Original Research Article

PRELIMINARY METABOLOMICS AND DETERMINATION OF A POTENT ANTI-INFLAMMATORY FRACTION OF FRESHWATER BROWN ALGAE

Abstract

Objective: The study aims to perform preliminary metabolomics and identify the potent anti-inflammatory fractions from freshwater brown algae extract.

Materials and Methods: The brown algae sample was harvested from the exposed rocks in Ebonyi River in Nigeria, and was subjected to cold maceration in a methanol-dichloromethane (2:1) solvent mixture for 72 hours, for extraction. The resulting extract was concentrated and subsequently fractionated via vacuum liquid chromatography (VLC) using solvent gradients of n-hexane and ethyl acetate. Acute toxicity was evaluated in Swiss albino mice according to Lorke's method, with mortality rates employed to determine the LD50. The anti-inflammatory potential of the fractions was assessed using both *in vitro* (protein denaturation and heat-induced hemolysis assays) and *in vivo* (acetic acid-induced vascular permeability and xylene-induced edema) models. Doses of 50 and 100 mg/kg as well as 1 and 2 mg of the fractions (3:7, 5:5, 7:3) were administered orally and topical for the *in vivo* models respectively, while doses ranging from 62.5 to 1000 µg were used for the *in vitro* test. Dexamethasone was used as the positive control. High-performance liquid chromatography (HPLC) dereplication was employed to identify the phytoconstituents of the fractions.

Results: Many anti-inflammatory compounds, including quercetin, ellagic acid and Kaempferol were identified in the fractions which would have accounted for the pronounced anti-

inflammatory properties observed. Fraction 5:5 showed a superior anti-inflammation compared to dexamethasone in the xylene-induced edema model, althoμgh not statistically significantly different.

Conclusion: These fractions, especially 5:5 can be further developed for the treatment of inflammation.

Keywords: Anti-inflammatory, Nigeria, Rivers, Animals, Methanol

Introduction

Inflammation is a vital protective mechanism which the immune system employs to defend the body against foreign pathogens, facilitate tissue repair, and restore homeostasis. However, when inflammation becomes dysregulated, acute inflammation can progress to a chronic state, contributing significantly to the development and exacerbation of various diseases such as Alzheimer's, cancer, diabetes, and inflammatory bowel disease [8]. While traditional anti-inflammatory drμgs, such as nonsteroidal anti-inflammatory drμgs (NSAIDs) and corticosteroids, are commonly used to manage inflammation, their long-term use is often associated with undesirable side effects, such as gastrointestinal irritation and immunosuppression [25]. As a result, there is increasing interest in exploring natural products as potential therapeutic alternatives, offering the prospect of safer and more effective treatment options.

Natural products have long been recognized as a rich source of pharmacologically active compounds, significantly contributing to drμg discovery. Among natural products, marine organisms, including algae, corals, and seaweeds, have garnered particular attention due to their

ability to produce a wide variety of bioactive secondary metabolites with anti-inflammatory properties [17].

Brown algae, or brown seaweed, represents an up-and-coming group of organisms, owing to its easy accessibility and diverse array of bioactive compounds. Most of the research on brown algae is focused on those found in the marine habitat. Studies on brown algae from freshwater habitats remain underexplored [27]. Notable bioactive compounds in marine brown algae include fucoidans [5], terpenes, polyphenols, and sulfated polysaccharides, with fucoxanthin—a carotenoid pigment responsible for the characteristic color of brown algaeemerging as a key compound with significant anti-inflammatory effects [9]. Fucoxanthin has been highlighted for its anticancer properties, particularly in breast, cervical, and lung cancers, depending on the tumor type and stage [18].

This study aims to investigate the anti-inflammatory agents derived from freshwater brown algae. By exploring the diverse bioactive compounds present in this organism, we hope to contribute to the growing body of research on natural product-based therapies for the treatment of inflammatory diseases.

Materials and Method

Materials:

Reagents and chemicals used included xylene (JHD, China), human blood, dexamethasone (Unicure), methanol, dichloromethane, ethyl acetate, n-hexane (JHD, China), silica gel, and other solvents. Equipment utilized included test tubes, a rotary evaporator (ByUyCHI Rotavapor Model R-215, Switzerland), isotonic buffer, vacuum liquid chromatography (VLC), and high-

performance liquid chromatography (HPLC) with an SPDM20A UV-DAD detector (Shimadzu, Japan), along with TLC plates (Merck, Germany) and an analytical balance (Oxoid, UK).

Animals:

Swiss albino mice (25–30 g) were sourced from the Department of Pharmacology, Enμgu State University of Science and Technology, Nigeria. The mice were acclimatized for one week prior to the experiments and were provided with food and water ad libitum. All animal experiments adhered to the NIH guidelines for the care and use of laboratory animals.

Test Material: Brown algae was collected from exposed rocks at the Ebonyi River in Ezza South Local Government Area, Ebonyi State, Nigeria, in January 2023.

Methods:

Extraction: The brown algae sample was pulverized and extracted using cold maceration with a 2:1 methanol-dichloromethane solvent mixture for 72 hours. The mixture was left to stand for 48 hours, sieved throμgh muslin cloth, and concentrated using a rotary evaporator. The resultant extract was filtered with Whatman No. 1 filter paper.

Vacuum Liquid Chromatography (VLC): Four grams of the extract was mixed with 2 mL of n-hexane, followed by the addition of silica gel (200-400 mesh). This mixture was loaded onto a column packed with silica gel, and fractions were eluted using solvent gradients of n-hexane and ethyl acetate (in ratios of 10:0 to 0:10), followed by methanol-ethyl acetate (1:9, 2:8, 10:0). Fractions were collected and concentrated at room temperature.

Acute Toxicity Study: Acute toxicity was assessed using Lorke's method. Phase 1 involved administering 10, 100, and 1000 mg/kg of the extract to three groups of three mice each, followed by 24-hour observation for signs of toxicity and mortality. In Phase 2, the highest non-lethal dose from Phase 1 was used to determine the LD50.

Evaluation of the anti-inflammatory activity of the fractions:

- ***In vitro* Assays**:
    - **Protein Denaturation Assay**: This test assessed the anti-inflammatory activity of the extract and fractions (3:7, 5:5, 7:3) testing its ability to inhibit protein denaturation. The final concentrations tested were 1000, 500, 250, 125, and 62.5 µg/mL, with dexamethasone and Tween 20 as controls.
    - **Heat-induced Hemolysis Assay**: The extract and fractions (62.5, 125, 250, 500, 1000 µg/mL) were tested for their ability to inhibit heat-induced hemolysis of red blood cells (RBCs). Indomethacin was used as a reference standard.
- ***In vivo* Assays**:
    - **Acetic Acid-Induced Vascular Permeability Test**: Mice were divided into eight groups (n=5/group) and treated with the fractions (3:7, 5:5, 7:3) orally at 50 and 100 mg/kg doses. Dexamethasone (100 mg/kg, p.o.) and vehicle (5% Tween 20) were used as controls. An hour after treatment, mice were injected intravenously with Evan’s blue solution, followed by an acetic acid injection. After 30 minutes, the peritoneal cavity was washed, and Evans blue concentration was measured spectrophotometrically at 610 nm.

- **Xylene-Induced Topical Edema**: Mice were treated topically with the fractions (1 mg and 2 mg/ear) and dexamethasone, while edema was induced by applying xylene to the ears. After two hours, ear plµgs were collected and weighed to measure edema. The percent inhibition of edema was calculated.

Ethical consideration

During the experimental procedures, experimental animals were handled and cared for according to the accepted NIH guidelines for the care and use of laboratory animals (National Institute of Health (2011) (Pub No: 85-23). Ethical clearance was requested and obtained from Enµgu State University of Science and Technology, Nigeria.

Approval number: ESUT/AEC/0246/AP198.

High-Performance Liquid Chromatography (HPLC):HPLC analysis of the fractions was performed using an Agilent 1260 Infinity II series equipped with DAD WR G71115A (DEAC606992) (California, US), column Oven of G7130 (DEAEQ22974), and Quad Pump VL G711A (DEAEY01907)(USA). Auto Sampler G7129A (DEAEQ22974)(USA). The samples were run throµgh a poroshell column 120 EC-C 18 4µm (4.6x150 mm), PN 693970-902 (T), SN USHKB12136, Agilent Technologies (Santa Clara, CA, USA). Fractions 5:5 and 3:7 were analyzed in reverse phase mode under isocratic conditions with a mobile phase of 20% acetonitrile and 80% purified deionized water. The flow rate was set at 0.6 mL/min, and the detection was by absorption spectroscopy at a wavelength of 280 nm. The column oven temperature was maintained at 40°C, and the run time was 30 minutes.

These methods facilitated the identification of bioactive compounds from the brown algae with potential anti-inflammatory properties, aiding the development of natural therapeutic agents.

Statistical Analysis

The data obtained were expressed as mean $\pm$ SEM. Mean statistical analysis was carried out using SPSS version 20. All data were analyzed by the Kruskal-Wallis ANOVA test. The differences between treatments were compared with multiple comparisons of mean ranks for all groups. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. Dose-response curves and regression analysis were done using Microsoft Excel 2016.

Results

Results of the anti-inflammatory assay (*in vitro* and *in vivo*) on three VLC fractions and the crude extract

- **Protein denaturation assay**

The median inhibitory concentrations of the extract and fractions in inhibiting protein denaturation are shown in the dose-response curves in Figs 1-5. This is the concentration at which there is 50% inhibition of protein denaturation, this is to say that the lower the IC50, the better the activity of the fraction.

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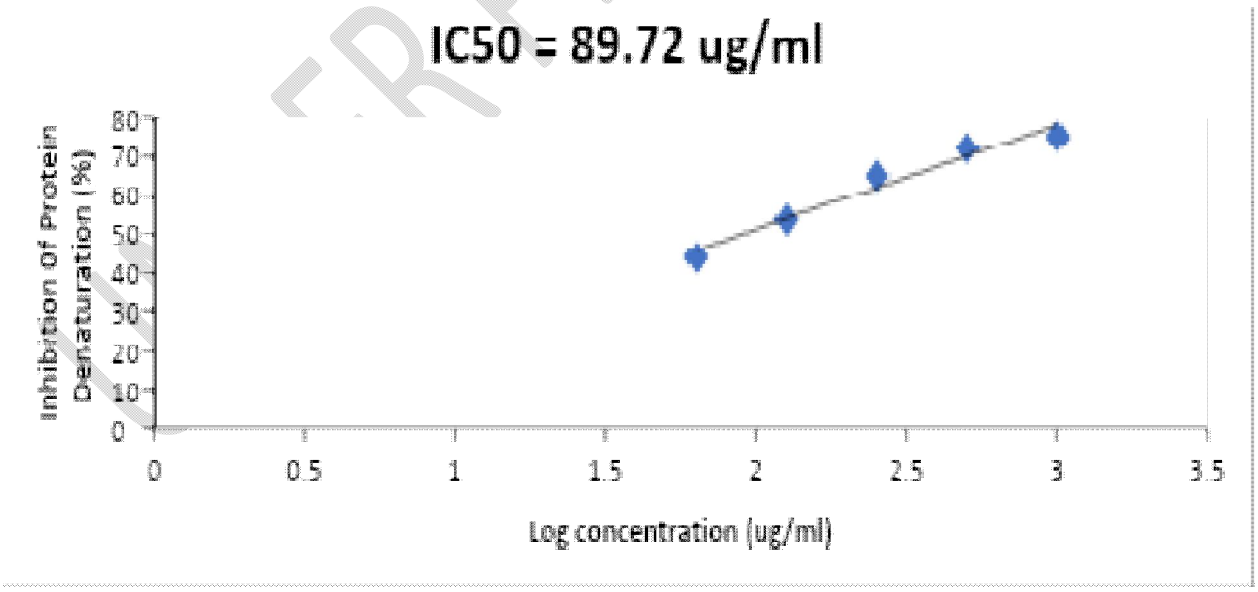

Fig 1: Dose-response curve for dexamethasone

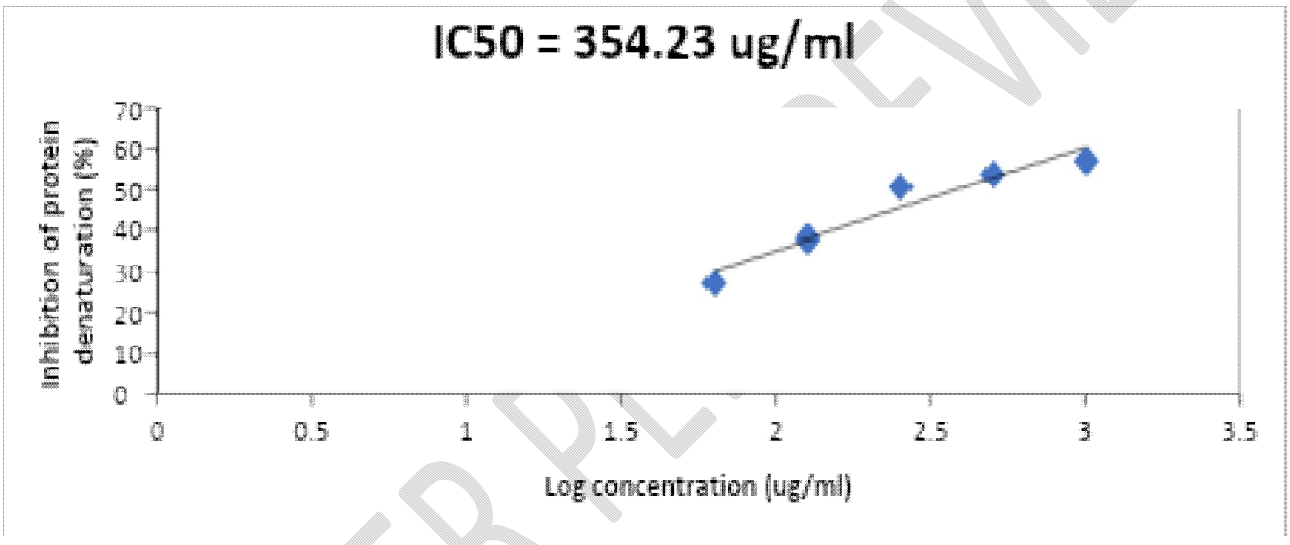

Fig 2: Dose-response curve for fraction 3:7

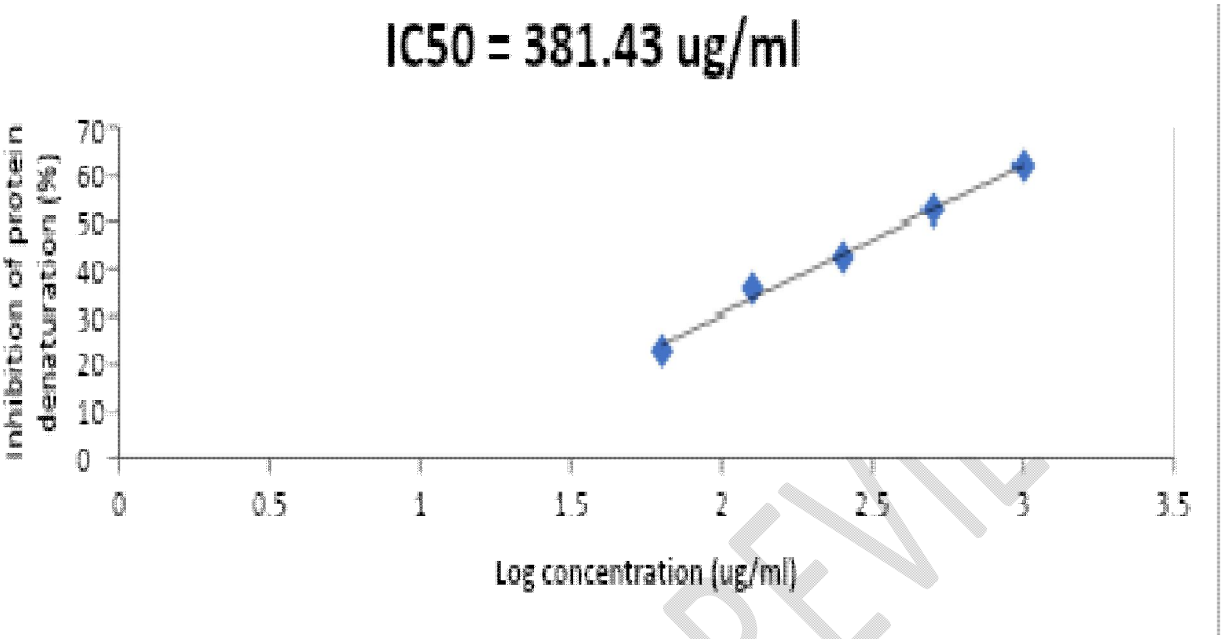

Fig 3: Dose-response curve for fraction 7:3

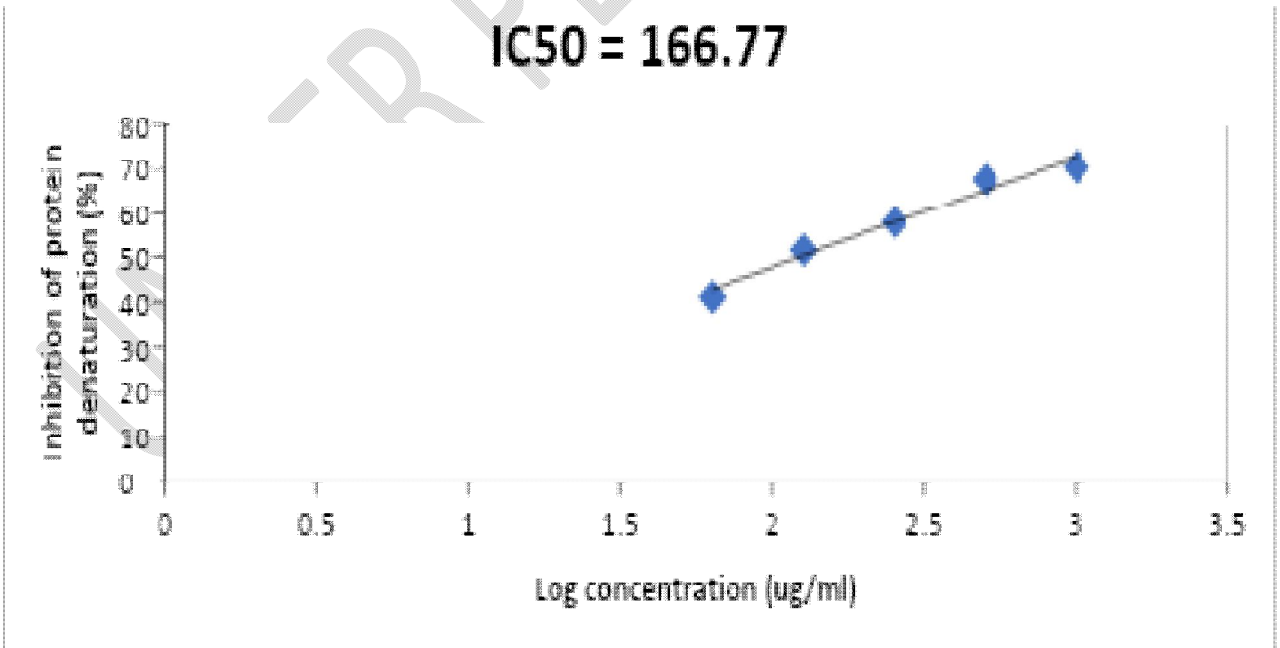

Fig 4: Dose-response curve for fraction 5:5

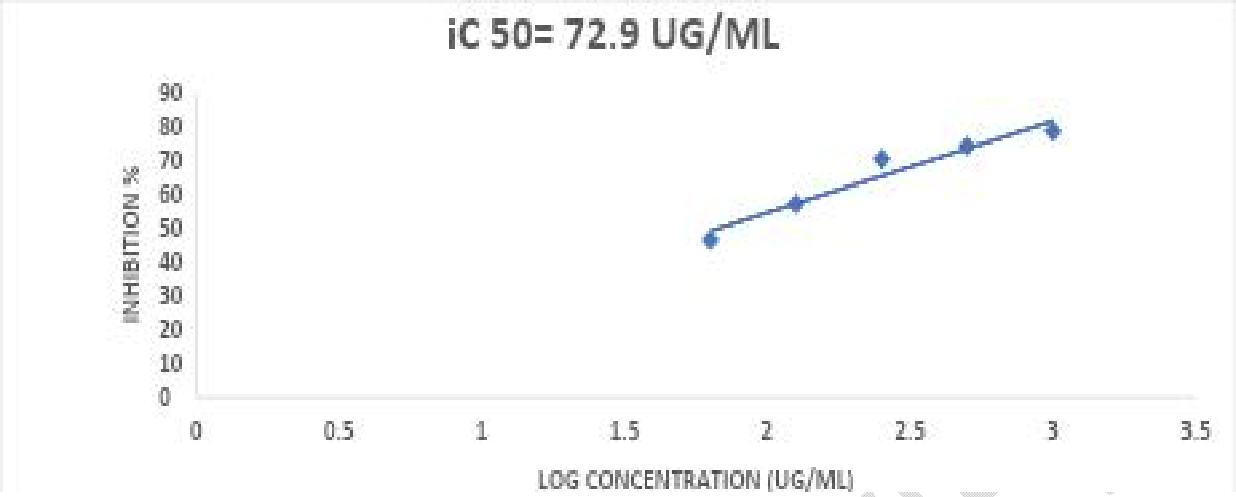

Fig 5: Dose-response curve for the crude extract

- **Heat-induced hemolysis assay**

The results show that fractions 3:7,5:5 and 7:3 all had a potent ability to protect the red blood cell's membrane against heat-induced hemolysis in a dose-dependent manner. The fractions (3:7, 5:5, and 7:3) inhibited the heat-induced hemolysis of RBCs membrane at an IC50 of 519.49 *µg/ml*, 318.77 *µg/ml*, and 842.28 *µg/ml* respectively, compared to the positive control (dexamethasone) with an IC50 of 250.56 *µg/ml* as seen in fig 6 – 10.

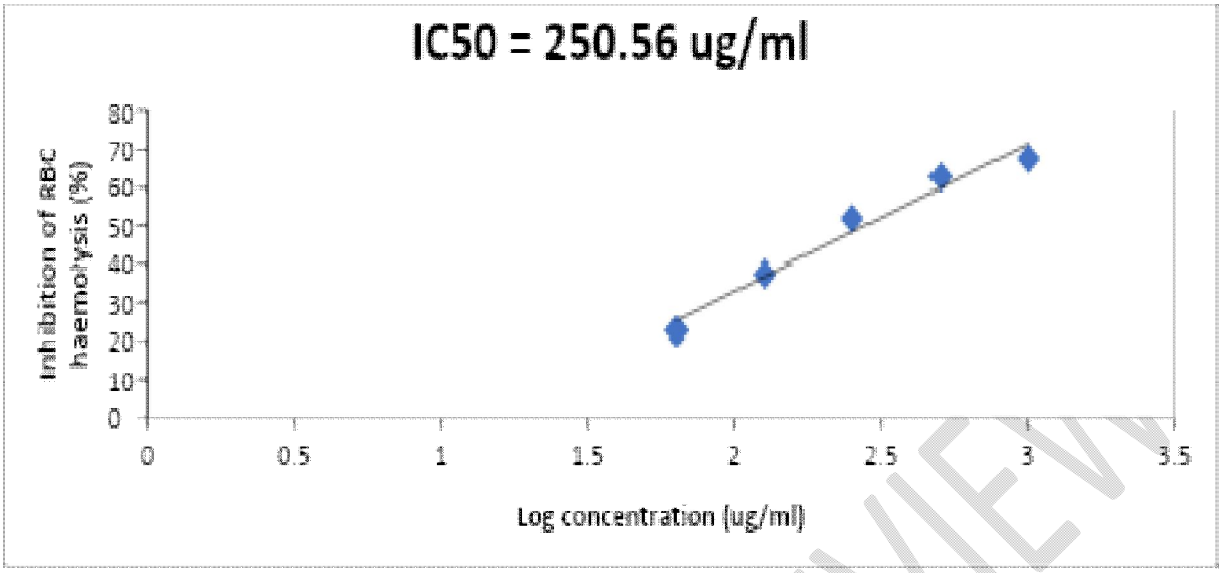

Fig 6: Dose-response curve for dexamethasone

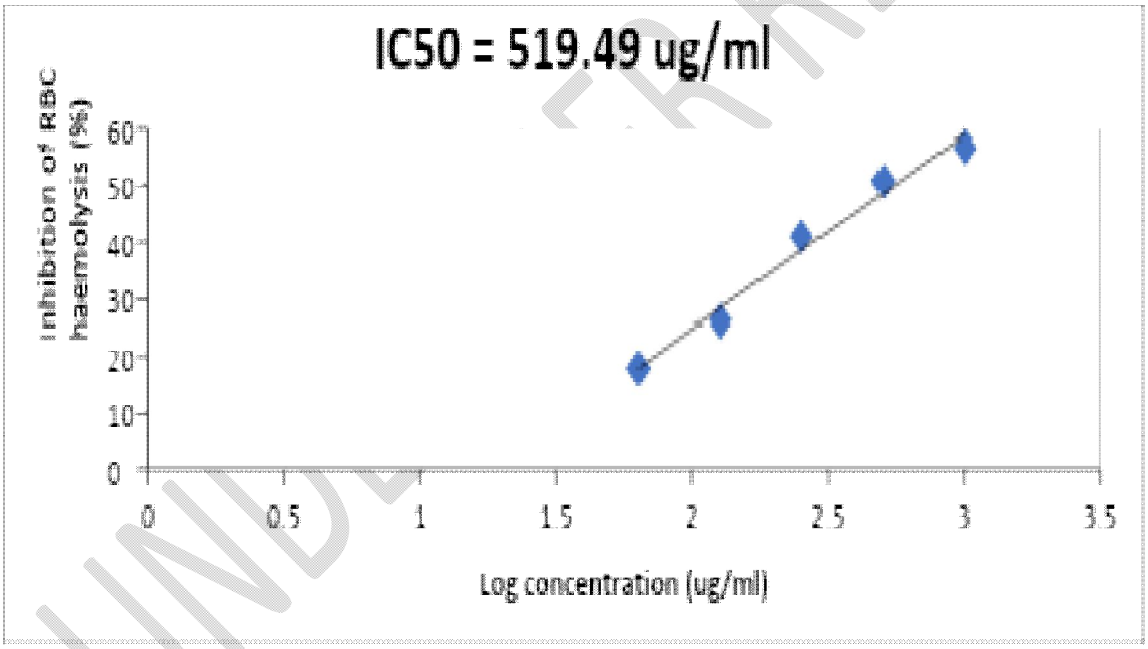

Fig 7: Dose-response curve for fraction 3:7

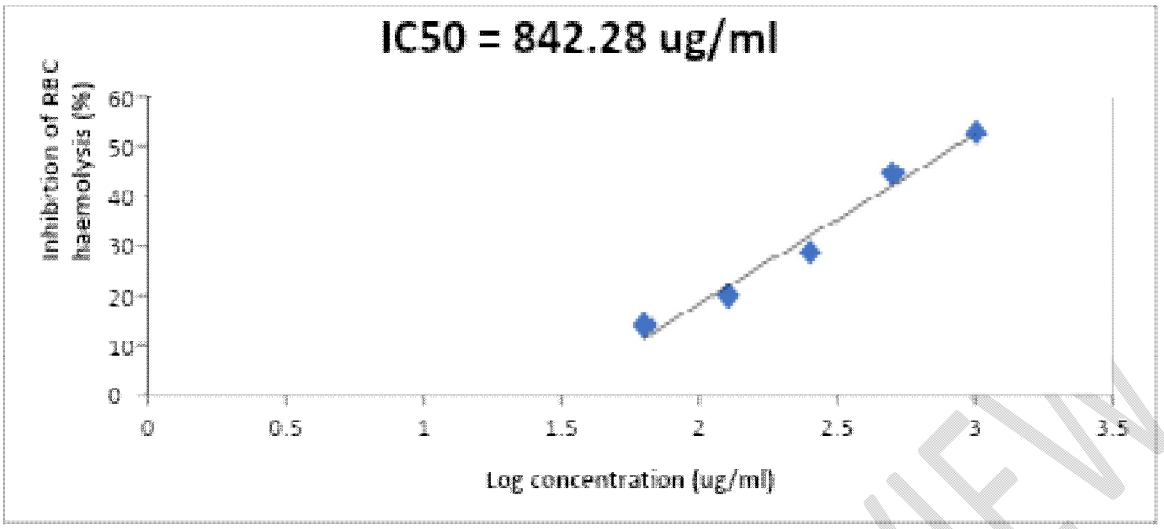

Fig 8: Dose-response curve for fraction 7:3

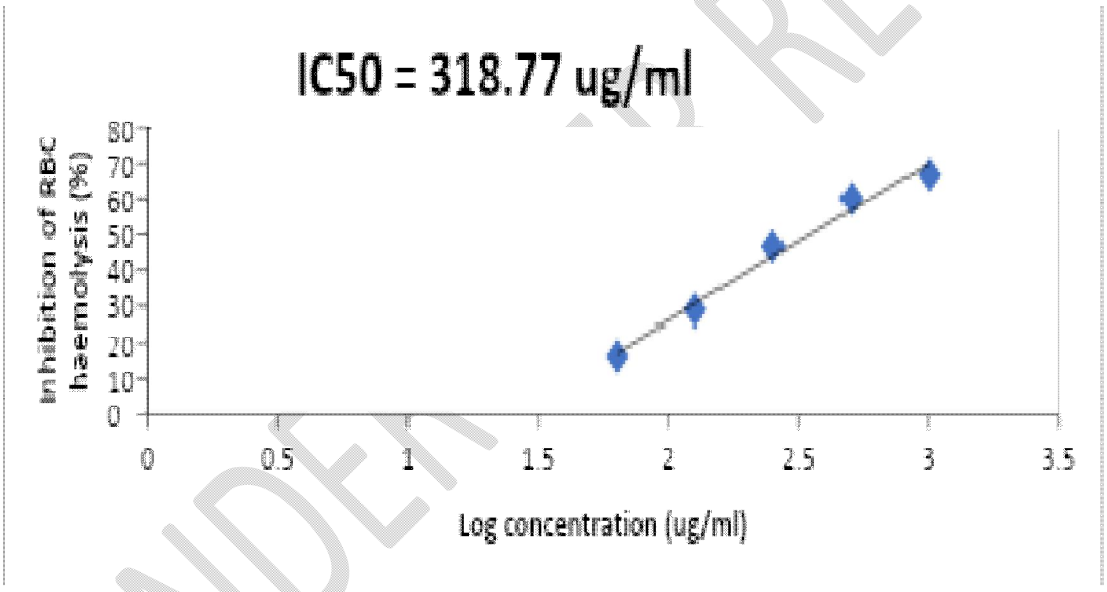

Fig 9: Dose-response curve for fraction 5:5

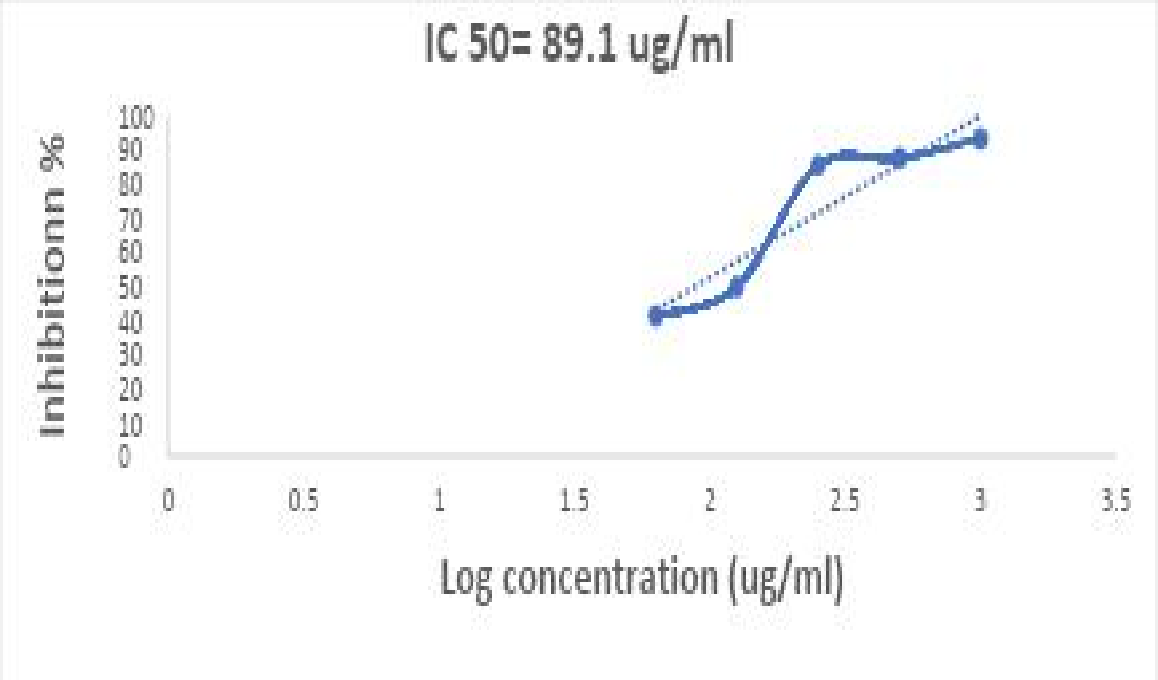

Fig 10: Dose-response curve for the crude extract

- **Xylene-induced topical edema**

The 3 fractions (5:5, 3:7, 7:3) were administered at 2 mg and 1 mg as shown in Fig 11. The analysis revealed that dexamethasone, the positive control, performed significantly better (P<0.05) than the vehicle and fraction 7:3 in inhibiting edema. The results also showed that the effects of fractions 5:5 and 3:7 showed no statistically significant difference from the positive control and thus showed similar activity. All fractions demonstrated a dose-dependent increase in their inhibitory effect on the edema, sμggesting that they can inhibit acute inflammation in a dose-dependent manner.

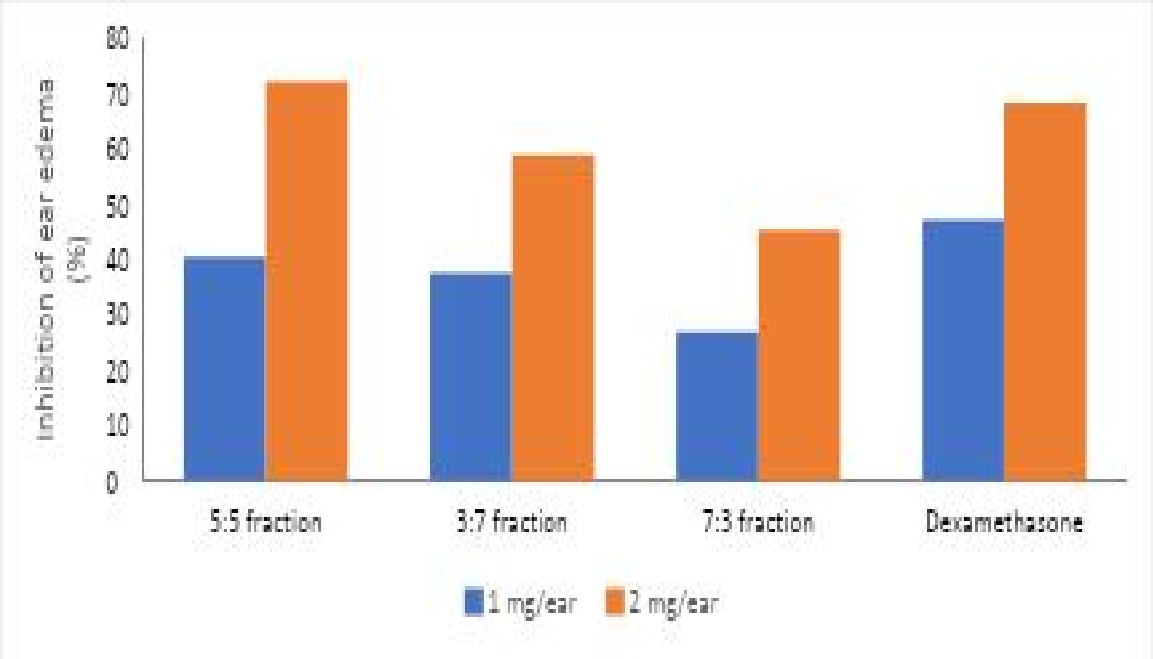

Fig 11 Bar graph showing fractionation in Xylene-induced topical edema

- **Acetic acid-induced vascular permeability assay**

The results in Fig 12 revealed the percentage inhibition of vascular permeability after induction with acetic acid and treatment with the various fractions. Here only one concentration of dexamethasone was used because it is the reference drµg. When compared with the vehicle, all the fractions just like dexamethasone produced a significant reduction in the Evans blue peritoneal concentration. However, when compared with the positive control (dexamethasone), the effect produced by the fractions was inferior. The effect of dexamethasone was significantly different from the fractions at $P < 0.05$.

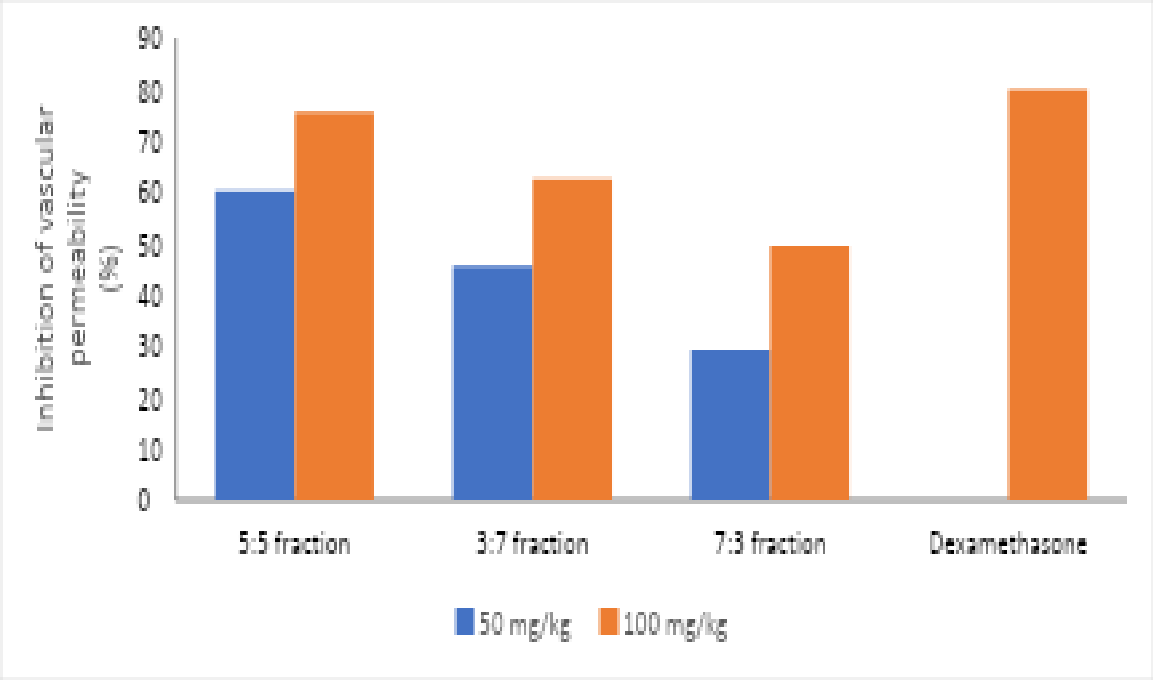

Fig 12 Bar graph showing fractionation in Acetic acid-induced vascular permeability assay

High-Performance Liquid Chromatography

The High-performance liquid chromatography analysis conducted on the fractions showed that Fraction 7:3 contained at least 16 different compounds represented by 16 distinct peaks (Fig 13) which were eluted at various retention times based on their affinity to the stationary phase. Among these compounds identified, 11 have been reported to possess significant anti-inflammatory properties. They include; eμgenol [11], benzoic acid [23], camphor, alpha-tumerone, epicatechin, alpha-pinene, quercetin [1], curcumin [12], demethoxycurcumin, limonene and germacrene [22]. Also, Fraction 5:5 contained 19 compounds (Fig 14) which consist of flavonoids, phenolic compounds, terpenes and fatty acid derivatives and they have all been reported to possess significant anti-inflammatory effects from previous studies.

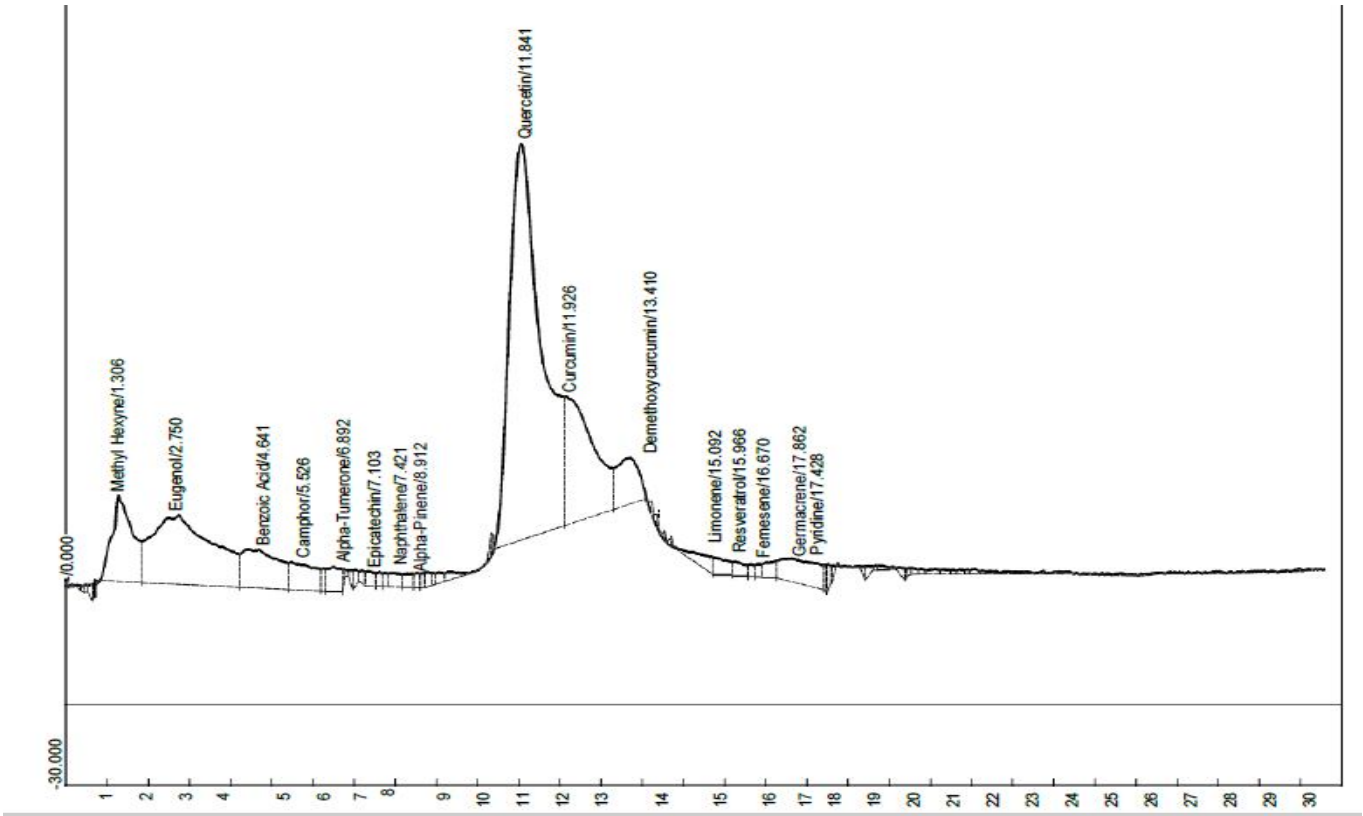

Fig 13: chromatogram for fraction 7:3

Component	Retention	Area	Height	External	Units
Methyl Hexyne	1.306	1101.4210	32.172	0.0000	%
Eugenol	2.750	2591.6021	25.578	318.8381	ppm
Benzoic Acid	4.641	882.4416	14.235	97.5292	ppm
Camphor	5.526	447.6140	10.207	0.0000	
Alpha-Tumerone	6.892	201.4231	9.258	0.0000	
Epicatechin	7.103	82.4251	5.385	0.0000	
Naphthalene	7.421	108.2205	5.087	0.0000	
Alpha-Pinene	8.912	80.2142	5.048	0.0000	
Quercetin	11.841	7541.1215	147.374	0.0000	
Curcumin	11.926	2441.2160	47.738	0.0000	
Demethoxycurcumin	13.410	655.4262	17.225	0.0000	
Limonene	15.092	177.4562	0.439	0.0000	
Resveratrol	15.966	166.2125	6.464	0.0000	
Fernesene	16.670	106.3681	5.271	0.0000	
Germacrene	17.862	119.4595	6.397	0.0000	
Pyridine	17.428	582.6421	8.544	0.0000	

Table 1: chromatogram table for fraction 7:3

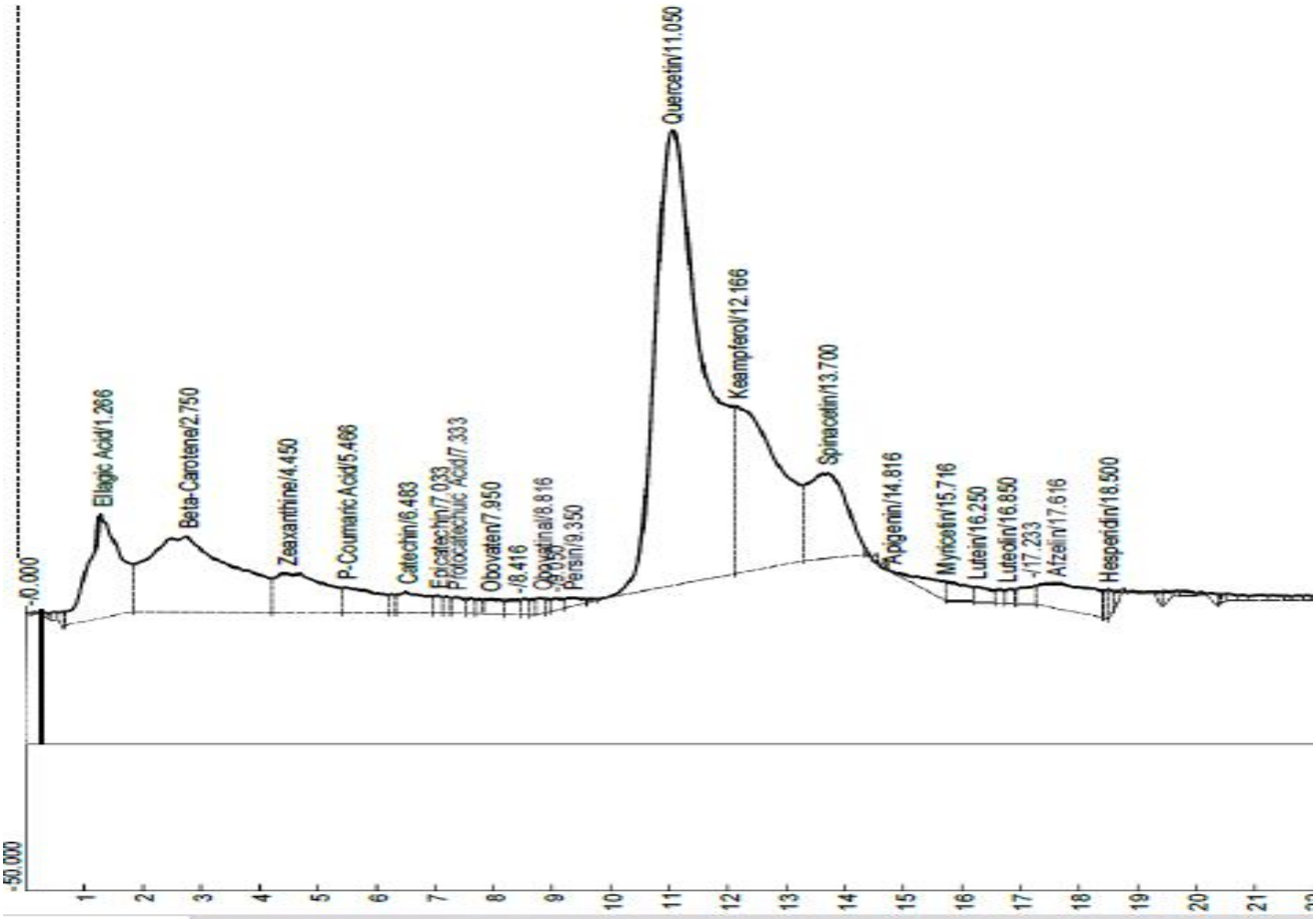

Fig 14: chromatogram for fraction 5:5

Component	Retention	Area	Height	External	Units
Ellagic Acid	1.266	1319.7430	35.630	0.0000	
Beta-Carotene	2.750	2648.9885	25.740	0.0000	
Zeaxanthine	4.450	799.5065	13.324	0.0000	
P-Coumaric Acid	5.466	353.3530	8.655	0.0000	
Catechin	6.483	241.1495	7.065	0.0000	
Epicatechin	7.033	50.8605	5.831	0.0000	
Protocatechuic Acid	7.333	81.9950	5.385	0.0000	
Obovaten	7.950	102.3055	5.087	0.0000	
Obovatinal	8.816	51.5560	5.194	0.0000	
Persin	9.350	53.9795	2.782	0.0000	
Quercetin	11.050	8719.2635	154.960	0.0000	
Keampferol	12.166	2915.1055	56.785	0.0000	
Spinacetin	13.700	1335.6985	29.027	0.0000	
Apigenin	14.816	175.3440	0.439	0.0000	
Myricetin	15.716	164.7435	6.464	0.0000	
Lutein	16.250	103.2780	5.271	0.0000	
Luteolin	16.850	51.7795	4.887	0.0000	
Afzelin	17.616	581.3260	8.544	0.0000	
Hesperidin	18.500	58.5210	9.773	0.0000	

Table 2: chromatogram table for fraction 5:5

DISCUSSION

The acute toxicity study of the fractions showed that the LD50 was greater than 5000 mg/kg, confirming the extract's relatively high safety margin. This data is crucial for determining safe dose ranges in subsequent studies and to ensure that the observations in subsequent investigations are not due to the toxic effect of the extract.

The VLC fraction (fractions 5:5, 3:7, 7:3 - n-hexane: ethyl acetate) showed significant anti-inflammatory activity compared with the negative control. Of the 3 fractions, fraction 5:5 exhibited consistent anti-inflammatory potency across all the models used and showed promising results.

One of the desired characteristics of drµggable substances is moderate polarity (Hydrophile: lipophile balance) such that they will be able to pass throµgh hydrophobic membranes as well as

hydrophilic mediums on their journey to the target site [21]. This can explain the consistent potency of the 5:5 fraction, which was eluted by a moderately polar mixture of ethyl acetate and hexane, across models. The HPLC dereplication studies showed that quercetin, kaempferol, beta-carotene, Zeaxanthine, spinacetin and ellagic acid are the major component of the fraction. The anti-inflammatory properties of these compounds have been reported and at least 3 of them have been isolated from our laboratory [23, 10, 19,17].

It was observed that fraction 5:5 at a dose of 2 mg/kg exhibited significantly (P<0.05) better inhibition of topically induced edema (72.1%) when compared with the positive control, dexamethasone, which elicited an inhibition of 68.3% at the same dose. There was also a significant difference between the effects of the fractions at 2 mg/kg and 1 mg/kg respectively showing that the anti-inflammatory properties of the fraction were dose-dependent. Considering the high safety profile of this fraction, its potential for further development as a herbal dosage form for the management of inflammatory conditions is high.

The xylene inflammatory model is an established and conventional model for the study of topical inflammation [16]. This assay is essential because inflammation is essential to the etiology of many diseases including cancer [6]. Chronic inflammation encourages tumor growth and metastasis which is linked to the onset and spread of cancer [14]. Therefore, by inhibiting inflammation-mediated tumor growth, compounds that exhibit anti-inflammatory actions may have therapeutic potential in cancer treatment.

All fractions reduced vascular permeability in a dose-dependent manner at the peritoneal level. Treatment with dexamethasone 100 mg induced a 79 % inhibition of vascular permeability while fractions 5:5, 7:3 and 3:7 at 100 mg induced 75.5%, 49.4 % and 62.4 % inhibition respectively. Again, the potency of fraction 5:5, a crude fraction was obvious at 75.5% inhibition compared to

pure dexamethasone at 79% inhibition. An increase in vascular permeability and localized inflammation, represent a critical step in the process of metastasis. The ability of anti-inflammatory fractions to reduce vascular permeability not only has implications for managing inflammation but also potentially impacts the process of metastasis by limiting the entry of cancer cells into the bloodstream and reducing the angiogenic support required for tumor progression[14].

The results in Figs 1-3 showed that the selected fractions all had the ability to inhibit protein denaturation in a dose-dependent manner. However, the IC_{50} of the most potent fraction (166.77 µg/ml) was almost twice the IC_{50} of dexamethasone, (89.72 µg/ml) the positive control, sµggesting that they were significantly less efficient than dexamethasone in the inhibition of protein denaturation. However, they were only crude fractions compared to purified compound. Further purification can easily increase and improve the IC_{50}. Protein denaturation is a key component of inflammatory response which serves as a target for inhibiting inflammation.

In the heat-induced hemolysis experiment, the fractions showed their ability to protect the red blood cells against heat-induced hemolysis in a dose-dependent manner with IC_{50}s shown in figs 6-9. Fraction 5:5 compared best with the positive control (dexamethasone) which returned an IC50 of 250.56 µg/ml. The lower IC_{50} values were correlated with higher RBC protecting ability, which represents the concentration of the fraction needed to inhibit 50% of the heat-induced hemolysis. It has been reported that agents that can protect the erythrocyte from heat-induced hemolysis and protein denaturation can stabilize the lysosomal membrane because the former and the latter are structural analogs. Stabilization of the lysosomal membrane will prevent the release of arachidonic acid which is the precursor of many inflammatory mediators [2, 20].

Also, the cytokine storm that occurs in COVID-19 leads to chronic inflammation and hemolysis (which is the early breakdown of red blood cells) [15]. So, discovering agents that inhibit the hemolysis of RBCs will help in the treatment of COVID-19 infection.

The findings revealed that the crude extract exhibited superior performance in inhibiting protein denaturation and heat-induced hemolysis in the *in vitro* assay. This could be due to the presence of a broader spectrum of bioactive compounds, including both polar and non-polar substances. Protein denaturation and hemolysis are processes that can be inhibited by various types of molecules, including phenolic compounds, polysaccharides, and proteins, which could be present in lower concentrations in the fractions [25]. These compounds are known to stabilize proteins and cell membranes by various mechanisms, such as direct binding to proteins, antioxidant activity, and membrane stabilization. The crude extract retains a more diverse array of these compounds, potentially providing a synergistic effect that enhances its ability to protect proteins from denaturation and red blood cells from hemolysis under heat stress.

Conclusion

The 5:5 fraction, with moderate polarity, exhibited the most consistent and potent activity, likely due to optimal hydrophile-lipophile balance and the validated anti-inflammatory properties of the phyto-constituents. It significantly inhibited xylene-induced edema and acetic acid-induced vascular permeability, closely matching dexamethasone's efficacy (with no statistically significant difference). Additionally, the 5:5 fraction showed strong HRBC membrane stabilization and protection against heat-induced hemolysis. These findings underscore the potential of moderately polar compounds in drµg development for anti-inflammatory and anticancer therapies, highlighting the importance of polarity in the design of effective pharmaceutical agents.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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