Journal of Medicinal Plants and By-products (2024) 1: 219-232
https://doi.org/10.22034/jmpb.2023.359374.1486

Original Article

Insights on the Anti-inflammatory Mechanism of the Polyphenolic-Rich Fraction of *Glyphaea brevis* (Spreng.) Monachino (Tiliaceae) Leaves

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Article History

Received: 14 July 2022
Accepted: 30 April 2023

Keywords
Glyphaea brevis
Phytochemicals
Anti-inflammation
Albumin Denaturation

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ABSTRACT

Glyphaea brevis (Spreng.) Monachino has been employed folklorically in West Africa for decades to manage inflammatory disorders such as peptic ulcer, edema, dyspepsia, and worm infestations; however, these pharmacological activities have not been scientifically proven. The study investigated the chemical composition and *in-vitro/in-vivo* anti-inflammatory capabilities of the polyphenolic-rich fraction of *G. brevis* leaves (PREG). *In-vitro* anti-inflammatory indices were evaluated using the human red blood cell (HRBC) methods. The acute lethality (LD_{50}) test was demonstrated in mice with graded dosages (10 to 5000 mg/kg body weight of PREG) via oral intubation. While *in-vivo* anti-inflammatory activity was determined using a mice model inflicted with an intraperitoneal injection of 0.1ml of undiluted fresh egg albumin paw edema using egg albumin. All parameters were assayed according to standard protocols. High levels of tannins, phenols, flavonoids, and minimum amounts of terpenoids, steroids, alkaloids, and saponins were observed in PREG. The LD_{50} test demonstrated no toxicity and mortality in mice up to 5.0 g/kg bw p.o. PREG. The anti-inflammatory assays showed that at the different concentrations (0.2 - 1.0 mg/ml), PREG effectively inhibited albumin denaturation, platelet aggregation, hypotonicity-induced hemolysis, protease, and phospholipase A2 activity, as the standard drugs (Aspirin and Prednisolone). Also, PREG suppressed significantly ($p< 0.05$) the progression of egg albumin-induced mice paw edema, and these increased with time (0.5 - 5h). The maximum percentage of edema inhibition (91.4%) was observed in mice administered with 400 mg/kg bw PREG, and this was close to that (94.49%) obtained in the group administered with the reference drug (Indomethacin). These give insights into the anti-inflammatory potential of PREG.

INTRODUCTION

Inflammation is a complex protective mechanism put forward by the immune system to protect living tissues from foreign entities and protect against cancerous cell growth [1]. However, uncontrolled and persistent inflammatory processes have been implicated as a causative factor in several long-term health disorders such as rheumatoid arthritis, asthma, osteoarthritis, colitis neurodegenerative, and cardiovascular diseases [2,3]. These diseases' substantial social and economic consequences on families and victims cannot be over-emphasized. Additionally, they can result in disability, disruption of the victim's social activities and quality of life, and sometimes death [4,5]. Inflammation is frequently manifested with pains, redness, swelling,

and fever due to increased vascular permeability, leukocyte migration, lysosomal leakages, protein denaturation, and tissue damage [6].

Synthetic antihistamine drugs, steroidal (corticosteroids) and non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), and immunosuppressants are the most widely recommended medications for the management of acute and chronic inflammatory diseases [7,8]. The effect of these drugs is to suppress inflammation, prevent joint destruction, provide pain relief, and restore disabled joint functions. However, their uses are associated with side effects such as gastrointestinal problems, immunosuppression, humoral changes, and many other life-threatening consequences [9]. These undesirable effects explain the need for new agents with better anti-inflammatory efficacy and high safety profile than the existing ones. Natural compounds of plant origin offer an unrivalled store of molecular variety to drug research and development [10].

Glyphaea brevis (Spreng.) Monachino (Tiliaceae) is an average-sized climber that can be seen in tropical Africa's forest regrowths, marshy areas, and rocky savannahs. It possesses irregular, sparsely stellate branchlets, ovate-oblong-shaped leaves, and lemon-yellow coloured flowers [11]. The plant *G. brevis* is also called masquerade stick (common name), Aloanyansi (Igbo), Dorina (Hausa), and Atori (Yoruba) [11,12]. Traditionally, plant materials treat gastric ulcers, edema, dyspepsia, and worm infestations. In contrast, root decoction manages chest pain, constipation, male infertility, and gastrointestinal disorders [12]. Studies have reported several pharmacological activities of *G. brevis* stem, root, and bark, such as anti-malaria antioxidant, anti-inflammatory, and antibacterial properties [13-15]. The chemical characterization of this plant has been extensively carried out in previous studies [16,17]. However, much has not been accomplished with the plant. The present study seeks to ascertain the *in vitro* and *in vivo* anti-inflammatory mechanism of ethanol extract of *G. brevis* leaves (PREG).

MATERIALS AND METHODS

Equipment and Reagents

All the chemicals and reagents used in the study were pure and analytical grade and products of British Drug House (BDH) England; Fluka Germany; Sigma Aldrich, USA; Harkin and Williams, England; Qualikems India; May Baker England; and Burgoyne India. The assay reagents in the study were commercialized kits and products of Teco (TC) and Randox, companies both in the USA.

Ethical Clearance

The study complied with ethical rules all through the study. The ethics committee of the Department of the Biochemistry University of Nigeria Nsukka reviewed and approved the research protocol and tendered document of ethical clearance and safe conduction of the experimental processes (approval number: UNN/BCH/9050). All human blood donors' informed consent and permission were properly solicited and obtained.

Identification of the Study Plant

The unsullied leaves of *G. brevis* were sourced from Nsukka metropolises of Enugu-Nigeria (6.8429° N, 7.3733° E) in June 2017. The leaf identification was performed by Mr. Alfred Ozioko (a specialist in BDCP). For further investigation, a specimen of the plant leaves was kept in the herbarium of the Bioresources Development and Conservation Program Research Centre, Nsukka, Enugu, Nigeria (voucher no: BDCP20210629).

Extraction Procedure

Fresh *G. brevis* leaves were collected and washed to remove any dirt. The leaves were then dried in the shade until crispy, turning them continuously to prevent rotting. A mechanical grinder was used to pulverize the dry leaves into powder. A measured amount (1.5 kg) of the pulverized leaves was macerated in 95% ethanol. The mixture was kept for 72 h with intermittent stirring and then filtered through a cotton cloth into a flask with a flat bottom. Further filtration was done with Whatman No.1 filter paper to eliminate fine residues and use a rotary evaporator at 45 °C. The filtrate was concentrated to obtain the crude ethanol extract. The resultant crude extract was stored at 2 – 8 °C in a well-labelled sterile screw-capped vessel.

Preparation of the Polyphenolic-Rich Fraction

The preparation procedures followed the methods described by Chu *et al*. [18] and Jesumani *et al*. [19]. A weighed amount (3 g) of ethanol extract was

introduced into a small flask containing 20 ml of 10 % H_2SO_4. The hydrolysis was facilitated by incubating in a water bath at 100 °C for 30 min, after which the reaction mixture was allowed to stay on ice for 15 min. The filtrate of the resulting precipitated solution was filtered and, was further dispensed into a warm (50 °C) volume of 95% ethanol (50 ml). The resulting mixture was then poured into a 100 ml graded flask and was made up to 100 ml using ethanol (95%) to provide the crude ethanolic extract rich in polyphenols. The residue from the ethanol extraction was mixed with 200 ml of distilled water at 65 °C under stirred conditions for 1h. Then the extract was subjected to centrifugation for 15 min at 15,000 rpm and the resultant supernatant was mixed with $CaCl_2$ and kept overnight at 4 °C to remove the alginate. A graded volume (3 ml) of 99% ethanol was mixed with the supernatant and was allowed to stay overnight at 4 °C to precipitate the polysaccharide content. The precipitate which is rich in polysaccharides was discarded, while the filtrate was concentrated with a rotary evaporator at 45 °C and reduced pressure to obtain the polyphenolic-rich fraction. The procedures were repeated several times to obtain a sufficient quantity of the rich fractions.

Phytochemical Analysis

Preliminary qualitative and quantitative phytochemical screening of PREG was performed using the following formula.

$$\text{Concentrations (mg/100 g)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Dilution factor}$$

$$\text{Dilution factor} = \frac{\text{Total volume}}{\text{Weight of extract}}$$

In vitro Anti-Inflammatory Assays

Phospholipase A_2 Activity

The human red blood cell (HRBC) method was adopted in assaying for the effect of the extract on Phospholipase A2 activity, following the laid down protocols by Vane [20]. The activated enzyme acts on the erythrocyte membrane, liberating phospholipids and ripping the lipid bilayer. These result in the leakage of haemoglobin into the reaction medium. As a result, the degree of activity of the enzyme is proportional to the haemoglobin concentration in the reaction milieu, read spectrophotometrically at 418 nm. Briefly, The HRBCs pellets, after centrifugation, were washed three times using an equivalent amount of normal saline. A 40% (v/v) reconstituted volume of packed cell was obtained using a phosphate-buffered solution (10 mM pH 7.4) and served as the enzyme substrate. Insets of three test tubes, graded amount (0.2 ml) of HRBC, were mixed with equal volume (0.2 ml) of 2 M $CaCl_2$ and crude enzyme (from a pure fungal culture of the *Aspergillus niger* strain). Each triplicate set of test tubes containing the reacting mixtures was mixed with normal saline and either the reference drug (prednisolone) or varied concentrations of the extract and was incubated for one hour. Except for the test samples, the red cell suspension, isolated enzyme, and $CaCl_2$ constituted the control test tube. A graded volume (0.2 ml) of the heat-induced denatured enzyme was included in the blank. The test tubes were centrifuged at 3000 rpm for ten minutes after incubation at ambient temperature. Following the distribution of 10 ml of normal saline into each graded volume of the supernatant (1.5 ml), the solutions' absorbance at 418 nm was read. The maximal enzyme activity and inhibition were calculated in percentage, thus:

$$\%\, maximum\ activity = \frac{Abs\ of\ test/t}{Abs\ of\ control/t} \times 100$$

$$\%\, inhibition = 100 - \%\, maximum\ activity\ of\ enzyme$$

Where Abs = Absorbance, t = time.

Protease Inhibition Assay

According to the method described by Oyedepo and Femurewas [21], this assay was done with minor modifications. The reaction milieu was composed of a graded amount of trypsin (0.06 mg), Tris HCl buffer (20 mM 1 ml, pH 7.4), and 1 ml of standard drug or varying concentrations (200-1000 µg/ml) of the extract. After 5 min of incubation at room temperature, 1 ml of 0.8% casein (w/v) was introduced. Afterwards, the mixture was heated further for 20 min following the addition of 70% perchloric acid (2 ml) to halt the reaction. The hazy suspension was spun at 3000 rpm for ten minutes, and the supernatant's absorbance was noted against the blank (buffer) at 210 nm. The test was done in triplicates, and the degree of proteinase inhibition was estimated thus:

$$\%\ Inhibition = \left(\frac{Abs\ control - Abs\ sample}{Abs\ control}\right) X\ 100$$

Inhibition of Albumin Denaturation

This was performed following the protocol described by Mizushima and Kobayashi [22]. The reaction mixture consists of the extracts or standard drug (Aspirin) and a 1% aqueous solution of albumin isolated from bovine. A few amounts of 1 N HCl were used to adjust the solution pH. After cooling for 10 min at ambient temperature, the mixture was heated at 51 °C for 20 min. The resultant mixture was cooled to room temperature, and the absorbance (Abs) was recorded at 660 nm. The test was done in triplicates, and the degree of proteinase inhibition was estimated thus:

$$\%\ Inhibition = \left(\frac{Abs\ control - Abs\ sample}{Abs\ control}\right) X\ 100$$

Calcium Chloride-induced Platelet Aggregation

Principle

This assay was performed according to the protocol described by Born and Cross [23]. Calcium chloride-induced platelet aggregation was used to examine the extract's anti-inflammatory properties *in-vitro*. Amplified platelet clumping in the reaction medium results in decreased absorption and increase light transmittance. The change in absorbance of the reaction medium read at 520 nm reflects the degree of inhibition exerted by the extract on $CaCl_2$-induced platelet aggregation.

Procedure

Graded volumes (5 ml) of whole blood samples were withdrawn intravenously from healthy human volunteers, transferred into EDTA bottles, and centrifuged for 10 min at 3,000 rpm in EDTA vials. The platelet-rich plasma (PRP) was obtained by collecting the supernatant and diluting it twice with normal saline. Afterwards, it was dispensed into the clean plastic tube and served as the PRP. The experiment was done in triplicates. A graded volume (0.2 ml) of PRP was dispensed into three test tubes, each consisting of 1 ml of the extractor of the reference drug (Aspirin) dissolved in normal saline at varying concentrations (200 - 1000 µg/ml). Normal saline, which served as the vehicle, was used to make the volume mark up to 2.2 ml. Graded volumes of PRP (0.2 ml) and normal saline (2 ml) were in the control test tube. The test tubes were heated before adding a graded volume (0.4 ml) of 1.47% $CaCl_2$ to induce platelet clumping. The difference in absorbance of the reaction mixture was noted at 520 nm, spectrophotometrically at 2-mins intervals for 8 mins. The blank always contained PRP void of extract or standard drugs. The % Inhibition of platelet clumping (X) was evaluated thus;

$$\%\ Inhibition\ of\ platelet\ aggregation\ (X) = \frac{A - B}{A} \times 100$$

Here; the control's aggregation rate maximum = A, while B is the test sample's aggregation rate maximum.

Hypotonicity-induced Haemolysis

This test was determined using the modified methods of Oyedepo *et al*. [24] and Ezekwesili and Nwodo [25]. A graded volume (3 ml) of whole blood sample was taken from a healthy volunteer, centrifuged at 3,000 rpm for 10 min, and washed three times with an equal volume of normal saline. The blood volume was reconstituted to 40 % (v/v) using normal saline. The extract and reference drug (indomethacin) was diluted with distilled water to obtain the hypotonic solution. An aliquot (1 ml) of varying concentrations (0.2 - 1.0 mg/ml) of the extract or standard drug was dispensed into five sets of three tests tube. The mixture volume in each test tube was made up to 4.9 ml mark using distilled water. One test tube represented the control and was made up of an equivalent volume (4.9 ml). After incubation, each tube's reaction mixture was centrifuged at 3000 rpm for 10 min, and the supernatant was measured spectrophotometrically at 418 nm. The tests were performed in triplicates. The respective blank for each test was a reaction medium made up of 1 ml of various concentrations of the extract or standard drug prepared up to 5.0 ml using normal saline and void of the RBCs. The control tubes' blanks were normal saline with no HRBC suspension. The degree of hemolysis inhibition exerted by the test samples was estimated by adopting the relation below:

$$\%\ Inhibition\ of\ Haemolysis = 100\ (1 - \frac{Abs2\ - Abs1}{Abs3 - Abs1})$$

Where Abs1 = Isotonic solution absorbance Abs2 = test sample absorbance, Abs3 = hypotonic solution absorbance.

In vivo Anti-Inflammatory Assay

Experimental Animals

Mature and healthy Swiss Albino mice of known weight ranges of 25 - 35 g, were employed in the study. The animals were purchased from the Faculty of Veterinary Medicine Animal House, University of Nigeria, Nsukka. The animals were acclimatized at the Department of Biochemistry's animal farm under standard laboratory conditions for a week before the experimental process, with 12 h light/dark cycles. They were administered *ad libitum* with standard (Vital) and clean water. Throughout the trial and experimental period, they were given human care in conformity with the laid down ethical protocol as enshrined in the revised NIH Guide for the Care and Use of Laboratory Animals [26]. They were sacrificed after the experiment, according to ethical guidelines.

Acute toxicity (LD_{50}) study

This was done using adult mice following the outlined protocols of Lorke [27]. The geometric average of the greatest non-fatal and minimum deadly dosages was used to determine the LD_{50}. Animals assigned into twelve groups (three mice per group) were deprived of food and water for 12 h before the experiment. Afterwards, they were administered (peroral) 10, 100, 1000, 1600, 2900, and 5000 mg/kg body weight of PREG, respectively, via gastric gavage. Twenty-four-hour post-treatments, the mice were observed for behavioural changes (in-coordination, dullness, and nervousness) or death. The mice were further observed for seven days for any latent toxicity. The result of the study was used to estimate the doses of PREG (100, 300, and 400 mg/kg bw p.o.) employed in the study.

Egg Albumin-Induced Mice Paw Edema.

Egg albumin-induced paw edema test is an effective *in vivo* model of inflammation used to evaluate the propensity of anti-inflammatory potentials of pharmacologic agents [27]. The edema induction is accompanied by increased vascular permeability, swelling of the paw, and fluid exudation. Inhibition of the attendant edema decreases the extent and magnitude of the epidemic paw and corresponds with the anti-inflammatory activity of the test compound. The change in mice paw volumes can be evaluated with a digital Plethysmometer device. In practice, the Plethysmometer device consists of a special water cell in which an animal paw is inserted. The change in resistance (calibrated in millilitres) following the paw immersion is displayed on the electronic monitor and corresponds with the volume displacement. The test procedure was similar to that documented by Okokon and Nwafor [28]. Briefly, animals were randomly assigned into five (5) groups of five mice each (n = 5). The vehicle (10 mg/kg bw per normal oral saline), varying concentrations (100, 300, and 400 mg/kg bw p.o.) of PREG and reference drug (10 mg/kg bw p.o. indomethacin), respectively, were administered to 24 h starved mice in the respective groups one h before the injection of a phlogistic agent. One hour post-administration, 0.1 ml of undiluted fresh egg albumin was injected intraperitoneally into the mice's right hind paw. The change in volume displacement of the paw of the mice (denoted as (Vt – Vo)) was measured using a digital Plethysmometer device instantly [29], at time zero of the experiment and 1 hr interval following egg albumin injection, for five (5) hours using the following formulae;

$$\text{Percentage (\%) inhibition of edema} = \frac{(\text{Vt} - \text{Vo})\ \text{control} - (\text{Vt} - \text{Vo})\ \text{treatment}}{(\text{Vt} - \text{Vo})\ \text{control}} \times 100$$

Where: Vo = mice paw edema volume at time zero, Vt = mice paw edema volume after various time intervals.

After the experiment was completed, the animals were euthanatized with halothane (an anaesthetic agent).

Statistical Analysis

Data obtained were analyzed with one-way and two-way ANOVA in SPSS (statistical product and service solutions) version 23. Using the Duncan *post hoc*, a significant difference ($p< 0.05$) between the groups where obtained. Results were expressed as Mean ± Standard deviation (from Descriptive tables). Values in the same row with the capital alphabet as superscripts and values in the same

column with lowercase alphabets as superscripts differed significantly.

RESULT AND DISCUSSION

In as much as the numerous synthetic drugs that inundate the market are not without several side effects, the need for alternative therapeutic agents from natural sources for disease management is strongly advocated for and has attracted a lot of public concerns [30]. Tremendous volumes of evidence exist on the use of the plant in folkloric medicines in several world societies, dating from the primordial age up to the contemporary era [31]. The upsurge in interest in alternative medicines has afforded insights and guides, modern scientists, to discover and design modern pharmaceutics and other therapeutics, including essential foods [5]. However, there is still a gap in scientific proof of the efficacy and mechanism of action of some of these medicinal plants [32]. Plants have evolved to withstand biotic and abiotic stress by synthesizing a variety of secondary metabolites, which help them survive these oxidative and environmental assaults [33]. As a result, the repository of bioactive secondary metabolites synthesized in plants allows for the isolation of possible drug-like compounds that could be exploited as pharmacological agents in disease management.

Phytochemical Examination of PREG

As shown in Table 1, the phytochemical screening showed that PREG contains high polyphenolic compounds such as tannins, flavonoids, and other phenols, with a moderate amount of terpenoids, steroids, alkaloids, and saponins also detected. This result corresponds with Oloruntobi and Olusola's [17] findings, who reported the presence of tannins, flavonoids, and other phytochemicals in the ethanolic extract of pulverized leaves *Glyphea brevis*. In consonance with this, Mbosso *et al*. [16] affirm the presence of appreciable levels of phenolic compounds in the leaf extract of *Glyphea brevis*. Many of these phytochemicals have been proposed to demonstrate anti-inflammatory potentials *in vitro* and *in vivo* studies [10,34]. For instance, the anti-inflammatory potentials of phenolics have long been reported by investigators [35-37]. Phenolic compounds include flavonoids, tannins, and other polyphenols. Of these groups, the most essential from a nutritional standpoint is said to be flavonoids. Based on structural differences, some classes of important flavonoids include; chalcones, anthocyanins, flavan-3-ols, flavonols, isoflavones, flavonols, and flavones, while aurones, flavan-3, 4-diols, coumarins, dihydroflavones are minor groups [38]. The numerous health benefits from the intake of phenolic compounds have been attributed to the bioactive end-product metabolites after enterobacteria actions. Numerous bacteria populating the gut convert food-derived phenolics; notable of them is the phylogenetically associated Eubacterium and Clostridium genera [39]. These gut bacteria transform food-derived phenolic compounds into absorbable forms, possibly due to strong phenolic-microbiota interactions. Evidence from *in vitro* studies has demonstrated the activities of intestinal microbes in the bioconversion of polyphenols from red wine, grape juice, and black tea [40]. It is also posited that a specific microbiota in the gut can eliminate the rutinosides in orange juice glycosides and transform them into aglycones, which are further absorbed into the body [40].

The analgesic and anti-inflammatory mechanism of flavonoids, which entails their direct suppression of the biosynthesis of eicosanoids which are established inflammatory mediators, has been demonstrated [41]. Flavonoids also act by reducing the levels of Ca^{2+} ions in the intracellular milieu and, as a result, repress the generation of pro-inflammatory mediators like TNF-α [36,42]. Studies have also documented that tannins are powerful cyclooxygenase-1 (COX-1) inhibitors and could also silence the activities of phlogistic agents [41]. Steroids, like corticoids, act via the steroid receptors as anti-inflammatory agents in mechanisms that involve either the positive (trans-activation) or negative (trans-repression) modulation of inflammation-related gene expression [43].

Effect of PREG on Albumin Denaturation

The degree of suppression of protein denaturation demonstrated by PREG and reference drugs are shown in Table 2. The result showed that PREG exhibited significantly ($p< 0.05$) high inhibition (in %) of albumin denaturation, following a trend that correlated positively with concentrations and was maximum (46.0%) at the greatest dosage (1.0 mg/ml). Furthermore, PREG exerted a similar inhibitory effect (34.0%) on albumin denaturation as the standard drug (26.0%), at a similar dosage (0.8

mg/ml), and the difference was not significant ($p > 0.05$).

Protein denaturation assay is one of the several testing procedures and biomarkers that have been deployed in the screening of anti-inflammatory potencies of various products [44]. Denatured proteins lose their bioactivity and induce the mobilization and increased activities of leukocytes [45]. Protein denaturation has been linked with the progression of several inflammatory disorders, including rheumatoid arthritis [46]. Therefore, the ability of pharmacological agents to inhibit the distortion of the native structure of proteins (albumin) *in vitro* may afford insights into its anti-inflammatory potential [47]. The result showed that the % inhibition of albumin denaturation exhibited by PREG was significantly ($p< 0.05$) higher than the reference drug and followed a trend that correlates positively with concentrations. These suggest that PREG competed favourably with standard drugs and could be adopted as a better anti-inflammatory formulation if purified.

Effect of PREG on Proteinase Activity

The degree of suppression (in %) of proteinase activity demonstrated by PREG compared with the standard drug is shown in Table 3. The result showed that PREG inhibited proteinase activity significantly ($p < 0.05$) in a fashion that correlates positively with concentrations. PREG demonstrated maximum % inhibition (42 %) of the enzyme activity at the highest dose (1.0 mg/ml). Besides, PREG exhibited significantly ($p < 0.05$) high (37.0 %) suppressive effect on the proteinase activity at mid-dose (0.8 mg/ml), and competed favourably with % inhibition (35.0%) of the reference drug at the same concentration (0.8 mg/ml) and the difference was not significant ($p> 0.05$).

The high suppressive effect (%) of proteinase activity exhibited by PREG, akin to the standard drug (Aspirin), suggests its potential as a good candidate for anti-inflammatory drug formulation. Proteinases abundantly exist in lysosomal granules of neutrophils, which predominates the inflammatory milieu a few minutes following tissue injury [48]. Studies have demonstrated the involvement of proteinase in the initiation and amplification of tissue injury during inflammation [6, 49]. Additionally, their denaturation of cellular and membrane-bound proteins marks them for phagocytic cell recognition and destruction, further resulting in increased mobilization of leukocytes. Their amplified activities increase cell death and tissue damage [50]. Bermúdez-Humarán *et al*. [51] reported that proteinase is implicated in several pathological conditions such as arthritis and results in intensified inflammation upon profuse production by immune cells and lysosomal leakages. A previous study has proposed that, since leucocyte proteases play critical roles in exacerbating tissue damage at the site of inflammation, agents that inhibit their disproportionate activity could proffer a significant degree of protection against inflammatory disorders [52].

Effect of PREG on the Activity of Phospholipase A2

The suppressive effect of PREG on phospholipase A2 activity compared with the standard drug is shown in Table 4. The result shows that PREG significantly ($p < 0.05$) suppresses the enzyme activity in a trend directly proportional to the dosages. PREG exhibited its greatest (51.33 %) inhibitory effect at the highest dosage (1.0 mg/ml). However, the standard drug (Prednisolone) exhibited significantly ($p< 0.05$) greater inhibition (73.50%) of the enzyme activity compared to that obtained with the extract (49.89%) at a similar concentration (0.8 mg/ml).

The observed inhibitory effect of the polyphenolic-rich fractions on this enzyme suggests its anti-inflammatory mechanism and resembles the principle of action of steroidal anti-inflammatory drugs. The lipase, upon activation, breaks down membrane-bound phospholipids and the resulting fatty acids liberated include arachidonic acid, a precursor substrate that feeds the cycloxygenases (COX) I and II and 5-lipoxygenase (5-LOX) pathways, and results in the biosynthesis of several potential inflammatory mediators [53]. The activity of COX on this substrate molecule leads to the synthesis of inflammatory mediators, including PGD2, PGI2, PGE2, and TXA2. Also, arachidonic acid is utilized by 5-LOX to produce leukotrienes such as LTB4, a chemo-attractant, and lipoxins, which are potent vasodilators. The resulting eicosanoids play different roles in initiating and amplifying inflammatory cascades [54]. Hence agents that inhibit eicosanoid biosynthesis are believed to proffer better anti-inflammatory effects

via mechanisms that nip at the bud of these inflammatory mediators by depriving COX and LOX of their substrate molecule [55]. They decrease vascular permeability, vasodilatation, chemotaxis, and tissue damage and are void of complications that accompany NSAIDs, such as mucosal erosion, peptic ulcer, and immunosuppression, resulting from mucosal erosion, peptic ulcer, and immunosuppression the over-suppression of COX I activity [56].

Effect of PREG on $CaCl_2$-induced Platelet Aggregation

The percentage inhibition of platelet aggregation exerted by PREG compared with the standard drug is presented in Table 5. The result showed that PREG suppressed platelet clumping significantly ($p < 0.05$) in a concentration- and time-dependent. The maximum inhibitory effect of PREG and the standard drug on $CaCl_2$-induced platelet aggregation was attained after the 8th minute of the experiment. Besides, PREG demonstrated a significantly ($p < 0.05$) more potent inhibitory effect (93.0%) on platelet clumping, compared to that obtained with the reference drug (81.0%) at the same dose (0.4 mg/ml).

These results imply that the purified polyphenolic-rich fraction could elicit even more anti-inflammatory potency than the reference drug. The anti-aggregatory effect of PREG could be due to its ability to suppress the action of PLA2 and COX, which are essential enzymes for the biosynthesis of thromboxane A2 (TXA2), a potent platelet aggregator. The eicosanoid TXA2 initiates platelet aggregation by inducing the elevation of intracellular Ca^{2+}, which facilitates the translocation of platelet granules toward its membrane. The subsequent exocytosis liberates inflammatory mediators, including ADP, enhancing platelet clumping. The anti-platelet aggregatory effect of PREG could also be due to reduced histamine and P-selectin activities, which upon platelet degranulation and aggregation, mediates vascular permeability and leukocyte extravasation [54].

Table 1 Phytochemical Composition of PREG

Phytochemical Composition	Relative abundance	Concentration (mg/100 g)
Phenols	++	459.200±44.195
Flavonoids	++	347.312±11.442
Tannins	+++	925.642±40.656
Terpenoids	++	41.868±13.605
Glycosides	+	ND
Saponins	+	0.126±0.004
Steroids	+	0.908±0.045
Alkaloids	+	18.878±0.382

Values are expressed in Mean ± SD; +, ++, and +++ = low, moderate and high abundance respectively.

Table 2 Effect of PREG on albumin denaturation

Treatments	Concentrations (mg/ml)	Absorbance at 660nm	% Inhibition of albumin denaturation
PREG	0.2	0.255±0.004 e	20.09±0.03 a
	0.4	0.245±0.005 d	23.11±0.01 b
	0.6	0.222±0.005 c	30.17±0.04 d
	0.8	0.210±0.007 b	34.02±0.04 de
	1.0	0.185±0.003 a	42.00±0.06 e
Aspirin	0.2	0.252±0.004 e	21.03±0.02 ab
	0.4	0.239±0.001 d	25.45±0.03 bc
	0.8	0.237±0.001 b	26.27±0.01 c

Results are expressed as Mean ± SD. Values in the same row with the capital alphabet as superscripts and values in the same column having different small-lettered alphabets as superscripts differ significantly ($p< 0.05$). n = 5.

Table 3 Effect of PREG on protease activity

Treatments	Concentrations (mg/ml)	Absorbance	% Inhibition of Protease activity
PREG	0.2	0.263±0.007 f	15.05±0.01 a
	0.4	0.223±0.006 d	28.39±0.03 c
	0.6	0.214±0.006 c	31.28±0.05 d
	0.8	0.194±0.003 b	37.47±0.03 e
	1.0	0.180±0.004 a	42.19±0.02 f
Aspirin	0.2	0.244±0.004 e	21.26±0.04 b
	0.4	0.221±0.002 cd	28.48±0.02 c
	0.8	0.199±0.001 b	35.07±0.06 e

Results are expressed as Mean ± SD. Values in the same row with the capital alphabet as superscripts and values in the same column having different small-lettered alphabets as superscripts differ significantly ($p< 0.05$). n = 5.

Table 4 Effect of PREG on phospholipase A_2 activity

Treatments	Concentrations (mg/ml)	Absorbance (418nm)	%Maximum	%Inhibition of Phospholipase A_2 activity
PREG	0.2	0.863±0.003 h	95.68±0.02 h	4.32±0.04 a
	0.4	0.766±0.001 g	84.92±0.10 g	15.08±0.01 b
	0.6	0.503±0.001 e	55.76±0.04 f	44.24±0.02 c
	0.8	0.452±0.002 d	50.11±0.07 e	49.89±0.07 d
	1.0	0.439±0.001 c	48.67±0.02 d	51.33±0.06 d
Prednisolone	0.2	0.534±0.003 f	59.20±0.04 c	40.80±0.03 c
	0.4	0.289±0.004 b	32.04±0.06 b	67.96±0.02 e
	0.8	0.239±0.002 a	26.50±0.09 a	73.50±0.04 f

Results are expressed as Mean ± SD. Values in the same row with the capital alphabet as superscripts and values in the same column having different small-lettered alphabets as superscripts differ significantly ($p< 0.05$). n = 5.

Table 5 Effect of PREG on calcium chloride-induced platelet aggregation

	Conc.	Δ Absorbance			
Treatment	(mg/m)	2 min	4 min	6 min	8 min
PREG	0.2	0.317±0.001 CB (81.0%)	0.322±0.003 EC (94.0%)	0.340±0.001 ED (99.0%)	0.314±0.001 CA (90.0%)
	0.4	0.326±0.002 EA (83.0%)	0.325±0.002 EA (95.0%)	0.332±0.002 DB (97.0%)	0.324±0.003 EA (93.0%)
	0.6	0.321±0.002 DB (82.0%)	0.317±0.002 DAB (93.0%)	0.316±0.002 CA (92.0%)	0.315±0.003 CA (91.0%)
	0.8	0.300±0.003 Bb (76.0%)	0.296±0.005 BAB (87.0%)	0.296±0.002 BAB (86.0%)	0.293±0.001 BA (84.0%)
Aspirin	0.2	0.301±0.000 BA (77.0%)	0.310±0.001 CB (91.0%)	0.316±0.001 CC (92.0%)	0.320±0.001 DD (92.0%)
	0.4	0.264±0.001 AA (67.0%)	0.271±0.001 AB (79.0%)	0.276±0.001 AC (80.0%)	0.282±0.002 AD (81.0%)

Results are expressed as Mean ± SD. Values in the same row with the capital alphabet as superscripts and values in the same column having different small-lettered alphabets as superscripts differ significantly ($p < 0.05$). n=5.

Table 6 Effect of PREG on hypotonicity-induced haemolysis

Treatments	Concentrations (mg/ml)	Absorbance	% Inhibition
PREG	0.2	0.145±0.002 f	22.50±0.07 a
	0.4	0.106±0.001 d	43.40±0.05 bc
	0.6	0.103±0.002 d	45.70±0.07 c
	0.8	0.088±0.001 c	53.30±0.01 d
	1.0	0.074±0.001 b	60.50±0.02 e
Indomethacin	0.2	0.112±0.005 e	40.20±0.06 b
	0.4	0.104±0.001 d	44.70±0.03 c
	0.8	0.065±0.002 a	65.30±0.01 f

Results are expressed as Mean ± SD. Values in the same row with the capital alphabet as superscripts and values in the same column having different small-lettered alphabets as superscripts differ significantly ($p< 0.05$), n=5.

Table 7 Acute lethality test (LD_{50}) of PREG

Dose (mg/kg b.w po)	Mortality rate
10 mg/kg b.w po PREG	0/3
100 mg/kg b.w po PREG	0/3
1000 mg/kg b.w po PREG	0/3
1600 mg/kg b.w po PREG	0/3
2900 mg/kg b.w po PREG	0/3
5000 mg/kg b.w po PREG	0/3

n=3; bw= body weight; po= per oral administration

Table 8 Effect of PREG on egg albumin-induced paw edema in mice

Groups		0.5 h	1 h	2 h	3 h	4 h	5 h
I	5 mg/kg bw normal saline	4.12±0.24 BA	4.56±0.24 BB	4.70±0.15 DBC	4.87±0.18 DBCD	4.99±0.19 DCD	5.08±0.18 DD
II	10 mg/kg bw indomethacin	3.40±0.20 AD (17.48%)	2.89±0.78 AD (36.62%)	1.42±0.44 AC (69.79%)	1.02±0.39 ABC (79.06%)	0.43±0.12 AAB (91.38%)	0.28±0.05 AA (94.49%)
III	100 mg/kg bw PREG	3.70±0.26 ABD (10.19%)	3.08±0.61 ACD (32.46%)	2.70±0.56 CBC (42.55%)	2.14±0.45 CAB (56.06%)	1.86±0.55 CA (62.73%)	1.56±0.37 BBA (69.29%)
IV	300 mg/kg bw PREG	3.61±0.60 ABC (12.38%)	3.21±0.63 AC (29.61%)	2.09±0.48 BB (55.53%)	1.88±0.60 BCA (61.40%)	1.45±0.39 BCA (70.94%)	1.23±0.31 BA (75.79%)
V	400 mg/kg bw PREG	3.38±0.54 AD (17.96%)	2.47±0.28 AC (45.83%)	1.63±0.16 ABB (65.32%)	1.39±0.07 ABB (71.46%)	1.18±0.24 BB (76.35%)	0.45±0.30 AA (91.14%)

Results are expressed as Mean ± SD. Values in the same row with the capital alphabet as superscripts and values in the same column having different small-lettered alphabets as superscripts differ significantly ($p< 0.05$). n=5.

Since platelets aggregation is implicated in the pathophysiology of myriads of diseases such as embolism, stroke, myocardial infarction, and other vascular thromboses [57], its inhibition by pharmacological agents affords credence for their possible adoption as an antithrombotic drug-like substance for the treatment of inflammatory and thrombotic disorders.

Effect of PREG on Hypotonicity-induced Hemolysis

The percentage inhibitory effect of PREG on hypotonicity-induced hemolysis compared with the standard drug is presented in Table 6. The result revealed that PREG effectively inhibited hypotonicity-induced hemolysis dose-dependent, akin to the reference drug (Aspirin). The maximum % inhibition (60.50%) of PREG was observed at the highest dose (1.0 mg/ml). Besides, the % inhibition (53.30%) exhibited by the extract was close to that obtained with the standard drug (65.30%) at a similar concentration (0.8 mg/ml).

This strongly supports the anti-inflammatory potential of PREG. Similarly, the submission of Enechi *et al*. [52] established that the capacity of plant extracts to foster the integrity of lysosomal membranes, protect against lysosomal leakage of proteolytic enzymes, and free radicals liberation, reflects its anti-inflammatory potential. Prolonged Inflammatory conditions, usually present with an unwarranted breakdown of the lysosomal membranes of phagocytic cells and the endothelium, which usually results in the release of hydrolytic enzymes that further instigate inordinate destruction of macromolecules and body tissues [58]. NSAIDs such as Aspirin exert their anti-inflammatory effects either by inhibiting the liberation of lysosomal protease or fostering the stability of the lysosomal membrane [59]. Following the exposure of red cells to deleterious agents such as heat and hypotonic media, hemolysis and oxidation of the free haemoglobin ensued [29]. The suspension of cells in a hypotonic medium results in the cell membrane rupturing due to the excessive accumulation of fluid

in the cell's cytoplasm [59]. Since the morphology of the lysosomal membrane resembles that of the red cells, agents that inhibit hypotonicity-induced hemolysis are believed to stabilize the lysosomal membrane and protect against lysosomal leakages [60].

Acute lethality test (LD_{50}) of PREG

As shown in Table 7, the lethality test showed that PREG caused no mortality or toxicity in mice, even at a high dose of 5000 mg/kg b.w po PREG. The acute toxicity (LD_{50}) study of PREG suggests that the plant polyphenolic-rich fraction is safe even at high dosages and could be administered orally without fear of toxicity, thus, representing a possible option for drug formulation, targeted toward disease that defiles the orthodox approach. Investigators have reported that pharmacological agents that demonstrate oral LD_{50} up to 5000 mg/kg could be deemed nonlethal and safe to use as a therapeutic agent [29,61].

Effect of PREG on Egg Albumin-induced Mice Paw Edema.

Furthermore, the *in-vivo* anti-inflammatory propensity of PREG was evaluated based on its ability to inhibit the progression of mice paw edema induced by the injection of a phlogistic agent (egg albumin) into the animal's hind paw [62]. The degree (%) of inhibition of paw edema exerted by PREG relative to the standard drug (indomethacin) is shown in Table 8. Animals in the groups administered with PREG before paw edema induction had significantly ($p< 0.05$) lower paw edema volume, thus, enhancing suppression of edemogenesis in a directly proportional manner to time. The decrease in paw size progressively occurred in a time-related manner. At the fifth hour, mice fed with 10 mg/kg bw indomethacin (group II) had the least edema volume, hence the greatest % inhibition of edema (68.30%) compared to other groups. However, there were no significant ($p > 0.05$) differences compared to the % inhibition (91.14%) demonstrated in *mice* administered with 400 mg/kg bw PREG (group V).

The subcutaneous injection of egg albumin in mice's hind paws results in edema formation, excessive exudation of tissue water, protein-rich plasma, and increased neutrophil extravasations [29]. The amplified paw edema volume in mice after egg albumin injection suggests the capacity of egg albumin to serve as a phlogistic agent. Egg albumin-induced edemogenesis follows two phases [29]. The early phase begins instantly after administering an irritant and lasts for about 2 h. This phase is typified by generating inflammatory mediators like histamine and serotonin, which increase blood flow, vasodilation, and leukocyte recruitment at the injured site. The later phase happens 3-5 h later and is typified by the amplified actions of bradykinin, protease, prostaglandins, and lysosomal leakage [63]. As revealed in the result, the time-dependent suppressive potential of PREG on paw edema progression in mice suggests its capacity to abrogate the various stages of the inflammation cascades. The decrease in the volume of mice paw edema observed in the result was akin to that obtained with the reference drug (indomethacin). These may involve the lessening of the early inflammation phase characterized by the excessive activities of histamine and serotonin and the inhibition of prostaglandin and kinin production which are usually apparent in the later phase [29]. These inflammatory mediators initiate increased vascular permeability, vasodilatation, and other inflammatory cascades [58]. Thus, the progressive reduction in the size of paw edema seen in the groups treated with various concentrations of PREG depicts its capacity to protect against inflammatory disorders. A comparative study by Dickson *et al*. [13] also reported that extracts from *G. brevis* leaves and stem bark had significant anti-inflammatory and antiallergic effects on carrageenan-induced foot edema. Obiri *et al*. [12] also demonstrated the antiallergic and antiarthritic activities of polyphenolic-rich fractions from *G. brevis* stem bark using Murine models.

CONCLUSION

The findings of the present study imply that polyphenolic-rich fraction of *G. brevis* leaves (PREG) possess significant anti-inflammatory activities and exert these effects following several mechanisms, which may include membrane stabilization and the inhibition of platelet aggregation, albumin denaturation, protease, and phospholipase A2 activity and egg albumin-induced mice paw edema. However, we recommend that further investigations be carried out to identify and probably isolate the bioactive compounds that elicit

the observed bioactivities and their unique possible mechanism of action.

Statements and Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests.

Credit Authorship Contribution Statement

Osmund Chukwuma Enechi: designed and performed most of the experiment and wrote the manuscript; Uchenna Collins Abonyi: helped perform the experiment and analysis with constructive discussions; Emmanuel Sunday Okeke: designed the experiments and wrote the manuscript; Goodness Doom Aker: helped perform the experiment and analysis with constructive discussions; Nnamdi Collins Eze: helped perform the experiment and analysis with constructive discussions; Chisom Jennifer Edeh: helped review the manuscript; Veronica Chisom Iloh: helped review the manuscript; Stephen Chijioke Emencheta: helped perform the analysis with constructive discussions and wrote the manuscript.

ACKNOWLEDGEMENT

We wish to acknowledge the entire academic and laboratory staff of the Department of Biochemistry of the University of Nigeria for their immense contribution towards the success of this work.

REFERENCES

1. Ahmed A.U. An overview of inflammation: mechanism and consequences. Front in Biol. 2011;6. http://dx.doi.org/10.1007/s11515-011-1123-9
2. Guo H., Callaway J.B., Ting J.P. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med. 2015;21:677-687. https://doi.org/10.1038/nm.3893.
3. Wu T. The effect of *Psidium guajava* hydroalcoholic extract on reducing the expression of Resistin and TNF-α genes in articular chondrocytes of patients with knee osteoarthritis. Cell and mol biol (Noisy-le-Grand, France). 2022;67(6): 74-81. https://doi.org/10.14715/cmb/2021.67.6.11
4. Larson E. Social and economic impact of infectious diseases--United States. Clin Perform Qual Healt Car. 1997;5:31-37.
5. Furman D., Campisi J., Verdin E., Carrera-Bastos P., Targ S., Franceschi C., Ferrucci L.W., Gilroy D., Fasano M.A., Miller G.W., Mantovani A.H., Weyand A., Barzilai C.M., Goronzy N., Rando J.J., Effros T.A.R.B., Lucia A., Kleinstreuer N., Slavich G.M. Chronic inflammation in the etiology of disease across the life span. Nat Med. 2019;25:1822-1832. https://doi.org/10.1038/s41591-019-0675-0
6. Chen L., Deng H., Cui H., Fang J., Zuo Z., Deng J., Li Y., Wang X., Zhao L. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017;9(6):7204-7218. https://doi.org/10.18632/oncotarget.23208
7. Kragballe K. Topical corticosteroids: mechanisms of action. Acta Derm Venereol Suppl (Stockh). 1989;151:7-10.
8. Zhu L., Zhang C., Chuck R.S. Topical steroid and non-steroidal anti-inflammatory drugs inhibit inflammatory cytokine expression on the ocular surface in the botulinum toxin B-induced murine dry eye model. Mol Vis. 2012;18:1803-1812.
9. Mpofu S. Steroids, non-steroidal anti-inflammatory drugs, and sigmoid diverticular abscess perforation in rheumatic conditions. Ann Rheum Dis. 2004;63:588-590. https://doi.org/10.1136/ard.2003.010355
10. Fürst R., Zündorf I. Plant-derived anti-inflammatory compounds: hopes and disappointments regarding the translation of preclinical knowledge into clinical progress. Mediat of Inflamm. 2014;1-9. http://dx.doi.org/10.1155/2014/146832
11. Osafo N., Boakye Y. *Glyphaea brevis* (Spreng.) Monach: A review of the ethno-medical, phytochemical and pharmacological investigations. Br J Pharm Res 2016;12:1-18. https://doi.org/10.9734/bjpr/2016/26689.
12. Obiri D.D., Osafo N., Abotsi R.E. Antiallergic and antiarthritic effects of stem bark extract of *Glyphaea brevis* (Spreng) Monachino (Tiliaceae) in murine models. ISRN Pharmacol. 2013;1–13. https://doi.org/10.1155/2013/874263.
13. Dickson R.A., Ekuadzi E., Annan K., Komlaga G. Antibacterial, anti-inflammatory, and antioxidant effects of the leaves and stem bark of *Glyphaea brevis* (Spreng) Monachino (Tiliaceae): a comparative study. Phcog Res. 2011;3:166-172.
14. Anjuwon T.M., Adepoju O.A., Adeniran S.M. A comparative study of antimalarial and toxicological effects of aqueous and methanol extracts of glphaea brevis leaves in mice. Afr J Biomed Res. 2019;22:309-314.
15. Olugbodi J.O., David O., Ojo O.A., Akinmoladun A.C. Influence of *Glyphaea brevis* twig extract on nucleus , tight junctions and expression of inhibin - β, stem cell factor, and androgen binding protein in TM4 Sertoli cells. Asia Pac J of Rep. 2019;8(4):157–166. https://doi.org/10.4103/2305-0500.262832
16. Mbosso E.J.T., Wintjensa R., Bruno Ndjakou Lentac B.N., Ngouelad S., Rohmere M., Tsamod E. Chemical constituents from *Glyphaea brevis* and *Monodora myristica*: chemotaxonomic significance. Chemist and Biodiver. 2013;10:224-232.

17. Oloruntobi J.A., Olusola J.O. Analysis of bioactive components in ethanolic extract of *Glyphaea brevis* leaves. J of EmergTren in Engineer and App Sci. 2015;6(4):263-266.
18. Chu Y.F., Sun J., Wu X., Liu R.H. Antioxidant and antiproliferative activities of common fruits. J Agric Food Chem. 2002;50:7449-7454.
19. Jesumani V., Du H., Pei P., Aslam M., Huang N. Comparative study on skin protection activity of polyphenol-rich extract and polysaccharide-rich extract from *Sargassum vachellianum*. PLo On. 2020;15(1): e0227308. https://doi.org/10.1371/journal.pone.0227308
20. Vane J.R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 1971;231:232–235. https://doi.org/10.1038/newbio231232a00
21. Oyedepo O.O., Femurewas A.J. Anti-protease and membrane stabilizing activities of extracts of *Fagra zanthoxiloides*, *Olax subscorpioides* and *Tetrapleura tetraptera*. In J Pharm. 1995;33: 65-69.
22. Mizushima Y., Kobayashi M. Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins. J Pharm Pharmacol. 1968;20:169-173. https://doi.org/10.1111/j.2042-7158.1968.tb09718.x.
23. Born G.V.R., Cross M.J. The aggregation of blood platelets. J Physiol. 1963;168:178-195.
24. Oyedepo O.O., Akindele V.R., Okunfolami OK. Effect of extracts of Parham, P. The immune system. New York (NY): Garland Publishing. 1997.
25. Ezekwesili C.N., Nwodo O.F.C. Anti-inflammatory properties of Vigna unguiculata seed extract. J Med Lab Sci. 2000; 9:141-145.
26. National Institutes of Health (US), author Guide for the Care and Use of Laboratory Animals. NIH Publication No. 85-23. Revised 1985.
27. Lorke D. A new approach to practical acute toxicity testing. Arch Toxicol. 1983;53:275-289.
28. Okokon J.E., Nwafor P.A. Antiinflammatory, analgesic and antipyretic activities of ethanolic root extract of *Croton zambesicus*. Pak J Pharm Sci. 2010;23:383-390.
29. Anosike C.A., Onyechi O., Ezeanyika L.U.S. The anti-inflammatory activity of garden egg (*Solanum aethiopicum*) on egg albumin-induced oedema and granuloma tissue formation in rats. Asian Pac J Trop Biomed. 2012;1:62-66.
30. Ekor M. The growing use of herbal medicines : issues relating to adverse reactions and challenges in monitoring safety. Front in Pharmacolo. 2014;4(January):1-10. https://doi.org/10.3389/fphar.2013.00177.
31. Šantić Ž., Pravdić N., Bevanda M., Galić K. The historical use of medicinal plants in traditional and scientific medicine. Medici Academi Mostariens. 2017;5(1-2):69-74.
32. Lim X.Y., Teh B.P., Tan T.Y.C. Medicinal plants in COVID-19: potential and limitations. Front. Pharmacol. 2021;12:611408. https://doi.org/10.3389/fphar.2021.611408
33. Di Marco G., Gismondi A., Panzanella L., Canuti L., Impei S., Leonardi D., Canini A. Botanical influence on phenolic profile and antioxidant level of Italian honeys. J Food Sci Technol. 2018;55:4042-4050.
34. Farooq S., Shaheen G., Asif H.M., Aslam M.R., Zahid R., Rajpoot S.R., Jabbar S., Zafar F. Preliminary phytochemical analysis: *in-vitro* comparative evaluation of anti-arthritic and anti-inflammatory potential of some traditionally used medicinal plants. Dos Respon. 2022;20(1):15593258211069720. http://dx.doi.org/10.1177/15593258211069720
35. Yang N.N., Zhang Y.F., Zhang H.T., Ma X.H., Shen J.H., Li P., Zhang Y.H. The *in vitro* and *in vivo* anti-inflammatory activities of triterpene saponins from *Clematis florida*. Nat Prod Res. 2020;1-4.
36. Yang T., Hu Y., Yan Y., Zhou W., Chen G., Zeng X., Cao Y. Characterization and evaluation of antioxidant and anti-inflammatory activities of flavonoids from the fruits of *Lyciumbarbarum*. Foods. 2022;11:306.
37. Waghole R.J., Misar A.V., Kulkarni N.S., Khan F., Naik D.G., Jadhav SH. *In vitro* and *in vivo* anti-inflammatory activity of *Tetrastigmasulcatum* leaf extract, pure compound and its derivatives. Inflammopharmaco. 2022;30:291-311.
38. Schauss A.G. Polyphenols and Inflammation. In: Watson RR and Preedy VR (eds.) Bioactive Food as Dietary Interventions for Arthritis and Related Inflammatory Diseases. San Diego: Academic Press, 2013, pp. 379-392.
39. Selma M.V., Espín J.C., Tomás-Barberán F.A. Interaction between phenolics and gut microbiota: role in human health. J Agric Food Chem 2009;12(57)(15):6485-6501. https://doi.org/10.1021/jf902107d
40. Gross G., Jacobs D.M., Peters S., Possemiers S., Duynhoven J.V., Vaughan E.E., Wiele T.V.D. *In vitro* bioconversion of polyphenols from black tea and red wine/ grape juice by human intestinal microbiota displays strong interindividual variability. J Agric Food Chem A-J. 2010. https://doi.org/10.1021/jf101475m
41. Attiq A., Jalil J., Husain K., Ahmad W. Raging the war against inflammation with natural products. Front in Pharmaco. 2018;9. http://dx.doi.org/10.3389/fphar.2018.00976
42. Mozaffarian D., Wu J.H.Y. Flavonoids, dairy foods, and cardiovascular and metabolic health. Circulat Res. 2018;122:369-384.
43. Timmermans S., Souffriau J., Libert C. A general introduction to glucocorticoid biology. Front Immunol. 2019;4(10):1545. https://doi.org/10.3389/fimmu.2019.01545

44. Gunathilake K.D.P.P., Ranaweera K.K.D.S., Rupasinghe H.P.V. *In vitro* anti-inflammatory properties of selected green leafy vegetables. Biomedicin. 2018;6:107 http://dx.doi.org/10.3390/biomed icines6040107
45. Dhama K., Latheef S.K., Dadar M., Samad H.A., Munjal A., Khandia R., Karthik K., Tiwari R., Yatoo M.I., Bhatt P., Chakraborty S., Singh K.P., Iqbal H.M.N., Chaicumpa W., Joshi S.K. Biomarkers in stress related diseases/disorders: diagnostic, prognostic, and therapeutic values. Front in Mol Biosci. 2019;6. http://dx.doi.org/10.3389/fmolb.2019.00091
46. Osman N.I., Sidik N.J., Awal A., Mara T. *In vitro* xanthine oxidase and albumin denaturation inhibition assay of *Barringtonia racemosa* L . and total phenolic content analysis for potential anti-inflammatory use in gouty arthritis. J of Intercult Ethnopharmaco. 2016;5(4). https://doi.org/10.5455/jice.20160731025522
47. Raju M.G., Srilakshmi S., Suvarchala NVLR. Anti-inflammatory, *in-silico* docking and adme analysis of some isolated compounds of *Tageteserecta* flower heads. Int J Pharm Sci Res. 2019;11:1358-1366.
48. Rosales C. Neutrophil: a cell with many roles in inflammation or several cell types? Front in Physiol. 2018;9. https://doi.org/10.3389/fphys.2018.00113
49. Chanchal S., Mishra A., Singh M.K., Ashraf M.Z. Understanding inflammatory responses in the manifestation of prothrombotic phenotypes. Front in Cell and Develop Bio. 2020;8. http://dx.doi.org/10.3389/fcell.2020.00073
50. Hirayama D., Iida T., Nakase H. The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis. Intern J of Molecu Sci. 2017;19(1):92. https://doi.org/10.3390/ijms19010092
51. Bermúdez-Humarán L.G., Motta J.P., Aubry C. Serine protease inhibitors protect better than IL-10 and TGF-β anti-inflammatory cytokines against mouse colitis when delivered by recombinant lactococci. Microb Cell Fact. 2015;14:26.
52. Enechi O.C., Okeke E.S., Abonyi U.C., Nwankwo N.E., Ndimele U.J., Benneth M.E. Evaluation of *in vitro* anti-inflammatory and antioxidant potentials of methanol leaf extract of *Fagarazanthoxyloides*. Indo Am J Pharm Sci 2019;6:14365-14371.
53. Hanna V.S., Hafez E. Synopsis of arachidonic acid metabolism: A review. J of Adv Res. 2018;11:23-32. https://doi.org/10.1016/j.jare.2018.03.005
54. Wang B., Wu L., Chen J. Metabolism pathways of arachidonic acids: mechanisms and potential therapeutic targets. Sig Transduct Target Ther. 2021;6:94 https://doi.org/10.1038/s41392-020-00443-w
55. Ricciotti E., FitzGerald G.A. Prostaglandins and inflammation. Arteriosclero, Thrombo, and Vascul Biolo. 2011;31(5):986-1000. https://doi.org/10.1161/ATVBAHA.110.207449
56. Takeuchi K. Pathogenesis of NSAID-induced gastric damage: importance of cyclooxygenase inhibition and gastric hypermotility. World J of Gastroenterol. 2012;18(18):2147–2160. https://doi.org/10.3748/wjg.v18.i18.2147
57. Fabre J., Gurney M.E. Limitations of current therapies to prevent thrombosis : a need for novel strategies. Mol Biosyst. 2010;6:305-315. https://doi.org/10.1039/b914375k
58. Umapathy E., Ndebia E.J., Meeme A., Adam B., Menziura P., Nkeh-Chungag B.N., Iputo J.E. An experimental evaluation of *Albucasetosa* aqueous extract on membrane stabilization, protein denaturation and white blood cell migration during acute inflammation. J Med Plant Res. 2010;4:789-795.
59. Kardile M.V., Mohamad A.S., Israf D.A. Membrane Stabilization assay for anti-inflammatory activity yields false positive results for samples containing traces of ethanol and methanol. World J Pharm Pharm Sci. 2016;5:493-497.
60. Yoganandam G.P., Mohamad A.S., Israf D.A. Evaluation of anti-inflammatory and membrane stabilizing properties of various extracts of *Punicagranatum* L. (Lythraceae). Int J Pharmtech Res. 2010;2:1260-1263.
61. Enechi O.C., Amah C.C., Okagu I.U., Ononiwu CP, Azidiegwu VC, Ugwuoke EO, Ndukwe EE. Methanol extracts of *Fagara zanthoxyloides* leaves possess antimalarial effects and normalizes haematological and biochemical status of *Plasmodium berghei*-passaged mice. Pharm Bio. 2010;57:577-585. http://dx.doi.org/10.1080/13880209.2019.165675
62. Omkar A., Jeeja T., Chhaya G. Evaluation of anti-inflammatory activity of *Nyctanthes arbour-tristis* and *Onosma echioides*. Pharmacogn Mag. 2007;2:258-260.
63. Kang H.S., Lee J.Y., Kim C.J. Anti-inflammatory activity of arctigenin from *Forsythiae fructus*. J Ethnopharmacol. 2008;116:305-310.