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Original Research Article

Invitro Evaluation Of The Hypoglycaemic, Anti-Inflammatory, And Antioxidant Activities Of *Tragia benthamii* Baker (EUPHORBIACEAE)

Gloria A. Ayoola, * Oluwatosin O. Johnson, John Aderounmu, Lukmon Raji, Rafiat B. Aremu, Seun Bankole

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Lagos, CMUL Campus, Lagos 100254, Nigeria

ABSRTACT

Tragia benthamii Baker (Euphorbiaceae) is a climbing herb with significant traditional uses in Africa, particularly in Western Africa. This study aimed to evaluate the *invitro* hypoglycemic, anti-inflammatory, and antioxidant effects of *Tragia benthamii* leaves. The alpha-amylase inhibition and membrane stabilization assays were employed to evaluate the *invitro* hypoglycemic and anti-inflammatory activities of the methanol extract, n-hexane, chloroform, and ethylacetate fractions. Additionally, the antioxidant activity of the methanol crude extract was investigated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging and ferric-reducing antioxidant power (FRAP) assays. The IC_{50} values obtained for the alpha-amylase inhibition were 63.52±0.069, 193.9±0.077, 913.19±0.044, and 3559.89±0.057 µg/mL for the methanol extract, n-hexane, chloroform, and ethylacetate fractions, respectively, while the reference drug sample, acarbose, exhibited an IC_{50} value of 455.30±0.063 µg/mL. IC_{50} values for the anti-inflammatory activity were 82.75±0.005, 75.06±0.02, 64.92±0.014, and 105.74±0.056 µg/mL for the methanol extract, n-hexane, chloroform, and ethylacetate fractions, respectively, and the reference drug, diclofenac, had an IC_{50} value of 53.26±0.002 µg/mL. Moreover, the IC_{50} values for the DPPH assay were 46.07±0.043, 53.78±0.008, and 7.43±0.008 µg/ml for the crude methanol extract, vitamin E, and vitamin C, respectively. The FRAP assay demonstrated that the leaf extract exhibited a total antioxidant activity of 185 µM Fe2+/g dry extract, whereas vitamin C (positive control) showed an activity of 405 µM Fe2+/g. Overall, this study indicates the potential hypoglycemic, anti-inflammatory, and antioxidant properties of *T. benthamii* leaf extracts.

Keywords: *Tragia benthamii*, Hypoglycemic, Anti-inflammatory, Antioxidant.

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Introduction

The exploration of medicinal plants for their potential health benefits has gained significant attention across the pharmaceutical, food, and cosmetics industries.[1] Plant extracts are known to possess a wide range of biochemical activities, including anti-inflammatory, antioxidant, anti-allergic, antimicrobial, antidiabetic, and anticancer properties.[2, 3] *Tragia benthamii* Baker (Euphorbiaceae) is a climbing herb commonly found in Western and Central Southern Africa.[4] Referred to as Eesii among the Yoruba tribe in Western Nigeria, *T. benthamii* is known for its irritant nature due to stinging hairs.[5]
The root extract of *T. benthamii* is traditionally used to alleviate childbirth pains and treat gonorrhea.[4] In addition, studies have shown that the ethanol extract of *T. benthamii* exhibits antimalarial activity comparable to the reference drug chloroquine.[6] Furthermore, the volatile oil from *T. benthamii* leaves has demonstrated significant antioxidant activity[7], making it a potential candidate for treating common headaches and migraines.[8]

*Corresponding author: E-mail: tosyn.villa@gmail.com

Tel: +2348033708829

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While there is limited information available on the biological activities of *T. benthamii* leaves, related species such as *T. involucrata, T. spathulata, and T. pungens* have been reported to exhibit antioxidant, anti-urolithiatic, cytotoxic, antidiabetic, and antibacterial properties.[9 – 13] To address the knowledge gap regarding the biological activities of *T. benthamii*, a recent review recommended further research on this plant.[14] Subsequently, a focused research effort was aimed to evaluate the *in-vitro* hypoglycemic, anti-inflammatory, and antioxidant potential of *Tragia benthamii* leaves. The research objectives are to perform a crude extraction of *Tragia benthamii* leaves using the cold maceration method. This will be followed by the fractionation of the crude extract into n-hexane, chloroform, and ethyl acetate fractions. Additionally, the study aims to identify the phytochemical constituents present in the crude extract. The antioxidant activity of the crude extract will also be evaluated, along with the assessment of hypoglycemic activity for each fraction using the in vitro alpha-amylase inhibition method. Furthermore, the anti-inflammatory activity of each fraction will be determined through the membrane stabilization method.

Materials and Methods

Collection and Identification of Plant Material
The whole plant, *Tragia benthamii* was obtained from Mushin market, Lagos State, Nigeria in September 2015. The plant's identity was confirmed by Mr. Oyebanji from the Department of Botany at the University of Lagos, Akoka, Lagos, Nigeria. A voucher specimen (LUH 6696) was then placed in the University herbarium.

Extraction and fractionation processes
The leaves were carefully plucked from the plant's stalk, thoroughly washed to remove contaminants and debris, and then laid out on the laboratory table to dry at a controlled room temperature of 25ºC. Once dried, the leaves were ground into a fine powder using a roller mill.

Approximately 600 g of the powdered leaf was cold macerated in 4500 mL of methanol for 72 hours, with filtration performed every 24 hours. The methanol extract was then concentrated and evaporated to dryness in a Stuart® rotary evaporator (RE3022C, Japan) at 40ºC. The resulting dried crude extract weighed 70.17 g, and was stored in a sample bottle in the refrigerator for subsequent analysis. A portion of the dried crude extract (50 g) was fractionated successively with n-hexane, chloroform, and ethyl acetate, leaving behind the water fraction. This process yielded four different fractions, which were concentrated to dryness in a Stuart® rotary evaporator at 40ºC.

Phytochemical Screening of Tragia banthamii leaves

Phytochemical screening of the methanol crude extract of the leaves was determined using the methods previously described.[15]

Ethical consideration on the use of experimental animals and human subjects

The membrane stabilization method protocol has been approved by the Health Research Ethics Committee of the College of Medicine, University of Lagos, Idi-araba, Lagos, Nigeria (CMULHREC) with approval number CMUL/HREC/05/24/1455. The protocol and safety guidelines meet CMULHREC policies for experiments involving human participation. Informed consent was obtained when the participant read and signed a consent form, which was then submitted to the Health Research Ethics Committee.

Determination of in-vitro hypoglycemic activity (inhibition of α-amylase assay)

The α-amylase inhibition assay was conducted following the method previously outlined in literature, with minor adjustments to the concentrations of the prepared samples.[16] 500 µL of the prepared concentrations (10, 50, 100, 200, 500, 1000 µg/mL) of the samples (fractions, methanol extract, and the standard drug Acarbose) were added to 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing porcine pancreatic α-amylase (0.5mg/mL; Sigma chemical company, St. Louis, MO, USA). The mixture was then stirred at 32°C for 10 minutes. Subsequently, 500 µL of 1% starch solution in 0.02M sodium phosphate buffer (pH 6.9 with 0.006M NaCl) was added to each prepared sample concentration. The reaction mixtures were incubated at 32°C for 10 minutes. Then, 1.0 mL of dinitrosalicylic acid color reagent was added, followed by incubating the reaction mixtures in test tubes in a boiling water bath for 5 minutes and then cooling them to room temperature. The reaction mixture was diluted with 10 mL of distilled water, and the absorbance was measured with J.P. Selecta, S.S. UV-1100D, Spain at 540 nm. The experiment was conducted in triplicate, with acarbose serving as the positive control.

The inhibition of α-amylase was calculated using Equation 1.

$$Percentage\ inhibition\ \% = \frac{(\text{ABcontrol} - \text{ABsample})}{\text{ABcontrol}} \times 100$$ ----------Equation 1

Where ABcontrol is the absorbance of blank sample and ABsample represents the absorbance of extract, fractions and standard respectively.

Determination of in-vitro anti-inflammatory activity (membrane stabilization assay)

Preparation of Human Red Blood Cell (HRBC)

The membrane stabilization assay method used was previously described in literature.[17, 18] Five milliliters of fresh whole blood were collected from a healthy human volunteer who had not taken non-steroidal anti-inflammatory drugs (NSAIDs) for at least two weeks. The blood was collected into a heparinized sample bottle and then centrifuged at 3000 rpm for 10 minutes. The red blood pellets were dissolved with an equivalent volume of normal saline. The volume of the red blood pellets was measured and then reconstituted to a 40% v/v suspension with an isotonic buffer solution (10 mM sodium phosphate buffer, pH 7.4). The reconstituted red blood cell supernatant was used to prepare the suspension.

Preparation of standard drug

Diclofenac, 200 µg/mL was prepared by dissolving an equivalent of 10 mg of diclofenac into 50 mL of 10 M Sodium Phosphate buffer (The buffer solution contained 0.2 g of NaH_2PO_4, 1.15 g of Na_2HPO_4 and 9 g of NaCl in 1000 mL of distilled water).

Preparation of control

Approximately 5 mL of Sodium Phosphate buffer (blank) was mixed with 0.1 mL of the blood suspension.

Membrane stabilization assay

The extract and fractions were each dissolved in an isotonic phosphate buffer solution. Centrifuge tubes containing 5 mL graded doses of the samples (100, 200, and 400 µg/mL) were arranged in triplicate. A set of control tubes prepared contained 5 mL of the buffer solution and 5 mL of 100, 200, and 400 µg/ml of diclofenac, respectively. 0.1 mL of HBRC suspension was added to each tube and mixed gently. One tube was incubated at 54°C for 20 minutes in a water bath, while another was maintained at -10°C in a freezer for 20 minutes. Afterward, the tubes were centrifuged at 1300 rpm for 3 minutes, and the hemoglobin content of the supernatant was estimated using a UV spectrophotometer (J.P. Selecta, S.S. UV-1100D, Spain) at 660 nm. The percentage inhibition was calculated using Equation 2.

$$Percentage\ inhibition\ \% = \frac{(\text{ABcontrol} - \text{ABsample})}{\text{ABcontrol}} \times 100$$ ----------Equation 2

Where ABcontrol is the absorbance of blank sample and ABsample represents the absorbance of plant samples and standard respectively.

Determination of in-vitro antioxidant activity

DPPH (1, 1-diphenyl-2-picryl-hydrazyl) assay

The methanol extract of *Tragia benthamii* was tested for *in-vitro* antioxidant potential against 1,1-diphenyl-2-picryl-hydrazyl (Sigma-Aldrich) radical by measuring UV absorbance at 517 nm.[19, 20] Various concentrations of the extract (0.02, 0.04, 0.06, 0.08, and 0.1 mg/mL) were prepared. Standards of ascorbic acid (Vitamin C) and α-Tocopherol (Vitamin E) were also prepared at the same concentrations. All solutions were prepared using methanol. Two milliliters of each prepared concentration were mixed with 0.5 mL of 1mM DPPH solution in methanol in test tubes. The experiments were conducted in triplicates. After 15 minutes of incubation at room temperature, the absorbance at 517 nm was measured with UV spectrophotometer (J.P. Selecta, S.S. UV-1100D, Spain). A blank solution containing the same amount of methanol and DPPH was also measured for absorbance. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The radical scavenging activity was calculated using the formula below.

$$DPPH\ scancenging\ effect\ \% = \frac{(\text{ABcontrol} - \text{ABsample})}{\text{ABcontrol}} \times 100$$ ----------Equation 3

Where ABcontrol is the absorbance of blank sample and ABsample represents the absorbance of plant samples and standards respectively.

Ferric reducing antioxidant power (FRAP) assay

A 1.5 mL of freshly prepared FRAP solution containing 25 mL of 300 mM acetate buffer, 2.5 mL of 10 mM 2, 4, 6 tripyridyl-5- triazine (TPTZ) in 40 mM HCL and 2.5ml of 20mM $Fecl_3.6H_2O$ was mixed with 1mL of extracts and the absorbance read at 593 nm. Calibration was prepared with $FeSO_4.7H_2O$. The results obtained are expressed in µM Fe^{2+}/g of dry plant material and compared with that of a standard drug, Ascorbic Acid.[21]

Statistical analysis

All experiments were conducted in triplicate. The percentage inhibition is presented as the mean ± standard deviation, while the IC_{50} values are shown as the mean ± standard error of the mean (SEM). Statistical analysis was performed using Microsoft Office Excel LTSC Professional Plus 2021.

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Results and Discussion

The phytochemical screening of the methanol leaf extract of *T. benthamii* revealed the presence of secondary metabolites such as cardiac glycosides, alkaloids, terpenes, saponins, reducing sugars, and flavonoids, while anthraquinone and tannins were absent. Comparatively with previous [6], the ethanol extract of the whole plant of *T. benthamii*, showed the presence of alkaloid, reducing sugar, tannins, and saponin while glycoside, protein, flavonoid, and sterols were absent.[7]

Alpha-amylase inhibition assay

The *in-vitro* alpha-amylase assay revealed hypoglycemic activity in *T. benthamii* leaves. The methanol extract showed the highest percentage inhibition at 79.41% when tested at 1000 µg/mL. In contrast, the ethyl acetate fraction exhibited the lowest inhibition of 0.51% at 20 µg/mL (Table 1). The methanol extract and n-hexane fraction demonstrated better activity, with values of 71.52±0.069 and 193.70±0.076 µg/ml respectively, compared to acarbose (455.30±0.063 µg/mL). The IC_{50} values for chloroform and ethyl acetate fractions were 913.19±0.044 and 3559.89±0.057 µg/mL respectively (refer to Figure 1).

Acarbose is known to inhibit the enzyme α-amylase, thereby impeding the release of glucose from starch and preventing a rise in glycemic levels. Similarly, *Tragia benthamii* leaf extract and fractions exhibited α-amylase inhibitory activity. This is consistent with findings from other Tragia species, such as *T. involucrata*, which showed significant activity with the chloroform, ethyl acetate, and aqueous fractions.[12] The presence of secondary metabolites like cardiac glycosides, alkaloids, terpenes, saponins, reducing sugars, and flavonoids may account for this activity. In particular, flavonoids and saponins have been identified as potential α-amylase inhibitors.[22-24]

Membrane stabilization assay

The *in-vitro* anti-inflammatory assay demonstrated potential anti-inflammatory activity in all the extract and fractions tested. The highest percent inhibition (99.02%) was shown at 100 µg/mL for diclofenac, while the least inhibition (72.59%) was shown in ethyl acetate fraction at 200 µg/mL (Table 2). The IC_{50} values were 82.75±0.005, 75.06±0.02, 64.92±0.014, and 105.74±0.056 µg/mL for the methanol extract, n-hexane, chloroform, and ethylacetate fractions, respectively. The reference drug, diclofenac, had an IC_{50} value of 53.26±0.002 µg/mL (see Figure 2). Inflammation is a normal protective response to tissue injury caused by various factors, and it involves a complex interplay of vasoactive, chemotactic, and proliferative factors. Inhibition of lysosomal enzymes released during inflammation is a key target for anti-inflammatory action.[25] Non-steroidal anti-inflammatory drugs exert their effects by inhibiting these enzymes or stabilizing the membrane. The prevention of hypotonicity-induced human red blood cell (HRBC) membrane lysis is used as a measure of the anti-inflammatory activity of drugs, as the HRBC membrane structure is similar to the lysosomal membrane structure. Certain flavonoids in plants have been associated with an acute stabilizing effect on lysosomal membranes *in-vitro* [20, 26], and this could be linked to the presence of flavonoids in *T. benthami* in this study. Further studies using a carrageenan-induced paw edema model in Sprague-Dawley rats revealed anti-inflammatory activity in methanol and chloroform extracts of the whole plant of *Tragia cannabina*. The percentage inhibition of carrageenan-induced paw edema by methanol extract (300 mg/kg, p.o.) and chloroform extract (300 mg/kg, p.o.) of *T. cannabina* was found to be 37.5% and 17.05%, respectively, comparable to the reference drug, ibuprofen (55.69%).[27]

Table 1: Percentage inhibition for *Tragia Benthamii* leave fractions and Standard drug, Acarbose

Conc(µg/ml)	N-Hexane Fraction	chloroform Fraction	Ethyl Acetate	Methanol Extract	Acarbose
			Mean ±SD%		
20	24.43±0.00	26.21±2.45	0.51±0.44	28.24±0.00	15.48±0.014
50	23.30±0.51	29.20±0.88	10.62±0.00	56.64±0.00	27.10±0.014
100	46.60±0.00	40.78±0.00	16.51±0.97	63.11±0.97	33.55±0.014
200	58.91±1.78	33.72±0.00	23.64±0.67	58.14±0.00	44.52±0.009
500	60.44±0.76	41.33±1.33	26.22±0.77	68.00±0.00	51.61±0.005
1000	66.18±0.00	56.37±0.85	41.18±1.47	79.41±0.00	56.13±0.015

Table 2: Percentage inhibition of *Tragia benthamii* leave fractions and Standard drug anti-inflammatory drug, Diclofenac

Conc (µg/ml)	N-Hexane Fraction	chloroform Fraction	Ethyl Acetate fraction	Methanol Extract	Diclofenac
			Mean ±SD%		
100	93.77±0.0002	92.56±0.004	86.63±0.0002	87.68±0.0007	99.02±0.0003
200	86.66±0.0005	97.01±0.0006	72.59±0.0003	89.57±0.0006	93.33±0.0002
400	91.63±0.002	92.81±0.0003	91.42±0.0007	88.54±0.0005	98.53±0.00015

Antioxidant assay

The percentage of inhibition increased with higher concentrations, as demonstrated in Table 3. The results from the IC_{50} analysis of the DPPH assay indicate that the plant extract (46.07±0.043 µg/ml) exhibits higher activity than Vitamin E (53.78±0.008 µg/ml), but lower activity compared to Vitamin C (7.43±0.008 µg/ml). The calibration curve of FeSO4 in Figure 3 is used to determine the FRAP value, with an absorbance of 0.3694 for *T. benthamii* and 0.8108 for vitamin C at 100 µM (Table 4). In the FRAP assay, the *T. benthamii* leaf extract demonstrated a total antioxidant activity of 185 µM Fe2+/g extract, while ascorbic acid showed 405 µM Fe2+/g. The DPPH test measures the ability of the test compound to function as a free radical scavenger. It is well-known that Vitamins C, and E are established antioxidants used in medicine. Furthermore, the volatile oil from *T. benthamii* leaves has been found to exhibit higher free radical scavenging activity (42.4%) than the reference standard, Vitamin C (13.8%), in the DPPH scavenging assay.[7] Therefore, the antioxidant capability of *T. benthamii* leaves is likely due to the presence of flavonoids and volatile oils in the leaves.

FRAP (Ferric reducing antioxidant power) is a rapid and useful test for routine analysis. This test estimates the antioxidant activity by measuring the increase in absorbance caused by the formation of ferrous

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ions from the FRAP reagent.[21] Furthermore, the reducing ability of the extract (185 µM Fe2+/g) indicates that it is less effective compared to Vitamin C (405 µM Fe2+/g).

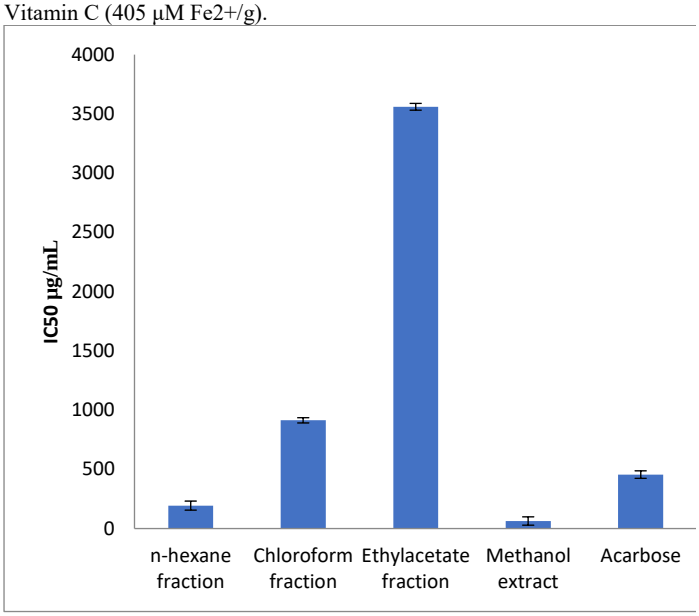

Figure 1: IC_{50} values for alpha-amylase inhibition by acarbose, extract and fractions from *T. benthamii* leaves.

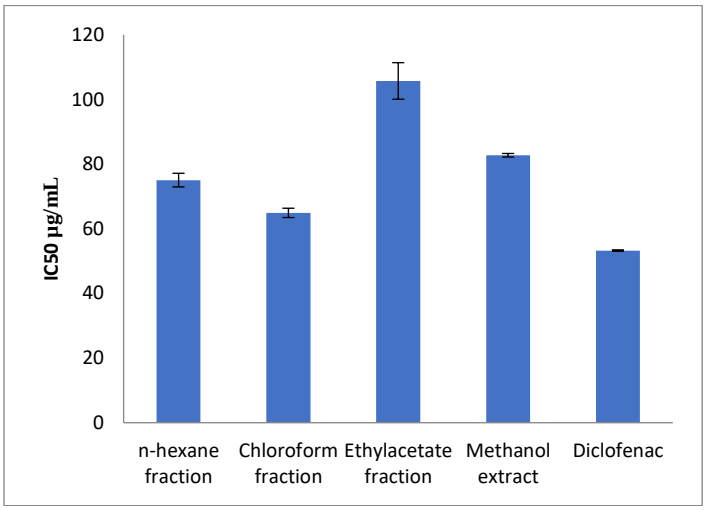

Figure 2: IC_{50} values for anti-inflammatory activity by diclofenac, extract and fractions from *T. benthamii* leaves

Table 3: Percentage inhibition of *Tragia benthamii* leaves extract and standard antioxidant drugs, Vitamin C and E

Conc (µg/mL)	20	40	60	80	100
			Vitamin E		
% Inhibition	55.34±0.23	54.65±0.117	55.78±0.077	59±0.058	54.63±0.034
			Vitamin C		
% Inhibition	94.29±0.04	96.69±0.038	96.62±0.076	98.88±0.019	98.75±0.009
			Tragia benthamii		
% Inhibition	50.67±0.16	56.26±0.002	64.45±0.138	68.5±0.079	74.92±0.146

Table 4: Absorbance of *T. benthamii* and Standard antioxidant, Vitamin C

SAMPLES	Concentration (µM)	Mean Absorbance
VITAMIN C	100	0.8108
Tragia benthamii	100	0.3694

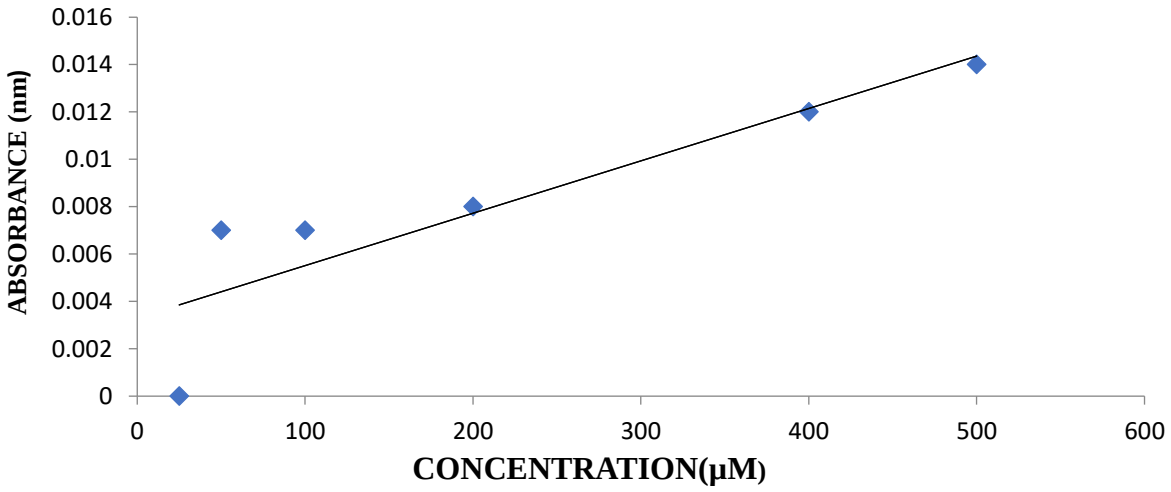

Figure 3: Graphical presentation of calibration curve of $FeSO_4$ used to determine the FRAP

Conclusion

The research reveals that *Tragia benthamii* leaves have exhibited *in-vitro* hypoglycemic, anti-inflammatory, and antioxidant properties compared to standard drugs such as acarbose, diclofenac, vitamin C and vitamin E, respectively. This may be due to the presence of secondary metabolites identified in the plant. The inhibitory concentrations (IC_{50}) of the extract and fractions can provide valuable guidance for traditional medicine practitioners in their practices. It is advisable to conduct further research to isolate and identify the bioactive compounds in the fractions that show pharmacological potential.

Conflict of Interests

The authors declare no conflict of interest

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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