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Research Article
Anti-Inflammatory Activity and Bioavailability Profile of Ethanolic Extract of *Dichaetanthera africana*

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Abstract

Background and Objective: *Dichaetanthera africana* (*D. africana*) is a traditional medicinal plant which is widely used in Cameroon, Gabon, Sierra Leone, Ivory Coast and Nigeria to treat various diseases such as coughs, chest ache and fatigue. This study aimed to evaluate anti-inflammatory activities and pharmacokinetic profile of the ethanolic extract of the stem bark of *D. africana* (EtOHDA). **Materials and Methods:** Ethanolic extract was evaluated for anti-inflammatory activities using *in vitro* and *in vivo* standard experimental models which included respectively protein denaturation, hypotonic-induced hemolysis and carrageenan paw edema assays. The characterization of the phytochemicals and bioavailability profiles of *D. africana* were investigated using high performance liquid chromatography-photodiode array detector (HPLC-PDA) analysis. The 16.0 SPSS windows software (SPSS Inc., Chicago, IL, USA) was used for the analysis of data. **Results:** The EtOHDA inhibited protein denaturation activity (3.12-82.59%) and protected the erythrocyte membrane against lysis induced by hypotonic solution (17.51-88.79%). Diclofenac sodium (78.22-94.53%) and indometacin (44.87-95.34%) showed the maximum inhibition. Significant reduction of paw edema ($p<0.05$) was observed in a dose-dependent manner after administration of the extract, especially at 400 mg kg^{-1} b.wt. These activities were comparable to the standard drugs while exhibiting no acute toxicity (LD_{50}>2000 mg kg^{-1}). Four major compounds were identified using HPLC, out of which only one was found in rat plasma. **Conclusion:** Results showed that EtOHDA has a significant anti-inflammatory activity. It is, therefore, a source of active compounds that might be used as anti-inflammatory agents.

Key words: *Melastomataceae*, egg albumin, red blood cells, edema, HPLC

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dampened inflammation causes serious harm to the ordinary tissues bringing about different disease conditions, for example, auto-inflammatory disorders, neurodegenerative distress and cancer[1]. Inflammation, a key physiological process that protects organisms against harmful stimuli, is characterized by an increase in blood influx to the affected tissue and is accompanied by pain, heat, swelling, redness and loss of function of the affected part. The main hemodynamic molecular and cellular mechanisms involved in inflammation include an alteration in blood flow, migration of leukocytes from the blood into the injured tissues and initiation of biochemical events that propagate the inflammatory response[2]. Anti-inflammatory drugs are used in order to alleviate this physiopathological situation. Anti-inflammatory drugs generally used are steroidal agents (SAs) and non-steroidal anti-inflammatory drugs (NSAIDs) on symptomatic effects[3]. However, a prolonged use of these drugs is followed by severe side effects such as gastro-duodenal and kidney damage, bone marrow depression, retention of salts and water, among others[4]. There is a clinical urgency to recognize novel molecules that are harmless, for the prevention and management of inflammatory disorders. Medicinal plants are a viable alternative to the discovery of new safer bioactive compounds. In fact, there are evidence that drugs derived from natural products modulate various inflammatory mediators, including their effects on the expression of pro-inflammatory mediators, such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), prostaglandin E_2(PGE_2), tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-10 and monocyte chemotactic protein-1 (MCP-1)[5,6]. Bioavailibility studies help incredibly in understanding the mode of action and confirming the efficacy of drugs so as to attain required pharmacological action and also to minimize the adverse-effects and toxicity. Therefore, it is valuable to perform bioavailability studies to evaluate the rationality and compatibility of combined prescriptions[7,8].

Dichaetanthera africana is a tree of 9-15 m high, found in riverine woodland from Sierra Leone to Congo and Angola[9]. The different parts of this plant are medicinally used in Cameroon, Gabon, Sierra Leone, Ivory Coast and Nigeria against coughs, chest ache and fatigue by oral decoction[10,11]. It has been documented that the species of *Melastomataceae* family to which *D. africana* belongs, exhibited *in vitro* and *in vivo* anti-inflammatory activities[12,13] as well as related activities such as an analgesic[14-16], antipyretic[12] and anti-lcer[17,18]. Apart from the use of *D. africana* in the treatment of inflammation and pain in folk medicine, there is no report about chemical and biological studies on this plant. Therefore, this study was designed to investigate the anti-inflammatory activity and bioavailability profile of *D. africana* stem bark extract.

MATERIALS AND METHODS

Collection of the plant material: The stem bark of *D. africana* was collected from the Littoral Region of Cameroon (Babimbi II) in February, 2012. Identification was done at the National Herbarium (Yaoundé, Cameroon), where a voucher specimen (No. 7157/SRFK) has been deposited.

Preparation of the crude extract: The air-dried and powdered stem bark of *D. africana* (2 kg) was macerated at room temperature in ethanol (5 L, 72 h) to obtain a crude extract (76.3 g) after evaporation under vacuum using a rotary evaporator (Büchi R200). The yield of the extraction (3.82%, w/w) was calculated with respect to the initial weight of the dry pulverized stem bark of the plant.

Phytochemical screening: The preliminary phytochemical screening was performed according to the methods described by Khandelwal[19].

Experimental animals: Nulliparous and non-pregnant wistar rats (180-200 g) were used and housed in plastic cages under normal laboratory conditions (12 h light/dark cycle, 25±2°C) for an acclimatization period of 7 days prior to the experiments. All animals were given food and water *ad libitum*. Animal care and handling was done according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). This guideline has been issued by the Ministry of Environment and Forests, Government of India. The animal study was performed at VNS Group of Institutions, Faculty of Pharmacy, Bhopal (Madhya Pradesh) with due permission from Institutional Animal Ethics Committee (Registration No. 778/PO/a/03/CPCSEA; 03.09.).

Acute oral toxicity: The LD_{50} was determined using the Up and Down Procedure[20]. Animals were divided into 3 groups of 6 animals each. The control group received water orally (10 mL kg^{-1} b.wt.) while test groups were administered with doses of extracts (1000 and 2000 mg kg^{-1} b.wt.). All animals were observed for toxic manifestations for the next 5 h and subsequently observed intermittently for signs of morbidity and mortality for 7 days.

Anti-inflammatory activity

Protein denaturation: Anti-inflammatory activity of the extract was evaluated by protein denaturation method as described by Padmanabhan[21]. Diclofenac sodium, a powerful non-steroidal, anti-inflammatory drug was used as standard drug. The reaction mixture consisted of 2 mL of extract or standard (50-1000 μg mL^{-1}), phosphate buffer saline pH 6.4 (2.8 mL) and 5% egg albumin (2 mL) and was incubated at 27°C for 15 min. Denaturation was induced by keeping the reaction mixture at 70°C in water bath for 10 min. After cooling, the absorbance was measured at 660 nm by using double distilled water as blank. Each experiment was done in triplicate. The percentage inhibition of protein denaturation was calculated by using the following equation:

$$\text{Inhibition of protein denaturation (\%)} = \frac{A_{sample} - A_{control}}{A_{control}} \times 100$$

Erythrocyte membrane stabilization: The membrane stabilizing activity of the extract was evaluated by using hypotonic solution induced hemolysis[22]. Whole blood was collected from anesthetized rats through orbital puncture using heparinized capillary tubes and was washed with an isotonic solution (154 mM NaCl) through centrifugation for 10 min at 3000 rpm. The volume of erythrocyte was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4). Thus, the suspension finally collected was the stock red blood cells suspension (RBCs). The test sample, consisting of RBCs (0.25 mL), was mixed with 2.5 mL of hypotonic solution (5 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) and 0.25 mL of the extracts or indometacin (50-1000 μg mL^{-1}). The control sample consisted of 0.25 mL of RBCs, was mixed with hypotonic buffered saline alone. The mixture was incubated for 10 min at room temperature and centrifuged for 10 min at 3000 rpm. The absorbance of the supernatant was measured at 540 nm. Each experiment was performed in triplicate. The percentage inhibition of hemolysis or membrane stabilization was calculated using the following equation[22]:

$$\text{Inhibition of hemolysis (\%)} = \left(1 - \frac{A_2 - A_1}{A_3 - A_1}\right) \times 100$$

Where:

A_1 = Test sample in isotonic solution
A_2 = Test sample in hypotonic solution
A_3 = Control sample in hypotonic solution

Carrageenan-induced paw edema: Paw edema was induced by injecting intraperitoneally 0.1 mL of 1% (w/v) carrageenan suspension into the subplanter region of the right hind paw of the rats[23]. Five groups of 6 rats each were orally treated 1 h before carrageenan injection with distilled water (10 mL kg^{-1} b.wt.), indometacin (10 mg kg^{-1} b.wt.) and extract (200 and 400 mg kg^{-1} b.wt.). The measurement of paw edema was carried out by displacement technique using a vernier caliper to find out the circumference of paw edema immediately before and after 1, 2, 3, 4 and 5 h, following the carrageenan injection. Total edema developed during this period was monitored as the area under the time-course curve (AUC). The inhibitory activity was calculated according to the equation:

$$\text{Inhibition of paw edema (\%)} = \left(\frac{(C_t - C_o)_{control} - (Ct - C_o)_{treated}}{(C_t - C_o)_{control}}\right) \times 100$$

Where:

C_t = Paw circumference at time t
C_o = Paw circumference before carrageenan injection
C_t-C_o = Edema or change in paw size after time t

HPLC fingerprint analysis: The experiment was performed using a high performance liquid chromatography apparatus (HPLC-Shimadzu chromatograph-LC-20 Avp series, Japan) equipped with two pumps (LC-20AD), degasser (DGU-14 A), photodiode array detector (SPD-M20A), oven column (CTO-20AD), rheodyne manual injector (loop 20 μL) and integrating CLASS (LC-20AD). The extract was dissolved in methanol and the solution (1 mg mL^{-1}) filtered with millipore membrane (0.45 mM pore diameter). The sample was eluted using a Phenomenex Luna reverse phase column C_{18} 5 μm (2) (250×4.6 mm) and Phenomenex C_{18} pre-column (4×3.0 mm) filled with similar material to the main column. The chromatographic separation of the compounds was carried out in gradient elution using the mobile phase methanol/water Milli-Q (45:55), at 40°C and flow of 1 mL min^{-1}. The elution time was 35 min and detection was carried out at λ_{max} 237 nm. The gradient trial which showed maximum compounds upon multiple replications was selected for the study[24].

Bioavailability study: Six rats were put into fasting for 12 h prior to the administration of the extract. Animals had free access to water during the experiment. After an oral administration of extract (400 mg kg^{-1}), blood samples (0.3 mL) were harvested into heparinized, Eppendorf tubes

from each rat through retro-orbital sinuses before dosing and at 0, 0.5, 1, 2, 4, 8 and 12 h. After centrifugation at 3000 rpm for 10 min, plasma samples were transferred to neat tubes and stored at -20°C until further analysis. Blank plasma was obtained from the rat without oral administration of plant extract. The retention time and area under the plasma concentration-time curve (AUC) were determined using HPLC-PDA[25,26].

Statistical analysis: Data were expressed as Mean±SD. Statistical analysis was carried out using the Newman-Keuls test. The 16.0 SPSS windows software (SPSS Inc., Chicago, IL, USA) was used for the analysis of data. Differences were considered significant at $p<0.05$.

RESULTS

Phytochemical screening: Phytochemical screening revealed the presence of alkaloids, glycosides, carbohydrates, tannins, flavonoids, resins and steroids. However, alkaloids, tannins and steroids were found in low quantity.

Acute oral toxicity: On day 7, no adverse reactions or mortality was seen at 1000 and 2000 mg kg^{-1} oral administration of ethanolic extract (LD_{50}>2000 mg kg^{-1}).

Anti-inflammatory activity: *D. africana* inhibited protein denaturation activity at the different concentrations tested with the percentage varying from 3.12-82.59% (Fig. 1). Diclofenac sodium showed the maximum inhibition (78.22-94.53%). At the different concentrations tested, *D. africana* protected the erythrocyte membrane against lysis induced by a hypotonic solution with the inhibition percentage of hemolysis ranging from 17.51-88.79% (Fig. 2). Indometacin exerted a higher inhibitory effect than extract (44.87-95.34%). The maximum inflammatory response induced by carrageenan occurred between 1 and 3 h. All doses of the extract tested were effective in reducing edema throughout the experiment with the percentage ranging from 22.64-41.86% (200 mg kg^{-1} b.wt.) and from 46.22-62.79% (400 mg kg^{-1} b.wt.) at 1 and 2 h, respectively. We found that 1 h post injection the dose of 400 mg kg^{-1} of *D. africana* significantly reduced the paw edema ($p<0.01$) compared to the control. *D. africana* at the dose of 400 mg kg^{-1} b.wt. showed a similar activity as indometacin (63.95-71.69%) between 1 and 2 h (Fig. 3).

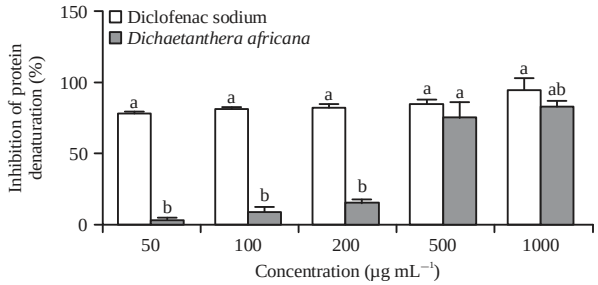

Fig. 1: Effect of *Dichaetanthera africana* on protein denaturation

[a,b]For the same concentration, values carrying different letters in superscript are significantly different at $p\leq0.05$ (Newman-Keuls test), Mean±SD

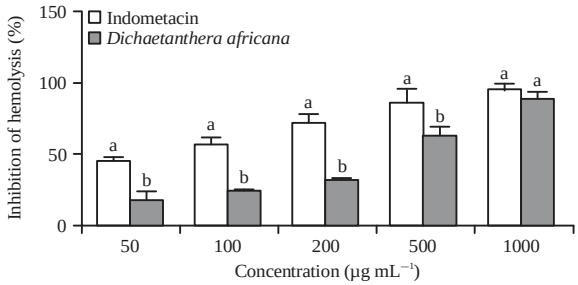

Fig. 2: Effect of *Dichaetanthera africana* on RBCs hemolysis

[a,b]For the same concentration, values carrying different letters in superscript are significantly different at $p\leq0.05$ (Newman-Keuls test) Mean±SD

HPLC fingerprint analysis: Four compounds were identified as the major components of the extract with a retention time of 7.488, 9.639, 12.562 and 27.608 min, respectively (Fig. 4).

Bioavailability study: One major compound of the ethanolic extract achieved peak plasma concentration at 60, 120, 240 and 480 min after oral administration of *D. africana* with retention time of 8.663 (AUC 31191), 8.994 (AUC 5689), 9.005 (AUC 7393) and 9.031 (AUC 9693) min, respectively (Fig. 5). However, other compounds were not found during pharmacokinetic study after oral dosing.

DISCUSSION

D. africana inhibited protein denaturation activity at the different concentrations tested with the percentage varying from 3.12-82.59%. Denaturation of proteins is a well-documented cause of inflammation[27]. Several anti-inflammatory drugs have shown dose-dependent ability to inhibit thermally-induced protein denaturation[28]. The mechanism of denaturation probably involves alteration of the

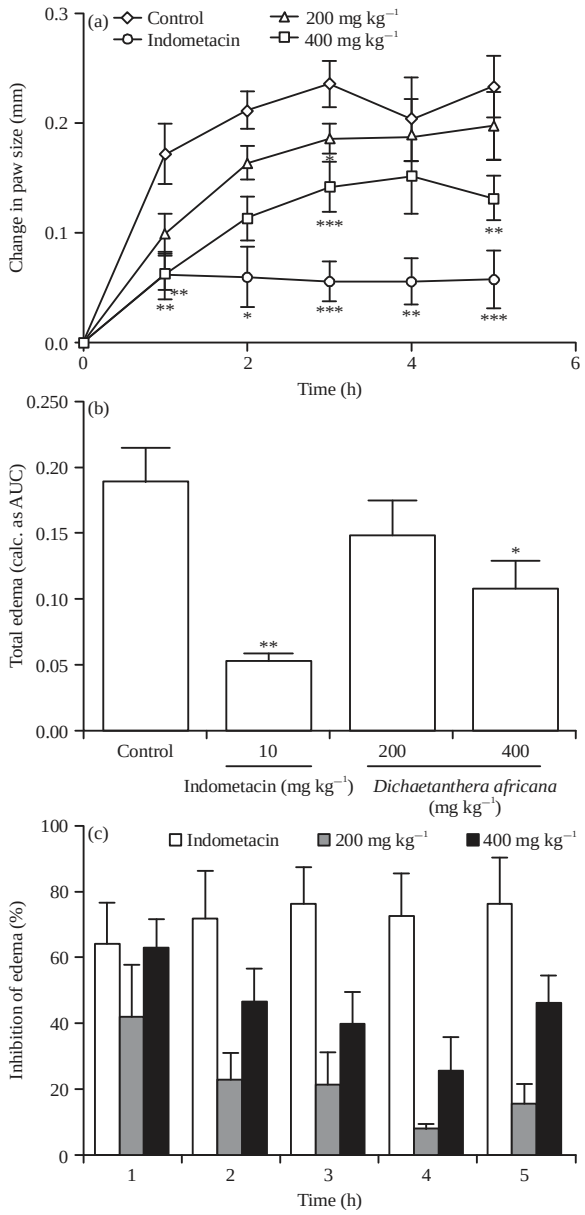

Fig. 3(a-c): Effect of *Dichaetanthera africana* on carrageenan-induced rat paw edema, (a) Change in paw size (mm), (b) Total edema (AUC) and (c) Inhibition of edema (%)

*p<0.05, **p<0.01, ***p<0.001 compared to vehicle-treated group (water), n = 6 (Newman-Keuls test), Mean±SD

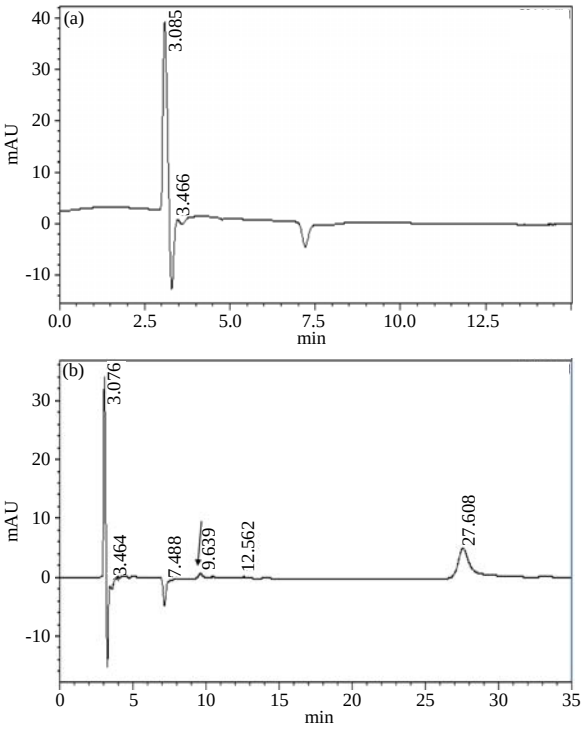

Fig. 4(a-b): HPLC Fingerprint detected at 237 nm, (a) Ethanol and (b) Ethanol extract of *Dichaetanthera africana*

electrostatic hydrogen, hydrophobic and disulfide bonding[29]. The ability of *D. africana* extract to bring down thermal denaturation of protein is possibly a contributing factor for its anti-inflammatory activity. At the different concentrations tested, *D. africana* protected the erythrocyte membrane against lysis induced by a hypotonic solution with the inhibition percentage of hemolysis ranging from 17.51-88.79%. The vitality of cells depend on the integrity of their membrane, exposure of RBCs to injurious substances such as hypotonic medium results in lysis of its membrane accompanied by hemolysis and oxidation of hemoglobin. The hemolytic effect of the hypotonic solution is related to the excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such damaging of RBC membrane will further render the cell more susceptible to secondary damage through free radical induced lipid peroxidation[30]. *D. africana* showed significant ($p<0.05$) membrane stabilizing property, which suggests that its anti-inflammatory activity observed in this study, may be related to the inhibition of the release of phospholipases that trigger the formation of inflammatory mediators. It is therefore expected that compounds with membrane-stabilizing properties, should offer significant protection of cell membrane against injurious substances[31]. The development of edema induced by carrageenan is a three phase event. The early phase (the first 90 min) involves the release of histamine and serotonin, the 2nd phase (90-150 min) is mediated by kinin and the 3rd phase (after 180 min) is mediated by prostaglandin[32]. The results from this study suggest that the extract of *D. africana* possibly acts by inhibiting the release and/or action of histamine, serotonin and kinin since the extract showed a significant inhibitory activity between the initial and middle

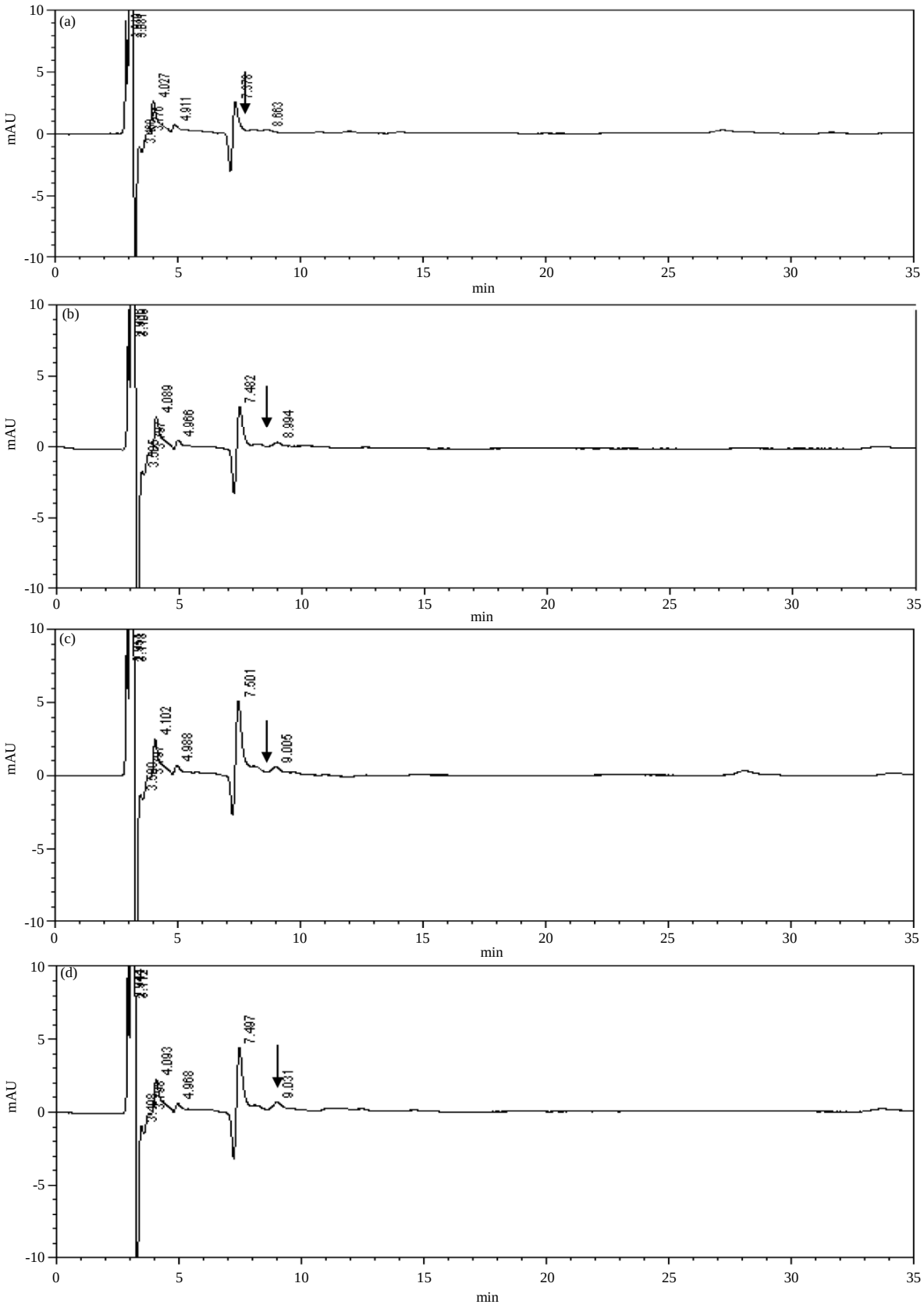

Fig. 5(a-d): HPLC Chromatograms plasma sample at (a) 60 min, (b) 120 min, (c) 240 min and (d) 480 min after oral administration of *Dichaetanthera africana*

phase (60-120 min). Compounds with membrane-stabilizing properties are recognized for their capability to hinder the release of phospholipases that trigger the formation of inflammatory mediators[33].

High-performance liquid chromatography is widely employed for screening the phytoconstituents for the quality management of herbal medicines. However, HPLC fingerprinting analysis of *D. africana* showed four major peaks which correspond to different phytoconstituents present in the ethanolic extract. The compounds detected were expected to be the major compounds present in the *Dichaetanthera* species.

Other investigators have reported the presence of these components in the *Melastomataceae* family to which belongs *D. africana*[18,34]. In addition, the branches of *Sakersia africana*, an isotype of *D. africana*, also revealed the presence of saponins, tannins (gallic tannins, catechic tannins), phenolic compounds, total flavonoids, carbohydrates, glycosides (digitoxin, digitoxigenin), alkaloids, coumarins, sterols and triterpenes[35]. *Dichaetanthera oblongifolia* (an allied species of *D. africana*) is reported to contain tannins, alkaloids etc[36].

It has been demonstrated that flavonoids are able to inhibit the enzymes such as aldose reductase, xanthine oxidase, phosphodiesterase, ATPase, lipoxygenase and cycloxygenase[37] as well as other mediators of the inflammatory process such as reactive C protein or adhesion molecules[38]. Apart from flavonoids, phenolic compounds, essential oils, glycosides, steroids, alkaloids etc.[39,40] are also responsible for the anti-inflammatory action.

Among the four compounds found through HPLC fingerprinting analysis, one compound was present in the rat plasma after oral administration of *D. africana*. This may be due to number of factors affecting the oral drug absorption. Into the gastric lumen, the drug may be metabolized by microsomal-enzymes and microbes, inactivated by gastric contents and eliminated in urine/feces. The fraction of the drug which is absorbed may be metabolized in the intestinal wall and in the liver. Hence, the amount of drug which reaches the systemic circulation is lesser as compared to the administered dose. The action of the drug depends on the rate and extent at which drug reaches the site (s) of action. Therefore, oral bioavailability data of the extract may be beneficial for its safe and effective use[41]. In addition, the results showed that the oral bioavailability of this compound decreased after 60 min. This may be due to its hydrophilic nature which subsequently results in its poor gastrointestinal absorption[42]. However, this compound would be one of the compounds responsible for the observed activities.

CONCLUSION

The present study showed that the ethanolic extract of *D. africana* had an appreciable anti-inflammatory activity and indirectly substantiated the traditional use of *D. africana* in some inflammatory and pain disorders by the local practitioners. At the same time, bioavailability study showed that one major compound was found in the plasma which could be one of the compounds responsible for the anti-inflammatory properties observed. Further studies should be carried out to characterize this compound.

SIGNIFICANCE STATEMENT

This study discovers the potential anti-inflammatory effect of *Dichaetanthera africana* that can be beneficial for carrageenan-induced paw edema rats. The compound found in the plasma could be the base of development of new anti-inflammatory agent. This study contributes in the short term to improving the health problems of populations by using medicinal plants as an alternative to conventional substances used in modern medicine. Thus, in the socio-economic context of developing countries, medicinal and food plants studies can lead to adequate and low cost therapeutic responses, coupled with proven scientific effectiveness and optimal cultural acceptability.

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