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IN VITRO ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF MARGARITARIA INDICA DALZEL

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Abstract

Margaritaria *indica* Dalzell. belongs to the family Phyllanthaceae, it is commonly known as: *Hennunanne*, *Kaalikudari* in kannada. It is distributed in Western Ghats at moist deciduous forests, up to 700 m altitude. Possible medicinal and economic value. Limited information is known about the medicinal properties of *Margaritaria indica*, Traditionally, Used to treat gastrointestinal problems, wound healing, fever and Malaria, coughs and bronchitis, rashes, itching, and dermatitis, swelling and inflammation.

Introduction

The deleterious effects of excess free radicals, or oxidative stress, have been reported to eventually lead to cell death. The overproduction of reactive oxygen and nitrogen species has been implicated in the development of various chronic inflammatory diseases Antioxidants inhibit the oxidation process, even at relatively low concentrations, and can protect cells against free-radical damage by delaying or preventing the oxidation of proteins, carbohydrates, lipids and DNA . antioxidants have the ability to donate electrons. Antioxidants that break the chain reaction are strong electron donors and react with free radicals before major molecules are damaged Inflammatory reaction, which is in close association with oxidant stress, is the process whereby blood cells and proteins enter tissues in response to injury, infection, stimuli that provoke an immune reaction . Inflammatory reaction is normally a self-limiting event that is beneficial to the organism in that it plays important roles in host defense to potentially dangerous microorganisms Margaritaria indica Dalzell. Belongs to the family Phyllanthaceae literature survey indicated not much work was reported on pharmacogenetic study and screening of Margaritaria Indica Dalzell. Bark extracts for in-vitro antioxidant and anti-inflammatory activities. Hence the present work was focused on the same

Key words: PHARMACOGNOSTIC EVALUATION, IN-VITRO ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF MARGARITARIA INDICA DALZELL.

Literature Review

Adeolu A. Adedapo et'al *Margaritaria discoidea* is a medicinal plant used for the treatment of various body pains in Central,
Eastern and Southern Africa. The aqueous extract of its stem bark was investigated for its anti-inflammatory and analgesic activities in animal models. The extract at 50, 100 and 200mg/kg body weight reduced significantly the formation of oedema induced by carrageenan and histamine. In the acetic acid-induced writhing model, the extract had a good analgesic effect characterized by a reduction in the number of writhes when compared to the control. Similarly, the extract caused dose-dependent decrease of licking time and licking frequency in rats injected with 2.5% formalin. These results were also comparable to those of indomethacin, the reference drug used in this study. Acute toxicity test showed that the plant may be safe for pharmacological uses. This study has provided some justification for the folkloric use of the plant in several communities for conditions such as stomachache, pain and inflammations. Rev. Biol. Trop. 57 (4): 1193-1200. Epub 2009 December 01.

Mehreen Jabeen et'al This research aimed to assess the anti-inflammatory and antioxidant potential of methanol extract of Wilckia maritima, a plant belonging to the family Brassicaceae, which is enriched with natural antioxidants. Qualitative phytochemical studies showed the presence of numerous compounds including glycosides, phenols, triterpenoids, and GC-MS studies revealed the presence of 35 bioactive components, including n-hexadecanoic acid (26.96%), 9,12,15 octadecatrienoic acid (cis) (25.52%), 3,5 di-hydroxy-6-methyl 2,3-di-hydro-4-pyran 4-one (14.35%), and 3-tertiary butyl-4-hydroxy-anisol (11.68%) as major components, which are thought to be responsible for anti-inflammatory and antioxidant potentials of methanol extract of W. maritima., flavonoids, steroids, tannins, and saponins.

John A. Asong et'al Although orthodox medications are available for skin diseases, expensive dermatological services have necessitated the use of medicinal plants as a cheaper alternative. This study evaluated the pharmacological and phytochemical profiles of four medicinal plants (Drimia sanguinea, Elephantorrhiza elephantina, Helichrysum paronychioides, and Senecio longiflorus) used for treating skin diseases. Petroleum ether and 50% methanol extracts of the plants were screened for antimicrobial activity against six microbes: Bacillus cereus, Shigella flexneri, Candida glabrata, Candida krusei, Trichophyton rubrum and Trichophyton tonsurans using the micro-dilution technique.

MATERIALS AND METHODS

Drugs and Chemicals:

All the Drugs and Chemicals were of analytical grade and obtained from S.D.Fine-Chem Ltd, Sigma-Aldrich and Hi-Media Laboratories Table 4.1 gives details of the supplier along with description.

Table List of chemicals used during experiment:

Sl. NO	List of Chemicals	Description
1	Ethanol, Methanol, Gelatin, Dimethylsulfoxide DMSO	S.D.Fine - Chem Ltd, Mumbai
2	Quercetin, Folin-Ciocalteu reagent, Tetracycline HCl Acrylamide- Bisacrylamide, Tetramethylethylenediamine (TEMED), Sodium dodecyl sulfate (SDS), Ammonium persulfate (APS), Acrylamide- Bisacrylamide, Diclofenac sodium	Sigma-Aldrich. Mumbai

3	Gallic acid, Aluminium chloride, Mannitol, Tris HCl, Gelatin,	Hi-Media Laboratories Mumbai

Collection Plant material:

Bark of *Margaritaria indica* Dalzell. were collected from Central Western Ghats of India. The location is Dandeli Uttara kannada, in the month of February, authentication of the plant was done by qualified taxonomist, Dr. Harsha Hegde, Scientist-E, ICMR National institute of traditional medicine, Belagavi. (Annexure 1) A herbarium specimen of the plant was kept in Department of Pharmacognosy, SET's College of Pharmacy, Dharwad. Herbarium accession No. (SETCPD/Ph.cog/herb/87/2024).

Preparation of the extract:

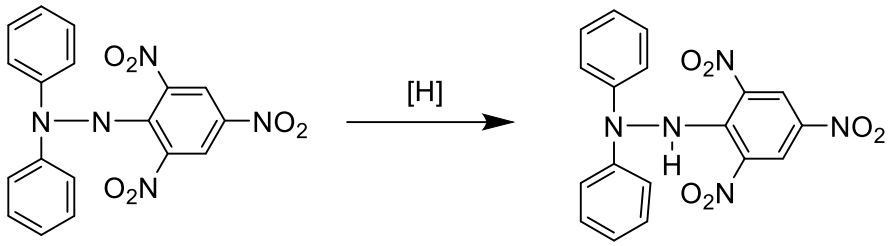
Ethanolic extract: *Margaritaria indica* Dalzell. bark powder was extracted with ethanol in a continuous hot extraction method using Soxhlet apparatus. The extracts were concentrated in a rotary flash evaporator *(Superfit Rotary Vacuuma)* and residue was dried in a vacuum desiccator over anhydrous calcium chloride to yield ethanolic extract [MIBE] The percentage yield of ethanolic extract was calculated.

Aqueous extract: *Margaritaria indica* Dalzell bark powder was macerated with chloroform water I.P. The mixture was filtered through muslin cloth and concentrated in vacuum under reduced pressure using rotary flash evaporator *(Superfit Rotary Vacuuma)* and then the extract was kept on water bath to obtain crude extract and finally residue was dried in a vacuum desiccator over anhydrous calcium chloride to yield aqueous extract [MIBA]. The percentage yield of aqueous extract was calculated.

IN-VITRO FREE RADICAL SCAVENGING ACTIVITY:

DPPH Assay:

DPPH (2, 2-Diphenyl-1-picrylhydrazyl)) assay is based on the measurement of the scavenging ability of antioxidant towards the stable DPPH radical. The free radical DPPH is purple in color in methanol and is reduced to the corresponding hydrazine, which is yellow in color, when it reacts with hydrogen donor.



2, 2-diphenyl-1-picryl-hydrazyl (DPPH)
Purple

2, 2-diphenyl-1-picryl-hydrazine
Yellow

It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in the absorbance is measured at 517 nm.

Preparation of standard solution: Required quantity of L-ascorbic acid was dissolved in methanol to give the concentration of 10, 20, 40, 60, 80 and 100 µg/ml.

Preparation of test sample: Stock solutions of samples were prepared by dissolving 10 mg of MIBE and MIBA in 10 ml of methanol to give a concentration of 1 mg/ml. Then prepared sample concentrations of 10, 20, 40, 60, 80 and 100 µg/ml.

Preparation of DPPH solution: 3.9 mg of DPPH was dissolved in 3.0 ml methanol; it was protected from light by covering the test tubes with aluminum foil.

Procedure:

Equal volume of 100µM DPPH in methanol was added to different concentration of fractions (10-100 µg/ml) in methanol, mixed well and kept in dark for 20 min. The absorbance at 517 nm was measured with or without extracts. Ascorbic acid (ASC) was used as standard for comparison. Different concentrations (1-5 µg/ml) of ASC were mixed with equal volumes of DPPH. Plotting the percentage DPPH scavenging against ASC concentration gave the standard curve. A linear correlation coefficient ($r^2 = 0.999$) was obtained.

$$\text{DPPH Scavenged (\%)} = \frac{(A_{cont} - A_{test})}{A_{cont}} \text{ X } 100$$

Where A_{cont} is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extract.

Hydroxyl radical scavenging activity:

Hydroxyl radical scavenging activity of the extractives was determined by the method of Halliwell et al. Steady state OH scavenging activity of MIBE and MIBA (20-100 µg/ml) was measured by degradation of deoxy-D-ribose method. Briefly, to the reaction mixture containing mannitol (0.1 mM), deoxy-D-ribose (3 mM), ferric chloride (0.1 mM), EDTA (0.1

mM), hydrogen peroxide (2 mM) in phosphate buffer (20 mM, pH=7.4), various concentrations of the extracts in a volume of 0.3 ml were added to give a final volume of 3.0 ml. After incubation for 30 min at ambient temperature, 1.0 ml of TCA-TBA reagent (Equal volumes of TCA-2.8% and TBA-0.5% in 4mM NaOH) was added, followed by boiling the tubes in a water bath for 30 min. The tubes were then cooled and the absorbance was measured at 532 nm. Mannitol was used as standard for comparison. Different concentrations (10-100 µg/ml) of mannitol were mixed as explained above. Plotting the percentage inhibition of OH scavenging against mannitol concentration gave the standard curve. A linear correlation coefficient (r^2 = 0.999) was obtained.

Reducing power assay:

For the measurement of the reductive ability, we investigated the Fe^{3+} Fe^{2+} transformations in the following the standard method. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Like the antioxidant activity, the reducing power of MIBE and MIBA increases with increasing concentration.

Preparation of standard solution: 3mg of L-ascorbic acid was dissolved in 3ml of distilled water. Dilutions of this solution with distilled water were prepared to give the concentrations of 50-300 µg/ml.

Preparation of test sample: Stock solutions of samples were prepared by dissolving 10mg of MIBE and MIBA in 10ml of methanol to give a concentration of 1mg/ml. Then sample concentrations of 50-300 µg/ml. were prepared.

Procedure: According to this method, the aliquots of various concentrations of the standard and test sample extracts (50-300µg/ml) in 1.0ml of deionized water were mixed with 2.5ml of (pH 6.6) phosphate buffer and 2.5ml of (1%) potassium ferricyanide. The mixture was incubated at 50°C in water bath for 20 min after cooling. Aliquots of 2.5ml of (10%) trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution 2.5 ml was mixed with 2.5ml distilled water and a freshly prepared 0.5ml of (0.1%) ferric chloride solution. The absorbance was measured at 700 nm in UV spectrometer (. A blank was prepared without adding extract[111-113].

ANTI-INFLAMMATORY ACTIVITY USING GELATIN ZYMOGRAPHY

(Detection of MMP-2 and MMP9)

Principle:

Zymography is a technique to assess the enzymatic activity of proteins either in situ or by separating them with electrophoresis. The enzyme converts the substrate (Gelatin) into a product which is detected by different staining methods. One of the most popular technique is by separating the protein mixture by Polyacrylamide gel electrophoresis in which a

substrate (Gelatin) is incorporated within the polyacrylamide gels. These protein substrates present in the gel are degraded by the proteases present in the sample which are activated during the incubation period. Staining with Coomassie blue shows the proteolytically cleaved sites as white clear bands on a dark blue background. Necessary precautions are taken to prevent the enzyme from denaturation during the process. Hence SDS is generally avoided. But in gelatinase zymography, SDS is used to activate the gelatinases[114].Required reagents and its composition:

1. Gelatin Substrate (20mg/ml): Weigh 20mg of gelatin and add 1ml of distilled water. Heat the solution till it becomes transparent. Cool it and store it at 4ºC.
2. Acryl amide Bis-acrylamide Solution (30:08): Weigh 7.5gm of Acrylamide +200mg of bis-acrylamide. Make it to 25 ml with distilled water. Store at 4ºC.
3. TEMED: Commercially available. Store at 4ºC.
4. 1.5% APS (Ammonium per sulphate): Weigh 75mg of APS and add 5ml of distilled water. Always prepare fresh.
5. Resolving Gel buffer stock (pH 8.8): 18.15gm of tris +24ml of 1M HCl Mix and brought to 50 ml final volume with distilled water. Filter, store at 4ºC.
6. Stacking gel buffer stock (pH 6.8): 6.0gm of Tris dissolved in 40ml of distilled water. Adjust the pH 6.8 with 1M HCl and brought to 100ml final volume with distilled water. Filter, store at 4ºC.
7. Reservoir Buffer stock (pH 8.3): 3.03gm of Tris+14.4 gm glycine+1gm SDS. Make it to 100ml with distilled water. Store at 4ºC. Working Solution: [10ml of Reservoir buffer stock + 90ml of distilled water].
8. 2.5% Triton x -100: Measure 2.5ml of Triton x-100. Make up the volume upto 100ml with distilled water store at 4ºC.
9. Incubation Buffer: Weigh 605mg of Tris HCL +222mg of Cacl2. Make it to 100ml with distilled water. Store at 4ºC.
10. 0.5 % Coomassie Brilliant Blue R250 [For 100ml]: Coomassie Blue 500mg + Methanol 50ml + Acetic acid 10ml + distilled water 40ml. Store at R.T.
11. De-staining Solution: Methanol 50ml + Acetic acid 10ml + distilled water 40ml store at R.T.
12. Storage buffer i.e. 5% Acetic acid: 5ml of Acetic acid + 95ml of distilled water. Store at R.T.
13. 10% SDS: Weigh 100mg of SDS +10ml of distilled water. Store at 4ºC.
14. 2X Non reducing buffer (For 10 ml): 197mg of 0.125M Tris HCL (pH 6.8), 400mg of 4% SDS, 2ml of 20% Glycerol, 4mg of 0.04% Bromophenol blue. Make up the final volume with distilled water. NOTE: Do not add any reducing agent.
15. 0.1% Agarose gel: 100mg agarose + 10ml of Normal Saline Boil the Solution till it becomes transparent. Store at R.T. or 4ºC.

Procedure:

1. Assemble the electrophoresis apparatus and seal the bottom with Agarose gel, leave it for 5-10 minutes.
2. Prepare the resolving gel as given below.

Preparation of 10% resolving gel (10ml)		
Sl. No	Reagents	Volume
1	Acryl amide – Bis-acrylamide	3.3ml
2	Resolving gel buffer stock	1.25ml
3	10% SDS	100µl
4	1.5% APS	500 µl
5	Gelatin	1ml
6	Water	3.8ml
7	TEMED	10µl

3. Mix the appropriate resolving gel mixture and pipette between the glass plates avoiding bubbles. Fill plates about 80% way up leaving space for the stacking gel and comb. Overlay with a small amount of water to achieve a completely flat interface between resolving gel and stacking gel. Allow to set for one hour.
4. While resolving gel is setting prepare the stacking gel

Preparation of 5% stacking gel (10ml)		
Sl. No	Reagents	Volume
1	Acryl amide – Bis-acrylamide	1.7ml
2	Stacking gel buffer	1.12ml
3	10% SDS	100µl
4	1.5% APS	500 µl
5	Water	6.5ml
6	TEMED	10µl

5. When resolving gel is set pour off the excess water and wash between the plates with distilled water.
6. Pour in stacking gel and insert comb avoiding bubbles. Allow to set for about one hour.

7. Preparation of MMP samples: The MMP samples will be collected from Squamous Cell Carcinoma of buccal mucosa Tissue. The collected tissue sample will be minced using 1-2ml of extraction buffer. The minced sample along with extraction buffer is transferred to a falcon tube. Sealed and stored at -20ºC . Before starting the experiment the stored samples are taken out from -20ºC, thaw it and centrifuged at 3000 RPM for 10-15mins. Supernatant is used for further experiment.
8. Preparation of extract: Weigh 50mg of test compounds in clean Eppendorf tubes and dissolve in 1ml of DMSO. Take 50 µl of this dissolved compound in another clean Eppendorf tube and add 50 µl of MMP sample (supernatant). Mix well.

Controls:

9. Negative control: Only 50µl of MMP sample (Supernatant)
10. Positive control: 50 µl of MMP sample (supernatant) +50 µl of Tetracycline HCL (20mg/ml). All the prepared extracts and controls (both positive and negative) are incubated for 1 hour at room temperature.
11. After incubation for one hour add equal volume of 2X non reducing buffer to all the test and control samples.
12. When stacking gel is set gently remove the comb, wash the wells with distilled water and assemble gel onto the electrode/gasket section of the gel apparatus. Fill top and bottom of the tank with reservoir buffer.
13. Load 25 µl of incubated sample in each well.
14. Connect the electrodes. Put lid on tank and plug cables into power supply.
15. Run at about 80V until the Bromophenol blue reaches the bottom of the plates.
16. After electrophoresis, dissemble the apparatus and gently remove the gel and put into a plastic dish and wash the gel with zymogram renaturing buffer i.e.2.5%Triton x-100 for one hour to remove SDS from the gel and allow proteins to denature.
17. Decant the zymogram renaturing buffer and incubate the gel in zymogram incubation buffer at 37ºC overnight.

Staining

1. Stain the gel with Coomassie Brilliant Blue R-250 for one hour.
2. Gels should be de-stained with an appropriate Coomassie R-250 destaining solution for about 2 hours.
3. After staining, the background stains blue with Coomassie stain where the gelatin is degraded into white bands indicating the presence of Matrix Metallo Proteinases or Gelatinases. The lower band is Gelatinase-A or Matrix

Metallo proteinase 2 (MMP-2) which is about 72 KDa while the upper band is Gelatinase-B or Matrix Metallo proteinase 9 (MMP-9) which runs at about 95 KDa.

4. Gel image was captured by Gel documentation system and exported to Total lab software (UK) for quantification of bands based on the intensity[115,116.]

STATISTICAL ANALYSIS:

The results were expressed as the triplicate and values were expressed as the Mean ± Standard Deviation (n=3) of the mean. Data were analyzed by one way ANOVA followed by Dunnet's multiple comparisons test using Graph Pad Prism version 6.0 Software.

Results

IN-VITRO STEADY-STATE FREE RADICAL SCAVENGING ACTIVITY

DPPH Scavenging activity:

DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517 nm, which is induced by different antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progress, results in the scavenging of the radical by hydrogen donation.

The results indicate both in the MIBE, MIBA and standard ascorbic acid showed DPPH free radical scavenging activity was dependent on concentration. The percentage of inhibition of MIBE and MIBA ranges from 25.33 to78.28 and 21.08 to 64.92 respectively, whereas standard ascorbic acid ranges from 38.81 to 87.00. The IC_{50} values for DPPH scavenging activity for MIBE, MIBA and ascorbic acid were 36.5µg/ml, 43.5 µg/ml and 28.65 µg/ml respectively.

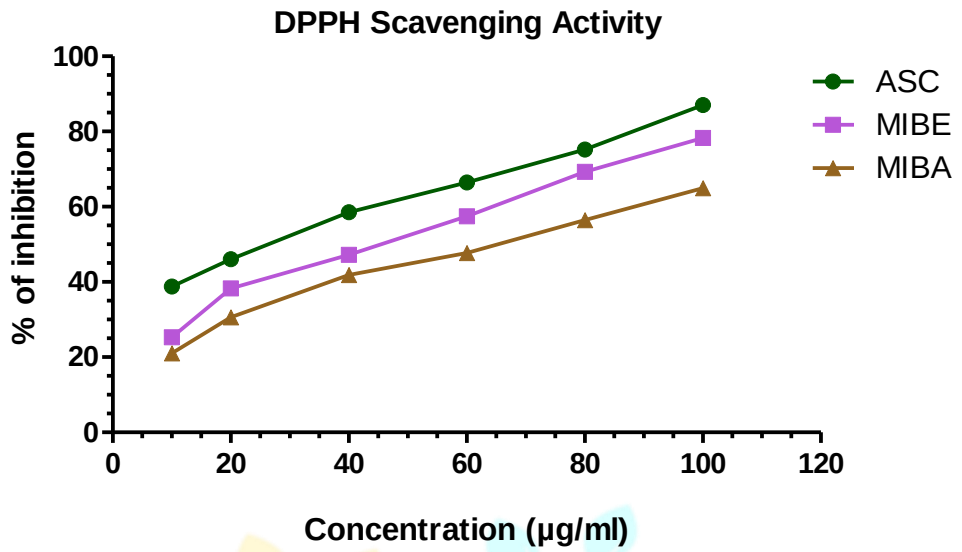


Figure Effect of MIBE and MIBA on DPPH scavenging activity

Hydroxyl radical scavenging activity:

The hydroxyl radicals are extremely reactive oxygen species that can react with every possible molecule in living organisms, especially with proteins, DNA, and lipids. Hydroxyl radicals are capable of rapid initiation of the lipid peroxidation process by extracting hydrogen atoms from unsaturated fatty acids.

The results indicate both in the MIBE, MIBA and standard Mannitol showed Hydroxyl radical scavenging activity was dependent on concentration. The percentage of inhibition of MIBE and MIBA ranges from 25.83 to 80.22 and 19.86 to 67.49 respectively, whereas standard ascorbic acid ranges from 36.56 to 87.24. The IC_{50} values for Hydroxyl scavenging activity for MIBE, MIBA and Mannitol were 37.04 µg/ml, 62.46 µg/ml and 20.02 µg/ml respectively.

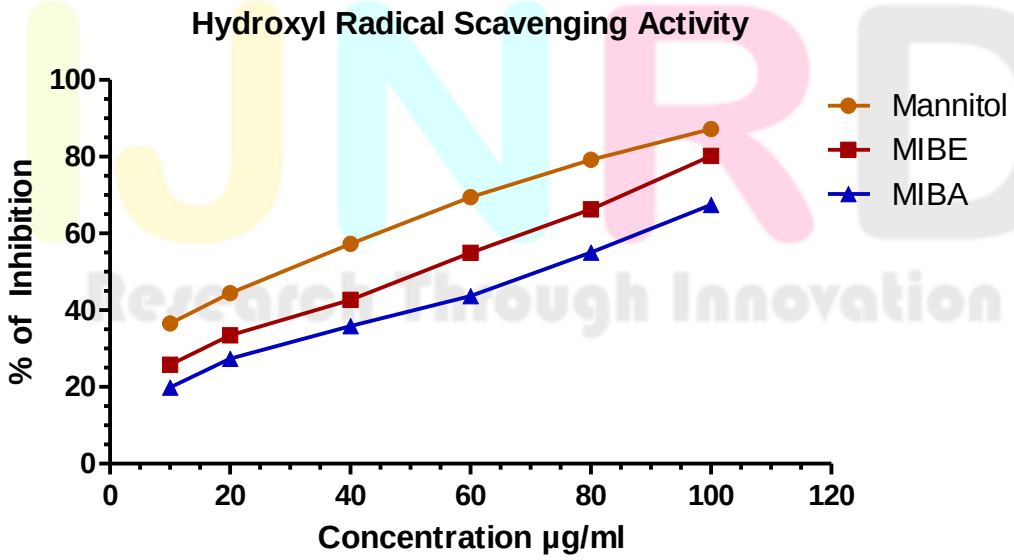


Figure Effect of MIBE and MIBA on Hydroxyl radical scavenging activity

Reducing power assay:

In this assay, Fe (III) reduction is often used as significant indicator of electron donation activity which is an important mechanism of phenolic antioxidant action by breaking the radical chain by donating a hydrogen atom.

The results indicate both in the MIBE, MIBA and standard Mannitol showed Reducing power activity was dependent on concentration. The percentage of absorbance of MIBE and MIBA ranges from 0.165 to 0.721 and 0.098 to 0.543 respectively, whereas standard ascorbic acid ranges from 0.245 to 0.783.

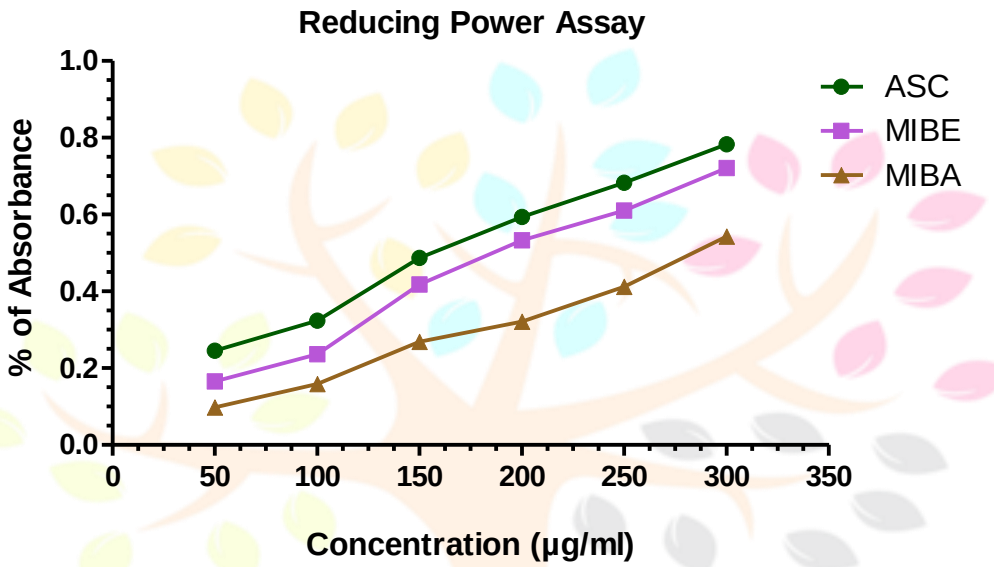


Figure Effect of MIBE and MIBA on Reducing power assay

IN-VITRO ANTI-INFLAMMATORY ACTIVITY

Gelatin Zymography: Detecting of MMP-9 and MMP-2

Coomassie blue staining of the gel revealed sites of proteolysis as translucent bands on a dark blue background. The background stains blue with coomassie stain where the gelatin is degraded, appearance of white bands indicats the presence of gelatinases. The upper bands are gelatinases B (MMP9) which runs at about 95 KB and the lower bands are gelatinases A (MMP-2)

Amongst both compounds, the ethanolic extract ie.MIBE has shown good anti-inflammatory activity against Matrix Metalloproteinase-9 (MMP-9) with percentage inhibition of 64% and Matrix Metalloproteinase-2 (MMP-2) with percentage inhibition of 78% as compared to aqueous extract ie. MIBA. Ethanol is suitable solvent for phytochemical extraction due to its ability to extract a high amount of phytochemicals. It can extract both polar and non-polar lipids, resulting in higher extraction yield. Because of this ability the inhibition of MMP-2 and MMP-9 is seen more in MIBE.

Table Inhibition of MMP-2 and MMP-9 by the MIBE and MIBA by Gelatin zymography method.

Sl. No	Name of the Extracts/drugs	% Bands of MMP		% Inhibition of MMP	
		MMP 2	MMP 9	MMP 2	MMP 9
1	Positive Control (Tetracycline HCL)	00	00	100	100
2	Negative Control (Supernatant of Squamous cell carcinoma)	100	100	00	00
3	MIBE	22	36	78	64
4	MIBA	96	88	04	12

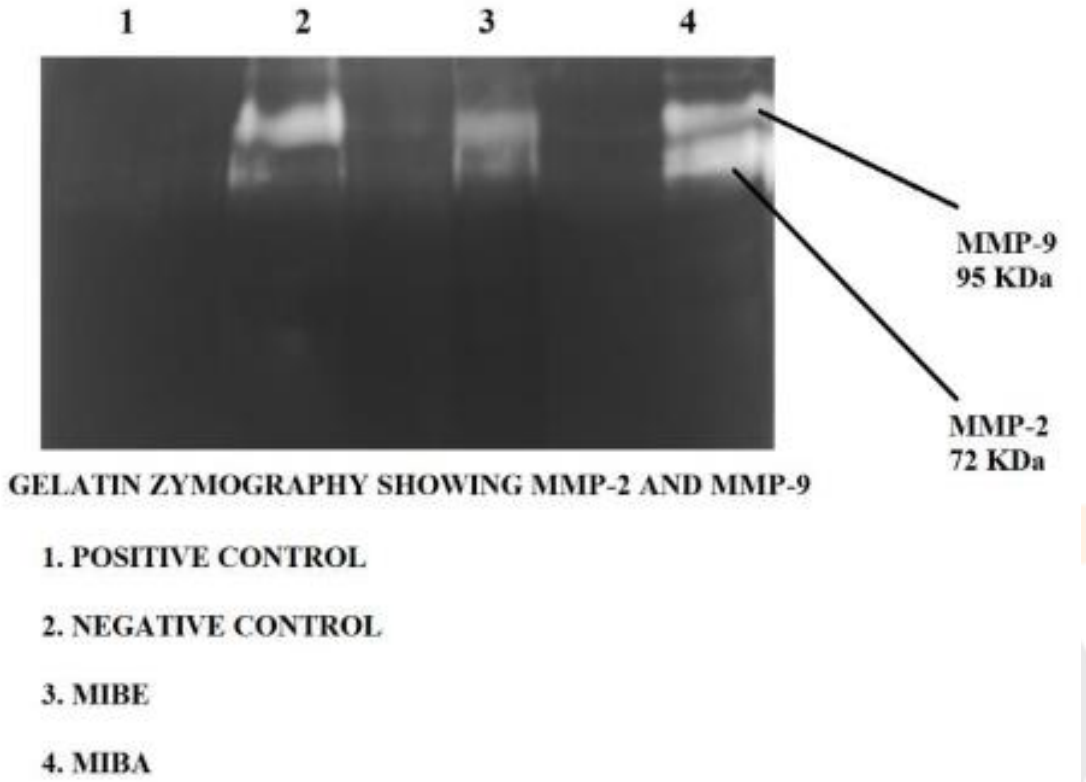


Figure Expression of MMP-2 and MMP-9 by gelatin zymography assay after the treatment of MIBE and MIBA

DISCUSSION

This study assessed the anti-inflammatory potential of the bark of Margaritaria indica. It is used to treat gastrointestinal problems, wound healing, fever and swelling and inflammation. In the present study, pharmacognostical evaluation of Margaritaria indica Dalzell bark and effect of MIBE and MIBA for in-vitro antioxidant and anti-inflammatory activities were evaluated. The results revealed that plant exhibits high potential role of treatment in monitoring of oxidative stress and inflammation.

Several techniques have been used to determine the antioxidant activity *in-vitro* in order to allow rapid screening of substances since substances that have low antioxidant activity *in-vitro*, will probably show little activity *in-vivo*. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms[128].

The electron donation ability of natural products can be measured by 2,2 -diphenyl-1- picrylhydrazyl radical (DPPH) purple-coloured solution bleaching[129]. The method is based on scavenging of DPPH through the addition of a radical species

or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test[130]. In the present study among all the fractions tested, MIBE showed significantly higher inhibition percentage and positively correlated with total phenolic content. Results of this study suggest that the plant extract contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage.

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell[131]. The hydroxyl radical is regarded as a detrimental species in pathophysiological processes and capable of damaging almost every molecule of biological system and contributes to carcinogenesis, mutagenesis and cytotoxicity[132]. Hydroxyl radicals were produced by the reaction of H_2O_2 and the ferrous that would react with 2-deoxyribose. The reaction was stopped by adding TBA reagent that would give a red colour if the malonaldehyde was formed as the result of the reaction between the radical and 2-deoxyribose. Hydroxyl radical scavenging capacity of an extract is directly proportional to its antioxidant activity which is depicted by the low intensity of red colour[133]. MIBE and MIBA when added to the reaction mixture actively scavenged the hydroxyl radicals and prevented the degradation of 2-deoxyribose.

In reducing power assay, the yellow colour of the test solution changes to green depending on the reducing power of the test specimen. The presence of the reductants in the solution causes the reduction of the Fe3+/ ferricyanide complex to the ferrous form. Therefore, Fe_2^+ can be monitored by absorbance measurement at 700 nm[134]. The reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain[135]. Increasing absorbance at 700 nm indicates an increase in reducing ability. The antioxidants present in the fractions of MIBE and MIBA caused their reduction of Fe_3^+ / ferricyanide complex to the ferrous form, and thus proved the reducing power.

Gelatinases present in a number of physiologic and pathologic conditions play a key role in inflammation and autoimmunity states[136]. Overproductions of MMPs (Matrix Metalloproteinase) are associated with tissue destruction in chronic inflammatory diseases such as periodontitis, rheumatoid, osteoarthritis, tumor cell invasion, and metastasis[137,138].

Gelatin zymography is a sensitive and powerful method used to detect proteolytic enzymes capable of degrading gelatin from various biological sources. It is useful for the assessment of the matrix metalloproteinase family, MMP-2(gelatinase A) and MMP-9(gelatinase B), due to its potent gelatin-degrading activity. MMPs are produced by activated inflammatory cells like neutrophils, macrophages, epithelial cells, fibroblasts, and vascular endothelial cells.[139,140].

This method is used to detect gelatinase activity, especially MMP-2 and MMP-9. remain inactive, while they are with their pro-domains and need denaturation to get activated. Then, it could be detected on gelatin zymograms as one or two white bands (pro and active forms) after staining with coomassie blue staining. The *in vitro* anti-inflammatory results are obtained from the % bands of MMP-2 and MMP-9 which was detected for each sample screened from the gelatin zymogram by gel electrophoresis apparatus[141].

The percentage of inhibition anti-inflammatory activity MMP-2 and MMP-9 for each samples is calculated by subtracting from 100 with the % of bands of MMP-2 and MMP-9. MIBE has shown good anti-inflammatory activity against MMP-9 with percentage inhibition of 64% and MMP-2 with percentage inhibition of 78%. MIBA showed moderate activity against MMP-2 and MMP-9 with percentage inhibition of 04% and 12% respectively.

MMPs are enzymes that degrade the extracellular matrix and basement membrane, and regulate the infiltration of inflammatory cells; consequently, they participate in tissue remodeling[142]. MMP-9 and MMP-2, members of the gelatinase family of MMPs, are recognized to play important roles in the turnover and degradation of extracellular matrix proteins during cellular recruitment in inflammation[143]. Whereas MMP-2 is constitutively expressed in many cell types, MMP-9 is strongly induced in airway epithelial cells by inflammatory cytokines, particularly TNF-α [144,145]. Thus, the relative expression

levels of MMP-9 were normalized to those of MMP-2. Our current findings indicate that MIBA and MIBE significantly decreases the MMP-9 activity in IT-stimulated BEAS-2B cells.

CONCLUSION

The present study demonstrates that bark extracts of Margaritaria indica Dalzell. possess powerful antioxidant and anti-inflammatory activities. MIBE and MIBA were found to have significant inhibitory effect on proteolytic enzymes viz. MMP-2 and MMP-9. MMP-2 seems to be more sensitive than MMP-9. Hence, MIBE and MIBA would be an important source of bioactive molecules in the treatment of inflammatory diseases. Further investigations should be conducted on the fractionation to determine the most active fraction, of active constituents isolation, binding studies and electrophysiological procedures may also be useful to completely elucidate oxidative stress, inflammation and specific mechanisms of Margaritaria indica Dalzell. bark involved in the anti-inflammatory mediated process

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