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Biophysical, Biochemical and Functional Characterization of Bioactive Compounds from *Tridax procumbens*

Ramesh Bandla[1,2,4], Ramesh Kande[5], Shanti Kumari Lunavat[1], Jaisatya Gowri Gogada[1,5], Navya Kalingapatnam[4], Navya Anthagiri[4] Abdul Qadeer Mohammed[4], Sandeepta Burgula[5], Praveen Boddana[3], Rajesh Medisetty[4], Raghu Gogada[1,2*]

[1]*Department of Molecular Therapeutics, Scigenebio Pvt Ltd, Hyderabad, Telangana, India*
[2]*Department of Biochemistry and Plant Physiology; M.S. Swaminathan School of Agriculture (MSSSoA), Centurion University of Technology and Management (CUTM), Parlakhemundi-761211, Odisha, India*
[3]*Department of Plant Pathology, M.S. Swaminathan School of Agriculture (MSSSoA), Centurion University of Technology and Management (CUTM), Parlakhemundi-761211, Odisha, India*
[4]*Analytical R&D Division, Biological E. Limited, Hyderabad, Telangana, India*
[5]*Department of Microbiology, Osmania University, Hyderabad, Telangana, India*

Abstract

Medicinal plant products have been used in health care for the treatment of human illnesses since old times. Various homegrown medicinal plants have been used the cure of blood-related issues due to the disturbance of the coagulation pathway. The goal of the study is to characterise the isolated plant leaf extracts of Tridax procumbens *that displayed strong anticoagulant properties. The objective of the study is to demonstrate haemolytic activity, wound healing activity, and assessment of the impurity in the partially purified leaf extract from the plant* Tridax procumbens. *The same fractions were assessed for pro- or anti-angiogenic properties as their therapeutic potential and cellular toxicity for the safety and efficacy of the purified plant extract. The purified leaf extracts contained 31% carbohydrates, 1.1% protein, and 0.9% lipids. The primary active component responsible for the anticoagulant activity is likely to be Azulene, a naphthalene-based derivative. A significant cellular migration was observed, assisting the wound healing. The anti-angiogenic was observed in the purified leaf extracts with no significant cellular toxicity by haemolysis and MTT assay. Traces of protein and lipid sources are present in the purified leaf extract fractions. About 31% of the leaf-extracted fraction has carbohydrates as an active anticoagulant content. The extract showed 90-60% cell migration, suggesting it to be a potent wound healing ability. The purified fractions did not show cytotoxicity at a concentration of 4-500 µg/mL in T47D cells.*

Keywords: *Anti-coagulation, Anti-angiogenic, Bioactive Compounds, Extraction and Characterization, Tridax Procumbens.*

Introduction

Tridax procumbens L. commonly known as coat buttons is a flowering herb that belongs to the family Asteraceae. It is native to tropical America but has widely spread across tropical and subtropical regions worldwide. This plant is recognized for its medicinal properties and has been widely used in traditional medicine. It is used as a drink to treat bronchial catarrh, diarrhea, dysentery, wound healing, hyperuricemia, oxidative stress, bacterial infection and liver diseases [1-6].

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****Corresponding Author: rgsl@uohyd.ac.in; raghu.gogada@roswellpark.org***

The phytochemicals found in *Tridax procumbens*, including flavonoids, tannins, saponins, and alkaloids, contribute significantly to its medicinal properties. These compounds exhibit a wide range of therapeutic effects, including antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, and cardioprotective activities. The traditional uses of *Tridax procumbens* are supported by scientific research, highlighting its potential in modern medicine [7-10].

Herbal medicine is prone to having vast occurrences of adverse effects and toxicities, as this is often related to improper formulations, excessive dosage, different combinations, nature of source material, geographical area, climate, and process of extraction [11]. Hence, proper assessment of safety and efficacy is highly warranted. When investigating the safety and efficacy of purified plant extracts for therapeutic use, it is crucial to evaluate their potential cytotoxic effects. In this study, we have evaluated the impurity profile, angiogenic properties, and cellular toxicity studies of the partially purified bioactive compound that showed properties of potential anticoagulation effects from *Tridax. Procumbens.*

Materials and Methods

Materials

All the reagents used for quantification of impurities, assessment for Chorio Allantoic Membrane (CAM) assay, and GC-MS analysis were of analytical grade. Folin & Ciocalteu's phenol reagent is from Sigma Aldrich, catalog number F9252. Bovine Serum Albumin standard ampules, 2 mg/mL were from Thermo Scientific™, Pierce™, catalogue number 23209. The lipid quantification kit was from Cell Biolabs Inc., catalog number STA 163. Vanillin is from Sigma, catalogue number V1104. Anthrone was from Sigma Aldrich, catalogue number 319899. RPMI 1640 medium was from Gibco, catalog number: 11875093 supplemented with 10% FBS (Thermofisher, catalog number 26140079), 1% penicillin streptomycin was from sigma, catalog Number: P4333. Typsin-EDTA was from Himeida, catalog number: TCL048).

Mass Composition of the Purified Leaf Extract Fraction by GCMS

GC-MS analysis of the partially purified leaf extract of Tridax Procumbens was performed using Thermo GC Trace Ultra Ver;5.0 and Thermo MS DSQ II equipment. A sample of 10 µL was injected with a split ratio of 10. The column used was the MEGA-1 MS 0.18mm, 0.40µm, GC-MS Capillary Column 50m. Helium was used as the carrier gas with a flow rate of 1.0 ml/min. The injector, column oven, transfer line, and detector operated at 250, 260, 280, and 290 °C, respectively.

Impurity Assessment

Protein Estimation

The total protein of the purified extract was estimated by the Lowry method [12]. Lowry method was used for determining protein concentrations. First, the peptide nitrogen reacts with the copper [II] ions under alkaline conditions and subsequently the Folin-Ciocalteu's reagent (phosphomolybdic phosphotungstic acid) is reduced to hetero-polymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The end product of the reaction has a blue color. The amount of protein in the samples was estimated by monitoring the absorbance at 750nm using a multimode reader (Spectramax I3x) against a standard curve. Briefly, 5 standard concentrations of BSA ranging from 50 to 10 µg/mL, test samples, and mid concentration of standard spiked to test sample (internal control) were used to determine the protein concentration. The recovery of the internal control shall not be more than ±10 %. The internal control was used as system suitability for the assay.

Carbohydrate estimation

The total sugar in the partially purified leaf extracts was estimated by the anthrone method. (Plant lab protocols, Biocyclopedia). The total carbohydrates present in the standard solutions or the test samples are dehydrated by conc. H_2SO_4 to form furfural or a derivative of furfural. The 0.2% anthrone solution in conc. H_2SO_4 reacts by condensing with the carbohydrate furfural derivative to give a green color in dilute and a blue color in concentrated solutions, which was determined at an absorbance of 630 nm using a multimode plate reader (Spectramax I 3x). Briefly, 1 mL of five working standards of glucose solution was taken along with the partially purified leaf extract fraction and 4 mL of 0.2% anthrone reagent in conc. H_2SO_4 was added to the standard and test solutions. These were incubated for 30 min in the water bath at 80 ºC. The resulting solutions were brought to room temperature and about 300 µL of the solutions were transferred to 96 well microplate. The reaction complex formed was measured at 630 nm using a plate reader [13].

Lipid Estimation

The total lipid content of the partially purified leaf extract was determined by the sulfo-phospho-vanillin method. The kit used for the quantification of lipids was from Cell Biolabs INC with a catalog number STA-613. The chemical basis for lipid estimation by sulfo-phospho-vanillin reaction contains three main steps. In the first step, unsaturated lipids react with sulfuric acid to produce a carbonium ion. In the second step, Vanillin reacts with phosphoric acid to produce an aromatic phosphate ester. In the last step, the carbonium ions from unsaturated lipids react with the activated carbonyl group of phospho-vanillin to produce a charged colored complex that is stabilized by resonance and absorbs at about 540 nm, which was monitored using a spectramax I3x multimode plate reader.

In-vitro Cell Migration (Wound Healing/Scratch) Assay

To investigate the anti-angiogenic properties of *Tridax procumbens* extract using MCF-7 cells, we performed an *in vitro* cell migration (wound healing/scratch) assay. MCF-7 cells (1 million) were cultured in DMEM with 10% FBS and antibiotics until reaching 80-90% confluence in a 6-well plate. A sterile pipette (10µL) tip was used to create a scratch in the cell monolayer. The cells were treated with 0, 50, 250, and 450 µg/mL concentrations of the partially purified leaf extract (Test) and Enoxaparin Sodium in a serum-free medium and incubated at 37°C with 5% CO_2 [14]. Wound closure was photographed at 0, and 24 hours using a Thermo Evos XL Digital Inverted Microscope. The wound area was analyzed using ImageJ software [15].

Cellular Toxicity

Hemolysis Activity Assay

Fresh human blood was collected into tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA). The collected blood was centrifuged at 500 x g for 15 minutes to separate the plasma from the red blood cells (RBCs). The supernatant was discarded, and the RBC pellet was washed by re-suspending in an equal portion of 1X Dulbecco's phosphate-buffered saline (DPBS). The washing process was repeated three times to ensure the removal of plasma proteins and other cellular debris. The RBC pellet was resuspended in 50 mL of DPBS for a 50-fold dilution. This suspension served as the substrate for evaluating the hemolytic activity of the test compounds.

Partially purified leaf extract of *Tridax procumbens* (test compound) was prepared at varying concentrations (50, 150, 250, 350, and 450 µg/mL) to evaluate their hemolytic potential. A positive control, consisting of 1% SDS, and a negative control, DPBS, were also prepared. In 1.5 mL Eppendorf tubes, 100 µL

of each test compound solution, the positive control, and the negative control were added to designated tubes. Subsequently, 100 µL of RBC suspension was added to each well, ensuring thorough mixing of the components. The tubes were incubated at 37°C for 24 hours to allow interaction between the RBCs and the purified leaf extract fraction (test compound). Following incubation, the tubes were centrifuged again at 500 x g for 15 minutes to pellet the intact RBCs. Care was taken to avoid disturbing the pellet during the subsequent step. From each tube, 100 µL of the supernatant was carefully transferred to a new 96-well plate. The absorbance of the supernatants was measured at 540 nm using a plate reader. The degree of hemolysis was quantified by calculating the percentage of hemolysis using the formula.

$$Hemolysis\ (\%) = \frac{(\text{Absorbance of Sample} - \text{Absorbance of Negative Control})}{(\text{Absorbance of Positive Control} - \text{Absorbance of Negative Control})} \times 100$$

Hemolysis percentages were categorized as follows: 0-5% indicating non-hemolytic activity, 5-10% indicating slight hemolysis, 10-20% indicating moderate hemolysis, and values exceeding 20% indicating strong hemolytic activity. These classifications provided a quantitative measure of the hemolytic potential of the test compounds [16].

Chick Embryo Chorio Allantoic Membrane (CAM) Assay

The Chorio Allantoic Membrane (CAM) assay provided valuable insights into the pro- or anti-angiogenic properties of the tested compounds, contributing to our understanding of their potential therapeutic applications. The CAM assay is a widely utilized *in-vivo* model for studying angiogenesis, tumor growth, and drug testing due to its rich vascular network, not requiring the approval of an institutional animal ethics committee. Fertilized chicken eggs were placed in an incubator set at 37.5°C and 85% humidity. The blood vessels were identified using an egg candler the day before inoculation. The Purified Leaf Extract (PLE) of *Tridax procumbens* (test substance) was prepared in sterile conditions, a small hole was made in the eggshell with a sterile needle. The PLE along with 1 million MCF-7 cells, were delivered in small volumes (100 µL) using a sterile insulin syringe into the CAM layer. After further incubation for 3 days, the CAM layer was placed onto a petri dish upon harvesting, and the neovascularization was quantified by counting the number of blood vessels in the treated area compared to control regions [17].

Cytotoxicity assay (MTT)

The T-47D (ATCC, HTB-133) epithelial cells were cultured in RPMI 1640 medium (Gibco, catalog number: 11875093) supplemented with 10% FBS (Thermofisher, catalog number 26140079), 1% penicillin-streptomycin (Sigma, catalog Number: P4333). The cells were harvested at 70-80% confluence using 0.25% Trypsin-EDTA (Himeida, catalog number: TCL048).

The 70-80% confluent cells grown in the T-25 culture flask (Corning, catalog number 353109) were harvested using 0.25% trypsin-EDTA solution. The cells were centrifuged at 1000xg (R.C.F) using a refrigerated centrifuge (Eppendorf, 5804 R). The cells sedimented were resuspended in 1mL of RPMI 1640 medium and counted using a hemocytometer. About 0.1 million cells (1 x 10-5) containing 100µL RPMI 1640 medium were seeded to 96 well tissue culture plates. The cells were incubated at 37°C with 5% CO_2 for 24 hrs. After 24 hrs, 100 µL of each two-fold serially diluted extract (concentrations ranging from 500 µg/mL to 3.91 µg/mL) in maintenance medium (RPMI 1640 with 1 % FBS) was added to the wells. Blank medium and cell without plant extract treatment were considered as medium control and cell control (positive control) respectively. Similarly, negative control (completely lysed; 100%) cells were taken as a control for cytotoxicity. These controls were also incorporated into the same plates. The PLE was serially diluted at

concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81 and 3.91µg/mL.

The 100 µL of plant extracts of treated cells were further incubated overnight (24±1 hr) at 37 °C in a 5 % CO_2 incubator and the cell viability was evaluated using the MTT colorimetric assay as described by Kumar [18]. Briefly, the cells treated with different concentrations of extracts along with the controls were washed with 100 µL of fresh media. Added 10 µL of the 12 mM MTT stock solution to the media in each well. Incubated the plate for 4 h at 37°C in a 5 % CO_2 and added 100 µL of solubilizing reagent (10% SDS in 0.01 N HCl). Allowed the plate to incubate overnight (16 ±1 h) at 37°C in a 5 % CO_2 and mixed the individual wells of the 96 well plate using a multichannel pipette and monitored absorbance at 570 nm using Spectramax i3x (Molecular Devices). The percent cell viability was calculated using the formula given below.

$$\%\ cell\ viability = \frac{\text{Absorbance at 570nm of the treated cells}}{\text{Absorbance at 570nm of the untreated cells (control)}} \times 100$$

An inhibitory Concentration of 50% (IC50) of plant extracts was determined by 4PL analysis.

Statistical Analysis

Results are presented as mean± standard deviation (SD) for sample size of n=6 or n=3. Student's t-test was used to determine the level of significance ($p<0.05$). The data for precision was represented in % relative standard deviation (%RSD).

Results and Discussion

The aerial parts of the plant and the leaves were independently treated with different extraction methods like solvent extraction, maceration, and steam distillation. Steam distillation and maceration crude extracts did not yield any anticoagulation activity by aPTT (data not shown). However, solvent extraction by Soxhlet extraction using a mixture of acetone and n-hexane solvent (50:50) as organic extract yielded mild anticoagulant activity by aPTT (data not shown). The sulfated polysaccharides isolated by Naqash and Nazeer showed prolonged anticoagulant activity for the time of 113 seconds by aPTT which was four times the control sample [19].

The crude leaf extracts upon purification by Ann ion exchange chromatograph showed anticoagulant activity. The fraction was considered as a partially purified leaf extract (PLE). The fraction was investigated for purity and identification by GCMS. The chromatogram is depicted in Figure 1, A major peak at around 5.935 minutes having an abundant mass of 268 Da was observed. The major identified moiety was Azulene, a derivative of naphthalene. This fraction showed positive anticoagulant activity. The partially purified fraction was assessed further for the presence of an impurity, angiogenetic ability, and cellular toxicity.

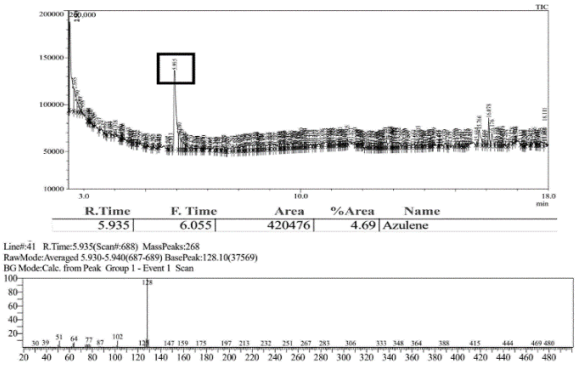

Figure 1. GC-MS Chromatogram of the Partially Purified Leaf extract (PLE). Where, the Chromatogram Represents a Major Peak, Identified as Azulene at the RT of 5.935 Seconds

The protein content of the partially purified leaf extract was 0.24 ± 0.016% (w/w) for an average of six preparations with a %RSD of 7.3 (Table 1). The %RSD serves as a measure of precision, illustrating the consistency or variability of replicate measurements within each preparation and these values remained within an acceptable range for precision. The baseline content found in the purified fraction is less than 1% for protein in the purified extract (Figure 2).

Table 1. Protein Content by Lowry Method in Purified Leaf extract

Leaf Extract Preparations	**Protein Content (%w/w)**	**% RSD within Experimental result**	**Remarks**
Preparation -I	0.25	8	Pass
Preparation -II	0.23	3	Pass
Preparation -III	0.22	3	Pass
Preparation -IV	0.26	4	Pass
Preparation -V	0.24	5	Pass
Preparation -VI	0.26	3	Pass
System Suitability	100	3	Pass
Average	**0.24**		
Standard Deviation	**0.016**		
Overall %RSD	**7.3**		

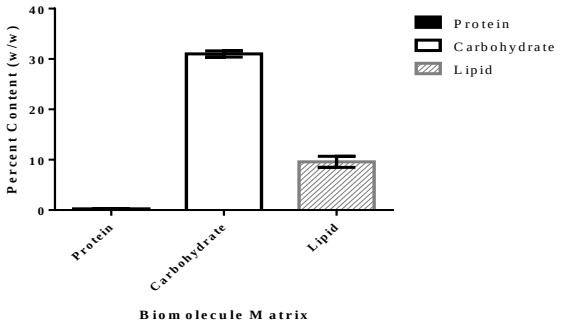

Figure 2. Percent Composition of Bioactive Material in Purified Leaf extract. Where the X-axis Denotes the Major Biomolecules (Protein, Carbohydrate, and Lipid) and the Y-axis denotes the Percent Composition in (w/w). The Error Bars in the Graph are the Representation of the Standard Deviation

The total carbohydrate content of the partially purified leaf extract was 31 ± 0.632 % (w/w) for the average of six preparations with a precession (%RSD) of 1.9 (Table 2). The prominent presence of carbohydrates in the purified fractions suggests the anticoagulant activity could be from the carbohydrate elements (Figure 2).

Table 2. Carbohydrate Content by Anthrone Method in Purified Leaf extract

Leaf Extract Preparations	**% Carbohydrate (w/w)**	**% RSD within Experimental result**	**Remarks**
Preparation -I	31	3	Pass
Preparation -II	32	1	Pass
Preparation -III	30	1	Pass
Preparation -IV	31	1	Pass
Preparation -V	31	3	Pass

Preparation -VI	31	1	Pass
Average	**31**		
Standard Deviation	**0.632**		
Overall %RSD	**1.9**		

The total lipid content of the partially purified leaf extract was 9.58 ± 1.113 % (w/w) for the average of six preparations with a %RSD of 11.6 (Table 3).

Table 3. Lipid Content by Phospho-vanillin Method in Purified Leaf extract

Leaf Extract Preparations	**% Lipid content (w/w)**	**% RSD within Experimental result**	**Remarks**
Preparation -I	9.6	3	Pass
Preparation -II	11.5	5	Pass
Preparation -III	8.2	3	Pass
Preparation -IV	9.4	2	Pass
Preparation -V	8.9	2	Pass
Preparation -VI	9.9	5	Pass
System Suitability	**98**	**2**	**25 µg out of 24 µg/mL (Pass)**
Average	9.58		
Standard Deviation	1.113		
Overall %RSD	11.6		

This suggests that the purified fraction has a presence of lipid elements in the purified leaf extracts (Figure 2).

The assessment of the cell migration was performed on the partially purified fractions of the *Tridax procumbens* showed a dose-dependent cell migration (Figure 3). At 24 hours, the percentages of wound closure for 450, 250, 50 and 0 (control) µg/mL for PLE (IA to I D) were 95%, 80%, 60%, and 45%, respectively. The control used for the assay was Enoxaparin sodium, which showed (II A to II D) the percentages of 95%, 75%, 70%, and 50%, respectively. This data indicates that higher concentrations of the extract resulted in greater cell migration of MCF-7. In the control group, MCF-7 cells exhibited significant migration of 50% and assisted in wound closure within 24 hours (Figure 4).

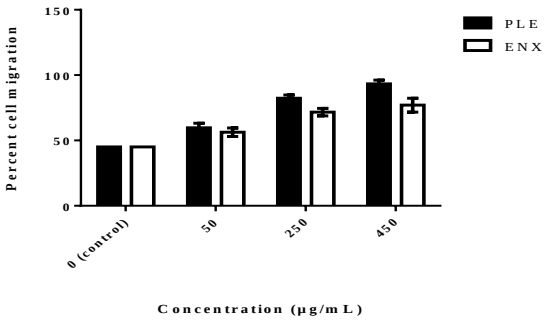

Figure 3. The Percent Cell Migration of Enoxaparin Sodium (ENX) and partially Purified Leaf extract (PLE). Where the X-axis denotes the concentration of Enoxaparin Sodium (ENX) and partially Purified Leaf (PLE) in µg/mL. The Y-axis denotes the percent cell migration. The error bars in the graph are the representation of the Standard Deviation

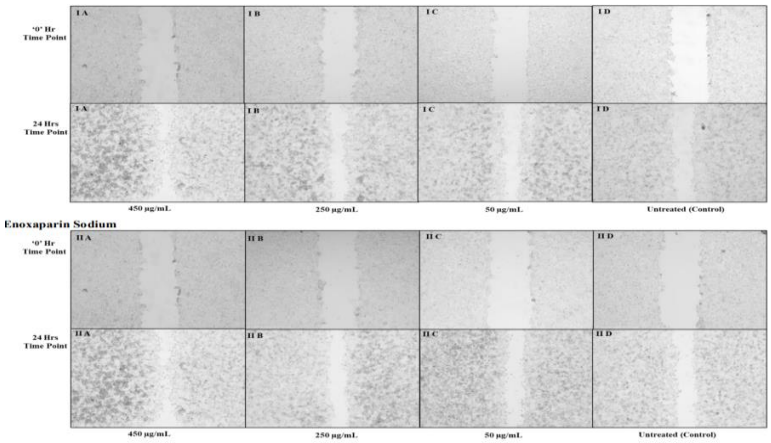

Figure 4. Pictogram of the Percent Migration in Partially Purified Leaf extract (PLE) of Tridax Procumbens and Enoxaparin Sodium. The panel for Tridax Procumbence and Enoxaparin sodium represents the control of '0' and migration at 24-hour timepoints in MCF-7 cells where IA to ID represents the concentrations of PLE and Enoxaparin sodium, at concentrations of 450 µg/mL, 250 µg/mL, 50 µg/mL and untreated control respectively.

Tridax procumbens extract has demonstrated significant cell migration enhancement in MCF-7 cell lines, suggesting its potential in a wound-healing treatment. The active compounds within the extract are likely influence cell movement and proliferation. However, further studies are necessary to elucidate the specific mechanisms and determine optimal therapeutic dosages. Additionally, scratch assays (wound healing assays), may promote cell migration and proliferation, accelerating wound closure. This effect could be attributed to extract modulation of signaling pathways favoring cell movement and growth, including matrix metalloproteinases (MMPs) and growth factor signaling [20].

The cellular toxicity of the partially purified fractions was assessed by the hemolytic assay, CAM assay, and MTT assay. The hemolytic activity of the PLE was assessed by measuring the absorbance of the supernatants at 540 nm. The percent hemolysis was calculated based on the relative hemolysis of positive and negative controls. The 1% SDS considered as positive control exhibited complete hemolysis, serving as a benchmark for maximum hemolytic activity. The DPBS showed negligible hemolysis was considered as a negative control, confirming the integrity of the assay. The PLE fractions showed low hemolytic activity, with hemolysis percentages ranging from 0 % to <10 % across all tested concentrations, classifying it as non-toxic and non-hemolytic (Figures 5 and 6, Table 4).

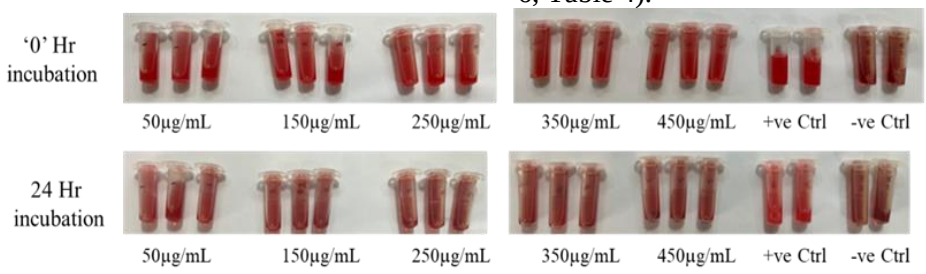

Figure 5. Hemolytic activity of the Partially Purified Leaf Extract (PLE) at a Time Interval of 24 hours. The Top and Bottom Panels in the Figure represent the Time Points of 0 and 24 hours respectively. The Positive and Negative Control used was 1% SDS and Dulbecco's Phosphate-buffered Saline (DPBS)

Table 4. Percent Hemolysis of the Partially Purified Leaf Extract at Different Concentrations using *In-vitro* Hemolysis Assay

S. No	Extract Concentration (µg/mL)	% Hemolysis			Average	Std Dev
		Rep-1	Rep-2	Rep-3		
1	50	6.773	9.727	8.097	8.199	1.480
2	150	6.650	7.414	9.124	7.729	1.267
3	250	6.773	6.880	6.163	6.606	0.387
4	350	7.882	8.007	9.909	8.599	1.136
5	450	12.438	11.210	10.997	11.548	0.778

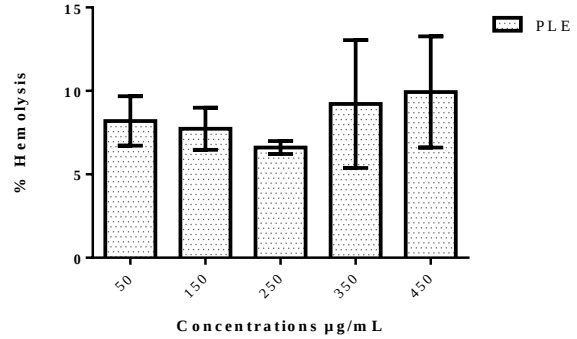

Figure 6. Hemolytic activity of the Partially Purified Leaf Extract (PLE). Where the X-axis denotes the Concentration of Partially Purified Leaf (PLE) in µg/mL. The Y-axis denotes the Percent Hemolysis. The Error Bars in the Graph are the representation of the Standard Deviation

The in vitro hemolysis assay is a crucial preliminary test for evaluating the safety of compounds intended for intravenous administration. Our results demonstrate minimal degrees of hemolytic activity among the tested concentrations, highlighting the importance of this assay in preclinical safety evaluation. Low hemolytic activity suggests a favorable safety profile for intravenous administration, as it remained within the non-hemolytic range at all tested concentrations. This indicates minimal disruption to RBC membranes, which is a desirable property for therapeutic agents [16].

The Chorio Allantoic Membrane (CAM) assay was used to evaluate the pro or antiangiogenic properties. Changes in blood vessel formation on the CAMs were observed and quantified post-incubation.

Basic fibroblast growth factor, bFGF (100 ng/mL), a known angiogenic agent was considered as a positive control which resulted in a significant increase in blood vessel density, thereby validating the assay by demonstrating a robust angiogenic response. In contrast, the negative control, phosphate buffer saline (PBS) did not show any significant changes in blood vessel formation, thus establishing the baseline level of vascularization inherent to the CAM. Another study found that intervention significantly reduced the growth of new blood vessels in response to Doxycycline treatment [21, 22]. The partially purified test fraction demonstrated various effects on cellular and molecular processes. In the context of the experiment, increasing concentrations of enoxaparin decreased blood vessel formation in this assay, showing a decrease compared to the control (MCF 7 cells only), indicating a dose-dependent anti-angiogenic effect (Figure 7). The CAM assay demonstrated that *T. procumbens* leaf extract, decrease in blood vessel formation with increasing concentrations in the CAM assay is likely due to its anti-

angiogenic properties, including the inhibition of growth factors, disruption of endothelial cell functions, and alteration of the extracellular matrix. Simultaneously, might promote efficient cell migration and proliferation, facilitating wound healing. These differential effects highlight the complex and context-dependent actions of *Tridax procumbens* on various cellular processes.

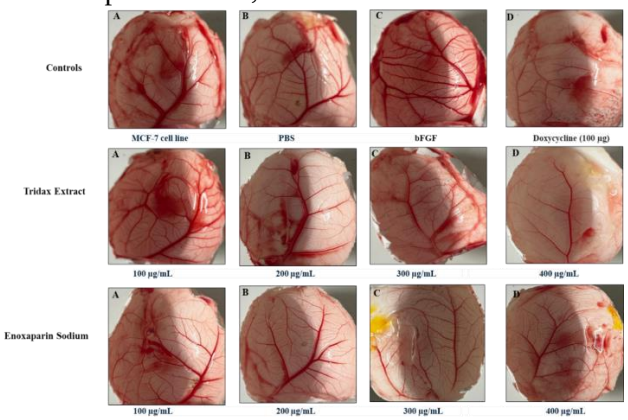

Figure 7. Evaluation of Angiogenic Properties by Chorio Allantoic Membrane (CAM) Assay

Panel for Controls: Represents (A) Treated only with HeLa cells, (B) treated with Phosphate buffer saline, (C) Treated with Basic fibroblast growth factor (D) Exposed to 100 µg/ml doxorubicin. Panel for *Tridax procumbence* leaf extract: A to D represents treatment at concentrations of 100, 200, 300, and 400 µg/mL for partially purified leaf extract. Panel for Enoxaparin sodium treatment: A to D represents treatment at concentrations of 100, 200, 300, and 400 µg/mL for Enoxaparin sodium.

The purified leaf extract exhibited no significant cellular toxicity on T47D cells at the concentrations range of 4 -500 µg/mL. The percent cell viability at a concentration range of 4-125 µg/mL and 250-500 µg/mL slightly decreased but stayed above 95% and 90% respectively (refer to Figure 8 & Table 5). The percent viability of the cells was comparable to the negative control. It is evident from the results that the partially purified extract can be safely used in potential therapeutic applications within the concentration range. The CC_{50} (cytotoxic concentration) assessed by Naqash and Nazeer was reported to be around 200 µg/mL for the sulphated residue isolated (19). Several other investigators have reported that plant extracts are cytotoxic [23].

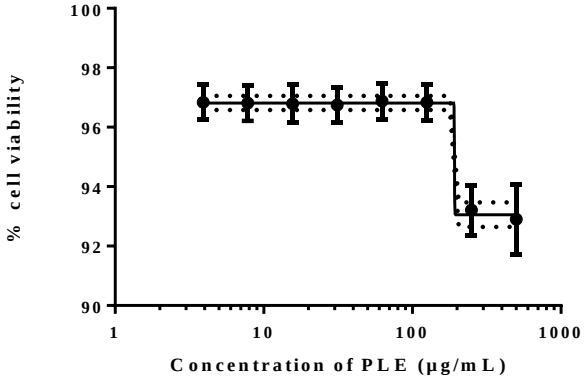

Figure 8. Cytotoxicity Assessment of the Purified Leaf extract on T47D cells by MTT Assay. Where the X-axis Denotes the Concentration of Partially Purified Leaf (PLE) in µg/mL. The Y-axis denotes the Percent Cell Viability. The Error Bars in the Graph are the representation of the Standard Deviation

Table 5. CC_{50} Determination for Assessment of Cellular Toxicity in T47D Cells for Purified Leaf extract

S. No	Conc (µg/mL)	Preparation						Result		
		1	2	3	4	5	6	Average	Std Dev	%RSD
1	500	93.84	93.69	90.93	92.04	93.78	93.15	92.91	1.180	1.3
2	250	93.99	93.71	91.68	93.07	93.73	93.08	93.21	0.837	0.9
3	125	97.05	97.19	95.76	97.35	97.15	96.5	96.83	0.601	0.6
4	62.5	97.26	97.16	95.81	97.39	97.17	96.47	96.88	0.613	0.6
5	31.25	97.12	97.15	95.79	96.9	97.25	96.26	96.75	0.588	0.6
6	15.625	97.41	96.75	95.78	97.27	97.21	96.33	96.79	0.636	0.7
7	7.8125	97.41	96.88	95.8	97.16	97.19	96.43	96.81	0.599	0.6
8	3.90625	97.39	97.07	95.79	97.18	97.1	96.49	96.84	0.594	0.6

Summary and Conclusion

This study was aimed to evaluate the safety and efficacy of the bioactive compounds isolated and purified from leaf extracts of *Tridax procumbens*. The fraction that showed the potential anticoagulant properties. It was assessed for angiogenic properties and cellular toxicity. The overall proportion of fraction was also quantified for total protein, carbohydrate and lipid.

The cell migration assay showed a dose dependency of 450 to 50 µg/mL with a migration of 95 to 60 % respectively suggesting that purified extract assisted in the wound healing ability. The hemolytic assay only showed 0-10% hemolytic activity and 90% of the cells were viable. The low hemolytic activity suggests a favourable safety profile. The anti-angiogenic activity of the purified extract was confirmed by the CAM assay, wherein no significant proliferative formation of blood vessels was seen at concentrations of 100-400 µg/mL. The MTT assay results indicated no significant cytotoxicity in T47D cells at concentrations ranging from 4 to 500 µg/mL. Cell viability remained high, suggesting that the extract is safe for further therapeutic exploration. The partially purified extract, rich in carbohydrates, shows potential as a safe and effective anticoagulant. These findings support further investigation and development of *Tridax procumbens* extracts as a natural anticoagulant agent. Future research is warranted in characterisation of specific bioactive compounds responsible for the anticoagulant activity and *in-vivo* studies are required to confirm the therapeutic potential of the purified fraction. The safety of the extracts purified shall be further investigated in the animal models.

Conflicts of Interest

Authors declare no conflicts of interest.

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References

[1]. Bhat, R. B., Etejere, E. O., Oladipo, V. T., 1990, Ethnobotanical studies from central Nigeria. *Economic Botany, 44*(3), 382-390.

[2]. Diwan, P. V., Tilloo, L. D., & Kulkarni, D. R., 1982, Influence of Tridax procumbens on wound healing. *Indian J Med Res, 75*, 460-464.

[3]. Jachak, S. M., Gautam, R., Selvam, C., Madhan, H., Srivastava, A., & Khan, T., 2011, Anti-inflammatory, cyclooxygenase inhibitory and antioxidant activities of standardized extracts of Tridax procumbens L. *Fitoterapia, 82*(2), 173-177. doi:10.1016/j.fitote.2010.08.016

[4]. Singh, C. P., Mishra, P. K., & Gupta, S. P., 2016, Design and Formulation of Tridax procumbens based Polyherbal Cream for Wound Healing Potential. *Pharm. Lett., 8*, 15-21

[5]. Taddei, A., & Rosas-Romero, A. J., 2000, Bioactivity studies of extracts from Tridax procumbens. *Phytomedicine, 7*(3), 235-238. doi:10.1016/S0944-7113(00)80009-4

[6]. Tiwari, U., Rastogi, B., Singh, P., Saraf, D. K., & Vyas, S. P., 2004, Immunomodulatory effects of aqueous extract of Tridax procumbens in experimental animals. *J Ethnopharmacol, 92*(1), 113-119. doi:10.1016/j.jep.2004.02.001

[7]. Cushnie, T., & Lamb, A., 2005, Antimicrobial activity of flavonoids *International Journal of Antimicrobial Agents* 26 (5): 343-56.

[8]. Ndebia, E., Kamgang, R., & Nkeh-ChungagAnye, B., 2007, Analgesic and anti-inflammatory properties of aqueous extract from leaves of Solanum torvum (Solanaceae). *African Journal of Traditional, Complementary and Alternative Medicines, 4*(2), 240-244.

[9]. Ramachandran, V., Kumar, G. V., Sugumar, S., Sundaram, V., & Ahamed, H. N., 2020, Cardioprotective effect of Tridax procumbens Linn in Isoproterenol Induced Myocardial Infarction in rats. *Research Journal of Pharmacy and Technology, 13*(4), 1923-1927.

[10]. Thalkari, A. B., Karwa, P. N., Shinde, P. S., Gawli, C. S., & Chopane, P. S., 2020, Pharmacological actions of Tridax procumbens L.: a scientific review. *Research Journal of Pharmacognosy and Phytochemistry, 12*(1), 27-30.

[11]. Balkrishna, A., Sharma, N., Srivastava, D., Kukreti, A., Srivastava, S., & Arya, V., 2024, Exploring the Safety, Efficacy, and Bioactivity of Herbal Medicines: Bridging Traditional Wisdom and Modern Science in Healthcare. *Future Integrative Medicine, 3*(1), 35-49.

[12]. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J., 1951, Protein measurement with the Folin phenol reagent. *J Biol Chem, 193*(1), 265-275.

[13]. Morse, E. E., 1947, Anthrone in Estimating Low Concentrations of Sucrose. *Analytical Chemistry, 19*(12), 1012-1013. doi:10.1021/ac60012a021.

[14]. Pasha, A., Kumbhakar, D. V., et al., 2022, Role of Biosynthesized Ag-NPs Using Aspergillus niger (MK503444.1) in Antimicrobial, Anti-Cancer and Anti-Angiogenic Activities. *Front. Pharmacol.,* 12 :812474.doi: 10.3389/fphar.2021.812474

[15]. Rodriguez, L. G., Wu, X., & Guan, J. L., 2005, Wound-healing assay. *Methods Mol Biol, 294*, 23-29. doi:10.1385/1-59259-860-9:023

[16]. Rajkumar, K., Mvs, S., Koganti, S., & Burgula, S., 2020, Selenium nanoparticles synthesized using Pseudomonas stutzeri (MH191156) show antiproliferative and anti-angiogenic activity against cervical cancer cells. *International journal of nanomedicine*, 4523-4540.

[17]. Ribatti, D., Vacca, A., Roncali, L., & Dammacco, F., 1996, The chick embryo chorioallantoic membrane as a model for in vivo research on angiogenesis. *The International journal of developmental biology, 40*(6), 1189-1197

[18]. Kumar, P., Nagarajan, A., & Uchil, P. D., 2018, Analysis of Cell Viability by the MTT Assay. *Cold Spring Harb Protoc, 2018*(6). doi:10.1101/pdb.prot095505

[19]. Naqash, S. Y., & Nazeer, R. A., 2011, Anticoagulant, antiherpetic and antibacterial activities of sulphated polysaccharide from Indian medicinal plant Tridax procumbens L. (Asteraceae). *Appl Biochem Biotechnol, 165*(3-4), 902-912. doi:10.1007/s12010-011-9307-y

[20]. Grada, A., Otero-Vinas, M., Prieto-Castrillo, F., Obagi, Z., & Falanga, V., 2017, Research Techniques Made Simple: Analysis of Collective

Cell Migration Using the Wound Healing Assay. *J Invest Dermatol, 137*(2), e11-e16. doi:10.1016/j.jid.2016.11.020

[21]. Ausprunk, D. H., Knighton, D. R., & Folkman, J., 1974, Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. *Developmental biology, 38*(2), 237-248.

[22]. Manjamalai, A., Kumar, M., & Grace, V., 2012, Essential oil of Tridax procumbens L induces apoptosis and suppresses angiogenesis and lung metastasis of the B16F-10 cell line in C57BL/6 mice. *Asian Pacific Journal of Cancer Prevention, 13*(11), 5887-5895.

[23]. Burgos-Pino, J., Gual-Orozco, B., Vera-Ku, M., Loria-Cervera, E. N., Guillermo-Cordero, L., Martinez-Vega, P. P., Gamboa-Leon, R., 2023, Acute oral toxicity in BALB/c mice of Tridax procumbens and Allium sativum extracts and (3S)-16,17-didehydrofalcarinol. *J Ethnopharmacol, 301*, 115840. doi:10.1016/j.jep.2022.115840