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Phytochemical screening and anti-inflammatory activities of different fractions from *Citrullus lanatus* leaves: A comprehensive study

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ABSTRACT

Inflammation, a protective immune response, can contribute to maintaining homeostasis but can also cause harm when chronic or excessive. Citrullus lanatus has been suggested to possess antioxidant and anti-inflammatory activities that may aid in preventing and treating oxidative stress and inflammation. The main objective of this study was to evaluate the levels of phytochemicals, as well as the antioxidant and anti-inflammatory activities of Citrullus lanatus leaves extract. The ethyl acetate, hexane, butanol fraction and aqueous methanol extract were subjected to various standard quantitative phytochemical analysis, while the hexane fraction and aqueous methanol extract were subjected to standard *in vitro* anti-inflammatory assays. Spectrophotometric and gravimetric methods were used in this study. The in vitro anti-denaturation (stabilization) assay was used for detecting a wide range of antioxidant compounds. The ethyl acetate fraction had the highest flavonoid and polyphenol levels, followed by hexane, butanol, and aqueous methanol extract, respectively. Quantitative phytochemical analysis showed the levels of flavonoids, polyphenols, tannins, alkaloids and saponins present in the different

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fractions of *C. lanatus.* The human erythrocyte membrane stabilization assay showed that extracts were able to stabilize human red blood cell membrane against hypotonic-induced hemolysis in five different samples. The extract of *Citrullus lanatus* was found to have the highest antioxidant activity. This study has shown that the leaves of *Citrullus lanatus* can be used in ethnomedicine for the prevention and treatment of ailments caused by oxidative stress and inflammation-related diseases.

Keywords: anti-denaturation properties, *Citrullus lanatus* leaves, Bovine serum albumin, Erythrocyte stabilization assay, Antioxidant activity

Introduction:

Watermelon contains carotenoids and lycope ne, which have been shown to protect against certain types of cancer. It is also rich in citru lline, an amino acid that helps remove toxins from the body. It is a species that grows on vi ne fruit and belongs to the Cucurbitaceae fam ily. It is known for its delicious taste, moistur izing properties, and health benefits. Waterm elon has anti-
inflammatory properties that are beneficial fo r conditions such as asthma, atherosclerosis, diabetes, cancer, and arthritis (3).

Figure 1: The picture shows a watermelon **(Citrullus lanatus) (3).**

Despite a considerable number of studies dedicated to the therapeutic values of watermelon, the leaves have been neglected in most cases. Therefore, the current study was designed to determine the antioxidant activity and reducing power of various fractions and an aqueous methanolic extract from Citrullus lanatus. This would help investigate their anti-inflammatory potential. Specifically, the purpose of this study was to shed light on the anti-inflammatory effect of the butanol and ethyl acetate fractions of the methanol extract of watermelon leaves. Not enough is known about these fractions as no such studies have been done comprehensively with adequate data (4). Therefore, as part of our anti-

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inflammatory studies, scientists had to purify the butanol and ethyl acetate fractions in their methanolic extracts of Citrullus lanatus leaves.

The extraction methods used here include liquid-liquid extraction and solid-phase extraction. When we received the fraction, we tested bovine serum albumin with anti-denaturation tests. These tests involved exposing the protein to inflammatory agents with subsequent determination of its degree of denaturation. The role of the butanol and ethyl acetate fractions in reducing or preventing BSA denaturation was assessed. Human red blood cells were also used for the erythrocyte stabilization tests. These tests evaluated the ability of the butanol and ethyl acetate fractions to stabilize red blood cells and prevent their destabilization caused by inflammatory stimuli.

Materials and Methods:

Citrus lanata leaves were collected from a farming area in Araromi, Ijesha village, Nigeria. The leaves were verified, along with other samples, at the University of Ibadan (UI) herbarium using reference code UIH-22656.

Additionally, leaves were plucked, separated from stems, aggressively cleaned under running water, dried at 40°C to maintain a consistent weight, and then manually ground in a grinder. 1,600 ml of 70% methanol were used to extract 200 grams of powdered leaf material, and the mixture was agitated intermittently for two days. This extract was filtered through a Whatman No. 1. Additionally, the filtrate was concentrated using a Stuart rotary evaporator RE300 set at 40°C. After being oven-dried, a concentrate was separated using n-hexane, n-ethyl acetate, and n-butanol, and it was then reconstituted with 200 ml of distilled water. These fractions were again concentrated at 40 degrees Celsius in a rotating evaporator. The fractions were then allowed to air dry at ambient temperature (40 °C); however, they were refrigerated at 4 °C until the next round of testing (5). The leftover fractions were then subjected to conventional quantitative methods for analyzing phytochemicals, antioxidants, and anti-inflammatory compounds.

QUANTITATIVE DETERMINATION OF PHYTOCHEMICALS

DETERMINATION OF TOTAL PHENOLIC CONTENT

The Folin-Ciocalteu assay, as reported by Zoyko et al. (6), was used to measure the total

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phenolic content of the ethyl acetate, butanol, and aqueous methanol extracts.

PROCEDURE: To prepare the extract, 0.5 ml (1 mg/ml) of the extract was combined with 2.5 ml of the Folin-Ciocalteu reagent (10% v/v) and 2 ml of sodium carbonate (7.5% w/v) in an aliquot. For color development, the test tubes were vortexed for 15 seconds and then left to stand at 40°C for 30 minutes. The standard was prepared by pipetting 100, 200, 300, 400, and 500 µl of the gallic acid solution into five different test tubes. The aforementioned process was then followed, but the standard solution was used in place of the stock sample. A UV spectrophotometer with dual beams was used to test the absorbance at a certain wavelength. The result was expressed as the equivalent of gallic acid by plotting a standard curve with an intercept of y=mx+c.

DETERMINATION OF TOTAL FLAVONOID CONTENT

Zhishen et al. (7) used the aluminum chloride colorimetric method to determine the total flavonoid content.

PROCEDURE: The standard solution was divided into test tubes at various concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 ml), and the ethanol was appropriately diluted to yield 1.0 ml. These tubes were filled with 0.2 ml of 2% AlCl3 solution, 0.2 ml of 1M potassium acetate solution, 5.6 ml of distilled water, and 3 ml of ethanol. For thirty minutes, every tube was left to stand at room temperature. Similarly, 1 milliliter of each fraction of the sample solution was substituted for the standard solution. This was mixed with 0.2 ml of 2% AlCl3 solution, 0.2 ml of 1M potassium acetate solution, 5.6 ml of distilled water, and 3 ml of ethanol. After combining the ingredients, they were left to stand at room temperature for half an hour. A UV (Shimadzu double-beam) spectrophotometer was used to measure the absorbance of the reaction mixture at 420 nm relative to a blank. The result was expressed as the quercetin equivalent by plotting a standard curve with an intercept of y = mx+c.

Determination of total tannin content

DETERMINATION OF TOTAL ALKALOID CONTENT

The alkaloid content was determined using the Harborne method (8).

Procedure: 5 g of powdered sample were mixed with 200 mL of 20% acetic acid in ethanol, covered, and left for 4 hours before

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filtering. Using a water bath, the filtrate was concentrated to a quarter of its initial volume. Drop by drop, concentrated aqueous ammonia was added to the extract until a precipitate formed. The precipitate was removed using pre-weighed filter paper, and the residue was cleaned with aqueous ammonia. After being dried for 15 minutes at 40˚C in the oven, the filter paper containing the precipitate was cooled and weighed again until a consistent weight was achieved. A record of the constant weight was made. This formula was used to determine the alkaloid content.

%Alkaloid= (weight of residue/weight of sample taken) x100

DETERMINATION OF TOTAL SAPONIN CONTENT

The total saponin content was determined using the Obadoni and Ochuko (9) method.

Two hundred (200) mL of 20% ethanol were used to disperse the 5g sample after it was weighed. The suspension was heated to 55˚C for four hours while being constantly stirred over a hot water bath. After the mixture was filtered, 100 milliliters more of 20% ethanol was used to extract the residue once more. Using a water bath heated to 90˚C, the combined filtrate was reduced to 20 ml. After transferring the concentrate into a 250 ml separating funnel, 10 ml of diethyl ether was added and thoroughly shaken. The purification procedure was repeated after recording the aqueous layer. N-butanol (20 ml) was added. Two rounds of washing the n-butanol extracts were conducted using 10 ml of 5% aqueous sodium chloride. We heated the leftover solution in a water bath.

The samples were dried in an oven to a consistent weight after evaporation. As a percentage of the sample's initial weight, the saponin content was computed.

$$\% \text{ Saponin} = \frac{Weight\ of\ final\ filtrate}{weight\ of\ sample} \times 100.$$

In vitro anti-inflammatory assay: A modified technique based on Sakat et al.'s work to investigate the extract's effects on protein denaturation (12). Bovine serum albumin (BSA) becomes denatured when heated, and this reaction is linked to antigens that cause type III hypersensitivity reactions and illnesses such as systemic lupus erythematosus, glomerulonephritis, rheumatoid arthritis, and serum sickness. The anti-denaturation (stabilization) properties of BSA were utilized in vitro to identify a variety

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of anti-inflammatory substances, reducing the necessity for animal experimentation during the initial phases of drug development. Test tubes were filled with reaction mixtures for the assay, which included test fractions, methanol as a control, and 50µl of different concentrations of the standard medication (Diclofenac sodium).

450µL of 5% w/v bovine serum albumin (BSA) was added to each tube. After 20 minutes of incubation at 37°C, the tubes were heated for three minutes at 57°C. Each tube received 2.5 mL of phosphate-buffered saline (pH 6.3) after cooling. A double-beam UV-visible spectrophotometer (T80 model PG instrument, UK) was used to measure the absorbance of the solutions at 660 nm. A linear regression equation was used to estimate the fifty per cent inhibitory concentration (IC50), which is the concentration needed to achieve 50% inhibition of BSA denaturation.

Erythrocyte Stabilization Assay: The in vitro anti-inflammatory activity was investigated using the human red blood cell (HRBC) membrane stabilization method (13). Using a 5mL hypodermic needle and venipuncture of the arm vein, a subject's 5mL of human blood was extracted. The blood was moved right away to a bottle containing ethylene diamine tetra acetic acid, an anticoagulant. After centrifuging the whole blood for ten minutes at 3000 revolutions per minute, the leukocytes and plasma-containing supernatant were carefully withdrawn. After washing the packed red blood cells in a recently made 0.9% w/v NaCl solution, centrifugation was performed once more until the clear supernatant was obtained. 0.9% w/v NaCl was used to prepare a 10% HRBC suspension.

One milliliter of sodium phosphate buffer (pH 7.0).4, 0.15 milliliters, two milliliters of 0.36% w/v NaCl, 0.5 milliliters of the stock HRBC suspension (10%, v/v), and 0.5 milliliters of the standard medication (Diclofenac sodium) or plant fractions at different concentrations in test tubes to form the assay mixture. To achieve maximal hemolysis, distilled water was used instead of NaCl (0.36% w/v) in the control tubes. The tubes were then incubated for a predetermined amount of time at 37˚C. After the incubation period, the tubes were centrifuged, and a 540 nm wavelength was used to measure the absorbance of the supernatant. The absorbance values were used to calculate the percentage inhibition of hemolysis.

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STATISTICAL ANALYSIS:

Statistical Package for the Social Sciences (IBM SPSS) Statistics version 23 was used in this study to calculate the mean and standard error of the mean, while Microsoft Excel was used for data computation and graphing.

Results

Summary of phytochemical components in fr uit leaves:

Table 1 provides the quantitative analysis of some phytochemical components in several p arts of watermelon leaves. Flavonoids, polyp henols, tannins, alkaloids, and saponins were examined. Tannin content is 0.073 mg TAE p er gram; Alkaloid content was measured as 2. 563% and saponin content as 27.283%.

Reduction Potential:

Figure 1. Absorption readings regarding the r edox potential of the extract and ascorbic aci d sample. In order of strength, this substance shows the highest reducing property with a v alue of 0.499 mg AAE/g, followed by hexane with a value of 0.441 mg AAE/g and 0.382 mg AAE /

Thiobarbituric acid inhibition:

Thiobarbituric acid and Comparison of ascor bic acid with different inhibition percentages (Figure 2). In the fractions, 60% inhibition w as found with ethyl acetate and 80% inhibitio n with hexane.

DPPH Radical Scavenging Efficacy:

Tip 3 displays the resulting images and introduces images depicting the percentage of DPPH radicals scavenged at different levels. The maximum inhibition of gallic acid is approximately 60%, ethyl acetate is 50%, and methanol-water dilution is 10%.

In vitro resistance to denaturation test:

The anti-denaturation assay's IC50 values are presented in Table 3. The aqueous methanol extract had an IC50 of 0.360 mg/ml, and the non-polar hexane fraction had an IC50 of 0.022 mg/ml.

Human Erythrocyte Stabilization Assay in Vitro:

The IC50 values for stabilizing human erythrocyte stabilization are also presented in Table 3. The IC50 values of the hexane fraction and aqueous methanol extract were 2.877 mg/ml and 3.026 mg/ml, respectively, compared to the standard diclofenac sodium's 0.523 mg/ml.

The study measured the amounts of flavonoids, polyphenols, tannins, alkaloids, and saponins in the leaves of Citrullus lanatus (watermelon) in different fractions. Considering that flavonoids and polyphenols

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have a well-established role in reducing inflammation and oxidative stress, and the high concentration of these compounds in the non-polar hexane fraction suggests potential antioxidant properties [1]. The presence of saponins and alkaloids may also indicate potential bioactive and therapeutic uses.

The ethyl acetate fraction exhibits strong antioxidant potential when the reducing capacities of various fractions. Additionally, evaluated. The results are consistent with the continuous scientific emphasis on antioxidants as a means of preserving general health. Given that a high reductive capacity is frequently indicative of strong antioxidant activity and that the ethyl acetate fraction—also known as EAF—was found to contain a sizable amount of polyphenols and flavonoids in recent reports by Kuma et al. This suggests that both the ethyl acetate fraction may have a strong antioxidant potential.

Furthermore, this study shows that there was a significant inhibition of thiobarbituric acid (TBARS) by the ethyl acetate and hexane fractions, indicating that they might be able to stop lipid peroxidation. These results are consistent with those of other studies that address the possibility of preventing lipid peroxidation by the ethyl acetate and hexane fractions. For example, Cederbaum (1989) showed that the ethyl acetate fraction reduced lipid peroxidation, indicating that it may have a role in preventing the formation of free radicals and the toxicity of 4-NQO. Nwuke & Ibeh (15) also demonstrated the methanol extract and its derived fractions to scavenge hydrogen peroxide, indicating their capacity to prevent lipid peroxidation. These fractions include n-hexane and ethyl acetate.

Rafiq (16) investigated the antioxidant and hepatoprotective properties of various leaf fractions, such as n-hexane and ethyl acetate, with a focus on their ability to inhibit lipid peroxidation.

Furthermore, the hexane and ethyl acetate fractions showed a noteworthy reduction in the DPPH radical scavenging activity compared to the other fractions, suggesting their capacity to stop lipid peroxidation. The different fractions and gallic acid displayed varying levels of inhibition, indicative of their ability to scavenge free radicals. The demonstrated inhibitory abilities of the various fractions against thiobarbituric acid are consistent with the findings of Demirel (17), who investigated the potential of natural compounds to alleviate inflammation and neuronal oxidative damage. These findings

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are consistent with the work of Mozzini, C., & Pagani, M. (3), who documented the potential advantages of natural antioxidants in preventing diseases linked to oxidative stress, including chronic illnesses and aging.

The hexane fraction demonstrated a low IC50 value in the current study as well, according to the results of the In Vitro Anti-Denaturation Assay. This suggests that it may be able to preserve protein structure and function, which is important for cellular health. These results advance our knowledge of natural substances that may find use in the biomedical and pharmaceutical industries, where protein denaturation is a problem.

Conclusion:

this study is supported by evidence that fruit l eaves have antioxidant properties and can prevent disease antibiotics.

Therefore, additional research into in vivo ox idation and anti-inflammatory effects is needed to determine t he impact of fruit leaves on the human body.

More studies on antioxidant drugs may help f ind out the role of ethyl acetate in preventing damage and other diseases.

Table 1: Quantitative Phytochemical Evaluation of *C. Lanatus* Leaves*

	QUANTITY PRESENT			
Fractions of *C. Lanatus* leaves	Total Flavonoid		Total Phenol	
Ethylacetate	0.177	± 0.0033 mg(QUE)/g	0.597	± 0.0049 mg(GAE)/g
Hexane	0.157	± 0.0064 mg(QUE)/g	0.463	± 0.0041 mg(GAE)/g
Butanol	0.046	± 0.0030 mg(QUE)/g	0.308	± 0.0045 mg(GAE)/g
Aqueous Methanol Extract	0.040	± 0.0029 mg(QUE)/g	0.183	± 0.0103 mg(GAE)/g

	Total Tannin	
C. Lanatus, total	0.073	± 0.0029 mg(TAE)/g

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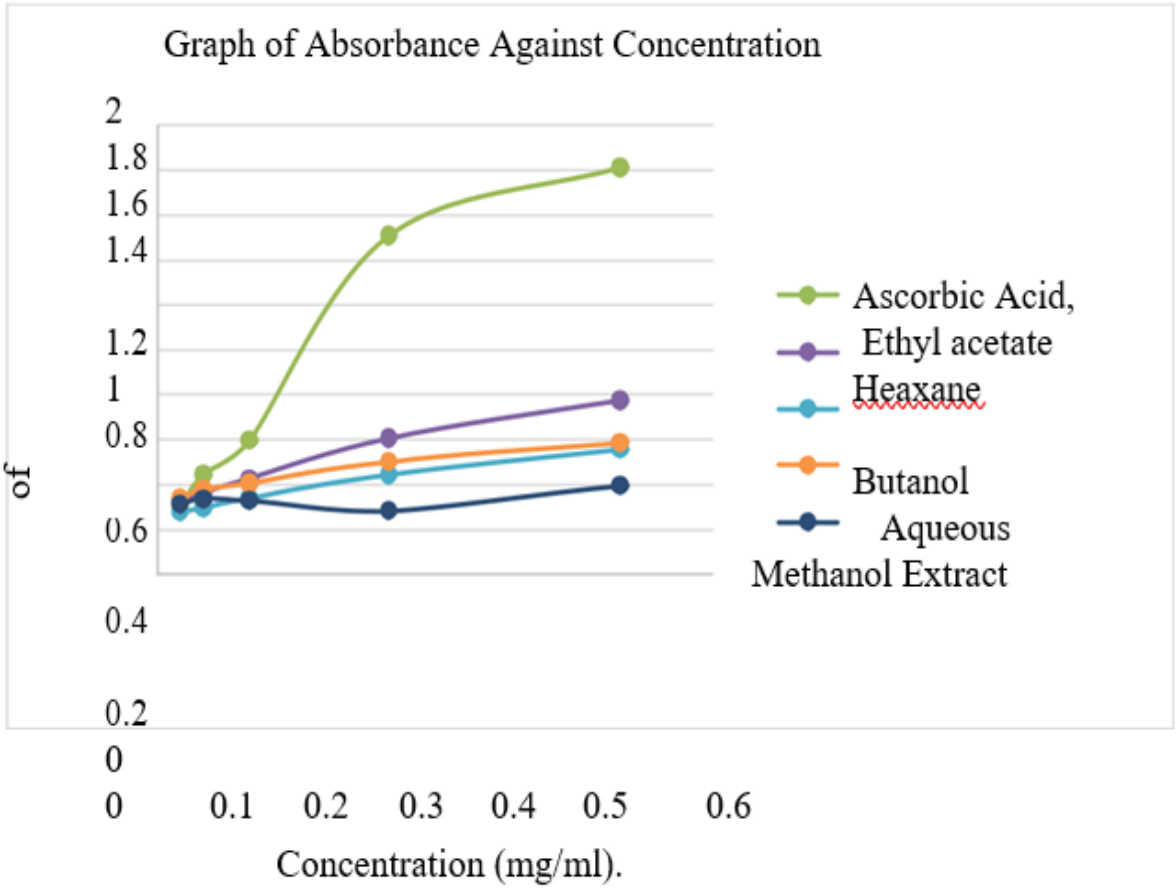
	Alkaloid	Saponin
C. Lanatus leaves	2.563 ± 0.817%	27.283± 0.768%

*Data = Mean ± SEM. QUE represents Quercetin Equivalent, GAE represents Gallic Acid Equivalent, and TAE represents Tannic Acid Equivalent.

Figure 1: The Reductive Capacities of Ethyl Acetate Fraction, Ethyl Acetate, Butanol Fraction, and Aqueous Methanol Extract with Ascorbic Acid Standard.



Graph of %inhibition against concentration:

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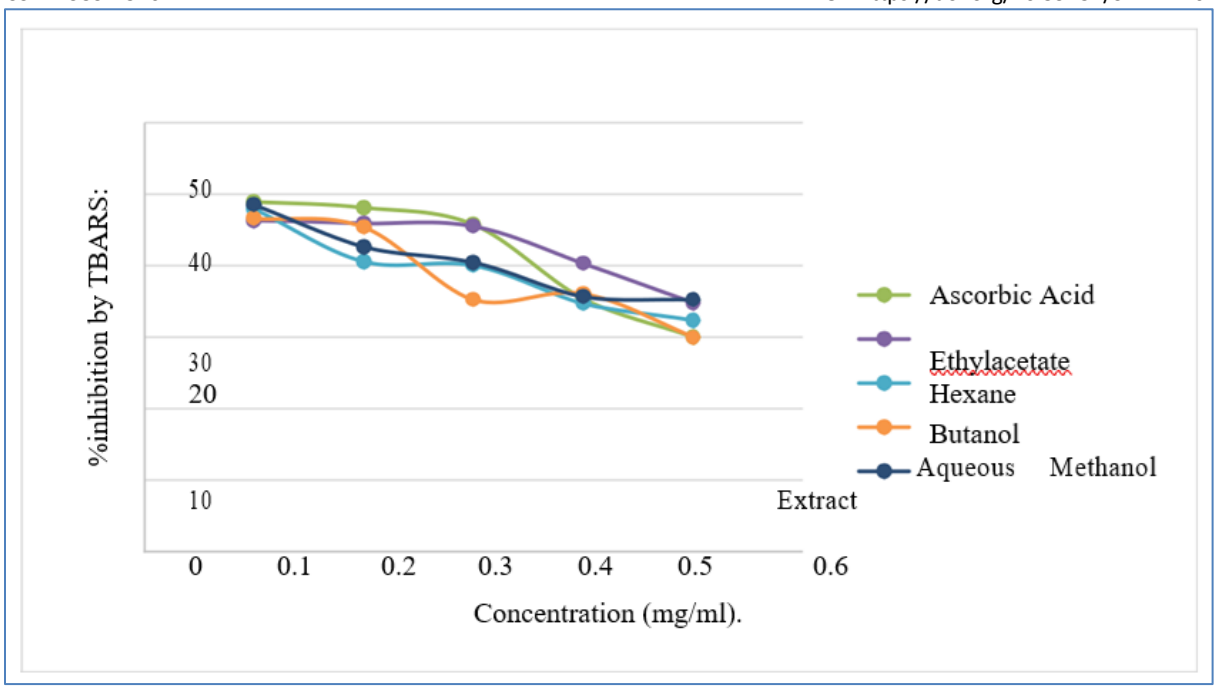

Figure 2: Percentage Inhibition of Thiobarbituric Acid by Ascorbic Acid, Ethyl Acetate Fraction, Hexane Fraction, Butanol Fraction, and Aqueous Methanol Extract.

Graph of %inhibition against concentration.

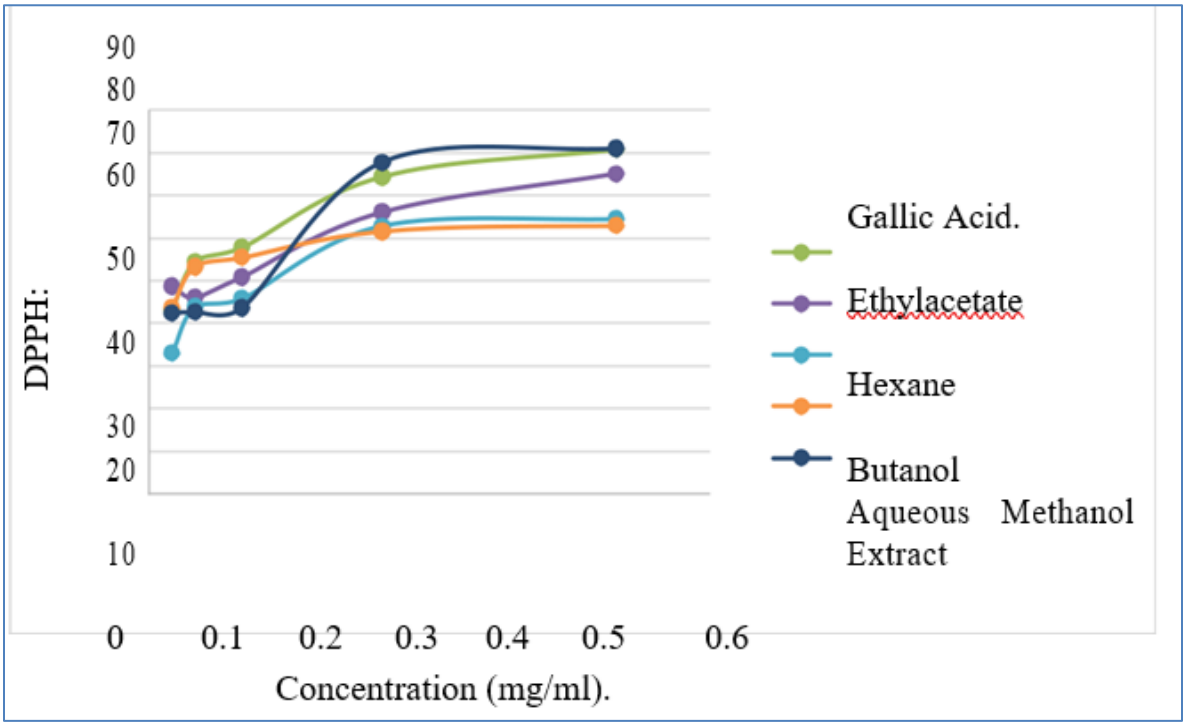

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Figure 3: Percentage inhibition of DPPH radical scavenged by Gallic Acid, Ethyl acetate Fraction, Hexane Fraction, Butanol Fraction, and Aqueous Methanol Extract.

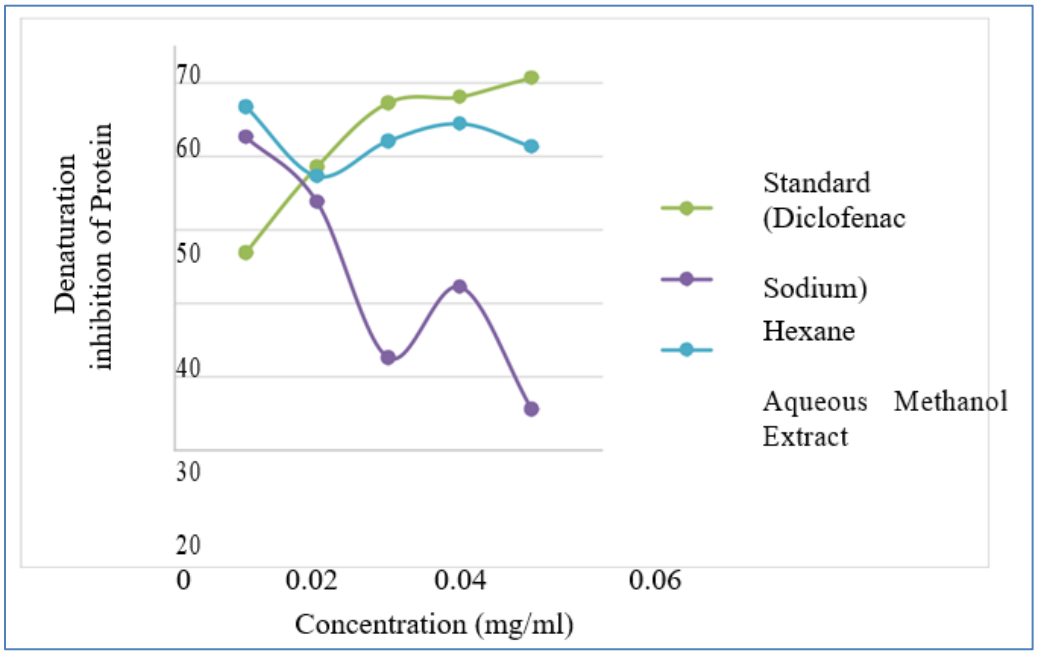

Figure 4: Percentage inhibition of thermally induced protein denaturation by the standard drug, diclofenac sodium, hexane fraction, and Aqueous Methanol Extract.

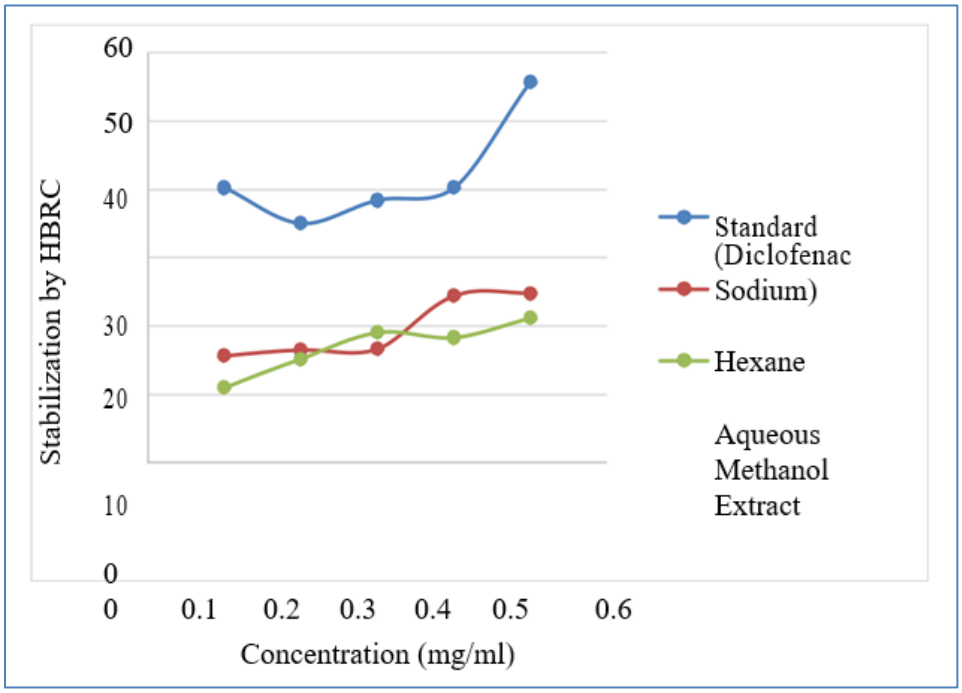

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Figure 5: Percentage Stabilization of HRBC at varying concentrations by the standard drug, diclofenac sodium, hexane fraction, and Aqueous Methanol Extract. *HRBC = Human red blood cell.

Table 3: Fifty per cent inhibitory concentration (IC_{50}) of the hexane fraction and aqueous methanol extract of *C. lanatus leaves in vitro* anti-denaturation assay. *

FRACTIONS	ANTI-DENATURATION (mg/ml)
Standard	0.008
Hexane	0.022
Aqueous Methanol Extract	0.360

*Data = IC_{50}. IC_{50}: Fifty per cent inhibitory concentration.

Table 4: Fifty per cent inhibitory concentration (IC_{50}) of the hexane fraction and aqueous methanol extract of *C. lanatus leaves in vitro* human erythrocyte stabilization assay. *

FRACTIONS	ERYTHROCYTE STABILIZATION (mg/ml)
Standard	0.523
Hexane	2.877 ± 0.851
Aqueous Methanol Extract	3.026 ± 0.785

*Data = Mean ± SEM; IC_{50}. IC_{50}: Fifty per cent inhibitory concentration

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