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LEUCAS ASPERA (WILD.) LINK ROOTS EXTRACT SECONDARY METABOLITES BESTOW ANTIOXIDANT, ANTI-BACTERIAL PROPERTIES AND INDUCE CELL CYTOTOXICITY TO HUMAN BREAST CANCER CELLS, MCF-7 BY APOPTOSIS

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Keywords:

Methanol extract, UPLC-MS, Antioxidant, Anti-biofilm, MCF-7 cells cytotoxicity, Apoptosis

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ABSTRACT: *Leucas aspera* species, auspicious in many cultures worldwide, are sources of novel bioactive secondary metabolites. The mechanism by which they exhibit therapeutic potential *via* antioxidant, antimicrobial and apoptotic properties is scant. The main objective of the current study was to evaluate the properties of activity-guided fractionated root methanol extract. As the methanol extract exhibited the highest antioxidant activity, it was evaluated for secondary metabolites and therapeutic properties UPLC-MS analysis of the extract identified the 15 and five secondary metabolites including oleanolic acid, 6-C-glucosyl- 8- C- arabinosylapigenin, 6- C-arabinosyl 8- C-glycosylapigenin, neohesperidin and beta-sitosterol were reported. Anti-bacterial activity by disc diffusion inhibited human pathogenic bacteria. Bacterial bio-film inhibition capacity of extract (750 µg) imaged by Confocal laser scanning microscopy revealed loss of microcolonies. Further, it quenched free radicals produced by Fentons' reagent studied by DNS-nicking assay, indicating its strong antioxidant property. The MTT assay results identified maximum cell death for human breast cancer cells, MCF-7 (EC_{50}, 60.31±2.71 mg/mL^{-1}). CLSM visualized a clear indication of apoptosis via chromatin condensation. The presence of novel bioactive compounds has uncovered possible therapeutic values by modulating antioxidants and apoptosis, leading to the development of potential alternative anticancer drugs.

INTRODUCTION: Human health care system is dependent on phytomedicines and considerable interest in elucidating the mechanism of their action to develop better medicines has been envisaged by the scientific community. Phenolic compounds, nitrogen compounds, vitamins, terpenoids, *etc*. in medicinal plants act as free radical scavenging molecules. Antioxidant and antimicrobial constituents in plants, spices, and herb tissues such as bark, seed, roots, flowers among other parts provide the curative constituents.

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Free radicals are generated as part of the body's normal metabolic process in the mitochondrial respiratory chain and liver mixed-function oxidases. They are part of metabolic activity from atmospheric pollutants, transitional metal catalysts, drugs, and xenobiotics. Uncontrolled production of oxygen-derived free radicals is associated with the onset of many diseases such as cancer, rheumatoid arthritis, cirrhosis, arteriosclerosis, and degenerative processes associated with aging. Scavenging of reactive oxygen species (ROS) protects DNA, proteins and lipids, reducing the risk of chronic diseases.

Leucas aspera (Wild.) Link belonging to the family of Labiatae is a common herb found in Asia-temperate and Asia-tropical countries. Traditionally, the whole plant is taken orally for analgesic,

antipyretic, anti-rheumatic, anti-inflammatory, and antibacterial treatment. Its paste is applied topically to inflamed areas. The entire plant is also used as an insecticide and indicated in traditional medicine for coughs, colds, painful swelling and chronic skin eruption. The plant also possesses wound healing properties and is used against cobra venom poisoning. Leaves of *L. aspera* are useful in chronic rheumatism, psoriasis, scabies and chronic skin eruptions, and their juice is used as antibacterial agent [1].

However, the mechanism by which they exhibit therapeutic potential via antioxidant, antimicrobial and apoptotic properties is scant. So, this study aimed to evaluate the antioxidant potencies of the *L. aspera* roots *via* standard DPPH and ABTS assay. Further, the capacity to quench the toxic radicals generated *via* Fentons` reagent nicking DNA was analyzed, a first of its kind. The secondary metabolite fingerprint of the extract was carried out to identify the polyphenols of the extracts confidently. A differential concentration of the extract was then applied to study the antimicrobial property against *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Bacillus subtilis,* and the results are presented in context to the observations. Inhibition of bacterial biofilm was tested. Cell cytotoxicity studies by MTT assay and apoptosis induced were assessed morphologically using acridine orange and propidium iodide staining.

MATERIALS AND METHODS:

Plants: The fresh roots (50 g) of the test plant were collected during winter (January, 2019) from Kigga region of Western Ghats in Chikmagalore District of Karnataka State, and herbarium specimen was deposited at the Institution of Excellence (*Leucas aspera* roots # IOE LP0014). Fresh roots were washed, shade-dried, ground to a powder and 100 g of powder were extracted three times by hexane, chloroform, and methanol (1:10 w/v) at room temperature, evaporated to yield dry extracts in Speed-Vac (Savant SPD 2010, Thermo Scientific), and stored under dark at 4°C until further use.

Chemicals and Reagents: Acridine orange, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 15-lipoxygenase (soybean), quercetin, 2,2-azinobis-(3-ethyl-benzthiazoline-6-sulfonic acid)(ABTS), trypsin, chloramphenicol, streptomycin, 2-(4-Iodophenyl)-3- (4- nitrophenyl)- 5- phenyltetrazolium chloride (INT), propidium iodide, ethidium bromide and 3-(4, 5-dimethylthylthiazol-2-yl)-2, 5-diphynyl tetra-zolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Ascorbic acid (AA), butylatedhydroxyl toluene (BHT), quercetin, L-glutamine, sodium bio-carbonate, non-essential amino acid, and minimum essential medium Eagle were purchased fromHi-Media (India). Plasmid (pBR322) and StepupTM 1kb DNA ladder were obtained from Merck Biosciences, Bangalore (India).

LC Analysis of Methanol Extract in Negative Mode: Synapt G2 (UPLC/MS separations with Quan-Tof) along with the Agilent zorbax SB-C18 (15cm, 3.5µm) column was coupled to an HCT-ultra ion trap MS detector was used for the qualitative analysis of the metabolites [2-3] according to manufacturers` protocol. The nebulizer pressure was 60 psi at a drying temperature of 350°C, and the nitrogen flow rate 10 $L.min^{-1}$ was used. The root methanol extract was filtered (0.2-micron syringe filters, Millipore, USA). An aliquot (15µL) was injected into the system with a dwell time of 420 msec, a flow rate of 0.4 $mL.min^{-1,}$ and finally, the temperature of the column was 25°C. The mobile phase consisted of (A) water + 0.1% acetic acid (v/v) and (B) acetonitrile containing 0.1% acetic acid (v/v). The used linear gradients included 10%, 50%, 95% (repeated twice), and 100% (repeated twice) (v/v) for the A solution and 0%, 25%, 45%, 55%, 60% for the B solution. The washing time was 75 min applying 0.3 mL/min at all times. The ESI-MS spectra in negative ionization mode mass spectra were acquired by electrospray, ESI ion source (Bruker Daltonik GmbH, Bremen, Germany). The collision gas helium was used for the fragmentation in the ion trap of the isolated compounds. The detection conditions were as follows: drying gas (N2) was 300°C at a dry gas flow rate of 35 $mL.min^{-1}$, nebulizing pressure (N2) of 30 psi, and capillary voltage 4 V. Control samples were prepared by diluting separate analytic stock solutions.

Determination of Free Radical Scavenging Capacity and Total Phenolic Content: Two methods were used for the evaluation of the

antioxidant activity for methanol extracts (50, 100, 250, 500 µg/mL, 1, 5, and 10 mg/mL): DPPH· Scavenging assay and 2,2-azinobis-(3-ethyl-benzthiazoline-6-sulphonic acid ($ABTS^{\cdot +}$) assay [4-5]. Results were compared with AA, BHT, and quercetin, the most used synthetic antioxidants. The antioxidant activity was expressed as IC_{50} values. The total phenol content of the sample was estimated using the Folin-Ciocalteu colorimetric method [6].

Bacterial Strains and Culture Conditions: Gram (−) bacteria, *Klebsiella pneumoniae*(MTCC 432), *Escherichia coli* (MTCC 724), *Pseudomonas aeruginosa* (ATCC 27853) and Gram (+) bacteria, *viz*., *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (MTCC 441), were procured from the Institute of Microbial Technology, Chandigarh, India. Bacterial inoculums were prepared by growing cells in Mueller Hinton Broth (MHA) (Hi-Media, India) for 24 h at 37°C. These cell suspensions were diluted with sterile MHB to provide initial cell counts of about 10^4 $CFU.mL^{-1}$.

Antibacterial Activity:

A. Agar–Disc Diffusion Method: Disc diffusion was carried out with the above-said samples. An aliquot (5 and 10µL; $1mg.mL^{-1}$) was introduced on the discs (0.7 cm; Hi-Media) for this experiment. The diameter of inhibition zones was recorded and was compared against streptomycin (positive standard; 3µL of $1mg.mL^{-1}$; Sigma-Aldrich St. Louis, MO, USA) and methanol (negative standard; 10 µL) under similar experimental conditions.

B. Minimum Inhibitory Concentration: Micro-dilution methods of broth were used for measuring minimal inhibitory concentrations (MIC). Muller Hinton Broth (MHB; Merck Biosciences, Bangalore, India) was used [7]. A twofold serially diluted extract (50µL) of *L. aspera* extract in methanol with a concentration range of 10, 5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, and 0.0390605 mg/mL was added to the 96-well microtiter plate. The bacterial suspension (50µL) in MHB ($10^4 CFU.mL^{-1}$) was introduced. Control wells were prepared with culture medium (sterility control), above said extract, MHB (50µL [negative control]), bacterial suspension in MHB (50µL positive control), and methanol (positive control). The contents of each well were mixed. The MIC was defined as the lowest concentration where no viability was observed after 24 h. This was assessed on the basis of metabolic activity visualized as purple color produced by the addition of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT; Sigma-Aldrich St. Louis, MO, USA) dissolved in water (10µL of INT, 2 mg/mL). The plates were incubated in the dark (37°C for 30 min) after the addition of INT. All measurements of MIC values were repeated in triplicate. Chloramphenicol was used as a positive control.

C. **Confocal Laser Scanning Microscopy Study to Assay Loss of Biofilm:** CLSM LSM 710 (Carl Zeiss, Germany) was used to observe the biofilm structure. The samples were prepared according to Zhang *et al*. [8] The overnight cultures of *P. aeruginosa* and *S. aureus* were prepared with the absorbance set at 1.5 ± 2.0 (600 nm). The assay was conducted in a 6-well plate. Coverslips were placed in all the wells, and above the coverslip, about 2ml of the media was added. The coverslip with media was maintained as control. To the test wells, overnight bacterial suspensions (750µl) were introduced. The extracts (200µL, 750µg) were added. The plates were incubated at 37 ºC for 24h. The biofilm formed on coverslips in wells was removed carefully. The biofilm was washed with PBS. The film was stained with acridine orange and ethidium bromide (20µL; 1 mg in 400µL; Hi-Media, India). After three washes, the coverslips with stained biofilm were placed on a glass slide in an inverted position. CLSM images of the samples were imaged, and results were presented.

DNA Strand Assay: A DNA-nicking assay was performed using supercoiled plasmid pBR322 (Merck Biosciences, Bangalore, India) [9]. A mixture of 5µl of extract (1mg/ml) and 2µl of plasmid was added to 5µl of Fenton's reagent (30mM H_2O_2, 50Mm ascorbic acid, and 80µM $FeCl_3$). The final volume was made up to15µl with sterile water followed by incubating for 30 min at 37°C. The DNA was analyzed on 1% agarose gel using ethidium bromide staining. The results were documented using XR+ Molecular Imager Gel documentation system (Bio Rad, USA).

Evaluation of *L. aspera* extract Induced Cell Cytotoxicity:

A. Cell Lines, Culture Conditions and MTT Assay: *In-vitro* experiments were done using

human cell lines, MCF-7 (human breast cancer cells). They were grown in DMEM, supplemented with 10% FBS as per reported protocol [10]. The cells were plated at 3×10^4 cells/cm^2, grown in humidified 5 % CO_2 with 95% air atmosphere at 37°C, and experiments were initiated 48h after plating. The culture medium was replaced two times a week. For the experiments, confluent cells were trypsinized and plated in 96-, 6-well plates or into tissue culture dishes (6 mm).

The cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT (1 mg/mL) was dissolved in sterile phosphate buffer saline (PBS; 0.05 M phosphate buffer, pH 7.2, 0.8% NaCl) at room temperature. The solution was further sterilized by passing through a 0.2-μm filter and stored at 4°C in the dark.

The cells were plated at 3×10^4 cells/cm^2, grown in humidified 5 % CO_2 with 95% air atmosphere at 37 °C, and experiments were initiated 48 h after plating. Different concentrations of *L. aspera* root methanol extract in the range from 1 to 200μg.mL^{-1} into the respectively labeled wells. After 48h of incubation, MTT (10μL) was added to each well and the plates were incubated at 37°C for 4 h in the dark. The supernatants were aspirated from the wells and washed thrice with PBS. DMSO (100μL) and 25μL of 0.1M glycine buffer (pH 10.5) were added to each well. After an incubation period of 15min, the absorbance was measured at 570 nm using multimode plate reader (Varioskan Flash Top, Thermo Fisher Scientific, Germany). Controls were consisting of the same concentration of cells without *L. aspera* extract was maintained. Any absorbance due to the reaction of the extract with MTT in wells devoid of cells was subtracted from the readings. Triplicate wells were assayed for each condition.

B. Apoptosis Assessed by Nuclear Morphology: Apoptotic nuclear morphological changes of MCF-7 cells after treatments with the extract were observed after dual staining with acridine orange/ propidium iodide (AO/PI). MCF-7cells (5×10^4 cells/well) were seeded in six-well plates on 0.01% poly-L-Lysine coated coverslips (24mm) and were treated with *L. aspera* (6mg.mL^{-1}) and camptothecin (2μg.mL^{-1}) for 48 h. Following incubation for 48 h, cells were washed with phosphate-buffered saline (PBS) twice and stained with AO/PI (1mg.mL^{-1}) mixture for 2–3min. Apoptotic nuclear morphological changes after treatments were observed by Confocal Laser Scanning Microscopy(CLSM) LSM 710 (Carl Zeiss, Germany).

Statistical Analysis: All experiments and measurements were made in triplicate. The values are expressed as the mean ± Standard deviation (SD). The results were subjected to analysis of variance followed by Tukey's test to analyze differences between the *L. aspera* root methanol extract and control samples. Statistically significant differences (P-value < 0.001) were shown.

RESULTS AND DISCUSSION:

Fingerprint Profiling *Leucas aspera* Root Methanol Extract for Polyphenols: Secondary metabolites of plants, including polyphenols, provide defense against several conditions and aggressive pathogens. Potential health benefits are attributed to these due to their therapeutic properties. Studies associated with meta-analyses suggest a rich diet of plant polyphenols provides broad-spectrum protection against the onset of diseases due to ROS, including inflammation leading to cancers and cardiovascular diseases, among others.

Quality control of plant extracts involving its standardization is an important topic, in particular when such extracts are used for medicinal purposes. The negative ion mode ESI-MS is particularly effective for the characterization of plant extracts [2]. Polyphenols, the secondary metabolites with health benefits, have been the center of several studies as they cannot be synthesized by human [11]. Fingerprint profiling *L. aspera* root methanol extract for polyphenols in the present study identified the presence of several molecules, and five could be identified and reported.

Polyphenol extraction was achieved using methanol as a solvent by applying sequential–liquid extraction. The LC-chromatogram obtained is shown in **Fig. 1**, and peaks were labeled according to the order of their retention time. Structural characterization was performed using the retention

time of standards, eluted metabolites, and published data [2-3]. (+)-catechin and (+)-epicatechin were used as standards in this experiment. The presence of unknown polyphenol, oleanolic acid, 6-C-glucosyl-8-C-arabinosylapigenin, 6-C-arabinosyl 8-C-glycosylapigenin, neohesperidin and beta-sitosterol were identified. **Table 1** and **Fig. 1** summarize the LC-MS and UV-vis data and polyphenols of *L. aspera* extract details.

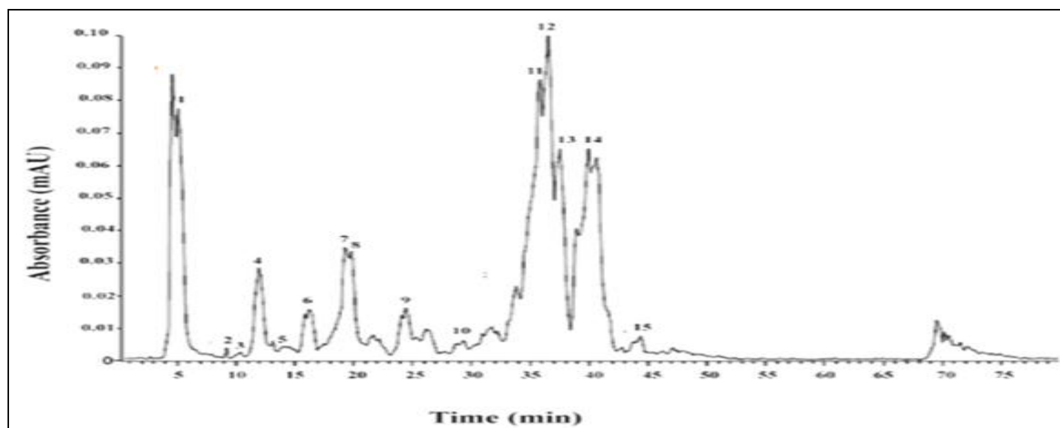

FIG. 1: UPLC-MS FINGERPRINT OF *LEUCAS ASPERA* ROOTS METHANOL EXTRACT

TABLE 1: UPLC-MS FINGERPRINT AND POLYPHENOLS IDENTIFIED FROM *LEUCAS ASPERA* ROOT METHANOL EXTRACT

Peak no.	Compound	Retention time (min)	References
1	unknown	5.143	[2]
2	unknown	9.257	[2]
3	Oleanolic acid	10.842	[3]
4	unknown	11.257	[2]
5	(+)-catechin	14.931	[2]
6	unknown	16.557	[2]
7	6-C-glucosyl-8-C-arabinosylapigenin	19.081	[2]
8	6-C-arabinosyl 8-C-glycosylapigenin	20.6	[2]
9	Neohesperidin	24.58	[2]
10	(-) - epicatechin	29.61	[2]
11	beta-sitosterol	35.596	[3]
12	unknown	37.51	[2]
13	unknown	38.23	[2]
14	unknown	40.15	[2]
15	unknown	44.03	[2]

The presence of beta-sitosterol was verified. The oleanolic acid, a natural triterpenoid, quenches ROS, 6-C-glucosyl-8-C-arabinosylapigenin and 6-C-arabinosyl-8-C-glycosylapigenin with potent antioxidant capacity [10], neohesperidin with strongest ROS scavenging capacity and anti-ageing effect [12], beta-sitosterol protection against 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis [13] are strongest natural antioxidant agents as indicated with references above. The presence of these polyphenols in the study material could also support the therapeutic property of *L. aspera* root for mentioned applications in Khare [14].

DPPH and ABTS Radical Scavenging Assay: Oxidative stress as an underlying condition for the onset of central nervous system disorders, cancer, diabetes and/or cardiovascular disease has been under scrutiny. The antioxidants and their role in the prevention and/or control of deleterious health conditions have gained considerable attention. The *Leucas aspera* root extract was assessed for its antioxidant activity *in-vitro* by decreasing the level of reactive oxygen species (ROS).

The percentage of DPPH decolorization is attributed to the hydrogen donating ability of test compounds. Variable DPPH and ABTS activities were recorded when compared to the reference standards ascorbic acid, BHT, and quercetin **Table 2**. Total phenol content is also reported.

TABLE 2: DPPH AND ABTS ANTIOXIDANT ACTIVITIES*. TOTAL PHENOL CONTENT OF *LEUCAS ASPERA* ROOT METHANOL EXTRACTS*

Samples	DPPH assay (IC_{50} value in µg.ml^{-1})	ABTS assay (IC_{50} value(µg.ml^{-1})	Total phenolic Content (GAE in µg.g^{-1} dry wt)
L. aspera root methanol extract	75±1.02	70±2.31	240
Ascorbic acid	64±1.32	56±1.89	
BHT	55±2.02	42±2.56	
Quercetin	23±1.4	15±2.4	

*Values represent the mean (n=3)

Antibacterial Activity: Value-added products for enhancing the value of food items by including an ingredient during processing and/or packaging are attractive. It is received more favorable by the consumer. They could be products such as breakfast cereals or extruded snacks, or ice creams.

Microbial contaminations can make considerable changes in food items and finally affect the quality of these value-added products. Microbial contaminations can adversely affect their shelf life. In contrast, the difference in intrinsic properties of vegetable oils can change the microbial load of vegetable oils. The fatty acid profile, phenolic compounds, tocopherol, and sterol contents of vegetable oils have a critical role in their intrinsic properties [15]. Benzoic acid, nitrites, sulfites as antimicrobial agents are in wide use. The antioxidants used include butylated hydroxyl-toluene (BHT), butylated hydroxyl anisole (BHA), and ascorbic acid. The uses of chemical preservatives are reported to be additive to major health hazardous and toxicity [16]. Thus, the use of natural preservatives has increased and finds acceptance by the consumers as a new approach to overcome on these adverse effects [17-18].

A. Agar-Disc Diffusion Method: In the present study, methanol extract of roots from *L. aspera* was examined for its anti-bacterial activity. Anti-bacterial activity by disc diffusion inhibited human pathogenic bacteria in the order: *Escherichia coli*> *Pseudomonas aeruginosa*>*Klebsiella pneumoniae* >*Staphylococcus aureus*>*Bacillus subtilis.* Zone of inhibition studies by the agar-disc diffusion method exhibited a clear antimicrobial effect of the extract. A statistically significant inhibition of both gram (-) and gram (+) bacteria was recorded **Fig. 2**.

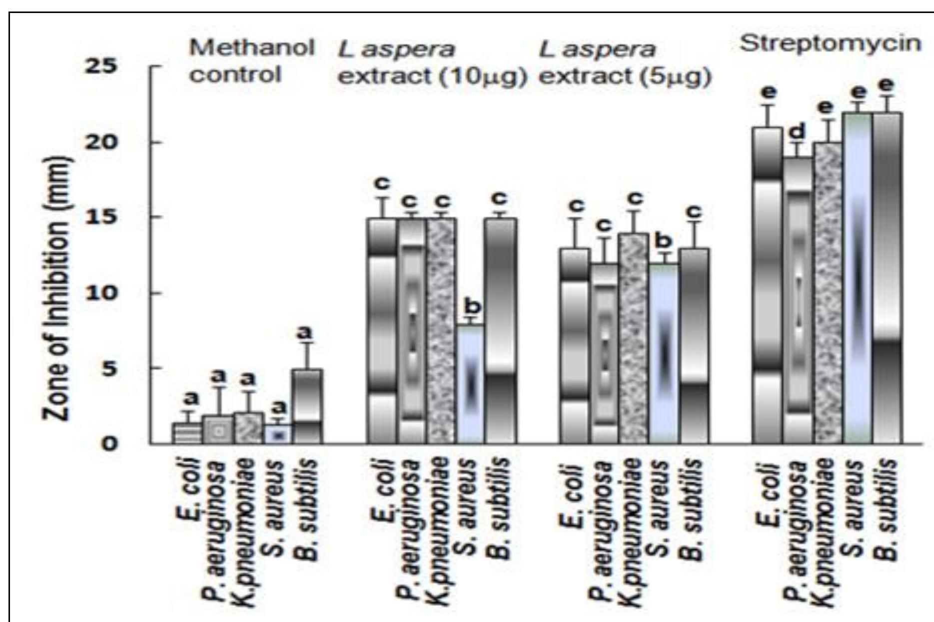

FIG. 2: ANTIBACTERIAL ACTIVITY ASSAYED BY DISC DIFFUSION METHOD AND ITS QUANTITATIVE RESULTS

B. Minimum Inhibitory Concentration: These extracts were assessed for their efficiency on the bacteria by MIC analysis using the broth micro-dilution method. It was observed that the extract was very effective against all the bacterial strains tested in **Table 3**. It could inhibit most of the *E. coli* growth with the best MIC of 103.7µg/mL.

TABLE 3: MINIMAL INHIBITORY CONCENTRATION* (µg/mL)

Samples	*Escherichia coli* (−ve)	*Pseudomonas aeruginosa* (−ve)	*Klebsiella pneumoniae* (−ve)	*Staphylococcus aureus* (+ve)	*Bacillus subtilis* (+ve)
L. aspera root methanol extract (5µg)	135.3[b]	143.6[c]	141.2[c]	148.9[cd]	136.4[b]
L. aspera root methanol extract (10µg)	103.7[a]	104.1[a]	103.6[a]	127.3[a]	110.1[a]
Chloramphenicol	22.9	31.4	15.3	62.1	31.4

*Values represent the mean (n=3)

CLSM (Confocal Laser Scanning Microscopy) Observation of Biofilm: Resistance to anti-microbials has posed an important global health issue, thus resulting in a huge gap between resistance and development of new antimicrobials. It is reported that several plant extracts may have good activity per se. They could also be sources of effective antimicrobial compounds that can act against pathogens' planktonic and/or biofilms.

Gram (+) strain, *Staphylococcus aureus* (ATCC 25923) and gram (-) strain, *Pseudomonas aeruginosa* (ATCC 27853), commonly reported as biofilm-forming strains, were included in this study. From sample observations of controls, *S. aureus* **Fig. 3a** and *P. aeruginosa* **Fig. 3c** formed an uninterrupted biofilm on the support. The addition of the extract (200µL, 750µg) resulted in a loss in the biofilm. CLSM studies visualized a significant biofilm inhibition. Patches and broken down of the biofilm architecture and decreased micro-colonies containing S. aureus Fig. 3b and P. aeruginosa Fig. 3d were recorded.

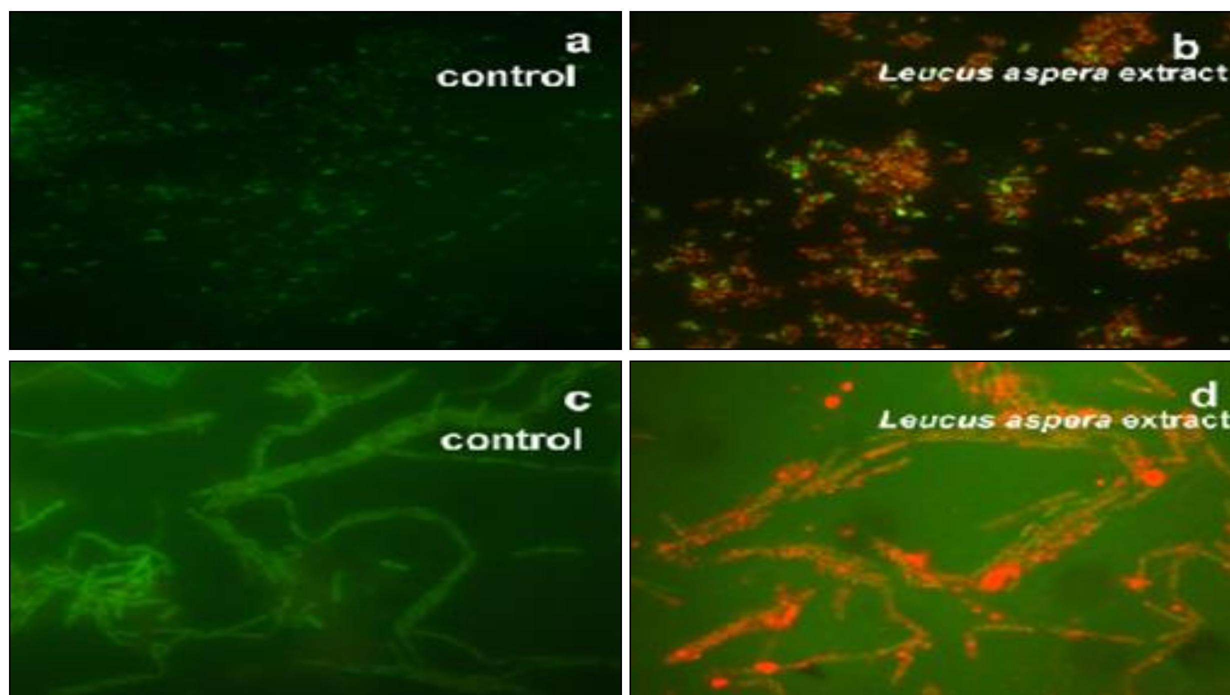

FIG. 3: BIOFILM INHIBITION ASSAY OF *LEUCAS ASPERA* ROOT METHANOL EXTRACT AS ANALYZED BY CONFOCAL LASER SCANNING MICROSCOPY. CONTROL SAMPLES OF TESTED BACTERIA, *STAPHYLOCOCCUS AUREUS* (FIGURE 3A) AND *PSEUDOMONAS AERUGINOSA* (FIGURE 3C) FORMED AN UNINTERRUPTED BIOFILM ON THE SUPPORT. A SIGNIFICANT BIOFILM INHIBITION AS VISUALIZED BY CLSM STUDIES WITH BREAKDOWN OF THE BIOFILM ARCHITECTURE AND WITH DECREASED MICRO-COLONIES ON THE COVER-SLIPS CONTAINING*S. AUREUS* (FIGURE 3B) AND *P. AERUGINOSA* (FIGURE 3D).

Phenols and flavonoids in the plant extracts exhibit anti-bacterial activity [19-20]. A few were identified in the current studies. However, several unknown compounds were still present that were not identified in this study. Thus, more work is required to identify these compounds that will be taken up in the future, revealing a potent anti-bacterial molecule.

DNA Strand Scission Assay: Protecting DNA from damage was investigated. The efficacy of the extracts was tested. The plasmid DNA (pBR322)

was exposed to a Fenton reagent used as a source of hydroxyl radical (30 min at 37°C). The plasmid DNA was damaged by the Fenton reagent resulting in supershift in native double-stranded DNA band (Form I) to single-stranded (as indicated by arrows), nicked DNA (Form II) as visualized in agarose gel electrophoresis **Fig. 4**. In comparison, when plasmid DNA was pre-incubated with 10µg of extract for 30 min and later exposed to Fenton reaction under similar conditions as above, *L. aspera* extract could prevent scission. It offered prominent protection to DNA.

The protective effect of the extract on DNA may be attributed to the presence of phenols and flavonoids, as they can prevent ROS production by complexing cations such as copper and iron that participate in hydroxyl radical formation.

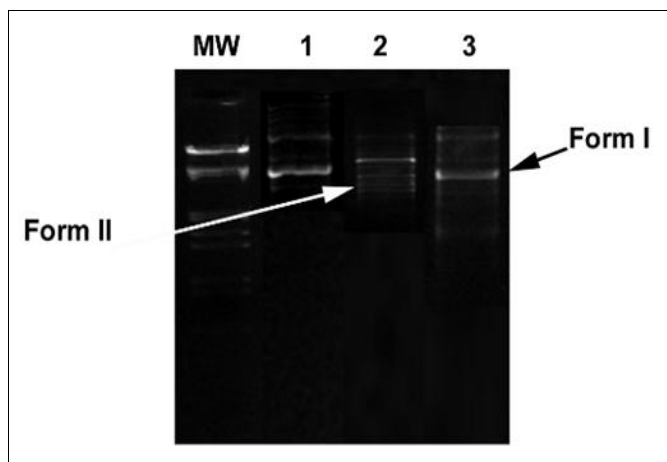

FIG. 4: DNA PROTECTION STUDIES: EFFECT OF *LEUCAS ASPERA* ROOT METHANOL EXTRACT ON THE PROTECTION OF DNA (PLASMID pBR322) DNA STRAND SCISSION INDUCED BY HYDROXYL RADICALS PRODUCED BY FENTON REAGENT. FENTON REAGENT INDUCED A SUPERSHIFT IN NATIVE DOUBLE-STRANDED DNA BAND (FORM I) TO SINGLE-STRANDED), NICKED DNA (FORM II) AS INDICATED BY ARROWS WAS CONFIRMED IN AGAROSE GEL ELECTROPHORESIS. M, DNA LADDER (1 kb); LANE 1, pBR322 (NATIVE PLASMID DNA); LANE 2, pBR322 DNA +FENTON REAGENT; LANE 3, pBR322 + *L. ASPERA* ROOT METHANOL EXTRACT + FENTON REAGENT.

Cytotoxicity Assay: EC_{50} is a useful parameter for quantification of drug effect on cell survival. The effect of increasing the concentration of extracts on the cytotoxicity to MCF-7 cells was measured by MTT assay. A dose-dependent curve identified the EC_{50} value of 6.31±2.71 $mg.mL^{-1}$ for the extract. The positive control, camptothecin, assayed for cytotoxicity under similar conditions, exhibited EC_{50} of 1.28 ± 3.11 mg/mL **Table 6**.

TABLE 6: CELL CYTOTOXICITY STUDIES ON MCF-7 CELLS

Samples	EC_{50}
L. aspera root methanol extract	6.31±2.71 $mg.mL^{-1}$
Camptothecin	1.28±3.11 $\mu g.mL^{-1}$

*Data are mean ± *SD* (*n* = 3)

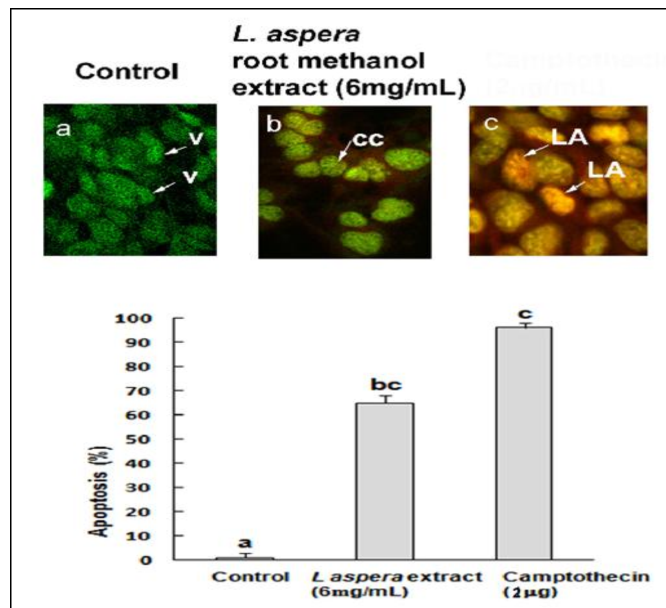

FIG. 5: *LEUCAS ASPERA* ROOT METHANOL EXTRACT AND CAMPTOTHECIN INHIBITED CELL GROWTH AND INDUCTION OF APOPTOTIC NUCLEAR MORPHOLOGY IN MCF-7 CELLS. MCF-7 CELLS WERE TREATED WITH THE INDICATED CONCENTRATIONS OF THE EXTRACT AND CAMPTOTHECIN. THEY WERE STAINED WITH AO/PI DUAL STAINING. V, VIABLE CELLS; CC, CHROMATIN CONDENSATION; LA, LATE APOPTOTIC CELLS ARE INDICATED IN THE REPRESENTATIVE IMAGES (n=3)

***L. aspera* Root Methanol Extract Induces Cell Cytotoxicity to MCF-7 Cells was Visualized by Acridine Orange (AO) – Propidium Iodide (PI) Staining for Morphological Observation of Cell Structure by Confocal Laser Scanning Microscopy (CLSM):** An in-depth study was also carried out by acridine orange(AO)/ propidium iodide (PI) double staining to decipher the mechanistic aspect of cancer cell death and to confirm apoptosis. The emission of green and orange fluorescent wavelengths by AO and PI respectively forms the basis of this assay. PI is a cell impermeable nucleic acid stain and is internalized only when the membrane is compromised. MCF-7 cells were stained with AO and PI dual stain wherein emission of green and orange fluorescent wavelengths by AO, and PI respectively was recorded by Confocal laser scanning microscopy. An increase in orange fluorescing nuclear stain **Fig. 5b, c** indicates

apoptosis with chromatin condensation for F. religiosa extract and camptothecin compared to controls Fig. 5a visualized green (indicating live cells) observed. Chromatin condensation confirms MCF-7 cell cytotoxicity by extract to be an apoptotic event. Chromatin condensation and DNA fragmentation by caspase-activated endonucleases are characteristics of apoptosis [21-22]. These events are observed during late apoptosis following caspases' activation and nuclear DNA degradation into nucleosomal units [23-24].

CONCLUSION: Thus, the knowledge of the signaling pathways along with physiological responses to the natural product, including activity guided fractionated medicinal plant extract, partially or characterized natural molecules, synthesized molecular entities and/or therapeutic drugs, defines the essentials to understanding the mechanism of toxicity. Thus, these studies were incorporated in the analysis of the therapeutic potential of *Leucas aspera* extract.

Hence, the excellent apoptotic and antioxidant activity demonstrated by the extract of *L. aspera* could indicate the potential of this species as the pharmaceutical source.

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CG performed the extraction and antioxidant study and analyzed the data. SS performed the antibacterial and MCF-7 cell cytotoxicity assays. Both drafted the manuscript and edited the same. SS planned and supervised the work and finalized the manuscript. Both the authors approved the final version of the manuscript.

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REFERENCES:

1. Kumar GV and Devanna N: An update of Leucas aspera- a medicinal plant. International Journal of Science and Research Methodology 2016; 5:1-19.
2. Choi JY, Desta KT, Lee SJ, Kim YH, Shin SC, Kim GS, Lee SJ, Shim JH, Hacımüftüoğlu A and Abd El-Aty AM: LC-MS/MS profiling of polyphenol-enriched leaf, stem and root extracts of Korean *Humulus japonicus* Siebold &Zucc and determination of their antioxidant effects. Biomedmedical Chromatography 2018; 32: 1-8.
3. Kumar BR: Application of HPLC and ESI-MS techniques in the analysis of phenolic acids and flavonoids from green leafy vegetables (GLVs). Journal of Pharmaceutical Analysis 2017; 7: 349-64.
4. Torre MP, Cavero RY, Calvo MI and Vizmanos JL. A Simple and a reliable method to quantify antioxidant activity *in-vivo*. Antioxidants 2019, 8: 1-11.
5. More GK and Makola RT: *In-vitro* analysis of free radical scavenging activities and suppression of LPS-induced ROS production in macrophage cells by *Solanum sisymbriifolium* extracts. Scientific Reports 2020; 10: 1-9.
6. Shivamallu A and Shailasree S: *Delonix regiaboj*. Ex. Hook raffin; family: Fabaceae, bark methanol extract phytochemical profile and potential therapeutic evaluation. Asian Journal of Pharmaceutical and Clinical Research 2020; 13: 100-05.
7. Klanˇcnik A, Piskernik S, Jeršek B and Možina SS: Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. Journal of Microbiological Methods 2010; 81: 121-26.
8. Zhang W, Wang Y, Tian RM, Bougouffa S, Yang B, Cao HL, Zhang G, Wong YH, Xu W, Batang Z, Suwailem AS, Zhang XX and Qian PY: Species sorting during biofilm assembly by artificial substrates deployed in a cold seep system. Scientific Reports 2015; 4: 6647-54.
9. Lee JC, Kim HR, Kim J and Jang YS: Antioxidant activity of ethanol extract of the stem of *Opuntia ficus-indica* var. saboten. Journal of Agriculture and. Food Chemistry 2002; 50: 1619-24.
10. Podio NS, Baroni MV, Pérez GT and Wunderlin DA. Assessment of bioactive compounds and their *in vitro* bioaccessibility in whole-wheat flour pasta. Food and Chemistry 2019; 293: 408-17.
11. Peterson J and Dwyer J: Flavonoids: dietary occurrence and biochemical activity. Nutritional Research 1998; 18: 1995-98.
12. Guo C, Zhang H, Guan X and Zhou Z: The anti-aging potential of neohesperidin and its synergistic effects with other Citrus flavonoids in extending chronological lifespan of *Saccharomyces cerevisiae* BY4742. Molecules 2019; 24: 1-14.
13. Baskar AA, Numair AKS, Paulraj GM, Alsaif MA, Muamar MA and Ignacimuthu S: β-sitosterol prevents lipid peroxidation and improves antioxidant status and histoarchitecture in rats with 1,2-dimethylhydrazine-induced colon cancer. Journal of Medicine and Food 2012; 15: 335-43.
14. Khare CP: Indian Medicinal Plants, An Illustrated Dictionary, 1st ed. Springer, New Delhi. 2007; 812.
15. Kalogianni AI, ThomaiLazou T, Bossis I and Gelasakis AI: Natural phenolic compounds for the control of oxidation, bacterial spoilage and foodborne pathogens in meat. Foods 2020; 9: 1-28.
16. Bhaskar A, Sekhar S and Javaraiah R; Antibiofilm and anti-inflammatory potential of lignans with collegial effect: Secoisolariciresinol diglucoside and sesamin as antimicrobial sources. Journal of Applied Biology and Biotechnology 2020; 8: 45-51.
17. Mei J, Ma X and Xie J: Review on natural preservatives for extending fish shelf life. Foods 2019; 8: 1-28.

18. Quinto EJ, Caro I, Villalobos-Delgado LH, Mateo J, De-Mateo-Silleras B and Redondo-Del-Río MP: Food safety through natural antimicrobials. Antibiotics 2019; 8: 1-19.
19. Panche AN, Diwan AD and Chandra SR: Flavonoids: an overview. Journal of Nutritional Science 2016; 5: 1-15.
20. Tungmunnithum D, Thongboonyou A, Pholboon A and Yangsabai A: Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: an overview. Medicines 2018; 5: 1-16.
21. Sekhar S: Caspases, significance in apoptosis. Research & Reviews in Pharmacy and Pharmaceutical Sciences 2016: 5: 104-105.
22. Saqib A, Pattar S, Karigar CS and Sekhar S: Caspase activators: phytochemicals with apoptotic properties targeting cancer, a health care strategy to combat this disease. Indian Journal of Pharmaceutical Education and Research 2020: 54: 43-55.
23. Jan R and Chaudhry GS: Understanding apoptosis and apoptotic pathways targeted cancer therapeutics. Advances in Pharmaceutical Bulletin 2019; 9: 205-18.
24. Azzwali AAAA and Azab AE: Mechanisms of programmed cell death. Journal of Applied Biotechnology and Bioengineern 2019; 6: 156-58.

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