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Full Length Research Paper

Anti-inflammatory and antioxidant activities of the hydroethanol extract and fractions of the bark of *Mimosa tenuiflora* (Willd.) Poir.

Marcel S. Nascimento[1], Igor O. Paiva-Souza[2], Ximene A. Fernandes[1], Sabrina Z. da C. de Moraes[1], Silvan S. Araújo[1], Andrea Y. K. V. Shan[1]*, Enilton A. Camargo[2], Antonio E. G. Santana[3], Brancilene S. Araújo [1] and Charles S. Estevam[1]

[1]Natural Products Chemistry Laboratory, Department of Physiology, Federal University of Sergipe (UFS), University City Prof. José Aloísio de Campos, Av. Marechal Rondon, s/n Jardim Rosa Elze, São Cristóvão, Sergipe, 49100-000, Brazil.
[2]Pharmacology Laboratory Inflammatory Process, Department of Physiology, Federal University of Sergipe (UFS), University City Prof. José Aloísio de Campos, Av. Marechal Rondon, s/n Jardim Rosa Elze, São Cristóvão, Sergipe, 49100-000, Brazil.
[3]Natural Resources Laboratory, Institute of Chemistry and Biotechnology, Federal University of Alagoas (UFAL), University City A.C.Simões, Av. Lourival Melo Mota, s/n Tabuleiro dos Martins, Maceió, Alagoas, 57072-900, Brazil.

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This study investigated the antioxidant and anti-inflammatory effects from the inner bark of *Mimosa tenuiflora*. The hydroethanol extract (HEE) and its hexane (HXF), chloroform (CLF), ethyl acetate (EAF) and hydromethanol (HMF) fractions were prepared and submitted to phytochemical screening and antioxidant activity. *In vivo* anti-inflammatory effect was investigated by using 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced edema and myeloperoxidase (MPO) activity in mice ears. Phytochemical prospection of HEE revealed the presence of flavonoids, tannins, xantones, triterpenes, esteroids and phenols. Higher total phenol content was found in EAF and higher percentages of inhibition of 2,2-diphenyl-1-picrylhydrazyl free DPPH radical were found for HEE, EAF or HMF. Lipid peroxidation (LP) induced by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was greatly inhibited by HEE or EAF, while inhibition of $FeSO_4$ - induced LP was higher for HMF. The coadministration of HEE (1 or 3 mg/ear) decreased edema ($p<0.001$) and MPO activity ($p<0.05$). All fractions reduced mice ear edema at the same extent, however, while EAF and HMF reduced MPO activity in mice ear at both 1 and 3 mg/ear ($p<0.001$ and $p<0.05$ respectively), CLF only at 3 mg/ear ($p<0.05$) and HXF did not affect this parameter. Taken together, these results demonstrate that inner bark of *M. tenuifolia* possesses antioxidant and anti-inflammatory effects.

Key words: *Mimosa tenuiflora,* oxidative stress; inflammation, edema, myeloperoxidase activity.

INTRODUCTION

Inflammation is considered a non-specific immune response that may be caused by an infectious agent, ischemia, injuries, and among others agents that can cause tissue injury associated with damage, which is generally related to free radicals (Serhan et al., 2010; Fernandes et al., 2015). The reactive oxygen (ROS) and nitrogen (RNS) species play an important role in modulating the inflammatory response, by stimulating the

release of cytokines and other chemotactic agents, as wells as the expression of adhesion molecules, thus contributing to the induction and maintenance of the inflammatory response (Rotelli et al., 2003).

The use of medicinal plants for the treatment of many diseases, including inflammatory conditions, is an ancient practice, representing the therapy of choice in various communities and, therefore, has become interesting for various research areas such as botany, pharmacology and phytochemistry in order to support the popular use. In Brazil, the market for herbal medicines is growing and presents a great pharmacological potential (Lourenzani et al., 2004). Some compounds found in medicinal plants, such as phenolic compounds, are associated with a high antioxidant capacity and reduced risk for chronic degenerative diseases (Upadhyay and Dixit, 2015). Flavonoids, a subclass of polyphenols, can be found in a variety of foods and are well known for their anti-inflammatory and antioxidant properties (Johnson et al., 2015). In turn, several studies have linked the presence of some antioxidant compounds to anti-inflammatory activity, thereby increasing the interest of researchers to investigate the relationship between these compounds and their anti-inflammatory effect (García-Lafuente et al., 2009).

Among the plants used in folk medicine in Brazil, *Mimosa tenuiflora* (Willd.) Poir. (Leguminosae) is popularly known as “jurema-preta”, a typical tree in the Brazilian semi-arid region, also found in other regions of Latin American, from the northeast of Brazil to the southern of Mexico, which is used for therapeutic purposes, mainly skin burns and inflammation (Maia, 2004; Bitencourt et al., 2014). Lozoya et al. (1990) described antimicrobial activity for the water and ethanol extracts of the bark of *M. tenuiflora*. The ethanol extract of the bark of this plant was also effective against *Staphylococcus aureus* (Padilha et al., 2008; Bezerra et al., 2010). On the other hand, Silva et al. (2013) showed no mutagenic activity for the ethanol extract of the bark of *M. tenuiflora.* Rivera-Arce et al. (2007) have shown that a hydrogel containing 5% of a crude ethanol extract of the bark of this plant was useful for the treatment of venous leg ulceration disease in patients.

Anton et al. (1993) identified triterpenoid or steroid saponins and other compounds (lupeol, campesterol, stigmasterol and beta-sitosterol) in the bark of this plant. Some saponins found in the bark of *M. tenuiflora* were also described as immunomodulatory (Jiang et al., 1992). A study has shown that *M. tenuiflora* aqueous extract reduces inflammatory response caused by *Titius serrulatus* scorpion venom (Bitencourt et al., 2014). A recent study showed the antinociceptive and anti-inflammatory activity for the ethanol extract of the bark of *M. tenuiflora* (Cruz et al., 2016).

Altogether, these studies suggest that the bark of this plant has a therapeutic potential and highlight the need for more information about its pharmacological activities. In this way, the present study aimed to evaluate the antioxidant activity and anti-inflammatory effect of the hydroethanol extract and fractions of the inner bark of *M. tenuiflora*.

MATERIALS AND METHODS

Collection, identification and processing of plant material

The bark of *M. tenuiflora* was collected in April 23, 2008, in the municipality of Piranhas, Alagoas, Brazil, (09º 37 '25 "S 37º45' 24" W). A specialist (Dr. Ana Paula Prata) identified a specimen and an exsiccate was deposited in the Herbarium of Federal University of Sergipe (ASE 13166). The bark was dried at room temperature, reduced to powder and submitted to the tests.

Preparation of the hydroethanol extract and fractions

The dried bark (4.828 kg) was subjected to extraction with 90% ethanol for five days, by exhaustive maceration. After this period, it was filtered and concentrated on rotaevaporator under reduced pressure at 50°C to give the hydroethanol extract (HEE) with a yield of 1.0%. A portion of HEE was dissolved in 40% methanol and subjected to liquid-liquid extraction, to obtain the hexane (HXF), chloroform (CLF), ethyl acetate (EAF) and hydromethanol (HMF) fractions, which were subjected to phytochemical screening, quantification of total phenols and antioxidant and anti-inflammatory activities.

Phytochemical screening and determination of total phenolic compounds

In the phytochemical evaluation, the presence of phenols, tannins, flavonoids, xanthones, catechins, saponins, pentacyclic triterpenoid and free steroid using was tested by using the methods described by Matos (2009).

The total phenol content (TP) was assayed according to the methodology by Sousa et al. (2007) with adaptions. An aliquot (100 μL) to the HEE or fractions (1 mg/mL in methanol) was mixed with 6 mL of distilled water and 500 μL of Folin-Ciocalteu (1 mol/L). After, it was added 2 mL of Na_2CO_3 (15%) and diluted with distilled water to a final volume of 10 mL. This mixture was incubated for 120 min at 23°C and the absorbance read in a spectrophotometer (Bioespectro UV-VIS model SP22), at a wavelength of 750 nm. The content of TP was determined by interpolating the absorbance of the samples against a calibration curve using gallic acid as standard (10 to 350 μg/mL), and results were expressed as mg of gallic acid per g of extract or fraction.

DPPH• free radical scavenging activity

The antioxidant activity was evaluated using DPPH• method

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

(Brand-Willams et al., 1995). Aliquots of a sample stock solution of 0.5 mg/mL of extract or fractions in methanol were added to a solution of DPPH• (40 µg/mL) to obtain final concentrations of 5, 10, 15, 20, 25 and 30 µg/mL of extract and fractions in a reaction volume of 3 mL. The blank was composed of a mixture of the sample analyzed and methanol; gallic acid was used as a positive control. The absorbance values at 515 nm, were measured at 1, 5 and 10 min, and then every 10 min up to 60 min. Results were expressed as percentage of remaining (REM) DPPH• calculated as follows:

$DPPH•_{REM}\% = [DPPH•]\ T/[DPPH•]\ T_0 \times 100$

In this equation, [DPPH•] T is the radical concentration after the reaction with the sample, and [DPPH•] T_0 the initial concentration of DPPH•. From the $DPPH•_{REM}\%$, the percentage inhibition (IP) was obtained over the 60 min. The antioxidant concentration necessary to decrease the initial DPPH• concentration by 50% inhibition (EC_{50}), as well the antioxidant activity index (AAI), were also used for the comparison of the antioxidant capacities of samples.

Lipoperoxidation

The ability to inhibit lipid peroxidation was determined by monitoring the production of thiobarbituric acid reactive substances (TBARS) in lipid-rich medium according to the description by Silva et al. (2007). A lipid solution (1% v/v) homogenizated in 20 mmol/L of phosphate buffer solution (pH 7.4) was obtained and mixed to solutions of the HEE or fractions at a concentration of 200 µg/mL. Lipid peroxidation was induced by the addition of 0.1 mL of AAPH (2,2-azobis (2-amidinopropane) dichloride, 0.17 mol/L) and 0.1 mL of $FeSO_4$ (0.17 mol/L). For the measurement of TBARS, the homogenate (0.5 mL) was incubated with 0.5 mL of saline solution (0.9%) and two milliliters of a thiobarbituric acid/trichloroacetic acid (0.67 and 15% respectively) mixture and boiled at 95°C for 10 min. Subsequently, this mixture was cooled at room temperature and centrifuged at 4 000 x *g* for 10 min. The whole supernatant was taken in spectrophotometer cuvette and read at 535 nm. Trolox was used as the positive control and methanol as negative control. The results obtained were expressed as percentage of inhibition of malondialdehyde (MDA) formation.

In vivo evaluation of anti-inflammatory activity

Animals

Male Swiss mice (20-30 g) were obtained from the Animal Center of the Federal University of Sergipe. Animals were kept at 21 to 23°C with free access to food and water under a 12:12 h light/dark cycle. All experimentation was conducted in agreement with the guidelines of the Brazilian College of Animal Experimentation and the internationally accepted principles for laboratory animal use and care. It was also approved by the Ethics Committee for Animal Use in Research at the Federal University of Sergipe (52/12).

Mice ear inflammation induced by TPA

Anti-inflammatory activity of HEE and fractions was evaluated by using TPA-induced ear edema and neutrophil accumulation. Ear edema induced by TPA was performed according to the method described by De Young et al. (1989), with minor modifications by our group (Bonfim et al., 2014). Animals were divided into five groups (n=6 each): Group 1 received TPA dissolved in acetone (vehicle); group 2 received TPA concomitantly to dexamethasone (0.05 mg/ear); groups 3, 4 and 5 received TPA concomitantly to the HEE (0.3, 1 or 3 mg/ear). In the second set of experiments, treated animals received TPA concomitantly to HXF, CLF, EAF or HMF (1 or 3 mg/ear each).

Briefly, animals were anesthetized with inhalatory isoflurane and 20 µL of TPA (1 µg/ear dissolved in acetone) was topically applied to the surfaces of the mice right ear with a polypropylene tip in the absence or concomitant presence of HEE, HXF, CLF, EAF or HMF, as well as dexamethasone. Each left ear received only the vehicle (acetone) application and each animal was used as its own control. Mice were euthanized by excess of inhalatory isoflurane after six hours of the induction of inflammation and eight millimeter-diameter biopsies were obtained from ears with a metal punch. The ear mass was measured and the edema was calculated by the difference of the mass of the right ear from the left ear in each group.

After this evaluation, the biopsies were submitted to the myeloperoxidase (MPO) activity measurement. Biopsies were immediately placed in a test tube in the presence of 0.5% of hexadecyltrimethylammonium bromide in 50 mmol/L potassium phosphate buffer (pH 6.0). Each tissue sample was homogenized and centrifuged at 12 000 x *g* for five min. After that, samples from the supernatants were submitted to the MPO assay by using a microliter plate scanner. This was performed by mixing 10 µL of sample with 200 µL of o-dianisidine solution (0.167 mg/mL of o-dianisidine dihydrochoride and 0.0005% hydrogen peroxide) and reading the changes in absorbance at 460 nm each 15 s over a period of three minutes. The MPO activity was expressed as MPO units (UMPO) per site (biopsy). One unit of MPO activity was defined as that degrading one µmol of peroxide per minute at 25°C (Bradley et al., 1982).

Statistical analysis

Results from *in vitro* experiments were expressed as mean ± SD. Data from *in vivo* experiments were expressed as mean ± SE. Comparisons were performed by one-way analysis of variance (ANOVA) followed by the appropriated post-hoc test and the results obtained were considered significant at $p < 0.05$.

RESULTS

Phytochemical screening of HEE and fractions

The phytochemical screening of HEE and fractions showed that all samples contained pentacyclic triterpenes and free steroids, while saponins were presented only in EAF and HMF and phenols, taninns, flavonoids and xanthones were presented in HEE, EAF and HMF. Besides, catechins were found neither in HEE nor in fractions.

Total phenolic content and *in vitro* antioxidant activity of HEE and fractions

From the analysis of phenolic content in HEE it was found that this extract contains about 156 µg of phenolic compounds/g of extract (Figure 1). Also, it was found that EAF and HMF possess higher contents of phenolic compounds when compared to HEE, HXF and CLF.

The highest antioxidant activities measured by DPPH• free radical scavenging method were observed in EAF

Table 1. Antioxidant activity of hydroethanol extracts (HEE) and hexane (HXF), chloroform (CLF), ethyl acetate (EAF), and hydromethanol (HMF) fractions of bark of *M. tenuiflora*.

Sample	EC_{50} (µg/mL)	IP (%)	AAI
HEE	7.63 ± 1.22^{b}	95.84	5.34
HXF	31.19 ± 0.84^{c}	51.40	1.28
CLF	44.98 ± 3.28^{d}	34.84	0.89
EAF	5.88 ± 0.19^{b}	94.66	6.79
HMF	6.21 ± 0.22^{b}	95.40	6.44
Galic Acid	1.05 ± 0.20^{a}	92.06	38.09

EC_{50}: Fifty percent effective concentration (mean ± SD; one-way ANOVA followed by Bonferroni´s test); IP: Percentage inhibition of free radical formation; AAI: Antioxidant activity index.

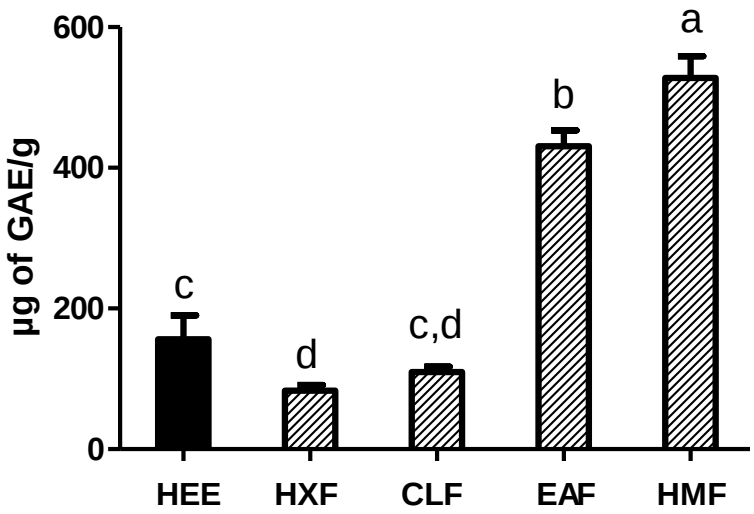

Figure 1. Total phenol content of hydroethanol extract (HEE) and hexane (HXF), chloroform (CLF), ethyl acetate (EAF) and hydromethanol fractions (HMF) of bark of *M. tenuiflora.* Differences of p <0.05 were indicated by symbols a, b, c and d (one-way ANOVA followed by Bonferroni´s test). GAE: Gallic acid equivalent.

and HMF, which did not differ statistically from HEE (Table 1). Lower antioxidant capacities were observed in HXF and CLF, when compared to other fractions or HEE. In addition, higher antioxidant activity was found for the gallic acid (control) when compared with HEE or fractions. Besides, HEE, EAF and HMF presented IP values higher than 90% and AAI above 2.0.

Lipid peroxidation induced by AAPH was greatly inhibited by HEE, EAF and trolox (95.1, 95.0 and 94.3% respectively), while CLF and HMF presented a partial inhibition (40.3 and 45.0% respectively). HXF caused only 5.9% of decrease of AAPH-induced lipid peroxidation. Differently, when the inducer of lipid peroxidation was $FeSO_4$, HMF and trolox produced the higher inhibitory effects (63.0 and 94.3%, respectively). The percentual of inhibition of $FeSO_4$-induced lipid peroxidation for HEE, HXF, EAF or CLF was 27.4, 38.8, 26.0 and 9.1%, respectively.

Effect of HEE and fractions of *M. tenuiflora* on TPA-induced ear edema and neutrophil accumulation in mice

The coadministration of HEE at 1 or 3 mg/ear, but not 0.3 mg/ear, significantly reduced ear edema ($p<0.001$) or MPO activity ($p<0.05$ for 3 mg/ear and $p<0.01$ for 1 mg/ear) induced by TPA (Figure 2A and B, respectively).

The inhibitory activity of fractions of HEE on the edema induced by TPA is shown in Figure 3. The coadministration of HXF, CLF or EAF at doses of 1 or 3 mg/ear significantly reduced the formation of ear edema ($P<0.001$), when compared to vehicle group. At the same doses, HMF also decreased the ear edema ($p<0.01$), in comparison to vehicle group. As an anti-inflammatory control, dexamethasone (0.05 mg/ear) significantly reduced ear edema ($p<0.001$). The percentage of inhibition of TPA-induced ear edema formation for the

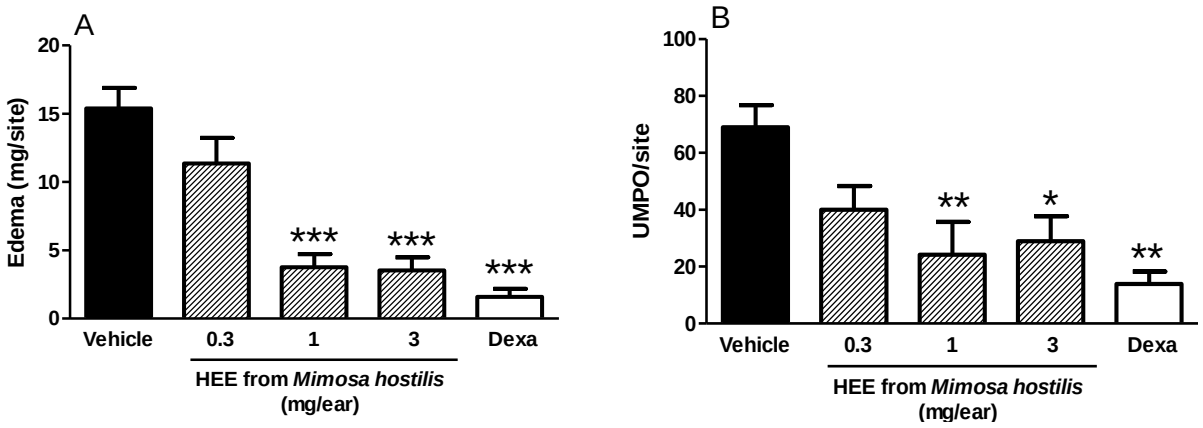

Figure 2. Anti-inflammatory effect of hydroethanol extract (HEE) from the bark of *M. tenuiflora* (0.3, 1 and 3 mg/ear) or dexamethasone (Dexa; 0.05 mg/ear) on TPA (1 μg/ear)-induced mice ear edema (Panel A) and myeloperoxidase (MPO) activity (Panel B). Data are mean ± SEM of edema (mg/site) or MPO (UMPO/site) for n=6 mice. *p<0.05, **p<0.01 or ***p<0.001 vs the respective vehicle group. One-way ANOVA followed by Dunnett´s test.

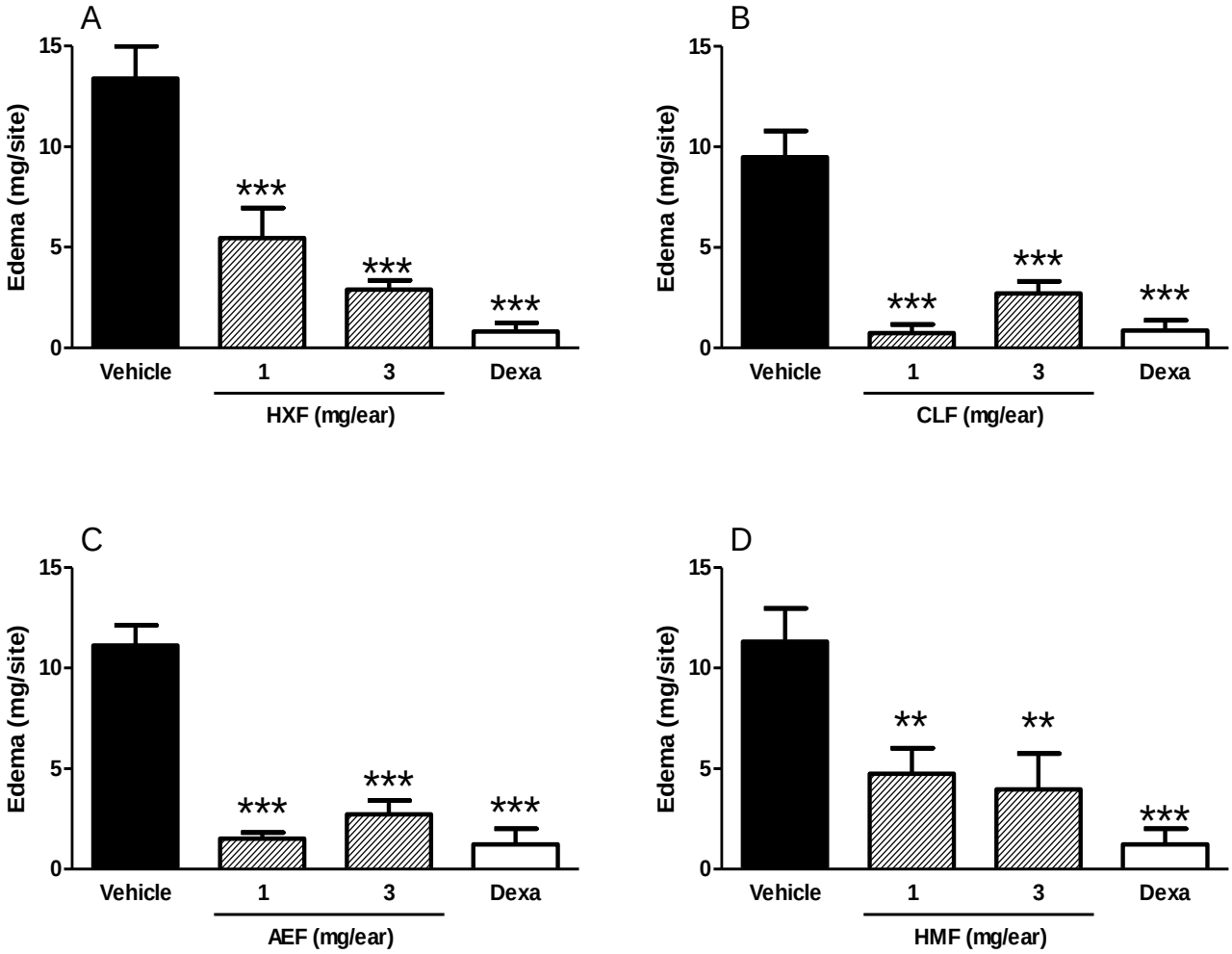

Figure 3. Inhibitory effect of hexane (HXF; Panel A), chloroform (CLF; Panel B), ethyl acetate (EAF; Panel C) or hydromethanol fractions (HMF; Panel D) of bark of *M. tenuiflora* or Dexamethasone (Dexa; 0.05 mg/ear) on TPA-induced ear edema. Data are mean ± SEM of edema (mg/site) for n=6 mice. **p<0.01 or ***p<0.001 vs the respective vehicle group. One-way ANOVA followed by Dunnett´s test.

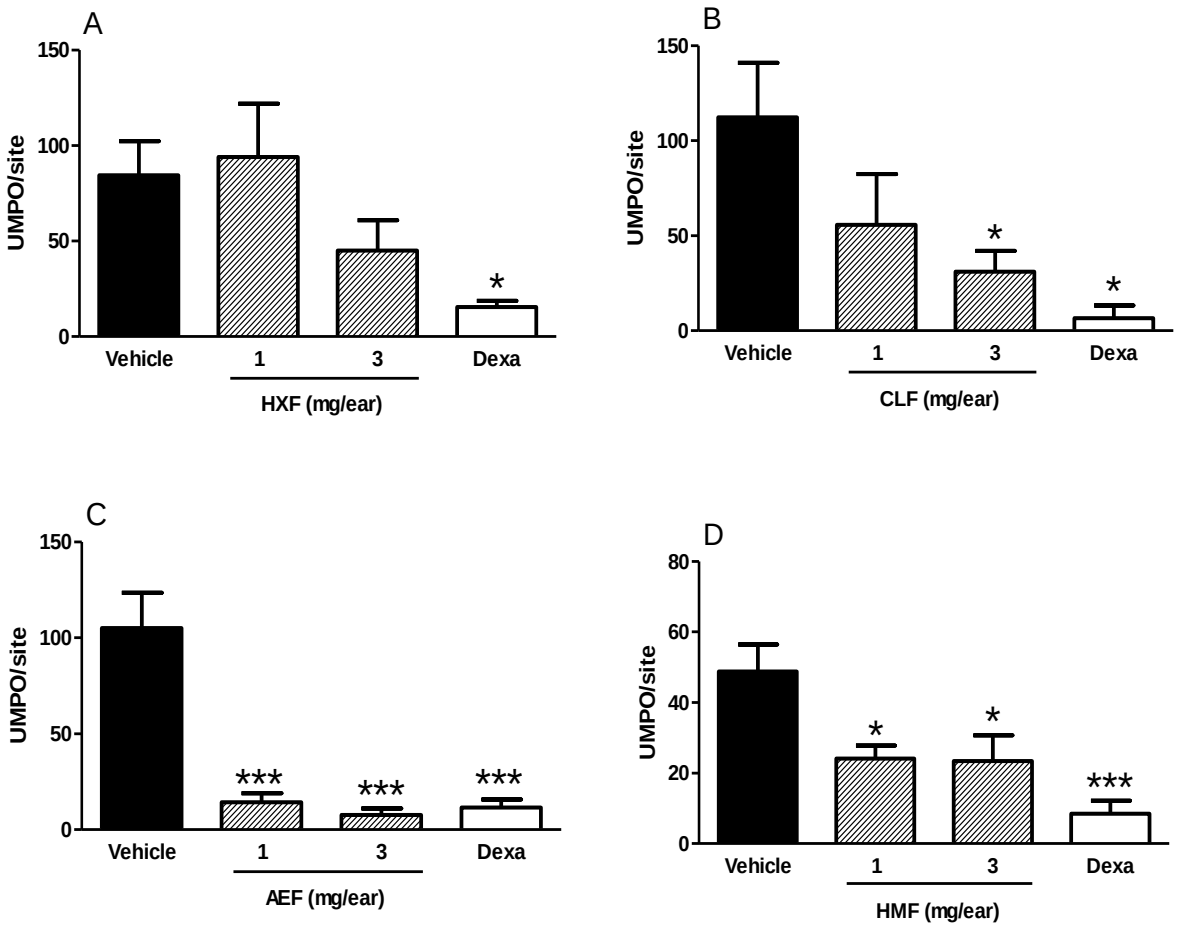

Figure 4. Effect of hexane (HXF; Panel A), chloroform (CLF; Panel B), ethyl acetate (EAF; Panel C) or hydromethanol fractions (HMF; Panel D) of bark of *M. tenuiflora* or Dexamethasone (Dexa; 0.05 mg/ear) on TPA-induced ear neutrophil accumulation. Data are mean ± SEM of MPO (UMPO/site) for n=6 mice. *p<0.05 or ***p<0.001 vs the respective vehicle group. One-way ANOVA followed by Dunnett´s test.

dose of 3 mg/ear of AEF, HMF, CLF and HXF were respectively 75.0, 64.9, 71.4 and 79.1%.

The activity of MPO was measured in the ear biopsies as an estimative of the neutrophil content. It was demonstrated that coadministration of HXF (1 or 3 mg/ear) did not change this activity (Figure 4A). Coadministration of CLF at 3 mg/ear, but not 1 mg/ear, to mice ears significantly reduced MPO activity ($p<0.05$), when compared to vehicle group. Doses of EAF or HMF (1 or 3 mg/ear) also diminished ($p<0.001$ or $p<0.05$ respectively) the activity of MPO, as did dexamethasone (0.05 mg/ear; $p<0.001$), when compared to vehicle group. The percentage of inhibition of TPA-induced MPO activity for the dose of for 3 mg/ear of AEF, HMF and CLF were respectively 92.7, 52 and 72.0%.

DISCUSSION

In this study, the preparation of HEE from the bark of *M. tenuiflora* and fractions was described, as well as the phytochemical, antioxidant potential and anti-inflammatory activity evaluation. The results indicated that the bark of this plant presents topical anti-inflammatory effect that was associated to the phenolic content and antioxidant capacity of HEE and selected fractions.

Few studies have evaluated the phytochemical composition of the bark of *M. tenuiflora.* Anton et al. (1993) have described the presence of saponins and terpenes in the stems of *M. tenuiflora.* Likewise, Sukanya et al. (2009) detected the presence of phenolic substances, as the C-glycosylated flavones, O-glycosylated flavones and tannins. In the present work, by using the phytochemical screening, we demonstrated that HEE from the barks of this plant, EAF and HMF contains phenolic compounds (phenols, tannins, flavonoids and xanthones). Accordingly, the higher concentrations of phenolic compounds were found in EAF and HMF, as assessed by Folin-Ciocalteu method,

although all fractions presented phenols. The highest concentrations of phenolic compounds in EAF and HMF results from the polarity of the extractor liquids that allow hydrogen bonds between the hydroxyl groups present in these compounds and the solvent, increasing its solubility. However, the HEE extracted by the high polar hydroalcohol mixture had low phenolic content when compared to EAF and HMF. The chemical diversity of the compounds, which exerts a competitive interaction with the reagents used in the assessment (Ainsworth and Gillespie, 2007) may explain this finding. No phenolic content was found in HXF and CLF in the qualitative tests and lower concentrations were found in the analytical test. We suggest that the low effective concentration of phenols when fractionated did not allow its detection by the analytical tests applied in HXF and CLF during the phytochemical screening.

Antioxidant activity against DPPH• free radical was correlated with phenolic content of HEE and fractions, because the higher the phenolic content of HEE or fraction, the best the DPPH• scavenging activity presented. According to Procházková et al. (2011), phenolic compounds are excellent radical scavengers, can donate hydrogen to the unpaired electron and remain stable due to the resonant phenoxyl ion formed. In addition, several other studies have shown that phenolic compounds significantly contribute to the antioxidant capacity of medicinal plants (Melo et al., 2010; Vaher et al., 2010; Moura et al., 2011; Silva et al., 2011). Free radical scavenging ability was higher for EAF and HMF, as evaluated by the lower values of AAI (Scherer and Godoy, 2009). These results seem to be primarily related to the abundance of flavonoids and tannins presented in these fractions.

In consonance with the phenolic content and DPPH• activity, the inhibition of lipoperoxidation caused by AAPH was more pronounced to EAF and HMF. However, $FeSO_4$-induced lipoperoxidation did not correlate with the other antioxidant data, since lipoperoxidation was more inhibited by HMF and HXF. The $FeSO_4$ can react with oxygen, superoxide anion radical and through the Fenton´s reaction can be dismutated to hydrogen peroxide, yielding hydroxyl radicals (Barreiros and David, 2006), which are responsible for initiating lipid peroxidation (Vasconcelos et al., 2007). Thus, these results suggest that the compounds present in HMF predominantly acts during the initiation of lipid peroxidation. Conversely, it can be suggested that EAF and HEE have compounds that acts mainly on the radicalar propagation phase. According to Silva et al. (2006), AAPH is decomposed at physiological temperature, in the presence of oxygen, and conducts to attacks to peroxyl radicals, that in turn scavenges hydrogen from lipids. These mechanist differences between AAPH and $FeSO_4$-induced lipoperoxidation can partly explain the dissimilarities in the results between the fractions (mainly HMF and EAF) from HEE.

Besides the antioxidant activity of HEE from bark of *M. tenuiflora* and its fractions, in this study the anti-inflammatory activity of the extract and fractions was investigated by using TPA-induced ear inflammation. This model of cutaneous inflammation was previously described as suitable for evaluating the activity of both steroidal and non-steroidal anti-inflammatory drugs after topical administration (Schiantarelli et al., 1982). Topical administration of TPA, an active compound isolated from croton oil (Saraiva et al., 2010), concomitant with HEE led to the findings that HEE reduced the ear edema and MPO activity in mice ear, which were indicatives of decreased plasma protein and liquid extravasation, as well as neutrophil accumulation, respectively. These results clearly indicated that HEE from bark of *M. tenuiflora* possesses topical anti-inflammatory effect. Corroborating our findings, the ethanol extract from bark of *M. tenuiflora* reduced inflammation induced by carrageenan or formalin in mice, as well as produced antinociceptive effect (Cruz et al., 2016). In this study it was shown that HEE contains high phenolic content and antioxidant activity against DPPH• radical, as well as reduces lipoperoxidation induced by $FeSO_4$ or AAPH. This antioxidant activity can be directly correlated with the anti-inflammatory activity of HEE. According to (Scalbert and Williamson, 2000) natural substances groups that scavenge free radicals, such as flavonoids, present biological effect on inflammation.

Many studies have described that phenolic compounds with antioxidant activity possess anti-inflammatory effect. A study performed with polyphenol resveratrol demonstrated that it inhibited the mRNA expression of cyclooxigenase-2, inducible nitric oxide synthase and some adhesion molecules (Rahman et al., 2006), that are important mediators of inflammation. Thus, HEE from bark of *M. tenuiflora* may present bioactive compounds that can interfere with the expression of these mediators, especially regarding to the inhibition of adhesion molecules, which are needed for neutrophil, as well as other leukocytes, migration to the focus of inflammation. However, we cannot discharge the possibility that other mechanisms are contributing to the anti-inflammatory effect of HEE, like its antioxidant activity.

Once the anti-inflammatory activity of HEE from bark of *M. tenuiflora* was established, the fractions prepared from HEE were also evaluated for this effect. Interestingly, it was observed that all fractions, when coadministrated with TPA, decreased the edema induced by this compound, but fractions with higher phenolic content and antioxidant activity caused more pronounced reduction of the MPO activity in mice ear. Comparatively, 1 mg/ear of EAF and HMF decreased the MPO activity, but for CLF it was necessary 3 mg/ear to reduce the MPO activity. Besides, for HXF, even this dose was not enough to reduce the MPO activity. Reduced MPO activity can also be related to the phenolic content of EAF and HMF and antioxidant activity. As our data indicate that these

fractions contain flavonoids, tannins and phenolic compounds, the anti-inflammatory activity can be attributed to their action. In this way, it is largely known that flavonoids are able to reduce the inflammatory response through inhibition of phospholipase A_2, cyclooxygenases, lipoxygenases and inducible nitric oxide synthase, besides other effects. These enzymes can generate inflammatory mediators that, in turn, may modulate the expression of adhesion molecules, such as ICAM-1 or VCAM-1 (Mutoh et al., 2000; Nijveldt et al., 2001; Raso et al., 2001; Chen et al., 2004; Peana et al., 2006; Rahman et al., 2006; Valério et al., 2009; García-Lafuente et al., 2009; Serafini et al., 2010).

Besides, the recent study by Cruz et al. (2016) has evaluated the hexane, dichloromethane, ethyl acetate and butanol fractions of the ethanol extract from bark of *M. tenuiflora* and found that hexane, dichloromethane and ethyl acetate caused anti-inflammatory effects in mice, although all fractions reduced nociception induced by various stimuli. However, it was not possible to establish a correlation among the polarity of the solvents used to obtain the fractions and the pharmacological effects from the study of Cruz et al. (2016), given the variability of the effects in the tests employed. This study also isolated a flavonoid called sakuranetin from *M. tenuiflora*, which also induced an anti-inflammatory and antinociceptive effect in mice.

The identification of phenolic compounds presented in HEE from *M. tenuiflora* or fractions was not yet performed, but in this study, the partition of HEE has shown that phenolic compounds may be of importance to the anti-inflammatory effects, mainly those related to the neutrophil migration.

Conclusion

In summary, this study demonstrated that HEE from *M. tenuiflora* and its fractions (mainly EAF and HMF) possess compounds with antioxidant properties and phenolic contents that seem to cause anti-inflammatory effect in mice ear. In this way, this plant may be of interest to search for new bioactive molecules for treating inflammatory conditions.

Conflict of Interests

The authors have not declared any conflict of interests**.**

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Experimental evaluation of wound healing activity of *Croton macrostachyus* in rat

Abraham Fikru Mechesso*, Ayele Tadesse, Reta Tesfaye, Wondmagege Tamiru, Tadesse Eguale

School of Veterinary Medicine, Hawassa University, Hawassa, Ethiopia.

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Croton macrostachyus **leaves are used for treatment of wounds by traditional healers in Ethiopia. Despite the use of this plant in the treatment of wound, there is limited data to support its medicinal use. The present study provides scientific evaluation for the wound healing potential of methanolic extract of *C. macrostachyus* leaves in rats. The leaves of *Croton macrostachyus* were studied for the presence of some secondary metabolites and wound healing activity. Ointments were made by incorporating the methanolic extract in simple ointment base B.P. in the concentration of 5 and 10% (w/w). Standard Nitrofurazone was used for comparison. Wound healing activity was studied, using excision and incision wound models. In excision wound model, percentage wound contraction, period of epithelization and morphological changes on the healed wounds were studied while incision wound model was used to determine breaking strength. The results were expressed as mean ± standard error of mean (SEM) and comparisons among treatment groups were made using one-way analysis of variance. Phytochemical screening of the methanolic extracts of leaves of *C. macrostachyus* showed the presence of different metabolites such as flavonoids and saponins which are reported to have significant wound healing activity. The results of epithelization period, percentage of wound contraction and morphological evaluation of groups of animals in the test groups showed significant ($p < 0.05$) wound healing activity compared to those treated with simple ointment. Similarly, the difference in breaking strength was significant ($p < 0.05$) for both 5 and 10% (w/w) methanol extract of *C. macrostachyus* ointment treated groups. Morphological evaluation showed a relatively better healing and growth of hair around the wound area in the 10% methanol extract of *C. macrostachyus* ointment treated group. Methanolic extract of *C. macrostachyus* enhanced wound healing significantly, corroborating the folk medicinal use of this plant.**

Key words: *Croton macrostachyus*, excision, incision, *in vivo*, wound healing.

INTRODUCTION

Wound is a major problem in developing countries, often having severe complications and involving high costs of therapy (Shenoy et al., 2011). Wound healing is a complex process which requires the collaborative efforts

*Corresponding author. E-mail: abrahamfikru@yahoo.com, abrahamf@hu.edu.et. Tel: +251-912-082845. Fax: +251-46-220-5421.

of different tissues of varying cell lineage (Suntar et al., 2011). It involves platelet aggregation, blood clotting, formation of fibrin, alteration in the ground substances, angiogenesis, and re-epithelialization. Healing is not complete until the disrupted surfaces are firmly knit by collagen (Guo and DiPietro, 2010). In spite of remarkable advances in pharmaceutical drug industry, the availability of drugs capable of stimulating the process of wound healing is still limited (Abraham et al., 2012). Conventional treatment for established wounds incorporates common principles that apply to the management of all wounds, including debridement of necrotic tissue, maintenance of a moist wound bed and control of infection. Unfortunately, there are no widely accepted, standardized protocols that define optimal standard treatment or the appropriate intensity of treatment delivery (Samson et al., 2004).

Medicinal plants are used extensively to enhance the healing of wounds (Suntar et al., 2011). *Croton macrostachyus* is a deciduous tree belonging to the family Euphorbiaceae. The leaves are large and green, turning to orange before falling. It is also characterized by creamy to yellow-white colored flowers with green (when young) to grey (at maturity) fruits. *C. macrostachyus* is commonly named as 'Bisana' in Ethiopia and is traditionally used for the treatment of wound (Giday et al., 2007; 2009; Teklehaymanot and Giday, 2007) malaria, rabies, and gonorrhea (Giday et al., 2007), *Tinea versicolor*, diarrhea, hepatitis, jaundice, and scabies (Teklehaymanot and Giday, 2007).

Studies revealed that *C. macrostachyus* has a wide range of activities which justifies its traditional use in the treatment of wound healing. The plant showed antimicrobial activity against pathogens which are common contaminants of wound, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Kalayou et al., 2012; Taye et al., 2011). The plant is also reported to have significant anti-inflammatory and analgesic activities in animal models (Kamanyi et al., 2009). Even though majority of the Ethiopian population uses traditional medicine, only limited studies were conducted on the traditional medicinal plants compared to the multicultural diversity and the diverse flora of the country (Tekalign et al., 2010). Therefore, based on the aforementioned ethnobotanical and *in vitro* studies which suggest the importance of the plant in enhancing wound healing, the present study was conducted to evaluate the *in vivo* wound healing potential of crude extracts of *C. macrostachyus* leaves using an excision and incision wound models.

METHODOLOGY

Preparation of the extract and ointments

C. macrostachyus was collected from Gondar, North Ethiopia. Botanical authentication was obtained and voucher specimen was also deposited at the mini-herbarium of Institute of Pathobiology, Addis Ababa University. The leaves were garbled, dried under shade and grinded prior to storage. The grinded plant material was extracted with 80% methanol by maceration for 48 h with frequent agitation (Debella, 2002) and the resulting liquid was filtered with filter paper (Whatman No. 1, Whatman Ltd., England). Maceration was repeated three times and the filtrates of all portions were combined in one vessel. The methanol was removed by evaporation using Rota vapor at a temperature of 40°C. Simple ointment which is composed of cetostearyl alcohol, wool fat, white paraffin, and hard paraffin was prepared according to the standard guide line (Gaur et al., 2009). Finally, ointments containing 5 and 10% (w/w) methanol extract of *C. macrostachyus* leaves were made using the simple ointment as a vehicle.

Preliminary phytochemical screening

The methanolic extract was tested qualitatively for different phytoconstituents using various chemical test procedures according to the standard methods described by Debella (2002).

Test for alkaloids

Two grams of thoroughly grinded plant material was treated in a test tube with 5 ml of 1% HCl for 30 min in a water bath. The suspension was filtered through cotton into a test tube and 5 drops of Mayer's reagent was added. The formation of whitish opalescence was regarded positive for the presence of alkaloids.

Test for anthraquinones

Test for free anthraquinones: The hydroalcholic extract of the plant material (about 100 mg) was shaken vigorously with 10 ml of benzene. The extract was then filtered and the filtrate was treated with 5 ml of 10% ammonia solution. The mixture was shaken and observed for the presence of a pink, red or violet color in the ammonia phase.

Test for O-anthraquinone glycosides: To about 5 g of powdered plant material, 10 ml of 5% H_2SO_4 was added and boiled for 1 h. The mixture was filtered, cooled and extracted with 10 ml of benzene. To 5 ml portion of the filtrate, equal volume of 10% ammonia solution was added and shaken. The formation of a pink, red or violet color in the aqueous phase (ammonia phase) indicates the presence of O-anthraquinone glycosides

Test for phytosterols (Salkowski reaction)

One gram of powdered plant material was macerated with hexane, filtered and concentrated. The concentrated residue was dissolved in chloroform. Three to five drops of concentrated H_2SO_4 was added carefully, the production of a red or reddish brown or violet color was regarded as positive for the presence of steroidal compounds.

Test for plyphenols (Phenolic compounds)

To 2 ml of filtered solution of aqueous macerate of a plant material, 3 drops of a mixture of 1 ml 1% $FeCl_3$ and 1 ml 1% $K_3Fe(CN)_6$ were added. The formation of a green blue color indicates the presence of polyphenols.

Test for saponins

Filtered solution of the extract (10 ml) was shaken in a large test

tube; the formation of honeycomb froth that persisted for half an hour indicates the presence of saponins.

Test for tannins

To 2 ml of the extract few crystals of sodium nitrate and 2 to 3 drops of 0.1 N HCl were added and observed for brown coloration.

Test for flavonoids

Diluted ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated H_2SO_4 (1 ml) was added and a yellow coloration that disappears on standing indicates the presence of flavonoids.

Experimental animals

Healthy albino rats (weight, 220 to 250 g) and mice (weight, 25 to 30 g) were procured from the Ethiopian Public Health Institute (EPHI). The animals were left for 8 days at room conditions for acclimatization. The animals were housed in polypropylene cages on standard pellet diet and water *ad libitum*. At the end of the experiment, animals were killed under halothane anesthesia. All experiments were carried out after the approval of the ethics committee at the Faculty of Veterinary Medicine, University of Gondar, Ethiopia and following the European guidelines for care and uses of laboratory animals (EEC Directive of 1986; 86/609/EEC).

Acute oral toxicity study

The acute toxicity study was carried out according to OECD guidelines – 425. Mice (25 to 30 g) were used to determine the safer dose. Distilled water was used as a vehicle to suspend the extract and administered orally at a dose of 2000 mg/kg. Animals were observed individually for changes in skin color, eyes and behavioral pattern.

During the experiment, the animals were weighed, food and water intake were monitored. Attention was also directed to observations of tremors, convulsions, diarrhoea, lethargy, sleep, and coma.

Observation was carried out at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter for a total of 14 days (OECD 425, 2008).

Wound healing activity

The animals were grouped into four groups, namely, control (positive and negative) and two test groups with six animals in each group. The animals in the negative and positive control groups were treated with simple ointment base B.P and Nitrofurazone (Galentic Pharma, India) ointment, respectively. Whereas, the test groups were treated with ointment containing 5 and 10% (w/w) methanol extracts of leaves of *C. macrostachyus*.

Circular excision wound model

The hair on the dorsal thoracic area of the rats was shaved and anesthetized with Halothane. Ethanol (70%) was used to clean the shaved area and a circular wound of 350 mm^2 was created on the depilated dorsal thoracic region of each rat.

Measurement of wound area

The ointment was topically applied to the wounds once daily until complete wound closure. The measurements of the wound areas were taken on the day of wound infliction (350 mm^2) and once every 2 days using transparent paper and a permanent marker. The recorded wound areas were measured using graph paper and the measurement was used to calculate the percentage wound contraction, considering the initial size of wound as 100% (Shenoy et al., 2011).

$$\text{Wound contraction (\%)} = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100$$

Measurement of epithelialization period

The epithelization period was determined by considering the number of days required for falling of the scab without any residual raw wound (Shenoy et al., 2011).

Morphological evaluation

Morphological evaluation was carried out to assess the change in skin surface, color, growth of hair, and presence or absence of unhealed wound among the test and control groups. Representative photographs were also taken during the wound healing period.

Determination of breaking strength

Linear incision wound model was used to assess the breaking strength of the healed wounds. The rats were anaesthetized with Halothane and shaved with shaving machine. Five centimeters long, linear-paravertebral skin incision was made on either side of the vertebral column at the distance of 1 cm from the midline. The incised skin was stitched with surgical thread and a curved needle at the intervals of 1 cm. The wounding day was considered as day 0. The 5 and 10% (w/w) ointments containing methanol extract of *C. macrostachyus* leaves, nitrofurazone and the simple ointment were applied topically once a day for a total of 9 days. The sutures were removed on the 9th day and breaking strength of each groups of animals were determined on the 10th day using continuous water flow technique. Briefly, anesthesized animals were secured on operation table in its natural position. Two forceps were firmly applied on the skin on either side of the incised and healed wound. The forceps on one side was hooked to a metal rod and fixed firmly to the operation table, while the other to a light plastic container with a string which passes over a pulley. Water was allowed to flow in to the plastic container at constant rate so as to build gradual pulling force necessary to disrupt the wound. As soon as the gaping of the wound was observed, the water flow was cut off. Further opening of the wound was avoided by removing the pulling force immediately, which was achieved by lifting up the plastic container. The volume of water in the plastic container was measured and converted to the corresponding weight assuming the density to be equal to ‘one’. The breaking strength was expressed as the maximum weight of water necessary to bring about breaking of the wound. Three such readings were recorded for a given incision wound and mean breaking strength for each animal was used to calculate the groups mean (Lee, 1968).

Statistical analysis

Percentage wound contraction was calculated as a percentage of

Table 1. Effect of topical application of methanol extract of *Croton macrostachyus* on percentage wound contraction of an excision wound.

Day	Wound area (mm^2) ± SEM (% Contraction)			
	Simple ointment	5% methanol extract	10% methanol extract	Nitrofutrazone
Day 2	288.67 ± 3.31 (17.53)	286.08 ± 13.07 (18.29)	286.67 ± 4.13 (18.1)	288.92 ± 4.59 (17.45)
Day 4	167.92 ± 14.25 (52.02)	158.17 ± 5.00 (54.81)	158.83 ± 4.78 (54.62)	120.04 ± 6.67** (65.7)
Day 6	77.67 ± 2.12 (77.81)	52.79 ± 1.89** (84.92)	23.29 ± 6.23*** (93.35)	49.54 ± 5.07** (85.87)
Day 8	45.50 ± 2.25 (87.02)	32.96 ± 2.14** (90.58)	19.67± 1.54*** (94.38)	17.96 ± 2.83*** (94.87)
Day 10	28.88 ± 1.25 (91.75)	16.50 ± 2.78*** (95.29)	7.33 ± 0.61*** (97.9)	3.83 ± 0.40*** (99.91)
Day 12	17.42 ± 1.90 (95.02)	7.58 ± 1.10*** (97.84)	1.17± 0 .42*** (99.67)	0.00 ± 0.00*** (100)
Day 14	9.38 ± 0.89 (97.32)	0.67 ± 0.42*** (99.81)	0.00 ± 0.00*** (100)	0.00 ± 0.00*** (100)

n = 6 animals in each group. ***P < 0.001; **P < 0.01; *P < 0.05.

Table 2. Effect of topical application of methanol extract of *Croton macrostachyus* on epithelization period and breaking strength.

Group	Epithelization period (days)	Breaking strength (g)
Nitrofurazone	11.67 ± 0.42***	547.33 ± 6.92***
5% methanol extract	14.17 ± 0.31*	484.33 ± 8.20*
10% methanol extract	12.67 ± 0.42***	500.83 ± 6.85***
Simple ointment	15.83 ± 0.31	445.17 ± 9.87

n = 6 animals in each group. ***P < 0.001; **P < 0.01; *P < 0.05.

the corresponding initial day (day 0) wound in mm^2. Data was processed using SPSS software Version 20.0. Results between treatment groups were compared using one-way ANOVA and the results were considered significantly different at P value < 0.05.

RESULTS

Preliminary phytochemical screening

Qualitative phytochemical analysis of methanolic extract of leaves of *C. macrostachyus* showed the presence of free anthraquinones, flavonoids, phytosterols, polyphenols, saponins and tannins; however, the extract was found to be negative for alkaloids.

Acute oral toxicity studies

The methanolic extract of *C. macrostachyus* was found to be safe up to 2000 mg/kg body weight by oral route. None of the animals showed behavioral, neurological and physical changes such as convulsion, coma, restlessness, lacrimation and diarrhea.

Excision wound study

The effects of methanolic extract of different doses of *C. macrostachyus* on percentage wound contraction and epithelization period are shown in Tables 1 and 2. The percentage of wound contraction of animals treated with ointment containing 5 and 10% (w/w) methanolic extract showed significant ($p < 0.05$) difference as of the 6th day after treatment as compared with the simple ointment treated group (Table 1). Similarly, the test groups required significantly shorter epithelization period (Table 2).

Gross morphological evaluation

Photographs of rats at 10th day post treatment with Nitrofurazone ointment, 10% extract ointment, 5% extract ointment and simple ointment are shown in Figure 1. The results showed progressive changes in percentage wound contraction in test groups as compared with the negative control (simple ointment treated) group. 10^{th} day photographs demonstrated that the 10% (w/w) extract of *C macrostachyus* ointment treated group demonstrated relatively better healing in which the color was close to the normal skin with smooth surface and relatively good growth of hair. Whereas, rats treated with ointment containing 5% (w/w) of the extract showed the presence of unhealed wound with insignificant growth of hair.

Breaking strength

Rats treated with 5 and 10 % (w/w) ointments of the

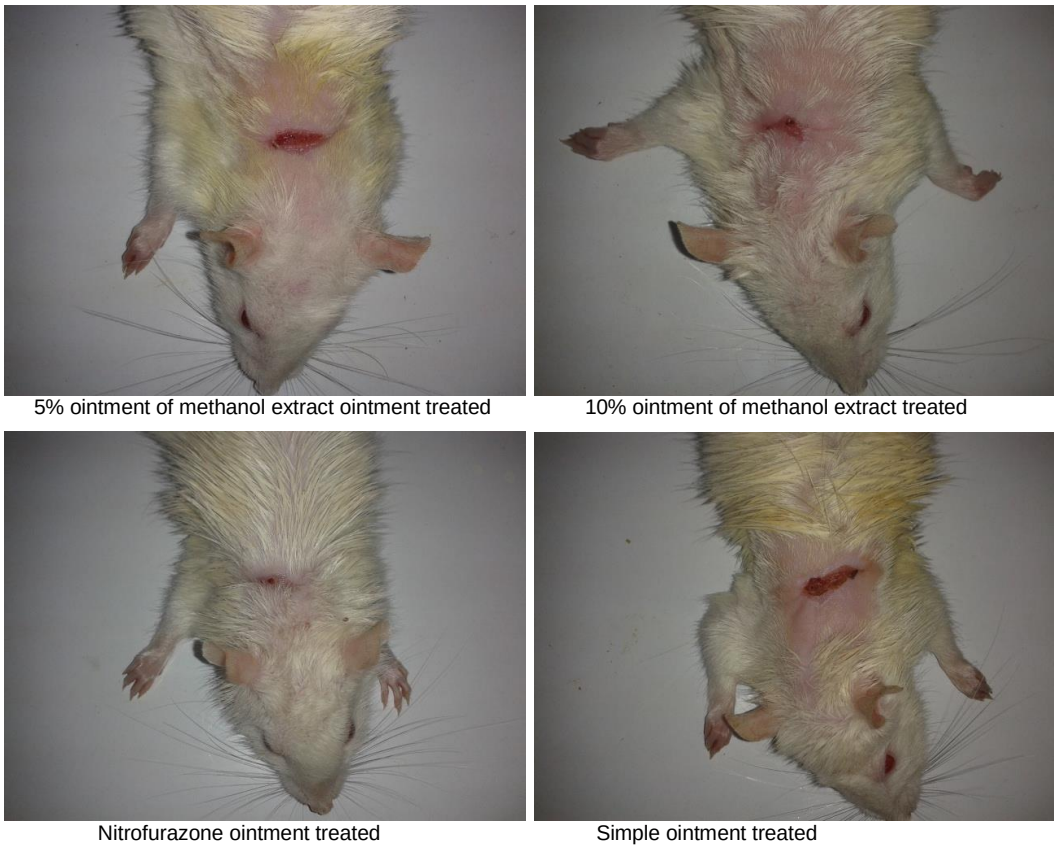

Figure 1. Photographs of rat dorsal wound at 10th day post treatment.

extract showed higher breaking strength, with the 10% ointment treated wounds being stronger than the 5% ointment treated wounds (Table 2).

DISCUSSION

Many studies indicated that plant products are potential agents for wound healing and largely preferred, because of the absence of unwanted side effects and their effectiveness (Joseph and Justin, 2011). In the excision wound model of this study, progressive reduction in wound area and enhanced period of epithelization were observed in both 5 and 10% ointment of methanolic extract of *C. macrostachyus* treated groups, while the fastest (12.67 days) and complete wound healing (100% wound contraction) were observed in the 10% (w/w) extract treated group, compared with the negative control group. The changes in the test groups might be attributed to the potential of the test extracts or its constituents to promote epithelization either by facilitating proliferation or by increasing the viability of epithelial cells (Mukherjee et al., 2013).

Wound has little breaking strength in the beginning, which increases rapidly during healing due to the synthesis and maturation of collagen (Wang et al., 2011). Collagen imparts strength and elasticity to healed skin. As the wound heals, collagen molecules are synthesized and laid down at the wound site. These molecules become cross-linked to form fibers (Pather et al., 2011). In this study, the strength of the repaired wound tissue might be the result of the remodeling of collagen and the formation of stable intra- and inter-molecular cross linking which is necessary for maturation of collagen as described by Pather et al. (2011) and Abraham et al. (2012) on different plant extracts. Accordingly, these results may suggest that the extracts could increase collagen synthesis and possibly aid in formation of cross linkages as the collagen matures.

In vitro and *in vivo* studies indicated the use of plant materials as topical antimicrobial agents to enhance wound healing (Akkol et al., 2011; Nayak et al., 2011). Similarly, *C. machrostachyus* has been reported to show antimicrobial activity against those pathogens which commonly infect wounds such as *Streptococcus pyogens* (Taye et al., 2011) and *Pseudomonas aeruginosa* (Wagate et al., 2010). Usually, the first aim of the wound management is to keep the wound free of infections and complications. Such types of agents are always required to contribute to the rapid healing of wound (Akkol et al., 2011). Consequently, the antimicrobial activity of the 80% methanol extract of *C. machrostachyus* might be

associated with the wound healing activity.

A study conducted by Teugwa et al. (2013) showed that methanolic extract of *C. machrostachyus* has an anti-oxidant activity. Earlier studies also indicated that antioxidant activity of plant extracts contributed significantly to wound healing activity (Pesin Suntar, 2010). Similarly, the antioxidant effect Vitamin C has been shown to contribute significantly during wound healing (Weeks and Perez, 2009). Therefore, the antioxidant activity of *C. machrostachyus* might contribute to its wound healing activity.

Secondary metabolites like anthraquinones, flavonoids, phytosterols, polyphenols, saponins and tannins were among the major phytoconstituents found in this plant. A number of secondary metabolites/active compounds isolated from plants have been demonstrated in animal models as active principles responsible for facilitating healing of wounds. Previous study on *Terminalia arjuna* showed that tannins enhance wound healing action by improving regeneration and organization of the new tissue (Chaudhari and Mengi, 2006). Flavonoids are known to reduce lipid peroxidation by preventing or slowing the onset of cell necrosis and by improving vascularity. Accordingly, a drug that inhibits lipid peroxidation is believed to increase viability of collagen fibrils by increasing strength of collagen fibres, increasing circulation, preventing cell damage and by promoting DNA synthesis (Pesin Suntar, 2010; Upadhyay et al., 2011). Flavonoids are also known for their astringent and antimicrobial property (Devipriya and Shyamala, 1999). Thus, wound-healing property of *C. machroystachus* may attribute it to the individual or additive effect of the phytoconstituents present.

In conclusion, the leaf extracts *C. macrostachyus* has remarkable wound healing activity and it may have a tremendous potential for treating wound. Further studies with purified constituents are needed to isolate active component (s) responsible for its wound healing activities and to understand the complete mechanism of wound healing activity.

Competing interests

The authors have not declared any conflict interests.

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Full Length Research Paper

Securing the pharmaceutical supply chain: A study of the use of Mobile Authentication Service (MAS) among the Nigerian populace utilizing antimalarials

Justine, Aisagbonhi[1] and Ilomuanya, Margaret O.[2]*

[1]West African Postgraduate College of Pharmacists 6, Taylor Drive, Medical Compound, P. M. B 2023, Yaba, Lagos, Nigeria.
[2]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy. University of Lagos, P. M. B 12003 Lagos, Nigeria.

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This study investigated the effectiveness of the Mobile Authentication Service (MAS) introduced on antimalarial medicines (AMs) in curbing the incidences of counterfeiting for this group of pharmaceuticals. The assessment of the knowledge, attitude and use of the technology by Nigerian citizens, uncovering areas of deficit as well as proffering recommendations on the current use pattern of MAS by Nigerians was carried out. Nine hundred (900) questionnaires were used to assess the awareness of the Nigerians on the availability of the MAS feature on AMs and their knowledge on the correct use of the feature to get needed information about the authenticity of the AMs. 78% of respondents claimed to have heard about Short Messaging Service to check for counterfeits in AM. 51.4% of the respondents who knew about MAS had actually scratched the tag or had been involved in the process of using the service. 48.8% of these respondents received positive responses as feedback upon sending the pin while 4.7% did not receive any feedback response. While MAS does not provide information about the biopharmaceutical properties of these medicines, it confirms the authenticity of the product source and supply chain, thereby guarding against the possibility of infiltration by counterfeiters. This advantage would be of high benefit for artemisinin-combination therapy (ACTs) medicines and other AMs if exploited properly. Nigeria is the first country where MAS has been deployed extensively for the security of pharmaceuticals thus the need to conduct corresponding studies in other countries to ascertain the effectiveness of the MAS.

Key words: Mobile authentication service (MAS), pharmaceutical supply chain, anti-malarial medications.

INTRODUCTION

The global supply chain especially for pharmaceuticals is complex, as many components of medicines and sometimes the entire pharmaceutical product are manufactured far from where they are being supplied and

*Corresponding author. E-mail: milomuanya@live.com, milomuanya@unilag.edu.ng.

consumed (Attaran et al., 2012; Mackey et al., 2012). In the public sector, procurement and distribution of medicines in Nigeria is both centralized and decentralized (Attaran et al., 2012; Iwokwagh, 2013). Most medicines are procured and stored by the individual health care institutions, while medications for HIV, malaria and tuberculosis are centrally procured and distributed from the Federal Central Medical Store (CMS) (Erhun et al., 2004). Each state in the varying geo political zones of Nigeria also has its own Central Medical Store, and procures and stores pharmaceuticals for their public facilities (Amin and Kokwaro, 2007; Onwujekwe et al., 2009). Private sector distribution is handled through pharmaceutical wholesalers although it is widely accepted that unlicensed wholesalers play a very significant role in drug distribution at this level of the supply chain (NAFDAC/Sproxil 2015).

The advancement in technology worldwide has increased the sophistication of pharma-counterfeiting and in turn compelled The National Agency for Food and Drug Administration and Control (NAFDAC) to employ technological measures in the fight against counterfeiting of medicines (Bansal et al., 2013; NAFDAC /Sproxil 2015). In the recent years, NAFDAC has introduced a range of fake drugs detecting technologies such as the TruScan device (based on Raman spectroscopy), Black eye, Radio Frequency Identification (RFID) and the Mobile Authentication Service (MAS) using Short Message Service (SMS) (Iwokwagh, 2013; NAFDAC /Sproxil, 2015).

The menace of counterfeit medicines especially AMs had assumed alarming proportions and the effects in terms of mortality rates was staggering (NAFDAC/Sproxil 2015). Given the prevalence of mobile technology throughout the world, it made sense to use a technology that was already in every customer's pocket pocket (Global medicine supply chain 2012; Damon Beres 2015). Through drug authentication, consumers can avoid purchasing counterfeit medication, while anonymously and passively providing key intelligence to law enforcement agencies regarding the location of fake drugs, anytime authentications fail repeatedly. The goal was to improve the consumers' overall health and quality of life (Iwokwagh, 2013; Osuolale, 2015).

MAS (also called Mobile Product Authentication) allows consumers to verify that the product they are buying is genuinely from the manufacturer, by using a mobile phone and a simple, free text message. If a fake product is found, a consumer is given a hotline number to call in order to report the fake product, so the issue can be directed to the appropriate authorities.

Among several approaches towards the eradication of fake/substandard pharmaceuticals NAFDAC employed the use of telecommunication in combating this menace. The Mobile Authentication Service (MAS) of NAFDAC is an attempt at turning the mobile phone which is now in the hands of majority of Nigerians into a tool of fighting the war against fake/substandard pharmaceuticals product proliferation in Nigeria (NAFDAC/Sproxil 2015; Osuolale, 2015).

Onwujekwe et al. (2009) in a paper assessing the quality of AMs provided by public and private healthcare providers in South-east Nigeria, observed that people seek treatment for malaria from public sector facilities and a range of formal and informal private sector facilities.

About 60% of all malaria episodes in sub-Saharan Africa are initially treated by private providers, mainly through the purchase of drugs from shops and drug peddlers (Onwujekwe et al., 2009). The "informal private sector", such as patent medicine dealers, is a main source of anti-malarial drugs, but the quality of treatment that they provide is questionable (Bate et al., 2008; Ilomuanya et al., 2012). A major problem with the treatment of malaria is the high level of treatment failures resulting in the large part from the high prevalence of counterfeit drugs bought by the patients (Bate et al., 2008; Snow et al., 2005). Hence the need for enforcement deadline given to pharmaceutical companies by NAFDAC for the implementation of MAS on all AMs and antibiotics sold in Nigeria which was July 2014 (NAFDAC/Sproxil, 2015; Osuolale, 2015; WHO Factsheet on the World Malaria Report 2013; World Health Organization: Medicines, 2010).

This study was therefore designed to evaluate the level of awareness of MAS among the Nigerian populace assessing the extent of utilization of MAS by the Nigerian public and assessing the degree of acceptance of MAS among community pharmacists in Nigeria.

This will be carried out by assessing the knowledge, attitude and use of the technology by the average Nigerian citizen. The study will also attempt to uncover areas of deficit as well as to proffer suggestions and recommendations on the current use pattern of MAS by the general public.

METHODS

Ethics

The Research and Ethics committee of the West African Postgraduate College of Pharmacist gave approval for the research to be carried out without a an ethical approval as the committee deemed that the project was an evaluation of the use of a service, that is, mobile authentication service (MAS) by the populace, that is a service evaluation. The evaluation of the use of MAS among the Nigerian populace utilizing AMs was assessed from April 2015 to July 2015 in Nigeria.

Setting

Situated between 4˚ and 13˚ Northern Latitude Nigeria has a suitable climate for malaria transmission throughout the country. Nigeria is made up of 36 states and a Federal Capital Territory which have been constitutionally divided into six geopolitical zones (Federal Republic of Nigeria Draft National Human Resources For

Health Strategic Plan 2008 to 2012). This study was carried using a state selected at random from each of the six geo-political zones in Nigeria and Lagos state. The states used from the respective zones are as listed as follows:

1. South-South Zone (SSZ): Rivers state
2. North-West Zone (NWZ): Jigawa state
3. South-East Zone (SEZ): Enugu state
4. North-Central Zone (NCZ): Nasarawa state
5. South-West Zone (SWZ): Ogun state
6. North-East Zone (NEZ): Taraba state
7. Lagos Zone (LZ): Lagos State

Data sampling

Sample size calculation was based on the assumption that 70% of the Nigerian population utilize mobile phones (Dorlo et al., 2012; Osuolale, 2013). The level of significance utilized for this study was 0.05 and a power of 0.7 was obtained based on the assumption of the number of Nigerians using mobile phones. Utilizing sample size calculator NSS version 12.0 (2013) Australian Bureau of Statistics, a sample size of 778 was obtained. The questionnaire was developed from previous studies developed in Survey Gizmo®, the survey however was online (Adibe, 2009; SurveyGizmo®, 2015; Strauss and Corbin, 1990). The questions captured the detail, rationale and application of methods used within the study design and populations.

The method utilized collection of primary data using informal questionnaires administered to the general population irrespective of gender, status or educational background. The questionnaires were administered via non probability cluster random sampling method by the researcher and research assistants across the states selected for the study. The informal questionnaires were used to assess the awareness of the Nigerian populace on the availability of the MAS feature on AMs and their knowledge on the correct use of the feature to get needed information about the authenticity of the AMs. The study locations utilized were NAFDAC registered Pharmaceutical retail premises which sold house hold products as well as Pharmaceutical products and services in the city centres of the States selected for the study. The respondents were randomly selected clients visiting these premises, every other client entering into the premise was asked to fill the questionnaire. This simple random sampling method is representative of potential clients who are utilizing AMs.

Pertinent areas highlighted in the questionnaire include observing the presence of the scratch tag on the AMs, conducting the authentication process, frequency of conducting this process and the outcome received from the process.

The study sample

A total number of nine hundred (900) questionnaires were produced for the process of data collection. An initial pre-test using 30 questionnaires was carried out in a small area in one of the study sites. This was done to assess the understanding of the respondents and to ensure that the questions posed did not mean different things to the respondents. Based on the findings of the pre-test, the questionnaires were modified and consequently administered in each of the study sites. One hundred (100) questionnaires were administered in each of the geo-political zones and three hundred (300) questionnaires in Lagos state. Seven hundred and seventy four (774) questionnaires were collected out of a total of nine hundred (900) a response rate of 86% was recorded which was adequate for the study.

Data collection

The questionnaires were self-administered by the literate respondents. The questions were carefully read and their responses were documented accordingly. For less literate respondents with whom reading the questionnaire or recording their responses posed some challenges, the researcher had to interpret the questions asked using a vernacular language such that they were not at variance from the words and statements contained in the questionnaire.

Data analysis

The tools used for data analysis were correlation coefficient and chi-square. SPSS version 21 (IBM corp. Amonk New York) statistical package was employed in the analysis.

RESULTS

Six geopolitical zones were used for the study with Lagos state as an additional zone on its own. LZ had the highest number of questionnaire retrieved accounting for 37.0%, NCZ, SWZ, SSZ and NEZ accounted for 13.4, 12.7, 12.1 and 10.9% of the questionnaires retrieved respectively. The NWZ accounted for the least number of questionnaires retrieved, that is, 4.5%. Descriptive statistics were utilized in reporting data for socio-demographics of the respondents 53.1% of the respondents were in the 25 to 45 years age bracket with 92.3% attaining secondary education or better. The frequency of antimalarial medicine use among the respondents was evaluated as shown in Table 1 where more than 62.5% of the respondents had used antimalarial medicine at least once a month, with 99.4% of respondents indicating use of AMs at least once this year. A high level of awareness of the use of MAS was seen through all the zones as shown in Table 2 with all the zones showing more than 70% of the respondents as being aware of MAS. However even though high levels of awareness was seen the extent of utilization was very poor as shown in Table 3, with 50.1% of the respondents not utilizing MAS even when they are aware of it use as reflected in Table 2. Table 4 showed a strong association between education by the pharmacist/pharmacy attendant and the respondents' actual use of the service, the outcome was that where the pharmacist or technician educated the patient about the tag the more likely it was for the patient to scratch the tag and send the pin number to the service provider, thus the high likelihood ratio and linear by linear association of these variables. Association between the response received from the MAS service by the respondents and their consistent, continued use of the service was investigated and the outcome was that a positive response correlated with continued use of the service as shown in Table 5 with a Pearson Chi-Squared value of 540.14. The association between the zones where the respondents were domiciled and the respondents' actual use of the service

Table 1. The frequency of use of antimalarial medicines among the respondents.

Antimalarial medicine use	Percentage of respondents
Did not indicate	0.6
Weekly	2.2
Monthly	14.2
Once in 3 months	28.0
Once in 6 months	19.1
Once a year	15.9
Can't remember frequency but has used antimalarial this year	19.9
Total	100.0

Table 2. Zonal distribution of the awareness of the respondents about the existence of the Mobile Authentication Service (MAS) scratch panel on antimalarial medicine products.

Zones where respondents are domiciled			Have you ever heard about sms texting on medicine to check for fakes?		Total
			Yes	No	
Zone	SSZ	Count	70	24	94
		% within Zone	74.5	25.5	100.0
	NWZ	Count	32	3	35
		% within Zone	91.4	8.6	100.0
	SEZ	Count	70	3	73
		% within Zone	95.9	4.1	100.0
	NCZ	Count	102	2	104
		% within Zone	98.1	1.9	100.0
	SWZ	Count	62	36	98
		% within Zone	63.3	36.7	100.0
	NEZ	Count	62	22	84
		% within Zone	73.8	26.2	100.0
	LZ	Count	206	80	286
		% within Zone	72.0	28.0	100.0
Total		Count	604	170	774
		% within Zone	78.0%	22.0	100.0

was investigated and the outcome is documented in in Table 6 showed a small linear by linear association between the two variables with a Pearson Chi-Square value of 67.27, the actual use of MAS was not dependent on the zones where the respondents were domiciled.

The highest percentage of respondents that had tried out the service never used it (47.3%). The percentage increases to 61.1% when using the service to check the product at the point of purchase is considered. Many reasons were proffered by the respondents for using or not using the service which include the need to confirm the quality of the product (26.4%) and not having much knowledge about the service (8.9%).

To determine the level of awareness of MAS among the Nigerian populace, cross tabulations were done between socio-demographic parameters (namely zone, age group, educational level, gender) and the awareness level of the respondents. Among the zones, the North-Central zone (NCZ) had the highest level of awareness (98.1%) while the South-West zone (SWZ) had the least awareness level of 63% (Table 2). Generally, there is a total level of awareness of 78% as against 22% of non-awareness of the service among the population out of 99.4 % of respondents who claimed to have purchased tagged antimalarial medicines.

The level of awareness was highest among the age group of 25 to 45 years with an awareness level of 82%. The age group of above 60 years of age presented the lowest percentage (64%) of awareness. Respondents with tertiary education had the highest level of awareness of MAS (85%) while those with no formal education had the least level of awareness. Gender-wise, the males seemed to be more aware of the service (79.1%) than the females (77.3%) (Table 7). Across the zones, respondents in the North-Central zone (NCZ) were most aware (98.1%) about the existence of the service while

Table 3. The extent of utilization of the Mobile Authentication Service (MAS) across the zones.

Zones where respondents are domiciled			Extent of utilization			Total
			No utilization	Average utilization	High utilization	
Zone	SSZ	Count	49	18	6	73
		% within Zone	67.7	23.7	8.6	100.0
	NWZ	Count	7	9	11	27
		% within Zone	23.5	35.3	41.2	100.0
	SEZ	Count	21	12	21	54
		% within Zone	39.1	21.7	39.1	100.0
	NCZ	Count	13	13	56	82
		% within Zone	15.4	16.3	68.3	100.0
	SWZ	Count	42	12	17	71
		% within Zone	58.7	17.4	23.9	100.0
	NWZ	Count	34	16	13	63
		% within Zone	53.7	25.6	20.7	100.0
	LZ	Count	120	46	34	200
		% within Zone	60.2	23.0	16.8	100.0
Total		Count	286	126	158	570
		% within Zone	50.1%	22.2	27.7	100.0

Table 4. Association between education by the pharmacist/pharmacy attendant and the respondents' actual use of the service.

Pharmacist/pharmacy attendant education of respondent			Scratching the tag to send the pin			Total
			Did not indicate	Yes	No	
Did a pharmacist/pharmacist attendant speak to you about the scratch tag on the product?	Did not indicate	Count	14	3	3	20
		% within Did a pharmacist/pharmacist attendant speak to you about the scratch tag on the product?	69.2	15.4	15.4	100.0
	Yes	Count	0	121	56	177
		% within Did a pharmacist/pharmacist attendant speak to you about the scratch tag on the product?	0.0	68.3	31.7	100.0
	No	Count	2	187	218	407
		% within Did a pharmacist/pharmacist attendant speak to you about the scratch tag on the product?	0.6	45.9	53.6	100.0
Total		Count	16	311	277	604
		% within Did a pharmacist/pharmacist attendant speak to you about the scratch tag on the product?	2.7%	51.4	45.9	100.0

Chi-square tests	Value	df	Asymp. Sig. (2-sided)
Pearson chi-square	483.050[a]	4	0.000
Likelihood ratio	155.597	4	0.000
Linear-by-linear association	84.436	1	0.000
Number of valid cases	604		

[a]1 cells (11.1%) have expected count less than 5. The minimum expected count is 0.71.

the South-West zone (SWZ) seemed least aware (36.7%) of the service as reflected in Tables 2, 3 and 8 showed the association between the zones where the respondents were domiciled and the respondents' actual use of the service was investigated and the outcome was that the south east zone (SEZ) had % within zone of 57.5% giving a yes response.

DISCUSSION

The extent of utilization of the MAS by the Nigerian public

Table 5. Association between the response received from the MAS service by the respondents and their consistent, continued use of the service.

Zones where respondents are domiciled			Scratching the tag to send the pin				Total
			No response	Always	Sometimes	Never	
Response received	No response	Count	25	1	2	240	268
		% within Response received	9.3	0.3	0.9	89.5	100.0
	Positive; medicine ok	Count	8	150	114	24	296
		% within Response received	2.6	50.8	38.6	7.9	100.0
	Negative; medicine not ok	Count	0	3	4	6	13
		% within Response received	0.0	23.5	29.4	47.1	100.0
	No response	Count	1	4	6	16	27
		% within Response received	5.6	13.9	22.2	58.3	100.0
Total		Count	34	158	126	286	604
		% within Response received	5.7	26.1	20.9	47.3	100.0

Chi-square tests	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	570.14[a]	9	0.000
Likelihood ratio	713.532	9	0.000
Linear-by-linear association	89.179	1	0.000
Number of valid cases	604		

[a]4 cells (25.0%) have expected count less than 5. The minimum expected count is 0.97.

Table 6. Association between the zones where the respondents were domiciled and the respondents' actual use of the service was investigated and the outcome is documented.

Zones where respondents are domiciled			Scratching the tag to send the pin			Total
			Did not indicate	Yes	No	
Zone	SSZ	Count	1	32	40	73
		% within Zone	2.1	43.6	54.3	100.0
	NWZ	Count	0	14	14	28
		% within Zone	0.0	51.4	48.6	100.0
	SEZ	Count	2	33	23	58
		% within Zone	2.7	57.5	39.7	100.0
	NCZ	Count	0	69	12	81
		% within Zone	0.0	84.6	15.4	100.0
	SWZ	Count	0	34	42	76
		% within Zone	0.0	44.9	55.1	100.0
	NEZ	Count	3	32	30	65
		% within Zone	4.8	48.8	46.4	100.0
	LZ	Count	10	97	116	223
		% within Zone	4.5	43.4	52.1	100.0
Total		Count	16	311	277	604
		% within Zone	2.7%	51.4	45.9	100.0

Chi-square tests	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-square	67.27[a]	12	0.000
Likelihood ratio	76.595	12	0.000
Linear-by-linear association	0.485	1	0.486
Number of valid cases	604		

researched into using the socio-demographic parameters reflected overall utilization showed that 50.1% of respondents do not use the MAS service at all. 22.2% expressed average utilization of the service while only 27.7% highly use MAS. The North-Central zone (NCZ) had the highest utilization percentage of 68.3% while the

Table 7. The socio-demographic data of the respondents in the study.

Gender	Frequency	Percent
Did not indicate	22	2.8
Female	383	49.5
Male	369	47.7
Total	774	100
Age group		
Did not indicate	7	0.9
15-24 years	230	29.7
25-45 years	410	53.0
46-60 years	102	13.2
Above 60 years	25	3.2
Total	774	100.0
Level of education		
Did not indicate	4	0.5
Primary School	25	3.2
Secondary School	187	24.2
University	527	68.1
No formal education	31	4.0
Total	774	100.0

Table 8. Association between the zones where the respondents were domiciled and the respondents' actual use of the service was investigated and the outcome.

Zones where respondents are domiciled			Scratching the tag to send the pin			Total
			Did not indicate	Yes	No	
Zone	SSZ	Count	1	32	40	73
		% within Zone	2.1	43.6	54.3	100.0
	NWZ	Count	0	14	14	28
		% within Zone	0.0	51.4	48.6	100.0
	SEZ	Count	2	33	23	58
		% within Zone	2.7	57.5	39.7	100.0
	NCZ	Count	0	69	12	81
		% within Zone	0.0	84.6	15.4	100.0
	SWZ	Count	0	34	42	76
		% within Zone	0.0	44.9	55.1	100.0
	NEZ	Count	3	32	30	65
		% within Zone	4.8	48.8	46.4	100.0
	LZ	Count	10	97	116	223
		% within Zone	4.5	43.4	52.1	100.0
Total		Count	16	311	277	604
		% within Zone	2.7%	51.4	45.9	100.0

Chi-square tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	67.272[a]	12	0.000
Likelihood ratio	76.595	12	0.000
Linear-by-linear association	.485	1	0.486
Number of valid cases	604		

[a]6 cells (28.6%) have expected count less than 5. The minimum expected count is 0.95.

South-South zone (SSZ) had the least usage of 8.6%. The higher percentage of utilization was recorded among the males (30.7%) while the usage among the females was put at 25.1%. The age group of 25-45 years gave the highest utilization percentage of 34.4%. The age group of 15-24 years had the least percentage (14.0%) of MAS utilization. In terms of the level of education of the respondents, those with tertiary educational level had the highest percentage of utilization (31.1%) while those without formal education showed the least percentage of MAS utilization (3.6%) as shown in Table 3. Though high percentages were obtained for awareness, it did not cut across educational levels and age groups thus the need to ensure awareness and enlightenment campaigns are brought to basic levels of language and media to reach the entire populace.

An association between the pharmacists' educational role in speaking to the respondents about the service and the respondents' willingness to use the service was investigated. 68.3% of the respondents who actually used the service claimed to have been spoken to by a pharmacist/pharmacy attendant about the service. This represents the largest percentage of those who had used the service. 53.6% of those who were aware of the service but still had not used it claimed they had received no education from the pharmacist or attendant at the outlet. A p-value of 0.00 was obtained upon chi-square analysis which indicates significance in the association as shown in Table 4.

A second association to confirm whether the response received from initially using MAS had any effect on the respondents' consistent and continued use of the service was investigated. 50.8% of the respondents who got a positive "medicine ok" response from the MAS service claimed to use the service repeatedly with each purchase. The higher percentage of 58.3% of the respondents who had initially used the service and received no response from the MAS claimed they never repeated the use of the service. A significant p-value of 0.00 was also obtained upon chi-square analysis as shown in Table 5.

A third association between the socio-demographic parameters and the use of the service was also investigated. Respondents in North-Central zone (NCZ) indicated the highest percentage of use with 84.6%. The highest percentage of those who had not used the service was found in the South-West zone (SWZ). 56% of those who had used the service had tertiary education level while 52.6% were males.

The use and extent of utilization of MAS for AMs appeared comparable with the level of awareness. The zone, age group, gender and educational level bracket with the highest levels of awareness of MAS also produced corresponding results of the highest use and extent of utilization of the service. The continued and consistent use of the service seemed affected by the response received from the MAS system upon initial use and whether the respondents had been spoken to/educated by the healthcare professional.

The level of awareness in the general population (78%) however, does not seem to translate to willingness of the population to use the service (54.4%) and much less to the regular, continued and consistent use of the service (26.1%) which is the intent of the introduction of the service and the premise on which the MAS service as a counterfeit deterrent is hinged upon. Among those who knew how to use the service, 61.1% do not use the service to check their products at the point of purchase.

While a sizeable proportion of the respondents (26.4%) indicated that their use of the service was to confirm the authenticity of the medicine, respondents presented various reasons for their unwillingness to use the service. 4.7% felt that once a pharmacy or representative was adjudged as 'standard', there was no need to authenticate the medicine's quality. Some stated that upon seeing the scratch tag on a product, it meant that the product was original even without employing the service. In most communities, the pharmacist is the first accessed healthcare professional. There is a need for community and hospital pharmacists to ensure that the patient is not only educated on the benefit of the service but is also encouraged and if need be, assisted to employ the service as it should be used.

There were also the issues of network failure and a proportion of respondents feeling that they could not be bothered about using the service. There is a challenge of technological support which needs to be looked into. MAS, as a tool relies on technology thus the need to liaise with telecommunication providers, not only for free MAS messaging, but to ensure that as much as is possible, network challenges are minimized.

By virtue of its design, the effectiveness of MAS in curbing counterfeiting is not just in its use but its regular use per unit, per purchase. In other words, every single scratch panel is meant to be scratched and pin sent to the default code to receive a response regarding the product's quality at the point of purchase. It does not matter if two individual packs are of the same brand or in one pharmacy. Each code is unique and is meant to protect the single unit pack bearing it and not the brand *per se* nor is it to endorse any outlet. This is what puts the power of safe guarding one's health literally in one's own hands.

With the actual consistent use of MAS at 26.1% of respondents, there appears to be a gulf between the awareness level and the regular utilization of the service. The extent of utilization was highest with the highest level of education and lowest with those without formal education. This may be an indication that the communication of the service, either in content or channels employed, may be occluded from a section of society.

Two decades ago, chloroquine had been the preferred drug of choice for malaria therapy (Ilomuanya et al.,

2012). Its effectiveness was overtaken by the emergence of resistance by the malaria parasites. Chloroquine-resistance has been traced to the use counterfeit and substandard medicinal products by patients (Onwujekwe et al., 2009). The last decade ushered in the newer and more effective artemisinin compounds which are now being used as first line antimalarial therapy in combination with existing AMs. It is mandatory in Nigeria that all artemisinin-combination therapy (ACTs) medicines utilize the MAS (Onwujekwe et al., 2009; NAFDAC /Sproxil, 2015), however while MAS does not provide information about the biopharmaceutical properties of these medicines, it confirms the authenticity of the product source and supply chain, thereby guarding against the possibility of infiltration by counterfeiters. This advantage is of high benefit for artemisinin-combination therapy medicines if exploited properly.

Nigeria is the first country where MAS has been deployed extensively for the security of pharmaceuticals. Apart from Nigeria, Kenya, Ghana and India have also used the service but on pilot scales. These countries have recently employed the service publicly on their pharmaceuticals thus the need for corresponding studies to ascertain MAS effectiveness. While MAS can guarantee the integrity of the supply chain of pharmaceuticals, the safety, efficacy and quality of the medicine are not taken into account. There must be a framework to ensure that the manufacturers/importers and their respective products are periodically assessed for quality and effectiveness.

Conclusion

Malaria medicines are constantly in high demand and unfortunately, they have become a target for pharma-counterfeiting. While MAS does not provide information about the biopharmaceutical properties of these medicines, it confirms the authenticity of the product source and supply chain, thereby guarding against the possibility of infiltration by counterfeiters. The strong association between education by the pharmacist/pharmacy attendant and the respondents' actual use of the service reflected that pharmacist involvement in patient care would increase the use of MAS. This advantage would be of high benefit for artemisinin-combination therapy (ACTs) and other AMs if exploited properly. Nigeria is the first country where MAS has been deployed extensively for the security of pharmaceuticals. There may be a need to have corresponding studies carried out in other countries to ascertain the effectiveness of the MAS.

Conflict of Interests

The authors have not declared any conflict of interests.

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