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Full Length Research Paper

Pharmacological evaluation of the aqueous stem bark extract of *Bombax buonopozense* in the relief of pain and fever

Essien, A. D.¹, Essiet, G. A.¹, Akuodor, G. C.^{2*}, Akpan, J. L.², Chilaka, K. C.³, Bassey, A. L.⁴, Ezeokpo, B. C.⁵ and Nwobodo, N. N.²

¹Department of Pharmacology, College of Medical Sciences, University of Calabar, Nigeria.

²Department of Pharmacology and Therapeutics, Faculty of Medicine, Ebonyi State University, Abakaliki, Nigeria.

³Department of Pharmacology and Therapeutics, College of Health Sciences, Nnamdi Azikiwe University, Awka, Nigeria.

⁴Department of Pharmacology and Toxicology, University of Uyo, Nigeria.

⁵Department of Internal Medicine, Faculty of Medicine, Ebonyi State University, Abakaliki, Nigeria.

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Bombax buonopozense is used in traditional medicine in Nigeria for the treatment of pains and feverish conditions. This study was orally carried out to establish the analgesic, anti-inflammatory and antipyretic properties of an aqueous stem bark extract of *Bombax buonopozense* in experimental animals. The analgesic activity was measured using the acetic acid-induced abdominal constriction and water tail immersion tests. The anti-inflammatory activity was assayed using the xylene and carrageenan-induced oedema tests, while the antipyretic activity was measured using the brewer's yeast and 2, 4 dinitrophenol-induced pyrexia. The stem bark extract (50, 100 and 200 mg/kg) at all doses used, was found to have significant ($P < 0.05$) analgesic, anti-inflammatory and anti-pyretic activities. Data shows that aqueous stem bark extract of *B. buonopozense* possesses constituents with therapeutic potential against pains and feverish conditions, thus supporting the use of the stem bark of this plant for similar ailments in traditional medicine.

Key words: *Bombax buonopozense*, stem bark, aqueous extract, pain, inflammation, fever.

INTRODUCTION

Bombax buonopozense P. Beauv which belong to the family bombaceae, is a large tropical tree which grows to 40 metres in height with large buttress roots which can spread 6 metres. The leaflets have entire margin and also large measuring between 8 to 23 cm in length and 3

to 8 cm in width. The undersides of the leaflets are glabrous or puberulous, and conical buds contain numerous seeds (Beentje and Sara, 2001).

B. buonopozense is widely distributed in African countries such as Nigeria, Ghana, Sierra Leone, Uganda

*Corresponding author. E-mail: goddyakuodor@yahoo.com, godwin.akuodor@ebsu-edu.net. Tel. +2348036725237.

and Gabon. Common vernacular names in Nigeria include: Akpu (Igbo), Kurya (Hausa), Ogbolo (Yoruba), and Vabga in Ghana. Different parts of the plant are used for different purposes (Dubost, 1984). The leaf, stem bark and root have been reported to possess antiplasmodial, antidiarrhoeal, pains, fever and anti-ulcer activities (Akuodor et al., 2011f; Akuodor et al., 2011g; Iwuanyanwu et al., 2012; Akuodor, 2011h; Nwagba et al., 2013). Earlier studies also have reported its nutritive and antimicrobial properties (Mann et al., 2003; Akuodor et al., 2011c; Mann et al., 2011). There is no doubt that Africa is blessed with abundant plants whose medicinal potentials are yet to be tapped. The interest in this plant was justified by its potential medicinal value against pains and feverish conditions. Therefore, the aim of this study was to investigate the analgesic, anti-inflammatory and antipyretic activities of the aqueous stem bark extract of *B. buonopozense* in experimental animals.

MATERIALS AND METHODS

Plant collection and extraction

The stem bark of *B. buonopozense* was collected in March, 2009 from a forest in Chaza village, Niger State, in Northcentral Nigeria. The plant material was identified and authenticated by a taxonomist in the Department of Medicinal Plant Research and traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. A voucher specimen (NIPRD/H/6402) was deposited in the herbarium of the Department. Fresh stem bark parts of *B. buonopozense* were air-dried in the laboratory until a constant weight was obtained and pulverized. The powdered plant material was then macerated in distilled water for 24 h. The filtrate was dried on a water bath and a yield 18.75% was obtained. The solid brown extract was subsequently reconstituted in distilled water to obtain appropriate concentrations for the study.

Experimental animals

Albino mice (20 to 22 g) and wistar rats (180 to 220 g) of either sex used in this study were obtained from the Animal House of Faculty of Health Sciences, Ebonyi State University, Abakaliki, Nigeria. The animals were kept in hygienic, properly ventilated compartments and maintained under standard environmental conditions. Animals were sustained on standard rodent pellet and water *ad libitum*. The experimental procedures adopted in this study were in accordance with the United States National Academy of Sciences Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, National Academy of Sciences, 2011).

Phytochemical screening

Phytochemical screening of the aqueous stem bark extract of *B. buonopozense* was carried out in accordance with standard procedures (Ajayi et al., 2011; Kasolo et al., 2010; Inyang-Agha, 2006).

Acute toxicity test

The acute toxicity of *B. buonopozense* stem bark extract was tested

to determine its safety adopting the guidelines of Organisation for Economic Co-operation and Development (OECD) (2010). The studies were done in two phases. Nine rats, randomized and divided into three were used in the first phase. The rats were orally administered with 100, 600 and 1000 mg/kg of the stem bark extract, respectively. The animals were observed for the first 4 h and 24 h for signs of toxicity and mortality. This was followed by the second phase in which 2000, 3000 and 5000 mg/kg of the extract was administered to the next three groups of three rats per cage. The signs of toxicity and mortality were observed for 24 h, 48 h and 72 h, respectively.

Pharmacological studies

Analgesic assay

Abdominal constriction test: Mice for this study were divided into five groups of six per cage. The different groups were orally treated with normal saline (20 ml/kg), *B. buonopozense* stem bark extract at doses of 50, 100, 200 mg/kg, acetylsalicylic acid (150 mg/kg). Thirty minutes later, mice were treated with 0.7% v/v acetic acid (10 ml/kg, i.p.). The number of writhes was then counted at 5 min interval for 30 min (Singh and Majumdar, 1995).

Water tail immersion test: Mice for this experiment were divided into five groups of six in each cage. The different groups of animals were orally treated with distilled water (20 ml/kg), *B. buonopozense* stem bark (50, 100, 200 mg/kg), and morphine (10 mg/kg). The initial reading was taken immediately before administration of test and standard drugs. Thirty minutes post drug administration, each mouse was restrained in a horizontal cylinder leaving the tail hanging freely and 4 cm portion of each mouse tail marked was then immersed in a water bath thermo-statically maintained at 50±1°C. However, the time taken for each mouse to remove its tail out of the water was recorded (Jansen and Jagenau, 1959; Akuodor et al., 2015). The tail flick latency was evaluated at 30, 60, 90 and 120 min.

Anti-inflammatory test

Xylene-induced ear oedema in mice

The mice used for this study were randomly divided into 5 groups of 6 in each cage. Mice in different groups were treated with normal saline (20 ml/kg, p.o.), *B. buonopozense* (50, 100 and 200 mg/kg, p.o.) and dexamethasone (4 mg/kg, p.o.). One hour post drug administration, oedema was induced in each mouse by applying 50 µL of xylene using a microliter pipette at the inner surface of the right ear. Thirty minutes after, the animals were sacrificed under light ether anaesthesia, and both ears were cut off to approximately equal size and weight. The mean difference between the right and left ear were determined for each group and recorded as an indication of inflammation (Junping et al., 2005).

Carrageenan-induced rat paw oedema

Rats for this experiment were divided into five groups of six in each cage and different groups were treated with normal saline (20 ml/kg, p.o.), *B. buonopozense* stem bark extract (50, 100, 200 mg/kg) and acetylsalicylic acid (150 mg/kg). One hour after administration, oedema was induced in rat by injection of 0.1 ml of freshly prepared carrageenan suspension (1%) in distilled water into the subplantar tissue of the right hind paw to all the groups (Gupta et al., 2005). The paw volumes were measured at 0.5, 1, 2, 3

Table 1. Effect of aqueous stem bark extract of *B. buonopozense* on acetic acid-induced writhing in mice.

Treatment	Dose (mg/kg)	Writhing	% Inhibition
Control	20 ml/kg	23.33±3.33	-
<i>B. buonopozense</i>	50	8.17±0.98	65*
	100	4.67±0.33	80*
	200	3.33±0.61	86*
Aspirin	150	3.00±0.73	87*

Values are expressed as mean ±SEM; *Significant at $p < 0.05$ when compared to control.

and 4 h respectively by using plethysmometer. The average swelling of paws in the groups of extract treated was compared with control group and the standard.

Antipyretic assay

Yeast-induced pyrexia

Rats were randomly divided into five groups of six rats each. The basal rectal temperatures of the animals were recorded over a period of 30 min. Ten ml/kg of brewer's yeast suspension (15 % in 0.5 % w/v methylcellulose) was injected subcutaneously into the rats to induce pyrexia. 24 h after yeast injection, the rectal temperatures of animals were taken and animals showing rise in temperature of less than 0.5°C were discarded. After the establishment of pyrexia, normal saline (20 ml/kg, p.o.); *B. buonopozense* extract (50, 100 and 200 mg/kg, p.o.), and acetylsalicylic acid (150 mg/kg, p.o.) were orally administered to rats in different groups. The rectal temperatures of animals were thereafter recorded at 1, 2, 3, and 4 h post-treatment (Agbaje et al., 2008; Akindele et al., 2012).

2, 4-Dinitrophenol-induced pyrexia

Rats employed for the study were randomized into 5 groups of 6 in each cage. Their basal temperature was taken and 10 mg/kg of DNP prepared in normal saline was subcutaneously injected to each rat to induce pyrexia. Thirty min after administration of DNP (confirmation of pyrexia), the animals in different groups were orally treated with normal saline (20 ml/kg), *B. buonopozense* stem bark extract (50, 100 and 200 mg/kg), and acetylsalicylic acid (150 mg/kg). Rectal temperature of each rat was thereafter taken at 1 h interval for 4 h (Essien et al., 2015; Okokon and Nwafor, 2010).

Statistical analysis

Results are expressed as mean ± SEM. The significant difference between mean was determined using one-way analysis of variance (ANOVA) to analyse results between groups. Statistical significance was established at $P < 0.05$

RESULTS

Phytochemical analysis

Phytochemical analysis of the aqueous stem bark extract

of *B. buonopozense* revealed the presence of alkaloids, terpenoids, flavonoids, tannins, saponins, reducing sugars and sterols.

Acute toxicity test

There was no mortality observed at any of the doses of aqueous stem extract of *B. buonopozense* examined. The animals were alive, healthy and active during the observation period.

The oral acute toxicity test of the leaf extract was estimated to be greater than 5000 mg/kg in rats. Thus, the experimental doses orally used (50,100 and 200 mg/kg) were within safe margin.

Acetic acid-induced writhing test

Table 1 shows the assessed analgesic profile of *B. buonopozense* aqueous stem bark extract using acetic acid induced abdominal constriction test in mice. The stem bark extract at all doses used, exhibited significant ($P < 0.05$) analgesic activity that lasted until the end of the experiment. Interestingly, all doses of the stem bark extract had analgesic activity which is comparable to aspirin, the standard drug.

Tail immersion test

Table 2 shows the antinociceptive activity of the stem bark extract of *B. Buonopozense* assessed using the tail immersion test in mice. The stem bark extract at different post treatment times significantly ($P < 0.05$) protected the animals from thermal stimuli. Morphine produced greater protection when compared to the extract at all post treatment times.

Xylene-induced ear oedema test

Table 3 shows the anti-inflammatory profile of the stem bark extract of *B. buonopozense* assessed using the

Table 2. Effect of aqueous stem bark extract of *B. buonopozense* on thermal stimuli response in the tail immersion test in mice.

Treatment	Dose (mg/kg)	Pre-treatment			Post-treatment		
		0 min	30 min	60 min	90 min	120 min	100 min
Control	20 ml/kg	7.17±0.87	8.17±0.40	9.67±1.65	9.83±1.62	11.67±0.71	13.33±1.22
<i>B. buonopozense</i>	50	7.67±0.49	10.0± 0.45	12.33±1.17	13.17±1.10	16.00±0.86*	14.67±0.08
	100	8.17±0.60	12.67±1.09	13.50±0.72	15.17±1.30	18.83±1.05*	15.83±1.05
	200	8.33 ±0.67	13.67±0.92	15.83±0.79	16.50±0.85	21.50±0.62*	17.00±0.62
Morphine	10	8.67±0.50	17.67±0.33	19.67±.50	20.67±0.26	23.67±0.10*	23.50±1.09

Values are expressed as mean± SEM; *Significant at p<0.05 when compared to control.

Table 3. Effect of aqueous stem bark extract of *B. buonopozense* on xylene induced ear oedema in mice.

Treatment	Dose (mg/kg)	Weight of right ear (g)	Weight of left ear (g)	Increase in ear weight (g)	% inhibition
Control	20 ml/kg	0.073±0.00	0.031±0.00	0.042±0.00	-
<i>B. buonopozense</i>	50	0.050±0.00	0.029±0.00	0.021±0.00	50*
	100	0.038±0.0	0.025±0.00	0.013±0.00	69*
	200	0.034±0.00	0.023±0.00	0.011±0.00	74*
Dexamethasone	4	0.029±0.00	0.020±0.00	0.009±0.00	79*

Values are expressed as mean ±SEM; *Significant at p<0.05 when compared to control.

Table 4. Effect of aqueous stem bark extract of *B. buonopozense* on carrageenan-induced paw oedema in rats.

Treatment	Dose (mg/kg)	Paw oedema volume (ml) versus time (h)						
		0 h	0.5 h	1 h	2 h	3 h	4 h	5 h
Control	20 ml/kg	1.25±0.02	1.62±0.01	1.70±0.01	1.78±0.02	1.85±0.02	1.91±0.02	2.00±0.02
<i>B. buonopozense</i>	50	1.26±0.02	1.57±0.02	1.51±0.02	1.45±0.03	1.39±0.03	1.32±0.02	1.26±0.02*
	100	1.24±0.02	1.57±0.02	1.49±0.02	1.43±0.02	1.37±0.02	1.30±0.02	1.23±0.02*
	200	1.22±0.02	1.54±0.02	1.47±0.03	1.41±0.02	1.33±0.02	1.27±0.02	1.20±0.02*
Aspirin	150	1.19±0.03	1.57±0.02	1.49±0.02	1.39±0.02	1.30±0.03	1.23±0.02	1.15±0.02*

Values are expressed as mean ± SEM; *Significant at p<0.05 when compared to control.

xylene induced ear oedema test in mice. The extract at dose levels used, exerted significant (P<0.05) and dose dependently reduced the oedema induced through topical application of xylene to the mouse ear. The standard drug, dexamethasone (4 mg/kg) exhibited greater effect than the stem bark extract (Table 3).

Carrageenan-induced paw oedema test

Table 4 shows the anti-inflammatory activity of the aqueous stem bark extract of *B. buonopozense* against carrageenan induced paw oedema test in rats. The observed activity of the extract at both dose levels used

were significant (P<0.05) dose dependent. However, data suggest more inhibitory activity with standard drug (Aspirin), than the stem bark extract.

Yeast-induced pyrexia test

Table 5 shows antipyretic profile of the stem bark extract of *B. buonopozense* assessed using brewer's yeast induced pyrexia in rats. The stem bark extract significantly (P<0.05) and dose dependently decreased the anal temperature of rats in all dose levels used in the test. The standard drug (aspirin), showed more activity than the extract.

Table 5. Effect of aqueous stem bark extract of *B. buonopozense* on yeast induced pyrexia in rat (Rectal temperature °C).

Treatment	Dose (mg/kg)	0 h	24 h	1 h	2 h	3 h	4 h	5 h
Control	20 ml/kg	35.39±0.06	37.75±0.04	37.88±0.03	37.69±0.03	37.51±0.03	37.32±0.02	36.72±0.02
<i>B. buonopozene</i>	50	35.27±0.03	37.32±0.06	36.99±0.11	36.45±0.03	36.18±0.01	35.54±0.04	35.24±0.02*
<i>B. buonopozene</i>	100	35.29±0.03	37.33±0.02	36.55±0.02	36.23±0.01	35.68±0.04	35.45±0.04	35.19±0.02*
<i>B. buonopozene</i>	200	35.25±0.02	37.35±0.03	36.49±0.05	36.24±0.04	35.55±0.03	35.32±0.02	35.17±0.01*
Aspirin	150	35.24±0.03	37.25±0.03	36.48±0.03	36.20±0.02	35.67±0.00	35.41±0.2	35.15±0.02*

Values are expressed as mean ± SEM; *significant at p<0.05 when compared to control.

Table 6. Effect of aqueous stem bark extract of *B. buonopozense* on 2, 4 Dinitrophenol-induced pyrexia in rat (Rectal temperature °C).

Treatment	Dose (mg/kg)	0 h	0.5 h	1 h	2 h	3 h	4 h	5 h
Control	20 ml/kg	35.37±0.05	37.52±0.04	37.80±0.02	37.63±0.02	37.42±0.02	37.29±0.02	36.69±0.03
<i>B. buonopozene</i>	50	35.29±0.02	37.33±0.05	36.86±0.09	36.40±0.03	36.14±0.04	35.49±0.02	35.24±0.01*
<i>B. buonopozene</i>	100	35.29±0.03	37.31±0.02	36.40±0.04	36.19±0.03	35.58±0.05	35.37±0.02	35.18±0.01*
<i>B. buonopozene</i>	200	35.28±0.04	37.29±0.03	36.51±0.04	36.23±0.02	35.54±0.05	35.32±0.04	35.13±0.02*
Aspirin	150	35.23±0.02	37.30±0.02	36.47±0.02	36.15±0.01	35.55±0.03	35.30±0.02	35.10±0.02*

Values are expressed as mean ± SEM; *significant at p<0.05 when compared to control.

2, 4 Dinitrophenol-induced pyrexia test

Table 6 shows the effect of the stem bark extract of *B. buonopozense* on 2, 4 dinitrophenol induced pyrexia in rats. The extract was found to have significant (p<0.05) and dose dependent antipyretic activity when compared to control.

However, standard drug (aspirin) produced more activity than the extract.

DISCUSSION

This study on the stem bark extract of *B. buonopozense* was conducted to establish potential pharmacological properties of the plant based on claims of its use in traditional medicine by Chaza community in Northcentral, Nigeria. The analgesic activity was tested using the abdominal constriction and tail immersion tests. The acetic acid induced writhing test is normally used to study the peripheral analgesic effects of drugs (Chang et al., 2011). The observed abdominal constrictions in this study are due to irritation of the peritoneal cavity induced by administration acetic acid (Vogel and Vogel, 1997). Prolonged irritation causes an increase in the levels of peritoneal prostaglandins, and the increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Ganesh et al., 2008; Gawade, 2012).

However, the constituents of the aqueous stem bark extract of *B. buonopozense* reduced reasonably the duration of the writhing in each mouse and consequently

the effects on arachidonate release and metabolism (Nuhu et al., 2007). This analgesic activity which was dose dependent may indicate the involvement of peripheral pathway. Moreso, the obtained results showed that the stem bark extract is effective in blocking the analgesic effect caused by the chemical stimulus in the writhing test.

To further confirm the analgesic action of the stem bark extract, tail immersion test was assessed. The activity of the extract in tail immersion confirmed its analgesic effect. It is well known that centrally acting analgesic agents elevate the pain threshold of mice towards heat and pressure (Akindele and Adeyemi, 2006). Significant activity in tail immersion test indicates involvement of central analgesic mechanism.

Topical application of xylene causes instant irritation of the mouse ear thereby leading to oedema formation and increase in myeloperoxidase enzymatic activity (Ravelo-Calzado et al., 2011). Such inflammation activity has been associated with increase in cystolic prostaglandin E₂, a potent vasodilator which synergizes with other inflammatory such as bradykinin and histamine which contributes to the redness and increased blood flow in areas of acute inflammation (Foyet et al., 2011). Agents with potential to inhibit effects associated with inflammation are considered to possess anti-inflammatory property. However, inflammation induction using xylene is considered to be associated with the role of phospholipase A₂ (PLA₂). Dexamethasone was used as the reference drug since the xylene induced ear oedema is less sensitive to non-steroidal anti-inflammatory drugs than steroidal anti-inflammatory

agents (Zaninir et al., 1992). A similar oedema inhibition observed, suggest the possible action of the stem bark extract with PLA₂ in its anti-inflammatory pathway.

The carrageenan induced rat paw oedema test which measures the ability of an agent to reduce local oedema induced in the rat paw by injection of an irritant agent such as carrageenan, has been widely used for screening for new anti-inflammatory drugs (Zakaria et al., 2008). The aqueous stem bark extract of *B. buopozense* caused marked inhibition at the early phase of inflammation indicating activity probably on histamine, serotonin and kinnins which are involved in the early stage of carrageenan induced oedema (Vane and Botting, 1987). The extract also reduced late phase of the oedema perhaps by inhibiting prostaglandin which is known to mediate the second phase of carrageenan induced inflammation (Necas and Bartosikova, 2013). The results obtained showed significant anti-inflammatory activity in the stem bark extract. This finding has scientifically confirmed the folklore use of *B. buopozense* in the treatment of ulcers in Nigeria (Nwagba et al., 2013).

The stem bark extract of *B. buopozense* was also found to possess antipyretic activity when assayed using the brewer's yeast and 2, 4 dinitrophenol induced pyrexia tests. It is well known that pyretic activity involves stimulation of the region in the hypothalamus, which controls body temperature through prostaglandins synthesized within the central nervous system (Uzcátegui et al., 2004) and the blood brain barrier prevents drug substances from having access to the central nervous system (CNS). The ability to cross the blood brain barrier may be one of the factors contributing to the antipyretic activity, and could also explain the observed central analgesic activity of the aqueous stem bark extract of *B. buopozense*. This finding is also in agreement with our earlier study of the methanolic leaf extract of *B. buonopozense* (Akuodor et al., 2011a).

The therapeutic importance of medicinal plants is often attributed to the combination of their active constituents. Different flavonoids isolated from medicinal plants have shown remarkable antipyretic, analgesic and anti-inflammatory activities (Sawadogo et al., 2006; Larkins and Wynn, 2004). The observed antipyretic, the analgesic and anti-inflammatory effects could be attributed to its flavonoids constituent, and this finding supported our recent observation on the stem bark extract of *B. buopozense*. The safety of the stem bark extract when taken orally is justified by the fact that oral administration of 5000 mg/kg in rats did not produce any mortality and visible toxic signs.

Conclusion

The present study indicates that *B. buonopozense* stem bark extract has peripheral and central analgesic, anti-inflammatory and antipyretic activities, thus confirming its

traditional use for the treatment of pain, inflammation and feverish conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of ethanolic extract of *Morinda citrifolia* Linn for antitumor activity

Gabriela Cavalcante da Silva*, Gibbelly Cavalcante da Silva, Waneska Alves Pereira da Silva, Alisson Macário de Oliveira, Matheus da Silva Mesquita, Maria da Conceição Cavalcanti de Lira, Evandro Valentim da Silva and Ivone Antônia de Souza

Laboratório de Farmacologia e Cancerologia Experimental, Centro de Ciências Biológicas, Departamento de Antibióticos, Universidade Federal de Pernambuco – UFPE, Recife-PE, Brazil.

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Given the concern about to the high incidence of cancer and low specificity of available antineoplastic drugs, *Morinda citrifolia* Linn is a tropical plant species with wide distribution, which had its consumption widely spread both because it is a source of nutrients and its phytotherapeutic properties as well as for its adaptation. The potential of tumor inhibition was done in vivo against Ehrlich Carcinoma and Sarcoma 180, a sharp reduction of weight of the tumors was obtained in comparison with the control group. The inhibition of tumor growth compared to Ehrlich carcinoma was more prominent in the dose 200 mg/kg/ip with inhibition percentage of 83.91%, on the other hand against Sarcoma 180. The greatest potential inhibition was detected at the dose of 100 mg/kg/ip with a percentage of 85.86%. Measurements of biochemical and hematological markers did not suffer drastic changes. Thus, the ethanol extract of *M. citrifolia* Linn showed promising antitumor effects towards the used varieties.

Key words: Bioactivity, antitumor, *Morinda citrifolia* Linn, Sarcoma 180, ehrlich carcinoma.

INTRODUCTION

Considered a problem of public health by 2030, the global burden will be 21.4 million new cancer cases and 13.2 million cancer deaths as a result of growth and population aging, the reduction in infant mortality and deaths from infectious diseases in development countries (Inca, 2013).

The use of plants with medical purposes is common, mainly in populations with low acquisition power, due to its accessible price. *M. citrifolia* Linn. is a tropical plant belonging to the family of Rubiaceae, it is characterized for being a brush of low to medium size (3 to 10 m tall)

and is largely distributed between the tropics, although it is native from southwest of Asia and Australia, and it has a history of use not only nutritional but also to promote health (Chan-blanco et al., 2006).

The content, composition and post harvest processes adopted in the preparation of *M. citrifolia* L juice, suffer great degree of variability (West et al., 2006). A commercial source *M. citrifolia* L juice, originated from French Polynesia, where it has been used as food and for medicinal purposes for over 2000 years; was approved as food by the European Union, European

*Corresponding author. E-mail: gcavalcante1988@gmail.com. Tel: +55 81 96504620.

Commission, 2003. For *M. citrifolia* L species has been assigned properties as antimicrobial, antipsychotic, antitumor, vasodilatory, antioxidant, among others (Hirazumi and Furusawa, 1999; Correia, 2011, Pandey et al., 2012; Brett et al., 2012; Ma et al., 2013).

Considering the fact that the location at which the plant grows is decisive for the presence and concentration of metabolites which are responsible for their biological properties and the high adaptability of said species to various soil types and climatic regions, it is important to evaluate the potential tumor inhibition of the fruit of *M. citrifolia* L.

MATERIALS AND METHODS

Collection and extraction of plant

The fresh fruits of *M. citrifolia* Linn were collected in February, 2014 in the city of Bezerros, Pernambuco, Brazil -8.24154246 latitude, longitude -35.76054788, and elevation 501 m. The voucher specimen of plant species has been identified and deposited in the herbarium Geraldo Mariz of the Federal University of Pernambuco under number 78155. After rinsing in sterile water and drying in an oven at 40°C temperature with circulation and air exchange for seven days; the fruits were sliced and seeds were extracted. The ethanolic extract of *M. citrifolia* L. (EEMC) was obtained from turbólise 500 g fruit in 1000 ml of ethanol at room temperature, which was filtered after 7 days for extracting plant wastes, such filtrate was concentrated on rotaevaporator and stored in the refrigerator at 4°C.

Antitumor activity animals

In order to investigate antitumor activity, albino male Swiss mice were used (*Mus musculus*), with approximately 60 days of age and weighing between 30 and 40 g. They were divided into six groups. These animals were kept under controlled lighting conditions (light / dark cycle of 12 h each) and temperature of 25±3°C in polypropylene cages. They received specific food and water *ad libitum*. The experimental protocol was approved by the Ethics Committee on Animal Experimentation (CEEAA) of the Biological Sciences Center of Universidade Federal de Pernambuco (UFPE), process number 23076.039925/2014-76.

Implantation of the tumor mass

Tumor cells (Ehrlich carcinoma and sarcoma 180) were removed from the donor animal by aspiration of ascitic form and introduced into recipient animals by subcutaneous route in subaxilar region, in a concentration of 25 x 10⁶ cells/ml (Stock et al., 1955; Koniya and Funayama, 1992). Forty-eight hours after the implant, the animals were divided into groups (n = 6) and treated for 7 days with; Cc: saline solution 0.9%/vo, Cp: Methotrexate 10 mg/kg/ip; C1: EEMC 100mg/ kg/vo, C2: EEMC 200 mg/kg/vo; C3: EEMC 100mg/kg/ip and C4: 200 mg/kg/ip to test the Ehrlich carcinoma and Sc: 0.9% saline/vo; Sp: 5- Fluoracil 25 mg/kg/ip; S1: EEMC 100mg/kg/vo; S2: EEMC 200 mg/kg/vo; S3: EEMC 100mg/kg/ip and S4: 200 mg/kg/ip) for the Sarcoma 180 line.

After treatment, the animals were weighed; blood samples were collected by cardiac puncture after anesthesia. They were then divided into two tubes, being one with EDTA for hematological analysis of erythrocytes, leukocytes and hemoglobin and blood indexes, such as: mean corpuscular volume (MCV), mean

corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). The other included a separating gel, which was centrifuged for 10 min at 3500 rpm to obtain serum for biochemical analyzes such as: urea, creatinine and transaminases. Afterwards, the animals were sacrificed in a chamber of CO₂ and tumors excised, weighed and the percentage of tumor inhibition calculated according to the equation:

Where:

$$TWI\% = \frac{C - T.100}{C}$$

TWI% = % of tumor inhibition

C = control group average tumor weight; T = test group average tumor weight.

Assays were performed as adapted from the method described by Geran et al. (1972).

Statistical analysis

The mean and standard deviation were evaluated by analysis of variance (ANOVA) by the test "t" by Student, being considered significant the values for p <0.05 (Morettin, 2010).

RESULTS AND DISCUSSION

The search for selective agents becomes important due to the non- specificity of various chemotherapeutics, which eventually promote damage to healthy cells as well as those which often can not reach the hypoxic cells in solid tumors. This possibly explains why the search for complementary therapies, by the patients with neoplasms, such as herbal medicines which act as adjuncts to primary treatment (Santos and Elisabetsky, 1999; Cassileth and Deng, 2004; Almeida et. al., 2005). Approximately 69% of the approved antitumor drugs were either natural products or were developed based on popular knowledge between the years 1989 and 2002 (Feng et al., 2011). The methotrexate and 5-fluorouracil here used as a standard drug for Ehrlich's carcinoma and Sarcoma 180 lines respectively have an important role in the therapy of certain cancers.

For some time the use of natural products as anticancer agents has been in process of growth, incorporating allopathic medicine, since some of the drugs currently used in cancer chemotherapy have been isolated from plant species. Therefore, the natural products research is still a successful strategy in the search for new drugs for anticancer therapy (Costa lotufo et al., 2005; Bezerra et al., 2008). Both the Sarcoma 180 and Ehrlich carcinoma are strains of murine tumors widely used in research of in vivo anti-tumor activity (Lee et al., 2003; Kintzios, 2004).

The weight of the tumors and the relation between tumor weight and the average weight of the animals (PT / PA) with Ehrlich's carcinoma showed a statistically significant decrease in the groups C2, C3 and C4 in contrast with CC group (Table 1). Considering the animals with

Table 1. Means and standard error of tumor weights and animal weights, including PT/PA ratio according to treatment groups with animals with Ehrlich Carcinoma.

Group	PT	PA	PT/PA
Cc	1.827±0.147 ^a	39.519±0.982	0.046±0.004 ^a
Cp	0.148±0.019 ^b	37.1±0.987	0.003±0.0004 ^b
C1	1.163±0.371 ^a	40.915±0.682	0.028±0.009 ^a
C2	0.510±0.095 ^b	38.767±1.605	0.013±0.003 ^b
C3	0.358±0.078 ^b	40.038±1.334	0.009±0.002 ^b
C4	0.294±0.209 ^b	37.054±2.053	0.009±0.006 ^b
p-value ¹	0.000*	0.268	0.000*

Cc: Saline solution, Cp: methotrexate 10 mg/kg/ip; C1: EEMC 100 mg/kg/vo, C2: EEMC 200 mg/kg/vo; C3: EEMC 100 mg/kg/ip and C4: 200 mg/kg/ip (n=6/groups) 1-P-value of ANOVA; * Statistically significant; Values in the same line followed by the same lowercase letter are not statistically different (p>0.05) Tukey Test

Table 2. Means and standard error of tumor weights and animal weights, including PT/PA ratio according to treatment groups with animals with Sarcoma.

Group	PT	PA	PT/PA
Sc	3.565±0.526a	46.917±1.336a	0.078±0.013a
Sp	0.4966±0.017b	39.63±0.40b	0.012±0.0005b
S1	0.924±0.269b	41.267±1.825b	0.022±0.006b
S2	0.683±0.166b	43.421±1.242b	0.016±0.004b
S3	0.504±0.123b	43.219±1.036b	0.012±0.003b
S4	0.695±0.268b	45.400±0.756b	0.015±0.006b
p-value ¹	0.000***	0.012***	0.000***

Sc: Solução salina, Sp: 5-Fluoracil 25 mg/kg/ip; S1: EEMC 100mg/kg/vo, S2: EEMC; 200mg/kg/vo; S3: EEMC 100mg/kg/ip and S4: 200 mg/kg/ip (n=6/groups). 1-P-value of ANOVA; * Statistically significant; Values in the same line followed by the same lowercase letter are not statistically different (p>0,05) Tukey Test

Sarcoma 180, all treatment groups demonstrated a significant decrease in contrast with Sc group, with respect to the weight of the tumors and the relation between tumor weight and the average weight of the animals (TP / BP), shown in Table 2.

According to Figure 1, the largest percentage of inhibition for Ehrlich carcinoma was 83.91% in group C4 with a dose of 200 mg/kg/ip, compared with the Cc group. The group C3 at a dose of 100 mg/kg/ip presented TW% of 80.41%, while the C1 group at a dose of 100 mg/kg/vo was able to inhibit only 36.34% of the tumor growth. According to Figure 2, the highest percentage inhibition for Sarcoma 180 was 85.86% in S3 group, at a 100 mg/kg/ip dose, compared with the control group. Group S4 showed TW% of 80.50%. Group S2 showed TW% of 80.84%, while the group S1 in the 100 mg/kg/vo dose was able to inhibit by 74.08% the tumor growth.

The toxicity in patients receiving chemotherapy is measured mainly by evaluating the complete blood count and tests of hepatic and renal functions. The liver and kidney are both the most important organs of

detoxification and excretion and very sensitive to chemotherapeutic drugs (Bezerra et al., 2008). With liver damage, hepatocytes can overflow in quantities greater than cytoplasmic enzymes such as AST aspartate aminotransferase and ALT alanine aminotransferase (Henry, 2008).

Amongst the hematological and biochemical assessments, in the antitumor testing of Ehrlich Carcinoma, the AST parameter showed a significant increase in the C1 and C2 groups compared to the control, which was reflected in the ALT parameter averaging 178.0±9.00 and 189.50±5.50 for the C1 and C2 groups, respectively (Table 3).

Regarding the biochemical and hematological results of animals with Sarcoma 180, the VCM measures showed no statistical difference from the Sc group. On the other hand, the groups S2, S3 and S4 significantly decreased their values compared to the Sp group (p = 0.037). The S1 and S2 groups showed a significant decrease in HCM index (p = 0.012) compared to the Sc group, averaging 15.83±0.24 and 14.97±0.30, respectively (Table 4).

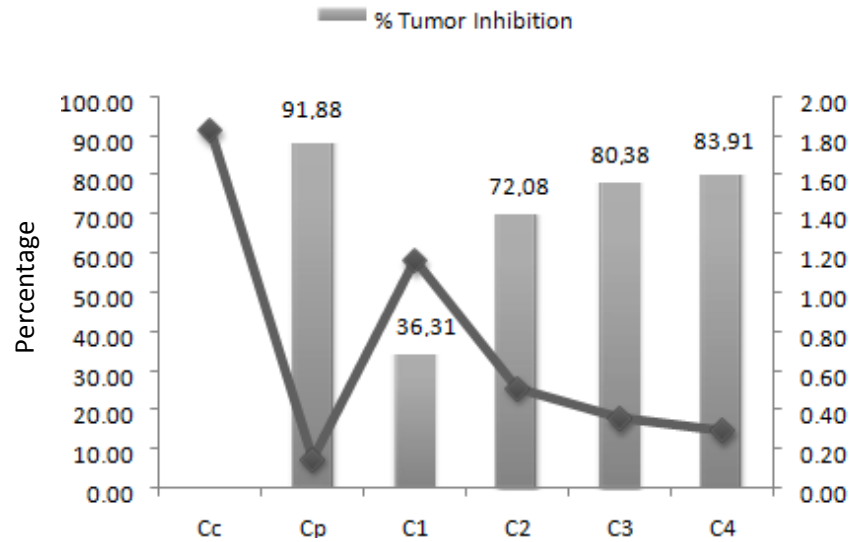


Figure 1. Comparison of the percentages of tumor Inhibition (%) and tumor weights (g) in animals with Ehrlich Carcinoma, treated with Cc: Saline solution, Cp: Methotrexate 10 mg/kg/ip; C1: EEMC 100 mg/kg/vo, C2: EEMC 200 mg/kg/vo; C3: EEMC 100 mg/kg/ip and C4: 200 mg/kg/ip (n=6/groups), respectively.

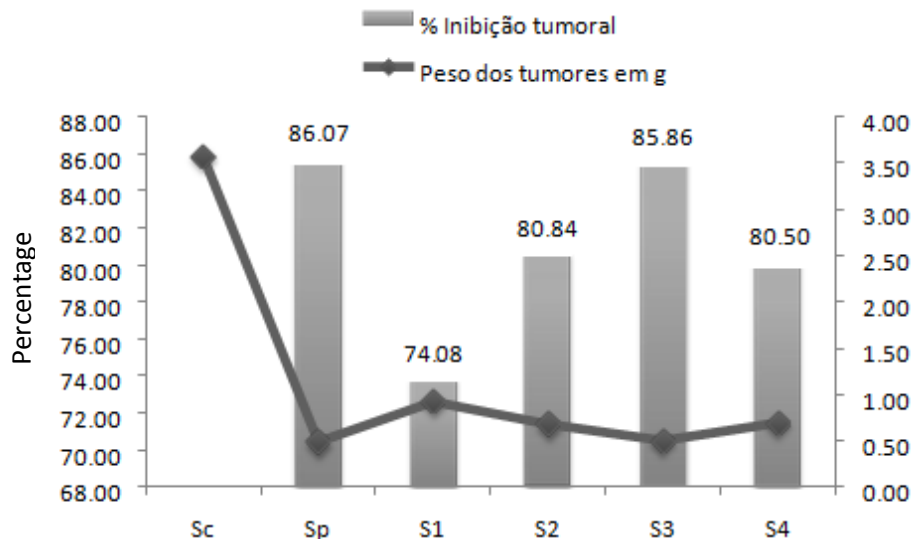


Figure 2. Comparison of the percentages of tumor inhibition (TW%) and tumor weights in animals with Sarcoma 180, treated with Sc: Saline solution, Sp: 5-Fluoracil 25 mg/kg/ip; S1: EEMC 100 mg/kg/vo, S2: EEMC 200 mg/kg/vo; S3: EEMC 100 mg/kg/ip and S4: 200 mg/kg/ip (n=6/groups), respectively.

With regard to the biochemical and hematological parameters in evaluation against Ehrlich carcinoma, there was a significant increase of AST in groups C1 and C2, orally administered, and in the C4 group, which accompanied a significant increase of ALT only in C1 and C2 groups. This suggests a possible liver overload principally with the treatment by the oral route. On the

other hand, the acceptable range of urea and creatinine in all treated groups, gives evidence that renal function did not change. In individuals with Sarcoma 180, biochemical markers of liver and kidney function showed no significant changes in the treated groups compared to the control, thus not influenced by therapy.

Among the blood abnormalities of patients with

Table 3. Means and standard error of biochemical and hematological results of animals with Ehrlich carcinoma according to the treatment groups.

Variable	Cc	Cp	Groups				p value ⁻¹
			C1	C2	C3	C4	
Urea	35.35±4.35	38.30±0.40	35.40±2.40	59.50±5.50	50.50±2.50	56.5±18.50	0.595
Creatinine	1,70±0.20	1.87±0.08	1.20±0.30	2.20±0.30	1.25± 0.25	2.35±0.85	0.361
AST	138.0±3.90 ^a	21.5±3.5 ^a	282±6.00 ^b	370.50±5.50 ^b	246±8.00 ^a	88±10.0 ^b	0.000
ALT	14.85±9.15 ^a	9.75±1.95 ^a	78.0±9.00 ^b	189.50±5.50 ^b	84.00±3.00 ^a	02.0±5.0 ^a	0.000
RBC	7.55±0.45	8.23±0.24	9.45±2.02	9.10±0.30	8.06±0.19	6.06±0.71	0.252
Ht	42.00±2.00	44.50±1.40	52.00±12.00	47.65±0.35	40.30±0.70	31.50±2.50	0.232
Hg	13.60±1.00	15.55±0.75	8.65±4.75	13.05±0.35	14.20±0.40	10.45±0.45	0.291
VCM	55.67±0.66	54.03±0.09	54.78±1.02	52.43±2.11	50.04±0.28	52.21±1.99	0.147
HCM	18.00±0.26	18.87±0.35	10.71±7.31	21.12±5.90	17.63±0.09	17.40±1.30	0.815
CHCM	32.34±0.84	34.93±0.59	19,80±13.71	27.38±0.53	35.23±0.38	33.27±1.22	0.440
Leukocytes	10.80±6.80	8.60±1.00	6.40±3.70	6.70±0.60	8.50±2.10	4.10±0.10	0.778

Cc: Saline solution, Cp: Methotrexate 10 mg/kg/ip; C1: EEMC 100 mg/kg/vo, C2: EEMC 200 mg/kg/vo; C3: EEMC 100 mg/kg/ip and C4: 200 mg/kg/ip (n=6/groups); 1-P-value of ANOVA; * Statistically significant; Values in the same line followed by the same lowercase letters are not statistically different (p>0.05) Tukey test.

Table 4. Means and standard error of biochemical and hematological results of animals with Sarcoma 180 according to treatment groups.

Variable	Groups						p-value
	Sc	Sp	S1	S2	S3	S4	
Urea	57.33±3.18	61.96±0.99	53.12±1.02	62.67±4.10	59.50±0.50	59.50±0.28	0.062
Creatinine	1.30±0.35	1.41±0.01	3.53±1.12	1.47±0.38	1.50±0.50	1.30±0.11	0.153
AST	320.00±86.93	271.66±10.37	353.00 ±96.88	262.67±64.67	431.00±25.00	176.00 ±9.23	0.332
ALT	122.33±63.36	107.3±1.55	106.75±30.59	86.00±18.04	165.00 ±21.00	55.00±4.61	0.435
RBC	5.40±0.22	6.07±0.07	5.80±0.77	6.63±0.22	6.29±1.13	6.85±0.68	0.634
Ht	31.40±0.30	35.03±0.82	31.30±4.70	34.70±1.56	33.73±5.67	36.93±3.64	0.833
Hg	9.50±0.37	10.56 ±0.40	9.20±1.25	9.93±0.22	9.86±1.75	10.60±1.05	0.897
VCM	57.95±2.42	57.67± 0.94 ^a	53.73±1.22	52.30 ±0.70 ^b	53.83±0. ^{78b}	53.90±0.46 ^b	0.037
HCM	17.59±0.28 ^a	17.41±0.74 ^a	15.83±0.24 ^b	14.97±0.30 ^b	15.63±0.22 ^a	15.43±0.15 ^a	0.012
CHCM	30.28±1.47	30.17 ±1.06	29.47±0.55	28.63±0.93	29.07±0.47	28.67±0.07	0.656
Leukocytes	4.77±0.23	3.87 ± 0.20	7.97±1.82	12.47±1.30	6.47±2.70	9.90±3.37	0.077

Sc: Saline solution, Sp: 5-Fluoracil 25 mg/kg/ip; S1: EEMC 100mg/kg/vo, S2: EEMC 200 mg/kg/vo; S3: EEMC 100 mg/kg/ip and S4: 200 mg/kg/ip (n=6/groups), respectively. 1-P-value of ANOVA; * Statistically significant; Values in the same line followed by the same lowercase letters are not statistically different (p>0,05) Tukey test.

neoplasias, a decrease in the levels of erythrocytes and hemoglobin can be mentioned, which may be the result of immunological reactions, suppression of hematopoiesis induced by the treatment, inadequate production of erythropoietin by the kidney and bone marrow intrinsic suppression (Zuckerman, 1998).

The negative influence of the tumor can be compensated by the presence of effective treatment, not producing toxicity in blood cells and hematopoiesis as a whole. Erythrogram patterns of animals with Ehrlich carcinoma, for example, the concentration of red blood cells and hemoglobin maintained the levels in relation to

the Cc control group, which reflects that these parameters were not affected. In the case of the white blood cell count, although not statistically significant, there was a higher leucopenia in treated groups compared to the control group, which provides greater vulnerability of animals in terms of immune competence.

Animals with Sarcoma 180 showed slight increase of erythrocyte number in the groups treated in relation to control. Even though it is not statistically significant, this fact can explain the statistically significant decrease in HCM parameter in the S1 and S2 groups if compared to Sc control, and VCM parameter in groups S2, S3 and S4

in relation to Sp group. In a different way to animals with Ehrlich carcinoma, those with Sarcoma 180, showed leukocytosis compared to the control group, which may suggest an improvement of immune competence.

The presence of tumor mass is a factor of stimulus for leukocytes proliferation (Sato et al., 2005; Lins et al., 2009). Another disadvantage of conventional chemotherapeutic treatments is that they induce leukopenia, which consequently makes the patient more vulnerable to infection. Examples of possible causes can be cited involution of organs such as the spleen and thymus (Bezerra et al., 2008). The weights of the tumors showed a decrease depending on the dose in relation to Ehrlich carcinoma line, both orally and intraperitoneally in the control group, which was statistically significant in groups C2, C3 and C4. The rate of tumor inhibition was more significant in the C4 group: 200 mg/kg/ip represented TW% 83.91%, which reflects considerable inhibition compared to the standard group Cp: Methotrexate 10 mg/kg/ip, which had TW% 91.88%.

Singh et al. (2013) performing an evaluation of Ehrlich carcinoma strain also found encouraging results using commercial freeze-dried product of *M. citrifolia* L. at doses of 250 and 500 mg/kg for 14 days. It was observed a decrease in weight and volume of the treated animals tumours, also managed to restore the levels of hemoglobin and red blood cells compared to the control, providing protective action of the hematopoietic system. Despite a longer treatment, the result agrees with the present study in terms to the weight of tumors. Taşkin et al. (2009) using an essay with cells of anti-proliferative ascites of Ehrlich carcinoma concluded that the inhibition is due to both the suppression of the proliferation and activation of apoptosis.

Regarding Sarcoma 180 strain, all groups had tumor weights which statistically decreased in relation to the control group. Nevertheless, the intraperitoneal route did not show a dose dependent relationship, both in the weight of tumors and in TW% tumor inhibition, once group S3: EEMC 100mg/kg/ip had a TW% of 85.86% and group S4: 200 mg/kg/ip had TW% of 80.50%. These results were very promising, since TW% of group Sp: 5-Fluoracil 25 mg/kg/ip was 86.07%.

Hirazumi and Furusawa (1999), Furusawa et al. (2003) found promising results in the treatment of ascitic Sarcoma, such as increased life time and cure rate, by combining a polysaccharide fraction of *M. citrifolia* L. with some immunomodulators such as interleukins 4 and 10, interferon gamma (IFN- γ) and chemotherapeutic drugs such as etoposide and vimcristine and 5-FU. Isolated compounds of *M. citrifolia* L. as Damnacanthal, Alizarin and morindone showed activity against the lung cancer and sarcoma (Patil et al., 2013).

Alkaloids, flavonoids and their derivatives found in most plant families are constantly associated to anti-tumor properties, as well as tannins, which are phenolic compounds, are also related to the prevention. Other classes cited as promising in anticancer therapy are the

coumarins, anthraquinones, triterpenes and essential oils (Patil et al., 2013).

Some of these compounds act primarily on suppression of inflammatory response left by transformation, hyperproliferation and initiation of the carcinogenesis process, which can lead to the suppression of final stages as angiogenesis and metastasis (Bhanot et al., 2011), are found in the classes as EEMC flavonoids, tannins, triterpenes and coumarins.

Conclusion

Treatment with EEMC has shown to be effective for the inhibition of tumor growth in animals with Ehrlich Carcinoma and Sarcoma 180. It is therefore necessary for the realization of tests to elucidate the possible mechanism of inhibition, and also more evaluation of cost concerning the monitoring of rates of kidney functions in Carcinoma lineage is important.

Conflict of interests

The authors have not declared any conflict of interests.

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