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Full Length Research Paper

Light spectra affect the morphoanatomical and chemical features of clonal *Phyllanthus tenellus* Roxb. grown *in vitro*

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Phyllanthus tenellus Roxb. is a widely distributed tropical medicinal plant and highly valued for its therapeutic properties. Since variable light conditions can significantly alter phenolic compounds that are the main therapeutic constituents of *P. tenellus*, including tannins and flavonoids, the development of this plant and its chemical metabolism in response to different light spectra were investigated. To accomplish this, *P. tenellus* was cultured in modified Murashige and Skoog (MS) medium for 60 days, and its development, leaf anatomy and phytochemistry were analyzed after exposure to white, red, green, yellow and blue light, as well as darkness. Compared to white light, the best *in vitro* morphogenic responses, including rooting percentage, shoot height, number of leaves, and number and length of branches, occurred under exposure to blue and yellow light. Plantlets developed under white and blue lights presented the greatest thickness of palisade and spongy parenchymas. Under dark condition, plantlets showed fragile aspect and the lowest thickness of leaf tissues. In contrast to other light treatments, chlorophyll and carotenoid contents were significantly lower in plants maintained under green light, whereas yellow light improved the production of phenolic compounds. These results highlight the influence of different light spectra on morphoanatomical features and suggest how different light spectra affect secondary metabolite production in the context of preserving this plant's therapeutic integrity.

Key words: *In vitro* culture, light spectra, phenolic compounds, photomorphogenesis, *Phyllanthus* sp., plant development.

INTRODUCTION

Phyllanthus tenellus Roxb. (Phyllanthaceae), a tropical

medicinal plant, is widely dispersed in Brazil. In particular,

this species, also known as quebra-pedra or erva-pombinha, is used in folk medicine to treat kidney disease, urinary bladder disturbances and hepatitis (Lorenzi and Matos, 2008). The therapeutic value of *P. tenellus* arises from its phenolic compounds, including tannins and flavonoids (Huang et al., 2003). Environmental, physical and biotic factors all interact to affect plant development, resulting in morpho-anatomical and physiological phenotypic plasticity (Catoni et al., 2015).

Of all these factors, light quality is preeminent by its ability to influence all phases of plant development, from germination to reproduction, as well as primary and secondary metabolism (Franklin, 2009). Different light qualities may also represent an abiotic stress for the plant, affecting its level of plastidial proteins by the degradation of rubisco enzyme, finally changing its metabolism (Feller et al., 2008). The light-dependent development of plants is a complex process that involves photoreceptor families of red-absorbing phytochromes and UV-A/blue light-sensing cryptochromes (Chen et al., 2004; Franklin, 2009). Some red light responses include adventitious shoot formation, primary leaf development, stimulation of seed germination, inhibition of internode elongation, induction of flowering by action on photoperiod, changes in leaf anatomy layers and synthesis of anthocyanin and phenolic compounds (Chen et al., 2004; Macedo et al., 2011; Victório et al., 2011).

Cryptochromes are flavoproteins involved in photorepair of UV-damaged DNA, and they regulate a wide range of responses in plants, such as inhibition of hypocotyl elongation and leaf expansion, pigment biosynthesis, growth of stems and internode elongation, stomatal opening, chloroplast migration, control of flowering time and phototropism (Franklin, 2009). Additionally, different light spectra interfere with secondary plant metabolism in the acetate-malonate and shikimate pathways to either promote or inhibit the production of phenolic compounds. However, by using tissue culture techniques, it is possible to evaluate how some previously selected conditions may influence plant development responses. Based on this hypothesis, the present study aimed to evaluate the development, leaf anatomy and production of phenolic compounds of *P. tenellus* under specific light qualities and darkness.

MATERIALS AND METHODS

Plant

Samples of the mother plant of *P. tenellus* Roxb. were obtained from the medicinal plant garden at the Biophysics Institute Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (Rio de Janeiro State, Brazil). The voucher specimen is deposited at the Herbarium of the National Museum of Rio de Janeiro under number

R 200872.

Tissue cultures

Cultures were established by Victório et al. (2010). Nodal segments (0.8-1.0 cm) were excised from *in vitro* culture seedlings and transferred to flasks (141 × 72 mm) containing 60 ml of basic MS (Murashige and Skoog, 1962) medium reduced to half of NH_4NO_3 and KNO_3 concentrations ($\text{MS}\frac{1}{2}\text{N}$). Plantlets were subcultured every 60 days and maintained at $25 \pm 2^\circ\text{C}$ with a photoperiod of 16 h. Light-quality experiments were performed in growth chambers (controlled environments) equipped with Sylvania® fluorescent tubes (F20 W T-12) [approximately $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR)] to provide different light spectra: white, blue, green, red, and yellow (Figure 1). White light and darkness conditions were used as control treatments to assess the effect of light on plantlets in the same medium formulation. Light intensities were measured by a quantameter (Biospherical Instruments Inc., QSL-100). Each treatment was completely randomized and consisted of four sets with at least 10 plantlets. Plantlet development was evaluated for 60 days according to the following parameters: number of shoots, number of branches; shoot height (main vegetative axis/main stalk); length of branches; rooting percentage; fresh and dry weights; and senescence percentage. For dry weight determination, groups of 10 plantlets from each treatment were dehydrated at 60°C for 24 h.

Anatomical analysis

Sixty-day-old clonal plantlets of *P. tenellus* exposed to different light qualities were used in histological studies. Branches were fixed in ethanol (70%) during 48 h. Then, leaf samples from the third node of branches of each group of three plantlets were dehydrated in an ethanol series (80, 90 and 95% each hour), infiltrated and embedded in basic Histo-resin (Leica Microsystems, Germany), then sectioned with a rotary microtome (820 Spencer Microtome, American Optical Corporation, USA). Sample sections 8 μm in thickness were stained in toluidine blue (0.05%) and prepared on permanent slides. The stained sections were examined, and drawings were performed using a Carl Zeiss optical microscope (model 4746.20-990) with a camera lucida attachment. Anatomical measurements were made using an optical Zeiss Axioskop 2 microscope (model DEI-750D, CE) equipped with computer and video and digital cameras (Optronics). About 30 measurements per treatment were carried out for each anatomical parameter. The thickness of adaxial and abaxial epidermis, including papillae and palisade and spongy parenchymas, was evaluated.

Photosynthetic pigments content

To determine total chlorophylls (a and b) and carotenoids, fresh leaves (50 mg) were macerated in dimethylsulfoxide (DMSO) and incubated in hot water at 60°C for 16 h, in semi-darkness. Determination of chlorophyll and carotenoid concentrations was obtained using a spectrophotometer (Spectronic Genesys 2 SERL 3N270093004). After filtering, absorbance of the extracted solution was measured at 649 nm (a chlorophyll), 665 nm (b chlorophyll) and 480 nm (carotenoids). Total concentrations of chlorophyll (chlorophyll a and b) and carotenoids on a fresh weight basis ($\mu\text{g mg}^{-1}$) were calculated according to the following equations

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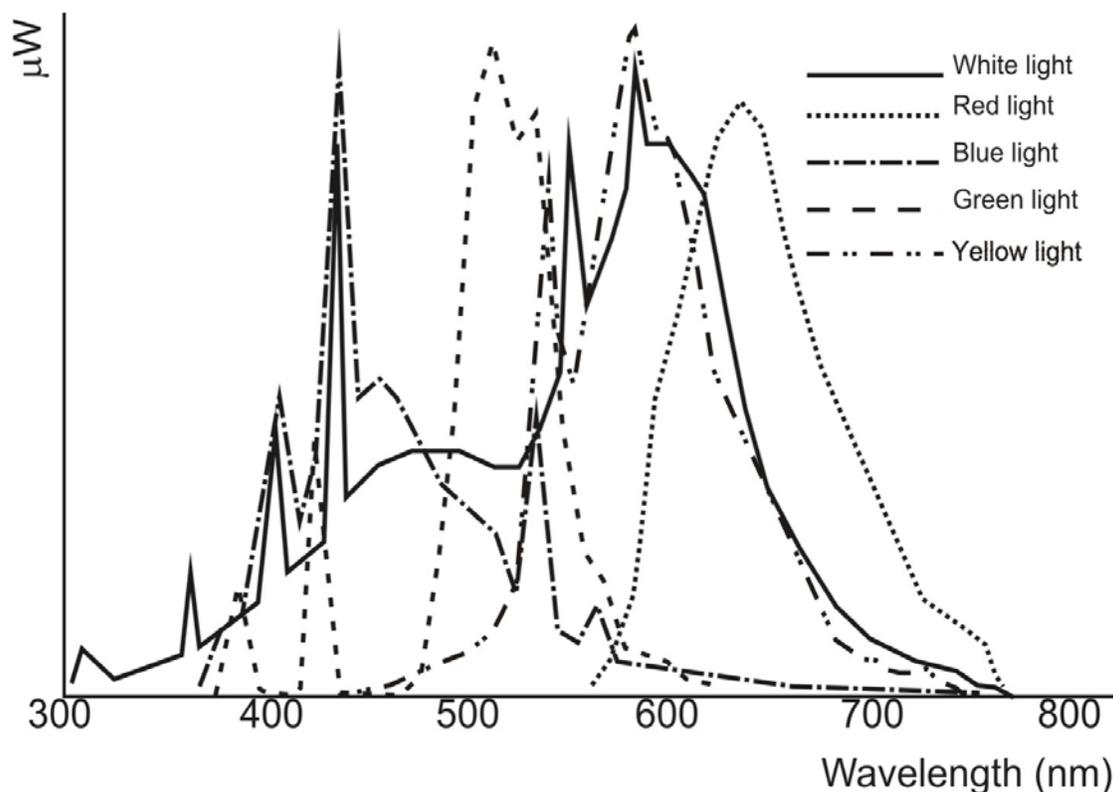


Figure 1. Emission spectra of the light sources used in the different treatments.

(Wellburn, 1994):

$$Ca = 12.19 A665 - 3.45 A649; Cb = 21.99 A649 - 5.32 A665; Cx+c = (1000 A480 - 2.14 Ca - 70.16 Cb) / 220$$

Where Ca and Cb are chlorophyll a and b concentration, respectively, and Cx+c is the total carotenoid content.

Chemical analysis

P. tenellus aqueous extract was obtained from dried leaves from about ten plants. Tubes containing a distilled water mixture with dried leaves at 1 g/20 ml were immersed in boiling water for 30 min. Crude extracts were filtered, dried using a lyophilizator, and then dissolved in MilliQ water at 10 mg/ml. High performance liquid chromatography-ultraviolet (HPLC-UV) analyses were performed on a Shimadzu setup equipped with a SPD-M10A diode array detector, LC-10AD pump and CBM-10 interface. Data were acquired and processed by a reversed phase column (Rexchrom®, 25 cm × 5 mm, 5 μm). Analytical separation was done in the following mobile phase: A – H₂O (MilliQ); B - KH₂PO₄ 0.1 M plus H₃PO₄ 0.1 M plus CH₃CN and C – MeOH with a gradient of solvent B where B = 50% (10 min), B = 50% - 100% (30 min) and B = 50% plus C = 50% (40 min) at a flow rate of 1 ml/min under ambient temperature. The injection volume was set at 20 μl. Detection was accomplished with a diode array detector, and chromatograms were recorded at 280 nm. Qualitative determinations were obtained after two separated extractions from plantlets of each light treatment, and samples were injected in duplicate. Methanol, phosphoric acid, acetonitrile, and

potassium dihydrogen phosphate were analytical grade. Geraniin was used as a standard of tannin.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and statistical average comparisons were made through Tukey's test at 5% significance level. A test of difference between two percentages was conducted, considering $P \leq 0.05$ by t-test.

RESULTS

Figures 2 and 3 show the morphogenic changes of 60-day-old *P. tenellus* plantlets under different light spectra and darkness. Under white light, plantlets presented a healthy aspect, showing a continuous growth that reached 2.6 cm and a rooting percentage of 73.2% within 60 days. Although no other light spectrum improved the number of shoots within 60 days, a greater number of nodes per shoot were obtained under blue light (4.9). The number of nodes may be used as an indicator of proliferation rate. In contrast to white light, shoots elongated more under yellow and blue light, but no change in inter-node elongation was observed among the different light spectra. The highest number of leaves was recorded under yellow light treatment (Figure 3E),

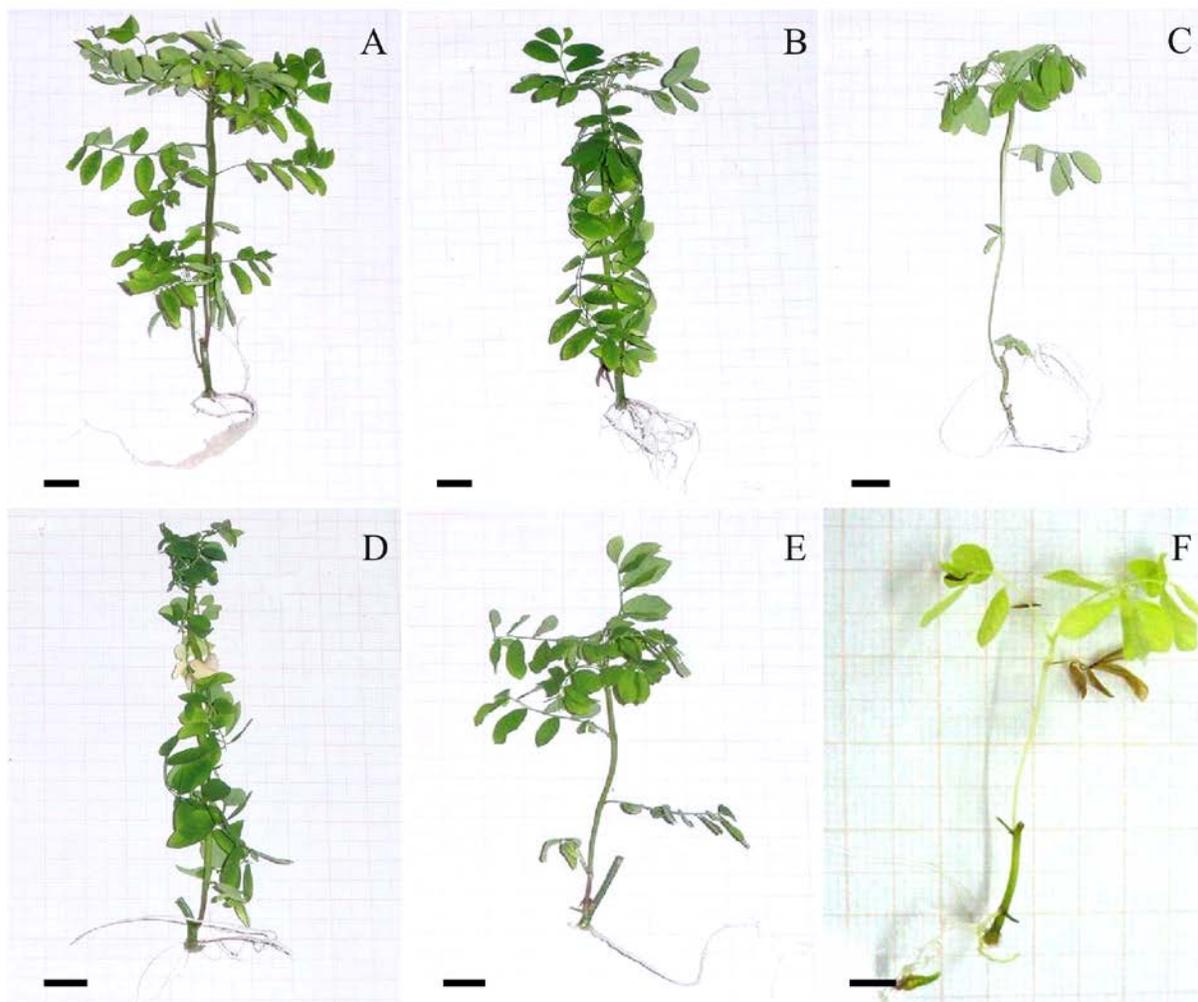


Figure 2. Sixty-day-old plantlets of *P. tenellus* cultured under different light spectra: (A) white, (B) blue, (C) red, (D) green, (E) yellow and (F) darkness. Scale bar=1 cm (A - E) and 20 cm (F).

showing significant differences compared with white light and darkness. Plantlets maintained under red light presented a green homogeneous color for the full width of their leaves, and the length of their branches was higher than that of plantlets under white light treatment. Cultures maintained under green light showed this spectral range as active in the morphogenesis process, resulting in an increased number of shoots and roots. Blue and green lights induced the greatest rooting (Figure 3F). Roots grew thicker under red light when compared with other light treatments. Rooting of plantlets cultured in darkness was 88.2% greater than the percentage of rooting cultured under white light (Figure 3F), thus confirming the ability of *P. tenellus* to establish rooting in darkness, as well as light.

Over the course of 60 days, it was verified that red light delayed leaf senescence of *P. tenellus* plantlets. Plantlets maintained under yellow light and in darkness presented

senescence rates greater than 50%, whereas the rates under green light were lower (15.4%) (Figure 3H). No correlation was confirmed between senescence and the decrease of chlorophyll and carotenoid contents (Figure 4). The statistical difference between chlorophyll and carotenoid contents was achieved by the effects of green light, which showed a reduction in carotenoid contents, as well as a and b chlorophylls (Figure 4). Fresh and dry weights of *P. tenellus* plantlets increased by the effects of blue light; these data are in agreement with the highest number of branches (Figure 3G). Transverse sections showed that leaves are amphistomatic with unistratified epidermis. Epidermal cells have an irregular shape and sinuous anticlinal walls. Papillae can be observed on both sides of the leaf blades. Under white, red and green light, the greatest variations in papillae were found on the abaxial side. In transverse sections of leaves of plantlets maintained under darkness, papillae were not observed

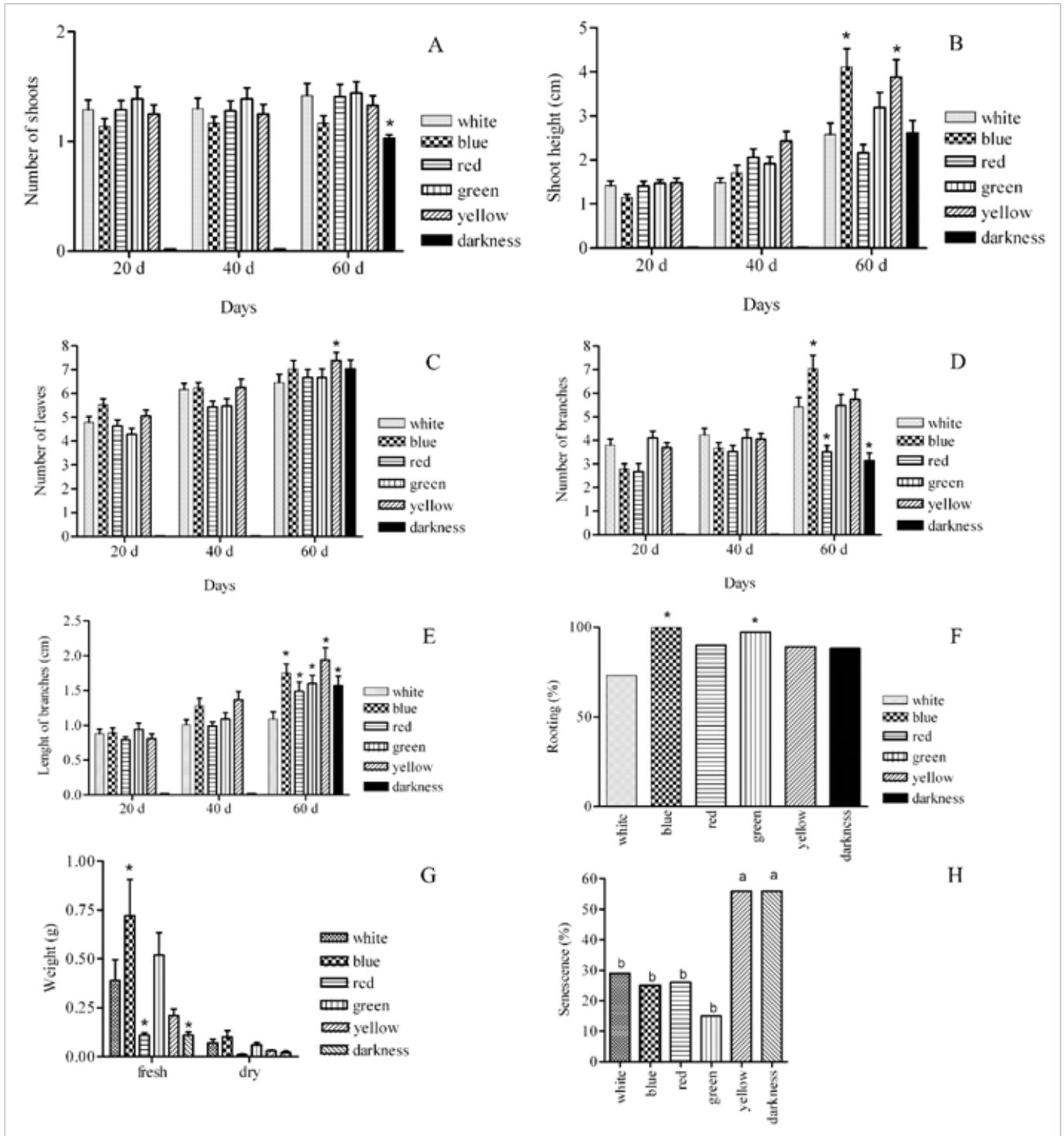


Figure 3. In vitro development of *P. tenellus* under different light spectra. Parameters were evaluated at 20, 40 and 60 days ($n \geq 30$). Rooting and senescence (F and H, $n \geq 30$); fresh and dry weight (G, $n = 10$). * $P < 0.05$: statistical differences in comparison with white light. Different letters denote statistical differences among treatments. Average \pm SD.

on either side (Figure 5). The mesophyll is dorsiventral. The palisade parenchyma is unistratified, and the spongy parenchyma consists of two layers of cells (Figure 5).

Thickness measured in the leaves of plantlets cultured under white light resulted in 8.8 μm (adaxial epidermis), 5.0 μm (abaxial epidermis), 19 μm (palisade parenchyma)

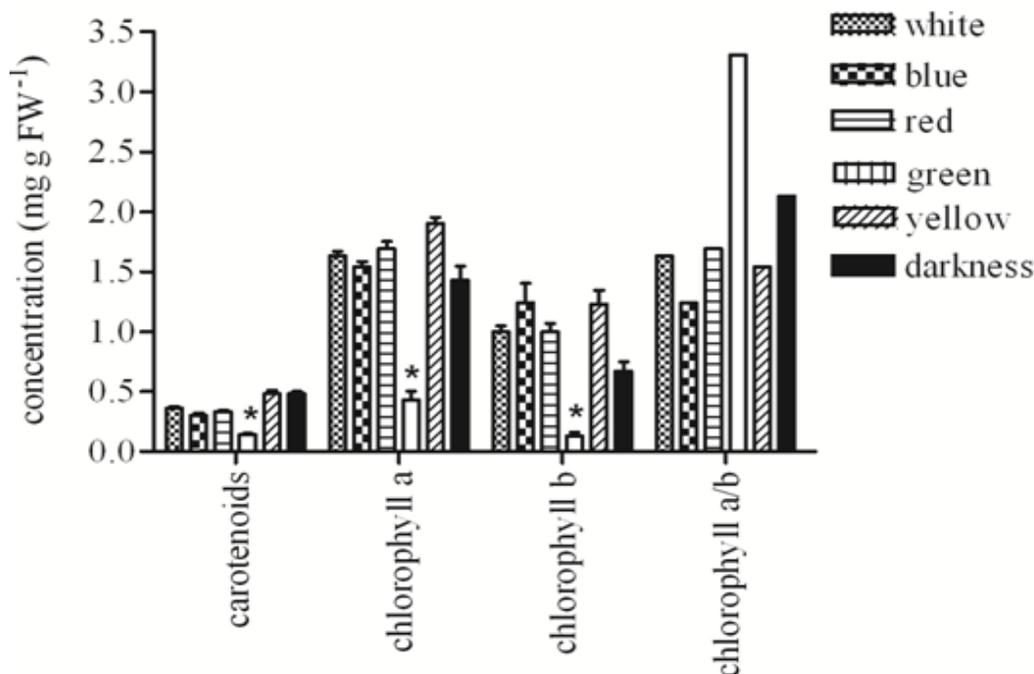


Figure 4. Photosynthetic pigment contents of *P. tenellus* cultured under different light spectra. *P<0.05: statistical differences in comparison with white light (Average \pm SD, n=10).

and 29 μm (spongy parenchyma) (Figure 6). By comparing anatomical features of plantlets cultured under different light qualities, it was found that the greatest thickness of adaxial epidermis was verified under red light, showing statistical differences compared to green light (Figure 6). Plantlets maintained under darkness presented lower measurements for all evaluated anatomical parameters compared with light treatments (Figure 6). Palisade parenchyma showed statistical difference when comparing white and other light qualities. The greatest palisade parenchyma thickness was verified under blue light (Figure 6).

In comparison with control treatment (white light), our analysis showed an improvement in the relative amount of compounds with phenolic features in aqueous extracts obtained from plantlets cultured under yellow light (Figure 7A). Geraniin, which is a hydrolysable tannin, was detected in *P. tenellus* extracts at 26 min (RT), a result obtained by comparison with the absorbance maximum of the geraniin spectrum (221 and 277 nm) (Figure 7B). Geraniin was found in plantlets cultured in all light treatments, but not in darkness. Also, under UV spectra flavonoid was found in abundance (Figure 7C).

DISCUSSION

Light spectra have been shown to be an important environmental factor influencing morpho-anatomical and phytochemical features of *P. tenellus* plantlets. With

respect to plant development, the number of nodes may be used as an indicator of proliferation rate such that each node marks the origin of a new plant. Accordingly, exposure to blue light, in contrast to control white light, resulted in plantlets with a greater number of nodes and induced the greatest elongation of *P. tenellus* shoots. However, this response is not in agreement with the results of previous researchers who have verified that blue light may either inhibit or increase stem elongation (Islam et al., 1999; Shimizu et al., 2005). We found that exposure to yellow light increased stem elongation, a finding which agrees with studies using *Cattleya walkeriana* (Islam et al., 1999). Also, according to Maas et al. (1995), changes in shoot elongation in response to blue and red light result primarily from cell wall extension, confirming the effects of yellow light, as shown in our study. The effects of yellow light were also shown to improve different morphological features in plantlets of *P. tenellus*, such as high production in the number of leaves together with high production of phenolic compounds, suggesting that phenolic content may improve morphological features. The effects of phenolic compound concentrations in either inhibiting or stimulating plant development have been reported by Ozyigit (2008). Plant phenolics are modulators of indole acetic acid (IAA) catabolism, and its concentration may either inhibit or stimulate enzymatic oxidation of auxin hormone, in turn affecting cell elongation and cell division, as well as subsequent plant growth and development. Moreover, plant phenolics increase the rigidity of plant cell walls, since

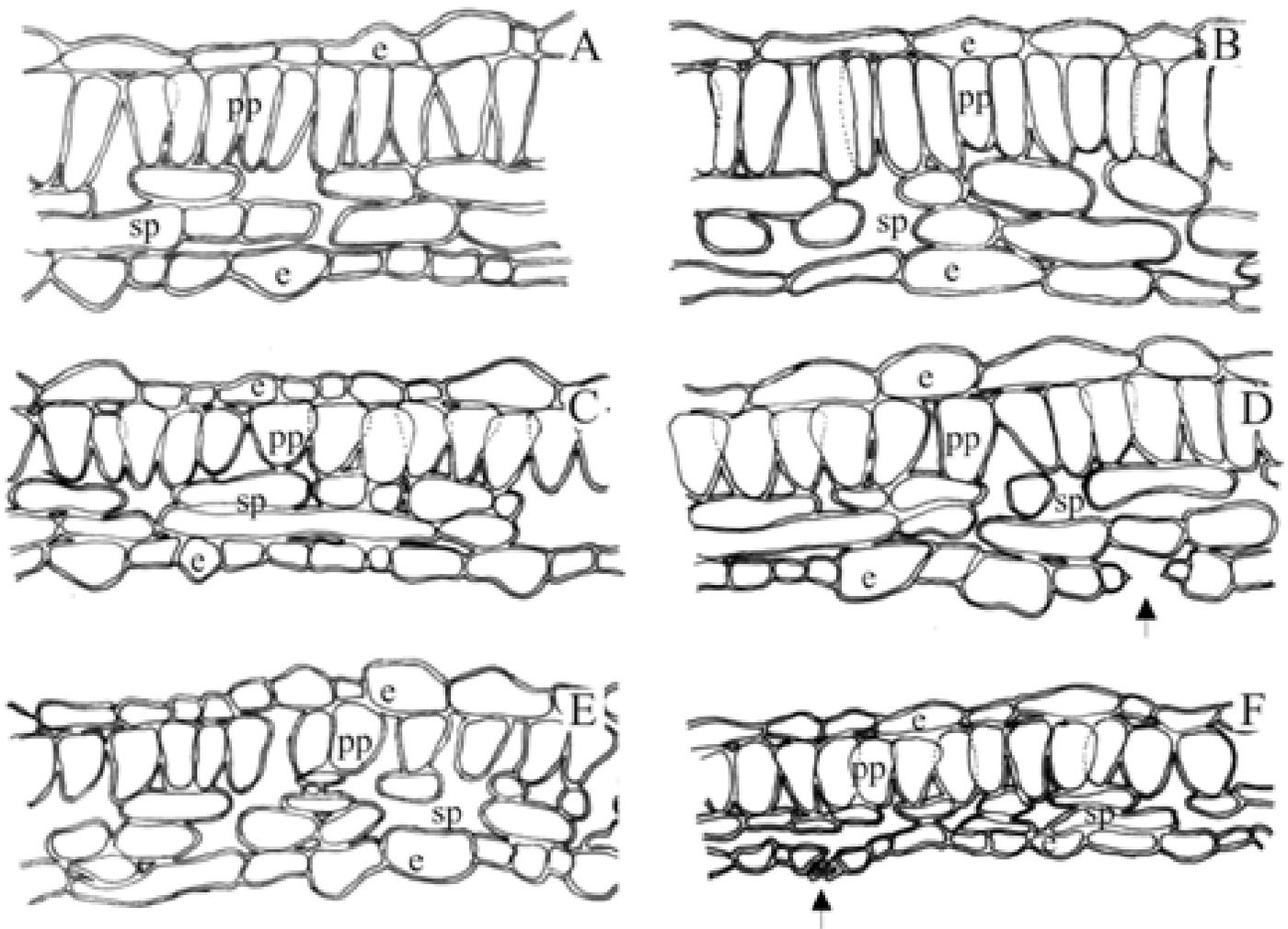


Figure 5. Leaf transverse sections of 60-day-old plantlets of *P. tenellus*. (A) white, (B) blue, (C) red, (D) green, (E) yellow, (F) darkness. e – epidermis, pp- palisade parenchyma, sp- spongy parenchyma. Arrows indicate stomata. Scale bar=50 μ m.

since they are precursors of lignin (Arnaldos et al., 2001; Ozyigit, 2008).

A higher number of leaves may result in increasing photosynthetic area and dry weight as a consequence of accumulating sucrose, which is an important energy source, but also a structural component of plant physiology and metabolite production. However, increase in the number of leaves under yellow light did not result in any statistical difference in either dry weight or chlorophyll content among the light treatments, except in relation to the green spectrum, as noted above.

The low production of carotenoid content and chlorophylls a and b under green light indicates that this light band is less efficient in photosynthesis. In support of this conclusion, Klein (1992) suggested that a

monochromatic green light was not significantly absorbed by chlorophyll a. However, this author also states that this light band continuously reflects a light wave from chloroplast to chloroplast, acting as an electron carrier.

Thus, after several cycles of reflection, the green light spectrum is still minimally effective in photosynthesis physiology.

Changes in morphogenesis by the effects of spectral green light showed that it is an important factor affecting plant development, contrary to some studies that reported green light to be innocuous to growth (Reichler et al., 2001). According to Folta and Maruhnich (2007), phytochromes and cryptochromes are responsible for the absorption of green light. Therefore, it is clear that studies reporting the effects of green light have drawn

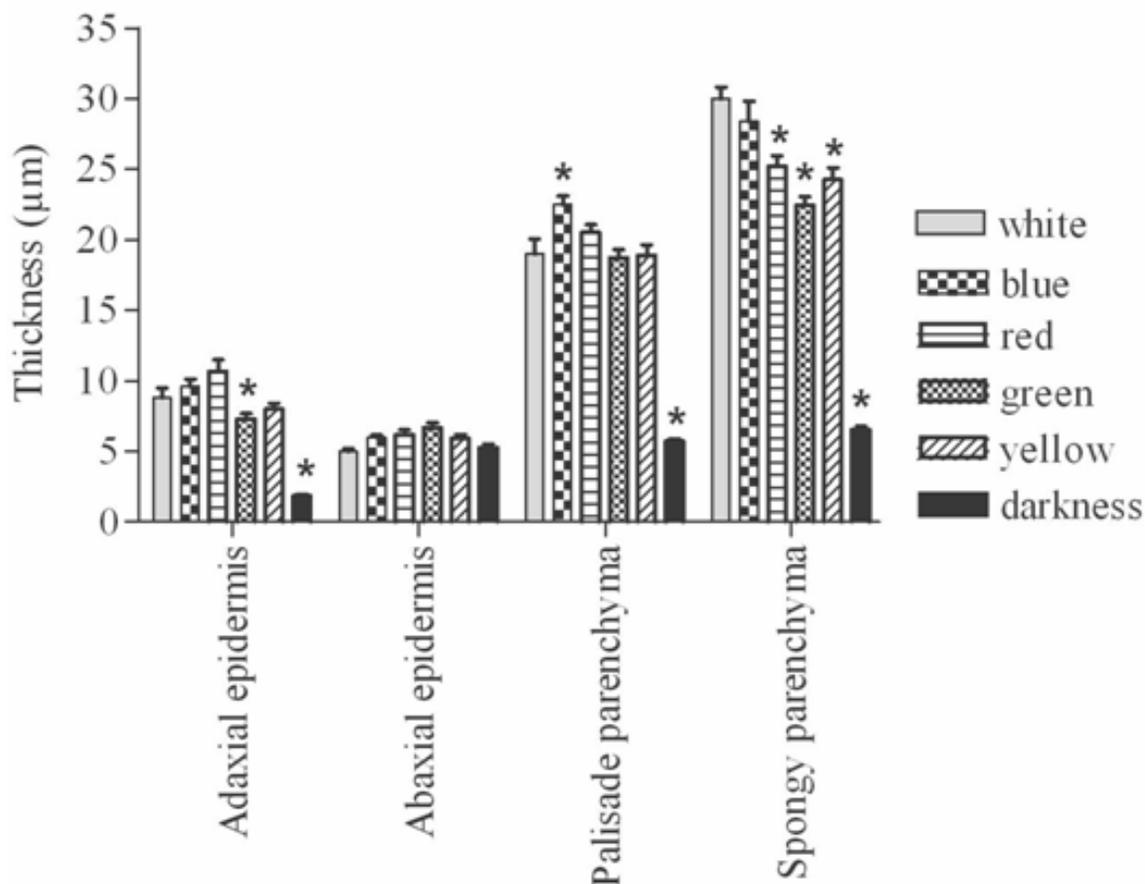


Figure 6. Leaf tissue thickness (μm) of *P. tenellus* cultured under different light spectra for 60 days. * $P < 0.05$: statistical differences in comparison with white light (Average \pm SD, $n=30$)

controversial conclusions. That is, this spectrum is sometimes found to be the least effective in promoting plant development, while, at other times, the opposite effect has been found, improving, for example, plant height (Dougher and Bugbee, 2001).

According to Economou and Read (1987), light may influence the rooting process, including the inhibition of root development. Our data showed the positive effects of blue light in improving rooting, while under red light, the roots presented a fragile aspect, results strongly suggesting the importance of light in the formation of plant structures. In contrast to our data, Hunter and Burritt (2004) verified that blue light reduced explant competence for organogenesis. It is important to underscore the fact that plant genotype is essential to fully evaluate the different responses obtained from the different effects of light spectra.

Senescence is controlled by internal signals, such as gene expression and plant hormones, and external factors, including light, that can delay or accelerate the death of cells, specific organs and whole plant (Lim et al., 2007). Initial stages of senescence involve the

chloroplast, responsible for photosynthesis, with gradual loss of chlorophyll, proteins and lipids associated with chloroplast (Hodges and Forney, 2000). Many different natural phenolic compounds in plants appear to function primarily in plant defense or as attractants of pollinators. They may also be involved in stages of plant development, and they are widely applied in therapeutic preparations.

In the current study, the correlation between senescence and decreasing chlorophyll and carotenoid contents was not verified, although decline in chlorophyll and progressive yellowing of the leaves from carotenoids became visible during senescence. Leaf senescence of the *P. tenellus* plantlets did not impair acclimatization. Several anatomical parameters were investigated in order to evaluate changes that might ultimately establish a developmental pattern in leaf tissues of *P. tenellus* exposed to different light spectra. For example, we found papillae in *P. tenellus*, as well as *P. urinaria* and *P. amarus*. The presence of epidermal papillae may, in fact, intensify light uptake reaching leaf tissues, thus improving photosynthesis (Vogelmann et al., 1996). When exposed

to radiation, leaf tissues may alter structures involved in light absorption. For example, changes in leaf thickness are commonly associated with the number of epidermal, hypodermal and parenchymal layers, particularly palisade parenchyma thickness. Red light increases the thickness of the adaxial epidermis, which, in turn, acts as a defense mechanism that reduces the penetration of radiation in the leaf mesophyll tissues, as observed by Yang et al. (2008). The effects of light qualities on epidermal thickness have also been described by Saebo et al. (1995) who found that blue light increased the area of epidermal cells and palisade parenchyma of *Betula pendula* cultured *in vitro*, resulting in greater leaf area. This finding confirms that light quality is an important factor in cell expansion. Plantlets maintained under darkness presented fragility also visualized by microscopic analysis showing reduction in thickness of leaf tissues and absence of papillae.

Environmental factors are decisive in controlling genetic regulation of plant development and production of secondary metabolites. Thus, the application of light qualities in tissue cultures could be a useful strategy for improving therapeutic metabolites under standardized conditions, and it is certainly useful in plantlet production. In our study, we showed that yellow light stimulated the production of phenolic compounds that are the main therapeutic constituents of *P. tenellus*, including tannins and flavonoids (Huang et al., 2003), suggesting that yellow light plays an important role in the biosynthesis of phenolic compounds in *P. tenellus*. The use of HPLC coupled to a diode array detector revealed a higher amount of phenolic compounds in crude aqueous extracts. Although yellow light has not been commonly used, this study revealed the unequivocal influence of this spectrum in plant development and the production of phenolic compounds.

Conclusion

This study adds important findings related to plant development of *P. tenellus* in response to different light spectra. The best *in vitro* development of *P. tenellus* was found under blue and yellow light. However, in the context of anatomical features, both white and blue light induced the greatest thickness of palisade and spongy parenchymas. Sixty-day-old plantlets of *P. tenellus* revealed a high production of phenolic compounds after yellow light exposure, which seems to be consistent with the better plantlet development observed under this light. This culture system study indicated the importance of light spectra as a simple means of inducing the production of phenolic compounds so important to the therapeutic properties of this medicinal plant.

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Conflict of interest

All authors declare that they have not conflict of interest.

REFERENCES

- Arnaldos TL, Munoz R, Ferrer MA, Calderon AA (2001). Changes in phenol content during strawberry *Fragaria x ananasa*, cv. Chandler) callus culture. *Physiol. Plant* 113:315-322.
- Catoni R, Granata MU, Sartori F, Varone L, Gratani L (2015). *Corylus avellana* responsiveness to light variations: morphological, anatomical, and physiological leaf trait plasticity. *Photosynthetica* 53(1):35-46.
- Chen M, Chory J, Fankhauser C (2004). Light signal transduction in higher plants. *Ann. Rev. Gen.* 38:87-117.
- Dougher TAO, Bugbee B (2001). Evidence for yellow light suppression of lettuce growth. *Photochem. Photobiol.* 73:208-212.
- Economou A, Read PE (1987). Light treatments to improve efficiency of *in vitro* propagation systems. *HortScience* 22:751-754.
- Feller U, Anders I, Demirevska K (2008). Degradation of Rubisco and other chloroplast proteins under abiotic stress. *Gen. Appl. Plant Physiol.* 34(1-2):5-18.
- Folta KM, Maruhnich SA (2007). Green light: a signal to slow down or stop. *J. Exp. Bot.* 58(12):3099-3111.
- Franklin KA (2009). Light and temperature signal crosstalk in plant development. *Curr. Opin. Plant Biol.* 12:63-68.
- Hodges DM, Fomey CF (2000). The effects of ethylene, depressed oxygen and elevated carbon dioxide on antioxidant profiles of senescing spinach leaves. *J. Exp. Bot.* 51(344):645-655.
- Huang RL, Huang YL, Ou, JC, Chen CC, Hsu FL, Chang CM (2003). Screening of 25 compounds isolated from *Phyllanthus* species for anti-human hepatitis B virus *in vitro*. *Phytother. Res.* 17(5):449-53.
- Hunter DC, Burritt DJ (2004). Light quality influences adventitious shoot production from cotyledon explants of lettuce (*Lactuca sativa* L.). *In Vitro Cell. Dev. Biol. Plant* 40(2):215-220.
- Islam MO, Matsui S, Ichihashi S (1999). Effects of light quality on seed germination and seedling growth of *Cattleya* orchids *in vitro*. *Jap. Soc. Hortic. Sci.* 68:1132-1138.
- Klein RM (1992). Effects of green light on biological systems. *Biol. Rev.* 67:199-284.
- Lim PO, Kim HJ, Nam HG (2007). Leaf senescence. *Ann. Rev. Plant Biol.* 58:115-136.
- Lorenzi H, Matos FJA (2008). *Plantas Mediciniais no Brasil: Nativas e Exóticas*. Nova Odessa, Instituto Plantarum, São Paulo.
- Maas FM, Bakx EJ, Morris DA (1995). Photocontrol of stem elongation and dry weight partitioning in *Phaseolus vulgaris* L. by the blue-light content of photosynthetic photon flux. *J. Plant Physiol.* 146:665-671.
- Macedo AF, Leal-Costa MV, Tavares ES, Lage CLS, Esquibel MA (2011). The effect of light quality on leaf production and development of *in vitro*-cultured plants of *Alternanthera brasiliana* Kuntze. *Environ. Exp. Bot.* 70:43-50.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Ozyigit II (2008). Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium hirsutum* L.) shoot tips. *Afr. J. Biotech.* 7:1145-1150.
- Reichler SA, Balk J, Brown ME, Woodruff K, Clark GB, Roux SJ (2001). Light differentially regulates cell division and the mRNA abundance of pea nucleolin during de-etiolation. *Plant Physiol.* 125:339-350.
- Saebo A, Krekling T, Appelgren M (1995). Light quality affects photosynthesis and leaf anatomy of birch plantlets *in vitro*. *Plant Cell Tiss. Org. Cult.* 41:177-185.

- Shimizu H, Ma Z, Tazawa S, Douzono M, Runkle ES, Heins, RD (2006). Blue light inhibits stem elongation of chrysanthemum. *Acta Hort.* 711:363-368.
- Victório CP, Henriques AB, Tavares ES, Esquibel MA, Lage CLS (2010). Standardized production of *Phyllanthus tenellus* Roxb. by plant tissue culture. *Rev. Cien. Agron.* 41:272-278.
- Victório CP, Leal-Costa MV, Tavares ES, Kuster RM, Lage CLS (2011). Effects of supplemental ultraviolet A on the development, anatomy and metabolite production of *Phyllanthus tenellus* cultured in vitro. *Photochem. Photobiol. B.* 87(3):685-689.
- Vogelmann TC, Nishio JN, Smith WK (1996). Leaves and light capture: light propagation and gradients of carbon fixation within leaves. *Trends Plant Sci.* 1:65-70.
- Wellburn AR (1994). The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *J. Plant Physiol.* 144:307-313.
- Yang Y, Yao Y, He H (2008). Influence of ambient and enhanced ultraviolet-B radiation on the plant growth and physiological properties in two contrasting populations of *Hippophae rhamnoides*. *J. Plant Res.* 121(4):377-385.

Full Length Research Paper

Effect of *Ginkgo biloba* extract on sperm quality, serum testosterone concentration and histometric analysis of testes from adult Wistar rats

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***Ginkgo biloba* extract is a widely consumed phytotherapeutic used mainly in the treatment of dementia and Alzheimer's disease, and it has also been employed to treat erectile dysfunction and as an aphrodisiac. This work was aimed at evaluating its effect on sperm quality, serum testosterone concentration and on the histometric analysis of the testes from adult male Wistar rats. Three-month-old Wistar rats were treated with distilled water (control group) and the aqueous extract of *Ginkgo biloba* at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0), once daily, for 56 consecutive days. Counting and morphological evaluation of sperm collected from cauda epididymis were analyzed. Histometric measures of the testes were also taken. This work shows that adult Wistar rats exposed to *Ginkgo biloba* presented histometric alteration in the volume of the Leydig cells and this finding suggests a possible functional deficit in these cells.**

Key words: *Ginkgo biloba*, phytotherapy, rats, sperm, testes, toxicity.

INTRODUCTION

The extract of *Ginkgo biloba* (GBE) is one of the most consumed phytotherapeutics in the world, being used to treat dementia and Alzheimer's disease, and to ameliorate peripheral blood flow (Oken et al., 1998; Mar and Bent, 1999; Stromgaard and Nakanishi, 2004). GBE is a mixture of approximately 300 components extracted from the leaves, ranging between 22 and 27% of flavonoid glycosides (flavones: quercetin, kaempferol and isorhamnetin; biflavones: bilobetin, ginkgetin, isoginkgetin and sciadopitysin), 5 and 7% of *terpene trilactones*

(ginkgolides: A, B, C e J) and less than 5 ppm of ginkgolic acids (WHO, 1999; Van Beek, 2002; Oh and Chung, 2004; Smith and Luo, 2004).

Other medicinal uses for GBE include treatment of erectile dysfunction as a result of chronic use of antidepressants (Kang et al., 2002; Mackay, 2004; Moyad et al., 2004; Wheatley, 2004; Tamler and Mechanick, 2007) and as an aphrodisiac (Malviya et al., 2011) which led to an increasing intake of the extract by men. Although, many plants are popularly used to regulate

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male fertility (Kamal et al., 2003), not many reports are specifically related to the effect of *G. biloba* extract. Al-Yahya et al. (2006), reported weight reduction of the cauda epididymis and the prostate in addition to reduced levels of nucleic acids, low gestational index and increased preimplantation loss in Swiss mice treated with the extract. Additionally, elevated doses of GBE reduced sperm penetration in the zona pellucida of hamster oocytes (Ondrizek et al., 1999a) and inhibited human sperm motility (Ondrizek et al., 1999b).

However, there were experimental studies which showed protective effects of GBE against testicular damage of many substances and other injuries: doxorubicin (Yeh et al., 2009), cadmium (Predes et al., 2011), cisplatin (Amin et al., 2012), diethylstilbestrol (Wang et al., 2008), carbon tetrachloride (Chávez-Morales et al., 2010), testicular torsion/detorsion (Kanter, 2011). In addition, Taepongsorat et al. (2008) showed that quercetin, one of the main components of GBE, ameliorated reproductive parameters such as motility, vitality and sperm concentration, and increased testicular weight in rats.

Many substances including environmental contaminants have been related to the occurrence of adverse effects on the male reproductive system for acting as endocrine dysregulators, for instance, the parabens (Hoberman et al., 2008; Tavares et al., 2009), organochlorine pesticides (Bretveld et al., 2007; Uzun et al., 2009; Victor-Costa et al., 2010), bisphenols (Sakaue et al., 2001) and phthalates (Hu et al., 2009; Saillenfait et al., 2009; Martino-Andrade and Chaoud, 2010) can interfere with the male reproductive system by mimetizing the endogenous estrogenic action or changing its metabolism. The estrogenic effects of GBE and its main isolated flavonoid components (quercetin, kaempferol and isorhamnetin) were reported by Oh and Chung (2004). They showed the affinity of these compounds to the human recombinant estrogenic receptors α (ER- α) and, especially to β (ER- β) *in vitro*.

Fransen et al. (2010), described the toxicological risks of GBE and alerted to the small number of animal assays so far developed. The authors mentioned the need for a multidisciplinary investigation in order to ensure a safe use of this phytotherapeutic. A recent study using GBE was developed by the National Toxicology Program in 2013 (NTP, 2013) using both F344/N rats and B6C3F1/N mice. The animals were treated five times a week during three months and two years in doses ranging 100, 300 and 1,000 mg of GBE per kilogram of body weight in rats and 200, 600 and 2,000 mg of GBE per kilogram of body weight in mice. The animals showed no lesions on male reproductive system and sperm concentration in a general analysis, although this study led to the conclusion that GBE caused cancer of the thyroid gland in male and female rats and male mice, and liver cancer in male and female mice.

Siegers (1999), mentioned that alquilfenols, represented by the ginkgolic acids, are toxic compounds that can cause allergies, and display mutagenic and carcinogenic properties

Koch et al. (2000), reported immunotoxicological effects of the extract in mice by causing lymphoproliferative reactions if applied parenterally on skin. For this reason, the extract should not have more than 5 ppm of alquilfenols (Siegers, 1999; WHO, 1999; Fransen et al., 2010). Sierpina et al. (2003) reported the inhibitory effect of the extract on the platelet activation factor and its potential anticoagulant effect.

Therefore, due to the fact that GBE intake by the population is not focused on its active constituents but on the extract, and owing to the possibility that the flavonoid compounds present in GBE may act as endocrine dysregulators and may alter the physiology of the organs of the male reproductive system, this work had the objective of evaluating the effect on the sperm quality, serum testosterone concentration and, specifically, on histometric analysis of the testes from adult male Wistar rats treated with GBE.

METHODOLOGY

The experimental protocol followed the international norms established in the manual about care and use of laboratory animals (National Research Council, 2003) and was approved by the Ethics Committee in Animal Experimentation of the Federal University of Juiz de Fora (UFJF) (protocol number 016/2010).

Ginkgo biloba extract

The extract of *G. biloba* was imported from China by JR Pharma pharmacy in Juiz de Fora City – Minas Gerais State, Brazil. The quality control carried out by Galena Laboratory showed that the extract is composed of 28.2% of ginkgoflavoglicosides (15% of quercetin, 10.9% of kaempferol and 2.3% of isorhamnetin), 8.3% of terpenolactones and less than 5 ppm of ginkgolic acids.

Animals

Eighty Wistar rats obtained from the vivarium of the Biology Center of Reproduction (CBR/UFJF) were used. The animals were three months old and weigh around 250 g. They were placed in polypropylene cages (49×34×16 cm) kept in acclimatized shelves (ALESCO®), with airflow, and under standard laboratory conditions, with a controlled temperature of 22 ± 2°C, and a 12 h light/dark photoperiod. They were fed on rat chow pellets and received water *ad libitum*. Each cage contained the maximum of five animals.

Experimental groups, doses of GBE, administration route and duration of treatment

The rats were randomly distributed into four groups: one control (n=20) and three treated groups (n=20/group). The control group received 1 ml of distilled water and the treated groups received the extract once daily at the concentrations of 3.5 mg/kg (GBE 3.5); 7.0 mg/kg (GBE 7.0) and 14.0 mg/kg (GBE 14.0). The lowest dose corresponded to the therapeutic dose used in the human being (WHO, 1999; Blumenthal et al., 2003; Sierpina et al., 2003; Smith and Luo, 2004). The doses of 7.0 mg/kg and 14.0 mg/kg were twice and four times higher than GBE 3.5, respectively. The extract was administered intragastrically for 56 consecutive days, which corresponds to the duration of spermatogenesis in the rat (Russell et al., 1990). One day after the end of treatment, the animals were

anesthetized with a combination of ketamine chloridrate (90 mg/kg) and xylazine chloridrate (10 mg/kg), administered intraperitoneally (Wolfensohn and Lloyd, 1994), following death by rupture of the diaphragm.

Testes weight

After euthanasia, the animals underwent laparotomy for the removal and weighing of the testes.

Sperm count

Sperm were collected from the epididymal secretion of the right epididymis cauda (Seed et al., 1996). The secretion from the epididymis was immediately placed in 50 μ L phosphate saline solution heated at 37°C and placed in a Petri dish. The sperm were counted in a hemocytometer with improved double Neubauer ruling and the total concentration was estimated according to the formula:

$$x = a \times 300 \times 10^4$$

where: x = final sperm concentration/mL

a = mean obtained from the eight lateral squares of the hemocytometer

Evaluation of sperm vitality

The sperm smear was stained by use of eosin-nigrosin staining technique (WHO, 2010). Only sperm with head and tail were considered. The data were expressed in percentage of live sperm.

Evaluation of the spermatic morphology

The sperm smear was stained by use of Shorr technique for counting and morphological classification in order to determine the abnormality index (WHO, 2010). The morphological alterations considered, according to Seed et al. (1996) and Perreault and Cancel (2001) were:

2.7.1 Amorphic head: loss of the typical hook shape of the rat sperm;

2.7.2 Cauda abnormality: coiled or broken.

Data were expressed in percentage of normal sperm for each animal.

Tissue preparation

After euthanasia, the animals underwent laparotomy for the removal and weighing of the testes. The gonadosomatic index that represents the testicular weight expressed as percentage of the body weight was determined using the formula below:

$$x = (a \div b) \times 100$$

where: x = gonadosomatic index (GSI); a = total weight of the testes (g); b = body weight (g)

The testes were fixed in Karnovsky modified fixative (4% paraformaldehyde: 4% glutaraldehyde a 0,1ml/L phosphate saline buffer, pH 7.4). Twenty four hours after the beginning of fixation, the tunica albuginea and the parenchyma of the right testicle were removed and weighed. The left testicle was embedded in histological resine and to conduct a light microscope examination, it was sectioned at 3 μ m thickness for toluidine blue – 1% sodium

borate staining. The histological sections were photographed for further analysis.

Testicular histometric analysis

Volumetric density of tubular and intertubular testicular compartments

2,660 points were counted with the program Image-Pro Plus[®] version 4.5.0.29 (Media Cybernetics – EUA) using a standardized grid of 266 points overlaying 10 images taken at random using 10 \times objective lens. The points were classified as belonging to the tubular or intertubular compartment. For the evaluation of the volumetric density of tubular and intertubular testicular compartments, the following formulas were used:

$$x = (a \div 2,660) \times 100$$

$$y = (b \div 2,660) \times 100$$

where: x = volumetric density of the tubular compartment; y = volumetric density of the intertubular compartment; a = total sum of the overlaying points in the tubular compartment; b = total sum of the overlaying points in the intertubular compartment

Volume of the tubular and intertubular testicular compartments and the tubulosomatic index

The specific testicular density was considered to be 1 (Johnson et al., 1981) therefore, the parenchyma weight (g) was considered equal to its volume (ml). For determination of the volume of the tubular and intertubular testicular compartments the formulas below were used, respectively:

$$x = (a \times z) \div 100$$

$$y = (b \times z) \div 100$$

where: x = volume of the tubular compartment (mL); y = volume of the intertubular compartment (mL); z = weight of the testicular parenchyma (g); a = volumetric density of the tubular compartment; b = volumetric density of the intertubular compartment

The tubulosomatic index (TSI) was also calculated using the formula below:

$$x = (a \div y) \times 100$$

where: x = tubulosomatic index (TSI); a = volume of the tubular compartment; y = body weight

Diameters of the seminiferous tubule and the tubular lumen, and the seminiferous epithelium height

Twenty transversal sections of the most circular seminiferous tubules were photographed (10 \times objective lens) for each animal and the diameter and radius were measured in each section. In addition, two measurements of the seminiferous epithelium height were taken at opposite positions and their mean value was considered (Figure 1). The luminal diameter was also determined considering the difference between the diameter of the tubule and the sum of the two heights of the seminiferous epithelium.

Total length of the seminiferous tubules per testicle and per testicle gram

After establishing the seminiferous tubules radius and volume occupied, the total length of the seminiferous tubules was taken by using the formula below (Attal and Courtois, 1963):

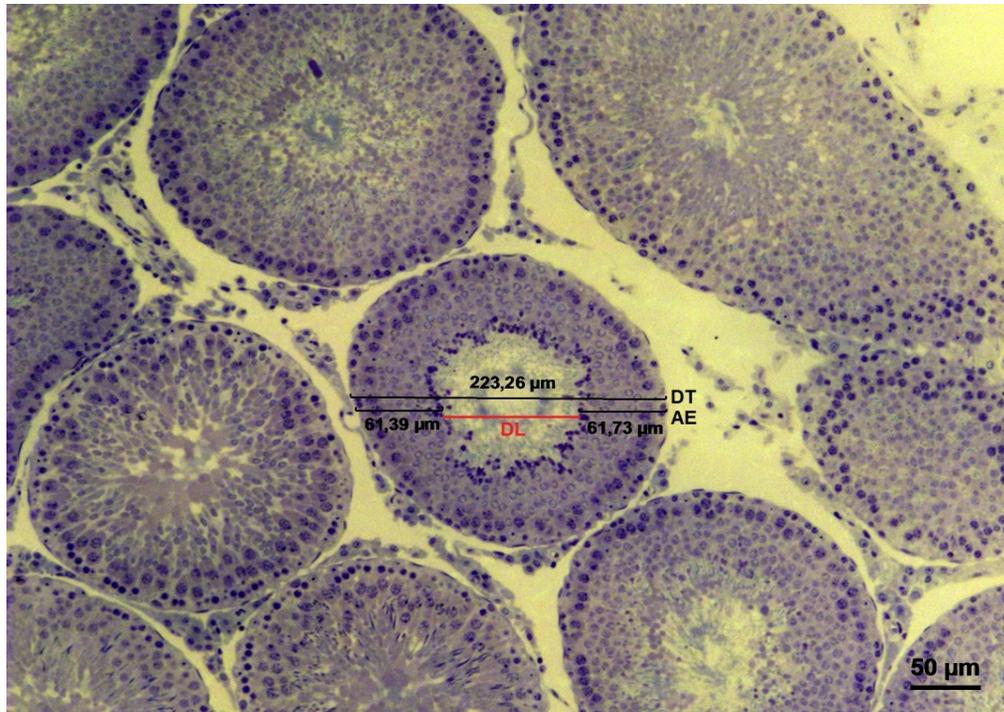


Figure 1. Determination of the histometric measures of seminiferous tubules from Wistar rat testicle. TD – Total tubular diameter; LD – Luminal tubular diameter determined by the difference between the total diameter and the sum of both measures of the epithelium height (EH1+EH2); EH – Height of the seminiferous epithelium determined by mean of two diametrically opposed measures (EH1 and EH2). Toluidine blue – 1% sodium borate staining. Thickness: 3 µm.

$$x = a \div (\pi \times r^2)$$

where: x = total length of the seminiferous tubules per testicle; a = volume tubular compartment; π = π value (= 3.14); r = seminiferous tubules radius.

The total length of the seminiferous tubules was divided by the total weight of the testes to obtain the length of the tubules per gram of testicle.

Volumetric density and volume of the elements from the intertubular tissue

One thousand points overlaying the images captured from the intertubular region in different histological sections from each animal testicle were counted with 40x objective lens and using a standardized grid of 609 points. The points were classified and quantified when found in the Leydig cells, blood vessels, lymphatic spaces, connective tissue and macrophages (Figure 2). The volumetric density of the elements was calculated according to the formula below:

$$x = (a \div 1,000) \times 100$$

where: x = volumetric density of the elements from the intertubular compartment; a = total sum of the overlaying points on each element of the intertubular compartment

Volume of the intertubular elements was calculated with the formula below:

$$x = (a \times z) \div 100$$

where: x = volume of the intertubular element

Hormonal concentration of serum testosterone

Blood was collected under anesthesia by cardiac puncture before the euthanasia and the serum was stored at the temperature of -80°C for posterior analysis of serum testosterone concentration. The serum from nine animals of each group were randomly collected to be used at analysis of testosterone concentration using Microreader ELISA plate ASYS HITECH GMBH® (Austria) and the kit Testosterone EIA Kit (Cayman Chemicals®).

Statistical analysis

The data were analyzed using the Levene test for evaluation of data distribution. Data showing normal distribution were analyzed with ANOVA followed by the Tukey test. Data without normal distribution were analyzed with the Kruskal-Wallis test, followed by the Mann-Whitney test. The level of significance considered was $\alpha = 0.05$. For multiple comparisons with the Mann-Whitney test, the significance level was 1%. The tests were performed using Statistical Package for the Social Sciences program (SPSS), version 13.

RESULTS

The mean weight of the left and right testes, the testicular structures such as tunica albuginea and parenchyma, and the gonadosomatic index (GSI) did not significantly

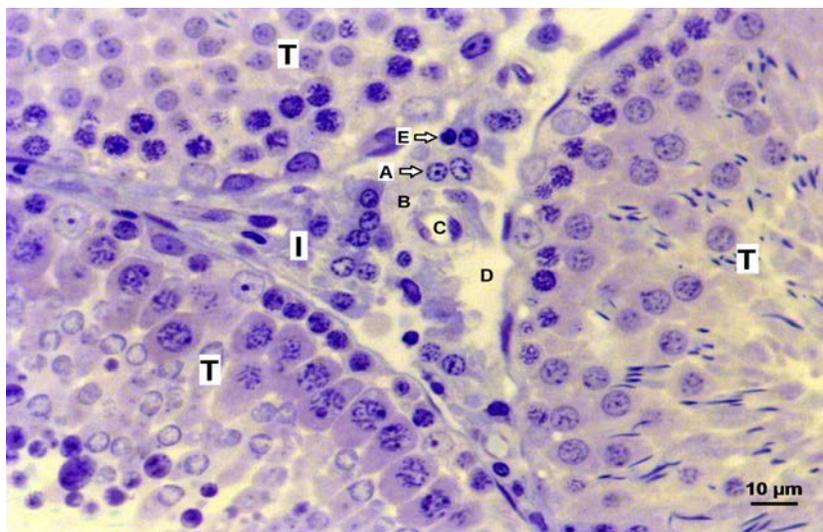


Figure 2. Photomicrography of histological sections of Wistar rat testicle. Identification of components from the intertubular testicular compartment I – Intertubular compartment composed of: A –Leydig Cells (arrow); B – Connective tissue; C – Blood vessel; D – Lymphatic spaces and E – Macrophage (arrow). T – Tubular compartment with seminiferous tubules. Toluidine blue – 1% sodium borate staining. Thickness: 3 µm.

between control and treated groups and between the treated groups (Table 1). The treatment with GBE at all dose levels for 56 days did not significantly alter the number, vitality and morphology of cauda epididymus sperm (Table 2). The differential analysis of the tubular and intertubular testicular compartments with respect to volumetric density, volume in the organ and the tubulosomatic index (TSI) were not significantly different between the groups (Table 3).

The total and luminal diameters, the height of the seminiferous epithelium, the total length of the tubule per testicle and length of the seminiferous tubule per testicle gram were not statistically different when comparing control and treated groups and between the treated groups (Table 4). There was no significant difference in the volumetric proportions of the Leydig cells, blood vessels, lymphatic spaces, connective tissue, macrophages, and blood vessel volume. However, there was a significant difference in the volume of the Leydig cells in which the groups GBE 3.5 and 7.0 had lower statistical means than the control and the GBE 14.0 groups (Table 5). Photomicrographs of cross histological sections of Wistar rat testicle showing Leydig cells are presented on Figure 4.

The testosterone serum concentrations exhibited great variations (Figure 3). However, there was no significant difference between the groups.

DISCUSSION

The toxicological evaluation of a substance in animal

models is primordial for determining the potential risk for human life and is a necessary step to ensure the safety of a medication (Asare et al., 2011). To this end, the international protocols provide useful guidelines and recommendations of tests to be employed (OECD, 2009). The assessment of the weight of the reproductive organs is one of the most sensitive parameters for detection of a substance influence on the male reproductive system (Mangelsdorf et al., 2003). Alterations in the testicular weight can indicate modifications in the seminiferous tubules or interstitial edema and consequently in the sperm production (Sellers et al., 2007).

There is a growing indication that the phytotherapies act as modulators of the estrogenic receptors (Patisaul and Adewale, 2009). Assinder et al. (2007), showed that a diet rich in genistein, glicitein and daidzein, flavonoids with estrogenic activity, increased the apoptotic rate of the testicular germinative cells and was capable of interfering with the spermatogenic process in the rat. In addition, Das et al. (2004) showed that the methanolic extract of *Vitex negundo*, a plant rich in flavonoids, had a negative action in the spermatid quality by reducing the number and motility of sperm. Studies with *G. biloba* extract evidenced reduced capacity of the sperm to penetrate the *zona pellucida* in the hamster oocyte (Ondrizek et al., 1999) and inhibition of motility of human sperm (Ondrizek et al., 1999). However, alterations in the gamete concentration, vitality and morphology were not observed in this study, suggesting that the extract did not exert a direct effect on the production and maturation of sperm. In this analysis, this study is in agreement with

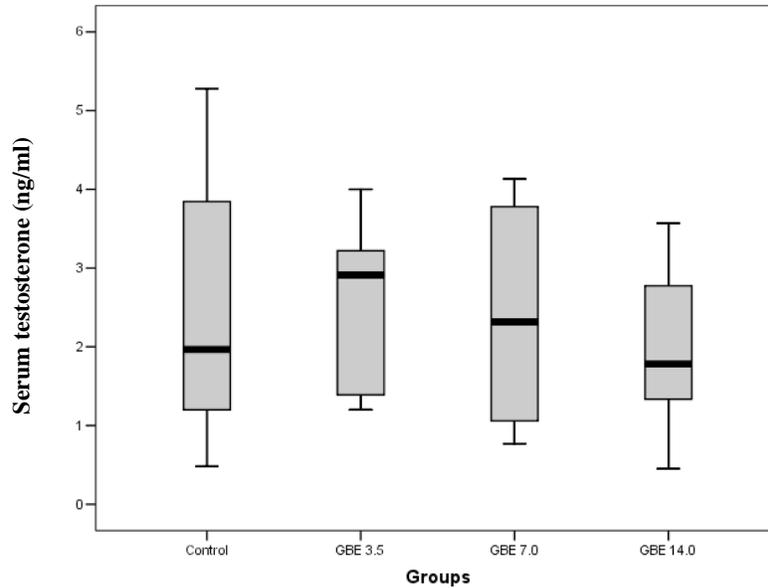


Figure 3. Total serum testosterone concentration (ng/ml) of Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data analyzed by ANOVA. $p=0.838$.

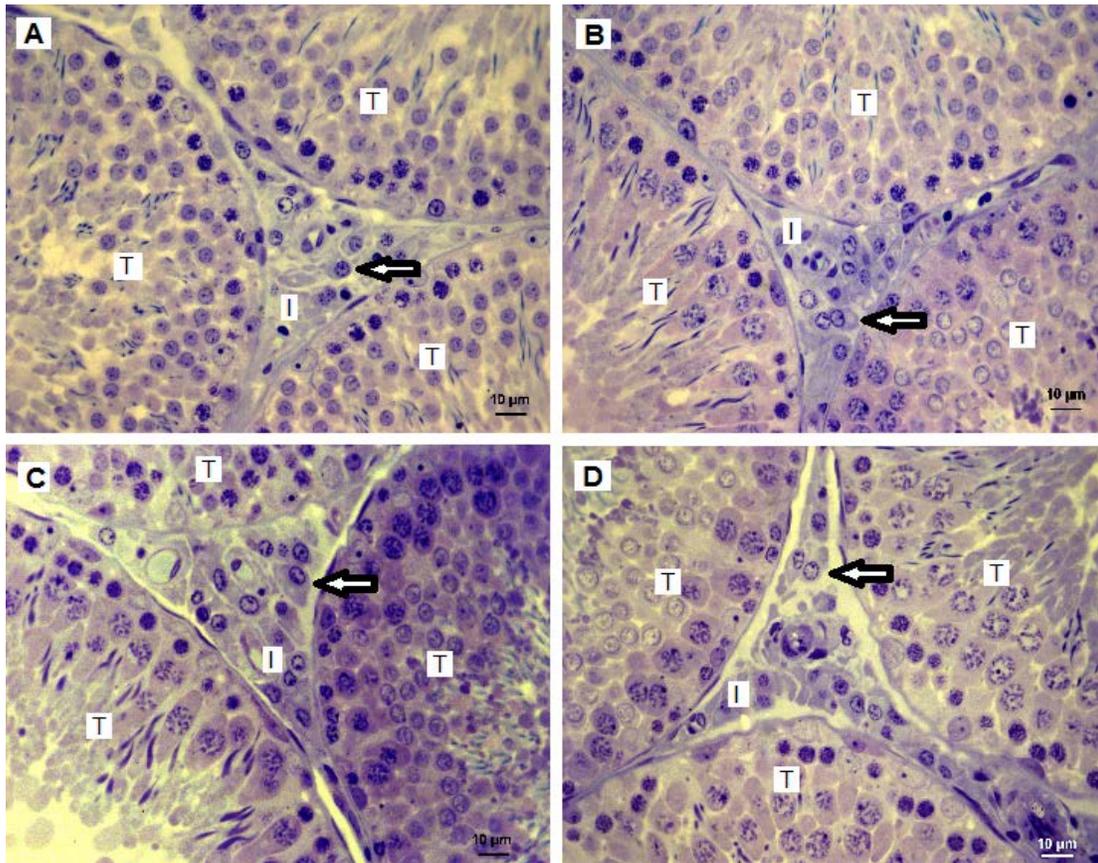


Figure 4. Photomicrographs of histological sections of Wistar rat testicle. A – Control group; B – GBE 3.5; C – GBE 7.0 and D – GBE 14.0. I – Intertubular compartment. T – Tubular compartment. Leydig Cells (arrows). Toluidine blue – 1% sodium borate staining. Thickness: 3 μ m.

Table 1. Mean weight of testes, right testicular tunica albuginea and parenchyma, and the gonadosomatic index (GSI) of Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean±standard deviation.

Parameter	Control (n=20)	GBE 3.5 (n=20)	GBE7.0 (n=20)	GBE 14.0 (n=20)	p
Testes (g)	1.39±0.10	1.33±0.11	1.38±0.15	1.40±0.11	0.253
Albuginea (mg)	42.85±3.50	40.15±4.11	42.75±4.38	41.35±4.45	0.132
Parenchyma (g)	1.21±0.11	1.16±0.12	1.21±0.11	1.21±0.13	0.406
GSI	0.84±0.09	0.81±0.07	0.88±0.07	0.84±0.08	0.075

Data analyzed by ANOVA. p = p-value. GSI - Gonadosomatic index

Table 2. Concentration, vitality and morphology of spermatids from Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean±standard deviation.

Parameter	Control (n=20)	GBE 3.5 (n=20)	GBE 7.0 (n=20)	GBE 14.0 (n=20)	p
SC (10^6 /ml)	232.20±59.60	224.06±53.38	263.31±84.49	234.93±61.81	0.266
SV (% live)	84.58±5.24	83.55±8.30	85.71±7.18	84.50±7.89	0.838
SM (% normal)	98.71±0.73	98.82±0.99	98.18±1.04	98.63±0.83	0.158

Data analyzed by ANOVA. p = p-value. Data analyzed by ANOVA. SC – sperm concentration (10^6 /mL); SV – spermatid vitality (% of live sperm); SM – spermatid morphology (% of normal sperm).

Table 3. Volumetric density, volume of testicular compartments and tubulosomatic index (TSI) of Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean ±standard deviation.

Parameter	Control (n=5)	GBE 3.5 (n=5)	GBE 7.0 (n=5)	GBE 14.0 (n=5)	p
VDT (%)	89.10±2.66	90.31±4.04	90.47±2.64	88.56±1.85	0.680
VDI (%)	10.90±2.66	9.69±4.04	9.53±2.64	11.44±1.85	0.680
VTT (ml)	1.07±0.03	1.04±0.05	1.08±0.03	1.07±0.02	0.376
VTI (ml)	0.13±0.03	0.11±0.05	0.11±0.03	0.14±0.02	0.582
TSI	0.32±0.01	0.32±0.00	0.33±0.01	0.32±0.01	0.058

Data analyzed by ANOVA. p = p-value. VDT – Volumetric density of tubular testicular compartment; VDI – Volumetric density of intertubular testicular compartment; VTT – volume of tubular testicular compartment (mL); VTI – volume of intertubular testicular compartment (mL); TSI – Tubulosomatic index.

Table 4. Histometric data of seminiferous tubules from Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean ±standard deviation.

Parameter	Control (n=5)	GBE 3.5 (n=5)	GBE 7.0 (n=5)	GBE 14.0 (n=5)	p
TD (μ m)	222.59±8.64	221.52±7.17	226.62±7.74	230.39±5.47	0.247
LD (μ m)	122.62±5.31	122.10±3.69	125.90±4.01	124.92±3.04	0.427
EH (μ m)	49.99±2.56	49.71±2.82	49.01±1.14	52.74±1.58	0.066
TL (m)	27.70±1.93	27.21±2.43	26.91±2.25	25.73±1.36	0.487
LS (m/g)	10.00±0.70	10.23±0.92	9.72±0.81	9.19±0.49	0.186

Data analyzed by ANOVA. p = p-value. TD – Total diameter (μ m); LD – Luminal diameter (μ m); EH – Epithelium height (μ m); TL – Total length of seminiferous tubules per testicle (m); LS – Length of seminiferous tubules per testicle gram (m/g).

Table 5. Volumetric density and volume of components of the intertubular testicular compartment from Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean \pm standard deviation.

Parameter	Control (n=5)	GBE 3.5 (n=5)	GBE 7.0 (n=5)	GBE 14.0 (n=5)	p
LEY (%)	44.30 \pm 3.91	49.52 \pm 7.44	50.08 \pm 2.69	45.32 \pm 3.53	0.173
BLV (%)	5.30 \pm 0.73	5.98 \pm 0.95	6.32 \pm 0.44	6.10 \pm 1.07	0.281
L YM (%)	20.18 \pm 1.60	17.48 \pm 1.72	19.38 \pm 4.79	20.24 \pm 4.13	0.550
COT (%)*	29.18 \pm 0.70	23.38 \pm 9.89	23.72 \pm 4.70	24.82 \pm 6.68	0.362
MAC (%)	2.36 \pm 0.54	1.98 \pm 0.27	1.90 \pm 0.42	1.88 \pm 0.29	0.228
VLEY (μ L)	61.00 \pm 6.78 ^e	46.00 \pm 6.93 ^{a,f}	46.40 \pm 1.82 ^b	64.40 \pm 6.88 ^{c,d}	**
VBLV (μ L)	7.00 \pm 1.58	6.00 \pm 2.12	7.60 \pm 1.67	8.20 \pm 1.30	0.245
VLYM (μ L)	24.80 \pm 6.06	16.60 \pm 3.72	22.40 \pm 9.76	28.60 \pm 9.74	0.140
VCOT (μ L)	33.80 \pm 6.69	25.60 \pm 11.52	26.20 \pm 6.54	34.60 \pm 10.67	0.283
VMAC (μ L)*	2.60 \pm 0.55	2.40 \pm 1.14	2.00 \pm 0.00	2.4 \pm 0.55	0.416

* Volumetric density of connective tissue and volume of macrophages analyzed by Kruskal-Wallis test. ** ^a Control group differs from GBE 3.5 ($p=0.006$ – *post hoc* de Tukey test); ^b Control group differs from GBE 7.0 ($p=0.007$ – *post hoc* de Tukey test); ^c Group GBE 3.5 differs from GBE 14.0 ($p=0.001$ – *post hoc* de Tukey test); ^d Group GBE 7.0 differs from GBE 14.0 ($p=0.001$ – *post hoc* de Tukey test); ^e Control group does not differ from GBE 14.0 ($p=0.808$ – *post hoc* de Tukey test); ^f Group GBE 3.5 does not differ from GBE 7.0 ($p=1.000$ – *post hoc* de Tukey test). LEY – Volumetric density of Leydig cells; BLV – Volumetric density of blood vessels; LYM – Volumetric density of lymphatic spaces; COT – Volumetric density of connective tissue; MAC – Volumetric density of macrophages; VLEY – volume Leydig cells (μ L); VBLV – volume blood vessels (μ L); VLYM – volume lymphatic spaces (μ L); VCOT – volume connective tissue (μ L); VMAC – volume of macrophages (μ L).

NTP (2013) which did not demonstrate any effect on sperm analysis and reproductive organs weight.

According to Sharpe (1998), the male reproductive system possesses estrogenic receptors α (ER- α) and β (ER- β). Oh and Chung (2004), reported the affinity of the main flavonoid constituents isolated from GBE (quercetin, kaempferol and isorhamnetin) with the human recombinant estrogenic receptors α (ER- α), particularly the β (ER- β) *in vitro*. Hence, an estrogenic overload would be expected to occur as a consequence of the binding of GBE constituents to the estrogenic receptors, resulting in alterations in the spermatogenic process as caused by other disruptors such as the parabens (Hoberman et al., 2008; Tavares et al., 2009), organochlorine pesticides (Bretveld et al., 2007; Uzun et al., 2009; Victor-Costa, et al., 2010), bisphenols (Sakaue et al., 2001) and phthalates (Hu et al., 2009; Saillenfait et al., 2009; Martino et al., 2010). However, the data observed in the testes of GBE treated-rats did not show any statistical difference in the mean gonadal weight, total and luminal diameters, and the height of the epithelium of the seminiferous tubules when compared to the control group and between the GBE treated groups. Testicular weight exhibits a strong correlation with the number of germinative cells present in the gonads (Russell et al., 1990) and, according to França and Russell (1998), there is also a positive correlation between the spermatogenic activity and the diameter of the seminiferous tubules and the height of the epithelium. The findings obtained in this study indicate that GBE did

not produce any modification in the morphology of the seminiferous tubules.

Substances exhibiting estrogenic effect can cause damage to the male reproductive system (Pflieger et al., 2004; Safe, 2004) by acting directly in the activity of the hormones of the hypothalamic-pituitary-gonadal axis or in the testicular structures. Shimomura et al. (2005), showed that the synthetic estrogens such as ethinylestradiol, used as female contraceptives and in the treatment of prostate hypertrophy and cancer, can bind to estrogenic receptors in the pituitary gland and in the hypothalamus, and reduce the secretion of the gonadotropin-releasing hormone (GnRH), the follicle stimulating hormone (FSH) and the luteinizing hormone (LH), and consequently reduce the levels of testosterone.

The reduction of the volume of the Leydig cells in the GBE treated-groups (GBE 3.5 and GBE 7.0), although not related to reduction of the serum testosterone level, could suggest some functional deficit of these cells. Johnson et al. (1992), have already demonstrated a reduction in Leydig cells volume and function in adult rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) - a toxic halogenated aromatic of industrial components - without a significant effect on spermatogenesis. In another study, Johnson et al. (1994) showed that the rats that received TCDD had a reduction in total volume of both Leydig cell smooth endoplasmic reticulum and mitochondria. Reduction in content of organelles that are responsible for various steps in steroidogenesis, could explain TCDD-reduced production of testosterone in rats.

Although, rats has had a decrease in serum testosterone concentration after being exposed to TCDD, these studies also further illustrate the reserve capacity of Leydig cell function to maintain spermatogenesis when the volume of these cells is significantly reduced.

In our study, other organelles could have reduced its volume but could have not influenced the testosterone production. This feature of reserve capacity of Leydig cell could explain why a decrease of serum testosterone concentration did not happen. This issue needs to be further investigated.

Conclusion

This study shows that adult Wistar rats treated with GBE displayed histometric alterations in the volume of the Leydig cells, although they did not exhibit macroscopic alterations in the organs of the male reproductive system or in the serum testosterone level, which could indicate some functional deficit of these cells.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

REFERENCES

- Al-Yahya AA, Al-Majed AA, Al-Bekairi AM, Al-Shabanah OA, Qureshi S (2006). Studies on the reproductive, cytological and biochemical toxicity of *Ginkgo biloba* in Swiss albino mice. *J. Ethnopharmacol.* 107:222-228.
- Amin A, Abraham C, Hamza AA, Abdalla ZA, Al-Shamsi SB, Harethi SS, Daoud S (2012). A standardized extract of *Ginkgo biloba* neutralizes cisplatin-mediated reproductive toxicity in rats. *J. Biomed. Biotechnol.* 2012:1-11.
- Asare GA, Addo P, Bugyei K, Gyan B, Adjei S, Otu-Nyarko LS, Wiredu EK, Nyarko A (2011). Acute toxicity studies of aqueous leaf extract of *Phyllanthus niruri*. *Interdiscip. Toxicol.* 4: 206-210.
- Assinder S, Davis R, Fenwick M, Glover A (2007). Adult-only exposure of male rats to a diet of high phytoestrogen content increases apoptosis of meiotic and post-meiotic germ cells. *Reproduction* 133:11-19.
- Attal J, Courot M (1963). Développement testiculaire et établissement de la spermatogénèse chez le taureau. *Ann. Biol. Anim. Biochem. Biophys.* 3:219-241.
- Blumenthal M (2003). *Ginkgo biloba*. In: Blumenthal M. *The ABC Clinical Guide to Herbs*. New York: Thieme. pp. 185-200.
- Bretveld R, Brouwers M, Ebisch I, Roeleveld N (2007). Influence of pesticides on male fertility. *Scand. J. Work. Environ. Health.* 33:13-28.
- Chávez-Morales RM, Jaramillo-Juárez F, Posadas del Río FA, Reyes-Romero MA, Rodríguez-Vázquez ML, Martínez-Saldaña MC (2010). Protective effect of *Ginkgo biloba* extract on liver damage by a single dose of CCl₄ in male rats. *Hum. Exp. Toxicol.* 30:209-216.
- Das S, Parveen S, Kundra CP, Pereira BMJ (2004). Reproduction in male rats is vulnerable to treatment with the flavonoid-rich seed extracts of *Vitex negundo*. *Phytother. Res.* 18:8-13.
- França LR, Russell LD (1998). The testis of domestic mammals. In: Martínez-García F, Regadera J (eds.), *Male reproduction – a multidisciplinary overview*. Madrid: Churchill Communications. pp. 198-219.
- Fransen HP, Pelgrom SMGJ, Stewart-Knox B, Kaste D, Verhagen H (2010). Assessment of health claims, content, and safety of herbal supplements containing *Ginkgo biloba*. *Food Nutr. Res.* 54:5221.
- Hoberman AM, Schreur DK, Leazer T, Daston GP, Carthew P, Re T, Loretz L, Mann P (2008). Lack of effect of butylparaben and methylparaben on the reproductive system in male rats. *Birth Defects Res. B Dev. Reprod. Toxicol.* 83:123-133.
- Hu GX, Lian QQ, Ge RS, Hardy DO, Li XK (2009). Phthalate-induced testicular dysgenesis syndrome: Leydig cell influence. *Trends Endocrinol. Metab.* 20:139-145.
- Johnson L, Petty CS, Neaves WB (1981). A new approach to quantification of spermatogenesis and its application to germinal cell attrition during human spermatogenesis. *Biol. reprod.* 25:217-226.
- Johnson L, Dickerson R, Safe SH, Nyberg CL, Lewis RP, Welsh TH (1992). Reduced Leydig cell volume and function in adult rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin without a significant effect on spermatogenesis. *Toxicology* 76:103-118.
- Johnson L, Wilker CS, Safe SH, Scott B, Dean DD, White PH (1994). 2,3,7,8-Tetrachlorodibenzo-p-dioxin reduces the number, size, and organelle content of Leydig cells. *Toxicology* 89:49-65.
- Kamal R, Gupta RS, Lohiya NK (2003). Plants for male fertility regulation. *Phytother. Res.* 17:579-590.
- Kang BJ, Lee SJ, Kim MD, Cho MJ (2002). A placebo-controlled, double-blind trial of *Ginkgo biloba* for antidepressant-induced sexual dysfunction. *Hum. Psychopharmacol.* 17:279-284.
- Kanter M (2011). Protective effects of *Ginkgo biloba* (EGb 761) on testicular torsion/detorsion-induced ischemia-reperfusion injury in rats. *Exp. Mol. Pathol.* 91:708-713.
- Koch E, Jaggy H, Chatterjee SS (2000). Evidence for immunotoxic effects of crude *Ginkgo biloba* L. leaf extracts using the popliteal lymphnode assay in the mouse. *Int. J. Immunopharmacol.* 22:229-236.
- Mackay D (2004). Nutrients and botanicals for erectile dysfunction: examining the evidence. *Altern. Med. Rev.* 9:4-16.
- Malviya N, Jain S, Gupta VB, Vyas S (2011). Recent studies on aphrodisiac herbs for the management of male sexual dysfunction – a review. *Acta. Pol. Pharm.* 68:3-8.
- Mangelsdorf I, Buschmann J, Orthen B (2003). Some aspects relating to the evaluation of the effects of chemicals on male fertility. *Regul. Toxicol. Pharmacol.* 37:356-369.
- Mar C, Bent S (1999). An evidence-based review of the 10 most commonly used herbs. *West. J. Med.* 171:168-171.
- Martino-Andrade AJ, Chahoud I (2010). Reproductive toxicity of phthalate esters. *Mol. Nutr. Food Res.* 54:148-157.
- Moyad MA, Barada JH, Lue TF, Mulhall JP, Goldstein I, Fawzy A (2004). Prevention and treatment of erectile dysfunction using lifestyle changes and dietary supplements: what works and what is worthless, part II. *Urol. Clin. North. Am.* 31:259-273.
- National Research Council (2003). *Manual sobre cuidados e usos de animais de laboratório*. Goiânia: Academy Press. 162p.
- National Toxicology Program (2013). NTP Technical report on the toxicology and carcinogenesis studies of *Ginkgo biloba* extract (CAS NO. 90045-36-6) in F344/N rats and B6C3F1/N mice (gavage studies). NIH Publication No. 13-5920. National Institutes of Health, Public Health Service, U.S. Department of Health and Human Services. Available at: www.ntp.niehs.nih.gov/ntp/htdocs/HT_rpts/TR578_508.pdf Accessed in 15 October 2013).
- OECD (2008). Test Guideline 452. OECD Test Guideline for Testing of Chemicals. Chronic Toxicity Studies. Available at: www.oecd.org/chemicalsafety/testing/41753317.pdf. Accessed 15 October, 2013.
- Oh SM, Chung KH (2004). Estrogenic activities of *Ginkgo biloba* extracts. *Life Sci.* 74:1325-1335.

- Oken BS, Storzbach DM, Kaye JA (1998). The efficacy of *Ginkgo biloba* on cognitive function in Alzheimer disease. *Arch. Neurol.* 55:1409-1415.
- Ondrizek RR, Chan PJ, Patton WC, King A (1999a). An alternative medicine study of herbal effects on the penetration of zona-free hamster oocytes and the integrity of sperm deoxyribonucleic acid. *Fertil. Steril.* 71:517-522.
- Ondrizek RR, Chan PJ, Patton WC, King A (1999b). Inhibition of human sperm motility by specific herbs used in alternative medicine. *J. Assist. Reprod. Genet.* 16:87-91.
- Patisaul HB, Adewale HB (2009). Long-term effects of environmental endocrine disruptors on reproductive physiology and behavior. *Front. Behav. Neurosci.* 3:1-18.
- Perreault SD, Cancel AM (2001). Significance of incorporating measures of sperm production and function into rat toxicology studies. *Reproduction* 121:207-216.
- Pflieger-Bruss S, Schuppe HC, Schill WB (2004). The male reproductive system and its susceptibility to endocrine disrupting chemicals. *Andrologia* 36:337-345.
- Predes FS, Monteiro JC, Matta SL, Garcia MS, Dolder H (2011). Testicular histomorphometry and ultrastructure of rats treated with cadmium and *Ginkgo biloba*. *Biol. Trace Elem. Res.* 140:330-341.
- Russell LD, Ettlin RA, Hikim APS, Clegg ED (1990). *Histological and histopathological evaluation of the testis*. 1st ed. Clearwater: Cache River Press.
- Safe S (2004). Endocrine disruptors and human health: is there a problem. *Toxicology* 205:3-10.
- Saillenfait AM, Sabaté JP, Gallissot F (2009). Effects of *in utero* exposure to di-n-hexyl phthalate on the reproductive development of the male rat. *Reprod. Toxicol.* 28:468-476.
- Sakaue M, Ohsako S, Ishimura R, Kurosawa S, Kurohmaru M, HAYASHI Y, AOKI Y, YONEMOTO J, TOHYAMA C (2011). Bisphenol-A affects spermatogenesis in the adult rat even at a low dose. *J. Occup. Health* 43:185-190.
- Seed J, Chapin RE, Clegg ED, Dostal LA, Foote RH, Mark EH, Klinefelter GR, Makris SL, Perreault SD, Scharader S, Seyler D, Sprando R, Treinen KDN, Veeramachaneni R, Wise A (1996). Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. *Reprod. Toxicol.* 10:237-244.
- Sellers RS, Morton D, Michael B, Roore N, Johnson JK, Yano BL, Perry R, Schaffer K (2007). Society of Toxicologic Pathology Position Paper: Organ Weight Recommendations for Toxicology Studies. *Toxicol. Pathol.* 35:751-755.
- Sharpe RM (1998). The roles of oestrogen in the male. *Trends Endocrinol. Metab.* 9:371-377.
- Shimomura K, Shimada M, Hagiwara M, Harada S, Kato M, Furuhashi K (2005). Insights into testicular damage induced by ethinylestradiol in rats. *Reprod. Toxicol.* 20:157-163.
- Siegers CP (1999). Cytotoxicity of alkylphenols from *Ginkgo biloba*. *Phytomedicine* 6:281-283.
- Sierpina VS, Wollschlaeger B, Blumenthal M (2003). *Ginkgo biloba*. *Am. Fam. Physician* 68:923-926.
- Smith JV, Luo Y (2004). Studies on molecular mechanisms of *Ginkgo biloba* extract. *Appl. Microbiol. Biotechnol.* 64:465-472.
- Stromgaard K, Nakanishi K (2004). Chemistry and biology of terpene trilactones from *Ginkgo biloba*. *Angew. Chem. Int. Ed. Engl.* 43:1640-1658.
- Taepongsorat L, Tangpraprutgul P, Kitana N, Malaivijitnond S (2008). Stimulating effects of quercetin on sperm quality and reproductive organs in male rats. *Asian J. Androl.* 10:249-258.
- Tamler R, Mechanick JI (2007). Dietary supplements and nutraceuticals in the management of andrologic disorders. *Endocrinol. Metab. Clin. North. Am.* 36:533-552.
- Tavares RS, Martins FC, Oliveira PJ, Santos JR, Peixoto FP (2009). Parabens in male infertility – Is there a mitochondrial connection? *Reprod. Toxicol.* 27:1-7.
- Uzun FG, Kalender S, Durak D, Demir F, Kalender Y (2009). Malathion-induced testicular toxicity in male rats and the protective effect of vitamins C and E. *Food Chem. Toxicol.* 47:1903-1908.
- Van Beek TA (2002). Chemical analysis of *Ginkgo biloba* leaves and extracts. *J. Chromatogr. A.* 967:21-55.
- Victor-Costa AB, Bandeira SM, Oliveira AG, Mahecha GA, Oliveira CA (2010). Changes in testicular morphology and steroidogenesis in adult rats exposed to atrazine. *Reprod. Toxicol.* 29:323-331.
- Wang W, Zhong XH, Ma A, Shi W, Zhang XS, Liu Y (2008). Effects of *Ginkgo biloba* on testicle injury induced by diethylstilbestrol in mice. *Am. J. Chin. Med.* 36:1135-1144.
- Wheatley D (2004). Triple-blind, placebo-controlled trial of *Ginkgo biloba* in sexual dysfunction due to antidepressant drugs. *Hum. Psychopharmacol.* 19:545-548.
- WHO (World Health Organization) (1999). *Folium Ginkgo*. WHO Monographs on selected medicinal plants. Geneva: WHO Press. pp. 154-167.
- WHO (World Health Organization) (2010). *WHO laboratory manual for the examination and processing of human semen*. 5th ed. Geneva: WHO Press.
- Wolfensohn S, Lloyd M (2013). *Handbook of laboratory animal management and welfare*. 4th ed. Oxford: Wiley-Blackwell.
- Yeh YC, Liu TJ, Wang LC, Lee HW, Ting CT, Lee WL, Hung CJ, Wang KY, Lai HC, Lai HC (2009). A standardized extract of *Ginkgo biloba* suppresses doxorubicin-induced oxidative stress and p53-mediated mitochondrial apoptosis in rat testes. *Br. J. Pharmacol.* 156:48-61.

Full Length Research Paper

In vitro* and *in vivo* evaluation of quinones from *Auxemma oncocalyx* Taub. on *Leishmania braziliensis

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The effect of a quinone fraction (QF) isolated from the heartwood of *Auxemma oncocalyx* Taub. was investigated *in vitro* and *in vivo* on *Leishmania braziliensis*. Although QF (1-10 µg/mL) showing marked *in vitro* anti-*Leishmania* activity (81 to 94%), the oral treatment with the compound did not protect hamsters against progressive *L. braziliensis* infection. When QF was administered intraperitoneally (20 mg/kg) the lesion size was reduced by 65%; however, it was not able to promote parasite eradication, as evidenced by the high number of parasites in draining lymph nodes. Quinones are highly redox active molecules and with their semiquinones radicals can lead to formation of reactive oxygen species (ROS). The generation of ROS could probably explain the *in vitro* leishmanicidal activity of the quinones, since promastigotes are susceptible to H₂O₂ lethal effect *in vitro*. In conclusion, although quinones seem to be effective against *Leishmania* parasites *in vitro*, they do not demonstrate a therapeutic effect in experimental leishmaniasis. In addition, it can be hypothesized that QF *in vivo* might possibly be converted into non-active metabolite(s) or be inactivated either by reduction or by interaction with serum proteins, losing its leishmanicidal activity.

Key words: *Leishmania braziliensis*, *Auxemma oncocalyx*, quinone, hamster, cutaneous leishmaniasis.

INTRODUCTION

Leishmaniasis are protozoan diseases which represent a risk for 350 million people worldwide, and 2 million new cases occur yearly (World Health Organization [WHO], 2010). Etiologic agents are intracellular parasites of the genus *Leishmania* that display a spectrum of a manifestation which goes from cutaneous involvement with

late destruction of mucous membranes to generalized systemic visceral disease with fatal outcome, if not treated (Pearson et al., 2000; Desjeux, 2004). There is still no effective vaccine to control the wide range of disease caused by different species of *Leishmania* (Oliveira et al., 2009). The disease may regress spontaneously or evolve,

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thus requiring treatment (Piscopo and Mallia, 2006). The antileishmanial first-line drugs are the pentavalent antimonials, meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®). They are generally effective during the acute infection stages but not against the late stages, and produce significant side effects due to high toxicity and tissue drug accumulation, which includes myalgias, nausea, vomiting, cardiac arrhythmia, hepatitis, or pancreatitis (Croft et al., 2006; Palumbo, 2009). Amphotericin B and pentamidine are better tolerated drugs, but require long courses of parenteral administration (Amato et al., 2008). Miltefosine and fluconazole have recently showed effectiveness against cutaneous leishmaniasis (CL) caused by *Leishmania braziliensis* (Machado et al., 2010; Sousa et al., 2011), but despite the lower toxicity, these second line drugs are not useful against other forms of leishmaniasis (Palumbo, 2009). Furthermore, the continuous use of ineffective drugs has led to the development of resistance to their compounds (Escobar et al., 2001), which have stirred an urgent need for novel, effective, and safe drugs for treatment of leishmaniasis.

Since plant derivatives are among the most active agents against infections (Delorenzi et al., 2001), many researchers have been looking for better effects and less toxicity to treat leishmaniasis in these substances, especially those already used by people living in endemic areas (Weniger et al., 2001). *Auxemma oncocalyx* Taub. belongs to the Boraginaceae family, it is known as “pau branco” (white wood), and is easily available by rural settlers in Northeastern Brazil. The stem bark of the tree is astringent and popularly used in the treatment of wounds (Braga, 1976).

Previous pharmacological studies reported that the hydroalcoholic extract of the stem presents antioxidant, analgesic and anti-inflammatory properties (Ferreira et al., 2004, 2008). Alantoin and β -sitosterol glycoside have been isolated from *A. oncocalyx* and are responsible for some of its pharmacological properties (Pessoa and De Lemos, 1997). Besides these compounds, at least six quinones were successfully isolated and oncocalyxone A, obtained in significant amounts, seems to be partially involved in the bioactivity of the plant (Leyva et al., 2000). Several quinones present antileishmanial (Sauvain et al., 1993; Sittie et al., 1999; Teixeira et al., 2001), antitumoral (Morello et al., 1995; Itoigawa et al., 2003), antifungal (Perry et al., 1991; Gafner et al., 1996), or antimalarial activity (Figueiredo et al., 1998), either *in vitro* or *in vivo*. The aim of the present work was to evaluate the *in vitro* and *in vivo* activity of the quinone fraction from *A. oncocalyx* Taub. against *L. braziliensis*.

MATERIALS AND METHODS

Plant extraction and purification of the quinone fraction

A. oncocalyx Taub. was collected in the city of Pentecoste, Ceará

State, Northeastern Brazil, and identified by Prof. A. G. Fernandes from the Biological Sciences Department. A voucher specimen has been deposited under the number 18459 at the Prisco Bezerra Herbarium of the Universidade Federal do Ceará, Brazil. The quinone fraction (QF) was prepared from grinded heartwood ethanolic extract through exhaustive aqueous extraction followed by lyophilization. Hydrosoluble components contained around 80% of oncocalyxone A, according to a previous characterization (Pessoa et al., 1993).

Parasites and animals

Three to four months adult female and male golden hamsters (*Mesocricetus auratus*), weighing 80 to 90 g, obtained from the central animal facility of Departamento de Patologia e Medicina Legal of Universidade Federal do Ceará (DPML/UFC), and housed in groups of six to eight per cage with free access to water and food. The Animal Care and Utilization Committee from UFC approved all experimental procedures (process n° 65/08). The *L. braziliensis* (MHOM/BR/94/H-3227) was originally isolated from skin lesions of a patient with CL from Ceará State, and previously typed using isoenzymes electrophoresis and monoclonal antibodies (De Oliveira et al., 2004).

The parasites, stored in liquid nitrogen, were thawed and cultured as promastigotes at 26°C in Schneider's insect medium (Sigma-Aldrich, Chemical Co., St. Louis, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma), 2% sterile normal human urine, 2 mM L-glutamine (Gibco BRL, Grand Island, NY), and antibiotics [100 U/ml penicillin, 100 µg/ml streptomycin sulfate (Sigma-Aldrich)]. Subcultures were made in the stationary phase of growth and parasites were used at no later than the fourth passage. Prior to infection, promastigotes were harvested from culture, washed in sterile saline, counted in Neubauer's chamber and adjusted to the appropriate concentration.

Anti-promastigote activity

For the tests *in vitro* against promastigotes in 96-well plates stationary-phase promastigotes were added at a concentration of 10^6 cells/well in Schneider medium supplemented with 10% heat-inactivated fetal calf serum (Sigma), 2% sterile normal human urine, 2 mM L-glutamine (Gibco), and 100 U/ml penicillin + 100 µg/ml streptomycin sulfate (Sigma-Aldrich) determined after counting in a Neubauer chamber. Drugs were diluted with DMSO 5% and placed at concentrations of 0.01 to 10 µg/mL. Amphotericin B was chosen as the control drug. The plates were incubated in a biochemical oxygen demand (BOD) at 24°C, and after that, 1 µCi per well of [3H]thymidine (Amersham International, Amersham, UK) was added and the cells were incubated for another 24 h and harvested. [3H]thymidine incorporation was measured in a β -counter (Pharmacia, Finland) after washing to distinguish the non-used thymidine from the one incorporated in DNA. Assays were done in duplicate and made a replica of each test. The inhibition of growth was expressed as the percent decrease of radioactive incorporation in treated cells when compared with untreated control. It was considered a good antileishmanial activity when values equal or greater than 70% were obtained. The formula for calculation of antileishmanial activity was:

$$\frac{\text{cpm of control cells (promastigotes)} - \text{cpm of treated cells (promastigotes + drugs)}}{\text{cpm of control cells (promastigotes)}} \times 100$$

$$\text{cpm of control cells (promastigotes)} \times 100$$

Infection, treatment and lesion development

Hamsters were infected subcutaneously in the right hind footpad with 10^6 stationary phase *L. braziliensis* promastigotes in 20 μ l of sterile saline. Treatment was initiated two weeks after inoculation when lesions were well defined. Animals were randomly divided into groups of six to eight and the drugs administered daily for 28 consecutive days. QF was dissolved in distilled water containing 0.1% of Tween 80 and 0.5% of carboxymethylcellulose (CMC) and administered using the following routes: (a) oral (p.o.), with 10 or 20 mg/kg by intragastric intubation; (b) intraperitoneal (i.p.) with 20 mg/kg. Alternatively, Glucantime® (Sanofi-Aventis Farmacêutica, São Paulo, Brazil) was injected at the dose of 60 mg/kg/day intramuscularly (i.m.). Control groups received p.o. or i.p. drugless vehicle (Tween 80 + CMC + H₂O) at equivalent volumes. Before treatment was conducted, a toxicity test using various concentrations of QF that demonstrated concentrations of 10 and 20 mg/kg did not kill the animals. Lesion sizes were measured weekly with a dial gauge caliper (Mitutoyo, 0.01 mm sensitivity) and expressed as the difference between the thicknesses (mm) of the infected and contralateral uninfected footpads.

Treatment outcome

The number of parasites in the popliteal lymph was quantified by the limiting dilution technique as previously described (Lima et al., 1997). Briefly, after the treatment, the animals were euthanized by inhalation of halothane (Sigma-Aldrich) and submerged in 3% iodized alcohol up to 3 min to allow decontamination. The lymph nodes were removed aseptically and macerated in a Petri dish with 2 ml of Schneider medium. After removal of debris by sedimentation for 5 min, the homogenates were serially diluted (1:10) in Schneider's medium supplemented with 100 U/ml of penicillin/ml, 100 μ g/ml of streptomycin/ml, 10% fetal calf serum and 2% sterile human urine. One hundred microliters of these dilutions was distributed into 96-well flat bottom plates containing agar-blood in 6 replicates per concentration. The plates were incubated at 25°C and observed under an inverted microscope (Nikkon, Japan) every 3 days, up to a maximum of 30 days, to record the dilutions containing promastigotes. The final number of parasites per tissue was determined using the ELIDA software, version 12c for window (Taswell, 1984).

Statistical analysis

The data are presented as mean \pm standard error of the mean. The significance of the results relating to the parasite load was calculated by Mann-Whitney test. The anti-promastigote activity and lesion sizes from treated and untreated animals were analyzed by the one-way analysis of variance (ANOVA) and complemented by the Bonferroni test for multiple comparisons. All analysis and graphs were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, USA). Values of $P < 0.05$ were considered significant.

RESULTS

QF displayed marked *in vitro* anti-promastigote activity at concentration of 1 and 10 μ g/mL (80.5 ± 2.80 and $91.0 \pm 0.65\%$, respectively) against *L. braziliensis*, being similar to Amphotericin B (Figure 1). To determine the effect of QF on *L. braziliensis* infection in hamsters, the animals days via oral. The results showed that hamsters treated with QF (10 or 20 mg/kg, p.o.) did not present significant

reduction of the lesion thickness as compared to untreated animals. Also, doses of 20 and 10 mg/kg (p.o.), did not produce significant reduction of parasite load in lymph nodes as compared to untreated group (Figure 2A and B). As expected, animals treated with glucantime showed antimonial drug effectively brought footpad sizes down to normal within 2 weeks of treatment and also suppressed parasite growth in lymph nodes (Figure 2A and B).

To investigate if a different administration route of the drug could be more effective on controlling *L. braziliensis* infection, hamsters were treated with 20 mg/kg QF intraperitoneally for 28 days (the highest dose, but yet not toxic to animals). The results showed that the lesion size of hamsters treated with QF i.p. decreased by 65% ($P < 0.05$), and parasite burden in the lymph nodes was significantly lower ($9.3 \pm 3.5 \times 10^5$) when compared with untreated controls ($1.98 \pm 2.0 \times 10^6$) (Figure 3A and B).

DISCUSSION

Antileishmanial activity has been reported in several compounds extracted from medicinal plants belonging to diverse chemical groups, including quinones (Chan-Bacab et al., 2001; Rocha et al., 2005). Other studies have found simple quinones isolated from dried trunks of *Jacaranda copaia* to present significant anti-promastigote and anti-amastigote activities *in vitro* against *L. amazonensis*, but only weak activity when tested against *L. amazonensis*-induced lesion in mice (Sauvain et al., 1993). Benzoquinones were found to be active *in vitro* against trypanosomes, but this has not been confirmed *in vivo* (Grady et al., 1984; Pahn et al., 1988).

In murine model, *Leishmania major*- and *Leishmania donovani*-infected BALB/c treated with buparvaquone formulation (BPQ), showed parasite burden decrease in lesions and liver, smaller and not ulcerated lesions, in comparison with untreated control (Garnier et al., 2007). Recently, a study using the molecular hybridization of a naphthoquinone core with a pterocarpan moiety (LGB-118) led to significant reduction in skin lesions development, swelling, ulceration and parasite burden of BALB/c *L. amazonensis*-infected (Da Cunha-Júnior et al., 2011). Also, acetylisolapachol, a hydroxyquinone derivative, showed *in vitro* activity against *L. braziliensis*, and *in vitro* and *in vivo* against *L. amazonensis* (Lima et al., 2004).

The leishmanicidal activity of a drug may be selective and direct against the parasite, or it may act indirectly by activating macrophage microbicidal mechanisms for instance. According to *in vitro* model systems, the macrophage microbicidal response to *Leishmania* infection can follow two distinct pathways. Upon infection, promastigotes elicit a respiratory burst with the generation of reactive oxygen intermediates such as hydrogen peroxide (H₂O₂), [•]OH radical, superoxide ([•]O₂⁻) were inoculated with promastigotes and treatment for 28

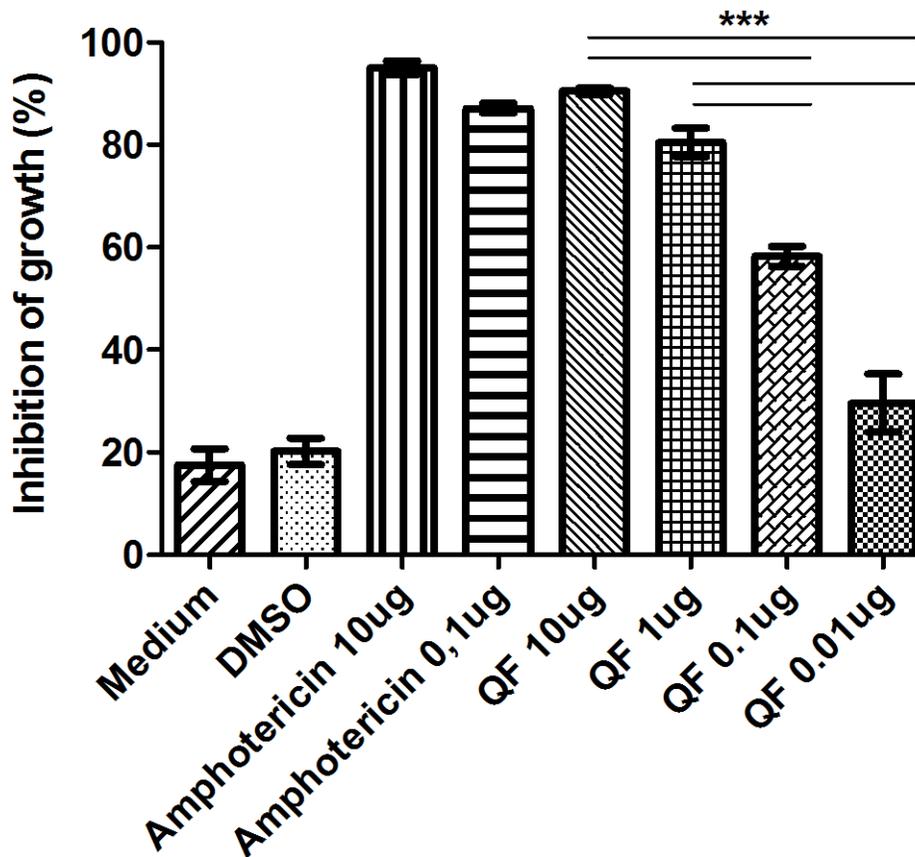


Figure 1. Antileishmanial activity *in vitro* of a quinone fraction (QF) isolated from the heartwood of *Auxemma oncocalyx* Taub. against promastigotes of *L. braziliensis*. The inhibition of growth was expressed as the percent decrease of radioactive incorporation in treated parasites when compared with untreated control. *** $P < 0.05$ (test ANOVA).

and peroxynitrate as part of an oxygen dependent mechanism to kill promastigotes, however, a small percentage of phagocytosed organisms can survive (Beaman and Beaman, 1984). Second, murine or human macrophages can be activated to kill intracellular amastigotes, the form present during established infection, by previous exposure to cytokines such as IFN- γ and TNF- α , which activate both oxidative and non- H_2O_2 -associated microbicidal mechanisms (Bogdan et al., 1990; Liew, 1992; Assreuy et al., 1994; McSorley et al., 1996; Panaro et al., 1999). Studies have demonstrated that both H_2O_2 -associated and non- H_2O_2 -associated pathways contribute to *Leishmania* killing and that their relative degree of importance may differ during initial promastigote invasion versus established amastigote infection (Chang, 1983; Murray and Nathan, 1999; Erel et al., 1999).

Quinones are highly redox active molecules and with their semiquinones radicals can lead to formation of reactive oxygen species (ROS), including $\cdot O_2^-$, H_2O_2 , and ultimately to hydroxyl radicals (Bolton et al., 2000). The formation of ROS could probably explain the *in vitro* leishmanicidal activity of the quinones in this study, since

promastigotes are readily susceptible to killing by H_2O_2 *in vitro* (Murray, 1981; Zarley et al., 1991). It has been shown that *Leishmania chagasi* promastigotes *in vitro* are susceptible to killing by both H_2O_2 and the redox-cycling compound menadione, a quinone that causes the generation of $\cdot O_2^-$ in the presence of promastigotes (Wilson et al., 1994). However, this source of quinone free radicals seems to be often more readily apparent *in vitro* than *in vivo*. Menadione providing an excellent example of this phenomenon, although presents anticancer activity *in vitro* in combination with other chemotherapeutic agents. However, menadione does not demonstrate the same activity *in vivo* even at high doses (Nestor et al., 1991; Djuric et al., 1995). Besides, in a BALB/c mouse model of leishmaniasis, sublethal concentrations of menadione caused *L. chagasi* promastigotes to become more virulent (Wilson et al., 1994).

When QF was administered intraperitoneally at a dose of 20 mg/kg, the lesion size was reduced by 65%, but it was however not able to promote parasite killing as evidenced by the mean number of parasites in the lymph nodes as compared to controls. Reduction in the lesion size induced by *Leishmania* infection can not necessarily

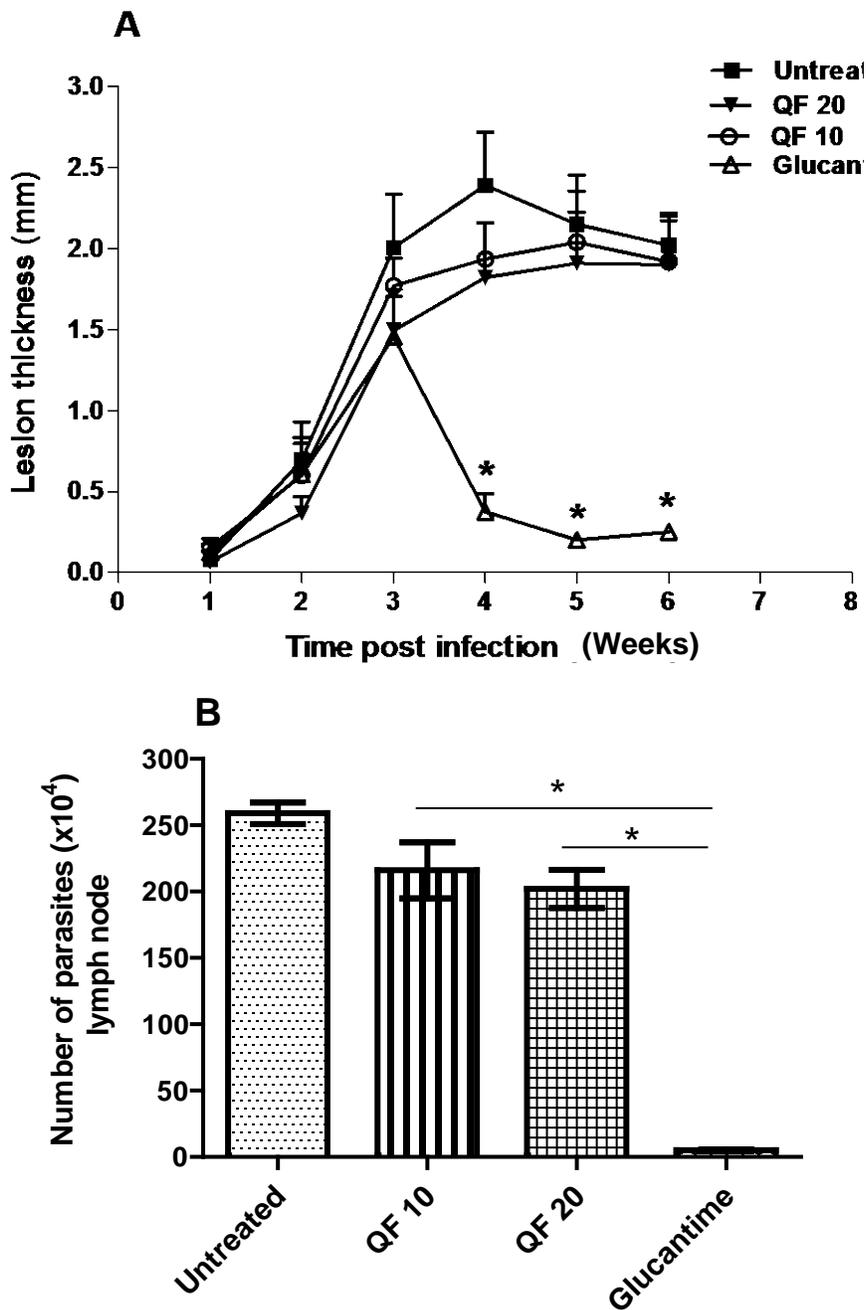


Figure 2. Effect of oral treatment with QF (20 or 10 mg/kg). (A) Lesion growth, and (B) Parasite load in lymph node. Hamsters were infected with 10^6 *L. braziliensis* promastigotes (8 per group). Animals were left untreated or were treated with daily dose of 20 mg/kg QF or with 10 mg/kg or 60 mg/kg/i.m. glucantime. Treatment started three weeks post infection. Lesion thickness was measured weekly using a dial gauge caliper (mean \pm SE, n = 8). Parasite load was evaluated after the treatment. *P < 0.05 (A, Glucantime versus untreated or QF treatment; B, Glucantime versus QF treatment).

mean a decrease in the parasite load, but only a decrease of the local inflammatory reaction. QF of *A. oncocalyx* has a wide range of biological effects including anti-parasitic, antitumoral and antiplatelet activities

(Leyva et al., 2000; Ferreira et al., 2008). It have also been shown that the compound presents anti-inflammatory and antiedematogenic activities, reducing the effect of carrageenan (Ferreira et al., 2004). Therefore, it

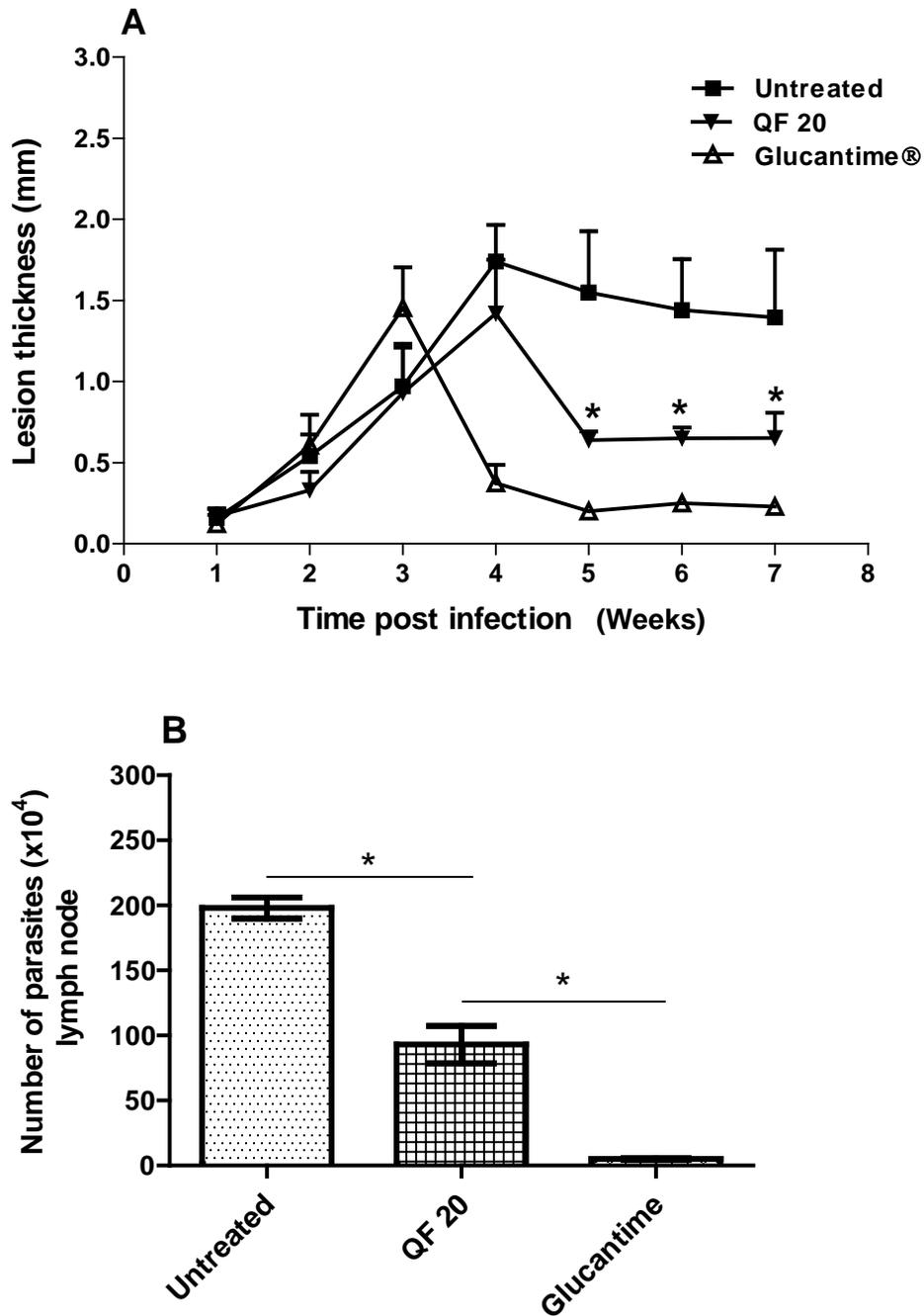


Figure 3. Effect of intraperitoneal treatment with QF (20 mg/kg). (A) Lesion growth, and (B) Parasite load in lymph node. Hamsters were infected with 10^6 *L. braziliensis* promastigotes (6 per group). Animals were left untreated or were treated with daily dose of 20 mg/kg QF or 60 mg/kg/i.m. glucantime. Treatment started three weeks post infection. Lesion thickness was measured weekly using a dial gauge caliper (mean \pm SE, n = 8). Parasite load was evaluated after the treatment. *P < 0.05 (A, QF treatment versus untreated; B, QF treatment versus untreated or Glucantime).

is suggested that the reduction in the lesion size produced by 20mg/kg, i.p. of QF in *L. braziliensis*-infected hamsters may probably be due to its anti-inflammatory effect. In addition, the observation that QF when used via

oral did not offer significant protection in hamsters infected by *L. braziliensis*, suggests that QF can possibly be converted into non-active metabolite(s) or otherwise be inactivated either by reduction or by interaction with

serum proteins. Quinones can be metabolized by various routes: substitution or reductive addition with nucleophilic compounds or one and two-electron reductions (Koster, 1991). Driscoll et al. (1974) found that the biological activity of some quinones, as lapachol and its analogs, is directly related to their chemical structures, thus any structural alteration *in vivo* will result in an inactive product or will abolish their biological activities (Teixeira et al., 2001).

Despite QF not to have demonstrated an anti-parasitic effect in *L. braziliensis*-infected hamsters, the production of quinones derivatives might insure their interest as antileishmanial candidate drugs. Furthermore, alternative therapy derived from medicinal plants opens new perspectives towards the development of effective, readily available and less-costly drugs for the treatment of the leishmaniasis in endemic areas.

Conflict of interest

The authors report no declarations of interest.

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REFERENCES

- Amato VS, Tuon FF, Bacha HA, Neto VA, Nicodemo AC (2008). Mucosal leishmaniasis. Current scenario and prospects for treatment. *Acta Trop.* 105(1):1-9.
- Assreuy J, Cunha FQ, Epperlein M, Noronha-Dutra A, O'Donnell CA, Liew FY, Moncada S (1994). Production of nitric oxide and superoxide by activated macrophages and killing of *Leishmania major*. *Eur. J. Immunol.* 24(3):672-676.
- Beaman L, Beaman BL (1984). The role of oxygen and its derivatives in microbial pathogenesis and host defense. *Annu. Rev. Microbiol.* 38:27-48.
- Bogdan C, Moll H, Solbach W, Rölinghoff M (1990). Tumor necrosis factor- α in combination with interferon- γ , but not with interleukin 4 activates macrophages for elimination of *Leishmania major* amastigotes. *Eur. J. Immunol.* 20(5):1131-5.
- Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ (2000). Role of quinones in toxicology. *Chem. Res. Toxicol.* 13(3):135-60.
- Braga R (1976). Plantas do Nordeste, especialmente do Ceará. 3. ed. Fortaleza: ESAM, p. 510.
- Chan-Bacab MJ, Peña-Rodríguez LM (2001). Plant natural products with leishmanicidal activity. *Nat. Prod. Rep.* 18(6):674-688.
- Chang KP (1983). Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. *Int. Rev. Cytol. Suppl.* 14:267-305.
- Croft SL, Seifert K, Yardley V (2006). Current scenario of drug development for leishmaniasis. *Indian J. Med. Res.* 123(3):399-410.
- Da Cunha-Júnior EF, Pacienza-Lima W, Ribeiro GA, Netto CD, do Canto-Cavalheiro MM, da Silva AJ, Costa PR, Rossi-Bergmann B, Torres-Santos EC (2011). Effectiveness of the local or oral delivery of the novel naphthopterocarpanquinone LQB-118 against cutaneous leishmaniasis. *J. Antimicrob. Chemother.* 66(7):1555-1559.
- De Oliveira CI, Teixeira MJ, Teixeira CR, Ramos de Jesus J, Bomura Rosato A, Santa da Silva J, Brodskyn C, Barral-Netto M, Barral A (2004). *Leishmania braziliensis* isolates differing at the genome level display distinctive features in BALB/c mice. *Microbes Infect.* 6(11):977-984.
- Delorenzi JC, Attias M, Gattass CR, Andrade M, Rezende C, da Cunha Pinto A, Henriques AT, Bou-Habib DC, Saraiva EM (2001). Antileishmanial activity of an indole alkaloid from *Peschiera australis*. *Antimicrob. Agents Chemother.* 45(5):1349-1354.
- Desjeux P (2004). Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* 27(5):305-318.
- Djuric Z, Corbett TH, Valeriotte FA, Heilbrun LK, Baker LH (1995). Detoxification ability and toxicity of quinones in mouse and human tumor cell lines used for anticancer drug screening. *Cancer Chemother. Pharmacol.* 36(1):20-26.
- Driscoll JS, Hazard GF Jr, Wood HB Jr, Goldin A (1974). Structure-antitumor activity relationships among quinone derivatives. *Cancer Chemother. Rep.* 4(2):1-362.
- Erel O, Kocyigit A, Bulut V, Gurel MS (1999). Reactive nitrogen and oxygen intermediates in patients with cutaneous leishmaniasis. *Mem. Inst. Oswaldo Cruz.* 94(2):179-183.
- Escobar P, Yardley V, Croft SL (2001). Activities of hexadecylphosphocholine (miltefosine), AmBisome, and sodium stibogluconate (Pentostam) against *Leishmania donovani* in immunodeficient *scid* mice. *Antimicrob. Agents Chemother.* 45(6):1872-1875.
- Ferreira MA, do Nascimento NR, de Sousa CM, Pessoa OD, de Lemos TL, Ventura JS, Schattner M, Chudzinski-Tavassi AM (2008). Oncocalyxone A inhibits human platelet aggregation by increasing cGMP and by binding to GP Iba glycoprotein. *Br. J. Pharmacol.* 154(6):1216-1224.
- Ferreira MA, Nunes OD, Fontenele JB, Pessoa OD, Lemos TL, Viana GS (2004). Analgesic and anti-inflammatory activities of a fraction rich in oncocalyxone A isolated from *Auxemma oncocalyx*. *Phytomedicine* 11(4):315-322.
- Figueiredo JN, Ráz B, Séquin U (1998). Novel quinone methides from *Salacia kraussii* with in vitro antimalarial activity. *J. Nat. Prod.* 61(6):718-723.
- Gafner S, Wolfender JL, Nianga M, Stoeckli-Evans H, Hostettmann K (1996). Antifungal and antibacterial naphthoquinones from *Newboudia laevis* roots. *Phytochemistry* 42(5):1315-1320.
- Garnier T, Mäntylä A, Järvinen T, Lawrence J, Brown M, Croft S (2007). *In vivo* studies on the antileishmanial activity of buparvaquone and its prodrugs. *J. Antimicrob. Chemother.* 60(4):802-810.
- Grady RW, Blobstein SH, Meshnick SR, Ulrich PC, Cerami A, Amirmoazzami J, Hodnett EM (1984). The *in vitro* trypanocidal activity of N-substituted p-benzoquinones imines: assessment of biochemical structure-activity relationships using the Hansch approach. *J. Cell Biochem.* 25(1):15-29.
- Itoigawa M, Kashiwada Y, Ito C, Furukawa H, Tachibana Y, Bastow KF, Lee KH (2003). Antitumor agents. 203. Carbazole alkaloid muryaquinone A and related synthetic carbazolequinones as cytotoxic agents. *J. Nat. Prod.* 63(7):893-897.
- Koster AS (1991). Bioreductive activation of quinones: a mixed blessing. *Pharm. Weekbl. Sci.* 13(3):123-126.
- Leyva A, Pessoa C, Boogaardt F, Sokaroski R, Lemos TL, Wetmore LA, Huruta RR, Moraes MO (2000). Oncocalyxones A and C, 1, 4-anthracenediones from *Auxemma oncocalyx*: comparison with anticancer 1,9-anthracenediones. *Anticancer Res.* 20(2A):1029-1031.
- Liew FY (1992). Induction, regulation and function of T-cell subsets in leishmaniasis. *Chem. Immunol.* 54:117-135.
- Lima HC, Bleyenbergh JA, Titus RG (1997). A simple method for quantifying *Leishmania* in tissues of infected animals. *Parasitol. Today* 13(2):80-82.
- Lima NM, Correia CS, Leon LL, Machado GM, Madeira Mde F, Santana AE, Goulart MO (2004). Antileishmanial activity of lapachol analogues. *Mem. Inst. Oswaldo Cruz* 99(7):757-761.
- Machado PR, Ampuero J, Guimarães LH, Villasboas L, Rocha AT, Schriefer A, Sousa RS, Talhari A, Penna G, Carvalho EM (2010). Miltefosine in the treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis* in Brazil: a randomized and controlled trial. *PLoS Negl. Trop. Dis.* 4(12):e912.

- McSorley S, Proudfoot L, O'Donnell CA, Liew FY (1996). Immunology of murine leishmaniasis. *Clin. Dermatol.* 14(5):451-464.
- Morello A, Pavani M, Garbarino JA, Chamy MC, Frey C, Mancilla J, Guerrero A, Repetto Y, Ferreira J (1995). Effects and mode of action of 1,4-naphthoquinones isolated from *Calceolaria sessilis* on tumoral cells and trypanosoma parasites. *Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol.* 112(2):119-128.
- Murray HW (1981). Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. *J. Exp. Med.* 153(5):1302-1315.
- Murray HW, Nathan CF (1999). Macrophage microbicidal mechanisms *in vivo*: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. *J. Exp. Med.* 189(4):741-746.
- Nestor KE Jr, Emmerson DA, Anthony NB, Nestor KE (1991). Research note: lack of an effect of high levels of menadione on tumor development in Japanese quail females. *Poult. Sci.* 70(11):2382-2385.
- Oliveira CI, Nascimento IP, Barral A, Soto M, Barral-Netto M (2009). Challenges and perspectives in vaccination against leishmaniasis. *Parasitol. Int.* 58(4):319-324.
- Pahn EM, Molina Portela MP, Stoppani AOM (1988). Efecto de quinonas y nitrofuranos sobre *Trypanosomas mega* y *Crithidia fasciculata*. *Rev. Argent. Microbiol.* 20(3):107-118.
- Palumbo E (2009). Current treatment of cutaneous leishmaniasis: a review. *Am. J. Ther.* 16(2):178-182.
- Panaro MA, Acquafredda A, Lisi S, Lofrumento DD, Trotta T, Satalino R, Saccia M, Mitolo V, Brandonisio O (1999). Inducible nitric oxide synthase and nitric oxide production in *Leishmania infantum*-infected human macrophages stimulated with interferon-gamma and bacterial lipopolysaccharide. *Int. J. Clin. Lab. Res.* 29(3):122-127.
- Pearson RD, Queiroz Sousa A (2000). Leishmania species: visceral (kala-azar), cutaneous, and mucosal leishmaniasis. In: Mandell GL, Bennet EJ, Douglas R (eds). *Principles and Practice of Infectious Diseases* 5th ed. Churchill Livingstone, New York, USA. pp. 2832-2845.
- Perry NB, Blunt JW, Munro MH (1991). A cytotoxic and antifungal 1,4-naphthoquinone and related compounds from a New Zealand brown alga, *Landsburghia quercifolia*. *J. Nat. Prod.* 54(4):978-85.
- Pessoa ODL, De Lemos TLG, Silveira ER, Braz-Filho R (1993). Novel cordiachromes isolated from *Auxemma oncocalyx*. *Nat. Prod. Lett.* 2:145-150.
- Pessoa ODL, De Lemos, TLG (1997). Allantoin and fatty acid composition in *Auxemma oncocalyx*. *Rev. Bras. Farm.* 78:9-10.
- Piscopo TV, Mallia AC (2006). Leishmaniasis. *Postgrad. Med. J.* 82(972):649-57.
- Rocha LG, Almeida JR, Macêdo RO, Barbosa-Filho JM (2005). A review of natural products with antileishmanial activity. *Phytomedicine* 12(6-7):514-535.
- Sauvain M, Dedet JP, Kunesch N, Poisson J, Gantier JC, Gayral P, Kunesch G (1993). *In vitro* and *in vivo* leishmanicidal activities of natural and synthetic quinoids. *Phytother. Res.* 7(2):167-171.
- Sittie AA, Lemmich E, Olsen CE, Hviid L, Kharazmi A, Nkrumah FK, Christensen SB (1999). Structure-activity studies: *in vitro* antileishmanial and malarial activity of anthraquinones from *Morinda lucida*. *Planta Med.* 65(3):259-61.
- Sousa AQ, Frutuoso MS, Moraes EA, Pearson RD, Pompeu MM (2011). High-dose oral fluconazole therapy effective for cutaneous leishmaniasis due to *Leishmania (Vianna) braziliensis*. *Clin. Infect. Dis.* 53(7):693-5.
- Taswell C (1984). Limiting dilution assay for the determination of immunocompetent cell frequencies III. Validity tests for the single-hit Poisson model. *J. Immunol. Methods* 72(1):29-40.
- Teixeira MJ, de Almeida YM, Viana JR, Holanda Filha JG, Rodrigues TP, Prata JR Jr, Coêlho IC, Rao VS, Pompeu MM (2001). *In vitro* and *in vivo* leishmanicidal activity of 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol). *Phytother. Res.* 15(1):44-48.
- Weniger B, Robledo S, Arango GJ, Deharo E, Aragón R, Muñoz V, Callapa J, Lobstein A, Anton R (2001). Antiprotozoal activities of Colombian plants. *J. Ethnopharmacol.* 78(2-3):193-200.
- Wilson ME, Andersen KA, Britigan BE (1994). Response of *Leishmania chagasi* promastigotes to oxidant stress. *Infect. Immunol.* 62(11):5133-5141.
- World Health Organization (WHO) (2010). Control of the Leishmaniasis, Technical Report Series No. 949. World Health Organization, Geneva. P 201.
- Zarley JH, Britigan BE, Wilson ME (1991). Hydrogen peroxide mediated toxicity for *Leishmania donovani chagasi* promastigotes: role of hydroxyl radical and protection by heat shock. *J. Clin. Investig.* 88(5):1511-1521.

Review

***Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz: A review of the biological activities and phytochemical composition**

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***Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz is a medicinal plant widely known in Brazil as “jucá” or “pauffero” that belongs to the Fabaceae family. The species is native to Brazil and is mainly found in the North and Northeastern regions. It has been studied for its biological activities and chemical composition. Scientific literature has reported that this species contains different extracts and/or isolated compounds which have antimicrobial, anti-inflammatory, analgesic, antioxidant and hypoglycemic properties, as well as others that are in popular use. The phytochemical literature reports on the presence of fatty acids, terpenoids, phenolic compounds and polysaccharides. However, further studies are necessary to find new bioactive molecules with biological relevance based on traditional medicine. The purpose of this review is to provide a broad and updated overview about the relevance of *Libidibia ferrea* species in regard to general aspects, traditional medicines, biological activities and chemical composition data.**

Key words: *Libidibia ferrea*, medicinal properties, phytochemistry.

INTRODUCTION

The Genus *Libidibia* (Fabaceae family) includes 500 species of trees distributed worldwide. It is characterized by its content of polyphenols, terpenes and steroids, as well as polysaccharide substances, which are principally responsible for its biological properties (Zanin et al., 2012). The main species of this genus are native to Brazil, and are distributed in different regions all over the country (Flora do Brasil, 2014).

Several uses in traditional medicine are described for different parts (barks, fruits, leaves, seeds and stems). This species is one of 71 species of medicinal plants

included in the national list of medicinal plants of interest to the Brazilian Public Health System (Relação Nacional de Plantas Mediciniais de Interesse ao Sistema Único de Saúde — RENISUS), due to its importance in public health and potential medicinal applications in Brazil (Brasil, 2009). In regard to the species *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz var. *ferrea*, the present work consists of a survey of the literature from several databases included original articles, books, sites and theses (Periodicos CAPES, PubMed, Science Direct, SciELO, SciFinder, Scopus and Web of Science)

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regarding general aspects, as well as chemical and pharmacological data about this species that has been published up until 2014.

Some key words used were *Caesalpinia*, *Caesalpinia ferrea*, *Libidibia ferrea*, Juca and Pau ferro, chosen because of the scientific name, synonyms, and main popular names of the species in accordance with the sites “Embrapa” (<http://www.cnpf.embrapa.br>) “Flora do Brasil” (<http://floradobrasil.jbrj.gov.br>), Tropicos (<http://www.tropicos.org>) and the plant list (<http://www.theplantlist.org>). The software MDL/Isis Draw Freeware Version 2.5 was used to draw the chemical structures.

GENERAL ASPECTS

Libidibia ferrea (Mart. ex Tul.) L. P. Queiroz var. *ferrea* belongs to the Fabaceae family, it is also called *Caesalpinia ferrea* and is popularly known as “júcá” or “pau ferro” (Lorenzi, 2002; Matos, 2007). With regard to its etymology, “*Caesalpinia*” was named after Andrea Caesalpinio (an Italian botanist); and “*ferrea*”, which means iron, is due to the high density of this wood (Embrapa, 2014). This species is considered a native tree to Brazil and is endemic to the North and Northeastern regions (Alagoas, Bahia, Ceará, Paraíba and Pernambuco), mainly in the geographic area dominated by the Caatinga (Brazilian savanna). Moreover, the species is cultivated in other countries for its use in the forestation of streets and parks and goes by the name of Brazilian ironwood (Matos, 2007). Therefore, the species plays an important role in environmental preservation and it can also have significant economic impact on the country (Embrapa, 2014). The *L. ferrea* tree is between 10 to 15 m in height and has a thin trunk with a diameter of 40 to 60 cm. The leaves are a bipinnate composite type measuring 15 to 19 cm in length, with opposite pinnae 5 to 11 and the leaflets have 8 to 24 pinnas. The fruits are indehiscent and have a hard pod and are dark brown in color (Figure 1) (Lorenzi, 2002).

BIOLOGICAL ACTIVITIES

Since 1960, the species has been studied for biological activities and most of these studies were based on popular information about the indications, usage mode, and parts of the plant used in the preparation.

Traditional medicine

Previous ethnopharmacological studies show that *L. ferrea* is used in many parts of Brazil for the treatment of a number of diseases and its resources are part of a traditional knowledge about this species (Albuquerque et

al., 2007). In this context, Barros (1982) related that the seeds and bark of *L. ferrea* have been used in traditional medicine in the form of tea and potions to lose weight and to clean injuries. The fruits are mainly used against anemia, lung disease (cough followed by bleeding) and diabetes. Some of its therapeutic activities have been described in scientific literature. Balbach (1972) described in his book that the infusion of stem bark of *L. ferrea* had been used for treating enterocolitis and diarrhea. Braga (1976), in the third edition of the book “Plantas do Nordeste, Especialmente do Ceará”; reports several therapeutic properties, including the treatment and alleviation of asthma, bruises, chronic cough and wounds, and the roots are antipyretic and antidiabetic. According to Lewis (1988), the roots of *L. ferrea* are used as an antipyretic and antidiarrheal and the decoction of the wood showed a healing and antisecretory effect. Thomas et al. (1998), gave the first description that the aqueous extract had anti-inflammatory and analgesic properties.

Some experiments in animals have shown the analgesic, anti-inflammatory and anti-ulcer activity of the fruit and bark extract of this species (Bacchi and Sertié, 1994; Bacchi et al., 1995; Carvalho et al., 1996). Also during the 90s, activities were described for treating respiratory tract diseases, dysentery and diabetes (Bragança, 1996). According to Maia (2004) the fruits are antidiarrheal and have healing effects and it was also noted that the roots are antipyretic.

Allelopathic activity

The study of Oliveira et al. (2012) was to evaluate the allelopathic potential of leaves, stem bark and mature pods of *L. ferrea* on seed germination and seedling development of lettuce (*Lactuca sativa*). The extracts of leaves and pods obtained, reduced the germination percentage of *L. sativa* and the authors concluded that the different extracts showed allelopathic activity.

Anti-inflammatory, analgesic and antinoceptive effects

Carvalho et al. (1996), conducted a preliminary study about the anti-inflammatory and analgesic activities of crude aqueous extract from the fruits of *L. ferrea* obtained by maceration. The crude extract (CE) showed inhibition in the formation of edema. In the intraperitoneal assay injection of acetic acid when treated with 10 mg/kg and 20mg/kg of the CE, the number of writhes was reduced. In addition, the carrageenan test induced rat hind paw edema showed inhibition when given a 300 mg/kg oral dose of the CE.

More recently, hydroalcoholic extract (95%) of pods (peels and seeds) obtained by agitation was assessed for anti-inflammatory and analgesic activities. The extract at

a dose of 50 mg/kg (body weight) showed inhibition of ear edema and of vascular permeability. The extract was also able to reduce cell migration to the peritoneal cavity. In addition, the induction test for acetic acid at doses of 12.5, 25 and 50 mg/kg reduced the number of contortions. Furthermore, the study reports that in the formalin test, the effects presented from the extract appeared only in the second phase (Lima et al., 2012).

The pods were used for total polysaccharide (TP) assay and this gave three polysaccharide rich fractions (FI, FII, FIII). The TP (1 mg/kg), inhibited the paw edema induced by carrageenan and FIII inhibited the inflammatory parameters in the paw edema induced by the following stimuli: bradykinin, carrageenan, compound 48/80, dextran, histamine, nitric oxide, serotonin and prostaglandin E₂. Furthermore, FIII inhibited the carrageenan-induced edema in animals. The model of peritonitis inhibited cell migration and protein leakage by carrageenan and N-formyl-methionyl-leucyl-phenylalanine (fMLP).

Finally, biochemical and hematological parameters were also observed during treatment with FIII (1 mg/kg) and according to the results, body weight loss, damage to heart, spleen or liver were not observed. However, hepatic markers were not affected and the level of urea showed high values (Pereira et al., 2012). The fruits were subjected to supercritical fluid extraction (SFE) using CO₂ and used in the development of wound dressings. Subsequently, tests of cytocompatibility and anti-inflammatory capacity were evaluated (Dias et al., 2013). The aqueous extract and lipid portion from *L. ferrea* seed were obtained and both presented anti-inflammatory and central analgesic properties. The authors indicated that lipids are responsible for the dose related antinociceptive action in models of nociception. In addition, they inhibited opioid, cholinergic receptors and COX-2 (Sawada et al., 2014).

The crude extracts of barks (acetone-water or aqueous turbo-extracts) were evaluated in relation to analgesic and anti-inflammatory activities. In the anti-inflammatory activity, the leukocyte migration model was used, and the carrageenan peritonitis showed a reduced amount of leukocyte migration. However, analgesic activity by the hot plate test and acetic-acid induction showed no positive results (Araújo et al., 2014).

Antimicrobial and antifungal activity

The antimicrobial activity of the methanol extract (80%, maceration) of the fruit was assessed by Minimal Inhibitory Concentration (MIC). The MIC values obtained were 25.0 to 100.00 µg/mL using American Type Culture Collection (ATCC) strains of *Candida albicans*, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus oralis* and *Lactobacillus casei*. Furthermore, the authors indicated the activity of the

extracts in biofilm formation, however, when compared with the control, low activity was reported (Sampaio et al., 2009).

For crude aqueous extract obtained from the seeds after treatment with NaCl and phosphate buffer, antibacterial activities were observed across *Staphylococcus aureus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Salmonella choleraensis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains; and antifungal activity was observed with *Aspergillus niger*, *Colletotrichum lindemuthianum*, *Colletotrichum truncatum*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium pallidroseum*, *Mucor sp.*, *Neurospora sp.*, *Penicillium herguei*, *Phomopsis sp.*, *Phytiumoli gandrums*, *Rhizoctonia solani* and *Thricoderma viridae*. In this same study, the extract showed cellulase and amylase activities, and important larvicidal activity against *Aedes aegypti* (Cavalheiro et al., 2009).

In the screening performed by Ferreira et al. (2013) the crude extracts from bark showed antifungal property, inhibiting the growth of ATCC yeast of *Candida* spp. The MIC obtained for *C. albicans* and *Candida krusei* was more effective in CEs obtained with ethanol: water or acetone:water; and was similar to *Cronobacter dublinensis* and *Candida glabrata*. The authors suggest the effectiveness of this plant against *Candida non-albicans* species. The Minimal Fungicidal Concentration (MFC) revealed that the ethanolic extract obtained reached an important value for the *C. dublinensis*.

Studies showed that the combined action of erythromycin with hydroalcoholic extract (70%) from fruits presented synergistic potential against to *S. aureus*, and the structural damage of staphylococcal DNA was reported (Silva et al., 2013). Ethanolic extract of fruits was analyzed for the existence of microorganisms and this was verified by the absence of *S. aureus*, *P. aeruginosa* and *Escherichia coli*. The antimicrobial assay for *S. mutans* and *S. oralis* was evaluated at 20 days and 140 days after preparation of extract, and also revealed effective results (Marreiro et al., 2014).

Antifungal effects of ethanolic extract showed activity of several levels against *Aspergillus parasiticus* and the extracts were effective in controlling the growth and production of aflatoxins by *A. parasiticus* (Martins et al., 2014). Araújo et al. (2014), also evaluated the antibacterial activity of the extracts from bark against Gram-positive strains (*S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*) and a clinical isolate of methicillin-resistant *S. aureus*) and Gram-negative strains (*E. coli*, *Salmonella enteritidis*, *Shigella flexneri* and *K. pneumoniae*). The CE of acetone: water (7:3) showed greater inhibition against most of the bacteria, whereas the CE aqueous showed greater activity against the *S. epidermidis*, *E. faecalis* and *Shigella flexneri*.

Regarding the MIC, the results indicated that acetone: water CE yielded better results compared to the aqueous, in addition, most Gram-negative bacteria were resistant

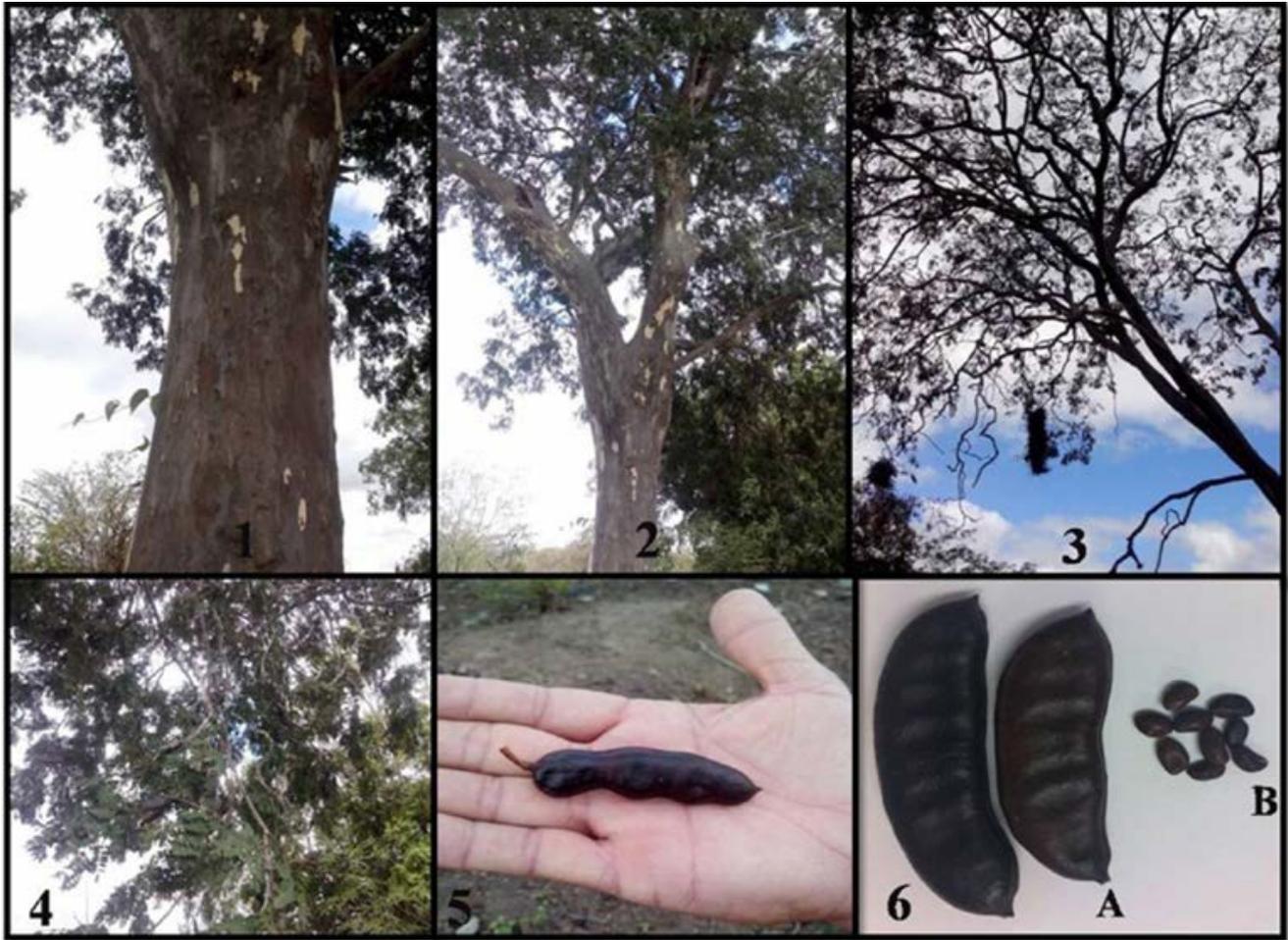


Figure 1. *Libidibia ferrea* Mart. ex. Tul. L. P. Queiroz (1. Stem; 2. Tree; 3. Tree with aerial parts; 4. Aerial parts; 5. Fruits; 6A. Fruits and 6B. seeds). Source: Paulo Aires, 2014.

to extract, especially *E. coli*.

Antidiabetic

The hydroalcoholic extract (80%) of fruits was partitioned, giving 1-BuOH extract, the residue and the fraction 7 showed better inhibitory activity against aldose reductase (Ueda et al., 2002). Ueda et al. (2004), tested the aldose reductase *in vitro* of ellagic acid and 2-(2,3,6-trihydroxy-4-carboxyphenyl)ellagic acid, from fruits of *L. ferrea*, and it was verified that both compounds dose-dependently inhibited sorbitol accumulation in erythrocytes, lens and sciatic nerve under *in vitro* incubation with glucose. On the other hand, studies by Carvalho et al. (2010) indicated that the aqueous extracts, when used in chronic treatment on the vascular reactivity of alloxan-induced diabetic rats, were not able to modify the contractions or relaxations. Vasconcelos et al. (2011), obtained the aqueous CE from the barks and evaluated the hypoglycaemic properties and the mechanisms of

reduction of glucose level in blood of diabetic rats via protein kinase B (PKB/Akt), AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC). The authors verified that the CE reduced blood glucose levels and improved the metabolic state of the animals. P-Akt was increased and P-ACC was reduced in the liver and skeletal muscle of the treated animals, P-AMPK was reduced only in the skeletal muscle. The biochemical parameters at a dose of 450 mg/kg/day presented reductions in levels of urea, uric acid, AST and ALT.

Antioxidant

In studies carried out by Silva et al. (2011), the ethanolic extract of fruits, exhibited strong antioxidant activity in the *in vitro* test and demonstrated a significant and linear correlation between the phenolic content and the antioxidant activity by phosphomolibdenum assay as well as the superoxide radical scavenging activity. On the other hand, the DNA nicking assay presented the ability

to inhibit the DNA degradation. A study on the antioxidant capacity by scavenging the application of free radical diphenylpicrylhydrazyl (DPPH) radical was performed by Port's et al. (2013), presented moderate activity, and when analyzed with the β -Carotene/linoleic acid system, the results showed high values.

Antitumor

Gallic acid and methyl gallate, isolated from fruits, were tested by the *in vitro* Epstein–Barr virus early antigen and were observed to decrease significantly the average number of papillomas, thus promoting the effects of 12-O-tetra-decanoylphorbol-13-acetate on skin tumor formation in mice (Nakamura et al., 2002; 2002). In 2003, the cancer chemopreventive activity of 2-(2,3,6-trihydroxy-4-carboxyphenyl)ellagic acid from fruits was evaluated and the two-stages of mouse skin papillomas induced were inhibited by this compound (Inada et al., 2003).

Others activities

The replication of herpes simplex virus (HSV) and poliovirus (PV) were evaluated by sulfated polysaccharide (seeds) and the authors proved the inhibition of virus absorption in the stages after penetration and the synthesis of viral protein (Lopes et al., 2013). The cardiovascular effect of aqueous CE (stem bark) demonstrates that it induces hypotension associated with tachycardia in normotensive rats. At a dose of 40 mg/kg, it induces transient bradyarrhythmias. The occurrence of vasodilatation in rat mesenteric artery mediated by ATP-sensitive K^+ channel openings was also reported (Menezes et al., 2007). To investigate the potential of the bark on the inhibition of DNA topoisomerase II, the compounds Pauferrol A, B and C showed inhibitory activities against human topoisomerase II and cell proliferation via the induction of apoptosis in human leukemia HL 60 cells (Nozaki et al., 2007). Bariani et al. (2012), have reported the use of trypsin inhibitors in seeds against the pathogenic fungi *Colletotrichum guaranicola*, *Corynespora cassiicola*, *Fusarium oxysporum* and *Sclerotium rolfsii*. The protein extracts were analyzed by SDS-PAGE and the effect in reducing sporulation and mycelial of these fungi was verified.

CHEMICAL COMPOSITION

Fatty acids and terpenoids

In the studies of Dias et al. (2013), the presence of fatty acids was reported in fruits after supercritical fluid extraction (SFE) using CO_2 . The analysis by gas chromatography

revealed that the fruits are composed of unsaturated and saturated fatty acids as well as terpenoids; there is also linoleic acid, palmitic acid, elaidic acid, gamma-sitosterol, stearic acid and lupenone. Moreover, 3,4-dimethylbenzadehyde and di-2-ethylhexylphthalate were also identified. Sawada et al. (2014), also identified the presence of fatty acids in the lipid portion of seeds, being linoleic, palmitic, oleic, estearic, palmitolenic and capric acids (Table 1).

Phenolic compounds

Among the phenolic compounds related to the different parts of *L. ferrea*, the condensed and hydrolysable tannins, and chalcones deserve special attention. Phytochemical studies by Thin Layer Chromatography (TLC) revealed the presence of coumarins, flavonoids, saponins, steroids (Gonzalez et al., 2004) and tannins (Gonzalez et al., 2004; Vasconcelos et al., 2011; Araújo et al., 2014). Ueda et al. (2002), isolated the ellagic acid and 2-(2,3,6-trihydroxy-4-carboxyphenyl) ellagic acid from fruits. According to Nakamura et al. (2002) gallic acid and methyl gallate were isolated from fruits. Also in fruits, the phenolic content was calculated from an obtained hydroalcoholic extract and values of 460 mg/g of gallic acid were found (Silva et al., 2011).

Sampaio et al. (2009), also calculated the content of polyphenols in methanolic extract, by the Prussian Blue Method and this was estimated at 7.3%. In addition, spectral analysis revealed hydroxy phenols and methoxilated compounds. using High-performance liquid chromatography (HPLC) analysis, revealed the presence of in aqueous crude extracts. The analysis of aqueous crude extract by HPLC revealed the presence of gallic acid, catechin, epicatechin and ellagic acid (Vasconcelos et al., 2011; Araújo et al., 2014)

In the methanolic extract of the fruits, described by Silva et al. (2013), gallic acid and methylated gallate derivative compounds were found. Port's et al. (2013), reported the presence of gallic acid and quercetin in methanolic extract of leaves. In the extractive solution of leaves, the polyphenolic content was calculated by Folin-Ciocalteu and the presence of gallic acid was identified and quantified by HPLC (Silva et al., 2014). Chalcones are also a substance that is present in this specie, the literature reports the presence of these compounds isolated from the stems of *L. ferrea*. The CE obtained with acetone when partitioned revealed the presence of Pauferrol A (Nozaki et al., 2007), Pauferrol B and C (Ohira et al., 2013).

Polysaccharides

The hydrocolloid extract purified from seeds contained 75% total carbohydrate and 9% protein. The 1D/2D NMR

Table 1. Main constituents from some parts of *Libidibia ferrea* (B: B; F: F; L: L; Se: Se; St: St).

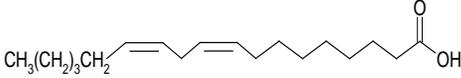
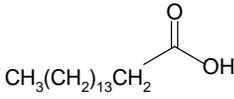
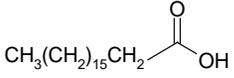
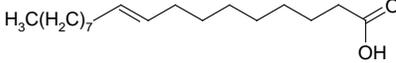
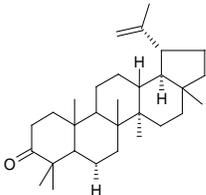
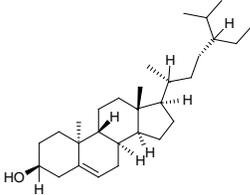
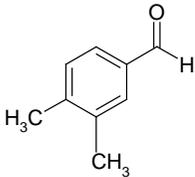
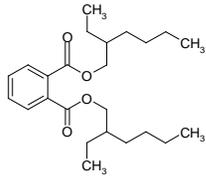
Compound	Structure	Plant part	Reference
Linoleic acid		F, Se	Dias et al., 2013; Sawada et al., 2014
Palmitic acid		F, Se	Dias et al., 2013; Sawada et al., 2014
Stearic acid		F, Se	Dias et al., 2013; Sawada et al., 2014
Elaidic acid		F	Dias et al., 2013
Lupenone		F	Dias et al., 2013
Gamma-sitosterol		F	Dias et al., 2013
3,4-dimethylbenzaldehyde		F	Dias et al., 2013
di-2-ethylhexylphthalate		F	Dias et al., 2013

Table 1. Contd.

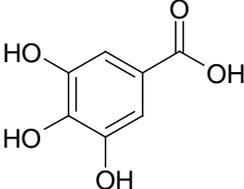
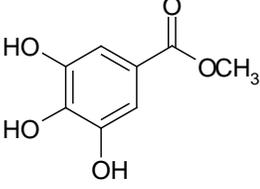
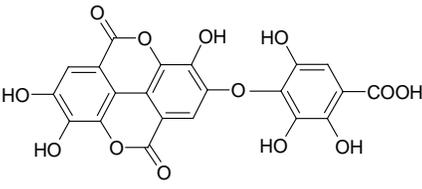
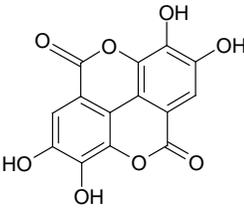
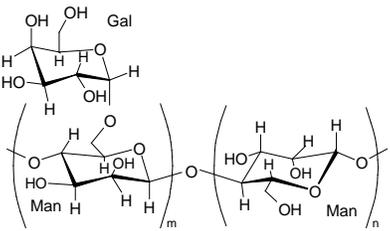
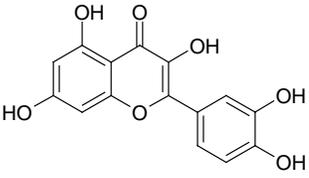
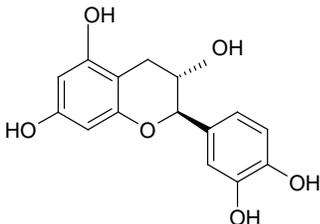
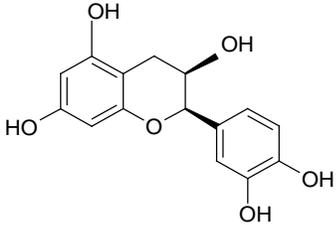
Gallic acid		F, L, Stand B	Nakamura et al., 2002a,b; Vasconcelos et al., 2011; Silva et al., 2013; Silva et al., 2014; Port's et al., 2013; Araújo et al., 2014
Methyl gallate		F	Nakamura et al., 2002a,b; Silva et al., 2013
2-(2,3,6-trihydroxy-4-carboxyphenyl)ellagic acid		F	Ueda et al., 2002; 2004
Ellagic acid		F, Stand B	Ueda et al., 2002; 2004; Vasconcelos et al., 2011
Galactomannan		F	Souza et al., 2010
Quercetin		L	Port's et al., 2013

Table 1. Contd.

D-galactose		Se	Lopes et al., 2013
D-mannose		Se	Lopes et al., 2013
Linolenic acid	$\text{H}_3\text{C}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH}$	Se	Sawada et al., 2014
Palmitolenic acid	$\text{CH}_3(\text{CH}_2)_4\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2(\text{CH}_2)_5\text{CH}_2-\text{COOH}$	Se	Sawada et al., 2014
Capric acid		Se	Sawada et al., 2014
Paufferol A		St and B	Nozaki et al., 2007
Paufferol B		St and B	Ohira et al., 2013
Paufferol C		St and B	Ohira et al., 2013

Table 1. Contd.

Catechin		St and B	Vasconcelos et al., 2011; Araújo et al., 2014
Epicatechin		St and B	Vasconcelos et al., 2011

spectra indicated the presence of galactomannan with a (1→4)-linked-β-D-mannopyranose, partially substituted at O-6 with single-unit α-D-galactopyranose side-chains. The splitting of three ¹³C signals in the region of the 4-O-Mannopyranose units revealed the α-D-Galpyranose units (Souza et al., 2010). Another study showed the presence of polysaccharides in aqueous extract from the seeds following sulfation. The analysis by ¹³C, ¹H NMR and FT-IR revealed the presence of d-Galactose and d-Mannose monosaccharides (Lopes et al., 2013). The presence of galactomannan in the seeds was also proved by Gallão et al. (2013), and they also indicated that galactomannan is located at the endosperm.

DISCUSSION

This paper presents a review of the biological and phytochemical aspects of the species *L. ferrea*, whose importance and relevance to traditional medicine stands out due to its biological properties against anemia, lung disease and diabetes. Antipyretic activity is also reported in this species. Several studies confirmed such activities as antidiabetic, anti-inflammatory and antimicrobial; and this can be attributed to the presence of the compounds (chalcones, flavonoids, polysaccharides, tannins and terpenes) obtained from several extracts from different herbal parts (leaves, fruits or stem barks). However, further studies are still necessary to demonstrate the potential of *L. ferrea* as a source of bioactive molecules or its use with standardized extracts with biological and pharmacological relevance. Despite the promising pharmacological and biological data on the species related in the literature, there are few toxicological studies. The investigation of the extract's safety plays an

important role in the establishment of its bioguided chemical profile with improved biological properties and low toxicology or side effects.

Conclusion

In conclusion, the data presented in this review about *L. ferrea* Mart. ex Tul. L. P. Queiroz, is a compilation of knowledge regarding its biological and phytochemical aspects, that may contribute as a basis to the development of research about its biological properties and chemical compounds, and further studies are needed to correlate the presence of such properties.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

REFERENCES

Albuquerque UP, Medeiros PM, Almeida ALS, Monteiro JM, Lins Neto EMF, Melo JG, Santos JP (2007). Medicinal plants of the *caatinga*

- (semi-arid) vegetation of NE Brazil: a quantitative approach. *J. Ethnopharmacol.* 114(3):325-354.
- Araújo AA, Soares LAL, Ferreira MRA, Neto MAS, Silva GR, Araújo Jr. RF, Guerra GCB, Melo MCN (2014). Quantification of polyphenols and evaluation of antimicrobial, analgesic and anti-inflammatory activities of aqueous and acetone-water extracts of *Libidibia ferrea*, *Parapiptadenia rigida* and *Psidium guajava*. *J. Ethnopharmacol.* 156:88-96.
- Bacchi EM, Sertié JAA (1994). Antiulcer action of *Styrax camporum* and *Caesalpinia ferrea* in rats. *Planta Med.* 60:118-120.
- Bacchi EM, Sertié JAA, Villa N, Katz H (1995). Antiulcer action and toxicity of *Styrax camporum* and *Caesalpinia ferrea*. *Planta Med.* 61:204-207.
- Balbach A (1972). *As Plantas que Curam*. São Paulo: Três Press. pp. 302-303.
- Bariani A, Gonçalves JFC, Chevreuil LR, Cavallazzi JRP, Souza LAG, Bentes JLS, Pando SC (2012). Partial purification of trypsin inhibitors from *Caesalpinia ferrea* and *Swartzia polyphylla* seeds and effect of protein extracts on pathogenic fungi. *Summa Phytopathol.* 38(2):131-138.
- Barros MAG (1982). *Flora medicinal do Distrito Federal*. Brasília: Brasil Florestal. 12(50):35-45.
- Braga R (1976). *Plantas do Nordeste, Especialmente do Ceará*. 2ª edição. São Paulo: Três Press. pp. 45-46.
- Bragança LAR (1996). *Plantas Mediciniais Antidiabéticas*. Niterói: EDUFF Press. 172p.
- Brasil (2009). "Fitoterapia: plantas de interesse ao SUS," Brazilian Health Ministry.
- Carvalho AA, Menezes IAC, Antonioli AR, Santos MRV (2010). Effect of the Chronic Treatment with Aqueous Extract of *Caesalpinia ferrea* and *Chrysobalanus icaco* on the Vascular Reactivity of Diabetic Rats. *Lat. Am. J. Pharm.* 29(5):845-848.
- Carvalho JC, Teixeira JRM, Souza PJ, Bastos JK, dos Santos Filho D, Sarti SJ (1996). Preliminary studies of analgesic and anti-inflammatory properties of *Caesalpinia ferrea* crude extract. *J. Ethnopharmacol.* 53:175-178.
- Cavalheiro MG, Farias DF, Fernandes GS, Nunes EP, Cavalcanti FS, Vasconcelos IM, Melo VMM, Carvalho AFU (2009). Biological and enzymatic activities of aqueous extract of seeds from *Caesalpinia ferrea* Mart., Leguminosae. *Rev. Bras. Farmacogn.* 19(2B):586-591.
- Dias AMA, Rey-Rico A, Oliveira RA, Marceneiro S, Alvarez-Lorenzo C, Concheiro A, Júnior RNC, Braga MEM, Sousa HC (2013). Wound dressings loaded with an anti-inflammatory jucá (*Libidibia ferrea*) extract using supercritical carbon dioxide technology. *J. Supercrit. Fluids* 74:34-45.
- Embrapa (2014). *Espécies Arbóreas Brasileiras*. http://www.cnpf.embrapa.br/pesquisa/efb/index_especies.htm
- Ferreira MRA, Santiago RR, Langassner SMZ, Mello JCP, Svidzinski TIE, Soares LAL (2013). Antifungal activity of medicinal plants from Northeastern Brazil. *J. Med. Plants Res.* 7(40):3008-3013.
- Flora do Brasil (2014). *Lista de Espécies da Flora do Brasil*. <http://www.floradobrasil.jbrj.gov.br> Accessed in: 20/10/2014.
- Gallão MI, Normando LO, Vieira IGP, Mendes FNP, Ricardo NMPS, Brito ES (2013). Morphological, chemical and rheological properties of the main seed polysaccharide from *Caesalpinia ferrea* Mart. *Ind. Crop. Prod.* 47:58-62.
- Gonzalez FG, Barros SBM, Bachi EM (2004). Atividade antioxidante e perfil fitoquímico de *Caesalpinia ferrea* MART. *Braz. J Pharm. Sci.* 40:79.
- Inada A, Nakanishi T, Murata H, Inatomi Y, Ueda H, Kawanishi K, Moriyasu M, Nishino H, Kuchide M, Takayasu J, Mukainaka T, Nobukuni Y, Okuda M, Tokuda H (2003). Cancer Chemopreventive Activity of 2-(2,3,6-trihydroxy-4-carboxyphenyl)ellagic Acid from the fruits of *Caesalpinia ferrea*. *Nat. Med.* 5(5):192-195.
- Lima SMA, Araújo LCC, Sitônio MM, Freitas ACC, Moura SL, Correia MTS, Malta DJN, Gonçalves-Silva T (2012). Anti-inflammatory and analgesic potential of *Caesalpinia ferrea*. *Rev. Bras. Farmacogn.* 22(1):169-175.
- Lopes N, Faccin-Galhardi LC, Espada SF, Pacheco AC, Ricardo NMPS, Linhares REC, Nozawa C (2013). Sulfated polysaccharide of *Caesalpinia ferrea* inhibits herpes simplex virus and poliovirus. *Int. J. Biol. Macromol.* 60:93-99.
- Lorenzi H (2002). *Árvores Brasileiras: Manual de identificação e Cultivo de Plantas Arbóreas Nativas do Brasil*. Nova Odessa: Instituto Plantarum. 162p.
- Maia GN (2004). *Caatinga: árvores e arbustos e suas utilidades*. São Paulo: D&Z Computação Gráfica, Leitura & Arte. 413p.
- Marreiro RO, Bandeira MFCL, Souza TP, Almeida MC, Bendaham K, Venâncio GN, Rodrigues IC, Coelho CN, Milério PSSL, Oliveira GP, Conde NCO (2014). Evaluation of the stability and antimicrobial activity of an ethanolic extract of *Libidibia ferrea*. *Clin. Cosmet. Invest. Dent.* 6:9-13.
- Martins M, Kluczkowski AM, Souza TP, Pacheco C, Savi GD, Scussel VM (2014). Inhibition of growth and aflatoxin production of *Aspergillus parasiticus* by guaraná (*Paullinia cupana* Kunth) and jucá (*Libidibia ferrea* Mart) extracts. *Afr. J. Biotech.* 13(1):131-137.
- Matos FJA (2007). *Plantas Mediciniais. Guia de Seleção e Emprego de Plantas usadas em fitoterapia no Nordeste do Brasil*. 3 ed. Fortaleza: Imprensa Universitária. 349p.
- Menezes IAC, Moreira IJA, Carvalho AA, Antonioli AR, Santos MRV (2007). Cardiovascular effects of the aqueous extract from *Caesalpinia ferrea*: Involvement of ATP-sensitive potassium channels. *Vasc. Pharmacol.* 47:41-47.
- Nakamura ES, Kurosaki F, Arisawa M, Mukainaka T, Okuda M, Tokuda H, Nishino H, Pastore Jr. F (2002b). Cancer chemopreventive effects of constituents of *Caesalpinia ferrea* and related compounds. *Cancer Lett.* 177:119-124.
- Nakamura ES, Kurosaki F, Arisawa M, Mukainaka T, Takayasu J, Okuda M, Tokuda H, Nishino H, Pastore Jr. F (2002a). Cancer chemopreventive effects of a Brazilian folk medicine, Juca, on *in vivo* two-stage skin carcinogenesis. *J. Ethnopharmacol.* 81:135-137.
- Nozaki H, Hayashi K-I, Kido M, Kakumoto K, Ikeda S, Matsuura N, Tani H, Takaoka D, Iinuma M, Akao Y (2007). Paufferol A, a novel chalcone trimer with a cyclobutane ring from *Caesalpinia ferrea* mart exhibiting DNA topoisomerase II inhibition and apoptosis-inducing activity. *Tetrahedron Lett.* 48:8290-8292.
- Ohira S, Takay K, Mitsui T, Kido M, Kakumoto K, Hayashi K-I, Kuboki A, Tani H, Ikeda S, Iinuma M, Akao Y, Nozaki H (2013). New chalcone dimers from *Caesalpinia ferrea* Mart act as potent inhibitors of DNA topoisomerase II. *Tetrahedron Lett.* 54:5052-5055.
- Oliveira AK, Coelho MFB, Maia SSS, Diógenes FEP (2012). Atividade alelopática de extratos de diferentes órgãos de *Caesalpinia ferrea* na germinação de alfaca. *Ciênc. Rur.* 42(8):1397-1403.
- Pereira LP, Silva RO, Bringel PHSF, Silva KES, Assreuy AMS, Pereira MP (2012). Polysaccharide fractions of *Caesalpinia ferrea* pods: Potential anti-inflammatory usage. *J. Ethnopharmacol.* 139:642-648.
- Port's PS, Chisté RC, Godoy HT, Prado MA (2013). The phenolic compounds and the antioxidant potential of infusion of herbs from the Brazilian Amazonian region. *Food Res. Intern.* 53:875-881.
- Sampaio FC, Pereira MSV, Dias CS, Costa VCO, Conde NCO, Buzalaf MAR (2009). *In vitro* antimicrobial activity of *Caesalpinia ferrea* Martius fruits against oral pathogens. *J. Ethnopharmacol.* 124:289-294.
- Sawada LA, Monteiro VSC, Rabelo GR, Dias GB, Cunha M, Nascimento JLM, Bastos GNT (2014). *Libidibia ferrea* mature seeds promote antinociceptive effect by peripheral and central pathway: possible involvement of Opioid and Cholinergic receptors. *BioMed. Res. Intern.* 508725.
- Silva FA, Ferreira MRA, Soares LAL, Sampaio EVSB, Silva FSB, Maia LC (2014). Arbuscular mycorrhizal fungi increase gallic acid production in leaves of field grown *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz. *J. Med. Plants Res.* 8(36):1110-1115.
- Silva LCN, Júnior CAS, Souza RM, Macedo AJ, Silva MV, Correia MTS (2011). Comparative analysis of the antioxidant and DNA protection capacities of *Anadenanthera colubrina*, *Libidibia ferrea* and *Pityrocarpa moniliformis* fruits. *Food Chem. Toxicol.* 49:2222-2228.
- Silva LCN, Miranda RCM, Gomes EB, Macedo AJ, Araújo JM, Figueiredo RCBQ, Silva MV, Correia MTS (2013). Evaluation of combinatory effects of *Anadenanthera colubrina*, *Libidibia ferrea* and *Pityrocarpa moniliformis* fruits extracts and erythromycin against *Staphylococcus aureus*. *J. Med. Plants Res.* 7(32):2358-2364.
- Souza AB, Souza LMS, Carvalho JCT, Maistro EL (2006). No clastogenic activity of *Caesalpinia ferrea* Mart. (Leguminosae) extract on bone marrow cells of Wistar rats. *Gen. Mol. Biol.* 29(2):380-383.

- Souza CF, Lucyszyn N, Ferraz FA, Sierakowski MR (2010). *Caesalpinia ferrea* var. *ferrea* seeds as a new source of partially substituted galactomannan. *Carbohydr. Pol.* 82:641-647.
- Thomas G, Araújo CC, Souza PS (1998). Avaliação das atividades anti-inflamatória, analgésica e antipirética dos extratos aquosos de *Caesalpinia ferrea*, *Plantago major*, *Polygonum acre* e *Pterodon polygaeflorus*. 10th Brazilian Symposium in Medicinal Plants. São Paulo, Brasil.
- Ueda H, Kawanishi K, Moriyasu M (2004). Effects of Ellagic Acid and 2-(2,3,6-Trihydroxy-4-carboxyphenyl) ellagic Acid on Sorbitol Accumulation *in Vitro* and *in Vivo*. *Biol. Pharm. Bull.* 27(10):1584-1587.
- Ueda H, Tachibana Y, Moriyasu M, Kawanishi K, Alves SM (2002). Aldose reductase inhibitors from the fruits of *Caesalpinia ferrea* Mart. *Phytomedicine* 8(5):377-381.
- Vasconcelos CFB, Maranhão HML, Batista TM, Carneiro EM, Ferreira F, Costa J, Soares LAL, Sá MDC, Souza TP, Wanderley AG (2011). Hypoglycaemic activity and molecular mechanisms of *Caesalpinia ferrea* Martius bark extract on streptozotocin-induced diabetes in Wistar rats. *J. Ethnopharmacol.* 137:1533-1541.
- Zanin JLB, Carvalho BA, Martineli PS, Santos MH, Lago JHG, Sartorelli P, Viegas Jr. C, Soares MG (2012). The Genus *Caesalpinia* L. (Caesalpinaceae): Phytochemical and Pharmacological Characteristics. *Molecules* 17:7887-7902.

Full Length Research Paper

Evaluation of membrane stabilizing, anthelmintic, antioxidant activity with phytochemical screening of methanolic extract of *Neolamarckia cadamba* fruits

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The current study was directed on methanolic extract of *Neolamarckia cadamba* fruits, belonging to the family Rubiaceae, to reveal the possible phytochemicals existence and also to evaluate the membrane stabilizing, anthelmintic, antioxidant properties. To estimate the membrane stabilizing activity, both heat and hypotonic solution induced haemolysis techniques were used. The anthelmintic test was conducted on earthworm *Pheritima phosthuma* using five different concentrations (10, 20, 40, 60, 80 mg/ml) of the extract and albendazole as standard drug (concentration 10 mg/ml). To investigate antioxidant property, two potential tests namely total phenolic content determination and the 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging assay were conducted. Phytochemical screening was carried out using different chemical group tests. The extract revealed good membrane stabilizing activity inhibiting both hypotonic solution and heat induced haemolysis in comparison to inhibition by standard acetyl salicylic acid. The methanolic extract showed potent anthelmintic activity at the highest concentration as it required less time for paralysis and death compared to the standard drug albendazole. The fruit extract showed potential antioxidant property. The analysis of phytochemicals reveals the presence of carbohydrate, phenol, phytosterol, protein and amino acid, terpene and glycoside. The results of the study showed that the plant extract has potential membrane stabilizing, antihelmintic, antioxidant activities along with the presence of significant phytochemicals.

Key words: *Neolamarckia cadamba*, membrane stabilizing activity, anthelmintic activity, antioxidant, phytochemicals.

INTRODUCTION

Neolamarckia Cadamba (Roxb.) Bosser is a well-known evergreen tropical flowering plant of Bangladesh, locally called "Kadam", belonging to the Rubiaceae family. It is

native to Bangladesh, Nepal, India, Myanmar, Sri Lanka, Philippines, Indonesia and Papua New Guinea (Uddin et al., 2013; Ganjewalaa et al., 2013). Since the prehistoric

time, different parts of this plant have been used as anti-diuretic, anti-pyretic, in the treatment of anemia, tumor as well as for the improvement of semen quality (Ahmed et al., 2011; Umachigi et al., 2007). Some previous studies evidenced that the plant also possesses antimicrobial, antioxidant, and wound healing (Umachigi et al., 2007) as well as anti-diarrheal properties (Alam et al., 2008). The plant is sometimes used in the treatment of various ailments like diabetes mellitus, inflammation, haemoptysis, cough, vomiting, ulcers and debility (Dubey et al., 2011). As the fruit is edible, its juice is given to children for the remedy of gastric irritability. Furthermore, its timber is used for making pulp and paper, boxes and furniture while its wood is used as fuel (Mishra, 2011).

A stabilized membrane is required to prevent oxidative damage and related inflammatory actions caused by free radicals produced within the body. Erythrocyte membrane stability test is an extensive study which highlights the effect of synthetic and herbal anti-inflammatory agents on erythrocyte membrane that is exposed to hypotonic solution and heat.

Due to the similarity of erythrocyte membrane with the lysosomal membrane, the effect of drug on erythrocyte stabilization can be compared to the lysosomal membrane stabilization (Sikder et al., 2010). To treat the consequences of oxidation and inflammation, there are many anti-inflammatory agents or drugs like nonsteroidal anti-inflammatory drugs (NSAIDs) available in the market. As these drugs are responsible for intestinal side effects and mucosal erosions, researchers have focused on medicinal plants for finding natural anti-inflammatory drugs with reduced side effects (Richard et al., 2011).

Helminthiasis is a macro parasitic disease that is very common among the developing countries all over the world including Bangladesh. Parasitic worms like Roundworms (Nematodes), Tapeworms (Cestodes) or Flukes (Trematodes) are responsible for this disease. According to the World Health Organization (WHO), about 2 billion people are affected by parasitic worm infection throughout the world because of poor management practices and insufficient control measures (Gaikwad et al., 2011). Oxidative stress occurs due to the increased formation of free radicals. It is a chain reaction that damages cell component like proteins, lipids and nucleic acids leading to cell death (Elmastas et al., 2007). Antioxidants have the ability to inhibit or delay the oxidation of an oxidizable substrate in a chain reaction. There are a number of synthetic antioxidants in the market which cause serious adverse effects on the body (Lobo et al., 2010). This is why finding natural antioxidants

without any adverse effects has gained importance. Phytochemicals are naturally occurring components in the medicinal plants that have various defence mechanisms and can protect us from various diseases. Phytochemical constituents present in medicinal plants can be useful in healing and assessing human diseases (Wadood et al., 2013).

Traditionally whole medicinal plant or different parts are used in the treatment of all kinds of diseases, and people prefer mostly traditional medicine because of its availability, cost effectiveness, non-toxic nature and high percentage of cure rate with single therapeutic dose (Rastogi et al., 2009). There are some evidences of various studies performed on different parts of *N. cadamba* plant but reports on fruits of the plant are very few.

Thus, our present study was designed to evaluate the membrane stabilizing, anthelmintic, antioxidant activity and also to identify the presence of phytochemicals in methanolic extract of *N. cadamba* fruits with the aim of developing new drugs.

METHODOLOGY

Plant collection and authentication

For this current investigation, the fresh fruits of the plant *N. cadamba* (Family: Rubiaceae) were collected from the surrounding campus of Noakhali Science and Technology University, Sonapur, Noakhali - 3814, Bangladesh in August, 2013 and identified by an expert botanist of the Bangladesh National Herbarium, Mirpur, Dhaka (DACB: Accession number: 38770).

Preparation of plant materials

The collected plant parts (fruits) were separated from undesirable materials of plants or plant parts. They were sun-dried for one week. The fruits were grounded into a coarse powder with the help of suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

Extraction procedure

550 g of dried powdered sample was submerged in 2500 ml of 90% methanol (Merck KGaA, Darmstadt, Germany) with sporadic shaking. After 15 days, the solvent was decanted and filtered using sterile cotton and Whatman® filter paper No. 1 (Sargent-Welch, USA) and then evaporated using rotary evaporator and freeze-dried (yield 28 g deep orange gummy extract) (Raju et al. 2013).

Membrane stabilizing activity

Membrane stabilizing activity was assessed by using hypotonic-

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solution and heat induced haemolysis of human erythrocyte by the method developed by Omale and Okafor (2008).

Collection of blood samples

Human red blood cells (RBCs) were collected for the study. 2 ml of blood was collected from each of the healthy Bangladeshi male human volunteers (aged 20 to 23 years) without a history of oral contraceptive or anticoagulant therapy and free from diseases (using a protocol approved by Institutional Ethics Committee). The collected RBCs were kept in a test tube with an anticoagulant Ethylenediaminetetraacetic acid (EDTA) under standard conditions temperature ($23\pm 2^\circ\text{C}$) and relative humidity ($55\pm 10\%$).

Preparation of erythrocyte suspension

To prepare the erythrocyte suspension, 2 ml of blood was obtained using syringes (containing anticoagulant EDTA) from male volunteers through puncture of the anti-cubital vein. The blood was centrifuged using centrifugal machine for 10 min at 3000 g and then it was washed three times using isotonic solution (0.9% saline). The volume of saline was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution (pH 7.4) which contained 1 L of distilled water: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.26 g; Na_2HPO_4 , 1.15 g; NaCl, 9 g (10 mM sodium phosphate buffer). Thus, the suspension finally collected was the stock erythrocyte (RBC) suspension.

Hypotonic solution-induced haemolysis

The test sample which consisted of stock erythrocyte (RBC) suspension (0.50 ml) was mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (2.0 mg/ml) or acetyl salicylic acid (0.1 mg/ml). The control sample which consisted of 0.5 ml of RBCs was mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm using UV spectrometer (Biswas et al., 2013). The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

$$\% \text{ Inhibition of haemolysis} = 100 \times (\text{OD1} - \text{OD2} / \text{OD1})$$

Where,

OD1 = Optical density of hypotonic-buffered saline solution alone (control) and

OD2 = Optical density of test sample in hypotonic solution

Heat-induced haemolysis

Aliquots (5 ml) of the isotonic buffer, containing 2.0 mg/ml of extract of the plant were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 μl) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54°C for 20 min in a water bath. The other pair was maintained at 0 to 5°C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm using UV spectrometer (Biswas et al., 2013). The percentage inhibition or acceleration of haemolysis in tests was calculated using the following equation:

$$\% \text{ Inhibition of haemolysis} = 100 \times [1 - (\text{OD2} - \text{OD1} / \text{OD3} - \text{OD1})]$$

Where, OD1 = test sample unheated; OD2 = test sample heated

and OD3 = control sample heated

In vitro anthelmintic activity

The anthelmintic test was carried out according to the method reported in Ajaiyeoba et al. (2001) with some requisite amendments. Adult earth worm (*Phertima posthuma*) was used to perform the test because of its anatomical and physiological resemblance with intestinal round worm parasite (Vidyarthi, 1967; Lakshmi et al., 2012). The worms were collected from the moist soil of Noakhali Science and Technology University area. Methanolic extract of *N. cadamba* fruit was taken at different concentrations (10, 20, 40, 60 and 80 mg/ml) separately. 100 mg of albendazole was dissolved in 10 ml water to prepare a concentration of 10 mg/ml which was referred as standard. A control group was established with distilled water for the test validation. Earthworms were placed into seven petri dishes in 7 groups, each containing five earthworms where five dishes were used for the five concentrations of methanolic extract of *N. cadamba* and one for the reference standard and another for the control group. The paralyzing time was counted only when there was no movement observed except that the worm was shaken vigorously. After ascertaining that the worms moved neither when vigorously shaken nor when dipped in warm water (50°C), the death time was recorded (Raju et al., 2013).

In vitro antioxidant activity

The in-vitro antioxidant activity test was done using two methods:

Determination of total phenolic content: The amount of total phenolic content present in plant extract was determined by using Folin-Ciocalteu reagent. As gallic Acid was used as standard, the total phenolic contents were expressed as mg/g of gallic acid equivalents (GAE). Concentration of 6.25, 12.5, 25, 50, and 100 mg/ml of gallic acid and concentration of 2 mg/ml of plant extract were also prepared in methanol. Then 0.5 ml of sample was introduced into test tubes and mixed with 2.5 ml of a 10 fold dilute Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The tubes were enclosed with para-film and allowed to stand for 30 min at room temperature and the absorbance was measured at 760 nm spectrophotometrically (UV-1800, Shimadzu, Japan). Total phenolic content was determined as mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve (Raju et al., 2013).

Free radical scavenging activity by DPPH method: The free radical scavenging activity of *N. cadamba* fruit extract was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (Diphenylpicrylhydrazyl) by using the standard method (Alam et al., 2008) at different concentrations (6.25, 12.5, 25, 50, 100 mg/ml), 2 ml of methanolic solution of sample (extract/standard) was mixed with 3.0 ml of a DPPH methanol solution (20 mg/ml). The mixture was kept in a dark place at room temperature for 30 min and later absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ Scavenging Activity} = (1 - A_{\text{test sample}} / A_{\text{control}}) \times 100.$$

Table 1. Effect of *N. cadamba* fruit extract on hypotonic solution and heat induced haemolysis of erythrocyte membrane.

Sample/Standard	% Inhibition on haemolysis	
	Hypotonic solution induced	Heat induced
-		
ME	34.36±0.31*	21.28±0.15*
ASA	52.22±0.54	40.02±0.37

ME= Methanolic extract, ASA= Acetyl salicylic Acid, Each value is presented as mean ± standard deviation (n = 5). Data are found to be significant by testing through one way ANOVA at 5% level of significance * P<0.05 when compared to the standard.

Here, A stands for Absorbance.

The graph plotted with inhibition percentage against extract/standard concentration; extract concentration providing 50% inhibition (IC_{50}) was calculated (Sikder et al., 2010).

Phytochemical screening

The freshly prepared crude extract was qualitatively tested to determine the presence of chemical constituents. Phytochemical screening of the fruit extract was performed using the following reagents and chemicals: Dragendorff's reagent for Alkaloid test, Mg and HCl for Flavonoid test, Ferric chloride and potassium dichromate solutions for Tannin and saponins with ability to produce stable foam and Libermann- Burchard reagent for Steroid test, Carbohydrates with Benedict's reagent. These chemical constituents were identified by characteristic colour changes using standard procedures (Howlader et al., 2012).

Statistical analysis

All data were presented as mean ± standard deviation (SD) and were analysed by One- way analysis of variance (ANOVA) (SPSS for windows, version 18.0, IBM corporation, NY, USA) and MS Excel for windows version 2010[®]. The values were considered significantly different at $p<0.05$.

RESULTS

Membrane stabilizing activity

The crude methanolic extract of fruits of *N. cadamba* was subjected to assays for membrane stabilizing activities by following standard protocols and the obtained results were statistically presented in Table 1. The results showed that the extracts (at concentration 2 mg/ml) were significantly ($p<0.05$) potent on human erythrocyte, adequately protecting it against hypotonic solution and heat induced lyses, when compared to the standard drug acetyl salicylic acid (0.10 mg/ml). In hypotonic solution and heat induced conditions, the extract was found to inhibit 34.36±0.31% and 21.28±0.15% haemolysis of erythrocyte membrane respectively, while in the same

conditions, acetyl salicylic acid inhibited 52.22±0.54 and 40.02±0.37% haemolysis of erythrocyte.

Anthelmintic activity

From the data in Table 2, it is observed that the gradual increase of sample concentration of methanolic extract of *N. cadamba* demonstrates paralysis as well as death of worms in fewer times. At the concentration of 80 mg/ml and 60 mg/ml, the methanolic extract showed paralysis time of 5.67±1.53 min, 10.00±1.00 min and death time of 8.67±1.53 min, 14.00±1.00 min respectively. These results were compared to that of the standard albendazole for which paralysis time was found as 8.66±0.58 min and death time 36.67±1.53 min at a concentration of 10 mg/ml.

Antioxidant activity

Determination of total phenolic content

Table 3 shows the total phenolic contents of methanolic extracts of *N. cadamba* fruits. Total phenolic compounds were reported as gallic acid equivalents by reference to a standard curve ($y=0.0125x+0.0521$; $R^2 = 0.9978$). The results showed that the total phenol content of methanolic extract was found to be 91.19±0.14 mg of GAE/g. of extract. The results of total phenolic contents suggest that the plant may possess good antioxidant activity.

Free radical scavenging activity by DPPH method

In this investigation, the crude methanolic extract of *N. cadamba* fruits showed the free radical scavenging activity with IC_{50} value of 1.01±0.01 mg/ml and the maximum inhibition was found as 92.68%. On the other hand, the standard ascorbic acid showed maximum inhibition of 95.86 and 50% inhibitory concentration (IC_{50}) was found as 1.53±0.02 mg/ml. Figure 1 shows the scavenging activity of fruit extract in a good way.

Table 2. Anthelmintic activity of crude methanolic extract of fruits of *N. cadamba* against *Pheretima posthuma*.

Test Substance	Concentration (mg/ml)	Time taken for paralysis (min)	Time taken for death (min)
Control (Distilled water)	-	-	-
Standard (Albendazole)	10	8.66±0.58	36.67±1.53
Sample 01	10	33.33±1.53***	46.66±1.53***
Sample 02	20	25.67±1.53***	31.33±1.53**
Sample 03	40	18.00±1.00***	22.00±1.00***
Sample 04	60	10.00±1.00*	14.00±1.00***
Sample 05	80	5.67±1.53*	8.67±1.53***

Each value is presented as mean ± standard deviation (n=5). * = p < 0.05, ** = p < 0.01, *** = p < 0.001. Data are found to be significant by testing through one way ANOVA at 5% level of significance (p<0.05) when compared to the control. (min = minute)

Table 3. Determination of total phenolic contents of *N. cadamba* fruits.

Extract	Absorbance of the sample	Average absorbance	Total phenolic content (mg of GAE/g) of Extracts
Methanol extract	2.43	2.33±0.06	91.19±0.14
-	2.34	-	-
-	2.22	-	-

Data represent mean ± standard deviation (n=3) of duplicate analysis.

Table 4. Phytochemical screening of the methanolic extract of *N. Cadamba* fruits.

S/N	Phytochemical constituents	Methanolic extract
1	Alkaloids	-
2	Carbohydrate	+
3	Glycosides	+
4	Saponins	-
5	Phytosterols	+
6	Phenols	+
7	Tannins	-
8	Flavonoids	-
9	Proteins and amino Acids	+
10	Terpenes	+

(+) = presence of constituents; (-) = absence of constituents

Phytochemical Screening

The preliminary phytochemical evaluation of methanolic extract of *N. cadamba* confirmed the presence of carbohydrates, glycosides, phytosterols, diterpenes, protein, amino acids and phenol, though alkaloid, flavonoid, saponin and tannin were absent (Table 4).

DISCUSSION

The present study was an attempt of investigating several

properties of methanolic extract of *N. cadamba* fruits and membrane stabilizing activity test was one of them. As we know, the vigour of cells depends on the integrity of their membranes, haemolysis of RBC on exposure to hypotonic or heated medium is an indication of its injurious membrane. It is therefore expected that membrane stabilizers should offer significant protection against hazardous substances and thereby elicit anti-inflammatory properties (Umukoro and Ashorobi, 2006). Previous reports showed that foods and fruits rich in flavonoids and other phenolic compounds have been associated with decreased risk of developing

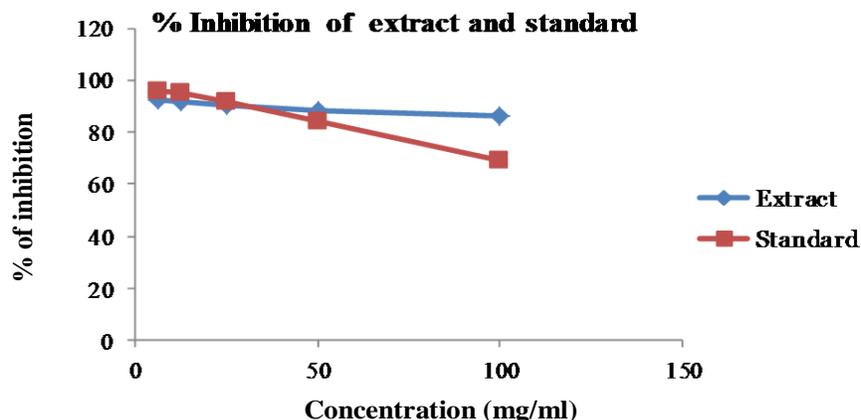


Figure 1. DPPH free radical scavenging activity of methanol extract of *N. cadamba* fruits and standard ascorbic acid.

inflammatory and other related diseases (Sies et al., 2005). Compounds with membrane-stabilizing properties are well known for their ability to interfere with the early phase of inflammatory reaction namely the prevention of the release of phospholipases that trigger the formation of inflammatory mediators (Saffoon et al., 2014).

In our study, we have also found the presence of phenols and terpenoids in the extract. Another research reported that phenolic compounds inhibit the activity of prostaglandin cyclooxygenase and thereby inhibit inflammatory mediators (Richter et al., 2003). The results of our investigation showed that the extract at a concentration of 2 mg/ml readily protected the lysis on human erythrocyte membrane induced by hypotonic solution as well as heat induced solution compared to the standard acetyl salicylic acid (0.1 mg/ml). This suggests that the plant extract may possess good membrane stabilizing activity.

The anthelmintic activity shown by the plant extract was dose dependant and was comparable to that of the standard drug albendazole. From the study, it was observed that the extract exhibited not only paralysis but also death of earthworms. It was also clear that the time for paralysis and the time for death of earthworms were inversely proportional to the concentrations of the extract.

Phytochemical analysis of the crude extract revealed the presence of phenols, terpenoids which are known to exhibit anthelmintic property. Previous studies showed that phenolic compounds can interfere with the energy generation in helminthic parasites by uncoupling oxidative phosphorylation (Athnasiadou et al., 2001) and also bind to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite leading to death (Salhan et al., 2011). Based on the above discussion, it can be assumed that terpenoids and phenolic compounds present in the fruit extract of *N.*

cadamba may be responsible for the anthelmintic activity.

In antioxidant activity test, the total phenolic content was determined considering gallic acid equivalent as reference by using the standard curve equation. Elmastas et al. (2007), reported that the phenolic compounds contain hydroxyl groups that may directly contribute to the antioxidant activity and play a critical role in scavenging free radicals. Some previous studies demonstrated that the higher the amount of total phenolic contents in a plant extract is, the higher is its antioxidant property (Madaan et al., 2011; Henríquez et al., 2010). Again it was found that absolute methanol is more effective than other solvents for extracting polyphenols from plant extracts (Lolita et al., 2012). In our study, we also found the methanolic extract of *N. cadamba* fruits rich in total phenolic components. These results are in accordance with previous reports which have shown that the fruit extract has a higher total phenolic components compared to the leaf extracts (Ganjewalaa et al., 2013) but lower than the bark extracts (Chandel et al., 2011).

Therefore, it may be said that the presence of higher total phenolic components may be responsible for demonstrating the antioxidant activity and free radical scavenging ability of the plant. We know free radicals are harmful chemical species that contain one or more uncoupled electrons which contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of different tissues and central nervous system, gastritis, cancer and AIDS (Kumar et al., 2010). For testing radical scavenging activity of various plant extracts, most commonly DPPH assay is considered as a quick method (Elmastas et al., 2007). In case of DPPH free radical scavenging activity, the extract exhibited a lower scavenging activity than the standard ascorbic acid. Figure 1 showed that methanolic extract contained significant ($p < 0.05$)

antioxidant and free radical scavenging activity. The data also revealed that the percentage of free radical inhibition increased with the increasing of concentration of extract.

Therefore, the study results support the authenticity of using the plant extract as a potent antioxidant. Isolation of phytochemical constituents on the fruit extract confirmed the presence of carbohydrates, glycosides, phytosterols, diterpenes, protein, amino acids and phenols, though alkaloid, flavonoid, saponin and tannin were absent (Table 4). From the previous study, it was confirmed that phenolic compounds have anti-oxidative, antidiabetic, anticarcinogenic, antimutagenic and anti-inflammatory activity (Arts and Hollman, 2005) and other phytochemicals present in fruit extract are also evident for having active properties against various diseases (Yadav and Agarwala, 2010).

Conclusion

In Bangladesh, like several other countries in the world, *N. cadamba* is an indigenous flowering plant. In the context of the above discussion, it can be concluded that the *N. cadamba* fruits contain important phytochemicals and possess various biological activities. The current study has confirmed that the crude methanolic extract of *N. cadamba* fruits showed potential membrane stabilizing, anthelmintic and antioxidant properties which indicates that *N. cadamba* fruits can play an important role in drug research. Therefore, the plant is a worthy contender for further systemic, chemical and biological studies to determine the active principle.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

REFERENCES

- Ahmed F, Rahman S, Ahmed N, Hossain M, Biswas A, Sarkar S, Banna H, Khatun MA, Chowdhury MH, Rahmatullah M (2011). Evaluation of *Neolamarckia cadamba* (Roxb.) Bosser leaf extract on glucose tolerance in glucose-induced hyperglycemic mice. *Afr. J. Tradit. Complement. Altern. Med.* 8:79-81.
- Ajaiyeoba EO, Onocha PA, Larenwaju OTO (2001). In vitro anthelmintic properties of *Buchholzia coriacea* and *Gynandropsis gynandra* extract. *Pharm. Biol.* 39:217-220.
- Alam MA, Akter R, Subhan N, Rahman MM, Majumder MM, Nahar L, Satyajit D (2008). Antidiarrhoeal property of the hydroethanolic extract of the flowering tops of *Anthocephalus cadamba*. *Rev. Bras. Farmacogn.* 18:155-159.
- Alam MA, Ghani A, Subhan N, Rahman M, Haque MS, Majumder MM, Majumder MEH, Akter RA, Nahar L, Sarkar SD (2008). Antioxidant and membrane stabilizing properties of the flowering tops of *Anthocephalus cadamba*. *Nat. Prod. Commun.* 3:65-67.
- Arts IC, Hollman PC (2005). Polyphenols are disease risk in epidemiological studies. *Am. J. Clin. Nutr.* 81:317-325.
- Athnasiadou S, Kyriazakis I, Jackson F, Coop RL (2001). Direct anthelmintic effects of condensed tannins towards different gastro intestinal nematodes of sheep: *In vitro* and *in vivo* studies. *Vet. Parasitol.* 99:205-219.
- Biswas MK, Mridha SA, Rashid MA, Sharmin T (2013). Membrane stabilizing and antimicrobial activities of *Caladium bicolor* and *Chenopodium album*. *IOSR J. Pharm. Biol. Sci.* 6:62-65.
- Chandel M, Kaur S, Kumar S (2011). Studies on the genoprotective/antioxidant potential of methanol extract of *Anthocephalus cadamba* (Roxb.) Miq. *J. Med. Plants Res.* 5:4764-4770.
- Dubey A, Nayak S, Goupale DC (2011). A review on phytochemical, pharmacological and toxicological studies on *Neolamarckia cadamba*. *Der. Pharm. Let.* 3:45-54.
- Elmastas M, Isildak O, Turkecul I, Temura N (2007). Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. *J. Food Compos. Anal.* 20:337-45.
- Gaikwad SA, Kale AA, Jadhav BG, Deshpande NR, Salvekar JP (2011). Anthelmintic activity of *Cassia auriculata* L. extracts-In vitro study. *J. Nat. Prod. Plant Resour.* 1(2):62-66.
- Ganjewalaa D, Tomara N, Kumar Guptaa AK (2013). Phytochemical composition and antioxidant properties of methanol extracts of leaves and fruits of *Neolamarckia cadamba* (Roxb.). *J. Biol. Active Prod. Nat.* 3:232-240.
- Henríquez C, Almonacid S, Chiffelle I, Valenzuela T, Araya M, Cabezas L, Simpson R, Speisky H (2010). Determination of antioxidant capacity, total phenolic content and mineral composition of different fruit tissues of five apple cultivars grown in Chile. *Chilean J. Agric. Res.* 70:523-536.
- Howlader MSI, Sayeed MSB, Ahmed MU, Mohiuddin AK, Labu ZK, Bellah SF, Islam MS (2012). Characterization of chemical groups and study of antioxidant, antidiarrhoeal, antimicrobial and cytotoxic activities of ethanolic extract of *Diospyros blancoi* (Family: Ebenaceae) leaves. *J. Pharm. Res.* 5:3050-3052.
- Kumar SVP, Kekuda TRP, Vinayaka KS, Sudharshan SJ, Mallikarjun N, Swathi D (2010). Studies on antibacterial, anthelmintic and antioxidant activities of a macrolichen *Parmotrema pseudotinctorum* (des. Abb.) Hale (Parmeliaceae) from Bhadra wildlife sanctuary, Karnataka. *Int. J. Pharm. Tech. Res.* 2:1207-1214.
- Lakshmi VK, Triveni KB, Anitha S, Shashidhara S (2012). In vitro anthelmintic activity of *Rotula aquatica* Lour bark. *Pharm. Sci. Monitor.* 3:2332-2339.
- Lobo V, Patil A, Phatak A, Chandra N (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacog. Rev.* 4(8):118-126.
- Lolita T, Zanda K, Ruta G (2012). Comparison of different solvents and extraction methods for isolation of phenolic compounds from horseradish roots (*Armoracia rusticana*). *World Acad. Sci. Eng. Technol.* 64:903-908.
- Madaan R, Bansal G, Kumar S, Sharma A (2011). Estimation of total phenols and flavonoids in extracts of *Actaea spicata* roots and antioxidant activity studies. *Indian J. Pharm. Sci.* 73:666-669.
- Mishra RP (2011). Antibacterial properties of *Anthocephalus Cadamba* fruits. *WebMD Centr. Ayurvedic Med.* 2:2073.
- Omale J, Okafor PN (2008). Comparative antioxidant capacity, membrane stabilization, polyphenols composition and cytotoxicity of

- the leaf and stem of *Cissus multistriata*. Afr. J. Biotechnol. 7:3129-33.
- Raju GS, Moghal MR, Dewan SMR Amin MN, Billah M (2013). Characterization of phytoconstituents and evaluation of total phenolic content, anthelmintic, and antimicrobial activities of *Solanum violaceum* Ortega. Avicenna J. Phytomed. 3:313-320.
- Rastogi T, Bhutda V, Moon K, Aswar PB, Khadabad SS (2009). Comparative Studies on Anthelmintic Activity of *Moringa Oleifera* and *Vitex Negundo*. Asian J. Res. Chem. 2:181-82.
- Richard SW, Marius L, Noya S, Pierre GI, Germaine NO (2011). Anti-inflammatory, analgesic and antipyretic effects of *Lepidagathis anobrya* nees (acanthaceae). Afr. J. Tradit. Complement. Altern. Med. 8(4):420-424.
- Richter LA, Salzar J, Rodriguez A (2003). A phytochemical analysis to suggest new applications of antiinflammatory plants from the Dominican Republic. Emanations 4:24-30.
- Saffoon S, Uddin R, Subhan N, Hossain H, Reza HM, Alam MA (2014). In vitro anti-oxidant activity and HPLC-DAD system based phenolic content analysis of *Codiaeum variegatum* found in Bangladesh. Adv. Pharm. Bull. 4(Suppl 2):533-541.
- Salhan M, Kumar B, Tiwari P, Sharma P, Sandhar HK, Gautam M (2011). Comparative anthelmintic activity of aqueous and ethanolic leaf extracts of *Clitoria ternatea*. Int. J. Drug Dev. Res. 3(1):62-69.
- Sies H, Schewe T, Heiss C, Kelm M (2005). Cocoa Polyphenols and inflammatory mediators. Am. J. Clin. Nutr. 81:304S-312S.
- Sikder MA, kuddus MR, Kaiser MA, Karna S, Rashid MA (2010). In vitro Membrane Stabilizing Activity, Total Phenolic Content, Free radical scavenging and cytotoxic properties of *Aphanamixis polystachya* (Wall.). Bangladesh Pharm. J. 13:55-59.
- Uddin MZ, Alam MF, Rhaman MA, Hassan MA (2013). Diversity in angiosperm flora of Teknaf wildlife sanctuary, Bangladesh. Bangladesh J. Plant Taxon. 20:145-162.
- Umachigi SP, Kumar GS, Jayaveera KN, Kishore DVK, Ashok-Kumar CK, Dhanpal R (2007). Antimicrobial, wound healing and antioxidant activities of *Anthocephalus cadamba*. Afr. J. Tradit. Complement. Altern. Med. 4:481-487.
- Umukoro S, Ashorobi RB (2006). Evaluation of anti-inflammatory and membrane stabilizing property of aqueous leaf extract of *Momordica charantia* in rats. Afr. J. Biomed. Res. 9:119-124.
- Vidyarthi RD (1967). *Pheritima phostuma*. In: A text book of Zoology. S. Chand and Co, New Delhi, India. pp. 329-370.
- Wadood A, Ghufuran M, Jamal SB, Naeem M, Khan A, Ghaffar R, Asnad (2013). Phytochemical analysis of medicinal plants occurring in local area of Mardan. Biochem. Anal. Biochem. 2:144.
- Yadav RNS, Agarwala M (2010). Phytochemical screening for active compounds in *Pedaliium murex* L. Recent Res. Sci. Tech. 2:110-114.



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