DELONIX REGIA BOJ. EX. HOOK RAFFIN; FAMILY: FABACEAE, BARK METHANOL EXTRACT PHYTOCHEMICAL PROFILE AND POTENTIAL THERAPEUTIC EVALUATION

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ABSTRACT

Objectives: The aim of this study was to evaluate the antioxidant, anti-inflammatory, and anti-cancer potencies of the *Delonix regia* bark, a first of its kind.

Methods: The bark was extracted sequentially in Soxhlet apparatus with hexane, chloroform, and methanol in the increasing order of polarity. These extracts were subjected to find its antioxidant activity and total phenol content. Antibacterial activity against human pathogenic bacteria was tested. The anti-inflammatory properties were elucidated by its capacity to inhibit 15-lipoxygenase (LOX) and human cyclooxygenase (COX)-2. Cell cytotoxic capacity was evaluated against MCF-7 cells breast cancer cell lines.

Results: Liquid chromatography (LC)-Mass Spectroscopy (MS) fingerprint of the methanol extract identified a total of 14 polyphenols, of which five were structurally characterized based on their mass-charge ratio $[M-H]^-$ peak, UV-vis absorption in comparison to published data. Antibacterial activity by disk diffusion inhibited human pathogenic bacteria. Bacterial biofilm inhibition capacity of extract (750 µg) imaged by confocal laser scanning microscopy revealed loss of microcolonies. Extract when tested for 15-LOX inhibition exhibited IC_{50} values of 94.5 ± 1.23 µg.mL^{-1} by enzyme kinetics studies using spectrophotometric techniques. Similarly, it could inhibit COX-2 enzyme at relatively lower concentrations (32.18 ± 1.91 µg.mL^{-1}). Further, it quenched free radicals produced by Fentons' reagent studied by DNS-nicking assay indicating its strong antioxidant property with the capacity to protect DNA. *In vitro* cytotoxicity was evaluated by 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphynyl tetrazolium bromide assay and apoptosis induced in MCF-7 cells was assessed morphologically.

Conclusion: Our data suggest that *D. regia* bark methanol extract exerts its therapeutic activity for further pharmaceutical evaluations. Further studies are necessary to determine the mechanisms of these pharmacological properties.

Keywords: LC analysis in negative mode, Antioxidant, 15-LOX, COX-2, Biofilm by Confocal laser scanning microscopy, MCF-7 cells cytotoxicity.

INTRODUCTION

Delonix regia, (Bojer. Ex. Hook) Raffin (Family: Fabaceae - Caesalpinioideae) (synonyms – *Poinciana regai*, Bojer ex Hook.), is known to common man as Gul mohar, It is a medium sized ornamental tree grown in parks, gardens, and road side and has yellow-brown wood. It is also called a flamboyant or flame ornamental tree. The Indian Medicinal Plant Dictionary describes the white bark with traditional application as febrifuge and in healing wounds with anti-rheumatic and spasmogenic capacity. The aqueous and alcoholic flower extracts were used for roundworm removal [1]. Some of the properties reported for bark include relieving knee pain, emetic effect, as bio-monitors, and bio-accumulants of atmospheric trace metals. A 5% chloroform was reported to have germicidal activity. The seeds were used in pyorrhea. The bark, fruits, and leaves have been used as anti-helminthic, reducing rheumatic pain and as purgatives in Pirojpur district, Bangladesh. The people of Yanadi (a tribal community in Andhra Pradesh, India) used flowers of *Delonix regia* in the treatment of dysmenorrhea [2,3].

Human body metabolism generates free radicals as part of the normal metabolic process in the respiratory chain of mitochondrial mainly from drugs, xenobiotics, pollutants from atmosphere and metal (transitional) catalysts. An uncontrolled production of oxygen derived free radicals is associated with the onset of many diseases such as inflammation, cancer, rheumatoid arthritis, cirrhosis, arteriosclerosis, and degenerative processes associated with aging. Scavenging of reactive oxygen species (ROS) provides protection to DNA, proteins, and lipids reducing risk of chronic diseases. The synthetic antioxidants used include butylated hydroxyl toluene (BHT), butylated hydroxyl anisole, and ascorbic acid.

Value added products for enhancing the value of items of food by inclusion of an ingredient during processing and/or packaging is found to be 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. Benzoic acid, nitrites, and sulfites as antimicrobial agents are in wide use.

However, the uses of chemical preservatives are reported to be additive to major health hazardous and toxicity [4]. Thus, the use of natural preservatives has increased and finds acceptance by the consumers as a new approach to overcome on these adverse effects [4,5].

Antioxidant and antimicrobial constituents in plant spices and herb tissues in bark, seed, roots, and flowers, among other parts provide these natural antioxidant and antimicrobial constituents.

Hence, the aim of this study was to evaluate the antioxidant, anti-inflammatory, and anticancer potencies of the *D. regia* bark, a first of its kind. The polyphenols finger print of extract was carried out to identify confidently the polyphenols of the extracts. A differential concentration 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. Other parameters screened were its possible anti-inflammatory activity, evaluated by its efficacy to inhibit 15-lipoxygenase (15-LOX) and human cyclooxygenase-2 (COX-2). The DNA protection property was evaluated by DNS-nicking assay using Fenton's reagent. Cell cytotoxicity studies by 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphynyl tetrazolium bromide (MTT) assay and apoptosis induced were assessed morphologically by combined acridine orange and ethidium bromide staining for detecting incidence of cell cytotoxicity.

METHODS

Plants
The fresh bark (50 g) of test plant was collected during winter (January, 2019) from Kigga region of Western Ghats in Chikmagalur District of Karnataka State, and a herbarium specimen was deposited at Institution of Excellence (*Delonix regia* bark # IOE LP0015). Fresh bark was washed, shade-dried, ground to a powder, and 100 g of powder were extracted 3 times by hexane, chloroform, and methanol (1:10 w/v) at room temperature, evaporated to yield dry extracts in SpeedVac (Savant SPD 2010, Thermo Scientific), and stored under dark at 4°C until further use.

Chemicals and reagents
Trypsin, chloramphenicol, acridine orange, and ethidium bromide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Linoleic acid, (+)-catechin, Luteolin, and 15-lipoxygenase (soybean) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards quercetin, ascorbic acid (AA), and BHT were purchased from HiMedia (India). Nonessential amino acid, sodium bicarbonate, Eagle minimum essential medium, and L-glutamine were purchased from HiMedia (India). StepupTM 1kb DNA ladder and plasmid (pBR322) were from Merck Biosciences, Bengaluru (India). COX-2 inhibition kit was obtained from Cayman Chemicals, Ann Arbor, MI, USA. Minimum essential medium Eagle, sodium bicarbonate, L-glutamine, and non-essential amino acid were purchased from HiMedia (India). pBR322, plasmid and StepupTM (1kb) DNA ladder were obtained from Merck Biosciences, Bengaluru (India).

Estimation of total phenolic content and free-radical scavenging capacity of the extract
The total phenol content of the sample was estimated using the Folin–Ciocalteu colorimetric method [6]. The methanol extracts (50, 100, 250, and 500 µg.mL^{-1}, 1, 5, and 10 mg.mL^{-1}) were evaluated for their antioxidant activity. Two methods used were DPPH. scavenging assay and 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS·+) assay [7,8]. The results were compared with the most commonly used synthetic antioxidants BHT, quercetin, and AA. The antioxidant activity was expressed as IC$_{50}$ values.

Bacterial strains and culture conditions
The Institute of Microbial Technology, Chandigarh, India, kindly provided the Gram (−) bacteria, *Klebsiella pneumoniae* (MTCC 661), *Escherichia coli* (MTCC 724), *Pseudomonas aeruginosa* (ATCC 27853), and Gram (+) bacteria, namely, *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (MTCC 441). Bacterial inoculums were prepared by growing cells in Mueller-Hinton Broth (MHA) (HiMedia, 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
Agar–disk diffusion method
Disk diffusion was carried out with the above said samples. An aliquot (5 and 10µL; 1 mg.mL^{-1}) was introduced on the disks (0.7 cm; HiMedia) for this experiment. The diameter of inhibition zones was recorded and was compared against streptomycin (positive standard; 3 µL of 1 mg.mL^{-1}; Sigma-Aldrich St. Louis, MO, USA) and methanol (negative standard; 10 µL) under similar experimental conditions.

Minimum inhibitory concentration (MIC)
Micro-dilution methods of broth were used for measuring MIC. Muller-Hinton Broth (MHB; Merck Biosciences, Bangalore, India) was used [9]. A two-fold serially diluted extract (50 µL) of *D. regia* extract in methanol with concentration range of 10, 5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, and 0.0390605 mg.mL^{-1} was added to the 96-well micro-titer plate. The bacterial suspension (50 µL) in MHB (10^4 CFU.mL^{-1}) was introduced. Control wells include wells with culture medium (sterility control) and the extracts. Wells with methanol were the positive control. MHB (50 µL) wells were negative control. Bacterial suspension in MHB (50 µL) was 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. It is the results produced by addition of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), and dissolved in water (10µL of INT, 2 mg.mL^{-1}). The plates were incubated in the dark (37°C for 30 min) after addition of INT. All measurements of MIC values were repeated in triplicate. Chloramphenicol was used as positive control.

Confocal laser scanning microscopy (CLSM) study to assay loss of biofilm
To observe the biofilm structure, CLSM, LSM 710 (Carl Zeiss, Germany) was used. The samples were prepared according to Zhang *et al.* [10]. The overnight cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were prepared with the absorbance set at 1.5 ± 2.0 (600 nm). The assay was conducted in a 6-well plate. Cover slips were placed in all the wells and above the cover slip about 2 ml of the media was added. The cover slip with media was maintained as control. To the test wells, overnight bacterial suspensions (750 µl) were introduced. The extracts (200 µL, 750 µg) were added. The plates were incubated at 37°C for 24 h. The biofilm formed on cover slips in wells were removed carefully. The biofilm was washed with PBS. The film was stained with ethidium bromide (20 µL; 1 mg in 400 µL; HiMedia, India). After three washes, the cover slips with stained biofilm were placed on glass slide in an inverted position. CLSM images of the samples were imaged and results were presented.

Anti-inflammatory activity
Lipoxygenase (15-LOX) Inhibition
A spectrophotometric assay for the determination of soybean 15-LOX (5 µg; Sigma- Aldrich, St. Louis, MO, USA) activity with 0.2 µM linoleic acid (substrate; Sigma-Aldrich, St. Louis, MO, USA) in buffer (0.2 M borate buffer (pH 9.0) was carried out. The extracts at 10, 25, 50, 75, and 100 µg.mL^{-1} were used for inhibition studies. The values of hydroperoxide content were estimated. The LOX activity was calculated as reported earlier [11]. The results were compared with AA, BHT, and quercetin, the most potent LOX inhibitors.

COX-2 inhibition
COX-2 inhibition kit was obtained from Cayman Chemicals, Ann Arbor, MI, USA. COX-2 inhibition by the extract was measured using a COX-2 inhibitor (human) screening kit (Cayman, Ann Arbor, MI, USA) by colorimetric estimation. The samples (100 µg) of the extract were used for inhibition studies as per manufacturer's protocol. The absorbance was read at 415 nm in a micro-titer plate reader (Varioskan Flash with SkanIt) with Software RE 2.4.3. The results were compared with AA, BHT, and quercetin, the most potent LOX inhibitors.

DNA strand scission assay
A DNA-strand scission assay was performed using super-coiled plasmid pBR322 (Merck Biosciences, Bengaluru, India) [12]. Mixture of 5 µl of extract (1 mg/ml) and 2 µl of plasmid were added to 5 µl of Fenton's reagent (30 mM H$_2$O$_2$, 50 µM ascorbic acid and 80 µM FeCl$_3$). The final volume was made up to 15 µl 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 (BioRad, USA).

Liquid chromatography (LC) analysis of methanol extract in negative mode

Synapt G2 (UPLC separations with QuanTof) was used for the qualitative analysis of the metabolites [13] according to manufacturer's protocol. The nebulizer pressure was 60 psi at a drying temperature of 350°C and the nitrogen flow rate 10 L/min was used. The methanol bark extract was filtered (0.2 micron syringe filters, Millipore, U.S.A) and an aliquot (5 μl) was injected into the system. The m/z 100–1000 in negative ionization mode mass spectra were acquired. The collision gas helium was used for the fragmentation in the ion trap of the isolated compounds. The detection conditions were as follows: Capillary voltage, 3500 V; skimmer voltage, –40 V; cap exit voltage, –158.5 V; Oct 1 DC, –12 V; Oct 2 DC, –2.45 V; trap drive level, 45.0; Oct RF, 150 Vpp; Lens 1, 5.0 V; and Lens 2, 60 V.

Cell cytotoxicity assay
MTT assay

MCF-7 (breast cancer cell lines) were maintained in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's salts. The cytotoxic evaluation was performed by MTT method. Trypsinized cells were washed with culture medium. Briefly, the cell cultures (5×10^5 cells.mL^{-1}) were prepared in a 96-well flat-bottomed plate and incubated for 48 h at 37°C in humidified 5% CO_2. Methanol extract (2, 4, 6, 8, 10, or 15 mg/mL filtered through 0.22-μm filters) and camptothecin (0.1, 1, 5, and 10 μg/mL) were added to the appropriate wells and incubated (48 h). MTT dissolved in PBS (5 mg/mL; 10 μL) was added to each well. They were incubated in dark (4 h). The supernatants were aspirated from the wells. Further washes were with Dulbecco's formula phosphate-buffered saline (PBS, pH 7.3). For solubilization of the resultant formazan product, DMSO (100 μL) and 25 μL of 0.1 M glycine buffer (pH 10.5) were added, followed by incubation (4 h at 37°C) and the absorbance (570 nm) was measured at (VarioskanTM Flash Multimode Reader, Thermo Scientific). Controls consisted of cells at same concentration. The IC$_{50}$ for sample was obtained from dose-effect curves (not shown) an average of three assays with six concentrations was reported.

Cytotoxicity of *D. regia* extract on MCF-7 cells visualization by acridine orange (AO) – ethidium bromide (EtBr) staining

The analysis of morphological changes to assess the cell death in the *in vivo* treated MCF-7 cells was performed using AO and EtBr combo-stain. Harvested MCF-7 cells (48 h) from control, *D. regia* bark methanol extract (6 mg.mL^{-1}) and camptothecin (1 μg.mL^{-1}) were washed with PBS washed 3 times in PBS (clears excess of staining components). Control consisted of cells with no treatment. The AO and EtBr stain (25 μL; 1 mg.mL^{-1} with 0.1% acetic acid and methanol) was added to the cell pellet in PBS (100 μL). The cells were placed on a microscopic slide. It was covered with a cover slip and sealed. The fluorescence was recorded using CLSM, LSM 710 (Carl Zeiss, Germany).

Statistical analysis

All experiments and measurements were made in triplicate. The values are expressed as the mean±S.E.M. The results were subjected to variance analysis followed by Tukeys' test to analyze differences between the *D. regia* extract and control conditions. Statistically significant differences (p<0.001) were shown.

RESULTS

LC analysis of methanol extract in negative mode

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 and published data [13]. (+)-catechin and (+)-Luteolin were used as standards in this experiment. The presence of unknown phytoconstituents, 1-caffeoylquinic acid, 3-Caffeoylquinic acid, Luteolin dihexoside, 6,8-C-C-diglucosyl apigenin, and diosmetin 7-O-diglucoside was identified. Table 1 and Fig. 1 summarize the LC-MS data and phytoconstituents of *D. regia* extract details.

DPPH and ABTS radical scavenging assay

The percentage of DPPH decolorization is attributed to 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 (252 ± 3.231 μg/g dry weight).

Antibacterial activity
Agar-disk diffusion method

Zone of inhibition studies by agar-disk diffusion method exhibited a clear antimicrobial effect of the extract. A statistically significant inhibition of both gram (−) and gram (+) bacteria was recorded (Fig. 2).

MIC

These extracts were assessed for their efficiency on the bacteria by MIC analysis by broth microdilution method. It was observed that the extract was very effective against all the bacterial strains tested (Table 3). It could inhibit most of the *E. coli* growth with the best MIC of 103.7 μg.mL^{-1}.
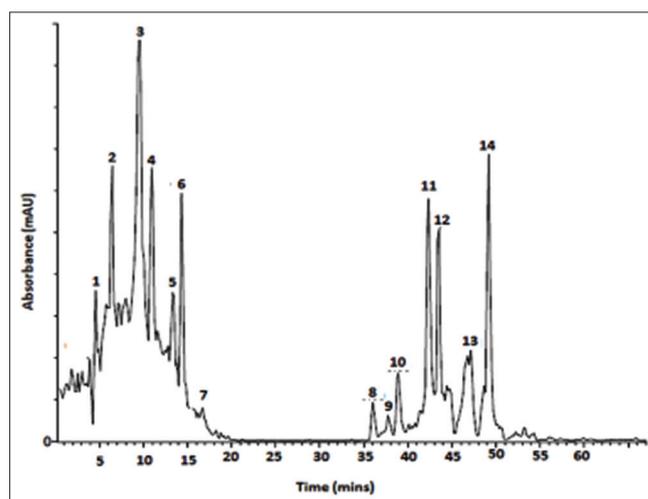

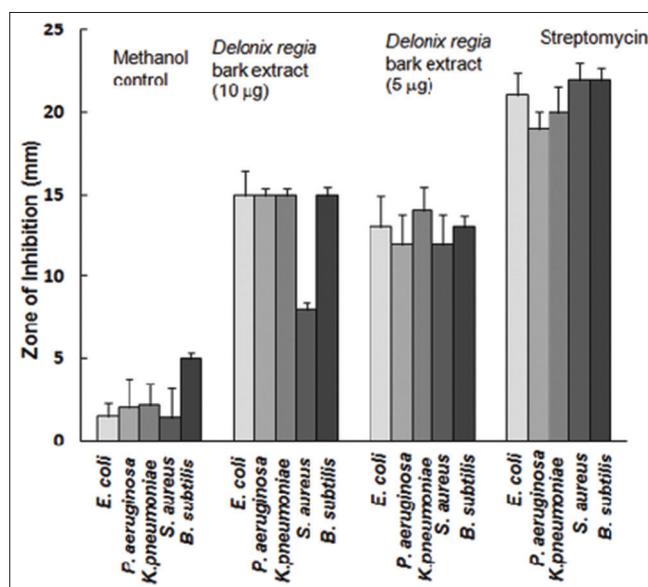**Fig. 1: LC analysis of *Delonix regia* barks methanol extract in negative mode.**

Fig. 2: Antibacterial activity assayed by disk diffusion method and its quantitative results. *Values are expressed as the mean±SEM (Standard Error Mean) (n=3): *Indicates p<0.001

CLSM observation of biofilm

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 biofilm on the support. The addition of the extract (200 µL, 750 µg) resulted in a loss in the biofilm. A significant biofilm inhibition was visualized by CLSM studies. Patches and broken down of the biofilm architecture and with decreased micro-colonies containing *S. aureus (Fig. 3b)* and *P. aeruginosa* (Fig. 3d) was recorded.

Anti-inflammatory activity

Lipoxygenase (15-LOX) Inhibition

The samples could inhibit 15-LOX to various capacities (Table 4). Of the reference standards, quercetin exhibited potent capacity inhibiting with IC_{50} 3.7±0.22 µg.ml⁻¹ followed by ascorbic acid and BHT. The *D. regia* extract exhibited IC_{50} of 114.5±0.53 µg.ml⁻¹ nearer to reference standard BHT indicating potent 15-LOX inhibition (Table 4).

COX-2 Inhibition

Inhibitory COX-2 effect (IC_{50}) of *D. regia* bark extract was at 62.18±3.14 µg.mL⁻¹ by *in vitro* enzymatic activities measured against human COX-2. However, the standards exhibited a lower IC_{50} values indicating their capacity to inhibit COX-2 (Table 5).

DNA strand scission assay for DNA protection studies

Protecting DNA from damage was investigated. The efficacy of the extracts was tested. The plasmid DNA (pBR322) was exposed to Fenton reagent used as source of hydroxyl radical (30 min at 37°C). Fenton reagent induced a super-shift 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. The plasmid DNA was damaged by Fenton reagent resulting in super shift 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 10 µg of extract for 30 min and later exposed to Fenton reaction under similar conditions as above, *D. regia* extract could prevent scission. It offered prominent protection to DNA.

Table 1: LC fingerprint and phytochemicals identified from *Delonix regia* bark methanol extract (n=3)

Peak no.	Compound	Retention time (min)	References
1	unknown	5.19	[13]
2	Unknown	7.94	[20]
3	1-caffeoylquinic acid	10.34	[13]
4	3-Caffeoylquinic acid	10.84	[21]
5	Luteolin dihexoside	13.76	[21]
6	(+)-catechin (STD)	14.93	[13]
7	6,8-C-C-diglucosyl apigenin	15.79	[21]
8	Diosmetin 7-O-diglucoside	35.26	[21]
9	Luteolin (STD)	36.92	[13]
10	unknown	39.59	[13]
11	unknown	42.26	[13]
12	unknown	43.12	[13]
13	unknown	46.14	[13]
14	unknown	49.79	[13]

*The identification of phytoconstituents was verified from three independent LC-MS spectra of the extract and is presented in the table.
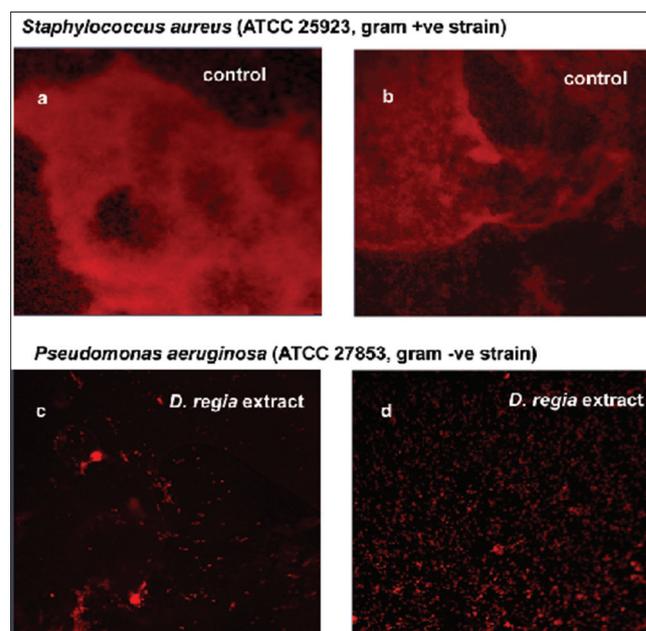

Fig. 3: Biofilm inhibition assay of *Delonix regia* bark methanol extract. Controls, *Staphylococcus aureus* (a); *Pseudomonas aeruginosa* (c). *D. regia* + *S. aureus* (b); *D. regia* + *P. aeruginosa* (d). The data are representative of three independent experiments (n=3).

Table 2: DPPH and ABTS antioxidant activities* (n=3). Total phenol content of *Delonix regia* bark methanol extracts (n=3)

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)
D. regia bark methanol extract	81±2.12	69±1.23	252±3.231
Ascorbic acid	66±3.12	59±2.09	
BHT	58±1.02	47±1.06	
Quercetin	28±1.32	19±2.03	

*Values are expressed as the mean±SEM (n=3): *indicates p<0.001

Table 3: Minimal inhibitory concentration* (µg.mL⁻¹)

Samples	*Escherichia coli* (−ve)	*Pseudomonas aeruginosa* (−ve)	*Klebsiella pneumoniae* (−ve)	*Staphylococcus aureus* (+ve)	*Bacillus subtilis* (+ve)
D. regia bark methanol extract (5 mg)	143.2[b]	147.72[c]	148.36[c]	153.82[cd]	142.32[b]
D. regia bark methanol extract (10 mg)	103.7[a]	114.06[a]	133.14[a]	131.14[a]	116.28[a]
Chloramphenicol	25.42	36.21	16.23	64.18	36.14

*Values are expressed as the mean±SEM (n=3): *Indicates p<0.001, one-way ANOVA, followed by Tukeys' test

M, DNA ladder (1 kb); Lane 1, pBR322 (native plasmid DNA); Lane 2, pBR322 DNA +Fenton reagent; Lane 3, pBR322 + *D. regia* bark methanol extract + Fenton reagent.

Cytotoxicity assay

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

Cytotoxicity of *D. regia* extract on MCF-7 cells visualization by acridine orange (AO) – ethidium bromide (EtBr) staining for morphological observation of cell structure by CLSM

MCF-7 cells, control groups, and post-treatment with *D. regia* extract were stained with combo of acridine orange (AO) and ethidium bromide (Etbr). The cell morphology was captured by a CLSM microscope. Control cells did not exhibit significant morphological changes and were green. However, the cells from the *D. regia* and camptothecin groups showed the signs loss of cell morphology and an uptake of Etbr indicating a loss of cell membrane morphology. The camptothecin (1 mg.mL^{-1}) treatment resulted in formation of fragmented nuclei with condensed chromatin (Fig. 5). *D. regia* (6 mg.mL^{-1}) treatment significantly decreased cell viability by 64.7% inducing apoptosis.

DISCUSSION

Quality control of plant extracts involving its standardization is an important topic, in particular, when such extracts are used for medicinal purposes [14]. The negative ion mode ESI-MS is particularly effective for characterization of plant extracts [13,15]. Polyphenols, the secondary metabolites with health benefits have been center of several studies as they cannot be synthesized by human [15]. Fingerprint profiling *D. regia* bark methanol extract for polyphenols in the present study identified the presence of several molecules and six could be identified and reported [16]. 1-caffeoylquinic acid and 3-Caffeoylquinic acid prevent

Table 4: Inhibition of 15-lipoxygenase* (n=3)

Samples	15-Lipoxygenase inhibition (IC_{50}, µg.mL^{-1})
D. regia bark methanol extract	114.5±0.53
Ascorbic acid	7.58±2.41
BHT	90.37±1.32
Quercetin	3.7±0.22

*Values are expressed as the mean±SEM (n=3): *Indicates p<0.001, one-way ANOVA, followed by Tukeys' test.

Table 5: Inhibition of cyclooxygenase-2* assayed by human COX-2 inhibitor screening kit (Cayman, Ann Arbor, MI, USA) by colorimetric estimation (n=2)

Samples	COX-2 inhibition (IC_{50}, µg.mL^{-1})
D. regia bark methanol extract	62.18±3.14
Ascorbic acid	21.1±2.11
BHT	5.65±2.02
Quercetin	15.65±3.11

*Values are expressed as the mean±SEM (n=3): *Indicates p<0.001, one-way ANOVA, followed by Tukeys' test.

Table 6: Cell cytotoxicity studies on MCF-7 cells

Samples	IC_{50}
D. regia bark methanol extract	6.31±2.71 mg.mL^{-1}
Camptothecin	1.28±3.11 µg.mL^{-1}

*Values are expressed as the mean±SEM (n=3): *Indicates p<0.001, one-way ANOVA, followed by Tukeys' test.

cell proliferation of carcinoma cells such as adenocarcinoma gastric (AGS) cell line [17], Luteolin dihexoside, a flavonoid, and suppresses cell survival pathways such as phosphatidylinositol 3′-kinase (PI3K)/ Akt, nuclear factor kappa B (NF-κB), and X-linked inhibitor of apoptosis protein (XIAP). It stimulates apoptotic pathways including those that induce the tumor suppressor p53 [18], Diosmetin 7-O-diglucoside protects against carcinogenesis [19] 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 *D. regia* bark for mentioned applications in Khare [3]. The previous studies have demonstrated antioxidant capacity of *D. regia* leaves extracts [1,2]. However, to date, no studies have extensively investigated the bioactivity of extracts of *D. regia* bark in terms of broad range pharmacological therapeutic capacity and elucidation of its mechanism.

In the present study, Soxhlet methanolic extract of bark from *D. regia* was examined for its activity. It quenched free radicals produced by Fentons' reagent studied by DNA-nicking assay indicating its strong antioxidant property with the capacity to protect DNA. *In vitro* cytotoxicity was evaluated by MTT assay and apoptosis induction was assessed morphologically. Thus, it had the capacity to suppress ROS and exhibited cytotoxicity to the tested cancer cell lines. Extract when tested for 15-LOX inhibition exhibited IC_{50} values of 94.5±1.23 by enzyme kinetics studies using spectrophotometric techniques. Similarly, it could inhibit COX-2 enzyme at relatively lower concentrations.

Antibacterial activity by disk diffusion inhibited human pathogenic bacteria. Bacterial biofilm inhibition capacity of extract (750 µg) imaged by CLSM revealed loss of microcolonies.
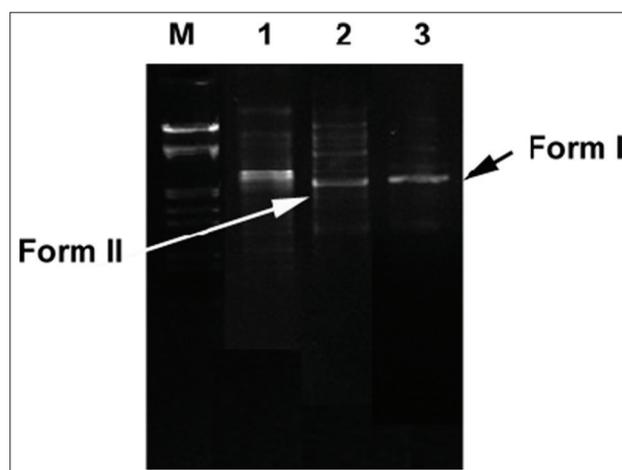

Fig. 4: DNA Strand Scission Assay for DNA protection studies. The data are representative of three independent experiments (n=3).
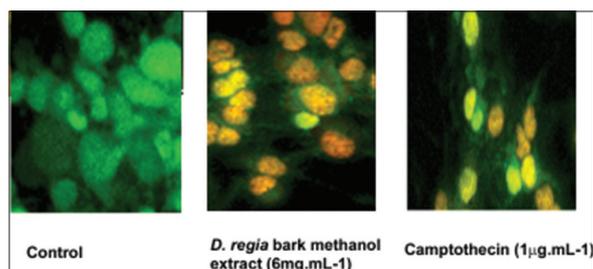

Fig. 5: Cell cytotoxicity analysis of MCF-7 cells. MCF-cells (control cells), MCF-7 cells + *D. regia* bark methanol extract (6mg.mL^{-1}) and MCF-7 cells + camptothecin (1 microg.mL^{-1}). Apoptotic characteristics of chromatin condensation were observed. The data are representative of three independent experiments (n=3).

Bacterial infections lead to pneumonia and tuberculosis, common respiratory tract infection in humans leading to an inflammatory condition of parenchyma tissue of lung. It is caused due to several bacterial pathogens such as *Streptococcus pneumoniae*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*. Pneumonia is currently identified as most widespread infection [22,23]. Finding alternative affordable treatments to local populations has been an important field in ethnopharmacological investigations [24]. Although the traditional use of the extract for antimicrobial activity is known, the need for identification of exact active phytoconstitutents is a requirement for standardizing traditional medicine [25]. Further work for toxicology assessment of the extract and formulations [26] is underway.

The selected plant had many proven therapeutic properties and, possibly, successively united onto the sort of potential therapeutic plants. Besides, isolation and discoveries will lead to the detection of certain novel compounds, which will be of potential medicinal value.

CONCLUSION

Taken together, our data suggest that *D. regia* bark methanol extract might exert its antioxidant activity, anti-inflammatory, and anticancer activity. Any study of the properties of medicinal plants should consider the qualitative and quantitative composition of phytochemicals present in the raw material. Its metabolism, bioavailability, absorption, and distribution on intake could be the next step for development of drugs from vast plant resources.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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AUTHOR'S CONTRIBUTIONS

AS performed the extraction and antioxidant study and analyzed the data. SS performed the anti-biofilm 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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