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Formulation and in Vitro Evaluation of The Anticancer Activity of A Hydrogel Containing A Bioactive Fraction Isolated From A Conventional 50% Ethanolic Whole-Plant Extract of *Calotropis Gigantea*

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Abstract: The present study was undertaken to formulate and evaluate a hydrogel containing a bioactive fraction isolated from a conventional 50% ethanolic whole-plant extract of *Calotropis gigantea*, with particular emphasis on its in vitro anticancer potential.. The whole plant was subjected to conventional extraction using 50% ethanol, followed by column chromatograpy gradient techniques used for the fractionations (F1-F4). The isolated fraction was subjected to utilized solvents like ethanol (F1), Petroleum Ether (F2), Ethyl acetate (F3) and Water (F4). All four fractions done invitro pharmacological studies and F3 have higher action The bioactive fraction (F3) was incorporated into a suitable hydrogel base using an appropriate formulation technique used to prepared Four formulations(H1-H4). In vitro release and diffusion studies were performed to assess the ability of the hydrogel to deliver the bioactive fraction from the formulation. The anticancer activity of the isolated fraction and/or the formulated hydrogel was investigated using an appropriate in vitro cancer-cell-line model. Cytotoxic activity was assessed at different concentrations, and the percentage of cell inhibition and, where applicable, IC_{50} values were determined. Appropriate untreated and positive-control groups were included for comparison. The study provides a systematic approach for exploring *Calotropis gigantea* as a potential source of bioactive compounds and for developing a plant-based hydrogel with promising anticancer activity.

Key Words: *Calotropis gigantean*, 50% ethanol, Fractions, Hydrogel, Anticancer, Cytotoxic and bioactive compounds

INTRODUCTION

Cancer is one of the major health challenges worldwide and is characterized by uncontrolled proliferation, abnormal survival, and progressive accumulation of genetically altered cells. Despite substantial advances in surgery, radiotherapy, chemotherapy, immunotherapy, and targeted treatment, the management of cancer remains challenging because of tumor heterogeneity, treatment resistance, recurrence, and adverse effects associated with conventional therapies. Therefore, systematic extraction and fractionation are important steps in obtaining fractions enriched in potentially biologically active constituents. *Calotropis gigantea* is a

medicinal plant belonging to the family Apocynaceae and is widely distributed in tropical and subtropical regions. The plant contains a variety of secondary metabolites, including cardenolides, flavonoids, terpenoids, steroids, phenolic constituents, and other bioactive compounds. These constituents have attracted scientific interest because of their reported pharmacological properties. However, the biological effects of *C. gigantea* may vary according to the plant part examined, extraction procedure, solvent system, concentration, and method used for biological evaluation. Conventional extraction is comparatively simple and widely applicable; however, the composition and biological activity of the resulting extract depend on extraction time, solvent-to-material ratio, temperature, particle size, and other processing variables. Subsequent fractionation of the crude extract can help concentrate selected groups of constituents and may provide a more appropriate material for pharmacological investigation. The identification of a bioactive fraction is particularly important in the development of plant-based formulations. A crude extract may contain numerous constituents, some of which may contribute to biological activity while others may have limited activity or affect the stability and formulation characteristics of the final product. Fractionation can therefore provide an opportunity to obtain a comparatively enriched fraction with greater biological relevance. Evaluation of the fraction through preliminary phytochemical screening and appropriate analytical methods can further support the identification and characterization of the constituents associated with the observed activity. Although naturally derived compounds may demonstrate promising anticancer effects, their practical therapeutic application can be limited by poor aqueous solubility, instability, inadequate retention at the site of administration, and difficulties associated with achieving an effective local concentration. These limitations have encouraged the development of novel drug-delivery systems for plant-derived bioactive compounds. Among the available delivery systems, hydrogels have received considerable attention because of their ability to incorporate active substances within a hydrated polymeric network and provide suitable topical application characteristics. Hydrogels are semisolid systems consisting of a polymeric matrix capable of retaining substantial quantities of water or aqueous fluid. Their properties can be modified by selecting appropriate polymers, concentrations, cross-linking conditions, and formulation components. Depending on the intended application, hydrogels may provide advantages such as ease of application, good spreadability, cooling and soothing effects, and prolonged contact with the application site. They may also facilitate controlled or sustained release of incorporated active constituents. Consequently, hydrogel systems have been investigated extensively for the delivery of pharmaceutical agents and natural bioactive compounds. The incorporation of a bioactive fraction obtained from *C. gigantea* into a hydrogel may offer an approach for combining the biological potential of plant-derived constituents with the formulation advantages of a topical delivery system. However, the preparation of such a formulation requires careful optimization because the physicochemical properties of the isolated Appropriate cancer cell-line models can be used to examine cellular responses following exposure to different concentrations of a test sample. Parameters such as cell viability, percentage inhibition of cell growth, morphological changes, and half-maximal inhibitory concentration (IC_{50}), where appropriate, can provide information about cytotoxic or antiproliferative activity. Comparison between the isolated fraction, hydrogel formulation, untreated control, and suitable reference treatment can help determine whether the formulation retains the biological activity of the incorporated fraction. The present investigation therefore focuses on the development of a hydrogel containing a bioactive fraction isolated from a conventional 50% ethanolic whole-plant extract of *Calotropis gigantea*.

MATERIALS AND METHODS

Plant Material

Fresh and healthy whole plants of *Calotropis gigantea* were collected from a suitable geographical location. The plant material was collected during the appropriate season and selected carefully to ensure that the specimens were free from visible fungal infection, insect infestation, disease, and mechanical damage. The collected material was transported to the laboratory in clean containers and processed as soon as possible after collection.

Preparation of Conventional 50% Ethanolic Extract

A predetermined quantity of the powdered *Calotropis gigantea* whole plant was accurately weighed and transferred into a clean, dry extraction vessel. A 50% ethanol-water mixture was prepared using

appropriate proportions of ethanol and purified water and used as the extraction solvent. The powdered plant material was sufficiently moistened with the solvent and subjected to conventional extraction. The plant-to-solvent ratio, extraction time, and other conditions were maintained consistently throughout the extraction process. The extraction was carried out under controlled conditions with intermittent agitation to facilitate contact between the plant material and extraction solvent. Following completion of the extraction period, the extract was filtered through suitable filter material to remove plant debris. The filtrate was collected and concentrated under reduced pressure using a rotary vacuum evaporator at a controlled temperature. Concentration under reduced pressure was performed to minimize excessive exposure of the extract to heat. The concentrated extract was further dried to obtain a semisolid or dry crude extract. The extract was weighed, and the percentage yield was calculated based on the original quantity of powdered plant material used. The dried extract was transferred to a clean, airtight, amber-colored container and stored under suitable conditions until further fractionation and analysis.

Fractionation of the 50% Ethanolic Extract

The concentrated 50% ethanolic whole-plant extract was subjected to column chromatographic fractionation to separate its constituents according to their physicochemical properties and differential affinity toward the stationary and mobile phases. A suitable chromatographic column was packed uniformly with an appropriate stationary phase, such as silica gel, according to the validated experimental procedure. Care was taken to avoid formation of air bubbles and cracks within the stationary phase. The concentrated extract was appropriately prepared and introduced onto the top of the packed column. A gradient solvent system was employed to facilitate sequential elution of constituents with different polarities. Fractions were collected separately in clean, labeled containers. The collected eluates were monitored using a suitable chromatographic technique, such as thin-layer chromatography, to identify fractions showing similar chromatographic profiles. Fractions exhibiting similar chromatographic patterns were pooled where appropriate. The pooled fractions were concentrated under reduced pressure at a controlled temperature and preserved for further phytochemical and pharmacological investigation. Four major fractions were obtained and designated as: F1 – Ethanol fraction F2 – Petroleum ether fraction F3 – Ethyl acetate fraction F4 – Aqueous fraction

In-Vitro Pharmacological Screening of Fractions

Antiodidant

Nitric oxide

Each fraction (F1, F2, F3 and F4) was accurately weighed and dissolved separately in a suitable solvent, preferably methanol or DMSO, to prepare a stock solution of 1 mg/mL (1000 µg/mL). The stock solutions were further diluted to obtain different concentrations, such as 25, 50, 100, 200 and 400 µg/mL. The final concentration of DMSO, if used, should be kept low and identical in all test and control tubes.A fresh solution of 10 mM sodium nitroprusside was prepared in phosphate-buffered saline (PBS), pH 7.4. The solution should preferably be freshly prepared and protected from excessive light. To each test tube, 1 mL of 10 mM sodium nitroprusside solution was added, followed by 1 mL of the respective concentration of F1, F2, F3 or F4. The reaction mixtures were incubated at 25 ± 2°C for 150 minutes under suitable illumination. A control was prepared in the same manner using the solvent instead of the test fraction. Ascorbic acid may be used as the reference standard antioxidant. After incubation, 1 mL of the reaction mixture was transferred into a fresh test tube and mixed with 1 mL of Griess reagent. The Griess reagent may be prepared freshly by mixing equal volumes of 1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride prepared in 2% phosphoric acid. The mixture was allowed to stand for approximately 10–15 minutes at room temperature for development of the pink/magenta-coloured azo dye. The absorbance was measured at 546 nm using a UV–Visible spectrophotometer against an appropriate reagent blank.

Hydrogen peroxide

Accurately weighed quantities of H1, H2, H3 and H4 hydrogels were dispersed separately in phosphate buffer (pH 7.4) to obtain appropriate test concentrations, for example 25, 50, 100, 200 and 400 µg/mL,

expressed in terms of the active fraction present in each hydrogel. If required, the dispersions were sonicated or vortexed to obtain uniform samples. A reaction mixture was prepared by mixing 1 mL of H_2O_2 solution (40 mM) with 1 mL of each concentration of H1, H2, H3 and H4. The mixtures were incubated at room temperature for 10 minutes. A control was prepared by replacing the hydrogel sample with the corresponding solvent or buffer. The absorbance of the reaction mixture was measured at 230 nm against an appropriate blank using a UV–Visible spectrophotometer

SRBC

Fresh sheep blood collected in an appropriate anticoagulant-containing tube was obtained from an authorized source. The blood was centrifuged at approximately 3000 rpm for 10 minutes to separate the erythrocytes. The packed erythrocytes were carefully separated and washed three times with isotonic saline (0.9% NaCl) until the supernatant became clear. A 10% v/v SRBC suspension was prepared by suspending the washed erythrocytes in isotonic saline or phosphate-buffered saline. The isolated fractions F1, F2, F3 and F4 were separately dissolved/dispersed in a suitable vehicle and prepared at selected concentrations, for 25, 50, 100, 200 and 400 µg/mL. imilarly, H1, H2, H3 and H4 hydrogels were accurately weighed and dispersed in the appropriate vehicle/buffer to obtain equivalent concentrations based on the amount of isolated fraction incorporated into each hydrogel. A known anti-inflammatory indomethacin used as the standard.

The reaction mixture was prepared by mixing: 1 mL of 10% SRBC suspension 1 mL of phosphate buffer 1 mL of hypotonic solution 1 mL of the respective test sample. The same procedure was followed separately for F1–F4 and H1–H4 at each concentration. For the control, the test sample was replaced with the vehicle. For the standard, the vehicle was replaced with the selected standard anti-inflammatory drug. All reaction mixtures were incubated at 37 ± 2°C for approximately 30 minutes. Following incubation, the tubes were centrifuged at approximately 3000 rpm for 10 minutes to sediment the intact erythrocytes.The absorbance of the supernatant was measured spectrophotometrically at approximately 540 nm against the appropriate reagent blank.

MTT assay

The isolated fractions F1–F4 should be dissolved in a suitable cell-culture-compatible vehicle and sterile-filtered where appropriate. The H1–H4 hydrogels should be dispersed/extracted in sterile culture-compatible medium or another validated vehicle so that the fraction & hydrogel concentration 6.25, 12.5, 25, 50, 100 and 200 µg/Ml.The exact concentration range should be selected following a preliminary cytotoxicity range-finding experiment. For meaningful comparison between fractions and hydrogels, report hydrogel concentrations based on the actual amount of F1–F4 incorporated/released, as well as the formulation concentration where appropriate. Cancer cells were maintained under their recommended culture conditions and harvested during the appropriate growth phase. The cells were seeded into sterile 96-well plates at a validated density suitable for the selected cell line and allowed to attach and stabilize. After cell attachment, the culture medium was replaced or supplemented with medium containing different concentrations of F1, F2, F3 and F4. A separate set of wells was treated with corresponding concentrations of H1, H2, H3 and H4**.** The cells were incubated with the test samples for an appropriate exposure period, commonly 24–48 hours, depending on the selected cell line and experimental design. After treatment, the medium was carefully removed and the cells were washed gently with PBS if required by the validated assay protocol. An appropriate concentration of MTT solution was then added to each well and the plate was incubated under cell-culture conditions for approximately 3–4 hours, allowing viable cells to convert MTT into purple formazan crystals. Following incubation, the MTT-containing medium was carefully removed. The formazan crystals were dissolved using an appropriate solubilizing solution such as DMSO. The plate was gently shaken to ensure complete dissolution of the crystals. The absorbance was measured using a microplate reader at approximately 570 nm

Preformulation study

The prepared samples were subjected to FTIR analysis over an appropriate spectral range, typically 4000–400 cm^{-1}, using an FTIR spectrophotometer. The spectra were recorded at an appropriate spectral

resolution, such as 4 cm^{-1}, with multiple scans accumulated to improve the signal-to-noise ratio. FTIR spectra were obtained for: Bioactive fraction F3. Polyvinyl alcohol. Glycerol. Glyceraldehyde and Sodium chloride

Hydrogel Formulation

A suitable hydrogel base was selected based on the physicochemical characteristics of F3 and the intended application of the formulation. Appropriate pharmaceutical-grade polymeric materials were used to develop the hydrogel. The selection of the polymer was based on its gelling capacity, compatibility, viscosity, stability, spreadability, and ability to facilitate release of the incorporated bioactive fraction. Four hydrogel formulations were prepared and designated as H1, H2, H3, and H4**.** The formulations contained different concentrations or combinations of the selected polymer and other suitable excipients to identify an optimized formulation. The required quantity of polymer was accurately weighed and dispersed or dissolved in an appropriate aqueous vehicle. The dispersion was allowed to hydrate adequately. Other formulation components were incorporated gradually with continuous mixing to obtain a uniform hydrogel base. The calculated quantity of F3 was separately dispersed or dissolved using a suitable vehicle or cosolvent, depending on its solubility characteristics. The bioactive fraction was then incorporated gradually into the hydrated polymeric base with continuous stirring to ensure uniform distribution. The formulation was allowed to equilibrate until a uniform hydrogel was obtained. Entrapped air bubbles were removed by allowing the preparation to stand or by using a suitable deaeration procedure. The final formulations were transferred into appropriate, well-closed containers.

Table:1: Formulation of hydrogel patches

Ingredients	**H1**	**H2**	**H3**	**H4**
Bioactive fraction	20mg	18mg	16mg	14mg
Polyvinyl alcohol	4%W/V(1ml)	3.5%W/V(1ml)	3%W/V(1ml)	2.5%W/V(1ml)
Glycerol	30%W/W(2.5ml)	28%W/W(2ml)	25%W/W(1.5ml)	23%W/W(0.7ml)
Glyceroldehyde	5%W/V(3ml)	4.5%W/V(2.8ml)	4%W/V(2.7ml)	3.5%W/V(2.5ml)
Sodium chloride	Required Quantity	Required Quantity	Required Quantity	Required Quantity
Water	q.s. to required volume	q.s. to required volume	q.s. to required volume	q.s. to required volume

In-Vitro Release Study

The in-vitro release behavior of the bioactive fraction from the hydrogel formulations was investigated using a suitable diffusion or membrane-based release apparatus. A predetermined quantity of each formulation was placed in the donor compartment or on an appropriate membrane, depending on the selected experimental method. A suitable receptor medium was maintained under controlled temperature and agitation conditions. Samples were withdrawn at predetermined time intervals and replaced immediately with an equal volume of fresh receptor medium to maintain sink conditions where applicable. The amount of bioactive fraction released at each time interval was determined using an appropriate analytical method. The cumulative percentage release was calculated and plotted against time. The release profiles of H1–H4 were compared to identify the formulation providing the most appropriate release characteristics.

Selection of Optimized Hydrogel

The four hydrogel formulations were compared based on their physical appearance, homogeneity, pH, viscosity, spreadability, extrudability, active-fraction content, and in-vitro release and diffusion characteristics. The formulation exhibiting the most desirable combination of physicochemical properties and release behavior was selected as the optimized formulation. The optimized formulation was subsequently subjected to further biological evaluation.

In-Vitro Anticancer Activity

The anticancer activity of the isolated bioactive fraction F3 and the optimized hydrogel formulation was evaluated using a suitable authenticated human cancer cell line. The selection of the cell line was based on the objective of the study and the biological relevance of the proposed application. Cells were maintained in an appropriate cell-culture medium supplemented with the required nutrients and serum under controlled incubation conditions. The cultures were maintained in a humidified incubator containing an appropriate concentration of carbon dioxide and maintained at physiological temperature. Before treatment, cells were seeded into suitable multiwell culture plates at a predetermined cell density and allowed to attach and stabilize. The test samples were prepared at different concentrations using an appropriate sterile vehicle.

The following experimental groups were considered where applicable: Untreated cell control Vehicle control Reference anticancer drug/positive control F3 bioactive fraction F3-loaded optimized hydrogel Blank hydrogel**,** where appropriate The cells were exposed to the test samples for the predetermined treatment period. Following treatment, cell viability was assessed using a suitable validated cytotoxicity assay, such as an MTT, resazurin, SRB, or equivalent assay appropriate for the selected cell line.

Assessment of Cell Viability and Cytotoxicity

Following exposure to the test samples, cell viability was determined according to the selected assay protocol. The absorbance or fluorescence response was measured using an appropriate microplate reader. The percentage of cell viability was calculated relative to the untreated control. Percentage inhibition of cell growth was subsequently determined A suitable equation was used: Cell viability (%) **=** Absorbance of treated cells / Absorbance of control cells × 100 Where appropriate: Cell inhibition (%) = 100 − Cell viability (%) The concentration producing 50% inhibition of cell viability was determined where sufficient concentration-response data were available and was expressed as the IC_{50} value.

Morphological Evaluation of Treated Cells

The morphological characteristics of untreated and treated cells were examined using an inverted microscope. Changes such as cell shrinkage, rounding, loss of adherence, membrane-associated alterations, reduction in cell density, and other visible morphological changes were recorded.

The morphological observations were compared among the control, positive-control, fraction, blank hydrogel, and loaded hydrogel groups where applicable.

RESULTS AND DISCUSSION

Plant Collection

Botanist authenticated 7 kg of plant material collected & weighed 6.5 kilograms after being dried shade remove moisture. 6.25 kilograms finished product were produced powdered.

Extraction and Yield

The product convention extraction process was 66.6 percent besides dry heat extraction 65.9 percent, used for the isolation and evaluation

Column Chromatograpy

The crude extract was successfully subjected to column chromatography and fractionated using solvents of increasing polarity, namely petroleum ether, ethyl acetate, ethanol, and water. This sequential extraction approach enabled the separation of phytoconstituents based on their polarity, facilitating better isolation of bioactive compounds.

Thin layer chromatographic profiling

Prepare for the chosen solvent system: the n-hexane Acetate d'Ethylema (4:1) The codes for the extracts included F1-Pet.ether, F2-Ethanol, F3-Ethyl acetate, and F4-Water. Based values were compared to standard values for the Rf value. Thin layer chromatography profiling was selected extracts in order to evaluate the phytochemical composition and separation pattern of different constituents present in the samples (Table:1 & Figures:1-4)

Nitric oxide

Among the isolated fractions, F3 exhibited the highest activity, increasing from 34.3 ± 1.8% at 10 µg/mL to 90.6 ± 6.66% at 320 µg/mL. F2 showed the next highest activity, increasing from 46.4 ± 0.3% to 75.2 ± 0.6% across the tested concentration range. F4 demonstrated moderate scavenging activity, increasing from 25.7 ± 2.4% at 10 µg/mL to 75.9 ± 7.58% at 320 µg/mL. F1 showed a progressive increase from 24.2 ± 1.4% to 70.5 ± 0.58%, indicating comparatively lower activity than F2 and F3 at higher concentrations. The standard ascorbic acid exhibited the strongest activity, increasing from 73.40 ± 0.70% at 10 µg/mL to 94.54 ± 0.06% at 320 µg/mL. At 320 µg/mL, F3 showed a marked scavenging effect of 90.6 ± 6.66%, approaching the activity observed with ascorbic acid.

SRBC

F1 exhibited an increase in inhibition from 18.59% at 200 µg/mL to 78.56% at 1200 µg/mL, with an IC_{50} value of 1020 µg/mL. F2 showed comparatively stronger activity, with inhibition increasing from 28.69% to 88.56% over the same concentration range and an IC_{50} value of 74.36 µg/mL. F3 demonstrated 42.19% inhibition at 200 µg/mL and increased progressively to 90.53% at 1200 µg/mL, with an IC_{50} value of 176.5 µg/mL. F4 showed inhibition ranging from 28.69% at 200 µg/mL to 88.56% at 1200 µg/mL, with an IC_{50} value of 66.84 µg/mL. Among the isolated fractions, F4 exhibited the lowest IC_{50} value, suggesting the highest membrane-stabilizing activity under the reported conditions. F2 also demonstrated appreciable anti-inflammatory activity, as indicated by its relatively low IC_{50} value. The standard showed a progressive increase in inhibition from 43.59% at 200 µg/mL to 92.53% at 1200 µg/mL, with an IC_{50} value of 150.2 µg/mL.

MTT assay

F1 showed inhibition ranging from 22.45% at 6.25 µg/mL to 78.56% at 100 µg/mL, with an IC_{50} value of 64.38 µg/mL. F2 demonstrated inhibition from 32.45% to 88.56% over the tested concentration range, with an IC_{50} value of 74.36 µg/mL. F3 exhibited the most pronounced activity, increasing from 7.35% at 6.25 µg/mL to 91.53% at 100 µg/mL, with an IC_{50} value of 37.26 µg/mL. F4 showed a progressive increase in inhibition from 27.45% to 88.56%, with a reported IC_{50} value of 66.84 µg/mL. The standard cyclophosphamide exhibited 2.25% inhibition at 6.25 µg/mL and increased markedly to 92.53% at 100 µg/mL, with an IC_{50} value of 34.52 µg/mL. Among the isolated fractions, F3 demonstrated the lowest IC_{50} value, indicating the strongest cytotoxic activity under the reported experimental conditions.

Fraction selection

Based on the above three activity indicated F3 have higher action selected for the hydrogel preparation.

Preformulation studies

Figure No. 5 represents the isolated F3 fraction, which was identified as the most active fraction among the fractions evaluated in the antioxidant, anti-inflammatory, and cytotoxicity studies. Figure No. 6 illustrates PVA, which was selected as a polymeric component for the development of the hydrogel formulation. Figure No. 7 represents glycerol, which can function as a plasticizing and humectant component and may contribute to improving the flexibility and moisture-retention properties of the formulation. Figure No. 8 represents glyceraldehyde, as indicated in the supplied figure description, although its specific role in the formulation should be clearly established from the experimental methodology. Figure No. 9 shows the combination of the bioactive F3 fraction with polyvinyl alcohol, demonstrating the incorporation of the selected bioactive fraction into the polymeric system.

Formulation

Figure No. 10 represents the H1 hydrogel formulation prepared using the bioactive F3 fraction isolated from the whole-plant extract of *Calotropis gigantea*. Figure No. 11 illustrates the H2 hydrogel formulation containing the selected F3 fraction incorporated into the polymeric hydrogel matrix. Figure No. 12 shows the H3 hydrogel formulation developed using the same bioactive F3 fraction with a modified formulation composition. Figure No. 13 represents the H4 hydrogel formulation prepared by incorporating the F3

fraction into the optimized polymeric system. All procedures and preparations followed by the table;1 in the materials and methods

Hydrogen peroxide

Hydrogen peroxide. F1 demonstrated the highest activity among the tested fractions, increasing from 31.25 ± 3.3% at 10 µg/mL to 92.2 ± 9.2% at 320 µg/mL. F2 also showed appreciable activity, increasing from 38.2 ± 3.9% to 89.3 ± 9.9% across the tested concentration range. F3 exhibited a gradual increase in activity from 31.25 ± 3.2% at 10 µg/mL to 76.2 ± 7.2% at 320 µg/mL. F4 showed a similar response to F3, with scavenging activity increasing from 31.25 ± 3.2% to 76.2 ± 7.2% as the concentration increased.

SRBC

H1 showed an increase in inhibition from 28.59% at 200 µg/mL to 88.56% at 1200 µg/mL, with a reported IC_{50} value of 997.5 µg/mL. H2 exhibited inhibition ranging from 29.69% to 89.56% and showed the lowest IC_{50} value of 77.86 µg/mL among the tested hydrogel formulations. H3 demonstrated 41.19% inhibition at 200 µg/mL, which increased progressively to 91.3% at 1200 µg/mL, with an IC_{50} value of 150.5 µg/mL. H4 showed inhibition ranging from 29.69% to 89.56%, with a reported IC_{50} value of 166.84 µg/mL

MTT assay

cytotoxic effects with increasing concentration. H1 showed inhibition ranging from 22.5% at 6.25 µg/mL to 78.6% at 100 µg/mL, with a reported IC_{50} value of 64.8 µg/mL. H2 exhibited inhibition from 33.5% to 89.6% across the tested concentrations and showed a reported IC_{50} value of 77.36 µg/mL. H3 demonstrated the most pronounced activity among the hydrogel formulations, increasing from 8.35% at 6.25 µg/mL to 91.53% at 100 µg/mL, with an IC_{50} value of 34.26 µg/mL. H4 showed a progressive increase in inhibition from 29.45% to 89.56%, with an IC_{50} value of 68.84 µg/mL.

Table 2: Thin layer chromatographic profiling

Detection	Rf	State	Extracts
UV at 254 nm& at 366 nm	0.79	Light yellowish sticky mass	Pet.ether
UV at 254 nm&at 366 nm	0.75	Light Brown sticky mass	Ethanol
UV at 254 nm& at 366 nm	0.82	Light yellowish brown	Ethyl acetate
UV at 254 nm& at 366 nm	0.65	Dark brown & Sticky	Water

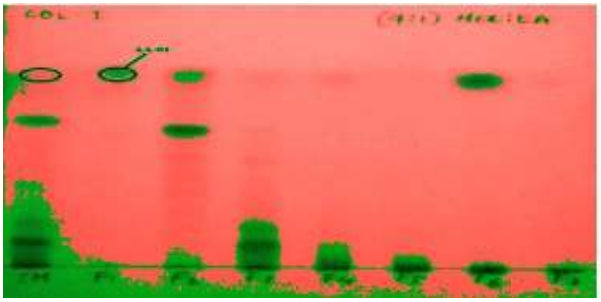

Fig 1: TLC For Various fractions: Visualization At UV 254 Nm

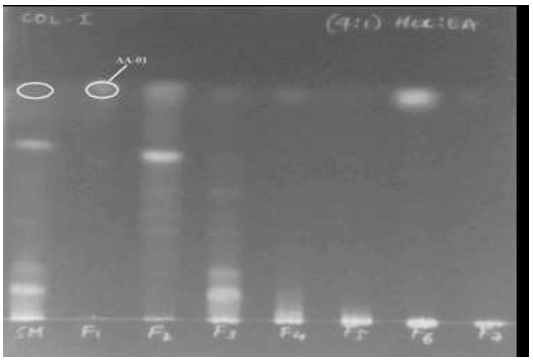

Fig 2: Visualization At UV 356 Nm

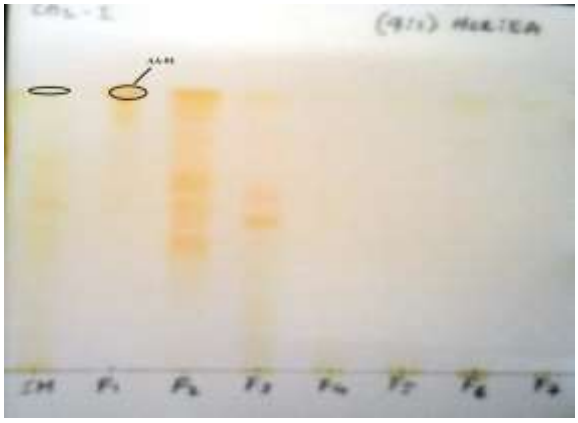

Fig 3: TLC Plate Normal

Table 3: Nitric oxide Radical Scavenging activity

Conc. (µg/ml)	Ascorbic acid	F1	F2	F3	F4
10	73.40±0.70	24.2±1.4	46.4±0.3	34.3±1.8	25.7±2.4
20	77.53±3.12	38.0±0.3	56.4±0.8	48.1±0.7	43.5±4.3
40	82.23±0.96	48.4±0.1	65.0±0.8	58.5±3.88	53.9±5.1
80	85.83±0.79	59.3±1.1	70.2±1.4	69.4±5.88	64.8±6.1
160	93.20±0.02	68.3±1.0	73.5±1.7	78.4±4.22	69.8±6.9
320	94.54±0.06	70.5±0.58	75.2±0.6	90.6±6.66	75.9±7.58

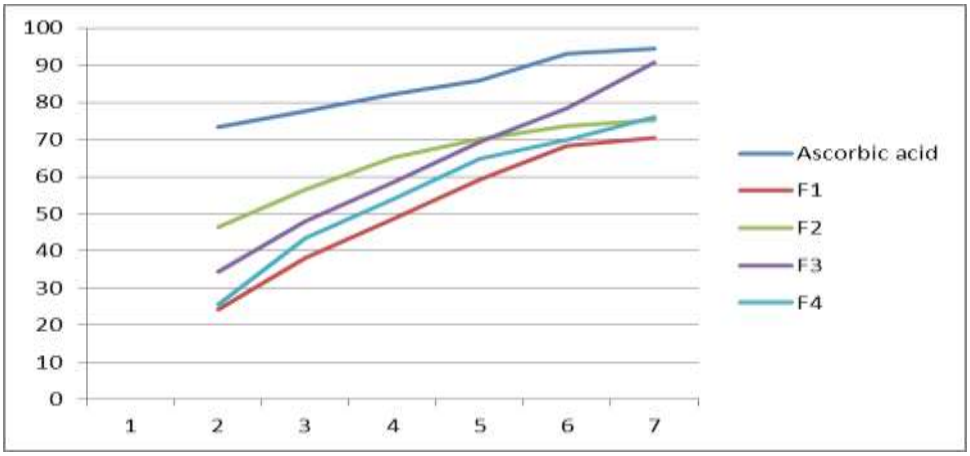

Fig 4: Nitric oxide Radical Scavenging activity

Table 4: *In vitro* SRBC anti-inflammatory activity of Fractions

Samples	Concentration (µg / ml)	% inhibition	IC50 (µ g / ml) Mean	SEM
Control	100	00	00	00
Standard	200	43.59	150.2	10.907
	400	56.83		
	800	65.94		
	1000	71.75		
	1200	92.53		
F1	200	18.59	1020	11.978
	400	35.21		

	800	43.95		
	1000	48.33		
	1200	78.56		
F2	200	28.69	74.36	8.762
	400	45.31		
	800	53.85		
	1000	58.73		
	1200	88.56		
F3	200	42.19	176.5	7.62
	400	55.23		
	800	66.34		
	1000	70.45		
	1200	90.53		
F4	200	28.69	66.84	6.75
	400	45.31		
	800	53.85		
	1000	58.73		
	1200	88.56		

Table 5: *In vitro* cytotoxic activity

Samples	**Concentration (µg/ml)**	**% inhibition**	**IC50 (µg/ml) Mean**	**SEM**
Control	100	00	00	00
Standard Cyclophosmaide	6.25	2.25	34.52	8.9057
	12.5	25.82		
	25	45.44		
	50	81.27		
	100	92.53		
F1	6.25	22.45	64.38	9.9738
	12.5	34.23		
	25	56.78		
	50	69.39		
	100	78.56		
F2	6.25	32.45	74.36	10.7632
	12.5	44.23		
	25	66.78		
	50	79.39		
	100	88.56		
F3	6.25	7.35	37.26	3.632
	12.5	25.12		
	25	83.44		
	50	86.27		
	100	91.53		
F4	6.25	27.45	66.84	6.725
	12.5	39.23		
	25	67.78		
	50	69.39		
	100	88.56		

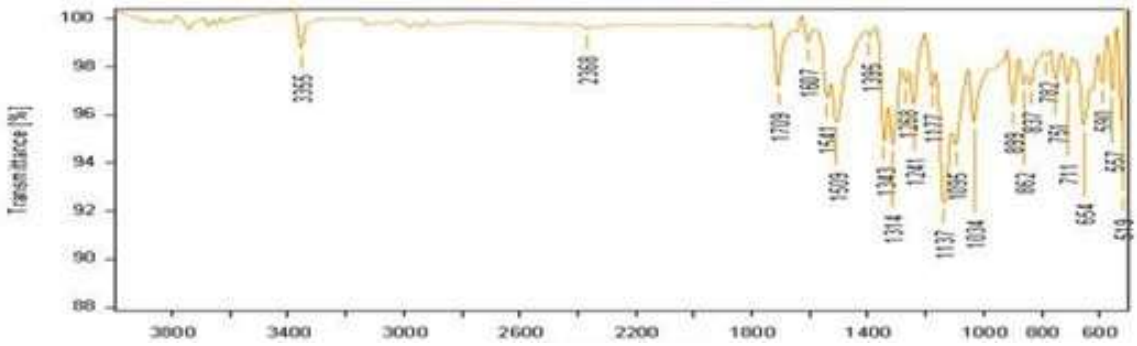

Fig 5: Bioactive F3 fraction

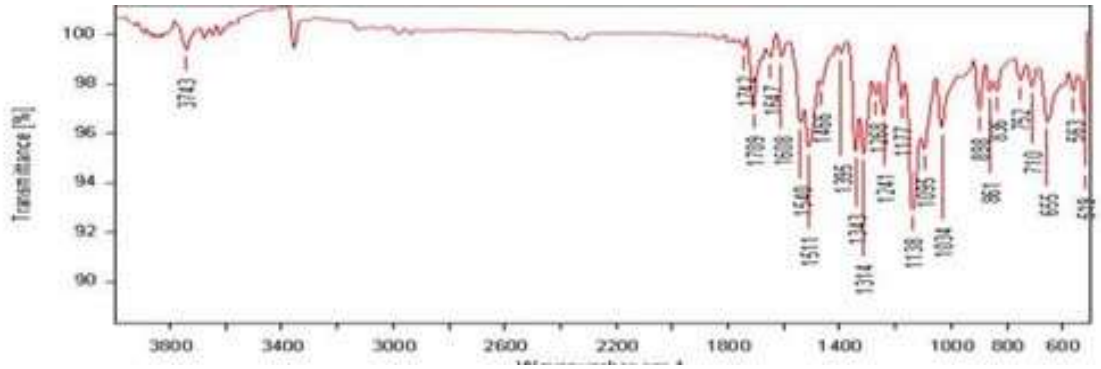

Fig 6: Polyvinyl alcohol

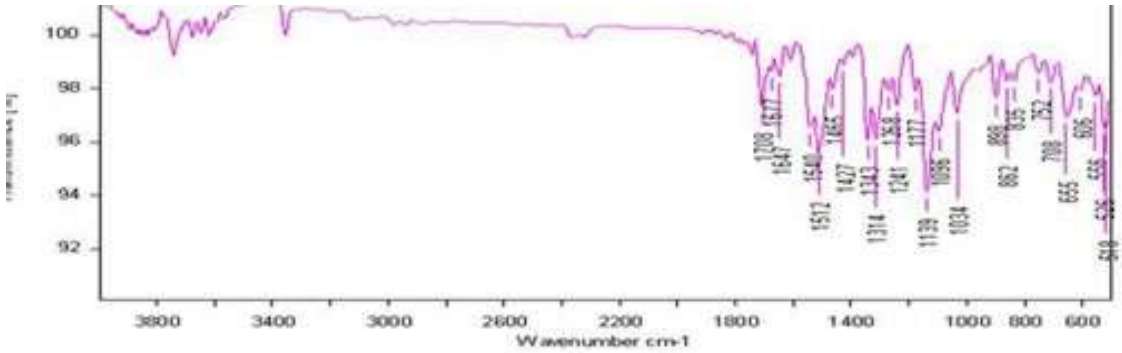

Fig 7: Glycerol

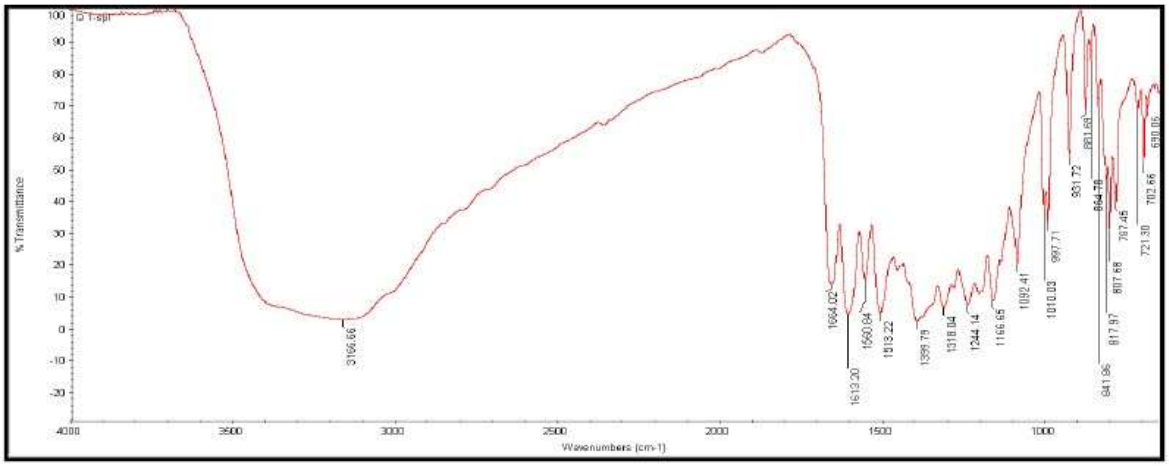

Fig 8: Glyceroldehyde

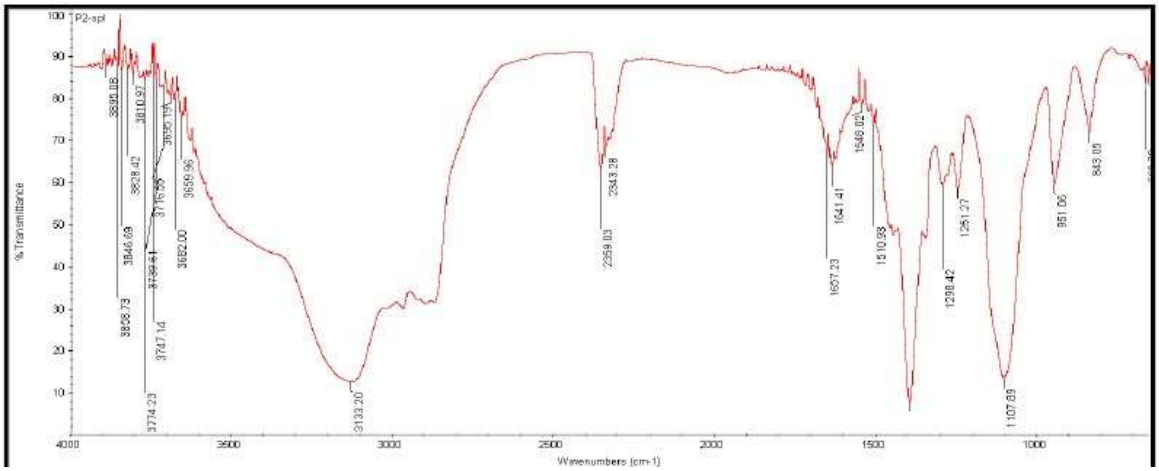

Fig 9: Bioactive F3 fraction+ Polyvinyl alcohol

Fig 10: F3 Make hydrogel H1 formulation

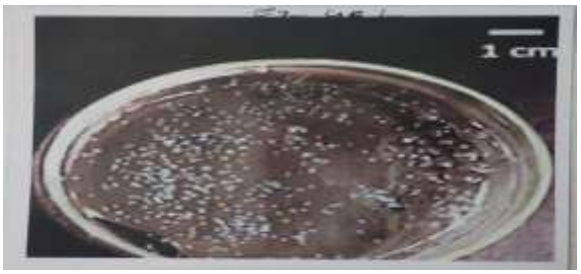

Fig 11: F3 Make hydrogel H2 formulation

Fig 12: F3 Make hydrogel H3 formulation

Fig 13: F3 Make hydrogel H4 formulation

Table 6: % cumulative release of H1-H4 hydrogel formulations

Formulation	% cumulative drug release at						
	0 min	**5 min**	**10 min**	**20 min**	**30 min**	**45 min**	**60 min**
F1	0.0	42.25 ± 0.74	68.14 ± 0.22	85.47 ± 0.93	94.18 ± 2.11	96.35± 0.39	96.58 ± 0.28
F2	0.0	48.38 ±3.03	68.41 ± 1.09	86.17± 0.64	92.14 ± 0.32	98.12± 1.13	98.35 ± 2.11
F3	0.0	50.36 ± 1.81	65.24 ± 0.28	80.15 ± 2.08	93.02 ± 1.76	95.17 ± 0.17	95.86± 0.41
F4	0.0	53.7 ± 3.65	64.26 ± 0.65	84.36 ± 0.87	96.15 ± 1.21	96.68 ± 0.65	96.86± 0.25

Table 7: Hyrogen peroxide Assay for hydrogel formulation

Conc.(µg/ml)	**Ascorbic acid**	**F1**	**F2**	**F3**	**F4**
10	26.2±3.2	31.25±3.3	38.2±3.9	31.25±3.2	31.25±3.2
20	60.4±7.5	75.54±7.6	70.62±6.1	62.54±6.6	62.54±6.6
40	66.2±7.6	84.22±7.5	74.74±7.76	64.22±6.5	64.22±6.5
80	68.4±7.2	87.77±8.5	79.4±7.56	67.77±6.5	67.77±6.5
160	77.4±8.0	88.24±8.3	87.5±9.99	74.24±7.3	74.24±7.3
320	90.8±9.0	92.2±9.2	89.3±9.9	76.2±7.2	76.2±7.2

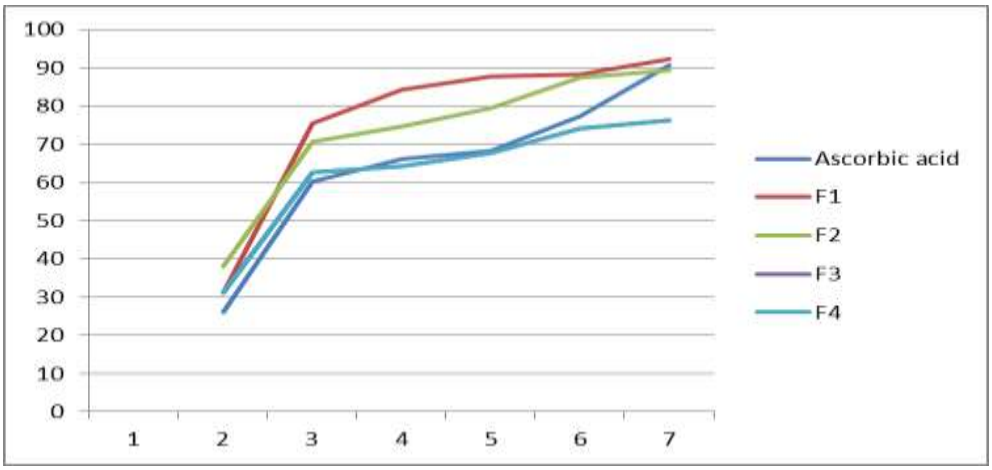

Fig 14: Hyrogen peroxide Assay for hydrogel formulation

Table 8: *In vitro* SRBC antiinflammatory activity of hydrogel formulatiom

Samples	Concentration (µg / ml)	% inhibition	IC50 (µg/ml) Mean	SEM
Control	100	00	00	00
Standard	200	42.9	140.8	7.97
	400	57.3		
	800	66.4		
	1000	77.5		
	1200	93.3		
H1	200	28.59	997.5	8.98
	400	45.21		
	800	53.95		
	1000	68.33		
	1200	88.56		
H2	200	29.69	77.86	8.72
	400	47.31		
	800	63.85		
	1000	68.73		
	1200	89.56		
H3	200	41.19	150.5	7.2
	400	54.3		
	800	65.4		
	1000	71.5		
	1200	91.3		
H4	200	29.69	166.84	12.75
	400	46.31		
	800	56.85		
	1000	59.73		
	1200	89.56		

Table 9: *In vitro* cytotoxic activity of hydrogel

Samples	Concentration (µg/ml)	% inhibition	IC50 (µg/ml) Mean	SEM
Control	100	00	00	00
Standard Cyclophosmaide	6.25	3.35	36.52	8. 7
	12.5	27.92		
	25	46.54		
	50	83.37		
	100	93.63		
H1	6.25	22.5	64.8	9. 8
	12.5	34.3		
	25	56.8		
	50	69.9		
	100	78.6		
H2	6.25	33.5	77.36	9.2
	12.5	45.3		
	25	67.8		
	50	80.9		
	100	89.6		

H3	6.25	8.35	34.26	3.2
	12.5	35.12		
	25	83.44		
	50	85.27		
	100	91.53		
H4	6.25	29.45	68.84	6.5
	12.5	38.23		
	25	70.78		
	50	71.39		
	100	89.56		

SUMMARY AND CONCLUSION

The present study was undertaken to investigate the extraction, isolation, biological evaluation, and hydrogel formulation of bioactive constituents obtained from the whole plant of *Calotropis gigantea*. The crude extract was subsequently subjected to column chromatography using solvents of increasing polarity, resulting in the separation of four fractions designated F1, F2, F3, and F4. Thin-layer chromatographic profiling using n-hexane acetate (4:1) demonstrated separation of the constituents present in the selected fractions and supported the fractionation process. The isolated fractions were subjected to nitric oxide scavenging, hydrogen peroxide scavenging, SRBC membrane stabilization, and MTT cytotoxicity assays to identify the fraction with promising biological activity. The fractions exhibited concentration-dependent antioxidant activity, while F3 showed particularly prominent nitric oxide scavenging activity, increasing from 34.3 ± 1.8% at 10 µg/mL to 90.6 ± 6.66% at 320 µg/mL. The SRBC membrane stabilization assay demonstrated appreciable anti-inflammatory activity among the isolated fractions, with F4 and F2 showing the lowest reported IC_{50} values of 66.84 and 74.36 µg/mL, respectively. The MTT assay demonstrated concentration-dependent cytotoxic activity, with F3 showing the strongest activity among the isolated fractions and a reported IC_{50} value of 37.26 µg/mL, compared with 34.52 µg/mL for cyclophosphamide. Based on the overall biological evaluation, F3 was selected as the bioactive fraction for further formulation development. The selected fraction was incorporated into a polyvinyl alcohol-based hydrogel system, and four formulations, H1–H4, were prepared using varying proportions of the bioactive fraction, PVA, glycerol, glyceraldehyde, sodium chloride, and water. The hydrogel formulations demonstrated concentration-dependent membrane-stabilizing and cytotoxic activities, with H2 showing the lowest reported SRBC IC_{50} value of 77.86 µg/mL and H3 exhibiting the strongest cytotoxic activity with an IC_{50} value of 34.26 µg/mL. Overall, the study successfully demonstrated the extraction and chromatographic isolation of bioactive fractions from *Calotropis gigantea*, identified F3 as a biologically promising fraction, and established its feasibility for incorporation into a hydrogel delivery system. The findings provide a scientific basis for further characterization of the active phytoconstituents, optimization of the hydrogel formulation, and detailed mechanistic investigation of its antioxidant, anti-inflammatory, and in-vitro cytotoxic properties.

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