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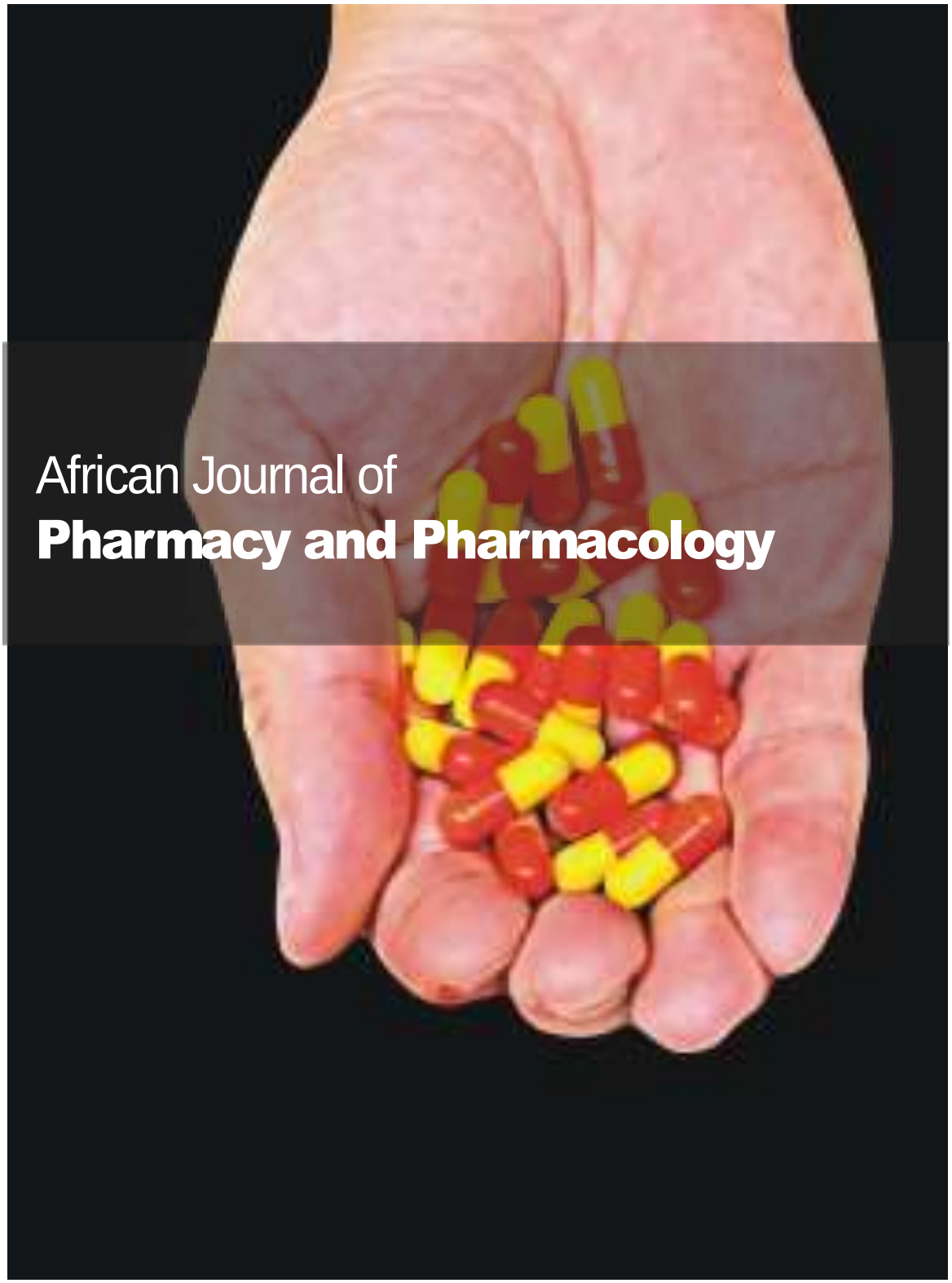

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Full Length Research Paper

Antibacterial activity of leaf and leaf callus extracts of *Anisochilus carnosus* (L) Wall.

Nissar Ahmad Reshi[1*], Sudarshana Mysore Shankarasingh[2] and Girish Vasanaika Hodiyala[2]

[1]Department of Botany, School of Science, Sandip University, Nashik, Maharashtra, India
[2]Department of Studies in Botany, University of Mysore, Mysuru, Karnataka, India.

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The present study was carried out to determine the antibacterial efficacy of chloroform, petroleum ether, ethyl acetate, methanol, ethanol and aqueous extracts of leaf and leaf derived callus of *Anisochilus carnosus* against *Bacillus cereus, Bacillus subtiltis, Staphylococcus aureus, Streptococcus pyogenes, Vibrio parahaemolytics, Enterobater aerogenes, Proteus mirabilis,* and *Klebseilla pneumoniae.* The leaf segments were cut into small pieces of size 1 sq.cm and were cultured on Murashige and Skoog solid medium supplemented with different auxins alone and in combination. Antibacterial efficacy was performed by disc diffusion method followed by minimum inhibitory concentration (MIC) determination by two fold serial dilution method. Leaf and leaf callus extracts were subjected to the qualitative phytochemical analysis. Maximum callus formation percentage was obtained from the leaf segments cultured on MS medium supplemented with 2, 4-D (3 mg/l). Methanolic leaf extract showed maximum antibacterial activity with 26 mm zone of inhibition against *K. pneumonia* with MIC value of 0.64 mg/ml. Out of the callus extracts, ethanolic callus extract showed the maximum bioefficacy against *K. pneumonia* with 20 mm zone of inhibition and MIC value of 1.25 mg/ml. Results revealed that both leaf and leaf derived callus are effective against *K. pneumoniae*. The bioefficacy study confirms the antibacterial potential of leaf and leaf derived callus of *A. carnosus*. The study paves a way for the production of secondary metabolites by establishing high potential cell lines of callus tissues.

Key words: Leaf, leaf callus, antibacterial efficacy, minimum inhibitory concentration (MIC), *Anisochilus carnosus.*

INTRODUCTION

Plants as a source of medicine have been inherited and form the backbone of health care system. From last few decades, there has been surge in the use of natural remedies and now a days traditional medicinal plants have become the focus of study in terms of validation of their traditional uses. Popular observation and scientific validation of medicinal plants will significantly contribute to the development of natural and novel drug system. In India, medicinal plants constitute 50% of the higher flowering plant species (Johnson et al., 2011). Till

*Corresponding author. E-mail: nissarreshi@gmail.com.

date, approximately 20% of the medicinal plants have been subjected to the different biological assays (Suffredini et al., 2004). Pharmaceutical industries are spending a considerable time and money in developing natural product based therapeutics. Number of efforts has been made to discover new antimicrobials of plant origin. Despite the advancement in science and technology infectious diseases claim millions of lives annually, especially in developing countries (York et al., 2011). The treatment of infectious diseases faces a serious issue of development of resistance by microbes against available antibiotics, which has necessitated a continuous search for the new antimicrobials of plant origin (Ghalib et al., 2012). Today, multi-drug resistant bacteria like methicillin resistant *Staphylococcus aureus* (MRSA), vancomysin resistant enterococci (VRA), *Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa* pose a serious threat to the human health. There is also an emergence of bacterial pathogens with intrinsic resistance to the available antibacterial agents thus making them obsolete (Tenover, 2006). A proper approach needs to be adopted to counter this menace of antibiotic resistance. The search for new antimicrobials of plant origin could possibly counter the infectious disease threat and may lead to the discovery of natural and novel drug. Plants are a rich source of antimicrobials and play an important role in the process of drug discovery and development (McGaw et al., 2008). In contrast to the synthetic antimicrobials, plant origin antibiotics are devoid of any ill effects and possess high therapeutic potential to counter infectious diseases. Screening of callus extracts for biological activities paves a way for the mass production of biologically viable secondary metabolites and thus provides an alternative to the *in vivo* plant material thereby minimising any possible future threat to the natural habitat of medicinal plants. *Anisochilus carnosus* (L) Wall. is an annual herb, 30-60 cm tall, robust and branched with fleshy leaves. It grows in the regions of Hong Kong, Macao, Shangai, Tianjin, India, Myanmar and Sri Lanka. The plant has been traditionally used for the treatment of gastrointestinal disorders, respiratory disorders, cough, cold and fever (Kamble et al., 2008). Its popular herbal preparation together with *Ocimum basilicum*, *Mentha piperita* and *Alpinia galanga* is used against the symptoms of influenza, dermatitis and the slight illness that derives from the bites of bugs (Subramanian and Nair, 1972). Essential oils have been extracted by hydrodistillation from the leaves and have been reported to be antimicrobial in nature (Senatore et al., 2003). The plant has been reported to have anti-inflammatory activity (Grover et al., 2001), antiulcer activity (Mohammed et al., 2008), antifungal property (Kulandhaivel et al., 2011) and anticancer property (Muthuraman et al., 2012). In the present study, an attempt was made to screen the leaf and leaf callus extracts of *A. carnosus* for their antibacterial efficacy.

MATERIALS AND METHODS

Collection of plant material and callus induction

A. carnosus plants were collected from the Nilgiri Hills, Western Ghats of Tamil Nadu, India and are being maintained in the medicinal plant garden of Department of Studies in Botany, University of Mysore, Mysuru. Healthy leaf explants were collected from the mother plant and were washed under running tap water for ten minutes to remove the soil and dust particles followed by treatment with fungicide bavistin (1% w/v) for 5 min. Explants were then washed thrice with sterile double distilled water and were treated with mercuric chloride (0.01% w/v) for 5 min followed by washing thrice with sterile double distilled water. Inside laminar airflow, leaf explants were cut into small pieces (1 sq.cm) and were inoculated onto the MS medium containing 3% sucrose, 0.08% agar and fortified with different concentrations of auxins. Prior to autoclaving at 121°C for 15 min, pH of the medium was adjusted to 5.8 using 1N NaOH and 1N HCl. The culture flasks were maintained in the incubation chamber under a 16 h photoperiod at light intensity of 25 $\mu mol/s^2/m^2$ for 4 weeks. Each experiment was performed with five replicates and repeated thrice. The callus cultures were maintained for the period of five months and were periodically subcultured with 2-3 weeks of interval onto the fresh MS medium for callus proliferation. Consequently, the callus was harvested at the transfer age of 3 weeks, kept in hot air oven at 60°C for 24 h till a constant dry weight was obtained and the callus was then further exploited for extraction and antibacterial evaluation.

Dried leaves and the *in vitro* leaf derived callus was powdered using electric homogenizer. Aqueous extraction was carried out by mixing leaf and leaf callus coarse powder separately with deionised water in the ratio of 1:5 (w/v) in conical flask and allowed to settle in an oven at 50°C for 72 h with occasional shaking. After 72hrs the extracts were filtered using Whatman filter paper (Harborne, 1998). The filtrate was lyophilised to dryness and stored in vials at 5°C for further use. Solvent extraction was carried out by taking 25 g of dry leaf and callus coarse powder and filled in a thimble separately and extracted sequentially with 200 ml of petroleum ether, chloroform, ethyl acetate, ethanol and methanol in Soxhlet extractor for 48 h. The solvent extracts were concentrated under reduced pressure and were stored at 5°C in vials for further use.

The test bacteria; Gram-positive bacteria: *Bacillus cereus* (MTCC 430)*, Bacillus subtilis* (MTCC 441)*, Staphylococcus aureus* (MTCC 1144)*, Streptococcus pyogenes* (MTCC 422)*.* Gram-negative bacteria: *Enterobacter aerogenes* (MTCC 111)*, E. coli* (MTCC 1687)*, Proteus mirabilis*(ATCC 7002), *K. pneumoniae* (MTCC 7407) were procured from MTCC Chandigarh, India. All the test bacteria were freshly subcultured once in a week during the course of work on nutrient agar slants (Himedia) and were incubated at 37°C for 24hrs.From 24hrs old slant culture, a loop of inoculum was transferred to the 8 ml nutrient broth (Himedia) and incubated for 24hrs at 37°C which was used as fresh suspension culture. The turbidity of bacterial suspension cultures was adjusted by adopting barium sulphate ($BaSO_4$) turbidity standard which is equivalent to 0.5 McFarland standard (Doughari*,* 2007), which gives 10^6 CFU/ml bacteria (1×10^6 cell/ ml). Antibacterial activity of leaf and leaf callus extracts was screened by adopting disc diffusion assay (Anonymous, 1996; Hoffman et al., 2004). Sterilised 20 ml nutrient agar medium was transferred to the pre-autoclaved petriplates (90 mm) and allowed to solidify.50 µl of broth suspension bacterial culture (24hrs old) containing 10^6 CFU/ml (0.5 McFarland standard)

Table 1. Influence of auxins on callus induction from the leaf segments of *A. carnosus.*

MS medium+Plant Growth Regulator mg/l	Mean percentage of callus induction	MS medium+Plant Growth Regulator mg/l	Mean percentage of callus induction
2,4-D		**IAA**	
0.5	68	0.5	43
1	86	1	47
1.5	90	1.5	56
2	90	2	62
2.5	92	2.5	64
3	98	3	70
3.5	72	3.5	76
4	70	4	79
4.5	74	4.5	82
5	68	5	78
IBA	32	**NAA**	30
0.5		0.5	
1	42	1	36
1.5	58	1.5	42
2	58	2	46
2.5	62	2.5	53
3	68	3	58
3.5	72	3.5	60
4	65	4	63
4.5	60	4.5	68
5	62	5	68

*All treatments with 5 replicates and were repeated thrice. Callusing percentage: (Number of explants callused/Total number of explants inoculated) x 100.

was put on to the solidified nutrient agar and was spread evenly using sterile glass spreader. Four impregnated discs (6 mm) were placed on inoculated nutrient agar in each petriplate, (i) leaf extract impregnated disc (10 mg/disc), (ii) leaf callus extract impregnated disc (10 mg/disc), (iii) standard streptomycin/gentamycin antibiotic disc (10 mg/disc) as reference drug, (iv) solvent impregnated disc (100 µl) as solvent negative control. The plates were kept in refrigerator at 5°C for 20 min to let extracts and controls diffuse into the agar. The plates were then incubated for 24hrs at 37°C. Antibacterial activity was evaluated by measuring the diameter of zone of inhibition after 24hrs around each disc against the test bacteria. The experiment was repeated thrice and statistical analysis was carried out by using SPSS, DMRT with P≤0.5.

The microplate (96 well) method was used to determine the minimal inhibitory concentration of leaf and leaf callus extracts (Eloff, 1998). Initially 100 µl sterilised nutrient broth was put in each well of microplate. All the extracts (leaf and callus) were tested at 5 mg/ml and were serially diluted two fold to 1 µg/ml after which 100 µl of bacterial cultures (10^6 CFU/ml) were added to each well. The final volume in each well was 200 µl. In each assay, streptomycin was used as reference drug (1 mg/l to 1 µg/ml). Control wells were prepared with one containing culture medium only and the other with normal bacterial suspension culture. The contents of each well were mixed on a microplate shaker at 900 rpm for 1 min prior to incubation for 24hrs (Klancnik et al., 2010). After 24hrs, 20 µl TTC solution (2,3,5-triphenyl tetrazolium chloride) prepared in deionised water (20 mg/ml) was added to each well and incubated for 1 h in dark (Eloff, 1998; Johnson et al., 2011). The microbial growth in each well was determined by reading the respective absorbance at 600 nm by using Universal Micro Plate Reader. The qualitative phytochemical analysis of leaf and leaf callus extracts was carried out by the method described by Zhang et al. (2016).

RESULTS

Callus induction was observed along the cut edges of leaf segments after two weeks of inoculation on MS medium supplemented with auxins. Callus induction percentage varied with respect to the concentration of the growth regulator used and the age of the explant. Young explants cultured on MS medium augmented with 2,4-D (1-3 mg/L) showed high percentage of callus induction and proliferation. IBA and NAA supplemented medium induced hard and compact callus with low percentage of proliferation (Table 1). Antibacterial activity was carried out by Disc Diffusion Assay and the results revealed the antibacterial property of both *in vivo* leaf extracts and *in vitro* leaf callus extracts. Both leaf and leaf callus extracts inhibited the growth of test Gram-positive and Gram-negative bacteria. Out of the six leaf extracts (petroleum

Table 2. Antibacterial activity (zone of inhibition in mm) of leaf extracts of *A. carnosus.*

	Leaf extracts						
Pathogen	**Aqueous**	**Ethanol**	**Methanol**	**E. Acetate**	**Pt. Ether**	**Chloroform**	**Streptomycin**
B. cereus	-	-	15±0.45	4±0.65	5±0.54	-	40±0.55
B. subtilis	-	6±0.65	9±0.34	`18±0.19	-	-	35±0.65
S. pyogenes	-	18±0.32	-	-	-	-	38±0.43
S. aureus	15±0.65	2±0.78	17±0.45	10±0.65	3±0.54	-	35±0.23
E. coli	-	12±O.67	20±0.34	-	-	-	40±0.53
P. mirabilis	-	8±0.65	15±0.36	-	-	-	38±0.54
K. pneumoniae	-	7±0.76	26±0.56	-	2±0.23	-	35±0.56
E. aerogenes	-	13±0.54	-	-	-	-	35±0.76

*Each test was repeated thrice. Each value represents Mean±S.D, statistical analysis done by SPSS, DMRT (P≤0.5), Pt Ether: Petroleum Ether; E. Acetate: Ethyl Acetate.

Table 3. Antibacterial activity (zone of inhibition in mm) of leaf callus extracts of *A. carnosus.*

	Leaf callus extracts						
Pathogen	**Aqueous**	**Ethanol**	**Methanol**	**E. Acetate**	**Pt. Ether**	**Chloroform**	**Streptomycin**
B. cereus	-	3±0.27	10±0.45	-	-	-	40±0.55
B. subtilis	-	8±0.25	2±0.54	`	-	-	35±0.65
S.pyogens	-	10±0.15	-	-	-	-	38±0.43
S. aureus	3±0.42	6±0. 62	3±0.45	12±0.54	-	-	35±0.23
E. coli	-	-	--	-	-	-	40±0.53
P. mirabilis	-	2±0.48	14±0.67	-	-	-	38±0.54
K. pneumonia	-	20±0. 41	14±0.52	-	-	-	35±0.56
E. aerogenes	-	-	-	-	-	-	35±0.76

*Each test was repeated thrice. Each value represents Mean±S.D, statistical analysis done by SPSS, DMRT (P≤0.5).

ether, chloroform, ethyl acetate, ethanol, methanol and aqueous extracts), maximum zone of inhibition (26 mm) was reported against *K. pneumoniae* in methanol extract. Chloroform leaf extract did not inhibit growth of any test bacteria. Antibacterial activity and zone of inhibition of leaf extracts against test bacteria is given in Table 2.

Leaf callus extracts also showed significant zone of inhibition against both Gram-positive and Gram-negative test bacteria. Non polar solvent callus extracts, that is, chloroform and petroleum ether did not show any bacterial growth inhibition against any test bacteria. The maximum zone of inhibition was reported in ethanolic callus extract against *K. pneumoniae.* The zone of inhibition of different leaf callus extracts against different test bacteria is given in Table 3. Disc diffusion method is the preliminary screening method for the assessment of the antibacterial property. The extracts (both leaf and leaf callus) which inhibited the growth of test pathogens as revealed by disc diffusion method were subjected to minimal inhibitory concentration (MIC) determination which provides quantitative data of minimum concentration of active extract which inhibits the bacterial growth. The MIC values of active leaf and leaf callus extracts was determined by two fold serial dilution microplate method. The MIC values of leaf and leaf callus extracts is presented in Tables 4 and 5 respectively.

DISCUSSION

The study was aimed to evaluate the antibacterial property of leaf and leaf callus extracts of *A. carnosus.* Effect of auxins on callus induction has been reported earlier by many tissue culturists (Rout et al., 1999; Jain et al., 2004; Shariff et al., 2006). In the present study, 2,4-D supplemented MS medium was found to induce maximum callusing in leaf segments. Our results are directly in concurrence with the earlier findings reported by Rout et al. (1999) and Manickam et al. (2000) in *Plumbago zeylanica* and *Withania somnifera* respectively.

In the present study, leaf and leaf callus extracts of *A. carnosus* inhibited the growth of both Gram-negative and

Table 4. Minimal inhibitory concentration (mg/ml) of leaf extracts of *A. carnosus* against test bacteria.

Extracts	Test bacteria							
	B. cereus	***B. subtilis***	***S. pyogenes***	***S. aureus***	***E. coli***	***P. mirabilis***	***K. pneumoniae***	***E. aerogenes***
Aqueous	-	-	-	2.50	-	-	-	-
Ethanol	-	2.50	1.25	2.50	1.25	2.50	2.50	1.25
Methanol	0.62	1.25	-	0.62	0.62	1.25	0.62	
Ethyl Acetate	2.50	1.25	-	1.25	-	-	-	-
Pt. Ether	5	-	-	2.50	-	-	2.50	-
Chloroform	-	-	-	-	-	-	-	-
Streptomycin	0.062	0.062	0.062	0.12	0.25	0.25	0.50	0.25

*Each test was repeated thrice. Each value represents Mean±S.D, statistical analysis done by SPSS, DMRT.

Table 5. Minimal inhibitory concentration (mg/ml) of leaf callus extracts of A. carnosus against test bacteria.

Extracts	Test bacteria							
	B. cereus	***B. subtilis***	***S. pyogenes***	***S. aureus***	***E. coli***	***P. mirabilis***	***K. pneumoniae***	***E. aerogenes***
Aqueous	-	-	-	1.25	-	-	-	-
Ethanol	5	2.50	1.25	2.50	-	2.50	1.25	-
Methanol	2.5	1.25	-	5	-	0.62	0.62	-
Ethyl Acetate	-	-	-	1.25	-	-	-	-
Pt. Ether	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-
Streptomycin	0.062	0.062	0.062	0.12	0.25	0.25	0.50	0.25

*Each test was repeated thrice. Each value represents Mean±S.D, statistical analysis done by SPSS, DMRT (P≤0.5).

Gram-positive test bacteria. Out of the extracts tested, non-polar solvent extracts (chloroform and petroleum ether) both from leaf and leaf callus showed little or no zone of inhibition against test bacteria. Moreno et al. (2006) explained in his studies that less polar compounds diffuse slowly into the medium as they are hydrophobic in nature which prevents their uniform diffusion through agar medium. Out of all the extracts tested, polar solvent (Ethanol, Methanol and Aqueous) extracts of both leaf and leaf callus showed promising results. Successful prediction of natural compounds from the plant or callus extract depends largely on the type of solvent used in the extraction process. Traditional practioners widely use water as a solvent, but in our study, we found that organic solvents like ethanol and methanol extracts exhibited more antibacterial activity against test bacteria than aqueous extract. Our studies are in concurrence with the earlier studies carried out by Martin (1995), Sanches et al. (2005), Aboaba et al. (2006) and Durmaz et al. (2006), who reported that generally water extracts of plants do not have much activity against bacteria. Eloff (1998) reported in his studies that ethanol and methanol extracts are most effective than water and other non-polar solvent extracts and the same is in concurrence with our study.

In the present study leaf and leaf callus extracts were more effective against the tested Gram-negative bacteria which are more complex than Gram-positive ones; their cell walls are more complex and make them less susceptible to antibacterial agents (Nostro et al., 2000; Tadeg et al., 2005). According to Gupta and Saxena (1984) who reported that the judgement of strong antibacterial activity is based on MIC values between 0.05 and 0.50 mg/ml, moderate activity on values between 0.6 and 1.50 mg/ml and weak activity on values above 1.50 mg/ml. Antibacterial activity of leaf callus extracts has been reported earlier in several medicinal plants earlier like *Barleria lupulina* (Moin et al., 2012). Callus cultures are considered to be very useful in the production of antimicrobial principles and are considered a *de novo* production of the needed metabolites through cell culture (Sokmen, 2001; Gulluce et al., 2003). Tejavathi and Rao (1996) reported in their study that callus and regenerated plants sometimes show enhancement of secondary metabolites when compared to *in vivo* plants. Hence an attempt was made to carry out

the antibacterial study of *in vivo* plant material and *in vitro* derived leaf callus of *A. carnosus.*

Conclusion

The study shows a strong correlation with the reported traditional medicinal use for the treatment of infectious diseases. Both leaf and leaf callus extracts exhibited antibacterial activity. The extracts were more effective against gram negative bacteria (*K. pneumoniae*). Callus mediated cell lines can be explored for the mass production of viable antibacterial secondary metabolites. Further studies need to be carry out to isolate and characterise the active principle from leaf and callus cultures.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Acute skin irritation, acute and sub-acute oral toxicity studies of *Rosmarinus officinalis* essential oils in mice and rabbit

Berhan Mengiste[1], Kassahun Dires[1], Ermias Lulekal[2], Mahlet Arayaselassie[3], Tizazu Zenebe[1], Gezu Feleke[5], Eyasu Makonnen[4] and Awol Mekonnen[1*]

[1]College of Medicine, Institute of Medicine and Health Science, Debre-Birhan University, P. O. Box 445, Debre-Birhan, Ethiopia.
[2]Department of Plant Biology and Biodiversity Management, College of Natural and Computational Sciences, Addis Ababa University, P. O. Box 34731(Private), Addis Ababa, Ethiopia.
[3]Department of Pathology, School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.
[4]Department of Pharmacology, School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.
[5]Department of Chemistry, College of Natural and Computational Sciences, Debre-Birhan University, P. O. Box 445, Debre-Birhan, Ethiopia.

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In Ankober, Northern Ethiopia, *Rosmarinus officinalis* has been commonly used for flavoring foods as a condiment; moreover, the plant has also been widely used for different medicinal purposes. The current study was undertaken to provide data on acute and subacute toxicity in mice as well as skin irritation of *R. officinalis* essential oil in rabbit. Acute dermal and oral toxicity tests were conducted using limited dose of 2000 mg/kg. In sub-acute study, 1000 mg/kg were given by gavage to mice for 28 consecutive days. The mice were weighed and various observations like mortality, behavior, injury, or any signs of illness were conducted once daily during the study period. At the end of each study, biochemical parameters were evaluated and kidney and liver were taken after sacrifice for gross findings and histological analyses. For dermal toxicity, 10% ointment formulation of oils was applied on the rabbit skin to evaluate whether the animals sustained significant skin damage. The LD_{50} of *R. officinalis* essential oil for both dermal and oral administration is greater than 2000 mg/kg. There was no significant difference ($p > 0.05$) observed in the body weights, biochemical parameters, and gross abnormalities, as compared to the control in subacute study. No mortality was recorded. Pathological studies showed that there were no any macroscopic changes in kidneys and liver and all of them have normal appearance. The data of acute skin irritation test demonstrated that 10% *R. officinalis* oils ointment formulation did not induce acute toxicity in the skin of the animals. Overall, the findings of this study indicate that *R. officinalis* essential oil is non-toxic.

Key words: *Rosmarinus officinalis*, skin irritation, acute dermal toxicity, acute oral toxicity, subacute toxicity, biochemical analysis, histopathology.

INTRODUCTION

Rosmarinus officinalis (Rosemary) is an evergreen shrub that belongs to the family of Lamiaceae. It is the most common household plants distributed in several parts of the world (Asressu and Tesema, 2014). Rosemary leaves are often employed for various medicinal uses in addition to its role as condiment for flavoring foods. Several ethno-medicinal study reports of Rosemary showed that the plant is widely used for management of headaches, inflammatory diseases, and physical and mental fatigue (Lulekal et al., 2013). Moreover, it has been reported for its use as a stimulant, hepatoprotective and mild analgesic agent (Raškovic et al., 2014). Ethnobotanical investigations on traditional use of *R. officinalis* in Ankober District, North-Central Ethiopia showed prominent use reports on utilizing leaves of *R. officinalis* to treat different human and livestock ailments. Moreover, people of the Ankober District frequently use crushed Rosemary leaves in salad, fish and meat as flavor and preventing food poisoning (Lulekal et al., 2013).

Various pharmacological studies on Rosemary reported its antitumour (Singletary and Nelshoppen, 1991), antispasmodic (Lis-Balchin, 1996), estrogenic (Zhu et al., 1998), antiulcer (Dias et al., 2000), antinephrotoxic (Makino et al., 2002), antitrypanosomal (Abe et al., 2002), and osteoclastic (Muhlbauer et al., 2003) properties. Moreover Hur et al. (2004) reported the use of Rosemary as an immune stimulant. Recent pharmacological investigations on Rosemary also indicated its diuretic (Haloui et al., 2007), antiproliferative (Hussain et al., 2010), anti-inflammatory (Minaiyan et al., 2011), analgesic (Martínez et al., 2012), psychostimulant (Alnamer et al., 2012), hepatoprotective (Raškovic et al., 2014), antioxidant (Takayama et al., 2016), and antimicrobial (Asressu and Tesema, 2014; Mekonnen et al., 2016) properties.

Toxicity is the expression of being poisoned that occurs due to the interaction between cells and toxicants (Jothy et al., 2011). For a very long time, several herbal based preparations are supposed to be safe. Yet, many herbal products have been shown to be highly toxic when given either acutely or repeatedly for management of aliments (Prasanth et al., 2015). For instance, ethnobotanical studies in Ethiopia reported traditional herbal preparations side effects such as diarrhea and skin necrosis (Limenih et al., 2015). Hence, it is a timely effort to assess toxicological effects of traditionally used medicinal plant extracts such as that of *R. officinalis,* preclinically or clinically. This help to identify any potential toxic effect so as to minimize or avoid health-risks. So far, in Ethiopia, studies carried out to evaluate toxicity of essential oils from *R. officinalis* are lacking. Hence, the present investigation is aimed at investigating acute, sub-acute and dermatotoxicity effects of *R. officinalis* leaves essential oil in order to increase the confidence in their safety to humans to treat various ailments.

MATERIALS AND METHODS

Collection and identification of plant material

R. officinalis fresh leaves were collected from Ankober Herbal Project Nursery Site which is located in North Shewa Zone, Amhara Regional State, Ethiopia and the identity of the plant specimen was confirmed by a taxonomist at the National Herbarium, College of Natural Science, Addis Ababa University, Ethiopia where a voucher specimen was deposited.

Preparation of essential oil

Thirty grams of shade dried *R. officinalis* fresh leaves were taken into a 1000-mL round bottomed flask and 300 mL of distilled water was added. Clevenger-type apparatus distilled the mixture for about 3 h to get colorless oil.

Preparation of drug formulation

Ointment (5 and 10% w/w) was formulated for acute dermal toxicity study and skin irritation test, where 5 and 10 g of the extract was incorporated in 100 g (for each) petroleum jelly base. Then physical parameters like physical appearance and homogeneity, viscosity, spreadability and extrudability were evaluated using procedures described elsewhere in Bora et al. (2014) and Nair et al. (2010).

Experimental animals

Female New Zealand rabbits (1.4 to 2.3 kg) and healthy adult Swiss Albino mice of both sexes (10 to 12 weeks of age) were obtained from the Ethiopian Public Health Institute and the School of Pharmacy, AAU animal house, respectively. All the animals were kept at room temperature (25 $\pm$ 2°C) in an air conditioned room at 12 h light/dark cycle and acclimatized for 5 days before the study. The animals were provided with water and food pellets *ad libtum* before and throughout the experimentation period. All the animals were cared for and treated humanely according to the Principles of Laboratory Animal Care (ILAR, 1996). Ethical approval for this study was obtained from Deber Birhan University IRB.

Rabbit skin irritation test

Acute dermal irritation tests were performed using OECD guideline 404 with little modification using two rabbits (OECD, 2002). The fur of the animal was removed by closely trimming the dorsal area of

*Corresponding author. E-mail: awolalim@gmail.com.

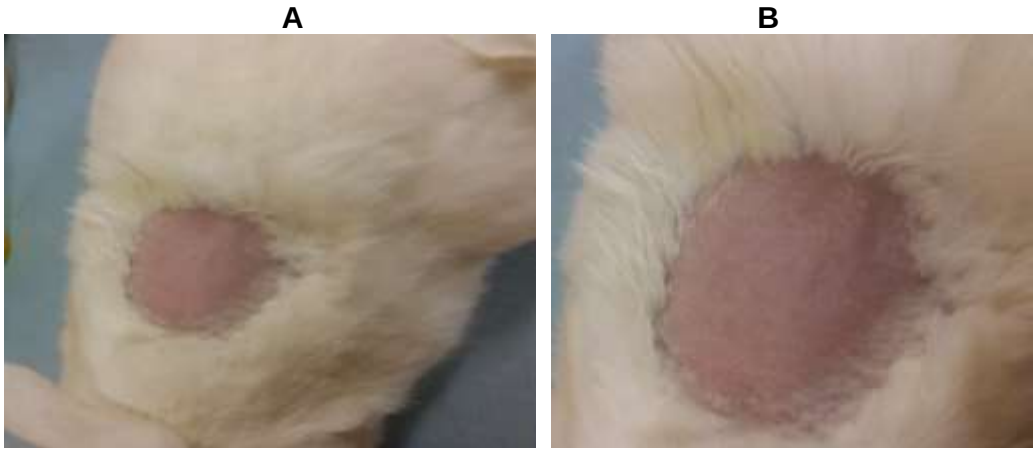

Figure 1. Photograph of skin of rabbit before application test ointment (A) and 1hr after removal of *R. officinalis* essential oils ointment formulation (B).

the trunk on different sites 24 h before the test (Figure 1). About 500 mg of 10% of *R. officinalis* essential oil ointment was applied to two sites and another site was used as a control. Sites were observed critically at 1 h after removal of test substance. The observation was repeated at 24, 48, and 72 h, for days 7 and 15th thereafter. Reactions like erythema and edema were assessed according to the scoring system for skin reactions (Kamkaen et al., 2007; OECD, 2002).

Acute dermal toxicity study

Acute dermal toxicity tests were performed using OECD guideline 434. About 10% of the body surface area fur was shaved 24 h before the study from the dorsal area of the trunk of five female rats showing normal skin texture. A limit test dose of 2000 mg/kg of formulation was applied uniformly over the shaved area for 24 h. Animals were housed individually and general behavioral changes; symptoms of toxicity and mortality were observed after treatment critically for the first 4 h, then over a period of 24 h. At the end of the exposure period, the remaining test substance was removed and the observation was continued daily thereafter for a total of 14 days (OECD, 2004).

Acute oral toxicity study

An acute toxicity study of *R. officinalis* essential oil was carried out in five female mice using the method of OECD guideline 425 (OECD, 2008). A limit test single dose of 2000 mg/kg of the test sample was given to mice using oral gavage after being deprived of food for 3 h. All the mice were observed for general behavioral changes; symptoms of toxicity and mortality after administration of essential oil for the first 4 h (critically), then over a period of 24 h, and thereafter daily for 14 days.

Sub-acute toxicity study

Sub-acute toxicity study was performed as per the OECD guidelines 407 (OECD, 2008). The treatment (5 male and 5 female) and control (5 male and 5 female) groups orally received *R. officinalis* essential oil at the dose of 1000 mg/kg and saline once daily for 28 days, respectively. All mice were observed once in day for any clinical signs of toxicity all the way during the experimental period and the body weight of each mouse was recorded once in a week. At 29th day, mice from both groups were properly anesthetized and blood was collected from a common carotid artery. The serum level of alkaline phosphatase (ALP), serum glutamic-oxaloacetic transaminase (SGOT), and serum glutamic-pyruvic transaminase (SGPT) were analyzed. After blood collection, mice from both groups were immediately sacrificed and vital organs of each mouse were examined for the evidence of gross lesions. Liver and kidney were preserved in 10% neutral buffered formalin solution to undertake histopathological examination. Tissues embedded in paraffin wax were sectioned 5 µm thick, stained with haematoxylin and eosin, mounted on glass slides and examined under a standard light microscope (Olaniyan et al., 2016).

Statistical analysis

The data were analyzed by using SPSS version 20 and $P < 0.05$ was considered as statistically significant. The results were expressed as mean ± standard deviation (SD).

RESULTS

R. officinalis oil ointment formulation

Table 1 shows physical evaluation of *R. officinalis* essential oil ointment formulation. The ointment formulation was found to be white color, smooth and free from grittiness. The physicochemical properties studied showed satisfactory results for spreadability, extrudability, washability and viscosity.

Rabbit skin irritation test

The effect of the essential oils on dermal irritation is

Table 1. Physical evaluation of 10% *R. officinalis* essential oil ointment formulation.

Formulation	Color	Viscosity (cP) at 100 rpm (Mean±SD)	Spreadability (g.Cm/min) (Mean±SD)	Extrudability (g) (Mean±SD)
R. officinalis oil ointment	White	8158.3±30.1	2420.7±77.9	0.137±0.015

Table 2. Score of erythema and edema after application of test materials on rabbit skin.

Reaction	1 h		24 h		48 h		72 h		7th day		15th day	
	Con	Treat	Con	Treat	Con	Treat	Con	Treat	Con	Treat	Con	Treat
Erythema	0	0	0	0	0	0	0	0	0	0	0	0
Edema	0	0	0	0	0	0	0	0	0	0	0	0

Score for skin reaction of 10% *R. officinalis* essential oil ointment (three treatment site) at different time interval after removing test ointment. Primary Irritation Index (PII) = 0/3, PII=0; Category of irritation based on PII is Negligible. Con: Control; Treat: treatment.

shown in Table 2. From the results of acute dermal irritation study, essential oils appeared to be safe after applying 10% ointment formulation. There was no evidence of any noticeable skin irritation (no erythema and edema) and inflammation in the study period when compared with the control (Figure 1). At 1 h after removal of test substance and thereafter on the skin sites where the test substance was applied, it was found that in all rabbits, erythema and edema score was “0”.

Acute dermal toxicity study

No toxic effect was observed on the behavioral response of rats treated with a dose of 2000 mg/kg *R. officinalis* essential oil. All rats were dosed once and observed for 14 days. Moreover, there were no signs of changes in the behaviour patterns, skin, eyes, salivation, and diarrhea of the rats. Neither mortality nor significant weight loss was also observed.

Acute oral toxicity study

Oral administration of *R. officinalis* essential oil at the dose of 2000 mg/kg did not show any signs of toxicity and mortality in any of the treated female mice for 14 days during observation period. Hence, the oral lethal dose 50 (LD_{50}) value of *R. officinalis* essential oil was found to be greater than 2000 mg/kg.

Sub-acute toxicity

General observation and effects on body weight

Oral administration of *R. officinalis* essential oil for 28 day caused no noticeable change in the general behavior of the mice and there were no significant changes in body weight or food intake of the mice as compared to the control group. Both the control and treatment groups appeared healthy during the whole study period. There was an increase in body weight of mice in both treatment and control groups. The percentage body weight gain, however, was higher in the control group (17.07%) as compared to treatment groups (7.85%) (Table 3).

The effect of R. officinalis oil on serum biochemical parameters

Sub-acute administration of *R. officinalis* essential oil effects on biochemical parameters are shown in Table 4. Administration of *R. officinalis* essential oil for 28 days did not show any significant changes in biochemical parameters such as SGOT, SGPT and ALPL when compared with the control groups. The serum level of SGOT, SGPT and ALP of treatment groups was lower as compared to the control group. Yet, the difference was not statistically significant.

Histopathology

Light microscopic examination of liver and kidney sections of control and treatment groups showed a normal histology (Figures 2 and 3) and absence of any gross pathological lesions after 28 days treatment with 1000 mg/kg dose of *R. officinalis* essential oil. The result showed normal hepatocellular morphology, normal peri-portal area with absence of necrosis and inflammation. All kidney sections showed normal glomerular architecture, no vascular necrosis or hyaline changes and unremarkable tubule interstitial paranchyma.

Table 3. Effects of *R. officinalis* essential oil on body weight of mice treated orally for 28 days.

Parameter	RO 1000 mg/kg	Control
Week 0 (g)	28±2.7	24.6±2.88
Week 1 (g)	28±2.7	24.6±2.88
Week 2 (g)	29±0.55	29±1.73
Week 3 (g)	30±2.61	28.8±1.09
Week 4 (g)	30.2±2.28	28.8±1.09
Body weight gain (%)	7.85	17.07

Values expressed as mean ± SEM, n = 10 animals/group, p > 0.05. RO: *R. officinalis.*

Table 4. Biochemical parameters of mice treated orally with *R. officinalis* oil for 21 days.

Parameter	RO 1000 mg/kg	Control
SGOT (U/L)	316±128	348±123
SGPT (U/L)	79.6±64.5	80.8±26.08
ALP (U/L)	265±124	267±33

Values expressed as mean ± SEM, n = 10 animals/group, p > 0.05. RO: *R. officinalis.*

DISCUSSION

Currently, concern regarding safety profile of traditional herbal preparations is increasing since there is no standard dosage and limited scientific studies on safety profile of traditional herbal products (Eran et al., 2016; Saad et al., 2006). Hence, adequate scientific knowledge of traditional herbal preparations oral toxicity is mandatory. This will help to reveal the possible clinical signs of adverse effect and identify doses that could be used by preparations under investigation. Irrespective of the pharmacological benefits of the *R. officinalis* essential oil, there are no detail knowledge about toxicity profile of this medicinal plant in Ethiopia. In the present acute toxicity study, administration of *R. officinalis* essential oil at 2000 mg/kg dose to the mice both orally and dermally did not reveal any signs of toxicity or mortality in any animal during the whole study period. Therefore, the LD_{50} of *R. officinalis* essential oil for both route of administration is considered to be greater than 2000 mg/kg. *R. officinalis* essential oil is safe since agents having LD_{50} value greater than 2000 mg/kg are considered as relatively safe (Nath and Yadav, 2015). This finding correlates with that of Fahim et al. (1999) and Nakavuma et al. (2016), who reported LD_{50} value of 5,000 g/kg and 4,723 mg/kg respectively. On the contrary, Alnamer et al. (2012) reported *R. officinalis* essential oil LD_{50} of 897.85 mg/kg; which is much lower than the LD_{50} value of the present study and previously reported value. The possible reason for this discrepancy could be variation in secondary metabolites due to agro-ecological difference. The main secondary metabolite of *R. officinalis* oil used in this study were α-pinene (50.83%), camphene (5.211%), ß-pinene (2.068%), ß-myrcene (0.683%), 1, 8- cineole (24.425%), camphor (3.845%), broneol (1..518%), and broneol acetate (1.628%). Verbenone (0.521%), linalool (1.262%), and limonene (1.729%) (Mekonnen et al., 2016). While the major constituents of *R. officinalis* oil used in the study of Nakavuma et al. (2016) were α-pinene (26.46%); 1, 8-cineole (24.20%), verbenone (9.41%), geraniol (3.38%), linalool (3.12%), and limonene (3.02%) (Un-published data) and Alnamer et al. (2012) study had composition of α-pinene (15.82%), camphene (6.80%), ß-pinene (4.75%), myrcene (1.70%), p-cymene (2.16%), 1, 8-cineole (50.49%), camphor (11.61%), broneol (2.58%), and broneol acetate (2.08%). Higher 1, 8-cineole (50.49%) content in the study of Alnamer et al. (2012) as compared to the present study and Nakavuma et al. (2016) study might play role for the observed difference.

To make sure people using pharmaceutical formulation are safe, assessment of skin irritancy potential to human skin of any chemicals or formulations is compulsory (Kamkaen et al., 2007). The absence of any toxic sign like erythema and edema reactions in present skin irritation test asserts the non-irritant of *R. officinalis* essential oil ointment. Based on the results, the essential oil primary irritation index was found to be 0 and it was concluded that the essential oil was non-irritating to the skin (PII<0.5). Low camphor content (3.845%) of *R. officinalis* essential oil might be responsible for absence of skin irritation since camphor is an irritant substance. This finding is in good agreement with that of Hamza et al. (2017), who reported *R. officinalis* essential oil was non-irritant to the skin though the study was conducted in rat.

Information on target organ toxicity, dosage regimens and observable sign of toxicity that possibly influence life span of experimental animals can be generated by sub-acute toxicity study (Hilaly et al., 2004). In toxicity study, changes in the body weight serve as a sensitive indicator of general health of experimental animals. There were no significant body weight changes of experimental animals after completing 28 days essential oil administration as compared to the control groups (Table 3). Both treatment and control group animals showed a steady increment in body weight throughout the study period.

Besides body weight, quantification of food and water consumption is necessary during toxicity study of an agent with medicinal value. In this study, *R. officinalis* essential oil did not affect food and water consumption showing that it did not interfere with the normal digestion and metabolism of animals. For better physiological status of the animal and response to the test substance

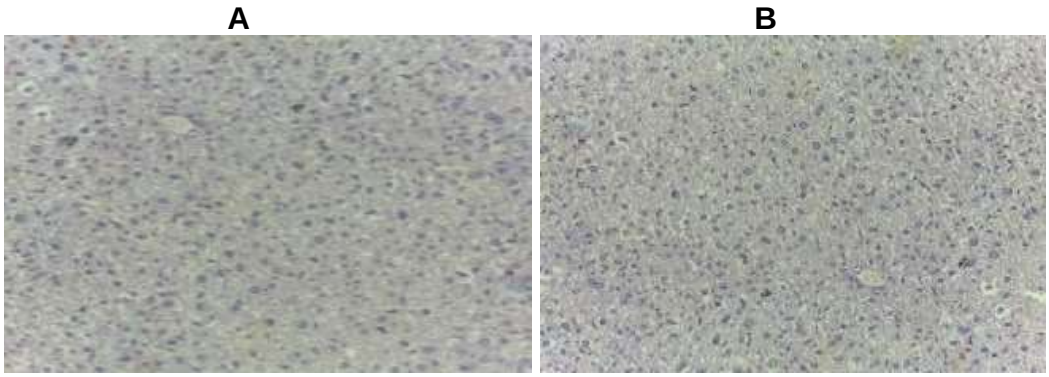

Figure 2. Liver histology of control and *R. officinalis* essential oil treated animals. (A) Liver section of control animals revealed normal architecture and hepatic cells; (B) Liver section of *R. officinalis* essential oil (1000 mg/kg) treated animals exhibited normal architecture of hepatocytes and hepatic cells after 28 days of treatment.

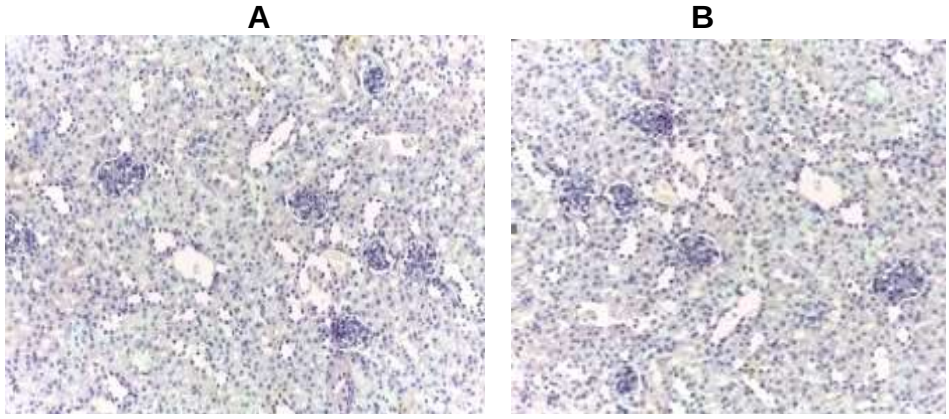

Figure 3. Kidney histology of control and *R. officinalis* essential oil treated animals. (A) Kidney section of control animal showed normal size of glomeruli with normal tubules; (B) Kidney section of *R. officinalis* essential oil (1000 mg/kg) treated animals exhibit normal size of glomeruli with normal tubules after 28 days of treatment.

under investigation, proper intake of supplements is needed (Kumar et al., 2014)

Serum biochemicals such as alkaline phosphatase, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase and creatinine can be used as biomarkers to identify potential toxic effect of drugs and xenobiotics to the vital organ (Brandt et al., 2009; Kumar et al., 2014). Higher blood level of both SGOT and SGPT enzymes are a good indicator of toxicity to the liver parenchymal cells (Kumar et al., 2014). In the present study, there was no statistically meaningful difference in all biochemical parameters measured between the treatment and control groups (Table 4). *R. officinalis* essential oil did not significantly change the levels of SGOT, SGPT and ALP in experimental animal highlights that it did not affect normal liver function and metabolism of the animals. Histological assessment of the organs (Figures 2 and 3) further confirmed serum biochemical observations. Normal structural features of kidneys histopathological investigation of both treatment and control group propose the normal renal function. Normal architecture of glomeruli and renal tubules portrays the absence of renal toxicity. Liver histopathology of both treatment and control group animals was almost the same with normal morphology of hepatocytes, central vein and portal triads. This finding is in good agreement with that of Nakavuma et al. (2016), who showed lack of gross and microscopic changes in the mice tissues and organs treated with *R. officinalis* essential oil.

Conclusion

The current finding asserts that *R. officinalis* essential oil is not toxic and do not produce any adverse effect in the acute and sub-acute oral toxicity investigations. The histology examination revealed no remarkable changes in kidney and liver of the mice in both control and treatment groups. The level of the marker enzymes in the vital organs was also found to be normal. Besides, the skin tolerance test on rabbit showed ointment of *R. officinalis* essential oil is not irritant and do not show any dermal toxicity. Overall, it can be concluded that the *R. officinalis* essential oil is well tolerated in daily dose at 1000 mg/kg for a period of 28 days and safe for traditional use and as food additive.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Healing effect of the microemulsion enriched with hydroalcoholic extract of *Abarema cochliacarpa* (Gomes) Barneby & J. W. Grimes (Fabaceae)

Antônio Santos Dias[1], Clívia Rolemberg Andrade[1], André Luiz Lima Meneses Santos[1], Sabrina Zelice de Moraes[1], Jymmys Lopes dos Santos[1], Silvan Silva de Araújo[1], Andrea Yu Kwan Villar Shan[1*], Jamylle Nunes de Souza Ferro[4], Marcelo Cavalcante Duarte[2], Emiliano de Oliveira Barreto[4], Anderson Carlos Marçal[3], Ricardo Luiz Cavalcante Albuquerque Júnior[5], Antonio Euzebio Goulart Santana[4], Brancilene Santos de Araújo[1] and Charles dos Santos Estevam[1]

[1]Department of Physiology, Federal University of Sergipe, São Cristóvão, SE, 49000-100, Brazil.
[2]Department of Pharmacy, Federal University of Sergipe, São Cristóvão, SE, 49000-100, Brazil.
[3]Department of Morphology, Federal University of Sergipe, São Cristóvão, SE, 49000-100, Brazil
[4]Institute of Biological and Health Sciences, Federal University of Alagoas (UFAL), Maceió, AL, 57072-900, Brazil.
[5]Institute of Chemistry and Biotechnology, Federal University of Alagoas (UFAL), Maceió, AL, 57072-900, Brazil.
[6]Tiradentes University (UNIT), Aracaju, SE, 49032-490, Brazil.

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Abarema cochliocarpa **is an endemic plant of Brazil and has been traditionally used as a popular medicine to treat various diseases and mostly as a healing agent. The aim of this study was to evaluate the healing effect of the microemulsion containing hydrometanic fraction of the inner bark (HMF) of the plant, as well as its antioxidant, antitumor and cytotoxic potential. The cytotoxicity of HMF was assessed by the viability of J774 macrophages cells and the antiproliferative activity in lines of human tumor cells. The antioxidant capacity of the formulation was evaluated *in vitro* by the ability to scavenge the DPPH and the healing effect was evaluated in *Rattus novergicus* in 7, 14, 21 days. The HMF significantly favored the viability of J774 macrophages in all tested concentrations and it was dose-dependent. The antiproliferative potential of FHM against human tumor cells was not satisfactory. This formulation presented CE_{50} of 24.87 ± 0.62 µg.mL^{-1}, and healing effect of 55.18 and 100% of wound retraction up to the 7th and 21st days of treatment, respectively. The bioactive compound of HMF isolated by HPLC was identified by ^{1}H and ^{13}C NMR and by literature data as (+)-catechin. The formulation has curative effect. It can be concluded that the phenolic compounds act in the reduction of oxidative stress and contribute to faster healing.**

Key words: Antioxidant, phenolic compound, formulation, healing.

INTRODUCTION

The role of natural products in the research of new drugs in both discovery and development is significant; about 60-75% of new drugs used to treat cancer and infectious diseases were from natural origins (Newman et al., 2016). At the beginning of the 21st century, almost ¼ of the world's best-selling drugs were obtained directly from

natural sources or their derivatives (Balunas and Kinghorn, 2005). In 2012, the market for herbal preparations worldwide was close to US $ 44 billion and the Brazilian one was between US $ 350 million and US $ 550 million (Brasil, 2012). The plant species *Abarema cochliacarpa* is endemic to Brazil, belongs to the Fabaceae family and in folk medicine is known as "Barbatimão". It is distributed in the Atlantic Forest, Caatinga and Cerrado biomes (Da Silva et al., 2010) with a wide distribution. Its inner bark is used in the form of tea and/or "garrafadas" made with white wine or cachaça to treat and cure leucorrhoea, gastritis, purulent wounds, pains, among others (Silva et al., 2009). Studies on their chemical composition have demonstrated the presence of catechins (their dimers and trimers) mainly, as well as saponins, tannins, phenols and proanthocyanidins (Da Silva et al., 2010; Dias et al., 2013). Currently, studies to discover its pharmacological potential are on the rise since even it being a medicinal species used by several Brazilian communities, there is still little scientific support. In this context, it is worth emphasizing that secondary metabolites such as flavonoids, catechins, tannins, saponins, among others may present several therapeutic benefits such as antioxidant, antitumor, antimicrobial, anti-inflammatory, antiasthmatic and cicatrizant action (Simões et al., 2007).

Healing is a tissue repair process that occurs after an injury, begins after the injury and has as purpose the formation of new tissue which is divided into three subsequent phases that are juxtaposed: inflammation, formation of granulation tissue and deposition of extracellular matrix, and remodeling (Mendonça and Coutinho-Netto, 2009; Isaac et al., 2010; Ruh et al., 2013). Over the years it has deserved researchers' attention, mainly in relation to the factors that delay or difficult its mechanisms (Santos et al., 2014). The release of ROS and RNS at the lesion site may play an important role in the modulation of the inflammatory response like the stimulus for the release of cytokines, adhesion molecules and chemotactic agents, further contributing to the maintenance of inflammation and subsequent delay in tissue repair. However, plants that demonstrate antioxidant role have been widely used in the various forms of preparation, with possible anti-inflammatory activity and assistants in the healing process (Da Silva et al., 2010; Piriz et al., 2014; Ebeling et al., 2014). In this sense, new healing agents for the treatment of cutaneous wounds, especially chronic ones, of natural origin would be of great value since they may act to decrease the undesirable effects of other drugs. Thus, this work evaluates the healing potential of a low cost formulation containing the hydrometanic fraction obtained from *A. cochliacarpa*.

MATERIAL AND METHODS

Collection, plant identification and obtainment of the active fraction

The plant sample (inner bark, 5 kg) was collected at morning from a native population, located in the Caípe Velho Village (11º0'49"S, 37º13'21"W), São Cristóvão, SE, Brazil. The botanical identification was performed by Dr. Ana Paula do Nascimento Prata from the Biology Department of the Federal University of Sergipe (UFS) and a specimen was deposited in the UFS Herbarium under the voucher 014639. This sample was dried in a stove with air circulation at 40°C and after complete dehydration, it was milled for the extraction of the active fraction (hydrometanic fraction - HMF), rich in phenolic compounds (Dias et al., 2013) and whose studies have proved their effectiveness in the wound healing process (Viera et al., 2008; Silva et al., 2010).Thus, the HMF was obtained by liquid-liquid extraction from the ethanolic extract resuspended with 40% (v/v) methanol and successive washes with hexane, chloroform and ethyl acetate, resulting in 40.6% yield in relation to the dry matter. After its preparation, HMF was used for chemical and biological analyzes and for the preparation of the healing formulation (microemulsion with 10% hydrometanic fraction –10% HMFM) with a patent registered under the registration BR1020150073810.

Isolation and identification of the bioactive constituent of the active fraction

HMF (70.2 mg) was initially subjected to flash chromatography using silica gel (70-230 mesh) and AcOet:MeOH (0 to 50%) in polarity gradient. 26 subfractions were obtained which were monitored in TLC using silica 60H and mobile phase buOH: acetic acid: water (65:15:20); being revealed with ceric sulfate and UV light and collected according to the similarity of the chromatographic profile, forming 4 groups. Among them, phenolic profile G-I (4g) was eluted with MeOH in column chromatography (CC) using Sephadex (LD-20), yielding 56 fractions which were concentrated in a rotary evaporator under reduced pressure. These fractions were monitored by TLC following the same procedure described previously and assembled into 4 subgroups (sub-I, sub-II, sub-III and sub-IV) analyzed on HPLC/DAD-UV-vis from 190 to 680 nm. Elution was on analytical (5 µm, 25.0 x 0.46 cm) Shim-pack® PREP-ODS C_{18} columns, by gradient (20 µL, water: 0-50% MeOH) for 30 min, with 1 $mL.min^{-1}$ flow. For elution, the subgroups were vacuum filtered using nylon membranes (2.5 cm x 0.45 cm d.i) and degassed in Ultracleaner 1400. Among them, the sub-I (67 mg) was eluted again on preparative C_{18} column (5 µm, 25.0 x 2 cm), but in isocratic condition (1000 µL, water: acetonitrile 0.5% acetic acid, 85:15), DAD-UV-vis detection at 280 nm, flow of 5 $mL.min^{-1}$with the duration of 30 min, isolating the major compound AC-HMF-1. This isolated compound was concentrated in N_2 gas and analyzed by ^{1}H and ^{13}C NMR in a Bruker 400 Ultrashield apparatus operating at 400 MHz for the ^{1}H nucleus and at 100 MHz for ^{13}C.

*Corresponding author. E-mail: andreashan@ymail.com.

Cell Viability of Macrophages J774

The viability of J774 cells (2 x 10^4 cells) was assessed in triplicate after 24 h of continuous exposure to HMF and measured by the colorimetric assay of tetrazolium salt 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction to formazan, according to Mosmann (1983). For this, after cell adhesion in a 96-well ELISA reader plate, the culture medium was replaced with 200 µL of culture medium containing the FHM diluted in 0.5% DMSO at respective concentrations of 10, 50 and 100 µg.mL^{-1}. The plates were then incubated for 24 h. After this time, the culture medium was replaced with 200 µL MTT 0.5 mg.mL^{-1}, pre-filtered on a 0.22 mm milipore membrane and the plates were incubated for 3 h. After, the supernatant was carefully aspirated and 200 µL of DMSO were added to each well for the solubilization of formazan. The entire contents were transferred to a new ELISA plate and read at 570 nm, whose absorbances were normalized according to equation:

% VC = [DO (treated cells) − DO (blank)/ DO (control) − DO (blank)] × 100.

Antiproliferative activity

The antiproliferative potential of HMF was verified by cytotoxicity analysis using the MTT method (Mosmann, 1983) against the following tumor cell lines: human promyelocytic leukemia (HL-60), human ovary (OVCAR-8), colon carcinoma (HCT-116) and glioblastoma (SF295), provided by the National Cancer Institute (NCI). In a 96-well plate, the cell lines were plated at the concentration of 0.1 × 10^6 cells.mL^{-1}and incubated for 24 h in a stove with 5% CO_2 at 37°C. Later, 50 µg.mL^{-1} of HMF was added to each well and incubated for 72 h in a 5% CO_2 stove at 37°C. Then, the plate was centrifuged (15 xg/15 min) at 4°C and the supernatants removed. Then 150 µL of the MTT solution (0.5 mg.mL^{-1}) was added and again incubated for 3 h. The plate was centrifuged (30 *g*/10 min) at 4°C, the supernatants were discarded and the pellets resuspended in 150 µL of pure sterile DMSO. For the formazan quantification by viable cells, the absorbance was read in an ELISA at 595 nm. Doxorubicin (Glenmark Laboratories) was used as a positive control (0.003 to 0.25 µg.mL^{-1}). HMF was found to cause more than 75% inhibition of growth in at least one of the cell lines tested and, therefore it was tested with increasing concentrations ranging from 0.024 to 50 µg.mL^{-1} to determine its IC_{50} (minimum inhibitory concentration capable of causing 50% of maximal effect) (Ribeiro et al., 2012). The analyzes were performed in triplicate and the IC_{50} and their respective confidence intervals (95% CI) were performed by non-linear regression.

DPPH radical-scavenging activity

The determination of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) sequestering ability was done with 10% MFHM and the 10% gallic acid Microemulsion (10% GAM) according to Brand-Wiliams et al. (1995) and the consumption of this free radical was monitored by decreasing the absorbances of test solutions in different concentrations. The samples concentrations used were: 10% HMFM at 15, 20, 40, 50 and 60 µg.mL^{-1}; 10% GAM at 30, 60, 80 and 100 µg.mL^{-1}. The absorbance measurements of the mixtures (0.3 mL of sample solution or positive control and 2.7 mL of DPPH stock solution at 40 µg.mL^{-1} were made at 515 nm in 1, 5 and 10 min and every 10 min until 1 h. The mixture of methanol (2.7 mL) and methanol solution of the extract (0.3 mL) was used as blank.

From the equation of the calibration curve (y = 110.547 – 0.02804a, 0.998) and the absorbance values in 30 min for each concentration tested, the percentages of remaining DPPH (%$DPPH_{REM}$) were determined. The expression of the results was made by the concentration required to decrease the initial concentration of DPPH by 50% (EC_{50}), by Inhibition Percentage (IP) and by the antioxidant activity index (AAI). EC_{50} was calculated by plotting the %$DPPH_{REM}$ at 60 min opposed to the sample concentrations being expressed in µg.mL^{-1} ± standard deviation. The IP was calculated with de 60 µg.mL^{-1} at 30 min. The AAI was calculated according to Scherer and Godoy (2009) in which AAI = %$DPPH_{REM}$ (end)/EC_{50} being considered unsatisfactory AAI < 0.5, moderate with 0.5 < AAI < 1.0, strong with 1.0 < AAI < 2.0 and very strong with AAI > 2.0.

Healing activity

The method was performed according to Martins et al. (2006) and Carvalho et al. (2009) with modifications and the tests described were approved by the Animal Research Ethics Committee of the Federal University of Sergipe (CEPA/UFS) under protocol 57/2012. The animals, *Rattus norvegicus* Wistar line (3.5 months, 200-300 g) were randomly selected, housed in cages at 22 ± 3ºC, with 12 h/12 h cycle, free access to food (Labina ®) and water *ad libitum*. They were divided into 3 groups: (i) No treatment Group (NTG, n = 15); (ii) Vehicle Group (VG, n = 15) treated with 100 µL of the formulation in the absence of MHF, and; (iii) 10% hydrometanic fraction microemulsion group (10% HMFMG, n = 15) treated with 100 µL of the microemulsion produced from the 10% hydrometanic fraction. Each group was subdivided into 3 subgroups (n = 15) according to the date of the sacrifice period: 7, 14 and 21 days after surgery. The animals were anesthetized intramuscularly with thiopental at a dose of 50 mg.kg^{-1} and wound formation was performed by means of a 0.8 mm diameter punch after the application of povidine 1% for local asepsis. The measurement of the area of the wound was done through a digital caliper and the formulation corresponding to each tested group was applied except NTG. At the end of the surgical procedure, the animals were housed in their cages and monitored for anesthetic recovery, as well as normalization of the respiratory rate and the beginning of the active search for water and food. The formulations were applied daily and the animals were examined for their mobility in the open field, presence or absence of secretions, crusts or necroses. On days 7, 14 and 21, animals were weighed, anesthetized with ether, euthanized by decapitation and the wounds measured by digital caliper. The wounds were measured on days 0 (immediately after surgery), 7, 14 and 21 postoperative (immediately after euthanasia) and the area of the wounds was calculated by the equation of Prata et al. (1998): A= π.R.r, where A is the area (cm^2); "R", the major radius and "r", the minor radius. The degree of contraction, expressed as percentage, was measured by the equation proposed by Ramsey et al. (1995): % of wound contraction= W0-Wi / W0 x 100, in which W0 = initial wound area on day 0 and Wi= area of the wound on the day of its excision and the results of area and contraction of the wounds were expressed as mean ± standard deviation, submitted to analysis of variance and tested by Tukey (p < 5). The animals were placed in plastic bags for infecting material, frozen in an appropriate freezer and collected through the collection of biological waste by the institution.

Microscopic wounds analysis

The surgical specimens obtained from NTG, VG and 10% GMFHM were submitted to 10 µm thick sections in cryostat, and then placed on glass slides and stained by the hematoxylin-eosin method (H-E)

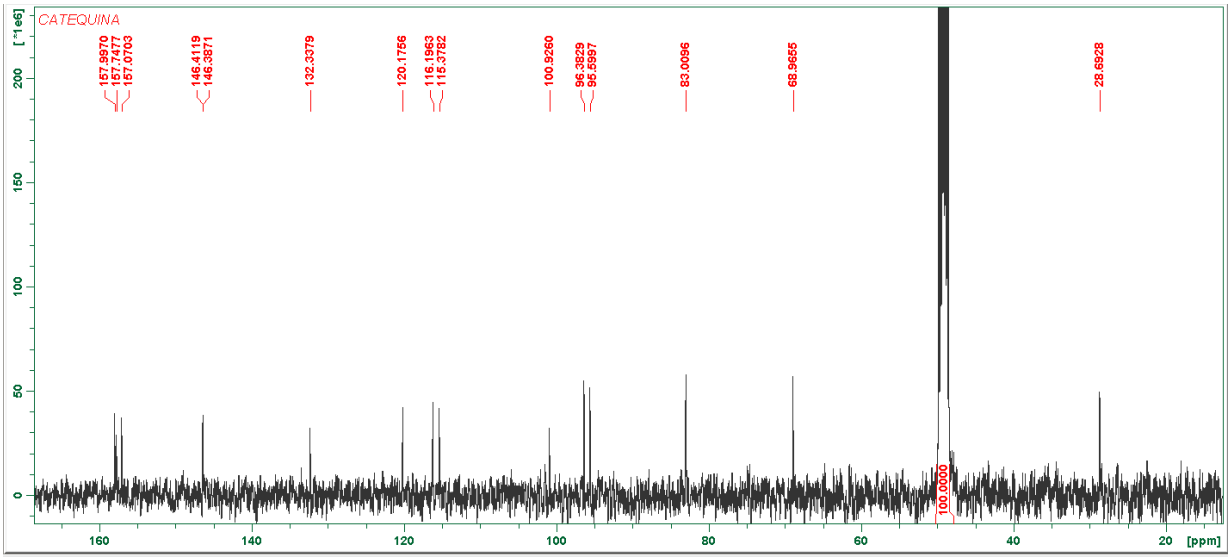

Figure 1. ^{13}C NMR spectrum (100 MHz, CD_3OD) of bioactive compound AC-FHM-1, of inner bark hydrometanic fraction *A. cochliacarpa*.

proposed by Luna (1968), for later microscopic observation of their respective granulation areas. The Sirius red staining method was also used for descriptive analysis of collagen deposition (Sweat et al., 1964) in which sections cut and stained were analyzed under polarized light. Collagen was classified as type I or III according to the birefringence presented, according to the morphological aspect of its fibers (striated or wavy, thin or thick, short or long) and arrangement of the bundles (reticular, interlaced or parallel).

Determination of total sulfhydrides (SH)

SH quantification was done according to Faure and Lafond (1995) in which aliquots of 200 µL of blood plasma were mixed with 800 µL of Tris-EDTA, pH 8,2. Thereafter, the first reading (A) was carried out at 412 nm. After the reading, the samples were transferred into test tubes and mixed in 20 µl of DNTB [10 mM 5,5-dithiobis (2-nitrobenzoic)] diluted in methanol (4 $mg.mL^{-1}$) and left to rest in the dark for 15 min. At the end of this time, the absorbance (A2) was measured for the second reading. The SH concentration was calculated according to the equation: (A2 - A1) - B x 1.57 mM x 1000.

RESULTS AND DISCUSSION

Isolation and identification of the majority constituent of the active fraction

In the analytical condition, the HMF chromatogram showed a major peak (AC-HMF-1) in the TR of 12.2 min and UV-Vis spectrum with band II at maximum absorption between 240-280 nm and I band between 300-550 nm, characteristic of flavonoid (Ugaz, 1994). This bioactive constituent was isolated on preparative HPLC (37 mg) and its melting range was determined at 170 to 175°C. The ^{13}C NMR spectrum (100mHz) showed characteristic signals for the basic structure of a flavonoid (Figure 1): δ 157.9 (C-7), δ 157.7 (C-9), δ 157.0 (C-5), δ 146.4 (C-3'), δ132.3 (C-1'), δ 120.1 (C-6'), δ116.1 (C-5'), δ 115.3 (C-2'), δ100.9 (C-10), δ 96.3 (C-6), δ 95.5 (C-8), δ 83.0 (C-2), δ68.9 (C-3), δ28.6 (C-4). The ^{1}H NMR spectrum (400 mHz) of the substance showed δ6.83 (d, J = 1.8 Hz, H-2'); δ6.76 (d, J = 8.1 Hz, H-5'), δ6.71 (dd, J = 8.1 e 1.8 Hz, H-6'), δ5.93 (d, J = 2.2 Hz, H-6), δ5.85 (d, J = 2.2 Hz, H-8),δ4.57 (d, J = 7.5 Hz, H-2), δ3.97 (m, H-3), δ2.85 (dd, J = 16.1 e 5.5 Hz, H-4α), δ2.51 (dd, J = 16.1 e 8.2 Hz, H-4β) (Figure 2). The ^{1}H and ^{13}C NMR signals showed chemical shifts of signals identical to those reported by Lôbo et al. (2008) as + (-) catechin (Figure 3). Thus, by the data shown above and in comparison, with the literature data it is concluded that AC-HMF-1, the major constituent of HMF is the same compound already identified by Sánchez-Fidalgo et al. (2013) in the specie. Although (+) - catechin is the major compound of this formulation and has an anti-inflammatory effect, studies have reported that such a substance is also an inhibitor of TGF-beta that is closely related to the process of wound contraction. In this sense, new studies are suggested only with the pure substance, since other components of the formulation probably act in the healing process.

Cytotoxicity in J774 Macrophages

HMF significantly favored the viability of J774 macrophages at all concentrations tested (10, 50 and 100

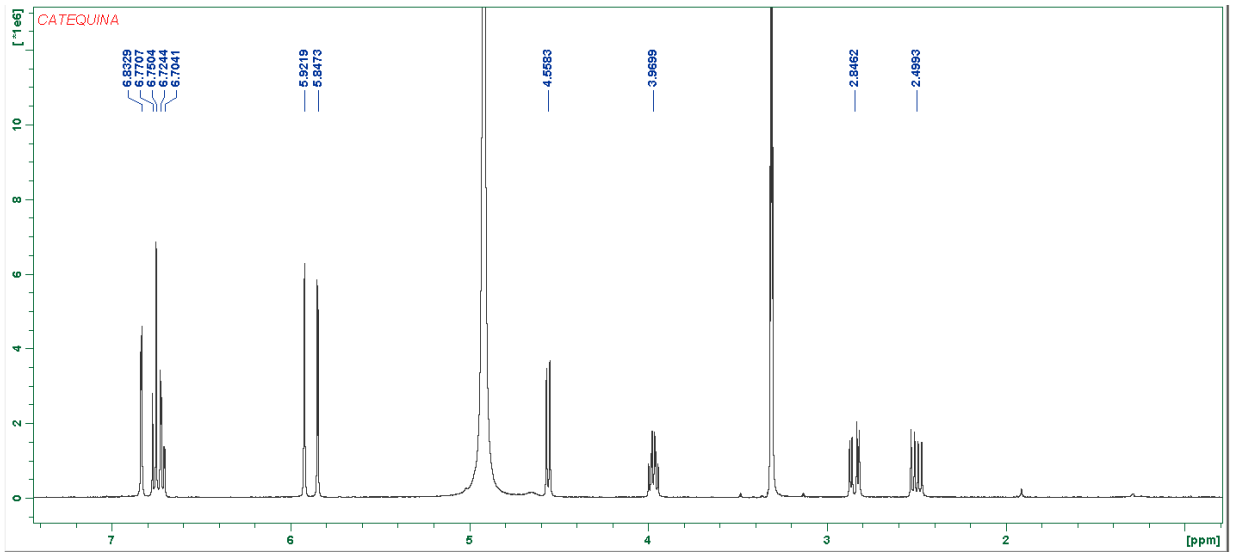

Figure 2. ^{1}H NMR spectrum (400 MHz, CD_3OD) of bioactive compound AC-FHM-1, of inner bark hydrometanic fraction *A. cochliacarpa*.

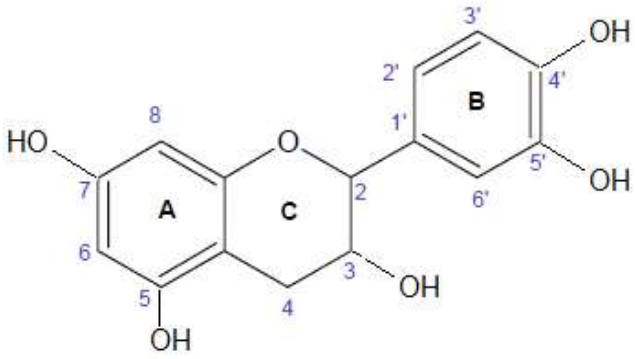

Figure 3. Catechin – bioactive compound in the inner bark hydrometanic fraction of *A. cochliacarpa*.

µg.mL^{-1}) (ρ <0.05) when compared to untreated cells (negative control) showing concentration-dependent behavior (Figure 4). According to Ribeiro et al. (2012), even showing a reduction was not equal to or greater than 75%, from which the plant extract can be considered cytotoxic. On the other hand, for Neri-Numa et al. (2014), an extract that inhibits more than 50% of cell growth and exhibits concentration-dependent behavior is anti-proliferative. The literature warns that this plant species has toxicity, however, such studies used very high concentrations and/or doses in comparison with the data from this study. In this context, Oliveira et al. (2013) demonstrated that *A. cochliacarpa* bark extracts caused hepatotoxicity in mice at concentrations of 125 to 1000 mg.mL^{-1}, higher than in this study. However, Lima et al. (2014) concluded that the ethanolic extract of *Pithecellobium cochliocarpum*, synonym of *A. cochliacarpa*, was a toxic agent in the *in vitro* tests against *Artemiasalina*, as well as in pre-clinical tests (acute) by intraperitoneal route. In this route, doses ranged from 160 (maximum dose free of mortality) to 414.72 mg.Kg^{-1} (minimum dose capable of leading to 100% death of the animals). However, when orally administered, it showed low toxicity; the doses used ranged from 1 to 5 g.kg^{-1}. Thus, exposure conditions, administered or absorbed dose, time and frequency of exposure and routes of administration are variables that should be considered.

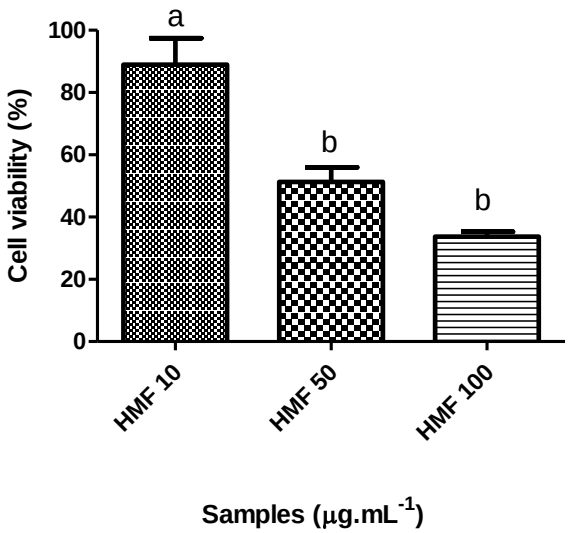

Figure 4. Effect of inner bark hydrometanic fraction *A. cochliacarpa* on viability of J774 macrophages using the 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide assay, MTT.

Antiproliferative activity

The antiproliferative potential of HMF against four human tumor cell lines, was not satisfactory for all tested strains without acceptable and compatible results with what is established by the National Cancer Institute of the United States (NCI, USA). According to this institute, the IC_{50} limit value for extracts considered active as or for anticancer agent should be ≤ 30 μg.mL^{-1} (Buriol et al., 2009; Ribeiro et al., 2012). Such concentrations, from a pharmacological point of view, are more feasible for potential clinical application (Rosa et al., 2014). In addition, HMF was not able to inhibit 75% survival of the cancer cell lines used, so their IC_{50} were not determined.

Antioxidant activity

The antioxidant effect of 10% MHMF, produced for wound treatment, was more effective than that used as a positive control (10% GAM) differing significantly ($\rho < 0.05$) (Table 1).However, when comparing the antioxidant action of this formulation with the antioxidant effect of unincorporated HMF, there is a significant difference when comparing them both, 4.06 ± 1.09 (Dias et al., 2013) which allows to extrapolate that in 6 times for this formulation to reach the same $EC_{50.}$ However, this result does not eliminate the effect of the 10% MFHM, since the AAI shows that the formulation had strong antioxidant activity, which was not observed with 10% GAM, whose potential was moderate.

Still, compared to the others EC_{50} observed in the literature for extracts of plants considered good antioxidants, it is lower, for example the one observed in the extract of *Acacia caesia* (L.), whose EC_{50} was approximately 109 μg.mL^{-1}, 4.5 times greater than 10% MFHM (Thambiraj and Paulsamy, 2012). The same behavior was observed with methanolic extracts from the leaves and bark of *Goniothalamus velutinus* (Airy Shaw), with EC_{50} of 155.32 and 203.96 μg.mL^{-1}, respectively (Iqbal et al., 2015). It is worth mentioning that, researches carried out in different databases, showed only one study with nanostructured formulation and the same biological purpose, but based on silicone, to which trans-resveratrol was incorporated and whose antioxidant potential was unsatisfactory, inhibiting 19.85% of the DPPH$^{\bullet}$. In this sense, both 10% MHMF and 10% GAM acted in the fight against the DPPH free radical action, presenting percentage of inhibition of 93.43 and 61.37%, respectively (Table 1).

Healing activity

Index of Clinical Wound Retraction (CWR) and area of granulation

According to Figure 5,both 10% HMFMG and VG showed improvement in wound healing at 7 days of treatment, being equal to each other ($\rho < 0.05$) and differing only in

Table 1. Antioxidant potential against the free radical 2,2-diphenyl-1-picrylhydrazyl of the microemulsion formulations of the 10% hydrometanic fraction of *A. cochliacarpa* and 10% gallic acid.

Sample	Analysis parameters		
	EC_{50}	AAI	IP (%)
10% hydrometanicfraction microemulsion	24.87 ± 0.62a	1.60	93.43
10% gallic acid microemulsion	57.36 ± 0.45b	0.7	61.37

EC_{50}, Efficient concentration to inhibit 50% of the free radical; AAI, Antioxidant activity index; IP, Inhibition Percent. The IP was calculated based after 30 min and concentration of 60 $\mu g.mL^{-1}$. Statistical differences were determined by the T test ($\rho < 0.05$).

relation to NTG. Wound contraction is closely related to myofibroblastic differentiation which is a phenotypic alteration suffered by fibroblasts that acquire contractile phenotype in response to TGF-β, and cytokine released by monuclear leukocytes (lymphocytes and macrophages/histiocytes) in the injured area (Thannickal et al., 2003). In this sense, the CWR and 10% HMFMG were the same, probably due to the presence of oleic acid in the base formulation, since fatty acids stimulate the synthesis of TGF-β and induce the expression of contractile proteins, such as α-actin of smooth muscle, in fibroblasts, differentiating them in myofibroblasts (Mishra and Simonson, 2008; Santos et al., 2014). In fact, several studies have highlighted that unsaturated fatty acids, including the oleic used in this formulation, act in this process by aiding tissue repair (Cardoso et al., 2011). Therefore, the similar behavior may suggest the low effectiveness of HMF because the compounds present did not act synergistically with oleic acid for this purpose, although there is a tendency. However, when the analysis of the granulation area is observed, it can be seen that at 7 days, the lowest area obtained was in 10% HMFMG, while the largest was VG. Namely, the granulation area or tissue is the point of reference for tissue repair. Its histological characteristic consists of the presence of new and small blood vessels, as well as in the proliferation of fibroblasts. Its size depends on the defect in the tissue created by the wound and the intensity of the inflammation (Santos et al., 2014). Thus, it is suggested that HMF compounds acted to reduce the inflammatory process, resulting in a smaller area of granulation. It is noteworthy that the 10% MHMF showed a strong antioxidant behavior, with a percentage of inhibition above 90% (Table 1), which reaffirms the possibility of being active in the reduction of inflammation since antioxidants have a positive correlation with the anti-inflammatory process (Oliveira et al., 2013). Furthermore, previous research by Da Silva et al. (2011) and Saturnino-Oliveira et al. (2014) showed the effectiveness of *A. cochliacarpa* in the anti-inflammatory action. At 14 days, it was observed that the three groups showed a similar pattern of CWR and granulation area ($p < 0.05$) (Figure 5). However, regarding the observations, at 7 days of treatment, there was an increase in the percentage of retraction of the wounds and an accentuated decrease in the areas of granulation. Such behavior in this period is expected since there is a reduction in the population of myofibroblasts, certainly induced by apoptosis (Ribeiro et al., 2009). However, at 21 days, it was observed that 10% HMFMG presented 100% retraction of the wound area and lower area of granulation differing significantly ($\rho < 0.05$) from VG and NTG, which obtained equal behavior. This event is probably associated with some of the chemical constituents of *A. cochliacarpa* HMF or their synergistic action, which may have increased the local production of TGF-β, thus increasing myofibroblastic differentiation. Vieira et al. (2008) demonstrated the positive action of flavonoids in the healing process and, later, Dias et al. (2014) studying the chemical profile of *A. cochliacarpa* have claimed it to be a species rich in flavonoids. In fact, the major constituent of 10% HMFMG, the + (-) catechin is already cited as a flavonoid with anti-inflammatory potential. However, it has already been cited as a TGF-β inhibitor, one of the growth factors closely related to the process of wound contraction, an effect not observed in this experiment. It is worth noting that at 17 days of this experiment, 10% HMFMG animals stopped receiving the topical application since their wounds were already fully contracted. Similar results were obtained in animals treated with the nanoemulsion eucalyptus oil, oil-in-water type, similar to the present study, at 16 days (Sugumar et al., 2014).

Microscopic wounds analysis

As for collagen deposition (Figure 6), the pattern observed was quite similar between groups over the experimental times, with predominance of collagen III in 7 days, depositing reticulated greenish fibers, characteristic of this type of collagen. On the 14th day, there was both the deposition of type I collagen, with golden and reddish fibers, well interlaced, as well as type III. In 21 days, it was possible to notice the resumption of collagen type III deposition in the three groups, mainly NTG and VG, in

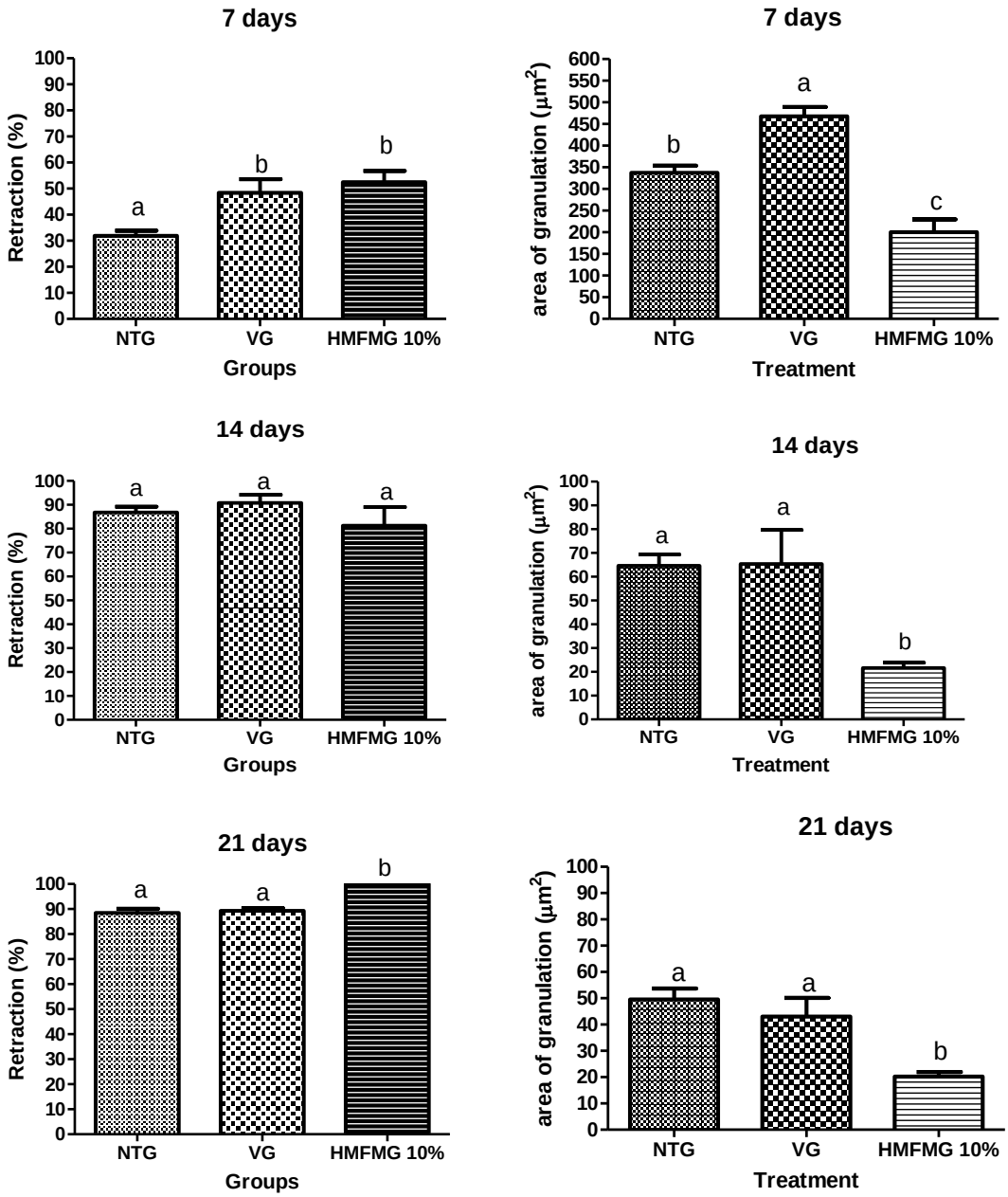

Figure 5. Index of Clinical Wound Retraction and area of granulation demonstrated by the treatment with *A. cochliacarpa* microemulsion, in open wounds of *R. novergicus*, during 7, 14 and 21 days. NTG, Group without treatment; VG, Vehicle group; 10%HMFMG, 10% Hydrometanic fraction microemulsion group compared by Tukey ($p<0.05$).

detriment of type I, as observed on the 7th day. This may be associated with the remodeling process of type I collagen and the synthesis and deposition of type III collagen. This resumption of collagen III deposition at the wound site is probably due to the papillary dermis which also synthesizes it (Isaac et al., 2010). Among the three groups analyzed, in 21 days, 10% HMFMG showed lower deposition of collagen I than the others. This fact is associated with a more accentuated repair process that should have been favored by the constituents of *A. cochliacarpa* HMF, which, as previously mentioned, caused an increase in the differentiation of myofibroblasts

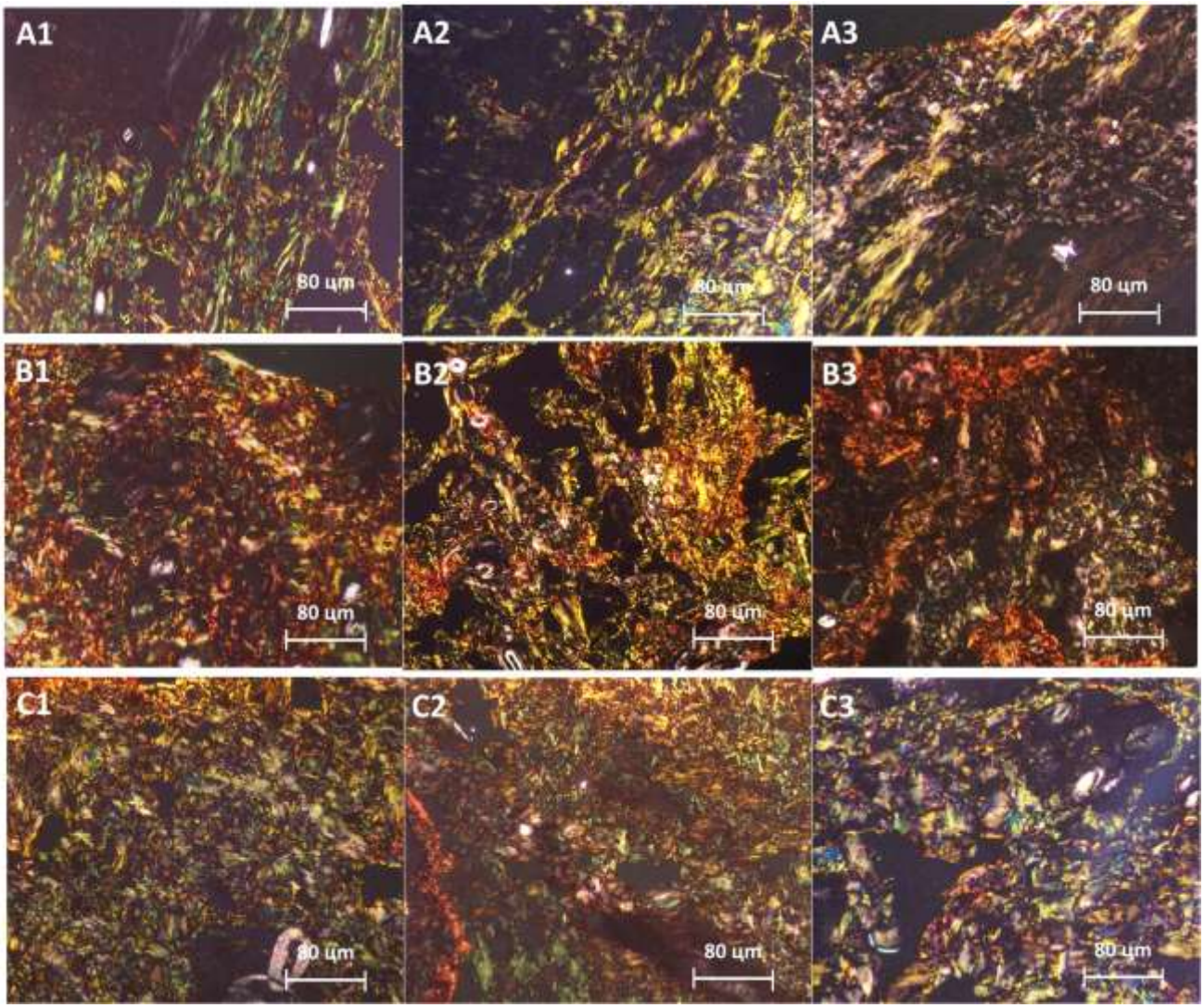

Figure 6. Collagen deposition of animals submitted to the healing activity treated with the 10% hydrometanic fraction microemulsion of the inner bark of *A. cochliacarpa*. A1, B1 and C1, Group without treatment in 7, 14 and 21 days, respectively; A2, B2 and C2, vehicle group in 7, 14 and 21 days, respectively; A3, B3 and C3, hydrometanic fraction Microemulsion of stem bark of A. *cochliacarpa* group coarser in 7, 14 and 21 days, respectively (Sirius Red/Polarized light - greenish birefringence: type III collagen; red-gold birefringence: type I collagen).

and accelerated the remodeling process with its successive stages of production, digestion and orientation of collagen fibrils (Hatanaka and Curi, 2007).

Determination of total sulfhydriles (SH)

According to Table 2, the concentration of SH on the 7th day presented a significant difference ($p<0.05$) between 10% HMFMG and NTG being higher in the first, and may be associated with a higher antioxidant effect. However, on the 14th and 21st days, there was no difference between any of the groups analyzed.

Thiol groups (SH) are structures associated with proteins and susceptible to oxidative damage. Its decrease in blood plasma is indicative of oxidative stress (Silva et al., 2014), and probably the largest area of granulation observed in both the NTG and VG groups is a result of a higher contribution of RNS inflammatory cells (phagocytes) to the wound site, thereby resulting in ROS and consequently causing depletion at SH levels. Among these species, the superoxide anion that reacts with nitric oxide peroxynitrite (ONOO-) was highlighted, whose presence in the tissues or body fluids leads to its rapid protonation, culminating, therefore, with SH depletion (Filippin et al., 2008; Silva et al., 2011). In contrast, the

Table 2. Levels of sulfhydryl (SH) in blood plasma of rats treated with 10% hydrometanic fraction microemulsion of the stem bark of *A. cochliacarpa* compared with vehicle group and group without treatment for 7, 14 and 21 days

Sample	SH (nmol.mg^{-1})		
	7thday	14th days	21th day
10% HMFMG	431.94 ± 89.58^{a}	164.9 ± 41.3^{a}	151.0 ± 81.7^{a}
VG	337.44 ± 66.48ab	185.3 ± 43.3^{a}	163.0 ± 19.8^{a}
NTG	277.54 ± 130.3^{b}	141.0 ± 39.4^{a}	226.1 ± 108.4^{a}

10% HMFMG, 10% hydrometanic fraction microemulsion group; GV, vehicle group; NTG, no treatment group. The results were represented in mean ± SD and analyzed by Tukey ($\rho < 0.05$).

higher concentration of SH present in the blood plasma of 10% HMFMG animals reflects the presence of antioxidant compounds, such as flavonoids present in its composition (Dias et al., 2013). It is noteworthy that at 7 days of treatment, it was found that 10% HMFMG showed a lower area of granulation and that this result was associated with the reduction of the inflammatory process probably caused by the active compounds of HMF.

Conclusion

The microemulsion elaborated with the hydrometanic fraction obtained from the inner bark of *A. cochliacarpa* at 10 %, has antioxidant and healing potential from its constituents, which need to be better explored separately for the knowledge of the biological effectiveness of each one.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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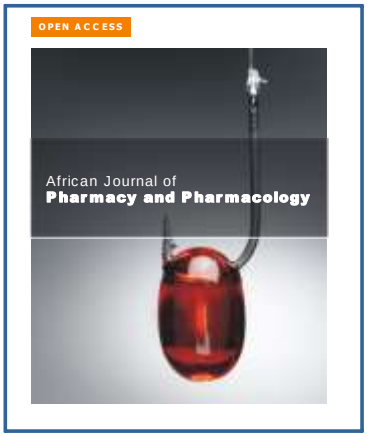

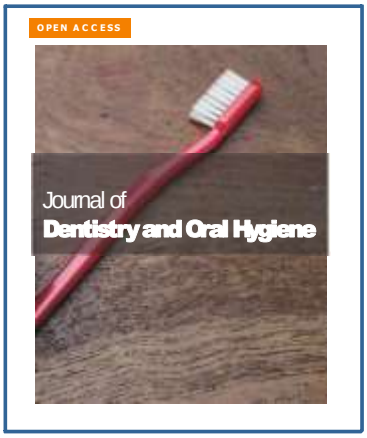

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