indicated for replantation of degraded areas (Lorenzi, 2000).

The fruits and seeds of *M. pubescens* are used to prepare a soap for the treatment of dermatitis and seborrhea and lice infestations and to also be used as insecticide (De Mesquita et al., 2009) and larvicidal (De Mesquita et al., 2009; Fernandes et al., 2005; Fernandes et al., 2007; Figueiredo et al., 2008; Vallotto et al., 2011). The roots infusions can also be used as a tranquilizer (De Mesquita et al., 2009).

Volatile compounds of this plant are associated with survival functions in the ecosystem; there chemical composition changes according to the gentic diversity and habitat of the plants (Siani et al., 2000).

Certain volatile compounds can also have antioxidant properties (Sacchetti et al., 2005). The most active and frequently occurring antioxidants of the plant origin are phenols, which include flavonoids (Pietta, 2000; Koleva et al., 2002; Pourmoradi et al., 2006). Flavonoids are the main flower chromophores, and flavonols (chalcones and aurones) are responsible for the yellow color of *M. pubescens* flowers (Brouillard and Harbone, 1998).

Plant secondary metabolites are important resources for biological applications, and their identification may contribute to the discovery of new biomolecules with potential applications on several areas of science (Sousa et al., 2007). *M. pubescens* has such potential; however, limited studies have been performed on this plant. The goal of the present study was to perform a phytochemical study of the main secondary metabolites to quantify the total flavonoids, evaluate the antioxidant activity of the flowers and leaves, and identify the volatile chemical composition of the flowers of *M. pubescens.*

METHODOLOGY

Collection of plant material

The flowers and leaves of *M. pubescens* were collected in Montes Claros, state of Minas Gerais, Brazil during spring in September of 2013. They were conservated in plant bags at -80°C and 0% relative humidity, in the dark. Voucher number: 106750- Herbarium of Montes Claros-HMC.

Plant extracts preparation

The leaves and flowers of *M. pubescens* were dried in an oven at 40°C (± 2°C) until a constant weight. The leaves were ground using a mill (Willey IKA A11B), and the flowers were ground using a mortar and pestle.

Crude leaf extracts (25%) were obtained through exhaustive maceration of the dried plant material (20 g) in ethanol: water (7:3) for seven days. Subsequently, the extract was filtered and evaporated.

The filtration residue was resuspended in the same solvent, and the extraction was repeated for three consecutive weeks. The resulting extract was stored in dark and cold (± 4°C) conditions until use.

Crude flower extracts (30%) were obtained by drying, grinding and homogenizing flowers (0.1 g ml^{-1}) in ethanol: water (6:4); subsequently, the samples were placed in an ultrasonic bath (UNIQUE) for 20 min. After 24 h of contact with the solvent, the extract was placed in an ultrasonic bath for an additional 20 min. Subsequently, the extract was filtered and evaporated. The resulting extract was stored in dark and cold (±4°C) conditions until use.

Chemical characterization

Qualitative tests to detect the contents of tannins, saponins, flavonoids, alkaloids, and terpenes were performed for the dry leaves and flowers: 10% neutral lead acetate and 2% iron choride reactions for tannins, 2% iron chloride and Shinoda reactions for flavonoids, Mayer, Bouchadart, Bertrand and Dragendorf reagents for alkaloids, Lieberman-Burchard reaction for sterols/triterpenoids, persistent foam test for saponins (Mouco et al., 2003; Barbosa., 2001).

Flavonoids content

The crude ethanol extracts (6:4) of flowers (0.33 g ml^{-1}) and leaves (0.38 g ml^{-1}) were diluted 400 times in the same solvent. Aliquots (0.5 ml) of the resulting solutions were transferred into tubes containing 0.5 ml 2% aluminum chloride (w/v). The mix was homogenized and left to stand for 30 min, and the absorbance was read at 410 nm using a spectrophotometer (Shimadzu). A calibration curve was obtained using a rutin commercial standard (Sigma Aldrich) at 0.01, 0.02, 0.04, 0.06 and 0.08 mg ml^{-1} (Fernandes et al., 2010). Measurements were performed in triplicate, and the results were expressed as rutin equivalent (EQ) g^{-1}. The statistic program used to calculate the standard deviation was Microsoft Office Exel 2007.

Antioxidant activity of leaves and flowers of *M. pubescens*

The antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH). 0.04% of DPPH solution reacted with crude ethanol (6:4) plant extracts (0.1 ml) at concentrations of 5, 10, 15, 20, 25 and 30 µg ml^{-1}. The mix was homogenized, and the free-radical scavenging capacity of the extract was measured as the absorbance at 517 nm using a spectrophotometer (Shimadzu) (Ramírez-Mares and De Mejía, 2003). Measurements were performed in triplicate, and the results were used to calculate the IC_{50}, which is the effective concentration at which 50% of the DPPH radicals are scavenged.The statistic program used to calculate the standard deviation was Microsoft Office Excel 2007.

Volatile characterization

The plant material (0.87, 0.96g, 0.67g, and 0.74 g) was stored individually in glass vials (20 ml) and placed in an auto sampler (HS combi-PAL). The flowers were homogenized at 500 rpm and incubated at 75°C for 5 min. The released volatiles were determined via headspace extraction and analyzed by gas chromatography coupled with mass spectrometry (GC-MS) under the conditions described in Table 1 (Aguiar et al., 2014).

GC-MS was performed using a gas chromatograph (Agilent

Table 1. Auto-sampler conditions for volatile extraction via static headspace (HS Combi-PAL).

Auto-sampler system for headspace extraction	Value
Injection volume (µl)	1000
Incubation temperature (°C)	75
Incubation time (m/s)	5 (300)
Syringe temperature (°C)	75
Agitation speed (rpm)	500
Fill speed ($\mu ls^{-1)}$	500
Fill strokes	0
Pullup delay (s)	500
Injection speed ($\mu ls^{-1)}$	500
Pre-injection delay time (ms)	0
GC run time (min)	47
Sample weight (g)	0.87, 0.96, 0.67, 0.74

7890A; Agilent Technologies), coupled with a mass spectrophotometer (MS 5975C) equipped with a fused silica capillary column HP-5 ms (30 m × 0.25 mm × 0.25 µm) using helium as the carrier gas (1 ml min^{-1}). Sample injection (1000 µl) was performed by split less injection using an auto injector (Combi PAL). The rate of temperature increase was 2°C min^{-1} from 35 to 80°C and then 4°C min^{-1} up to150°C, with a total run time of 42 min. The system was operated in the scan mode (monitoring) with electron impact ionization at 70 eV and scan mass range of 40 to 550 (*m/z*) (Aguiar et al., 2014).

The resulting data were analyzed using the software MSD Chemstation along with the National Institute of Standards and Technology Mass Spectral Library (NIST, 2009). The relative abundance (%) of the constituents was calculated from peak areas of the gas chromatogram (CG) and organized according to the order of elution. The percentage of each component was calculated using the normalized means of the chromatogram areas, and the compounds were identified through a comparison with the spectra of compounds deposited in the mass spectral library (NIST 2.0, 2009).

RESULTS AND DISCUSSION

Phytochemical screening, flavonoid quantification and antioxidant activity of *M. pubescens*

A qualitative evaluation of secondary metabolites, tannins, alkaloids, flavonoids, saponins and terpenes from the flowers and leaves of *M. pubescens* is described in Table 2. The leaves and flowers, showed a strong positive result for saponin heterosides and hydrolysable tannins was obtained and the presence of gallic tannins was observed. Isoflavonoids and chalcones were only identified in the leaves, while flavonols, flavones and flavonones were only found in the flowers.

Total tannins were moderately detected in the leaves, and total flavonoids were observed in the flowers. Total alkaloids were weakly detected in the leaves and flowers, and gallic tannins were observed in the flowers. Certain tests did not detect the presence of tannins and alkaloids in the leaves and flowers.

In a previous study of *M. pubescens* leaves, a negative result was observed for steroids/triterpenoids, a strong positive result was observed for saponins, a positive result was observed for tannins, a weak positive result was observed for alkaloids, and a negative result was observed for flavonoids (Silva et al., 2010). To our knowledge, studies on the phytochemistry of *M. pubescens* flowers have not been performed.

In the same study, a negative result was obtained for flavonoids using the Shinoda test (Silva et al., 2010), which is consistent with the results of the present study,wherein a negative result was observed for flavonoids using the Shinoda test and a positive result was observed using the aluminum chloride test (Table 2).

Regarding flavonoid concentration, the results for flowers and leaves was 12.67 ± 0.05 mg rutin EQ. g^{-1} and 11.81 ± 0.05 mg rutin EQ. g^{-1}, respectively. These values are similar to those reported in a study analyzing the aqueous extracts of *Achillea millefolium*, an herb with medicinal potential (Eghdami and Sadeghi, 2010; Masika and Alfalayan, 2003).

The antioxidant potential of the leaves and flowers was evaluated through their capacity to inhibit the oxidation of DPPH free-radicals, and the potential was expressed as the IC_{50} (Huang et al., 2005), which was 18.14 ± 0.02 µg ml^{-1} for the leaves, 31. 19 ± 0.05 µg ml^{-1} for the flowers, and 1.47 µg ml^{-1} for the gallic acid standard.

In addition, *Aristolochia bracteata* has been reported to be an accessible source of natural antioxidants and exhibited IC_{50} values similar to the ones observed for *M. pubescens* (Farias et al., 2013).

Volatile profile in flowers of *M. pubescens*

Twenty volatile compounds were identified in *M. pubescens* flowers (Table 3), and they belonged to five different classes: furanoids (3.9%), esters (9.3%), alcohols (43.8%), aldehydes (24.5%) and hydrocarbons

Table 2. Phytochemicalscreening of the leaves and flowers of *Magoniapubescens.*

Class	Test	Leaf	Flower
Tannins	Ferric chloride	++	+
	Alkaloid aqueous solution	-	-
	Neutral lead acetate	+++	+++
	Copper acetate	++	++
	Tanninspecific	+++	+
Saponins	Persistent foam	+++	+++
Flavonoids	Shinoda	-	+++
	Aluminum chloride	+++	++
Alkaloids	Mayer reagent	-	+
	Bouchadart reagent	+	-
	Bertrand reagent	+	+
	Dragendorf reagent	+	+
Triterpenes	Liebermann-Burchard reaction -	+++	++

(-) Negative, (+) Weak positive, (++) Moderate positive, (+++) Strong positive.

(18.5%).

Benzyl alcohol (8) is a methylated derivative of benzenoid, and it was the most abundant compound (17.9%), thus indicating the presence of O-methyltransferase, an enzyme that catalyzes the transfer of methyl groups to hydroxyl or carboxyl groups within a vast range of receptor molecules. Benzenoids are involved in the biosynthesis of odoriferous substances (Alves et al., 2005) and many of them are important to the cosmetics industry.

Volatile compounds are widely used as a flavoring for foods, confections and spices and as a fragrance in perfumes and cosmetics. They are also used in the production of several skin products because of the complexity of their active compounds, significant aromatic properties and market value (Zellner et al., 2009)

Linalool [10 (1.9%)] and methyl salicylate [13 (7.8%)] are important compounds for the perfume industry (Sell, 2003; Lapczynski et al., 2007). Methyl salicylate is more abundant than linalool, and it is found in several pharmaceutical forms and used in cosmetic and non-cosmetic products, such as cleaning products (Lapczynski et al., 2007). Methyl salicylate is responsible for the refreshing character in the scent of oil, and octanal [7 (1.7%)] is responsible for citrus scents (Zellner et al., 2009; Mahattanatawee et al., 2007).

Linalool oxide [9 (3.9%)] occurs n *M. pubescens* flowers and is a common chemical in floral aromas that is known to be an important mediator of pollination (Knudsen and Tollslen, 1993), particularly with bees for *M. pubescens* (Dewick, 2009).

During evolution, plants developed the ability to attract pollinators as well as defense mechanisms against herbivores and pathogens. These chemical defenses are composed of compounds that are produced and stored for immediate lease following an attack and through the synthesis of new compounds induced in response to attacks (Almeida et al., 2003).

Styrene is the second most abundant compound found in *M. pubescens*, and it appears to possess defensive properties. The high production of styrene compared with other compounds is not clearly explained in literature, and there are almost no studies directly reporting the effects of styrenein plants (Gatehouse, 2002).

Styrene [4 (13.9%)] is a toxic hydrocarbon with a balsamic scent (Stolarska et al., 2010), and it has deleterious effects on the health of organisms in general and is a potential carcinogen. In high concentrations, styrene has an inhibitory effect on seed germination. Winter wheat grown under atmospheric concentrations of styrene between 570 and 2.280 mg m^{-3} was observed to grow more slowly than controls and exhibit a number of anatomical changes (Leffingwell and Alford, 2005).

Methyl salicylate [13 (7.8%)], is a salicylic acid ester that may also be associated with the defense mechanisms of *M. pubescens*. Methyl salicylate occurs widely in plants (Jayasekara et al., 2002), because its production is associated with induced resistance to phytopathogens (Seskar et al., 1998).

A repellent effect of this compound higher than 80% was observed in the larvae of *Boophilus microplus* (Novelino et al., 2007), and terpenoid volatile compounds

Table 3. Volatile constituents detected in flowers of *Magonia pubescens* by gas chromatography coupled to mass spectrometry (GC-MS).

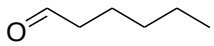
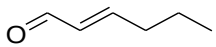
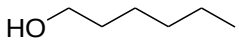
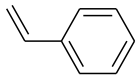
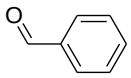
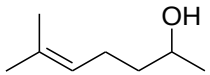
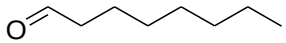
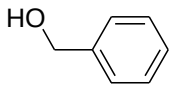
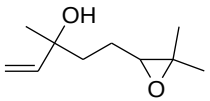
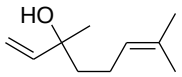
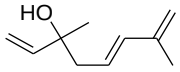
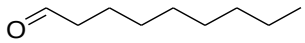
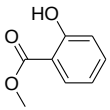
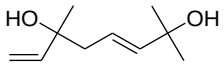
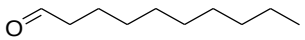
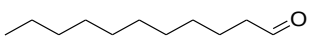
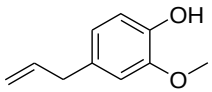
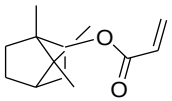
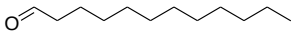
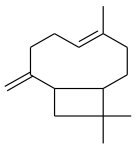
S/N	R.T.[a]	Compounds	Area (%)	M.F.[b]	M.S.[c]	C.I.[d]
1	6.5	Hexanal	10.4	$C_6H_{12}O$		82 (22), 72 (23), 67 (18), 57 (53), 56 (87), 45 (18), 44 (87), 43 (51), 41 (73), 40 (100)
2	8.8	2-Hexenal	3.2	$C_6H_{10}O$		98 (M+35), 83 (83), 80 (18), 70 (24), 69 (83), 57 (51), 55 (88), 43 (23), 42 (68), 39 (71)
3	9.7	1-Hexanol	13.1	$C_6H_{14}O$		84 (5), 69 (36), 56 (100), 55 (53), 54 (4), 45 (4), 43 (48), 42 (33), 41 (36), 39 (12)
4	10.6	Styrene	13.9	C_8H_8		104 (M+100), 103 (49), 102 (9), 78 (40), 77 (22), 75 (4), 52 (6), 51 (17), 50 (8), 40 (7)
5	14.9	Benzaldehyde	2.3	C_7H_6O		106 (M+86), 105 (100), 78 (21), 77 (81), 51 (25), 50 (18), 40 (22), 39 (8)
6	17.2	6-methyl 5-hepten-2-ol	1.8	$C_8H_{16}O$		128 (M+12), 110 (22), 95 (100), 71 (19), 69 (33), 68 (13), 67 (19), 55 (17), 53 (14), 45 (19)
7	18.0	Octanal	1.7	$C_8H_{16}O$		85 (30), 84 (66), 82 (48), 81 (68), 57 (77), 56 (70), 55 (26), 45 (35), 43 (46), 41 (100)
8	20.1	Benzyl alcohol	17.9	C_7H_8O		108 (M+100), 107 (68), 91 (15), 80 (9), 79 (100), 78 (12), 77 (61), 51 (22), 50 (9)
9	23.6	Trans-Linalool oxide	3.9	$C_{10}H_{18}O_2$		155 (15), 111 (38), 94 (65), 93 (32), 81 (23), 79 (15), 68 (31), 67 (26), 59 (100), 55 (41)
10	24.7	Linalool	1.9	$C_{10}H_{18}O$		121 (31), 93 (76), 80 (24), 77 (18), 71 (100), 69 (47), 67 (21), 55 (47), 43 (49), 41 (45)
11	24.9	Hotrienol	2.3	$C_{10}H_{16}O$		82 (81), 81 (18), 79 (12), 71 (100), 67 (31), 55 (12), 53 (11), 44 (8), 43 (35), 41 (11)

Table 3. cont'd

12	25.1	Nonanal	3.1	$C_9H_{18}O$		82 (53), 81 (40), 70 (55), 57 (100), 56 (89), 55 (61), 44 (49), 43 (71), 42 (36), 41 (81)
13	29.8	Methyl salicylate	7.8	$C_8H_8O_3$		153 (4), 152 (M^+ 54), 121 (29), 120 (100), 93 (14), 92 (59), 65 (17), 64 (10), 63 (10)
14	29.9	2,6-dimethyl-3,7-Octadiene-2,6-diol	4.9	$C_{10}H_{18}O_2$		85 (5), 83 (6), 82 (100), 72 (4), 71 (64), 67 (40), 55 (7), 43 (41), 41 (10), 40 (5)
15	30.7	Decanal	1.2	$C_{10}H_{20}O$		71 (67), 70 (74), 68 (54), 57 (97), 56 (58), 55 (88), 44 (41), 43 (83), 41 (100), 40 (18)
16	35.1	Undecanal	1.4	$C_{11}H_{22}O$		126 (30), 95 (51), 82 (75), 81 (55), 71 (50), 68 (45), 57 (100), 55 (84), 43 (65), 41 (66)
17	36.6	Eugenol	1.9	$C_{10}H_{12}O_2$		164 (M^+100), 149 (37), 137 (21), 131 (44), 103 (34), 91 (33), 77 (28), 51 (13) 39 (16)
18	37.4	Isobornyl acetate	1.5	$C_{13}H_{20}O_2$		136 (52), 121 (58), 108 (32), 95 (100), 93 (46), 69 (38), 67 (26), 55 (73), 43 (5), 41 (16)
19	38.8	Dodecanal	1.2	$C_{12}H_{24}O$		140 (24), 97 (43), 83 (46), 82 (88), 69 (82), 67 (63), 57 (100), 56 (46), 55 (91), 41 (86)
20	38.9	Caryophyllene	4.6	$C_{15}H_{24}$		204 (M^+8), 133 (88), 120 (48), 107 (49), 105 (56), 93 (100), 91 (86), 79 (72), 69 (65), 41 (56)

Total compounds (%)	
Furanoid	3.9
Esters	9.3
Alcohols	43.8
Aldehydes	24.5
Hydrocarbons	18.5

[a]Retention time, [b] Molecular formula, [c]Molecular structure, [d]Characteristic ion, [e]Molecular weight according to the NIST 2.0 library.

such as methyl salicylate [13 (7.8%)] were reported to repel aphids and other insects by inhibiting their attraction to host plants (Norin, 2001). Methyl salicylate appears to have a number of functions and is involved in different chemical signaling pathways (Norin, 2001).

A repellent effect was also observed for isobornylacetate [18 (1.5%)], and it has been suggested that the toxicity of essential oils to insects depends on the chemical composition, including the presence of isobornylacetate [18 (1.5%)] (Lee et al., 2001). A repellent effect of linalool [10 (1.9%)] has been described for several insects (Labinas and Crocomo, 2002; Castro et al., 2006; Lima et al., 2009; Wang et al., 2011; Niculau et al., 2013), and this compound has been found to exhibit higher toxicity than other tested compounds in certain studies (Wang et al., 2011; Niculau et al., 2013).

Eugenol [17 (1.9%)] was found in the same percentage as linalool, and it is also a strong repellent that causes behavioral reactions in a number of insect species (Krell and Kramer, 1998). In addition, several volatile compounds, especially eugenol [17 (1.9%)], are associated with plant stress (Silva et al., 2012).

Caryophyllene [20 (4.6%)] is known for its anti-inflammatory and anti-fungal properties, and it is used as local anesthetic and was observed to have cytotoxic effects on a wide range of cell lines (Fernandes et al., 2007; Ashour et al., 2007). Anti-fungal effects were also observed for the aldehydes hexanal [1 (10.4%)] (Almenaret al., 2007; Neri et al., 2006; Baggio et al., 2014) and nonanal [12 (3.1%)], which prevented the germination of *Penicillium digitatum* and *Penicillium italicum* conidia at high concentrations (Droby et al., 2008). At low concentrations, however, it favored conidia development.

To our knowledge, the volatile composition of *M. pubescens* flowers has not been studied. Benzaldehyde [5 (2.3%)], hexanal [1 (10.4%)], and nonanal [12 (3.1%)], which were observed in *M. pubescens* flowers in this study, have been previously observed through head space extraction of the volatiles of fresh, unheated, and desalinated (NaCl) samples and in heated and salted (NaCl) samples of the fruits of *Nephelium lappaceum* (Sapindaceae)(Laohakunjit et al., 2007). Linalool [10 (1.9%)] and 1-hexanol [3 (13.1%)] were also found in cultivars of *Dimocarpus longan* (Sapindaceae) (Zhang et al., 2009). The remaining detected compounds (2, 6, 11, 14, 15, 16, 19) are involved in the synthetic pathways of these volatiles, few studies are focused on these type of compounds.

Conclusion

The leaves and flowers of *M. pubescens* have high bioactive potential that could be related with the presence of some secondary metabolites detected in the present study. Saponins, flavonoids and tannins were the main classes of compounds detected, having the last two classes effective action against free radicals. The volatile compounds identified in the present study are promising molecules for the cosmetic and pharmaceutical industries and may be involved in interactions that promote the reproductive success of *M. pubescens*.

Conflict of Interests

The authors have not declared any conflict of interests.

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