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Full Length Research Paper

Phytochemical and efficacy study on four herbs used in erectile dysfunction: *Mondia whiteii*, *Cola acuminata*, *Urtica massaica*, and *Tarenna graveolens*

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There is a resurgence in the use of herbal medicine in the developed countries, with even much more in low developed Countries and especially for conditions such as erectile dysfunction. Studies thus need to be conducted to scientifically validate claims on certain medicinal plants reported to be efficacious in traditional medicine. This current study was conducted to determine the phytochemical composition and efficacy of four herbs *Mondia whiteii* (Hook. F.) roots locally called (Mulondo), *Cola acuminata* (P. Beauv.) fruits locally called (Engongoli), *Urtica massaica* (Mildbr) leaves, locally called (Engyenyi) and *Tarenna graveolens* (S. Moore) roots, locally called (Munywamaizi) in Runyankole which have been reported as remedies for the management of erectile dysfunction in South-western Uganda. Phytochemical screening was conducted following methods described in Kokate, Trease and Evans. Sexual function was tested using both the contact and non-contact model. Arginine was present in all the extracts. Aqueous extract of *Tarenna graveolens* significantly improved testosterone levels but none of the extracts had significant effects on mounting frequency. Aqueous extract of *Tarenna graveolens* could be useful in management of erectile dysfunction associated with hypogonadism.

Key words: Erectile dysfunction, efficacy, phytochemical, testosterone, *Mondia*, *Cola*, *Urtica*, *Tarenna*.

INTRODUCTION

Medicinal plants are used worldwide as an alternative or complementary medicine to treat various conditions and

as a result, interest in medicinal herbs is increasing as precursors of pharmacological actives. The use of herbal

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medicine to treat sexual dysfunction among males is on the rise in Uganda and studies have documented several plants that have been used, including *Mondia whitei* (Hook. F.) roots locally called (Mulondo), *Cola acuminata* (P. Beauv) fruits locally called (Engongoli), *Urtica massaica* (Mildbr) leaves, locally called (Engenyeni) and *Tarenna graveolens* (S. Moore) roots, locally called (Munywamaizi) in Runyankole (Kamatenesi-Mugisha and Oryem-Origa, 2005). The wide spread use of herbs is compounded by the fact that sildenafil, the standard drug for treatment of erectile dysfunction (ED) is very expensive, only effective for about 70% of those with erectile dysfunction (NIH, 2003; Magoha, 2000) and there is high availability of brands that are not very effective.

The estimated range of men suffering from ED worldwide is from 15 to 30 million (NIH, 2003). According to a survey done in three countries of Nigeria, Egypt and Pakistan by Shaeer et al (2003), the age-adjusted prevalence rates of ED among men attending primary care clinics was 57.4% in Nigeria, 63.6% in Egypt, and 80.8% in Pakistan, while prevalence rate in Uganda is not known.

Although not documented, erectile dysfunction seems problematic among Ugandan men especially as noted by the rate of advertisement and sale of herbs for the condition in Uganda. The NIH Consensus Development Conference on Impotence (NIH; 1992) defined impotence as "male erectile dysfunction", the inability to achieve or maintain an erection sufficient for satisfactory sexual performance. Although reported at 16% (Isis-WICCE; 2011), sexual dysfunction might be contributing more to prevalence of domestic violence in Uganda and indeed the rest of the world. Several medicinal plants including those studied in this current work are sold within Uganda for the treatment of erectile dysfunction.

Mondia Whitei is used traditionally as an antacid and to treat indigestion; as a tonic; to stimulate appetite; and infusions of the root are used in Zimbabwe for treating anorexia and bilharzia. Fits in children and stress and tension in adults are also to be treated with this plant. The roots are used as an aphrodisiac and for the treatment of erectile dysfunction and impotence (<http://www.plantzafrica.com> accessed 6.6.2015) and for appetite and libido, as a galactagogue, fertility medication, and as an antidepressant (Oketch-Rabah, 2012). *Urtica massaica* is reportedly used for treatment of diarrhea in Rwanda and commonly eaten by Mountain Gorillas (Alphonse et al., 2008). *Cola acuminata* is reported to have stimulant action apart from the caffeine content, valuable nervine, heart tonic, and a good general tonic (www.botanical.com) and no other medicinal use of *Tarenna graveolens* was found other than its use in the management of erectile dysfunction in south-western Uganda (Kamatenesi-Mugisha et al., 2005). Earlier studies on the phytochemical compositions indicates that *M. whitei* has reducing sugars, triterpenes, steroids, phenolic compounds and flavonoids (Watcho et al., 2007). Sonibare et al. (2009) reported that aqueous

extract of *Cola acuminata* contains; alkaloids, saponins, tannins, cardenolides and no anthraquinones. Studies on the hydromethanolic extract of *U. massaica* indicated that anthocyanes, flavonoids, saponins and tannins were present while alkaloids, quinines and sterols were absent (Nahayo et al., 2008).

The aim of this current study was to determine separately, the effects of these four herbal remedies on sexual function in male rats by specifically; (i) conducting qualitative phytochemical screening on the four plants extract, (ii) evaluating their effects on erection and mounting frequency and to ascertain the effects of the extracts on testosterone levels in male rats.

MATERIALS AND METHODS

Study design

This was a short term prospective experimental study that was conducted in a period of 2 months.

Materials

120 Male and forty female albino Wistar rats, 4 month of age were used in the experiment. Sildenafil citrate (Pfizer), estrogen and progesterone (sigma Aldrich), extract of four different plants that were studied, oral cannulas for drug and extract administration, transparent glass observation cages, ketamine for anesthesia.

Experimental animals

Male Wister Rats 4 months old were secured for use in the experiment, 120 for erectile function test. There were five main groups of 30 animals each for the four plants extracts, and a control group; and the five main groups were divided into five sub-groups of six each for the efficacy study. The animals were kept at standard conditions following the NIH guidelines for animal handling in teaching and research (Guide for care and use of Laboratory Animals, 2011)

Extract preparation

Plant collection

The parts of the various plants used were collected from various villages within Western Uganda. These included *M. whitei* (Mulondo) roots, *C. acuminata* (Engongoli) fruit, *U. massaica* (Engenyeni) leaves, and *T. graveolens* (Munywamaizi) roots. Herbarium specimens were prepared and taken for botanical identification, at Science Laboratory Technology/Biology, at Mbarara University of Science and Technology and voucher specimens were deposited in the herbarium at the Faculty of Science.

Plant processing and extraction

The different plant parts once brought to laboratory were washed under running water, shade dried at room temperature for two weeks then crushed into powder form and extracted by warm maceration. About 4 kg of the various plants powder were extracted

at once for both phytochemical screening and efficacy evaluations using water.

Separation of crude extract from extracting solvent

The crude extract that were obtained was evaporated at controlled temperatures of 60°C to reduce them to semi solid states and then transferred to a desiccator with a hygroscopic substance to absorb the remaining moisture and the final powder were collected and stored ready for the tests.

Phytochemical screening

Phytochemical screening was conducted following the methods described by Trease and Evans (2009) and Kokate et al. (2010). All were conducted using chemicals of analytical grade, secured from reputable companies.

Efficacy study

Non-contact erections

A non-contact erection was conducted following the method described by Sachs et al. (1994). Female rats were placed in behavioural oestrous by the administration of ethinyl oestradiol orally at the dose of 100 µg/animal 48 h prior to the pairing and progesterone injected subcutaneously, at the dose of 1 mg/animal 6 h before the experiment. The receptivity of the female animals was confirmed before the test by exposing them to male animals, other than the control, test and Standard drug group animals. The most receptive females were selected for the study.

On the day of experiment, male rats in group A, B and C for each of the four extracts were treated with 250, 500 and 1000 mg/kg respectively. The control groups D was treated with Sildenafil Citrate at the dose of 10 mg/kg, while Group E was only given distilled water. Groups in 250 mg/kg for all the extracts were tested in one day and likewise for the other dose levels. All male animals in each group were treated with extracts 1 h before pairing with the females. For the non-contact model, Male and female rats were placed in different chambers of the glass observation cages which had a dividing wall with a perforation that separated the two halves. The perforation was to allow the passage of auditory, visual and pheromonal cues between the male and female animals in either side of the observation cage. The males were then observed and recorded for general sexual characteristic behavior for 1 h.

Contact model

In this model, the male rats were initially treated once a day with the extracts orally. On the third day, each male was treated and after one hour they were paired up with the receptive female and the number of successful mounting recorded for 1 h.

For the next four days, each male rat was treated with the same dose up to a total of 7 days of treatment. On day 8, the animals were sacrificed, their blood samples removed and then analyzed for the testosterone levels.

Ethical consideration

The proposal was submitted to Mbarara University of Science and Technology Institutional Review Committee (MUST-IRC), approval number MUIRC 01/02-13 and Uganda Nation Council for Science

and Technology (UNCST), approval number HS 1557. Animals were deeply anesthetized with ketamine at the end of the experiment to prevent pain.

Testosterone analysis

Testosterone analysis was performed using the AXSYM Testosterone reagent by Abbott AXSYM system. The system is based on Microparticle Enzyme Immunoassay (MEIA) technology for the quantitative determination of testosterone in human serum and plasma. The AXSYM Testosterone assay displaces bound testosterone from the protein and measures total testosterone. Measurement of results by the AXSYM system is based on the Beer's Law. The unit of measurement that was used in this case was ng/ml. (Abbott AxSYM system Operation Manual, 1996).

Data analysis

Data obtained from the study were analyzed using graph pad prism software version 5.0. The testosterone levels in the five rat groups was compared using one way analysis of variance (ANOVA) followed by Turkey multiple comparison test. In all, results were considered statistically significant if the p value is less than 0.05.

RESULTS

Phytochemical screening test

Phytochemical screening test Indicated the presence of saponins, Tannins, Arginine, Reducing sugars and phenolic compounds in all the four plant extracts. Glycosides was present in all extracts except *C. acuminata*, protein and free amino acids was present in all extracts except *M. whiteii*, terpenoids was present in all except *T. graveolens* and *U. massaica*, triterpenoids was present in all except *C. acuminata*, flavonoids was present in *M. whiteii*, *C. acuminata* and absent in *U. massaica* and *T. graveolens*, alkaloids was only present in *U. massaica* extract and absent in all the other three extracts and finally steroids and phlobatannins which were absent in all the four plants extracts (Table 1).

Effects on mounting frequency

None of the four plants extracts showed any positive increase in mounting frequency in the male rats except the standard drug sildenafil at 10 mg/kg with a $p=0.0002$ (Table 2).

Effects on testosterone levels

Of the four plants extracts, only *T. graveolens* caused a significant increase in testosterone levels with maximum effects achieved at 250 mg/kg ($p=0.0038$) while 500 mg/kg though significant indicated decrease in testosterone levels ($p=0.0385$) (Table 3).

Table 1. Phytochemical test results for the four plants extracts studied.

Phytochemical	<i>M. whitei</i> (Omulondo)	<i>C. acuminata</i> (cola nut)	<i>U. massaica</i> (Engenyeni)	<i>T. graveolens</i> (Munywamaizi)
Alkaloids	-	-	+	-
Flavonoids	+	+	-	-
Saponins	+	+	+	+
Tannins	+	+	+	+
Phlobatanins	-	-	-	-
Steroids	-	-	-	-
Terpenoids	+	+	-	-
Triterpenoid	+	-	+	+
Protein	-	+	+	+
Amino acids	+	+	+	+
Arginine	+	+	+	+
Glycosides	+	-	+	+
Reducing sugars	+	+	+	+
Phenolic compounds	+	+	+	+
Anthraquinones	-	-	-	-

+ = Preset, - = absent.

Table 2. Effects of the four plants extracts on mounting frequency.

Extract and dose	Mean mount in 1 h	Std. dev	P-Value	95% CI
Mondia 250	8.33	20.41	0.5808	-13.09 to 29.75
Mondia 500	13.33	13.81	0.3452	-1.16 to 27.82
Mondia 1000	5.33	8.29	0.7970	-3.36 to 14.03
Cola 250	20	15.88	0.1271	3.33 to 36.67
Cola 500	10	11.08	0.5199	-1.63 to 21.63
Cola 1000	9.5	5.32	0.5627	3.92 to 15.08
Urtica 250	0	0	0	0
Urtica 500	6.2	10.67	0.7357	-5.03 to 17.36
Urtica 1000	8.83	6.71	0.6064	1.79 to 15.87
Tarenna 250	5.83	7.81	0.7778	-2.36 to 14.03
Tarenna 500	5.5	8.17	0.7905	-3.07 to 14.07
Tarenna 1000	7.83	9.35	0.6564	-1.98 to 17.64
Sildenafil 10mg	36	2.37	0.0002***	33.52 to 38.48
Distilled water	10.33	11.43		-1.66 to 22.33

*= Significance levels, Mondia = *M. whitei*, Cola = *C. acuminata*, U.= *U. massaica* and Tarenna = *T. graveolens*. The table above indicates that none of the extracts improved mounting frequency in the male rats.

DISCUSSION

This current study was conducted to determine the phytochemical constituents in the extracts from four plants *M. whitei*, *C. acuminata*, *T. graveolens* and *U. massaica*. Screening test revealed the presence of a number of phytochemicals, some of which have been known to have effects on erection. Of particular importance is the presence of arginine in all the extracts and sugar. Arginine has been reported as a regulator of

penile erection and those disorders that reduces its synthesis or release in its penile tissues results in erectile dysfunction (Burnett., 1997, 2004). Saponins were also found present in all the four extracts. Available information implicates steroidal saponins as possible causes of increase in testosterone levels in animals (Walid et al., 2007). Studies by Gauthaman et al. (2002; 2008) also reported increase in testosterone levels caused by steroids, specifically Protodioscin isolated from *Tribulus Terrestris* extract. However, this was a

Table 3. Effect of the four plants extracts on testosterone levels.

Extract and dose	Mean testosterone levels (ng/ml)	Std. dev	P-Value	95% CI
Mondia 250	1.882	1.454	0.2370	0.356 to 3.408
Mondia 500	0.370	0.368	0.9997	-0.016 to 0.756
Mondia 1000	1.423	0.469	0.5013	0.215 to 2.631
Cola 250	4.117	5.202	0.1175	-1.343 to 9.576
Cola 500	3.460	3.524	0.0950	-0.238 to 7.158
Cola 1000	2.508	3.975	0.2607	-1.664 to 6.680
Urtica 500	1.270	0.788	0.6631	0.443 to 2.097
Urtica 1000	2.013	1.581	0.1983	0.354 to 3.673
Tarenna 250	4.428	2.180	0.0038**	2.141 to 6.761
Tarenna 500	3.100	2.042	0.0385*	0.957 to 5.243
Tarenna 1000	3.197	3.305	0.1107	0.272 to 6.666
Sildenafil 10 mg	1.492	0.463	0.3957	-0.48 to 1.72
Distilled water	1.425	0.383		1.199 1.717

*= Significance levels, Mondia = *M. whitei*, Cola = *C. acuminata*, U = *U. massaica* and Tarenna = *T. graveolens*. The table above indicates that only *T. graveolens* significantly improved testosterone levels.

qualitative study, making it not possible to estimate how much of each was present in the extract. It was noted that the extract of *Mondia whitei* had reducing sugars, triterpenes, phenolic compounds and flavonoids. This was in agreement with studies conducted by Watcho et al. (2007). It was also noted in this current study that the dried and grounded powder extract of *Mondia whitei* displayed a hygroscopic characteristic when left in the open uncovered. This current study also found similar constituents in aqueous extracts of *Cola acuminata* as in Sonibare et al. (2009) where; alkaloids, saponins tannins were found present. On *U. massaica*, only saponins and taninns occurred in the extracts tested as in the case of Nahayo et al. (2008) and the extracts in this current study. No study was however found conducted on the aqueous extract of the root bark of *Tareena graveolens*, making this probably the first of its kind. Common to all this extracts was however the presence of free amino acids and specifically arginine, which has been reported to have aphrodisiac effects as it is converted into nitric oxide which is an important vasodilator in the penile cavernosal tissue (Burnett, 2004).

The extract did not induce erection in the animals, indicating that all are not inducers of erection. Although mounting was noted in some of the animal groups treated, the results were not statistically significant in comparison to those of the control groups (Table 2). Despite the presence of arginine and saponins in the extract and their being reported as substance known to regulate erectile function and increase in testosterone levels respectively

T. graveolens at the dose of 250 mg/kg significantly increased testosterone levels in males rats (Table 3) more than that reported for *Citropsis articulata* leaf extract (Vudriko et al., 2014) and *Citropsis articulata* root bark extract (Oloro et al., 2014). Although not

quantitatively determined, the increase in testosterone levels could suggest that there was a higher level of saponins in the extract of *T. graveolens* (Koumanov et al., 1982). This result thus indicates that the aqueous extract of *T. graveolens* could be useful in treatment of those with erectile dysfunction resulting from hypogonadism. More so, the increase in testosterone levels indicated by the aqueous extract of *T. graveolens* did not correspond to the mounting frequency, contrary to many reports including (Vudriko et al., 2014; Oloro et al., 2014). The extract of *T. graveolens* as indicated in this study could be improving sexual activity in men with suspected erectile dysfunction by increasing their testosterone level. The experiment however could not show how this is achieved, whether by displacement of bound testosterone from the sex hormone binding proteins, inducing its synthesis or inhibiting its conversion to other metabolites.

Conclusion

The result of this study indicated that aqueous extract of *T. graveolens* improves testosterone levels in male rats and that none of the four herbs studied, *T. graveolens*, *M. whiteii* roots, *C. acuminata* fruit and *U. massaica* leaves, neither induce erection nor improved mounting frequency in male Wistar rats.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

***In vitro* antibacterial and antioxidant activities of alcoholic extract from the leaves of *Podocarpus neriifolius* D. Don**Rashaduz Zaman¹, Mohammad Parvez¹, Md. Jakaria^{1*}, Minhajul Islam¹, Md. Sekendar Ali¹ and Md. Aslam Hossain²¹Department of Pharmacy, Faculty of Science and Engineering, International Islamic University Chittagong, Chittagong 4203, Bangladesh.²Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka 1000, Bangladesh.

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In the present investigation, the methanol leaves extract of *Podocarpus neriifolius* D. Don was assessed for antibacterial and antioxidant activities. The antibacterial action was evaluated through agar disc diffusion method against seven pathogenic and non-pathogenic bacterial strains at various concentrations and compared against the standard (kanamycin 30 µg/disc). The antioxidant activity of the extract was investigated by means of a variety of *in vitro* assays and results were compared with standard drugs. The experiment indicates that the extract was effective against all the organisms and highest effectiveness showed against *Salmonella paratyphi* at 1000 µg/disc concentration. It also exhibited significant antioxidant activity. The total phenolic content was 168.2±6.4 mg gallic acid equivalents/g of extract. The reducing power of this extract increases with the raise of concentration and the value of IC₅₀ of 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity was 4.73 µg/ml (IC₅₀) value of standard ascorbic acid is 5.99 µg/ml. The results provided that the studied plant might indeed be possible basis of natural antioxidant as well as antimicrobial agents.

Key words: *Podocarpus neriifolius*, antioxidant, antibacterial, 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power, total phenol.

INTRODUCTION

Herbal medicines have been used for aid of symptoms of the disease from a long period of time (Asadbeigi et al., 2014). Traditional medicines are in trend in the remote areas of the developing and underdeveloped countries because of availability and low cost (Jakaria et al., 2015).

The therapeutically important plants are frequently used in the treatment of different pathological conditions (Hasan et al., 2015). In recent years, an increased resistance development of bacterial pathogens against antibiotics has become a difficult concern caused by the

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haphazard use of up to date antibiotics (Rahman et al., 2008). Plants contains a wide diversity of secondary metabolites; these are called phytochemical constituents including tannins terpenoids, alkaloids, flavonoids etc. and some of these constituents show a broad range of *in vitro* antibacterial as well as antifungal activities (Dahanukar et al., 2000; Cowan, 2008).

Free radicals have the potential to harm cells. The gaining or losing of an electron from a molecule or atom can lead to creation of a free radical. At inferior concentration, the effect of free radicals can be minimized by body's homeostasis system but higher concentration of it can hazardously damage DNA, RNA and even cell membrane. The DNA damage may play a role in the cancer formation, ensuing in mutations that can harmfully affect the cell cycle and potentially direct to malignancy and additional fatal diseases including stroke, myocardial infarction and diabetes etc. For example, the atherosclerosis as a cardiovascular disease can be accredited to free-radical induced oxidation of numerous of the chemicals making up the body. Moreover, free radicals are responsible for alcohol-induced liver damage (Lobo et al., 2010; Ali et al., 2013). Various phytochemicals such as phenolic acids, flavonoids, anthocyanins, tannins and carotenoids have potent antioxidant activity and this potentiality may be used as pharmacologically active products (López et al., 2007).

Podocarpus neriifolius D. Don is a fairly large tree and medium sized to which can make up to 35 (-45) m tall. It is infrequently spurred or even buttressed while the surface of bark is grayish-brown. It is the most prevalent species of genus, geographically distributed from Nepal, India, Indo-China and Thailand, throughout malesia, towards the Solomon Island and Fiji; it is also planted in garden (Lambert, 1824; Zaman et al., 2015a). Leaves have been reported to be used as boiled water for bathing and bark decoction is applied with cotton on herpes (Zaman et al., 2015a).

In total eleven phytochemicals isolated from *P. neriifolius* were recognized as C₃₄H₆₉OH, β-sitosteryl stearate, β-sitosterol, sciadopitysin, podocarpusflavone B, robustaflavone-7"-methyl ether, podocarpusflavone A, robustaflavone, p-hydroxyl benzoic acid, 2"-O-rhamnosylscopariu, 2"-O-rhamnosyl vitexin (Li-zhen et al., 1993). *P. neriifolius* is reported to have antiproliferative (Shrestha, 2011), cytotoxic and thrombolytic activities. To the date, there is no scientific record concerning the antibacterial and antioxidant activities of *P. neriifolius*. So, the methanol leaves extract of the plant was assessed for antibacterial and antioxidant activities.

MATERIALS AND METHODS

Plant

For this study, fresh leaves of *P. neriifolius* were gathered from the local region of Chittagong, Bangladesh and authenticated by

Professor Dr. Sheikh Bokhtear Uddin, Department of Botany, University of Chittagong, Bangladesh. The leaves were dried at room temperature for 7 days and in hot air oven for 2 days.

Preparation of extract

The dried leaves powdered and extracted with methanol for 7 with standardized shaking by using rotary shaker machine. The solvent was completely separate by filtering through No. 1 Whatman filter paper and water bath was used at 40°C to the drying of filtrates. The attained dried crude extracts were used for experiments.

Phytochemical screening

The phytochemical assessment of alcoholic extract of *P. neriifolius* was performed with the methods of Zaman et al. (2015b).

Antibacterial screening

Test organisms

Pure cultures of bacterial strains obtained from the microbiology laboratory of the Department of Pharmacy, International Islamic University Chittagong. were used as test. The crude extract was tested against *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, and *Salmonella paratyphi*.

Preparation of test solution

In the case of test solution preparation, 33.33 mg of weighed crude extract was dissolved in 1 ml of distilled water then mixed thoroughly by vortex mixture. 30 µl of test solution added to each disc was corresponding to the concentration of 1000 µg/disc. Similarly, 24 and 15 µl of test solution in each disc were corresponding to 800 and 500 µg/disc respectively.

Bacterial assay

The screening for the antibacterial activity was usually performed by disc diffusion method described by Bauer et al. (1966) and Sarker et al. (2010). The extract was tested at different concentrations 500, 800 and 1000 µg/disc, the diameters of the zone of inhibition fashioned as a result of the compounds were compared with the standard antibiotic (Kanamycin, 30 µg/disc). The tests were done in triplicate.

Antioxidant activity

DPPH radical scavenging activity

The free radical scavenging activity of the methanol extract of *P. neriifolius* leaves was quantified according to the protocol of Ali et al. (2013). In this method, antioxidant activity was determined based on the scavenging activity of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). This activity of the crude extract was investigated at different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml). First of all, 2 ml crude extract was added to 3 ml of a 0.004% methanol solution of DPPH. Secondly, after 30 min absorbance was taken at 517 nm and the percentage inhibition of activity was calculated by using the following equation:

Table 1. Antibacterial activity of alcoholic extract of *P. neriifolius* leaves.

S/L code	Name of the bacteria	Zone of inhibition in mm			
		Kanamycin disc (Standard)	<i>P. neriifolius</i> leaves (methanol extract)		
		30 µg/ disc	500 µg/ disc	800 µg/ disc	1000 µg/disc
B1	<i>B. subtilis</i>	29	10.5±0.00	13.67±0.33	14.33±0.33
B2	<i>S. aureus</i>	30	10.5±0.00	12.67±0.33	14.33±0.67
B3	<i>E. coli</i>	32	15.67±0.33	14.33±0.33	14.33±0.67
B4	<i>P. aeruginosa</i>	27	11.25±0.00	15±0.58	14.67±0.33
B5	<i>S. typhi</i>	30	16.67±0.33	17.67±0.33	18.67±0.33
B6	<i>B. cereus</i>	28	16.33±0.33	16±0.58	16.33±0.33
B7	<i>S. paratyphi</i>	29	22.67±0.33	23±1.00	26.67±0.88

Values represent the average of three different values ± S.E.M.

% of the scavenging activity = $[(A_0 - A_1)/A_0] \times 100$

Where A₀ denotes the absorbance of the control and A₁ denotes the absorbance of the extract. The curves were arranged and the IC₅₀ value was calculated from the graph.

Reducing power activity

The reducing power activity was evaluated according to the method described previously by Lee et al. (2009). The several concentrations of extract (62.5, 125, 250, 500 and 1000 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer solution (2.5 ml, 0.2 M, pH 6.6) plus potassium ferricyanide, that is, K₃Fe(CN)₆ (2.5 ml, 1% w/v). Then the obtained mixture was incubated at 50°C for 20 min. A fraction (2.5 ml) of 10% trichloroacetic acid was added to the mixture, which was after that centrifuged at 3000 rpm for 10 min. The upper layer of the 2.5 ml solution was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1% w/v) then the absorbance was determined at 700 nm. The increased reducing power confirmed with the increased absorbance of the reaction mixture. Ascorbic acid was used as the reference as well as phosphate buffer (pH 6.6) used as blank solution.

Total phenol content

Total phenol content of the extract was investigated by using Folin-Ciocalteu reagent according to the method of Ali et al. (2013) and Singelton et al. (1999). The sample (200 µg/ml) was mixed with 400 µl of the Folin-Ciocalteu reagent along with 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and made up to 10 ml with distilled water and then allowed to stand for 2 h. Afterward the absorbance was taken at 765 nm wavelength. The concentration of total phenol content in the extract (200 µg/ml) was then determined as mg of gallic acid equivalent by using an equation that was obtained from the standard gallic acid graph. The concentration of total phenolic compounds in sample was determined as milligram of gallic acid correspondent by using the following equation:

$$A = (cxv)/m$$

Where, A = Total phenol content (mg/ml gallic acid equivalent); c = X/1000 = Concentration of gallic acid (mg/ml); v = Quantity of extract (Volume); m = Mass of the extract (g).

RESULTS

Phytochemical screening

The phytochemical screening of methanol extract of *P. neriifolius* leaves showed the presence of terpenoids, phenols, tannins, flavonoids, alkaloids, carbohydrates, saponins, cardiac glycosides, and steroids.

Antibacterial assay

The antibacterial activity of this extract was presented in Table 1. The extract demonstrated different zones of inhibition at different concentrations (500, 800 and 1000 µg/disc) against the tested bacteria.

The extract showed the best activity at 1000 µg/disc in all the tested organisms. The maximum zone of inhibition of 26.67±0.88 mm was recorded at 1000 µg/disc concentration against *S. paratyphi*. The minimum zone of inhibition of 10.5 mm was found at 500 µg/disc concentration against *B. subtilis* and *S. aureus*.

Activity against oxidation

The DPPH radical scavenging activity

The percentage of the scavenging activity of extract and ascorbic acid at different concentration is shown in Figure 1. The value of IC₅₀ tested extract as well as ascorbic acid (positive control) was found to be 4.73 and 5.99 µg/ml, respectively.

Activity of reducing power

In this test, the yellow color of the test solution transforms to different shades of green and blue depending upon the reducing power of present compounds. The potentiality of the reducing power of the extract rose with the raise in

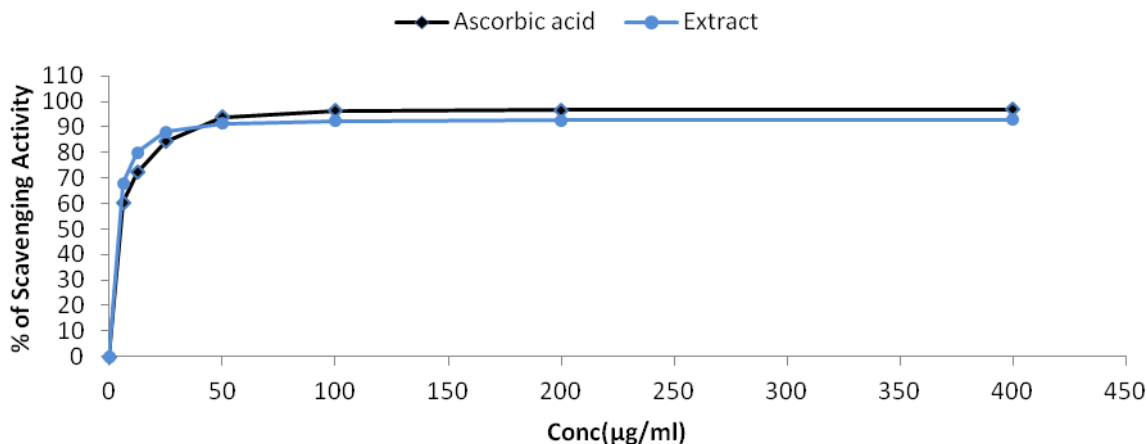


Figure 1. % of scavenging activity of the methanol leaf extract of *P. neriifolius* compared with those ascorbic acid.

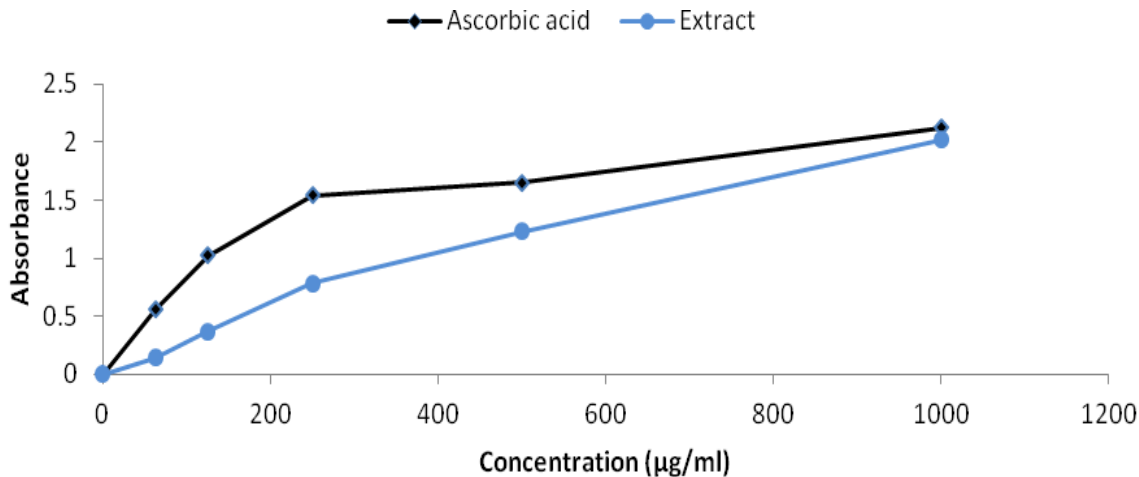


Figure 2. Reducing powers of Ascorbic acid and Methanol extracts of *P. neriifolius*.

concentration of methanol leaves extract of *P. neriifolius* and the important increasing absorbance was found to be 2.018 at 1000 µg/ml (Figure 2).

Total phenol content

The quantitative estimation of the phytochemical constituents of *P. neriifolius* shows that the medicinal plant is rich in total phenols. The estimated total phenol was 168.2 ± 6.4 mg GAE/g extract.

DISCUSSION

The study aimed to inspect the antibacterial and antioxidant activities of alcoholic extract from the leaves of *P. neriifolius*.

The investigations of the extract aimed to find the active phytochemical constituents present in this extract and valuable compounds founded in this extract. Then, antibacterial activity was assessed against. According to the result of antibacterial activity tests, this extract inhibits the growth of bacteria. Among the seven bacteria, this extract produced greatest inhibition against *S. paratyphi* and *S. typhi*. Literature revealed that, herbal products from the natural resources represent a promising source of antimicrobial agents for the reason that they are natural and affordable, particularly for rural communities (Ghosh et al., 2008). Approval of medicines from herbal sources as another form of medical care is increasing since they are serving as sources of novel antibiotic prototypes. It has been reported that various phytochemical active compounds including glycoside, saponin, tannin, flavonoids, terpenoid, and alkaloids are responsible for antimicrobial activity in various plant species

(Ebi and Ofoefule, 1997; Devi et al., 2012; Itoandon et al., 2012). This study suggests that, *P. neriifolius* possesses antibacterial activity because of these phytochemical constituents.

The investigation of antioxidant activity and this activity was also found in this extract. *In vitro* antioxidant tests permit quick estimation and identification of compounds as antioxidant agents because the substances have low antioxidant (Saeed et al., 2012). Regarding the results of the DPPH radical scavenging activity it is recommended that the plant extract contain phytochemical constituents that were capable of donating hydrogen to a free radical to scavenge the probable harm. The presence of reductants has reducing activity of a compound, which showed antioxidative property probable by breaking the free radical chain, donating a nitrogen atom. The attendance of reductants in *P. neriifolius* extracts results in the reduction of the Fe²⁺ ferric cyanide compound to the ferrous form. The phenolic compounds from the plants were also very significant because their hydroxyl groups confer scavenging ability. From the results of total phenolic content test, this extract was affluent of total phenol also responsible for antioxidant activity.

This research study suggests that the methanol extract of *P. neriifolius* leaves possesses antibacterial and antioxidant activity. But through which mechanism the extract inhibits the bacterial growth is unknown. Additional studies would be essential to estimate the involvement of active chemical constituents for the experimental antibacterial activity as it still remains to be determined which compounds were accountable for these effects. In near future this plant might be a contributor in the field of allopathy and/or naturopathy as a potent therapeutically active drug.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Chronic toxicity assessment of crude ethanolic extract of *Wissadula periplocifolia* (L.) C. Presl. leaves in albino Wistar rats

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Wissadula periplocifolia (L.) C. Presl. is a medicinal plant widely distributed in USA, but it has not yet been investigated extensively. Some pharmacochemical studies were performed at the Federal University of Paraíba, and the components of the crude ethanolic extract were identified; one of them was a tiliroside, which has already been shown to have excellent anti-inflammatory activity that can inhibit the arachidonic acid cascade and can inhibit the release of prostaglandins, prostacyclins, and leukotrienes from leukocytes and neutrophils. This study aimed to verify the possible long-term toxicity of this plant. Chronic toxicity tests were performed, including all recommended parameters; Wistar rats of both sexes were treated with 3 doses of the ethanolic extract of *W. periplocifolia* (L.) C. Presl leaves, and 2 satellite groups and a control group were set. The platelet and white blood cell counts for all treated males were higher than those in the controls. The anatomopathological tests did not reveal any alterations which were consistent with those obtained from the biochemical tests.

Key words: Chronic toxicity test, medicinal plants, phytotherapeutic, *Wissadula periplocifolia* (L.) C. Presl.

INTRODUCTION

The World Health Organization (WHO) has expressed a great interest in documenting the use of medicinal plant

products from populations across the world (Macedo et al., 2007). Many developing countries have increased

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their documentation of ethno-medicinal data, and scientific research is being conducted using medicinal plants. However, the popularity and traditional use of medicinal plants are not sufficient to validate their effectiveness and safety. Therefore, evaluating the risks and benefits associated with their use is necessary by conducting toxicological studies (Lima et al., 2014).

Members of the family, Malvaceae are known to exert several pharmacological effects and are used mainly as anti-tumor agents and in the treatment of ulcers, wounds and, especially, inflammatory diseases (Chaves et al., 2013). *Wissadula periplocifolia* (L.) C. Presl. is a rarely studied species of the family Malvaceae. Previous studies have shown that this species has higher antioxidant potential than several plants from the same family (Oliveira et al., 2012); its extracts have been shown to exert antibacterial effects against *Enterococcus faecalis* (Teles et al., 2014).

The pharmacological potential of *W. periplocifolia* is related to its production of different secondary metabolites. Previous phytochemical studies on *W. periplocifolia* have suggested the presence of flavones, flavonoids, glycosylated phenolic acids, chlorophyll derivatives, steroids and triterpenes (Teles et al., 2014, 2015). However, many plant products and phytochemicals can produce toxic and adverse effects. Thus, investigating the potential toxicity of natural products used in popular medicines is necessary (Bussmann et al., 2011). Other studies indicated that the *W. periplocifolia* CEE showed no relevant toxicity in the pharmacological screening at a dose of 2000 mg/kg, and no deaths were observed during the acute study (Guedes et al., 2016). The results of other tests with *W. periplocifolia* demonstrated a significant antiinflammatory activity, comparable to that of indomethacin which was used as standard. However, this effect was not CEE dose-dependent (Guedes et al., 2016).

The investigation of chronic toxicity with CEE of the leaves of *W. periplocifolia* (L.) C. Presl., by observing behavioral parameters, weight, temperature, hematological, biochemical, anatomy and pathological study is the objective of this assay.

MATERIALS AND METHODS

Plant materials

W. periplocifolia (L.) C. Presl plants were collected from Pedrada Boca (PB), municipality of Ararúna-PB, Brazil, in August, 2005. Botanical identification was performed by Prof. Dr. Maria de Fátima Agra of the Natural Products Research Center, UFPB. A specimen was archived in the herbarium of Prof. Lauro Pires Xavier at the Center of Exact and Natural Sciences, Federal University of Paraíba, under number 6498. The collection authorization number is SISBIO 46923-2.

Preparation of the extract

The leaves (3 kg) were dried in an oven at 40°C for 96 h and

then ground to powder in a mechanical mill; subsequently, the powder was macerated with ethanol (EtOH) for 3 days. This procedure was repeated to maximize extraction. The ethanolic extract was concentrated using a rotary evaporator at 50°C, and 705 g of crude EtOH extract (CEE) was obtained. The CEE (200 g) was solubilized in EtOH : water (9:1) solution.

Animals

Adult albino Wistar rats (*Rattus norvegicus*), males and nulliparous non-pregnant females, weighing between 200 and 300 g, were supplied by the vivarium, courtesy Prof. Thomas George, UFPB.

The animals were grouped in polyethylene cages, maintained under controlled temperature at 27±2°C, without the use of any medication, and provided food (Purina® ration pellets) and drinking water *ad libitum*; water was provided in graduated polyethylene bottles placed in metal grids in the upper part of the cages. The animals were maintained under a 12 h light-dark cycle. Before the start of the experiment, the animals were placed in the working environment for at least 30 min. The experimental protocol was approved by the Ethics Committee for Animal Experimentation (CEUA) of the UFPB (Process no., 169/2015).

Toxicological assay

The animals were divided into 6 groups; one control and 5 test groups, containing 20 animals each (10 males and 10 females) and treated orally (gavage) daily in the morning for 90 days. The control group was administered water, which was the vehicle used for the preparation of *W. periplocifolia* CEE. The 3 treatment groups received the following doses of CEE: the lowest dose of 10 mg/kg, an intermediate dose (3x) of 30 mg/kg, and a higher dose (9x) of 90 mg/kg. The minimum dose was calculated from a previous study of carrageenan paw edema induced by *W. periplocifolia* CEE (Guedes et al., 2016). The fifth and sixth groups (satellite groups) received doses of 30 and 90 mg/kg, respectively. All animals were killed 30 days after the end of the experiment to assess the reversal of possible toxicities condition.

The effects of prolonged administration of the *W. periplocifolia* CEE were evaluated using the following parameters: temperature, water and food consumption, weight evolution, exploratory activity (open-field test), and motor activity of the animals (Rota-rod test). Hematological and biochemical assays and anatomy pathological examinations were performed using rat organs. The results were compared with those obtained from the control group, according to previous studies (Castello et al., 2011).

Temperature

The body temperature was measured using a digital thermometer (model MC-3BC®; OMRON, China). Temperature was measured by lubricating the thermometer's thermosens or with petroleum jelly and introducing it up to 5 cm in the rectum, reaching the colon. This parameter was measured weekly in all animals.

Consumption of water and food and weighted assessment

The consumption of water and food pellets was evaluated in all the animals. Every day, 200 g of food and graduated bottles containing 250 mL of water were placed in the cages. On the following day, in

the morning, the water volume ingested by the animals was measured and assessed cumulatively. The consumed food was evaluated in a similar manner. The animals were weighed daily to calculate the administered dose.

Behavioral evaluation

Open-field test

The open-field apparatus (Insight, Brazil) was used for behavioral evaluation following the protocol described by Carlini et al. (1986) to analyze the exploratory activity of animals by spontaneous movement (locomotion). The test registered the number of crossings with 4 paws between the grid and across the square crossings of the field and quantified self-cleaning, rinsing, and defecation (number of fecal cakes) behaviors.

The parameters were used as the index to determine the influence of CEE on the emotional behavior of the animals; it indicates the changes in the central nervous system. Every fortnight, one hour after CEE administration, the parameters described above were assessed in the treated and control animals (3 min for each animal) (Mansur et al., 1971).

Rota-rod test

Further, every fortnight, one hour after CEE administration, the treated and control animals were placed on a rotating rod of the Rota-rod apparatus (Acceler Rota-Rod (Jones & Roberts) for rats; 7750; Ugo Basile, Italy), which was rotated at a constant speed of 9 rpm, and the riding time on the device was recorded with 3 repetitions.

Laboratory assessment of hematological parameters

After the chronic toxicity experiment, blood samples were collected from the brachial plexus of the animals treated with CEE (10, 30 and 90 mg/kg/day) and the control group. After a 12 h fasting period, blood was collected in tubes containing EDTA for the assessment of hematological parameters (hemogram and platelet count) and in tubes containing separator gel; they were centrifuged for 10 min at 3,500 rpm to obtain serum, which was subjected to biochemical analysis for glucose, urea, creatinine, transaminases, aspartate amino transferase (AST), alanine amino transferase (ALT), cholesterol, triglycerides, uric acid, alkaline phosphatase, total proteins and fractions, calcium, magnesium, sodium and potassium levels.

Anatomy-pathological examination

The test animals were killed by administering high levels of the anesthetic (xylazine and ketamine at 30 and 255 mg/kg, respectively) followed by cervical action as recommended by the scientific community, and the viscera were collected and examined macroscopically, with resection and consecutive weighing. The heart, liver and kidney were sectioned by sagittal incision, and the lungs were subjected to perfusion through the trachea by using 10% formaldehyde.

The organs of the animals treated with the highest dose (90 mg/kg) were sectioned and immersed in a fixing solution. After 12 h of fixation, the samples for histopathological processing were obtained by paraffin embedding and stained using hematoxylin and eosin and subjected to Masson trichrome.

Satellite groups

Thirty percent of the male and female animals treated with 30 and 90 mg/kg CEE were kept alive for 30 days after the remaining were killed, to assess the reversibility of the possible toxic effects caused by the administration of CEE.

RESULTS

Administration of CEE at the stipulated doses did not cause mortality in any of the animals, but bone marrow signs of toxicity were observed.

Temperature

Small changes in body temperature were observed in both sexes. As compared to that in the control group, the temperature increased during the first week after treatment with 10 mg/kg CEE in males, whereas it increased during the third week at doses of 30 and 90 mg/kg in females.

Consumption of water and food

Water consumption significantly increased in the fourth week in males treated with 90 mg/kg CEE, whereas it increased in the tenth week in females after treatment with 30 mg/kg CEE and in the tenth, eleventh and twelfth weeks in females treated with 90 mg/kg CEE. The cumulative water and food consumption in males and females is shown in Figures 1, 2 and 3.

Weight evolution

No change was observed in the weight of control rats or those treated with the CEE.

Behavioral (open-field test) and motor (Rota-rod test) assessment

In the open-field test, changes were observed only in 2 instances in males treated with 90 mg/kg on the first day, in which an increase in behavioral activity was noted. However, in the Rota-rod test, the riding time on the rotating rod decreased only in one of the males treated with 90 mg/kg on the fourth day. All other results were normal in all the groups.

Clinical blood assessment

At 90 days in the treated groups and 120 days in the 2 satellite groups, whole blood was collected for biochemical measurements and hematological assessment.

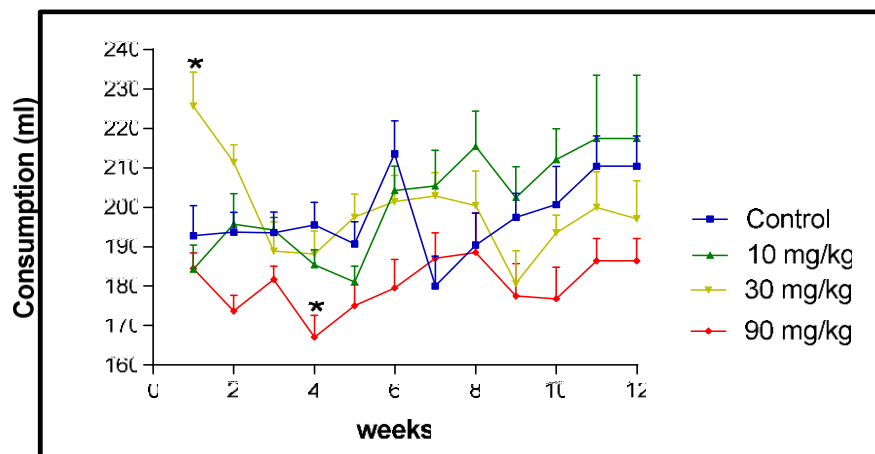


Figure 1. Water consumption by male Wistar rats treated with *W. periplocifolia* CEE during the chronic toxicity test. The values are expressed as mean \pm standard error of the mean (n = 10 One-way analysis of variance (ANOVA)/Tukey test (* P <0.05).

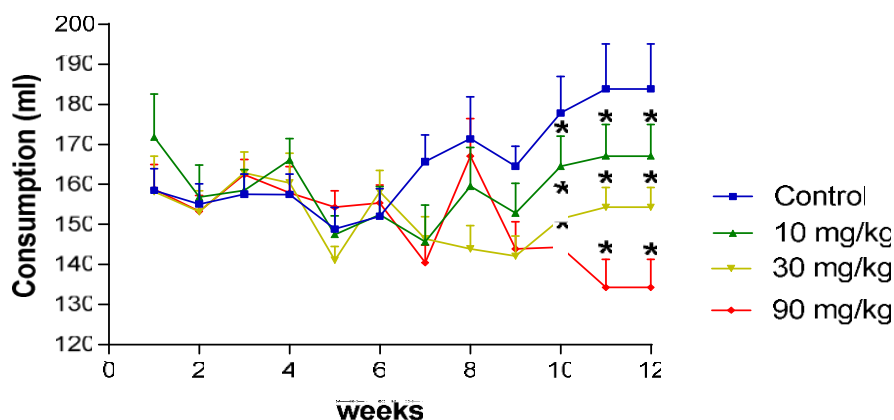


Figure 2. Water consumption by female Wistar rats treated with *W. periplocifolia* CEE during the chronic toxicity test. The values are expressed as mean \pm standard error of the mean (n=10) One-way analysis of variance (ANOVA)/Tukey test (* P <0.05).

Hematological parameters

Statistically significant changes were observed in the hematological parameters. In both sexes, the hematocrit value of the satellite groups treated with 30 and 90 mg/kg reduced. However, the hematocrit value was slightly higher in males than in females treated with 30 and 90 mg/kg CEE; this increased the mean corpuscular hemoglobin concentration (MCHC) in the 90 mg/kg satellite group as compared to that of the 90 mg/kg female treatment group. Significant differences in leucopenia mainly at the expense of neutropenia were noted in the male treated groups and the satellite groups, but no tin females as we can see in the Table 1.

Biochemical parameters

Some parameters (total protein, albumin, calcium and uric

acid) were significantly different in the treated groups than in the control group as we can see in Table 2.

Anatomy pathological study

Macroscopically, the organs showed no significant anatomical changes, with minimal variations in weight. None of the animal organs showed histological peculiarities, except the lung **Figure 4A, B, C, D**) that showed minor changes such as discreet pulmonary infiltrates, which was related to the oral administration by gavage.

DISCUSSION

Toxicological studies involving evaluations of efficacy and



Figure 3. Food consumption by male Wistar rats treated with *W. periplocifolia* CEE during the chronic toxicity test. The values are expressed as mean \pm standard error of the mean (n=10). One-way analysis of variance (ANOVA)/Tukey test (* P <0.05).

Table 1. Hematological parameters of Wistar rats treated with *W. periplocifolia* CEE during the chronic toxicity test.

	Control	10 mg/kg	30 mg/kg	90 mg/kg	30 mg/kg satellite	90 mg/kg satellite
Males						
Red blood cells ($10^6/\mu\text{L}$)	8.43 \pm 0.19	8.35 \pm 0.17	8.25 \pm 0.22	8.51 \pm 0.14	7.87 \pm 0.11	6.61 \pm 0.83
Hemoglobin(g/dL)	16.5 \pm 0.5	1.9 \pm 0.3	15.6 \pm 0.4	16.3 \pm 0.2	15.9 \pm 0.4	14.0 \pm 1.8
Hematocrit (%)	45.8 \pm 1.3	45.2 \pm 0.9	44.5 \pm 0.7	44.4 \pm 0.7	41.5 \pm 0.6	34.3 \pm 4.5*
MCV (μ^3)	53.1 \pm 0.6	53.2 \pm 0.3	53.6 \pm 0.6	53.4 \pm 0.5	52.7 \pm 0.5	51.9 \pm 0.9
MHC (μg)	19.2 \pm 0.3	18.7 \pm 0.2	19.2 \pm 0.3	19.1 \pm 0.2	2.2 \pm 0.5	19.6 \pm 0.3
MCHC (%)	36.1 \pm 0.3	35.1 \pm 0.3	35.9 \pm 0.3	35.8 \pm 0.2	38.4 \pm 0.5*	37.8 \pm 0.3*
Leukocytes (mm^3)	6573 \pm 630.1	3789 \pm 281.1*	3087 \pm 471.7*	3054 \pm 31.2*	4550 \pm 1245	2920 \pm 209.3*
Neutrophils (mm^3)	1948 \pm 183.3	1155 \pm 111.7*	927.0 \pm 191.2*	800.1 \pm 132.8*	1228 \pm 399.2	898.7 \pm 118.9*
Eosinophils (mm^3)	8.94 \pm 8.94	11.55 \pm 8.893	9.62 \pm 9.62	13.28 \pm 5.802	16.70 \pm 16.70	6.00 \pm 6.00
Lymphocytes (mm^3)	3759 \pm 371.5	2380 \pm 147.9*	2017 \pm 283.2*	2038 \pm 250.3*	2989 \pm 818.0	1854 \pm 150.3*
Monocytes (mm^3)	644.8 \pm 71.1	242.0 \pm 39.9	133.2 \pm 41.5*	111.0 \pm 12.1*	316.1 \pm 143.0	161.8 \pm 24.0
Platelets($10/\text{mm}^3$)	647.6 \pm 0.31	609.6 \pm 0.24	497.4 \pm 0.30*	456.4 \pm 0.32*	622 \pm 0.47	585.2 \pm 0.73
Females						
Red blood cells ($10^6/\mu\text{L}$)	7.75 \pm 0.12	7.46 \pm 0.15	7.25 \pm 0.60	7.52 \pm 0.12	6.38 \pm 0.63	6.14 \pm 0.73
Hemoglobin(g/dL)	15.4 \pm 0.2	14.4 \pm 0.3	15.7 \pm 0.7	14.8 \pm 0.3	13.0 \pm 1.2	12.8 \pm 1.4
Hematocrit (%)	40.9 \pm 0.7	39.3 \pm 0.7	40.6 \pm 0.5	39.8 \pm 0.6	33.9 \pm 3.5*	32.6 \pm 3.5*
MCV (μ^3)	52.8 \pm 0.2	52.7 \pm 0.4	52.0 \pm 0.4	53.0 \pm 0.4	53.0 \pm 0.6	53.4 \pm 1.3
MHC (μg)	19.6 \pm 0.1	19.4 \pm 0.2	19.2 \pm 0.3	19.62 \pm 0.3	20.4 \pm 0.3	20.9 \pm 0.4
MCHC (%)	37.0 \pm 0.2	36.8 \pm 0.5	36.9 \pm 0.3	37.0 \pm 0.4	38.5 \pm 0.5	39.1 \pm 0.6*
Leukocytes (mm^3)	3784 \pm 399.7	4017 \pm 461.5	3206 \pm 573.5	4322 \pm 973.6	3908 \pm 916.6	2600 \pm 668.4
Neutrophils (mm^3)	1125 \pm 113.9	1447 \pm 168.5	850.0 \pm 209.4	1893 \pm 888.3	1219 \pm 248.4	739.5 \pm 246.5
Eosinophils (mm^3)	10.9 \pm 7.1	6.1 \pm 4.4	2.5 \pm 2.5	2.7 \pm 2.7	14.1 \pm 14.1	11.1 \pm 11.1
Lymphocytes (mm^3)	2436 \pm 280.3	2336 \pm 266.8	2178 \pm 511.5	2299 \pm 262.1	2419 \pm 585.6	1654 \pm 343.6
Monocytes (mm^3)	213.0 \pm 32.8	232.6 \pm 61.6	118.3 \pm 29.3	127.4 \pm 31.4	255.8 \pm 74.3	195.3 \pm 88.0
Platelets($10/\text{mm}^3$)	480.6 \pm 63.6	603.1 \pm 18.2	530.2 \pm 24.2	517.6 \pm 28.5	46.7 \pm 50.4	490.0 \pm 80.5

The values are expressed as mean \pm standard error of the mean (n=10).One-way analysis of variance/ Tukey test * p <0.05.MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV mean corpuscular volume

Table 2. Biochemical parameters of Wistar rats treated with *W. periplocifolia* CEE during the chronic toxicity test.

	Control	10 mg/kg	30 mg/kg	90 mg/kg	30 mg/kg Satellite	90 mg/kg Satellite
Males						
Glucose(mg/dL)	135.7±11.3	144.8±11.2	139.4±8.7	123.0±8.8	126.0±16.2	89.5±4.2
Urea (mg/dL)	39.2±1.2	38.4±1.4	40.9±1.2	38.6±1.0	40.7±1.5	37.2±1.9
Creatinine(mg/dL)	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.10	0.6±0.0
Cholesterol(mg/dL)	53.0±2.5	49.7±2.3	54.9±2.4	54.8±3.5	63.0±1.5	47.3±2.4
Triglycerides(mg/dL)	99.4±17.1	84.8±8.6	93.9±14.0	103.9±9.2	89.7±14.7	71.5±17.7
Uricacid(mg/dL)	1.2±0.2	1.1±0.1	0.8±0.1*	0.7±0.0*	0.8±0.1	0.8±0.1
AST(U/L)	169.5±15.2	150.9±10.2	151.4±7.8	132.0±7.9	135.7±21.2	132.0±9.3
ALT(U/L)	55.6±4.4	60.1±2.1	59.3±3.2	60.3±2.3	63.7±3.2	46.8±1.1
ALK phosphatase(U/L)	150.6±12.9	122.5±3.8	141.9±8.6	159.3±12.2	175.0±6.6	108.5±15.9
HDL (U/L)	29.0±1.3	29.1±0.9	30.6±0.8	31.1±1.1	36.0±1.5	25.0±1.4*
Total proteins (g/dL)	6.3±0.1	6.3±0.1	6.3±0.1	6.3±0.1	5.9±0.12	5.7±0.1*
Albumin(g/dL)	2.6±0.1	2.8±0.1	2.7±0.1	2.7±0.1	2.5±0.1	2.4±0.1*
Globulin(g/dL)	3.6±0.12	3.5±0.1	3.6±0.1	3.6±0.1	3.5±0.10	3.3±0.1
Na ⁺ (mEq/L)	137.0±0.6	137.6±0.7	138.8±0.4	139.1±0.5	136.3±1.2	139.0±0.1
K ⁺ (mEq/L)	5.8±0.2	5.1±0.2	5.3±0.3	5.7±0.2	5.7±0.0	5.5±0.2
Ca ²⁺ (mEq/L)	10.0±0.1	10.0±0.1	9.2±0.1*	9.3±0.1*	9.2±0.2	9.2±0.1
P ³⁻ (mEq/L)	6.3±0.2	5.9±0.3	6.2±0.1	6.6±0.2	6.6±0.2	6.5±0.4
Females						
Glucose(mg/dL)	115.1±5.3	115.9±4.5	102.6±6.4	98.7±4.4	123.4±13.3	83.2±5.7
Urea (mg/dL)	38.8±1.5	44.7±1.8	43.0±1.2	40.4±1.5	33.0±2.8*	41.0±2.6
Creatinine(mg/dL)	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.02	0.6±0.02	0.6±0.0
Cholesterol(mg/dL)	62.4±4.1	57.1±3.0	54.3±2.9	53.9±2.7	53.2±5.4	52.3±4.4
Triglycerides(mg/dL)	64.4±7.2	77.6±7.4	67.0±6.5	53.7±7.6	43.0±6.7	47.7±7.2
Uric acid (mg/dL)	1.0±0.0	1.0±0.1	1.1±0.0	0.9±0.1	1.8±0.2*	1.1±0.1
AST (U/L)	141.7±10.2	163.6±13.1	178.4±19.3	127.1±6.6	150.0±10.2	246.8±69.4*
ALT(U/L)	59.4±3.3	48.1±5.1	58.4±4.8	55.3±2.9	63.8±9.1	73.8±11.8
ALK phosphatase(U/L)	101.1±10.0	96.5±9.2	107.6±7.6	121.0±22.7	84.2±13.2	124.9±22.3
HDL(U/L)	34.1±1.3	32.9±1.3	31.1±1.1	31.2±1.2	29.0±2.5	28.5±2.3
Tot.proteins (g/dL)	6.5±0.1	6.5±0.1	6.4±0.1	6.2±0.1	6.2±0.2	5.9±0.1
Albumin (g/dL)	3.0±0.0	3.0±0.1	2.9±0.1	2.8±0.1	2.6±0.1*	2.6±0.4
Globulin (g/dL)	3.5±0.1	3.4±0.1	3.4±0.1	3.5±0.2	3.4±0.1	3.4±0.10
Na ⁺ (mEq/L)	137.4±0.6	133.3±0.4	137.4±0.1	138.1±0.4	136.4±0.6	135.0±1.0*
K ⁺ (mEq/L)	4.2±0.1	4.5±1.0	5.1±0.2*	4.4±0.2	5.2±0.3	4.6±0.2
Ca ²⁺ (mEq/L)	10.0±0.8	10.2±0.1	9.51±0.1*	9.1±0.1*	9.0±0.1*	8.9±0.1
P ³⁻ (mEq/L)	4.8±0.2	5.2±0.3	5.45±0.2	5.1±0.2	5.4±0.4	6.0±0.3

The values are expressed as mean±standard error of the mean (n=10). One-way analysis of variance (ANOVA)/Tukey test* (p< 0.05). ALT, alanine transaminase; AST, aspartate transaminase; ALK phosphatase, alkaline phosphatase.

safety are fundamental to the development of medicinal plant products. In a previous study, the changes in the organs and body weights of rats were considered clear indications of damage caused by the test substance (Berenguer-Rivas et al., 2013). Treatment with *W. periplocifolia* CEE reduced the consumption of water and food in females and males at some doses as compared to that in the control group. However, the weights of the animals did not vary significantly between

the control and treated groups; thus, CEE was thought to have low toxicity after chronic exposure (Berenguer-Rivas et al., 2013). With regard to motor and behavioral tests, the animals were active and responsive to stimuli, without any clinical signs that could be associated with local or systemic toxic effects. Normality was noted, and the behavior of the animals remained normal (Castello Branco et al., 2011).

The hematopoietic system is one of the more sensitive

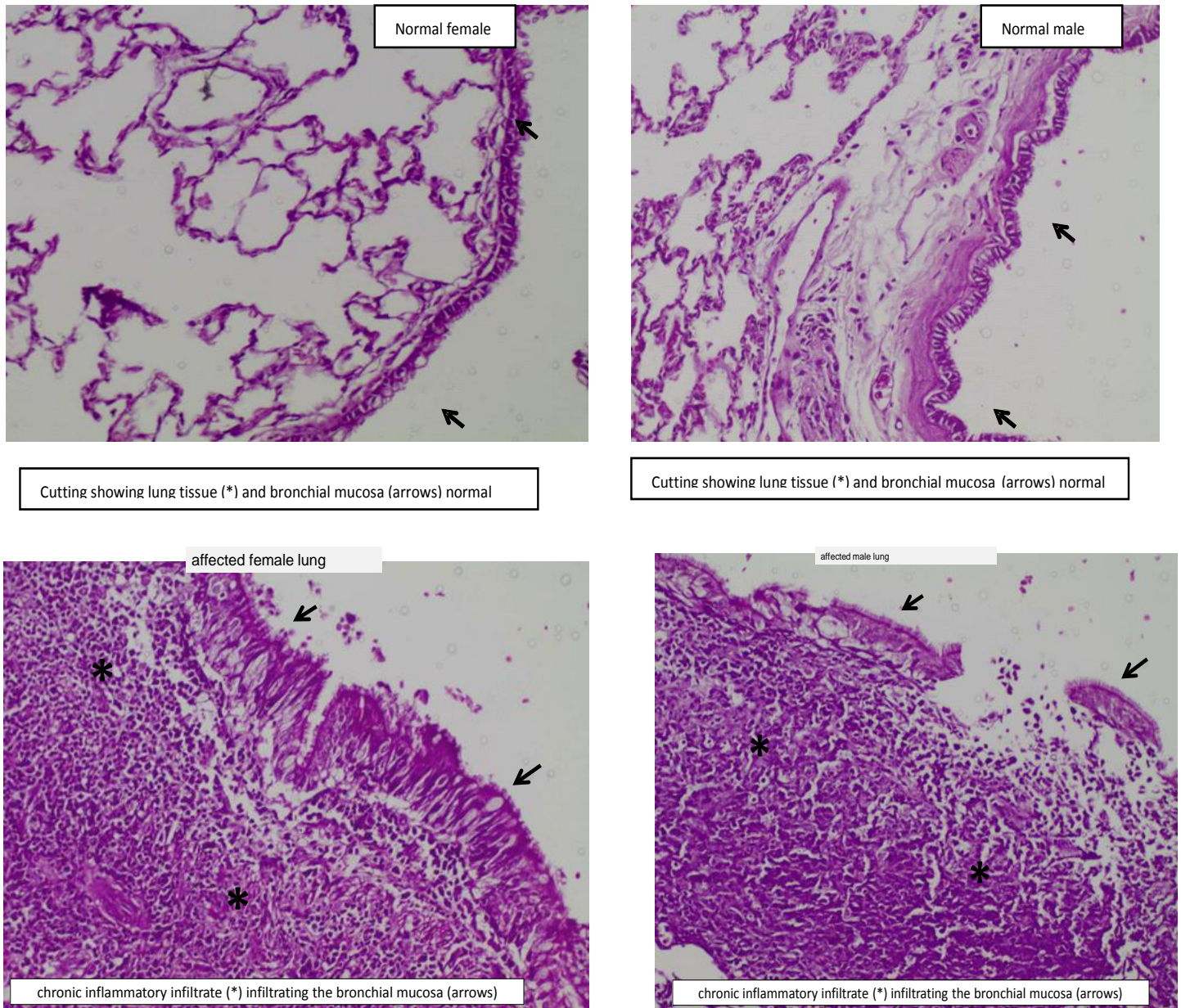


Figure 4. A and B: Photomicrographs of bronchial parenchyma of normal female and male controls, showing no significant change. C and D: Photomicrographs of the bronchial parenchyma of a male and a female from the treated groups showing mucous infiltrates in the membranes.

targets for toxic compounds and is an important index to measure the physiological and pathological states of treated animals. Changes in these parameters in animal studies have a greater predictive value for human toxicity to a substance (Olson et al., 2000). For the assessed hematological parameters, statistically significant alterations were observed as compared to published data (Castello Branco et al., 2011).

However, in the red series of both sexes, the hematocrit value decreased in the 30 and 90 mg/kg satellite groups,

which was slightly more pronounced in males than in the satellite groups. These alterations increased the MCHC in the 90 mg/kg satellite group as compared to that of the 90 mg/kg group of females, which was as expected, since erythrocytes are known to remain viable in the peripheral blood for 90 to 120 days (Castello Branco et al., 2011). Unlike that in previous studies (Castello et al., 2011; Giknis and Clifford, 2006), significant difference was noted among males of the 10, 30, and 90 mg/kg groups and those of the 30 and 90 mg/kg satellite groups, which

showed leukopenia mainly at the expense of neutropenia; this was not observed in females. Regarding platelet count, no statistically significant changes were noted among females whereas, in males, a significant reduction was observed in the groups treated with 30 and 90 mg/kg CEE, although this count was within the normal limits for the rats studied (Castello Branco et al., 2011; Giknisand Clifford, 2006).

The CEE had been showed to have a potential-inflammatory effect (Telles et al., 2015) and can inhibit the arachidonic acid cascade, thereby inhibiting the release of prostaglandins, prostacyclins, and leukotrienes from leukocytes and neutrophils (Guedes et al., 2016). The CEE of *W. periplocifolia* also included a potential-inflammatory substance, tiliroside, which according to previous studies, selectively inhibits the spinal release of platelets and granulocytes in male rats after prolonged use (Telles et al., 2015).

Some enzymes and proteins (ALT, AST, gamma-glutamyl transferase and bilirubin; OCDE2008b) (Brandt et al., 2009) and biomarkers of renal function, such as blood creatinine, urea and nitrogen, can be considered indicators of hepato cellular alterations (Lameire et al., 2005). Of the 12 biochemical indicators of transaminases, AST and ALT are the 2 enzymes that are associated with hepato cellular damage and are thus used as biomarkers to predict possible toxicity (Huang et al., 2006; Ojiako and Nwanyo, 2006). Although, both are common hepatic enzymes, and their concentrations in hepatocytes is high, only ALT is noticeably specific to liver function, since AST is mainly present in the myocardium, skeletal muscle, brain and kidneys (Sacher and McPherson, 1991; Witthawaskul et al., 2003). No statistically significant difference was noted in the hepatic or renal parameters between the treated and control groups. Some parameters (total protein, albumin, calcium and uric acid) were statistically significant from those in the control group. Initially, hypoalbuminemia was noted in males treated with 90 mg/kg CEE and in females treated with 30 mg/kg CEE as compared to that in rats of the 90 and 30 mg/kg satellite groups. This might have been caused by the reduction in food intake. The reduction in albumin also justifies the reduction in calcium, since 50% of calcium is bound to albumin in the peripheral blood, and its levels are measured and interpreted by comparing the levels of free calcium and those bound to albumin. However, according to Giknis and Clifford (2006), the reduction noted in this study was not clinically significant and could be considered as a physiological variation in both males and females (Castello Branco et al., 2011).

Qualitative macroscopic analyses revealed that none of the tested doses produced changes in the vital and reproductive organs of the treated animals. Similarly, in the histopathological analysis, no changes suggesting toxic effects were noted. These results were in accordance with the obtained data from biochemical analyses. The chronic toxicity study showed the

occurrence of major and significant changes primarily at the level of blood parameters and warrants additional studies for confirming these results in the long term.

Conflict of Interests

The authors have not declared any conflict of interests.

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