



Evaluation of the Organ Microscopy, Chemical Composition and Anti-inflammatory Potentials of Methanolic Leaf Extract and Fractions of *Diaphanante bidens*

Immaculeta C. Umeyor^{1*}, Chukwuebuka E. Umeyor², Cyril O. Ogbue¹, Felix A. Onyegbule³ and Festus B. C. Okoye³

¹Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.

²Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.

³Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author ICU performed the study, analyzed the results, and prepared the first draft of manuscript. Author CEU managed literature search and result analysis. Author FAO supervised the study. Author FBCO designed the study, wrote the protocols, supervised the study, read and corrected the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/APRJ/2021/v8i130166

Editor(s):

(1) Dr. Shiamala Devi Ramaiya, Universiti Putra Malaysia, Malaysia.

Reviewers:

(1) Kamal Mohamed, Oswego State University, New York, US.

(2) Irene Ávila-díaz, Universidad Michoacana de San Nicolás de Hidalgo, México.

Complete Peer review History: <https://www.sdiarticle4.com/review-history/70857>

Original Research Article

Received 07 May 2021
Accepted 13 July 2021
Published 19 July 2021

ABSTRACT

Aim: The study aims to investigate high-pressure liquid chromatographic (HPLC)-based chemical composition and anti-inflammatory activity of methanolic leaf extract and fractions of *D. bidens*.

Study Design: The study is a laboratory-based research involving collection, identification and preparation, of plants, extraction, characterization and evaluation of the anti-inflammatory potential of *D. bidens*.

Place and Duration of Study: Department of Pharmacognosy and Traditional Medicine, Faculty of

Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria between January 2016 and June 2019.

Methodology: Powdered leaves of *D. bidens* was macerated in methanol and the obtained crude extract was fractionated using *n*-hexane, ethylacetate, butanol, and water respectively. The acute toxicity profile of the extract was determined. Spectroscopic factors of the plant were assayed using HPLC-DAD and the anti-inflammatory activity of the extract and its fractions were evaluated.

Results: Acute toxicity study showed that the crude extract has an LD₅₀ > 5,000 mg/kg. HPLC-DAD analysis revealed the presence of compounds including apigenin monoglycoside, quercetin 3-O-(6''O-acetyl) galactopyranoside, kaempferol 3-O-(6'' O-malonyl) glucoside, genistein 8-C-glucoside, while anti-inflammatory study showed that the extract and fractions inhibited cellular activities of inflammatory mediators (86 – 97 %) in comparison with the diclofenac (53 %); thus, supporting its ethnomedicinal use in the treatment of inflammation.

Conclusions: Methanolic extract of *D. bidens* has wide margin of safety. It has sufficient deposit of flavonoids which might be responsible for its strong anti-inflammatory activity that is comparable with diclofenac.

Keywords: *Diaphanathe bidens*; microscopic; phytochemical; anti-inflammatory activity.

1. INTRODUCTION

Plant materials have been used for thousands of years as traditional medicines for the treatment and prevention of diseases. During the last decade, medicinal plants have gained increasing attention and their use have expanded globally across different cultures as various traditional medicine systems such as Indian Traditional Medicine (ITM), Chinese Traditional Medicine (CTM), Japanese Traditional Medicine (JTM), and African Traditional Medicine (ATM) have been documented. The use of these plants has continued both among the poor rural dwellers in developing countries as recognized by the World Health Organization (WHO) and in developed countries where conventional or synthetic medicines is predominant [1]. This trend could be attributed to the rising drug resistance and therapeutic failures associated with synthetic drugs, and perceived low untoward effects, affordability, and availability of plant-based medicines. Medicinal plants have important chemical constituents called secondary metabolites or phytochemicals, which are used by these plants for protection and defence against predators and pathogens [2]. In addition, these chemical agents are mobilized in living tissues in response to inflammatory processes, involving a cascade of events elicited by various stimuli including antigen-antibody interaction or infectious agents [3]. Generally, inflammation (generalized or localized) is a complex physicochemical reaction involving enzyme activation, cell migration, tissue breakdown, and mediator release. It is a response of living tissues to injury (physical or chemical) and it facilitates healing process because in the absence of

inflammation, the injury would never heal and the survival of the tissues might be compromised [4]. Chronic inflammation is associated with the pathogenesis of various diseases such as carcinomas, cardiovascular diseases, Alzheimer's disease, diabetes mellitus and other metabolic disorders [5]. Unfortunately, most of the classical synthetic drugs used in the treatment of inflammatory conditions including non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, etc. have recently received low clinical application due to serious adverse effects such as gastric discomfort and bleeding, bone marrow suppression, and fluid and electrolyte retention, which worsen the disease condition, increases healthcare costs, and could be life-threatening [6]. These challenges have made the use of synthetic medicines unacceptable for the treatment of inflammatory diseases thereby highlighting the urgent need for increasing search for alternative available, affordable, and natural anti-inflammatory medicines from plants.

Diaphanathe bidens (Afzel. Ex. Sw) Schltr. is an epiphytic plant species of the family Orchidaceae and the order of Asparagales which is characterized by tough wiry stems. It has an elongate, pendent stem of about 50 cm long and this enables the plant to hang gracefully from the branches of forest trees and adorned with salmon pink, yellowish pink or white flowers. It is commonly called wax orchid by the English, and 'bombins' by Sierra Leoneans. The Yoruba tribe of South-West Nigeria call it 'ikori', but the plant has no known common among the Igbo tribe of South-East Nigeria. However, locals of South-East Nigeria use decoctions of the plant for the treatment of asthma, diabetes mellitus, and

inflammatory diseases [7]. So far, a study has investigated the anti-hyperglycaemic activity of the methanol leaf extract of *D. bidens* in normoglycaemic and streptozotocin-induced hyperglycaemic rats [7], but there is no more scientific study carried out to confirm its anti-inflammatory activity.

Therefore, this study was undertaken to evaluate the anti-inflammatory activity of *D. bidens*, and the chemical constituents responsible for the activity with the aim of establishing its efficacy as well as the pharmacological basis of its ethnomedicinal use in the management of inflammation.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material

Fresh, green whole plant (2 kg) of *Diaphanthe bidens* (Afzel. Ex. Sw) Schltr. was collected from the wild in Ede-Oballa in Nsukka Local Government Area, Enugu State, Nigeria in the month of June, 2016. Mr. Felix Nwafor of the Department of Botany, Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria authenticated the plant material, and a voucher specimen (PCG 520/A/056) was deposited at the herbarium of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. The plant name was checked with <http://www.theplantlist.org/> on 13 July 2016, and confirmed to be an accepted name. It is called wax orchid by the English, and 'bombins' by Sierra Leoneans. The Yoruba tribe of South-West Nigeria call it 'ikori'.

2.2 Reagents

The chemical reagents used include: methanol, butanol, ethylacetate, *n*-hexane (JHD, China), silica gel 200-400 (Mesh Kemlight Laboratories Pvt. Ltd., India), xylene (Fluka, Germany), polysorbate (Tween®) 80 (BDH, Poole, England). All other reagents used were of analytical grade.

2.3 Animal Care and Use Protocols

Wistar rats (150 – 200 g) and white albino mice (20 – 29 g, BALB/c strain) were purchased from the animal house of the Department of Pharmacology and Toxicity, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. The animals were

housed in standard laboratory conditions and fed with commercial rodent pelletized feed (Guinea Feeds Nigeria Ltd) and had free access to clean drinking water prior to experiments. All animal experiments complied with the ARRIVE guidelines and were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. Cage-side clinical observations of the mice were made throughout the study period. The study protocols were approved by the Animal Care and Use Committee of our institution.

2.4 Extraction of Plant Material

Fresh plant materials were washed with clean water, cut into tiny pieces, dried under shade for about four (4) months, and was mechanically pulverized into powders. The powders (1 kg) were cold macerated in methanol (3.5 L) for 48 h with intermittent shaking at 2 h interval, followed by decantation, and use of fresh solvent so as to maintain the extraction gradient. The resulting extract was filtered using Whatman filter paper (No.1), and the filtrate was concentrated to dryness using rotary evaporator at 40 °C under reduced pressure to obtain the crude methanol extract (CME) which was stored in a refrigerator at 10 °C until further use.

2.5 Fractionation of Crude Methanolic Extract

Crude extract was subjected to liquid-liquid partitioning. The extract (80 g) was reconstituted in 20 ml methanol and the dispersion made up to 200 ml using distilled water. The dispersion was shaken for 15 min and partitioned successively using *n*-hexane (500 ml×2), ethylacetate (500 ml×2), and butanol (500 ml once) in increasing order of polarity with 1,000 ml separating funnel. The fractions were concentrated *in vacuo* using rotary evaporator at 40 °C to obtain *n*-hexane, ethylacetate, butanol, and aqueous fractions. The fractions were stored in a refrigerator at 10 °C until time of use.

2.6 Plant Microscopic Studies

The microscopic features of the leaf, stem, root, and petiole of *D. bidens* were studied using binocular zoom light microscope (LABO, Deutschland, Germany). Cross-sections of the plant parts were prepared by free hand thin transverse and longitudinal sectioning, cleared with chloral hydrate, stained with freshly

prepared safranin dye, alcohol was used to enhance visibility, and the plant material was observed for general and specific microscopic features [8]. Images of the cytological characters present in the plant materials examined were taken using a digital camera (Samsung, UK).

2.7 HPLC-DAD Analysis

HPLC analysis was carried out on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector (DAD) (UVD340S, Dionex Softron GmbH, Germering, Germany). Detection was at 235, 254, 280 and 340 nm. The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol were used as eluent. The compounds were detected by comparing the retention times and spectra of compounds in the in-built library.

2.8 Phytochemical Analysis

Qualitative phytochemical analysis was carried out on the crude methanol extract and fractions of *D. bidens* to determine the presence of various constituents. Similarly, quantitative phytochemical analysis was carried out to determine the presence of alkaloids, tannins, saponins, and flavonoids in the crude extract. The tests were carried out according to standard methods previously described [9, 10].

2.9 Acute Toxicity Test

This test was carried out according to guidelines prescribed by Organization of Economic Co-operation and Development (OECD) with slight modification [11]. Six albino mice of either sex in four groups selected randomly were used in this study. The first group served as the negative control while the other groups served as the test groups. The animals were fasted over-night with free access to water. Before dose administration, the body weight of each animal was determined and the dose was calculated according to the body weight. The crude methanol extract (CME) of *D. bidens* at 2000, 3000, 4000 and 5000 mg/kg were administered to the test groups of mice by lavage to determine the toxic dose while the control group received normal saline. The mice were observed closely for obvious signs of toxicity and mortality for 24 h and thereafter, for a total of 7 days.

2.10 Determination of Anti-Inflammatory Effect Using Xylene-Induced Oedema Model

Topical anti-inflammatory activity was evaluated following a slight modification of previously reported method [12]. Thirty albino mice (20 – 29 g) of either sex were randomly selected and kept in separate cages. The mice were divided into six groups (A – F) of five animals each. The treatment groups (A – E) received crude methanol extract (CME), and *n*-hexane, ethylacetate, butanol and aqueous fractions dissolved in xylene (5 mg per ear) applied topically on the anterior surface of the right ear. Topical inflammation was induced on the posterior surface of the same ear by application of xylene (0.05 ml). Control (group F) received either equivalent volume of the phlogistic agent (xylene) or diclofenac dissolved in xylene (5 mg per ear). Two hours after application, the mice were sacrificed and both ears harvested. Circular sections (5 mm) of both the right (treated) and left (untreated) ears were punched out using a cork borer, and weighed. Oedema was quantified as weight differences between the two earplugs. The anti-inflammatory activity was evaluated as percent oedema inhibition in the treated animals relative to the control animals using the relation:

$$\text{Oedema reduction (\%)} = \left[1 - \frac{R_t - L_t}{R_c - L_c} \right] \times 100 \quad \dots \text{Eqn. 1}$$

where R_t is the mean weight of right earplug of treated animals, L_t the mean weight of left earplug of treated animals, R_c the mean weight of the right earplug of control (vehicle treated) animals and L_c the mean weight of the left earplug of control (vehicle treated) animals.

2.11 Evaluation of Anti-Inflammatory Activity Using Egg Albumin-Induced Rat Paw Oedema Model

The test was carried out as previously described [3] with a slight modification. Thirty-five Wistar rats (155 – 200 g) of either sex were randomly selected, and kept in separate cages. The rats were divided into seven groups (A – G) of five animals each. They were fasted for 5 h and deprived of water only during the experiment to ensure uniform hydration and to minimize variability in oedematous response. Groups A – E received 250 and 500 mg/kg of the crude methanol extract and fractions solubilized in 10 % Tween®80 by intraperitoneal (i.p) injection.

Group F received 100 mg/kg of diclofenac as a standard drug which serves as the positive control, and Group G (the negative control) received 0.2 ml 10 % Tween®80. All substances were administered 30 min before intraperitoneal injection of the phlogistic agent (0.1 ml of fresh undiluted egg albumin) in the plantar region of the right hind paw. Paw volume was measured at 0.5, 1, 2, 3 and 4 h after induction of oedema by water displacement method. The anti-inflammatory effect was calculated at each time of observation as percentage inhibition of oedema in the animals treated with the test substances in comparison with the vehicle treated animals. The percentage inhibition of oedema was calculated as:

$$\text{Inhibition (\%)} = \frac{V_o - V_t}{V_o} \times 100 \quad \dots\dots\dots \text{Eqn. 2}$$

Where V_t is the volume of oedema of test rats corresponding time, while V_o is the volume of oedema of control rats at the same time

2.12 Determination of Effect on Formaldehyde-Induced Arthritis

This study was carried out according to a previously described method [13]. Thirty-five Wistar rats (150 – 200 g) of either sex were randomly selected and kept in separate cages. The rats were divided into seven groups (A – G) of five animals each. Groups A – E received 250 and 500 mg/kg of the crude methanol extract and fractions of *D. bidens* solubilized in 10 % Tween®80 by intraperitoneal (i.p) injection on day one. One hour later, arthritis was induced by i.p injection of 0.1 ml of 2.5 % formaldehyde solution at the right hind paw of the rats. Group F received 100 mg/kg of diclofenac (i.p) as a standard drug which serves as the positive control, and Group G received 0.2 ml of 10 % Tween®80 as the negative control. The global oedematous response was quantified as the area under curve (AUC) of the time course of the arthritic event. The AUC was calculated using the trapezoid rule to show the degree of inhibition of arthritis using the following equation:

$$\text{Inhibition (\%)} = \frac{AUC_c - AUC_t}{AUC_c} \times 100 \quad \dots\dots\dots \text{Eqn. 3}$$

Where AUC_c = AUC of the control groups, AUC_t = AUC of the treated groups.

2.13 Statistical Analysis

Data analysis was done using Graphpad Prism version 5.0 for Windows software and results were presented as mean ± standard deviation (SD). Significance between control, crude and fraction treated groups were determined using student *t*-test and one-way analysis of variance (ANOVA). Differences between means were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Plant Microscopy

Transverse and longitudinal microscopic examination of the plant revealed the presence of epidermal cells, calcium oxalate crystals, pith cells, phloem and xylem vessels, trichomes, parenchyma cells, cellulosic cells, paracytic stomata, and guard cells as shown in Figs. 1 – 5. Epidermal cells serve as an impermeable barrier which control exchange of materials between plants and their environment as well as provide mechanical strength, support growth and flexibility of the plant [14]. Calcium oxalate crystals are vital indicators for identification and standardization of crude drugs usually found inside the vacuoles of parenchyma cells [15]. Pith cells are essential parenchyma centres for storage of starch or sugar in the root, and play important roles in regeneration and wound healing of plants [16]. The xylem transports dissolved mineral nutrients and water from the roots (unidirectional) using the tracheids and vessel elements. Phloem transports sugars, proteins and minerals both up and down (multi-directional) the stem of plants [17]. Trichomes are excellent diagnostic features which defend plants from phytogous insects, and are vital for water and temperature regulation. Stomata increases photosynthetic potential of plants, regulate water and heat losses from plants [15]. Guard cells surround each leaf stoma and regulate uptake of carbon dioxide and the release of oxygen and water vapour by the stomata [18].

3.2 HPLC-DAD Analysis

The ethylacetate, *n*-hexane, butanol, and aqueous fractions from the CME of *D. bidens* were subjected to HPLC-DAD analysis and compound identification was through a comparison of the ultraviolet (UV) scan of each of these compounds with an in-built library. The HPLC chromatograms of the identified

compounds in the fractions are shown in Figs. 6 and 7. The result revealed the presence of compounds that are mainly phenolic in nature. Ethylacetate fraction revealed the presence of six (6) compounds labelled A – F as follows: A – Apigenin monoglycoside (Retention time, R_t = 22.64 min), B – Quercetin 3-O-(6"O-acetyl) galactopyranoside (R_t = 23.78 min), C = Kaempferol 3-O-(6"O-malonyl) glucoside (R_t = 25.44 min), D – Genistein 8-C-glucoside (R_t = 27.29 min), E – Isoflavone (R_t = 27.81 min), and F – Rocaglamide S (R_t = 28.68 min). In the *n*-hexane fraction, HPLC-DAD analysis revealed the presence of two (2) compounds labelled G and H as follows: G – Cytosporone C (R_t = 28.92 min), and H – Aureonitol (R_t = 38.35 min). Butanol and aqueous fractions showed the presence of Apigenin monoglycoside (R_t = 22.53 min and 19.81 min) already labelled as A. The result indicates that majority of the identified compounds have higher solubility in ethylacetate than in any other fractionating solvent. Compound A (Apigenin monoglycoside) was identified as the most abundant compound in the plant being present in three of the fractions (ethylacetate, butanol, and aqueous). Apigenin monoglycoside is a glycol-flavone which has been identified as a very potent anti-inflammatory compound [19 – 21]. Its anti-inflammatory activity is due to inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (Cox 2) enzymes [20]. Quercetin 3-O-(6"O-acetyl) galactopyranoside is a derivative of quercetin, a flavonol glycoside which has been well documented to have antioxidant and anti-inflammatory activities [22, 23]. Quercetin inhibits inflammatory enzymes including cyclooxygenase, nitrous oxide synthase, lipooxygenase, as well as reduces the level of C-reactive protein, thereby decreasing inflammatory mediators such as prostaglandins and leukotrienes [24]. In addition, quercetin produces its antioxidant activity by scavenging ROS, activating antioxidant enzymes e.g. superoxide dismutase and catalase, inhibition of oxidases, and mitigation of oxidative stress caused by NO [25,26]. Kaempferol 3-O-(6"O-malonyl) glucoside is a kaempferol derivative, an abundant polyphenol compound found in most plants. It has been reported to have many pharmacological activities including anti-inflammatory and antioxidant activities [27,28]. Genistein 8-C-glucoside is an isoflavone glycoside prominent as an active antioxidant compound [29]. Genistein produces its antioxidant activity by inhibiting oxidative stress due to reactive oxygen species which triggers

cellular anomalies such as lipid peroxidation and deactivation of antioxidant enzymes [30]. It has also been reported to have anticarcinogenic effect due to its antioxidant activity [31]. Isoflavone is a derivative of flavonoids which are ubiquitous phenolic bioactive compound abundant in plants, and have been reported with efficient antioxidant activity [29]. Rocaglamide S is a derivative of rocaglamides or flavaglines which are tetrahydrobenzofurans widely reported to have very potent anti-inflammatory property. This could be due to its ability to inhibit proinflammatory factors which activate the immune system inappropriately, leading to abnormal expression of genes encoding inflammatory cytokines and tissue destructive enzymes. It has also been reported to have immunosuppressive activity [32,33]. Cytosporone C is a derivative of cytosporones which are phenolic lipids reported to have antioxidant and anti-inflammatory activities. This could be due to their amphiphilic nature which is attributed to the presence of non-isoprenoid side chains bound to their hydroxybenzene ring [34]. Finally, Aureonitol is a tetrahydrofuran derivative reported to have antioxidant activity [35]. In addition, reports have shown that it has efficient anti-viral activity *in vitro* because it inhibited influenza viral replication [36].

3.3 Phytochemical Tests

Qualitative phytochemical profiles of the crude methanol extract and fractions of *D. bidens* revealed the presence of various secondary metabolites (data not shown). Furthermore, quantitative phytochemical analysis of the extract shown in Table 1 recorded flavonoids as the constituent with the highest concentration (20 %), while saponins and tannins, which occurred in equal concentrations, were the least (14 %). These constituents have been shown to have biological activities including antimalarial, anti-inflammatory, antioxidant, among other activities [37]. The high content of flavonoids confirms the data from HPLC-DAD analysis which revealed that majority of the compounds (apigenin monoglycoside, quercetin 3-O-(6"O-acetyl) galactopyranoside, kaempferol 3-O-(6"O-malonyl) glucoside, genistein 8-C-glucoside, isoflavone, rocaglamide S, cytosporone C) identified in the *D. bidens* fractions were phenolic in nature with anti-inflammatory and antioxidant activities. Therefore, it could be safe to submit that flavonoids were mainly responsible for the anti-inflammatory activity of *D. bidens*.

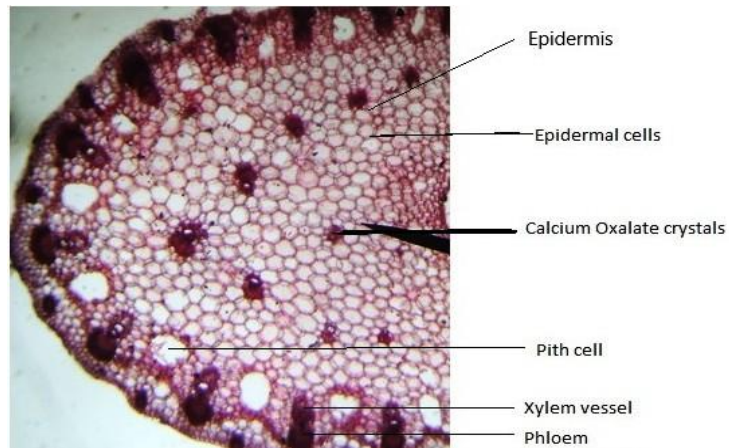


Fig. 1 Microscopic features of transverse section of the leaf of *D. bidens* (x200)

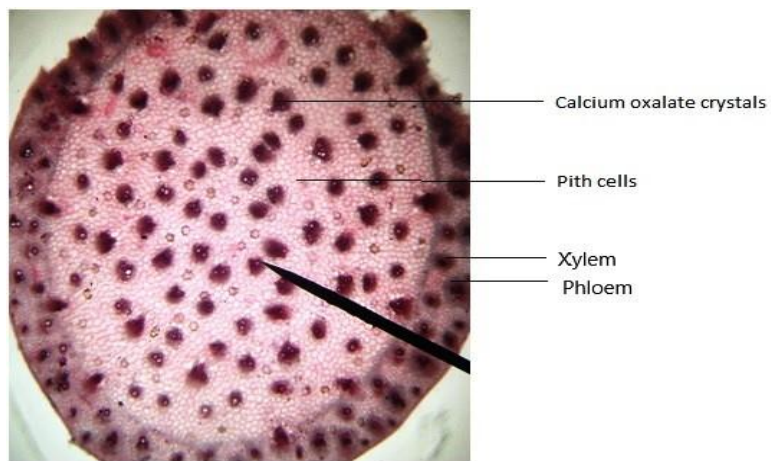


Fig. 2 Microscopic features of transverse section of the stem of *D. bidens* (x200)

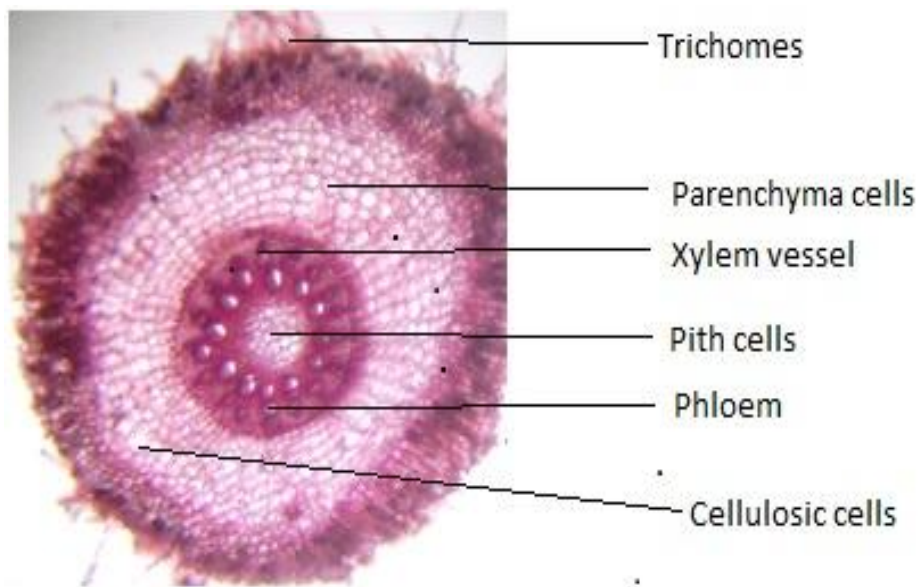


Fig. 3 Microscopic features of transverse section of the root of *D. bidens* (x200)

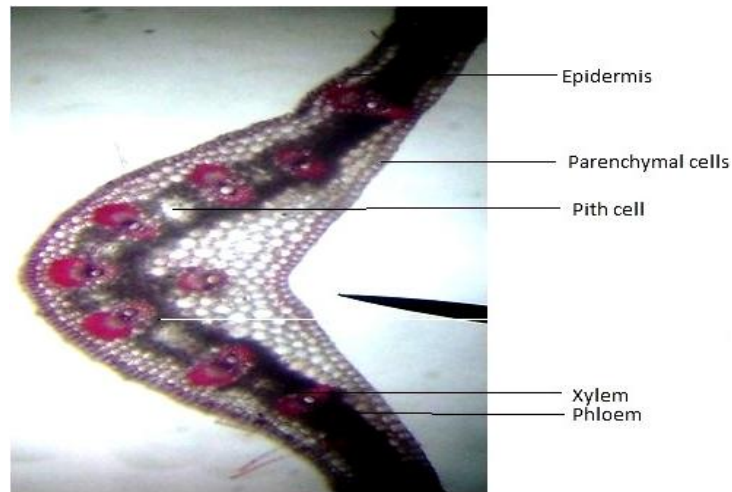


Fig. 4 Microscopic features of longitudinal section of the petiole of *D. bidens* (×200)

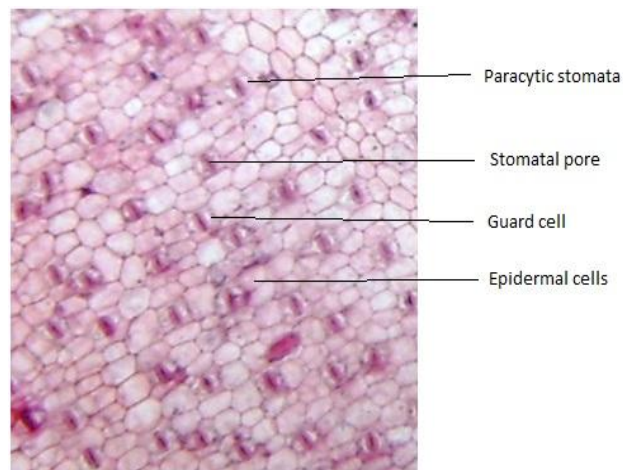


Fig. 5. Microscopic features of leaves of *D. bidens* (×200)

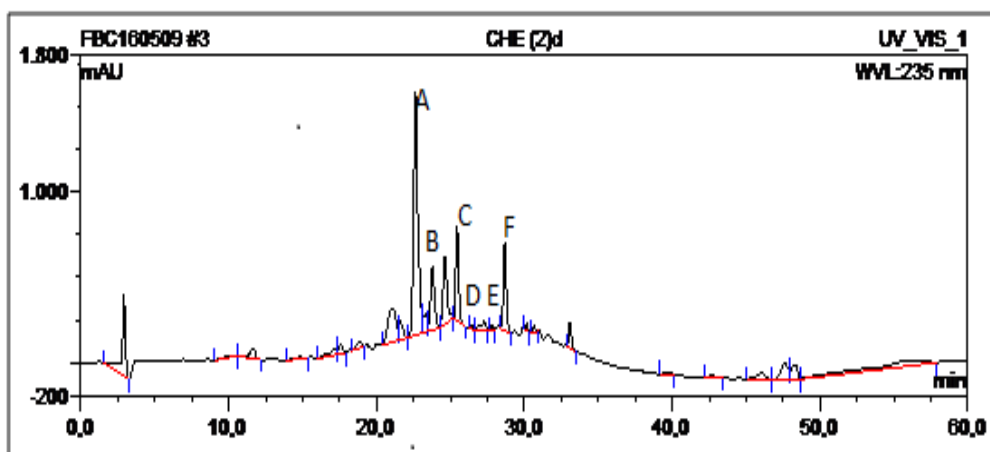


Fig. 6. HPLC chromatogram of ethylacetate fraction of *D. bidens* leaf methanol extract showing six (6) compounds identified and their retention times: A – Apigenin monoglycoside (Retention time, R_t = 22.64 min), B – Quercetin 3-*O*-(6''*O*-acetyl) galactopyranoside (R_t = 23.78 min), C = Kaempferol 3-*O*-(6''*O*-malonyl) glucoside (R_t = 25.44 min), D – Genistein 8-*C*-glucoside (R_t = 27.29 min), E – Isoflavone (R_t = 27.81 min), and F – Rocaglamide S (R_t = 28.68 min)

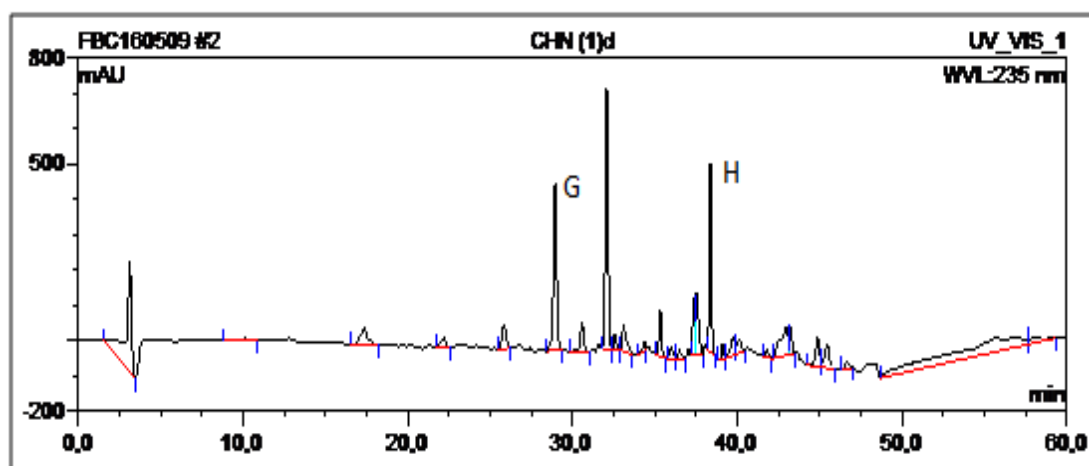


Fig. 7. HPLC chromatogram of *n*-hexane fraction of *D. bidens* leaf methanol extract showing two (2) compounds identified and their retention times: G – Cytosporone C ($R_t = 28.92$ min), and H – Aureonitol ($R_t = 38.35$ min)

Table 1. Quantitative phytochemical constituents of CME of *D. bidens*

Phytochemical	Composition (%)
Alkaloids	15.2
Saponins	14.1
Tannins	14.1
Flavonoids	20.5

3.4 Acute Toxicity Test

Acute toxicity test results from animals are highly essential in judging the safety of crude drugs. The acute toxicity study showed that the crude methanol extract of *D. bidens* is safe with wide safety or therapeutic margin since no death was recorded in the study animals used at a maximum dose of 5,000 mg/kg. Furthermore, no significant change in behaviour of the animals was observed. From the foregoing, the crude extract from *D. bidens* will be assigned class 5 status ($LD_{50} > 5,000$ mg/kg) which is the lowest toxicity class, according to the chemical labelling and classification of acute toxicity recommended by OECD [11].

3.5 Anti-inflammatory Activity

Result of the topical anti-inflammatory activity on xylene-induced oedema as shown in Table 2 indicates that the crude extract and fractions produced significant ($p < 0.05$) percentage inhibition against inflammation ranging from 86 – 97 % in contrast to the positive control (diclofenac) with 53 % inhibition and no (zero) inhibition obtained for the negative control. Ethylacetate fraction gave the highest (97 %) inhibition of inflammation; *n*-hexane and butanol fractions produced equal inflammatory inhibition

(94 %), while aqueous fraction recorded the least (86 %) oedema inhibition.

The highest anti-inflammatory activity produced by the ethylacetate fraction could be due to the presence of the six (6) phenolic compounds (apigenin monoglycoside, quercetin 3-*O*-(6''-*O*-acetyl) galactopyranoside, kaempferol 3-*O*-(6''-*O*-malonyl) glucoside, genistein 8-*C*-glucoside, isoflavone, roscoglanide S) identified in it through HPLC-DAD analysis, and all of which have been shown to possess potent anti-inflammatory activity [19,20,23,24,28,33].

This is followed by the compounds (cytosporone C, aureonitol, and apigenin monoglycoside) identified in the *n*-hexane and butanol fractions. The high concentration and content of the phenols in the ethylacetate fraction could be responsible for the 97 % inhibition of inflammation observed as six (6) compounds were identified in it compared to three (3) identified in the *n*-hexane and butanol fractions together which produced 94 % inhibition each. In addition, the recorded topical anti-inflammatory activity could be attributed to the highly lipophilic nature of the compounds in *D. bidens* which improved their ability to permeate the dermal barrier of the skin to produce their anti-inflammatory effect [12].

Table 2. Effect of crude extract and fractions of *D. bidens* on xylene-induced inflammation

Treatment	Dose (mg/ear)	Oedema (Mean \pm SD)	Inhibition (%)
Crude extract	5.0	0.0073 \pm 0.0	95.33
<i>n</i> -HF	5.0	0.0086 \pm 0.0	94.74
EAF	5.0	0.0075 \pm 0.0	97.37
BUF	5.0	0.0071 \pm 0.0	94.74
AQF	5.0	0.0065 \pm 0.0	86.11
Diclofenac	5.0	0.0118 \pm 0.0	53.21
Vehicle	0.05 ml	0.0350 \pm 0.0	—

^aResult presented as Mean \pm standard deviation (SD). EAF = ethylacetate fraction, *n*-HF = *n*-hexane fraction, BUF = butanol fraction, AQF = aqueous fraction. '—' means zero inhibition

Result of the systemic anti-inflammatory activity on egg albumin-induced rat paw oedema at different time intervals as shown in Fig. 8 indicates a progressive and steady decrease in oedema for all fractions with time which was dose-dependent. Ethylacetate fraction at 500 mg/kg produced the highest ($p < 0.05$) oedema inhibition in comparison with the controls (diclofenac and Tween® 80) and the crude extract, while the aqueous fraction produced the least percentage inhibition of oedema. This further confirms the effectiveness of the phenolic compounds (already listed above) identified in the ethylacetate fraction to modulate the pro-inflammatory activities of inflammatory cells. This suggests that the extract and/or its fractions could be used in the effective management of arthritic and rheumatoid inflammations since the phenolic compounds which are highest in content in the ethylacetate fraction might have inhibited the active release and/or physiological activity of chemical inflammatory mediators such as histamine, prostaglandin, which are prominent inducers of acute inflammation though this assertion remains inconclusive at this level [3].

However, since the highest inhibition of rat paw oedema was recorded at the last study hour (4th hour), it could be inferred that the phenolic compounds of ethylacetate fraction might have inhibited the second phase of inflammation where there is elevated production of prostaglandins, oxygen-derived free radicals, and the production of inducible cyclooxygenase [38].

Result of the effect on formaldehyde-induced arthritis shown in Fig. 9 suggests that paw oedema was generally high on day 1 for all groups shown by high AUC values, including the group administered the standard control, diclofenac. However, it was observed that there was significant ($p < 0.05$) and steady decrease in paw oedema from day 2 for all groups with decreased AUC values indicating that the

fractions and extract at 250 and 500 mg/kg inhibited global oedematous response to formaldehyde-induced arthritis comparable with the standard positive control, diclofenac at 100 mg/kg, except the negative control group which sustained its inflammatory potential throughout the duration of study. A comparison of the extract and fractions shows that ethylacetate fraction produced the best resolution of inflammation with the least AUC value for arthritis throughout the study period followed by the *n*-hexane fraction, then butanol fraction, crude extract, and aqueous fraction. It should be noted that the decreased inflammation recorded for the fractions and crude extract on day 7 was very significant ($p < 0.05$) compared to oedema recorded on day 1. The high potential for arthritic resolution of the crude extract and fractions of *D. bidens* might be attributed to the secondary metabolites content especially flavonoids (phenolics) which have been reported as potent inhibitors of inflammation [12,39]. This is especially true for the ethylacetate fraction which has the highest concentration of phenolics and its highest arthritic inhibition is consistent with earlier observations. The results indicates that the ethylacetate fraction of *D. bidens* is best suited for topical application in the management of arthritis. Since the fractions and extract produced their best inhibition of oedema on day 7, it is possible that the phenolic compounds might have produced their anti-arthritic effect by inhibiting the late stage of inflammatory response characterized by the *in vivo* mobilization and migration of neutrophils to the site of chemotactic stimulus which is a vital stage in the pathogenesis of acute and chronic inflammatory episodes [13]. Thus, inhibition leucocytes migration to the site of inflammation might be an important mechanism of action of the anti-inflammatory effect of the fractions of *D. bidens* especially ethylacetate fraction which contains the highest concentration of the phenolics; however, this is yet to be clarified.

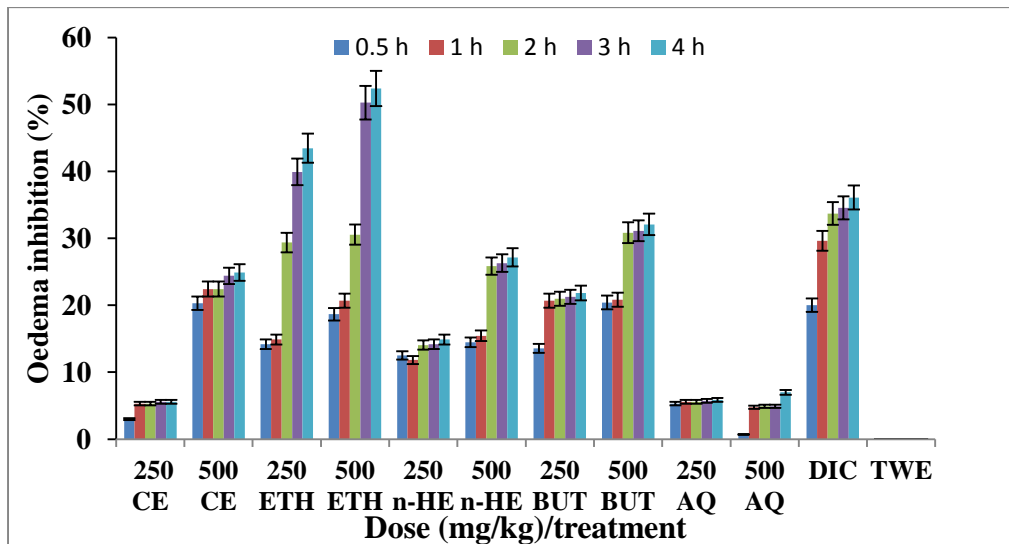


Fig. 8. Percentage inhibition of rat paw oedema by the crude extract and fractions of *D. bidens* at different time intervals

****Key:** Crude extract (CE), ethylacetate fraction (ETH), n-hexane fraction (n-HE), butanol fraction (BUT), aqueous fraction (AQ), diclofenac (DIC), Tween[®]80 (TWE)

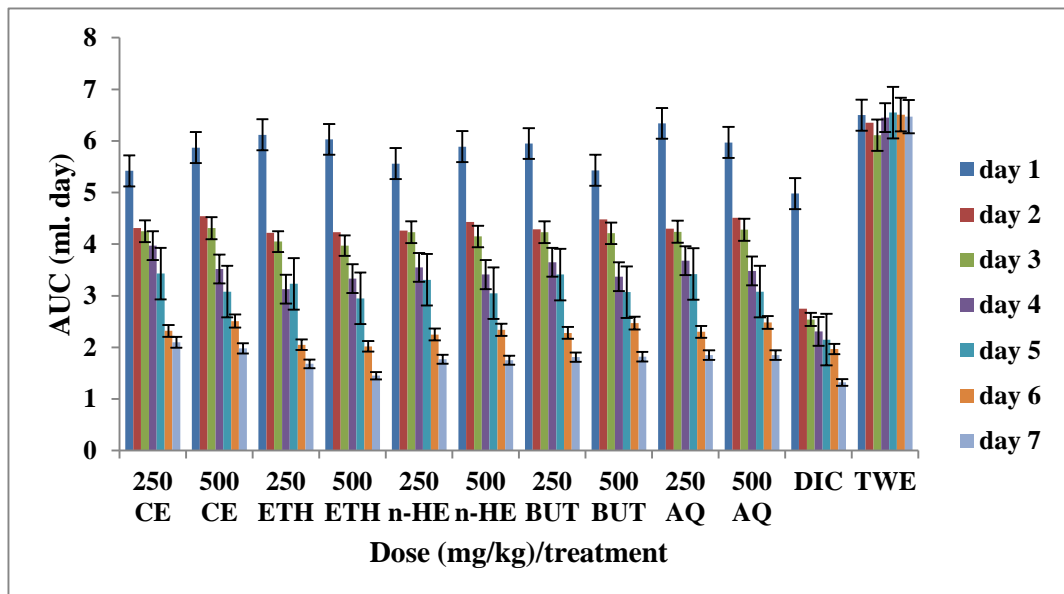


Fig. 9. Percentage inhibition of arthritis by crude extract and fractions of *D. bidens* at different time intervals

*****Key:** Crude extract (CE), ethylacetate (ETH), n-hexane (n-HEX), butanol (BUT), aqueous (AQ), diclofenac (DIC), Tween[®] 80 (TWE)

4. CONCLUSIONS

The microscopic and chemical composition studies embodied in this report represent a good attempt to profile the diagnostic and chemical characteristics of *D. bidens* with the aim of establishing quality control parameters to ensure the identity, quality, safety, and efficacy of the plant material during use. The empirical data

obtained from the anti-inflammatory study of the crude extract and fractions of *D. bidens* highly supports the ethnomedicinal use of the plant in the management of acute and chronic oedema and other inflammatory disorders. The study showed that the ethylacetate fraction of *D. bidens* has the largest content of phenolic compounds which inhibit inflammation probably by inhibiting *in vivo* mobilization and migration of

leucocytes to the site of inflammation. However, further work is required to isolate and elucidate the structure of these constituents in *D. bidens* and determine the exact mechanism(s) of action of its anti-inflammatory activity.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

- World Health Organization. National policy on traditional medicine and regulation of herbal medicines. Report of a WHO global survey; 2005
- Singh R. Chemotaxonomy: A tool for plant classification. *J Med Plants Sci.* 2016;4(2): 9–93
- Osadebe PO, Okoye FBC. Anti-inflammatory effects of crude methanolic extract and fractions of *Alchornea cordifolia* leaves. *J Ethnopharmacol.* 2003; 89: 19–24
- Galam NZ, Gambo IM, Rabi A, Chinelo N, Dami S. Anti-inflammatory effect of aqueous extract of coffee plant leaves (*Coffea canephora*) in rats. *J Nat Sci Res.* 2013; 3(7): 191–193
- Campbell AW. Inflammation: the root of our chronic diseases. *Altern Ther.* 2015; 21(6): 8–9
- Rodrigues G, Lima DM, Montenegro CDA. Database survey of anti-inflammatory plants in South America: A review. *Int J Mol Sci.* 2011; 2692–2749
- Anaga AO, Onuoha A, Igboeli OO, Ezeja M. Antihyperglycemic effects of the methanol leaf extract of *Diaphanthe bidens* in normoglycemic and streptozotocin-induced hyperglycemic rats. *Asian Pac J Trop Med.* 2012:192–196
- Alam F, Saqib QN. Pharmacognostic standardization and preliminary phytochemical studies of *Gaultheria trichophylla*, *Pharm Biol.* 2015;53(12): 1711–1718
- Harbourne JB. *Phytochemical methods: A guide to modern techniques of plant analysis.* 3rd ed. London, Chapman and Hall; 1998.
- Trease GE, Evans W. *Pharmacognosy.* 13th ed. London, Bailliere Tindal; 1996.
- Jothy SL, Zakaria Z, Chen Y, Lau YL, Latha LY, Sasidharan S. Acute oral toxicity of methanolic seed extract of *Cassia fistula* in mice. *Molecules.* 2011;16:5268–5282.
- Okoye FBC, Osadebe PO, Nworu, CS, Okoye NN, Omeje EO, Esimone CO. Topical anti-inflammatory constituents of lipophilic leaf fractions of *Alchornea floribunda* and *Alchornea cordifolia*. *Nat Prod Res.* 2011;25(20):1941–1949
- Okoye FBC, Osadebe PO. Studies on the mechanisms of anti-inflammatory activity of the extracts and fractions of *Alchornea floribunda* leaves. *Asian Pac J Trop Med.* 2009;2(3):7-14.
- Glover BJ. Differentiation in plant epidermal cells. *J Experim Bot.* 2000; 51(344):497–505
- Ambi AA, Nuru GF, Mora AT, Ahmad A. Pharmacognostic studies and elemental analysis of *Cassytha filiformis* Linn. *J Pharmacogn Phytotherap.* 2017;9(8):131–137
- Brooker RJ, Widmaier EP, Graham LE, Stilling PD. *Biology.* New York, McGraw-Hill; 2008.
- Raven PH, Evert RF, Eichhorn SE. *Biology of plants,* 7th ed. W.H. Freeman and Company, New York; 2005.
- Kerr S. *Plant development I: tissue differentiation and function.* Georgia Technol Bull. 2016;1–20.
- Ngwoke KG, Akwagbulam AG, Erhirhie EO, Ajaghaku DL, Okoye FBC, Esimone CO. Antioxidant, anti-inflammatory, analgesic properties and phytochemical characterization of stem bark extract and fractions of *Anthocleista nobilis*. *Pharmacognosy Res.* 2018;10(1):81–87.
- Ali F, Rahul Naz F, Jyoti S, Siddique YH. Health functionality of apigenin: A review. *Int J Food Prop.* 2017;20(6):1197–1238
- Qiulan H, Yiran G, Rao F, Tong P, Yuting Z, Fang C. Antioxidant activity of flavonoids from leaves of *Jatropha curcas*. *ScienceAsia* 2014;40:193–197
- Materska M. Quercetin and its derivatives: chemical structure and bioactivity – a

- review. Polish J Food Nutr Sci. 2008; 58(4): 407 – 413
23. Panat NA, Amrute BK, Bhattu S, Haram SK, Sharma GK, Ghaskadbi SS. Antioxidant profiling of C3 quercetin glycosides: Quercitrin, quercetin 3-β-D-glucoside and quercetin 3-O-(6''-O-malonyl)-β-D-glucoside in cell free environment. Free Rad Antioxid. 2015; 5(2): 90 – 100
 24. David AVA, Arulmoli R, Parasuraman S. Overviews of biological importance of quercetin: A bioactive flavonoid. Pharmacogn Rev. 2016; 10(20): 84 – 89
 25. Prochazkova D, Bousova I, Wilhelmova N. Antioxidant and prooxidant properties of flavonoids. Fitoter. 2011; 82: 513–523
 26. Lakhampal P, Rai DK. Quercetin: A versatile flavonoid. Int J Med Update. 2007; 2: 22 – 37
 27. Dehshahri S, Wink M, Afsharypuor S, Asghari G, Mohagheghzadeh A. Antioxidant activity of methanolic leaf extract of *Moringa peregina* (Forssk) Fiori. Res Pharm Sci. 2012; 7(2): 111 – 118
 28. Riaz A, Rasul A, Hussain G, Zahor MK, Jabeen F, Subhani Z, et al. Astragaloside: A bioactive phytochemical with potential therapeutic activities. Adv Pharmacol Sci. 2018; 1 – 15
 29. Kinfe HH, Long HS, Stander MA, van Wyk BE. The major phenolic compound of the roots and leaves of *Rafina amplexicaulis* (Fabaceae), a liquorice substitute and traditional tea used in Cape herbal medicine. South Afr J Bot. 2015; 100: 75 – 79
 30. Kaur, IP, Geetha T. Screening methods for antioxidants. A review. Minirev. Med Chem. 2006; 6: 305 – 312
 31. Antosiak A, Milowska K, Maczynska K, Rozalska S, Gabryelak T. Cytotoxic activity of genistein-8-C-glucoside from *Lupinus luteus* L. and genistein against human SK-OV-3 ovarian carcinoma cell line. Med Chem Res. 2017; 26(1): 64 – 73
 32. Proksch P, Giaisi M, Treiber MK, Palfi K, Merling A, Spring M, et al. Rocaglamide derivatives are immunosuppressive phytochemicals that target NF-AT activity in T cells. J Immunol. 2005; 174: 7075 – 7084
 33. Li-Weber M. Molecular mechanisms and anticancer aspects of the medicinal phytochemicals rocaglamides (= Flavaglines). Int J Cancer. 2015; 137: 1791 – 1799
 34. Oliveira RJ, Navarro SD, de Lima DP, Meza A, Pesarini JR, Gomes RS, et al. A novel cytosporone 3-heptyl-4, 6 –dihydroxy – 3H – isobenzofuran – 1 – one: synthesis, toxicological, apoptotic and immunomodulatory properties, and potentiation of mutagenic damage. BMC Cancer. 2015; 15: 561
 35. Li H, Liao Z-B, Tang D, Han W-B, Zhang Q, Gao J-M. Polyketides from two *Chaetomium* species and their biological functions. J Antibiot. 2018; 71(7): 677 – 681
 36. Sacramento CQ, Martorelli A, Fintelman-Rodrigues N, de Freitas CS, de Melo GR, Rocha MEN, et al. Aureonitol, a fungus-derived tetrahydrofuran, inhibits influenza replication by targeting its surface glycoprotein hemagglutinin. PLoS ONE. 2015; 10(10): e139236
 37. Ismail M, Haq NU, Ali S, Ali I, Shaheen R, Murtaza G. Qualitative analysis for phytochemicals of selected medicinal plants from Gilgit-Baltistan, Pakistan. Asian J Chem. 2017; 29(9): 1929 – 1932
 38. Ajaghaku DL, Ilodigwe EE, Okonta MJ, Ogbue CO, Okafor MA, Igboeme SO. Mechanism of anti-inflammatory activity of the leaf extract and fractions of *Millettia aboensis*. Int Res J Pharm. 2013; 4(9): 54 – 59
 39. Arzi A, Olapour S, Yaghooti H, Karampour NS. Effect of royal jelly on formalin-induced inflammation in rats' hind paw. Jundishapur J Nat Pharm Prod. 2015; 10(1): e22466

© 2021 Umeyor et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:

The peer review history for this paper can be accessed here:
<https://www.sdiarticle4.com/review-history/70857>