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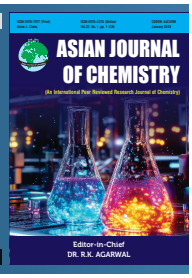

Phytochemical Screening, GCMS-LCMS Analysis of Bioactive Compounds from Methanolic Extract of *Eichhornia crassipes*: *In vitro* Cytotoxic Effect against MCF7 Cell Line

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Eichhornia crassipes, commonly referred to as water hyacinth, is an invasive aquatic plant that has not undergone comprehensive research about its possible medicinal properties. The present study aims to utilize its rapidly growing potential for medical purposes. The leaves of *Eichhornia crassipes* were extracted in a sequential manner using hexane, methanol and water using Soxhlet apparatus. The preliminary phytochemical analysis has confirmed the presence of bioactive phytochemicals in the methanolic extract of *E. crassipes* leaves. The methanolic extract showed different types of high and low molecular weight compounds by GCMS-LCMS analysis. The GC-HRMS analysis revealed the presence of *n*-hexadecanoic acid, stigmasta-3,5-diene and vitamin E. The HR-LCMS analysis identified the presence of khivorin, cymarin and salicin using positive electrospray ionization (ESI) mode and rhoifolin and digitoxin using negative ESI mode. The isolated and identified compounds in the crude extract exhibit following bioactivities like antimicrobial, antidiabetic, hypocholesterolemic, antioxidant, anti-inflammatory and cytotoxic effects. The extract exhibited cytotoxic effect in the experiments against MCF7 breast cancer cell line, resulting in a reduction in cell viability to 48.29 ± 1.39% with an IC_{50} value of 770.90 µg/mL. Further, studies on DNA ladder assay demonstrated that the cytotoxic effect of extract is due to DNA fragmentation and alteration of DNA properties.

Keywords: *Eichhornia crassipes*, Phytochemical analysis, GCMS-LCMS, Bioactive compounds, DNA ladder assay.

INTRODUCTION

The use of herbal drugs in treatment of diseases is found among all sections of people in India. *Eichhornia crassipes*, commonly referred to as water hyacinth, is an aquatic plant that flourishes in warm aquatic environments. The plant is indigenous to the Amazonian basin in Brazil and belongs to the family Pontederiaceae. The plant has a wide distribution in multiple countries, including Africa, Asia, North America, Canada and other locations. This weed is classified as one of the top 10 most invasive and hazardous plants in the world because to its rapid spread and detrimental effects on aquatic environments [1]. The primary emphasis in agriculture is the usage of water hyacinth as a fertilizer, animal feed and as a medical treatment for wounds in cattle [2]. Various indigenous groups frequently employ this plant for the production of ropes, baskets and other forms of accessories. According to studies, people in specific regions of Thailand incorporate this aquatic plant into their diet [3]. *E. crassipes* serves as a reservoir of phytochemicals, which possess the potential to be utilized as therapeutic substances. A variety of phytochemicals, including flavonoids, alkaloids, saponins, sterols, phenolic compounds, terpenoids, phenalene derivatives, carbohydrates, organic acids, quinones and anthraquinones, have been recorded in the literature [4,5].

Incidences of cancers are rising world-over, getting closer to becoming the latest developing countries epidemic. A good number of cancer cases (~16%) are infection-caused and the infections that lead to cancers are reported to be more prevalent in developing countries [6]. In this regard, there is need to keep on the fight against cancers through rational drug discovery and development from more available and cheaper resources such as plants. The plant *E. crassipes* is reported to have various health benefits including nutrients, mineral metals, antimicrbial activities, antioxidant activities, as well as cytotoxic activities [7]. Although the plant is known in traditional medicine for its medicinal properties, there is a lack of scientific research that has thoroughly investigated its purported therapeutic effects.

The present work seeks to investigate the qualitative and quantitative composition of phytoconstituents in *E. crassipes* leaf extracts using the Soxhlet extraction method. This study is motivated by the necessity to optimize the usage of waste resources. Furthermore, the study aims to evaluate the biological properties and examine the chemical constituents of the raw methanolic extract utilizing gas chromatograph with a high-resolution mass spectrometer (GC-HRMS) and high-resolution liquid chromatograph mass spectrometer (HR-LCMS). The results of this study may uncover innovative uses of this plant and previous research has examined the safety assessment of this plant in albino rats [8]. Further the cytotoxic effect of the methanolic extract was assessed by anti-proliferation (MTT) assay for MCF7 breast cancer cell line and the genotoxic effects by studying genome DNA integrity and properties by DNA ladder assay and UV-Vis spectrophotometric method.

EXPERIMENTAL

Plant material collection: The leaves of *E. crassipes* were gathered from the vicinity of the Tungabhadra river in Kampli (15.407ºN 76.61ºE), India. An herbarium specimen of the same plant, with voucher number 89/NPH/BSH/KLETech, was stored at Department of Botany, PC Jabin Science College, Hubballi, India. The plant sample was verified and confirmed to be *E. crassipes* (Mart.) Solms. The leaves were promptly and meticulously rinsed with water to eliminate any dust and other substances that were sticking to them. Then, the material was dried in open air and then subjected to oven drying at 40 ºC for 2 days. The leaves were pulverized into a coarse powder to enhance the surface area using a mixer grinder and were then stored at 4 ºC.

Preparation of plant extracts: The powdered leaf material was extracted using a Soxhlet extractor. A quantity of 40 g of leaf powder was measured and mixed with 400 mL of solvent. The solvent extraction process was conducted in a sequential manner employing hexane, methanol and aqueous solvents in sequence of increasing polarity. This approach was employed to enhance the extraction of a diverse variety of phytoconstituents. The extraction process involved adjusting the temperature of the extractor to a level just below the boiling temperatures of the solvents. The three samples were gathered and the solvents were subsequently removed through evaporation using a rotary evaporator. The resulting solutions were then stored in Amber-coloured bottles in a refrigerator at 4 ºC until they could be further analyzed.

Qualitative analysis of phytochemicals: A preliminary qualitative phytochemical study was conducted to verify the existence of several phytochemicals in the leaf extracts. The hexane, methanol and aqueous extracts were examined for the presence of phytochemicals using the established techniques [9]. The additional material contains a comprehensive explanation of the procedures used. The methanol and aqueous extracts exhibited a greater abundance of phytochemicals in comparison to the hexane extract. The methanol extracts exhibited a greater concentration of phytochemicals (as shown by colour intensity) compared to the aqueous extracts. Methanol is frequently employed as an extraction solvent due to its significant polarity, which results in substantial extraction yields [10,11]. Consequently, we chose to only focus on the methanol extract of *E. crassipes* leaves for subsequent investigations.

Quantitative analysis of phytochemicals in methanolic extract: Spectrophotometric techniques were employed to measure the concentration of phytochemicals in the methanolic extract.

Estimation of total phenolic content: The Folin-Ciocalteau colorimetric technique was used to measure the total phenol concentration of the methanolic extract [12]. The standard utilized was gallic acid and the results were represented as milligrammes of gallic acid equivalent (GAE) per gramme of extract. Various quantities (ranging from 0.02-0.10 mg/mL) of gallic acid were produced in distilled water for the purpose of creating a standard graph. In test tubes, 0.5 mL of plant extract and various concentrations of the standard solution were combined with 2 mL of Folin-Ciocalteu reagent (diluted 1:10 in deionized water). Then, 4 mL of saturated solution of sodium carbonate (7.5% w/v) was added. The test tubes were sufficiently sealed and incubated for 30 min at room temperature with periodic agitation. The emergence of blue colour was detected and quantified using spectrophotometric analysis utilizing a UV-Visible spectrophotometer. The measurement of absorbance was performed at a wavelength of 765 nm.

Estimation of total flavonoid content: The quantification of total flavonoid concentration was conducted using the $AlCl_3$ colorimetric test method, as described earlier [13]. $AlCl_3$ undergoes a chemical reaction with the keto groups and hydroxyl groups present in flavonoids, resulting in the formation of complexes that are resistant to acid and exhibit an orange colour [14]. The standard utilized in the study was quercetin and the results were reported as milligrammes of quercetin equivalent (QE) per gramme of extract. Concentrations ranging from 0.02-0.1 mg/mL of quercetin were generated in distilled water for the standard graph. To conduct the analysis, 0.5 mL portions of the plant extract and various concentrations of the standard solution were placed in separate test tubes. Then, 2 mL of distilled water was added. Afterwards, 0.15 mL of a sodium nitrite solution containing 5% $NaNO_2$ by weight was added and mixed. After a duration of 6 min, 0.15 mL of a solution containing 10% $AlCl_3$ (wt./vol.) was introduced and allowed to incubate for another 6 min. Subsequently, 2 mL of a solution containing 4% NaOH (wt./vol.) was added to the reaction mixture. The ultimate volume was modified to 5 mL using distilled water, well mixed and then incubated for 15 min. The spectrophotometric measurement was used to quantify the produced orange colour. The measurement of the absorbance of each mixture was conducted at a wavelength of 510 nm. Blank measurements were conducted using appropriate solvents.

Estimation of total alkaloid content: The total alkaloid content of extract was measured using the bromocresol green solution (BCG) method [9,15]. The total alkaloids were determined by reacting the alkaloids present in the plant extract with bromocresol green dye. A yellow-colour compound with absorption peaks at 470 nm was synthesized. The compound might be extracted using chloroform [16].

A 10^{-4} M BCG solution was developed by dissolving 6.98 mg of bromocresol green in a mixture of 0.3 mL of 2 N NaOH and 0.5 mL of distilled water. The resulting solution was then diluted to a final volume of 100 mL with distilled water. A solution of sodium phosphate (2 M) was utilized to develop a phosphate buffer solution with a pH of 4.7. The pH was adjusted by adding 0.2 M citric acid. Caffeine was employed as the benchmark and the outcomes were quantified as caffeine equivalent (CE) per gramme of extract. Various amounts (0.02-0.10 mg/mL) of caffeine were prepared in distilled water for the purpose of creating a standard graph. The analysis was conducted by combining 1 mL of plant extract with varying doses of the standard solution in separate test tubes. Then, 5 mL of phosphate buffer (pH 4.7) and 5 mL of BCG solution were added. Finally, 4 mL of chloroform was added and then the mixture was incubated for 5 min and subsequently left undisturbed at the ambient temperature. The yellowish precipitate generated was cautiously collected and the absorbance was measured at 470 nm relative to a blank sample.

Estimation of total carbohydrate content: Carbohydrates are the fundamental metabolites of utmost significance. The phenol-sulphuric acid method was used to assess the total carbohydrate content in the methanolic extract, with glucose serving as standard [17]. The results were quantified in terms of milligrammes of glucose equivalent (GE) per gramme of extract. A range of glucose concentrations (0.02-0.10 mg/mL) were generated in distilled water in order to develop the standard graph. For analysis, 1 mL of the plant extracts and various concentrations of the standard solution were used. A total of 1 mL of 5% solution of phenol and 5 mL of conc. H_2SO_4 were mixed and allowed to incubate for approximately 10 min. The spectrophotometric measurement was used to quantify the development of the reddish-orange colour. The optical density of each mixture was measured at a wavelength of 490 nm. Respective solvents were used for blank measurements.

Evaluation of biological activities: An assessment of the biological activity was conducted on the unrefined methanolic extract derived from the leaves. The crude extract was assessed for its antibacterial, antifungal, antidiabetic, antioxidant, anti-inflammatory, hypocholesterolemic and cytotoxic properties.

Antimicrobial susceptibility assays: The antimicrobial efficacy of methanol extract of *E. crassipes* leaves was evaluated against specific strains of Gram-positive bacteria, Gram-negative bacteria and yeast. The selected quality control organisms are *Escherichia coli* MTCC 723, *Pseudomonas aeruginosa* MTCC 424, *Salmonella typhi* MTCC 3224, *Bacillus subtilis* MTCC 441, *Bacillus cereus* MTCC 430 and *Staphylococcus aureus* MTCC 3160. The yeast culture used was *Candida albicans* MTCC 3958. In addition, the extract was used to assess the minimum inhibitory concentrations (MIC) on specific bacterial and fungal strains.

Agar-well diffusion method: To conduct initial screening, bacterial cultures were introduced and placed in nutrient broth at 37 ºC for a period of time, typically overnight, in order to achieve the necessary level of cloudiness. A volume of 100 µL from the aforementioned bacterial solution was applied onto nutritional agar and distributed evenly using sterile cotton swabs. The agar plates were prepared with wells and the diameter of the wells was measured. The wells were filled with 100 µL of methanol extract of *E. crassipes* and the plates were kept at 37 ºC for 24 h. In the negative control, the only difference in the protocol was the substitution of 100 µL of methanol for the plant extract. The measurements of the widths of the zones of inhibition were measured around the well in millimetres [7]. Ciprofloxacin and nystatin are used as positive controls for antibacterial and antifungall studies respectively.

Determination of minimum inhibitory concentration (MIC): The minimum inhibitory concentration (MIC) is the lowest concentration of a plant extract that prevents the development of microorganisms in a liquid medium. This can be determined by observing the absence of turbidity, which indicates that the broth is not contaminated [7]. The minimum inhibitory concentration (MIC) of the methanolic extract was assessed on certain bacteria and fungus identified during the initial screening phase, employing the microdilution method.

Determination of MIC for bacteria: The minimum inhibitory concentration (MIC) of methanolic extract was obtained by testing its effects on both Gram-positive and Gram-negative bacterial strains, specifically *S. aureus* MTCC 3160 and *E. coli* MTCC 723. A series of concentrations ranging from 156.25-5000 µg/mL was synthesized in DMSO solvent. The bacterial solution, containing 4×10^7 CFU/mL, was prepared by diluting the original culture with Muller Hinton broth. Ciprofloxacin served as the reference standard and the plant extracts were included at the aforementioned concentrations. The bacterial solution, with a concentration of 4×10^7 CFU/mL, was thoroughly mixed. This suspension was then added to each well of the microtiter plate, resulting in a total volume of 200 µL for both the compounds and the media. The microtitre plate was placed in an incubator at 37 ºC for 24 h. During this time, the growth of bacteria was examined. The concentration of the plant extract in the vial, which had the same level of cloudiness as the broth without any added organisms, was determined to be the minimum inhibitory concentration (MIC) for that particular organism.

Determination of MIC for yeast: The microdilution method was used to determine the minimum inhibitory concentration (MIC) of the methanolic extract on *Candida albicans* MTCC 3958. The concentration range of 156.25-5000 µg/mL was evaluated again. The yeast culture was cultivated using Czapek Dox broth. Each well was supplemented with the culture solution to achieve a final volume of 200 µL. The plates were cultured for 96 h at ambient temperature. Amphotericin served as a conventional antifungal medication.

Antidiabetic activity: The α-amylase inhibitory assay method was used to assess the *in vitro* antidiabetic activity of *E. crassipes* [18]. The substrate employed was a 0.5% (w/v) solution of starch and the samples were prepared by boiling starch in deionized water for 15 min. The enzyme preparation was achieved by dissolving 1 mg of α-amylase in a 20 mM phosphate buffer solution (100 mL, pH 6.9). Various concentrations ranging from 2-10 mg/mL of plant solutions were prepared in methanol. The DNS reagent was employed to terminate the reaction. The experiment was conducted in the following

manner: 1 mL of plant extract at various concentrations and 1 mL of α-amylase enzyme solutions were mixed in a test tube and incubated at 25 ºC for 30 min. A 1 mL of 0.5% starch solution was added to the above mixture. The mixture was then kept at 25 ºC for 3 min. Subsequently, 1 mL of DNS solution was introduced to halt the reaction and the tubes were subjected to a 15 min heating process in a boiling water bath. Following the chilling process, 9 mL of distilled water was introduced to the reaction mixture in order to dilute it and then vortexed. The measurement of absorbance was conducted at a wavelength of 540 nm using a UV-Vis spectrophotometer. When it comes to blank, there is a complete absence of any reaction. Therefore, the DNS solution was introduced before to the starch solution and the subsequent steps were identical to those carried out in the experiment. A negative control was prepared by substituting 1 mL of methanol in the reaction mixture instead of the plant extract, while maintaining the identical procedural steps. Acarbose, a drug used to treat diabetes, was employed as a reference standard with a concentration range of 0.00156-0.10 mg/mL. The % inhibition was determined using the following formula:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100 \quad (1)$$

where A_c = absorbance of control; A_s = absorbance of sample.

Anti-inflammatory activity: The anti-inflammatory activity of the leaf extract of *E. crassipes* were investigated using the Bovine serum albumin (BSA) denaturation method [19]. The reaction mixture consisting 2 mL of test plant extracts with varying concentrations (ranging from 0.4-2 mg/mL) prepared in methanol, 0.2 mL of 1% aqueous solution of bovine albumin fraction and 2.8 mL of phosphate buffer saline at pH 6.4. The reaction mixture was initially incubated at 37 ºC for 20 min, followed by a further incubation at 60 ºC for 20 min. Finally, all the samples were cooled to the ambient room temperature. Additional absorbance was quantified at a wavelength of 660 nm using a UV-Vis spectrophotometer. For blank samples, BSA solution was added at the conclusion of the experiment, while the rest of the method was carried out as previously described. In order to serve as a negative control, the plant extract was substituted with distilled water. Ibuprofen, a non-steroidal anti-inflammatory medicine, was employed as a positive control at the same concentration range (0.4-2 mg/mL). The percentage inhibition of BSA denaturation was determined using eqn. 2:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100 \quad (2)$$

where A_c = absorbance of control; A_s = absorbance of sample.

Antioxidant activity: The DPPH free radical scavenging method was used to investigate the antioxidant activity of the methanol extract of *E. crassipes*. A 0.1 mM DPPH solution was prepared by dissolving 3.94 mg of DPPH in methanol. The reaction mixture comprised 3 mL of DPPH solution and 1 mL of plant extracts at varying concentrations ranging from 200-1000 µg/mL. The solution was vigorously agitated and let to stand at ambient temperature for 30 min. The measurement of absorbance at 517 nm was conducted using a UV-Vis spectrophotometer [20]. The DPPH assay quantifies the ability of the plant extract to donate a hydrogen atom or one electron, hence assessing its antioxidant activity. The reduction of DPPH to diphenyl picryl-hydrazine was determined using a spectrophotometer at a wavelength of 517 nm. In order to establish a negative control, the plant extract was substituted with methanol. Ascorbic acid was employed as a positive control, with a concentration range of 200-1000 µg/mL. The percentage of DPPH scavenging activity was determined using the following formula:

$$\text{DPPH scavenging activity (\%)} = \frac{A_c - A_s}{A_c} \times 100 \quad (3)$$

where A_c = absorbance of control; A_s = absorbance of sample.

Hypocholesterolemic activity: The hypocholesterolemic action of *E. crassipes* leaves extracts was investigated using the *in vitro* lipase enzyme inhibitory activity method, as described by Maqsood *et al.* [21] with minor modifications. The substrate used was *p*-nitrophenyl acetate (*p*-NPA). Under the above reaction conditions, the lipase enzyme catalyzes the hydrolysis of *p*-NPA, resulting in the liberation of *p*-nitrophenol, a coloured compound with an absorbance of 410 nm. Lipase was dissolved in tris-buffer at a concentration of 0.5 mg/mL. The tris-buffer had a pH 8 and a concentration of 50 mM. The substrate *p*-NPA was produced in 2-propanol at a concentration of 3 mM. To perform the analysis, 1 mL of plant extracts with varying concentrations (ranging from 200-1000 µg/mL) were combined with 0.5 mL of lipase solution. The mixture was then incubated for 30 min at 37 ºC. Subsequently, a 1 mL of substrate *p*-NPA was introduced into the solution, followed by an incubation period of 2 h at 37 ºC. The absorbance was measured at a wavelength of 410 nm relative to a blank sample. To establish a negative control, the plant extract was substituted with methanol. Orlistat was employed as a positive control, utilizing the identical concentration range of 200-1000 µg/mL. The percentage of inhibition was determined from eqn. 4:

$$\text{Hypocholesterolemic activity (\%)} = \frac{A_c - A_s}{A_c} \times 100 \quad (4)$$

where A_c = absorbance of control; A_s = absorbance of sample.

Evaluation of cytotoxic effect of plant extract by MTT assay and neutral red uptake (NRU) assay: The methanolic extract of *E. crassipes* leaves was evaluated for its cytotoxic activity against MCF7 breast cancer cell lines. The cell line was cultivated in a low glucose DMEM medium, enriched with 10% foetal calf serum and antibiotic-antimycotic 100X solution. The cells were incubated in a CO_2 incubator at a constant temperature of 37 ºC, with a CO_2 concentration of 5% and humidity of 95%, until the experiment was finished. The cells were placed in a 96-well microplate at a density of around 5×10^3 cells per well. They were then kept in an incubator at 37 ºC with a humidity level of 95% and a CO_2 concentration of 5% for the duration of the night. Subsequently, various quantities (800, 400, 200, 100, 50, 25 µg/mL) of plant extract were administered. The cells were placed in an incubator for an additional 48 h. Next, the cells in the wells were rinsed twice with phosphate buffer solution. Then, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining solution (5 mg/mL in phosphate buffer solution) was added to

the wells. The plate was then placed in an incubator at 37 ºC. After 4 h, a 100 µL of DMSO with a concentration lower than 1.5% was introduced into each well in order to dissolve the formazan crystals. The absorbance at a wavelength of 570 nm was then measured using a microplate reader, as described by Bhat *et al.* [22]. Paclitaxel, a commonly used anticancer drug, was employed as a reference standard. The percentage of viable cells was determined using eqn. 5:

$$\text{Surviving cells (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD of negative control}} \times 100 \quad (5)$$

A modified version of the reported procedure was used to conduct the neutral red uptake test [23]. Five different quantities (ranging from 0-800 µg/mL) of methanolic leaf extract of *E. crassipes* were generated by diluting the real extract with the maintenance medium. These amounts were then put to 96-well plates, each containing 5×10^3 MCF7 cells per well. The microplate cells were subsequently cultured for 72 h at 37 ºC, with a CO_2 concentration of 5%. Following incubation, the DMEM medium was extracted from each well and then each well was rinsed with 150 µL of phosphate buffered saline (PBS) once. Subsequently, 100 µL of neutral red solution (40 µg/mL in DMEM media) was added to the well. The plates were thereafter incubated for 2 h to facilitate the absorption of dye by the lysosomes of the cells. Afterwards, the mixture was taken out and the neutral red dye that had been combined was extracted by pouring 150 µL of destaining solution for neutral red (ethanol: glacial acetic acid: water, 50:1:49, v/v/v) into each well. The absorbance measurement was taken at a wavelength of 540 nm using a micro-plate reader manufactured by BioTek, specifically the EPOCH model. The experiments were conducted three times to ensure accuracy and reliability.

Evaluation of cytotoxicity by DNA ladder assay: The genomic DNA of MCF7 breast cancer cell lines before and after treatment with methanolic sample was extracted using Hi-Media (INDIA) genomic DNA extraction kit. The extracted genomic DNA was precipitated by ethanol precipitation and separated by centrifugation at 14000 rpm (at 4 ºC) for 15 min and the DNA pellets were purified. The purified genomic DNA samples were electrophorized using 0.7% agarose gel electrophoresis at 150 V for about 90 min to determine the extent of apoptotic DNA fragmentation [24]. The electrophorized genomic DNA and their fragments on agarose gel were detected by treatment with ethidium bromide and the florescence produced by UV light was photographed and analyzed using gel documentation system (Alpha Imager, France). The hypochromacity evaluation was carried out for the purified methanolic extract to assess the possibility of active ingredients from methanolic extracts to intercalate with genomic DNA (MCF7 cell line), whose absorption decreases upon binding to ingredients from plant extract at 260 nm (DNA hypochromicity). The comparison was performed against untreated genomic DNA samples and the treated DNA samples.

Identification of compounds from the plant extract: The methanolic extract of *E. crassipes* was analyzed for bioactive chemicals using a GC-HRMS system. The GC system used was an Agilent 7890 with a FID detector, while the MS system used was a Jeol AccuTOF GCV with a time of flight analyzer. The mass range of the analyzer was 10-2000 amu and it had a mass resolution of 6000. A 70 eV high-energy electron was utilized for spectrometric detection. A methanol solution was produced using a plant extract at a concentration of 1 mg/mL. The carrier gas employed was helium, with a flow rate of 1 mL/min. The instrument was maintained at a temperature range of 50-150 ºC, with a heating rate of 3 ºC per min. It was then kept at a constant temperature for 10 min. Subsequently, the temperature was raised to 300 ºC with a pace of 10 ºC per min. The sample was injected with a split ratio of 20:1 using 2 µL [25]. Gas chromatography is a process that separates the compounds in the extract. The MS spectrum obtained will be compared to the MS spectrum of recognized substances in the NIST database, which has over 62,000 patterns. Analyzed using HR-LCMS, the methanolic extract of *E. crassipes* leaves was subjected to high-resolution liquid chromatography-mass spectrometry using an Agilent (6550 I Funnel Q-TOFs) instrument. The equipment consisted of a binary pump, Hip sampler, column component, Q-TOF with dual ion source and electrospray ion generation (ESI) with Agilent Jet Stream (AJS). The chromatographic separations were conducted by injecting a 5 µL methanolic sample, along with needle wash, onto an Agilent 1290 infinite UHPLC system that was equipped with a Hypersil GOLD C18 column (100 × 2.1 mm-3 µm). The separation process was carried out at a flow rate of 200 µL/min. The column was run with 95% solvent A (water) and 5% solvent B (acetonitrile) for 2 min. This was followed by a 20 min step gradient from 5% B to 95% B. Finally, there was a 5 min period with 5% A and 95% B. The elution was achieved by using a linear gradient ranging from 5% A to 95% A over a period of 4 min. Electrospray ionization was performed with positive ion polarity using the following settings: capillary voltage of 3500 V, capillary temperature of 250 ºC, nebulizer pressure of 35 psi and drying gas flow rate of 13 L/min. These settings were maintained during the whole MS experiment. The data gathering and mass spectrometric analysis were performed using the Agilent Mass Hunter Qualitative Analysis B.06 software [26].

Statistical analysis: The experiments were conducted three times to ensure accuracy and reliability. The results were presented as the mean value plus or minus the standard error of the mean (SEM). The data was analyzed using Microsoft Excel (2010) and GraphPad Prism 8.

RESULTS AND DISCUSSION

Qualitative analysis of phytochemicals from leaf extract: Phytochemical analysis of *E. crassipes* leaf extract detected medically important phytochemicals in the hexane, methanol and aqueous extracts (Table-1). The results suggest that methanol is a superior solvent for extracting phytochemicals from *E. crassipes* leaves, as it produces larger concentrations of phytochemicals compared to hexane and aqueous extracts. As a result, the methanolic extract was selected for further quantitative studies. The methanolic extract exhibited a substantial concentration of flavonoids, alkaloids, quinones, terpenoids, phenols and carbohydrates. The qualitative analysis findings aligned

TABLE-1
PHYTOCHEMICAL SCREENING DATA OF *E. crassipes* LEAF EXTRACTS

Phytochemicals tested	Tests performed	Hexane extract	Methanol extract	Aqueous extract
Carbohydrates	Molish's test	Moderate intense	Highly intense	Moderate intense
Reducing sugars	Fehling's test	Absence	Moderate intense	Slight positive
Tannins	$FeCl_3$ test	Absence	Absence	Highly intense
Saponins	Foam test	Absence	Moderate intense	Moderate intense
Flavanoids	NaOH test	Slight positive	Highly intense	Moderate intense
Alkaloids	Mayer's test	Absence	Absence	Absence
	Hager's test	Moderate intense	Highly intense	Moderate intense
	Wagner's test	Moderate intense	Highly intense	Moderate intense
Quinones	H_2SO_4 test	Absence	Highly intense	Slight positive
Glycosides	Molish's test	Absence	Absence	Absence
Cardiac glycosides	Keller-kiliani test	Slight positive	Moderate intense	Slight positive
Terpenoids	Salkowski test	Slight positive	Moderate intense	Absence
Diterpenoids	Copper acetate test	Absence	Slight positive	Slight positive
Triterpenoids	Salkowski test	Slight positive	Moderate intense	Slight positive
Phenols	$FeCl_3$ test	Absence	Moderate intense	Slight positive
Coumarins	NaOH test	Slight positive	Absence	Slight positive
Sterols	Liberman-Buchard test	Absence	Moderate intense	Slight positive
Phytosterols	Liberman-Buchard test	Slight positive	Absence	Absence
Phlobatanins	HCl test	Absence	Absence	Absence
Anthroquinones	Borntrager's test	Absence	Absence	Absence
Amino acids	Ninhydrin test	Absence	Moderate intense	Absence

with the results reported by Haggag *et al*. [7], which indicated the existence of alkaloids, flavonoids, terpenoids and tannins in the methanolic extract. Through the utilization of the sequential solvent extraction method in this study, a wide range of phytoconstituents was isolated. A previous report [27] undertook a study to determine the phytoconstituents found in the extracts of *E. crassipes* roots, leaves and petioles. The research indicated that the leaf extract included a greater concentration of potential phytochemicals, making it an attractive plant component for extraction.

The research carried out by Cornelius *et al*. [28] showed a lesser amount of phytochemicals in comparison to the results of this investigation. This difference can be attributed to the variation in the proportion of plant material to solvent, with a ratio of 1:20 in the prior study and a ratio of 1:10 in this analysis. They reported the comparable findings, suggesting that dichloromethane would be a more effective solvent due of its greater concentration of phenolic compounds in comparison to present work.

Quantitative analysis of phytochemicals in leaf extract: An analysis was conducted on the methanolic extract to determine its overall phenolic, flavonoid, alkaloid and carbohydrate levels. Phenolics had the greatest quantity, whilst alkaloids had the lowest amount. The total phenolic content was determined by applying the regression equation ($y = 5.969x + 0.001$, $R^2 = 0.998$). The plant extract was found to have a phenolic content of 46.124 ± 0.341 mg GAE/g. The plant extract was found to have a total flavonoid concentration of 37.192 ± 1.079 mg QE/g. The number was derived by computing it using the regression equation of the calibration curve of quercetin, which was $y = 5.049x + 0.04$, with an R^2 value of 0.984. The plant extract was shown to have the lowest overall alkaloid concentration, particularly 22.682 ± 1.739 mg CE/g. The regression equation ($y = 3.324x + 0.027$, $R^2 = 0.997$) used caffeine as reference. The plant extract was analyzed and found to contain a total carbohydrate content of 180.104 ± 2.581 mg GE/g of plant extract. This determination was made using the regression equation of the calibration curve of glucose ($y = 7.247x + 0.064$, $R^2 = 0.972$). Previous studies have shown a strong link between the total phenolic content and the antioxidant activity obtained from the plant extract. The scientific data serves as the basis for developing a correlation between the antioxidant activity and the overall phenol content in the plant extract [29]. Carbohydrates are the primary compounds engaged in metabolism and are therefore anticipated to be present in greater quantities compared to secondary metabolites [30].

Evaluation of biological activities: A biological activity assessment was performed on the raw methanolic extract. The crude extract was evaluated for its antibacterial, antifungal, antidiabetic, antioxidant, anti-inflammatory, hypocholesterolemic and cytotoxic properties. The results obtained are as follows:

Antimicrobial susceptibility test: The qualitative assessment of the antibacterial efficacy of the methanolic extract was conducted using the well-diffusion method on three types of Gram-positive bacteria and three types of Gram-negative bacteria. Furthermore, the minimum inhibitory concentration (MIC) values were established for *E. coli*, *S. aureus* and *C. albicans*. The antibacterial activity was assessed using the agar well diffusion method against three types of Gram-negative bacteria and three types of Gram-positive bacteria. The Gram-negative bacteria used were *P. aeruginosa*, *E. coli* and *S. typhi*. The experiment utilized three Gram-positive bacteria: *B. cereus*, *B. subtilis* and *S. aureus*. The widths of the zones of inhibition were measured in mm against both Gram-positive and Gram-negative bacteria (Table-2).

The antibacterial activity of the extract was attributed to the presence of flavonoids and phenolics. The results of the antibacterial study are still applicable within the defined experi-

TABLE-2
ZONES OF INHIBITION OF METHANOLIC EXTRACT ON VARIOUS MICROORGANISMS

Test organism	Zone of inhibition (mm)
Escherichia coli	6
Salmonella typhi	4
Pseudomonas aeruginosa	4
Bacillus cereus	8
Staphylococcus aureus	5
Bacillus subtilis	5
Candida albicans	4

mental conditions. The methanolic extract was tested to determine its minimum inhibitory concentration (MIC) against Gram-positive and Gram-negative bacterial strains, namely *E. coli* and *S. aureus*, as well as *C. albicans*, an opportunistic fungal pathogen. The minimum inhibitory concentration (MIC) values observed for *E. coli*, *S. aureus* and *C. albicans* were 2500 µg/mL, 5000 µg/mL and 5000 µg/mL, respectively. Both bacterial strains were shown to have a MIC of 25 µg/mL against the conventional antibiotic ciprofloxacin. Similarly, the standard antifungal amphotericin exhibited a MIC of 50 µg/mL. The crude preparation of the plant extract resulted in high MIC values against both bacterial and fungal strains. Based on the fact that the MIC of *E. coli* is lower than that of *S. aureus*, it may be inferred that the methanolic extract is more effective in inhibiting Gram-negative bacteria compared to Gram-positive bacteria. The present results aligned with the results of Haggag *et al.* [7], which also reported the inhibition of both Gram-positive and Gram-negative bacteria. The investigation revealed that the methanolic extract exhibited a decreased MIC value against *E. coli*, whereas it demonstrated an increased MIC value against *Lactobacillus casei*. The fluctuations in the MIC measurements might also be attributed to the particular conditions of the experiment.

During this experiment, an inoculum with a density of 4×10^7 CFU/mL, which is four-fold higher, was employed. As a result, a larger amount of plant extract is necessary to effectively impede the survival of organisms. The results of the MIC investigation retain their validity under defined experimental settings. Shanab *et al.* [5] utilized the disk-diffusion method to investigate the antibacterial properties of crude *E. crassipes* extract. The findings demonstrated that the extract shown notable antibacterial activity against *E. coli*, *S. aureus* and *C. albicans*. Panthagada *et al.* [31] conducted a separate experiment to evaluate the effectiveness of the complete extract of *E. crassipes* against *B. subtilis*, *E. coli* and *S. aureus* using the disc diffusion method. The results demonstrated zone of inhibition measurements of 5 mm, 8 mm and 6 mm, respectively, which are consistent with the results of this present investigation. The plant extract's antibacterial effect can be linked to its capacity to disrupt the bacterial membrane, causing alterations in the membrane potential and heightened permeability of the inner membranes of the bacteria [32].

Antidiabetic activity: The antidiabetic activity of *E. crassipes* formulations was evaluated *in vitro* using the α-amylase inhibitory assay. The inhibitory effects of methanolic extracts were evaluated at concentrations ranging from 2000-10000 µg/mL. The percentage of inhibition was calculated by comparing the results to acarbose as reference standard (Fig. 1).

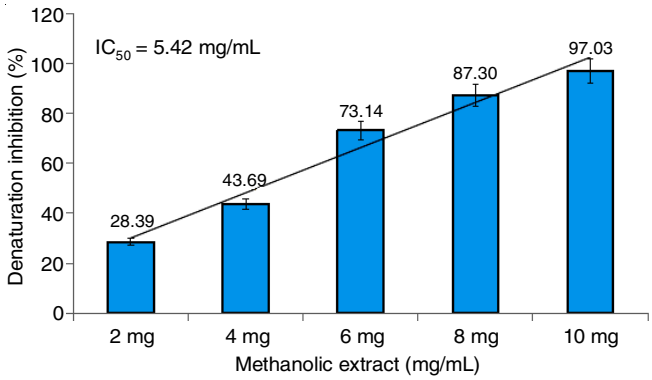

Fig. 1. *In vitro* Antidiabetic assay of methanolic extract of *E. crassipes* leaves (Standard: acarbose)

The methanol extract showed a range of α-amylase inhibition percentages from 28.39 ± 2.10% to 97.03 ± 1.73%. The inhibitory concentration (IC_{50}) value of the extract was determined to be 5.42 mg/mL. When compared, acarbose had a lower IC_{50} value of 0.005032 mg/mL. The larger IC_{50} value of the methanolic extract, in comparison to the standard, can be attributed to the fact that acarbose is a singular, refined compound, whereas the extract is an unrefined combination including several components. This study provides evidence of the herb's remarkable antidiabetic capabilities. The extract contains flavonoid compounds, which is the cause of this phenomenon.

Anti-inflammatory activity: The anti-inflammatory effectiveness of *E. crassipes* extracts was evaluated by studying their ability to inhibit the heat-induced denaturation of albumin protein at different concentrations (range from 400-2000 µg/mL). The percentage of inhibition to denaturation was calculated and compared to the reference ibuprofen (Fig. 2).

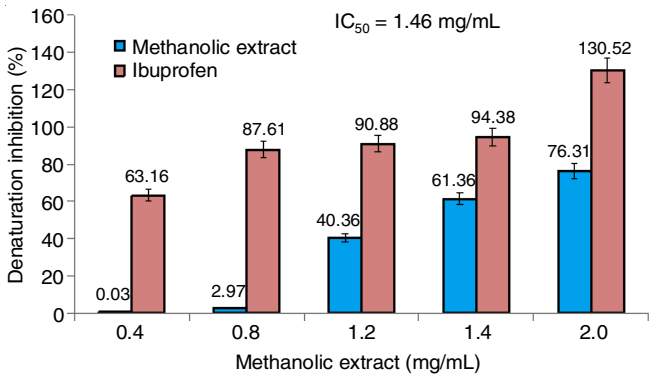

Fig. 2. *In vitro* anti-inflammatory activity of methanolic extract expressed as denaturation inhibition (%), (Standard used: ibuprofen)

An increase in the concentration of plant extract led to a proportional rise in the percentage of inhibition. The maximum level of inhibition, measuring 76.31 ± 3.27%, was obtained when the dosage was 2 mg/mL. Nevertheless, the level of inhibition observed was reduced in comparison to ibuprofen, which yielded a higher inhibition rate of 97.85 ± 1.48% at the identical concentration of 2 mg/mL. The IC_{50} value of the plant extract was

1.46 mg/mL, which was higher than that of ibuprofen, precisely 0.23 mg/mL. The anti-inflammatory action of the extract can be linked to the presence of flavonoids and tannins. The previous study conducted by Panthagada *et al.* [31] found slight variations in the anti-inflammatory properties. At a dosage of 5 mg/mL, there was a precise inhibition rate of 77%. Additionally, when subjected to 50 ºC, denaturation took place. However, in the present inquiry, the temperature was set at 60 ºC.

Antioxidant activity: The antioxidant activity of *E. crassipes* was assessed *via* the DPPH free radical scavenging approach. The scavenging activity percentage was assessed across the concentration range of 200-1000 µg/mL. A comparative analysis was performed to assess the antioxidant efficacy of methanolic extract in contrast to the standard ascorbic acid (Fig. 3).

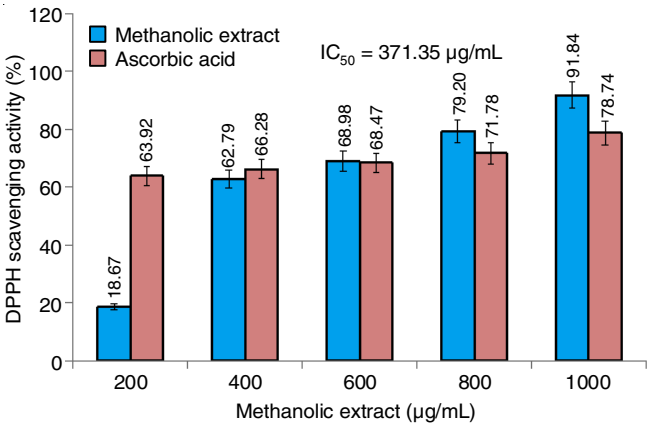

Fig. 3. Antioxidant activity of methanolic extract of *E. crassipes* leaves expressed as DPPH free radical scavenging (%), (Standard: ascorbic acid)

The highest observed scavenging activity was found to be 91.83 ± 1.48%, surpassing the scavenging activity of ascorbic acid (78.74 ± 3.14%) at the identical concentration of 1000 µg/mL. The IC_{50} value of the plant extract for DPPH scavenging activity was found to be 371.35 µg/mL, whereas ascorbic acid had an IC_{50} value of 91.62 µg/mL. The DPPH scavenging activity exhibited a dosage-dependent increase. The research conducted by Tyagi & Agarwal [27] reported that the IC_{50} value for the DPPH scavenging activity of *E. crassipes* was 742 ± 0.02 µg/mL, which is greater than the IC_{50} value observed in this work. A lower IC_{50} value signifies a medicine that is more efficacious. The difference can be ascribed to the elevated concentrations of flavonoids and phenolic compounds observed in the present investigation, which exhibit remarkable antioxidant properties [33,34].

Hypocholesterolemic activity: The methanolic extract of *E. crassipes* was tested *in vitro* using the lipase inhibitory assay to determine its hypocholesterolemic activities. The lipase inhibition % was determined at several concentrations ranging from 200-1000 µg/mL (Fig. 4). The highest degree of inhibition, with a value of 87.82 ± 1.36%, was observed at a concentration of 1000 µg/mL. The extract showed an IC_{50} value of 539.1 µg/mL, while orlistat had an IC_{50} value of 52.18 µg/mL. The ability of methanolic extract to lower cholesterol levels may be attributed to the presence of phytochemicals such sterols and terpenoids. However, there is a lack of studies that have recorded the cholesterol-lowering properties of *E. crassipes* up till now.

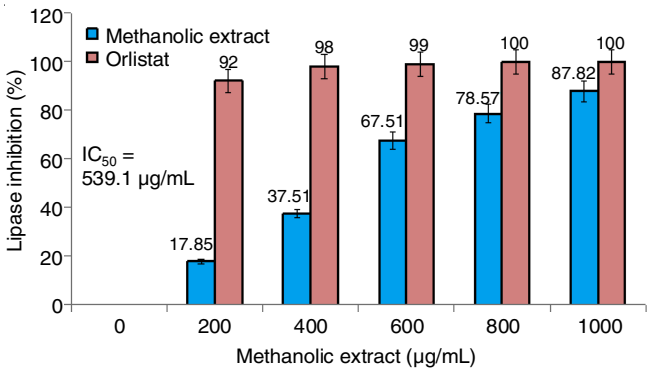

Fig. 4. *In vitro* hypocholesterolemic activity of the methanolic extract of methanolic extract of *E. crassipes* leaves (Standard: orlistat)

Cytotoxic activity: This study shows that the methanolic extract of *E. crassipes* leaves has an IC_{50} value of 770.90 µg/mL against the MCF7 breast cancer cell line. By comparison, the conventional medication Paclitaxel exhibits an IC_{50} value of 298.86 µg/mL for the identical cell line. The concentration of 800 µg/mL resulted in a drop in cell viability to 48.29 ± 1.39%. The findings indicate that the methanolic crude extract exhibits substantial cytotoxicity when compared to paclitaxel, a standard drug. Fig. 5 illustrates the correlation between the concentration of methanolic extract (µg/mL) and the percentage of cell viability. The microscopic image of cytotoxic effect of plant extract on MCF7 cell line after 48 h of treatment has shown significant cellular damage and cell deformation (Fig. 6).

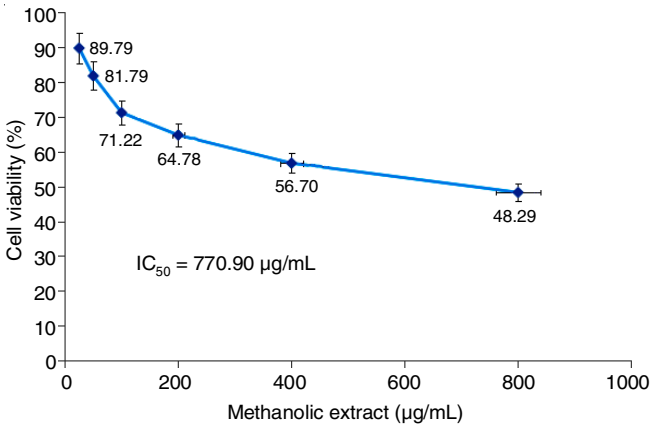

Fig. 5. Anticancer activity of the methanolic extract of *E. crassipes* leaves against MCF-7 cell line. Cell viability measured after 48 h by MTT assay

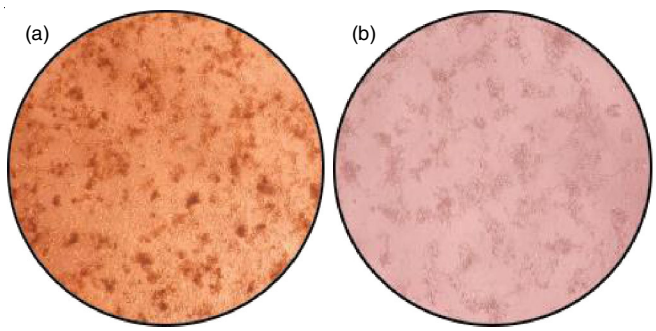

Fig. 6. Microscopic images of MCF-7 cell-line after 48 h of treatment with (a) methanolic extract and (b) negative control indicating its anti-proliferative effect

The analysis revealed that the extract had a direct correlation with the viability of cells, meaning that the higher the dose of extract, the greater the impact on cell viability. The NRU assay was employed to determine cell viability following exposure to methanolic leaf extract. The findings indicated that the extract exhibited cytotoxic effects at various concentrations, ranging from 200-800 µg/mL, in a way that was depending on the dosage administered. The NRU experiment established a direct relationship between the concentration of leaf extract and its ability to kill cells. The observed outcomes can be ascribed to the presence of various secondary plant chemicals that are detrimental to the MCF7 cell-line.

DNA ladder assay: Initial microscopic studies of the cells treated with methanolic extract of *E. crassipes* showed significant morphological changes of the MCF7 cells. Further the probable pro-apoptotic effect of the plant extract was investigated by DNA ladder assay. The results have shown that, there is significant DNA framgmentation in the cells treated with the plant extract compared to control (Fig. 7). The probable pro-apoptotic changes may be condensation of chromatin, cell shrinkage and other cellular alterations, characteristics of apoptotic cells. The DNA fragmentation assay or DNA ladder assay is qualitative indicator of apoptosis. Cells which are undergoing apoptosis characteristically exhibit fragmentation of the genomic DNA into small oligo-nucleosomal fragments, a hallmark of apoptosis [35]. The results obtained confirmed that the methanolic extracts induced DNA ladder formation, which is the characteristic feature of apoptosis. Distinctive ladder pattern due to induction of apoptosis was also reported with aqueous ethanol seed extract of *Ziziphus mauritiana* in HL60 cells [36].

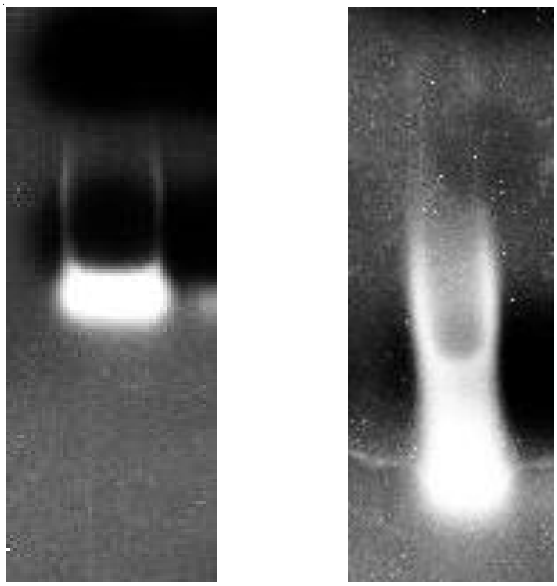

Fig. 7. DNA Ladder assay: Control - genomic DNA untreated cells and Test: genomic DNA methanolic leaf extract treated cells

Identification of compounds by GC-HRMS & HR-LCMS analysis: The mass spectrum of the unknown molecule in the raw extract was compared to the spectrum of the known compounds in the NIST library using the retention time values. The gas chromatogram of the methanolic extract was obtained (Fig. 8). Table-3 displays the findings from the GC-HRMS analysis of the methanolic extract, indicating the existence of 12 significant phytochemicals.

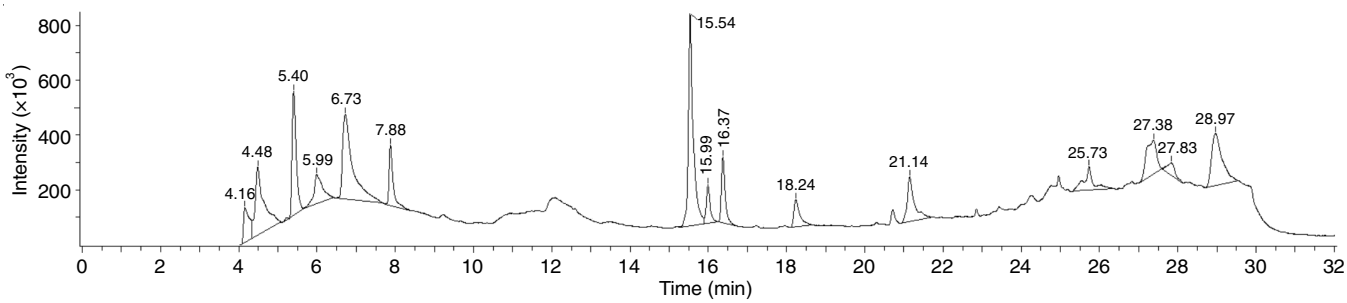

Fig. 8. GC Chromatogram of methanolic extract indicating eluted compounds at different retention times

TABLE-3
MAJOR COMPOUNDS PREDICTED ON THE BASIS OF RETENTION TIME BY GC-MS IN THE CRUDE METHANOLIC EXTRACT OF *E. crassipes* LEAVES

S. No.	Name of the compound	Retention time (min)	Area [intens*sec]	m.f.	m.w.
1	2,4-Diamino-6-hydroxypyrimidine	4.48	3878905.51	$C_4H_6N_4O$	126
2	3,5-Dihydroxy-6-methyl-2,3-dihydro-4*H*-pyran-4-one	5.42	3284519.14	$C_6H_8O_4$	144
3	1-Methyl-1-(3-methylbutyl)oxy-1-silacyclobutane	6.00	1622199.19	$C_9H_{20}OSi$	172
4	5-Hydroxymethylfurfural	6.73	5542302.70	$C_6H_6O_3$	126
5	1-(2-Hydroxy-5-methylphenyl) ethanone,	7.88	1530559.64	$C_9H_{10}O_2$	150
6	Phytol	15.54	6761861.98	$C_{20}H_{40}O$	296
7	*n*-Hexadecanoic acid	18.24	1046190.95	$C_{16}H_{32}O_2$	256
8	*cis*,*cis*,*cis*-7,10,13-Hexadecatrienal	21.15	2045649.73	$C_{16}H_{26}O$	234
9	9,12-Octadecenoic acid (Z)-, phenylmethyl ester	25.73	1371458.33	$C_{25}H_{40}O_2$	372
10	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate	27.37	2253708.30	$C_{32}H_{52}O_2$	468
11	Stigmastan-3,5-diene	27.87	599761.99	$C_{29}H_{48}$	396
12	Vitamin E	28.96	3565446.18	$C_{29}H_{50}O_2$	430

Tyagi & Agarwal [37] examined the ethanolic leaf extract and identified the presence of phytol, *n*-hexadecanoic acid, stigmasterol, 9,12-octadecadienoic acid and ethyl ester. The findings align with the current analysis. In addition, the extract contained vitamin E. The investigation using GC-HRMS has revealed phytoconstituents that demonstrate a wide range of biological activities.

A HR-LCMS analysis was performed on the crude methanolic extract of *E. crassipes* utilizing both positive and negative electrospray ionization (ESI) modes. The unknown chemicals in the raw extract were identified and their molecular weights were measured. Moreover, a liquid chromatogram of the methanolic extract (Fig. 9), only 14 phytochemical compounds are detailed which are associated with the most significant LC peaks (Table-4).

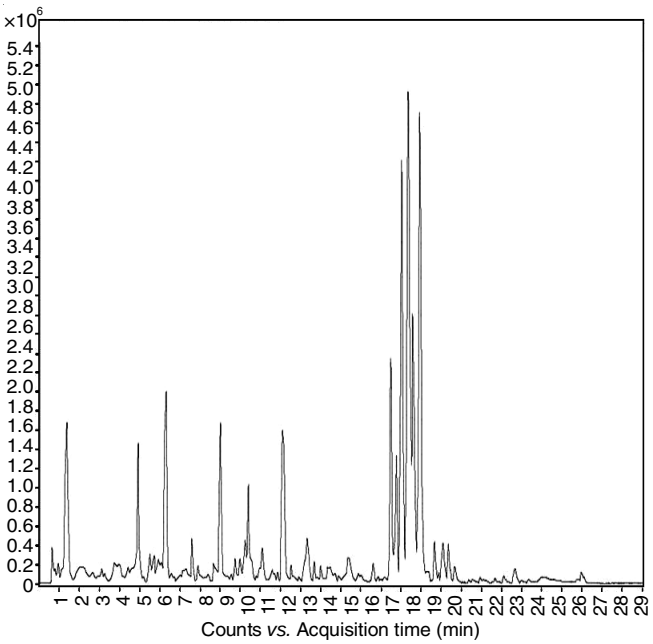

Fig. 9. LC Chromatogram of methanolic extract indicating eluted compounds at different retention times

Conclusion

The present study has demonstrated promising therapeutic applications of the plant *E. crassipes*. The present study lies in the comprehensive investigation successive solvent extraction and evaluation of phytoconstituents using advanced GC-HRMS and HR-LCMS techniques. The methanolic extract showed the presence of unique phytochemicals such as khivorin, known for its anticancer properties and rhoifolin, known for its antioxidant, anti-inflammatory, antibacterial, hepatoprotective and anticancer properties. Thus, the methanolic extract exhibits significant *in vitro* antibacterial, antidiabetic, anti-inflammatory, antioxidant, hypocholesterolemic and cytotoxic properties. The methanolic extract showed significant anti-proliferative effect against MCF7 cell-line and demonstrated apoptotic effect on cancer cells by fragmentation & intercalation of genomic DNA. The isolation and standardization of herbal extracts could be a good beginning for an interdisciplinary approach for drug discovery.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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TABLE-4
MAJOR COMPOUNDS PREDICTED ON THE BASIS OF RETENTION TIME AND MASS FRAGMENTS IN HR-LCMS IN THE CRUDE METHANOLIC EXTRACT OF *E. crassipes*

S. No.	Compound name	Retention time (min)	Mass	Formula
	Positive ESI mode			
1	Khivorin	18.049	586.2714	$C_{32}H_{42}O_{10}$
2	Cymarin	20.352	548.2906	$C_{30}H_{44}O_9$
3	Methyl reserpate	12.332	414.2193	$C_{23}H_{30}N_2O_5$
4	5-Methylthioribose	4.765	180.0442	$C_6H_{12}O_4S$
5	Salicin	7.912	286.1055	$C_{13}H_{18}O_7$
6	Benzenemethanol, 2-(2-aminopropoxy)-3-methyl-	6.332	196.1125	$C_{11}H_{16}O_3$
7	12-Oxo-9-octadecynoic acid	13.196	294.2262	$C_{18}H_{30}O_3$
8	C16 Sphinganine	10.111	273.2736	$C_{16}H_{35}NO_2$
	Negative ESI mode			
9	(S)-Ureidoglycolic acid	4.706	134.0340	$C_3H_6N_2O_4$
10	Retusin dimethyl ether	1.128	312.1001	$C_{18}H_{16}O_5$
11	Rhoifolin	4.945	578.1527	$C_{27}H_{30}O_{14}$
12	9S,10S,11-Rtrihydroxy-12Z-octadecenoic acid	9.278	330.2385	$C_{18}H_{34}O_5$
13	11-Hydroperoxy-12,13-epoxy-9-octadecenoic acid	9.134	328.2229	$C_{18}H_{32}O_5$
14	Digitoxin	15.478	764.4445	$C_{41}H_{64}O_{13}$

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