Pharmacological Screening of Phragmites "*Karka Extract*" To Treat Wounds With Different Screening Models

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Abtract-

Phytochemical studies carried out on the *Phragmites karka* plants have demonstrated a wide variety of the biomolecules present in the plants which may be responsible for their use in traditional medicine. Through various bioassays, different biologically active compounds were identified. Since bio autography was used to monitor the wound healing activity of the extract, this shows that bioassays can be used as a guide during isolation. Although a p-coumaric acid compound isolated in this study was not identified due to a contaminant, this study shows thatthere is a biologically active compound belonging to the phenolic derivative. Which may be unique Further work is required to identify the specific phenolic and to determine if it is indeedunique and a good candidate for the treatment of infected wounds.

Keywords- *Phragmites karka*, Antioxidant Activity, Wound healing, Glycosides, Scavenging Activity.

INTRODUCTION

The skin is the largest organ of the body. The skin and its derivatives (hair, nails, sweat and oil glands) make up the integumentary system.[1] One of the main functions of the skin is protection. It protects the body from external factors such as bacteria, chemicals, and temperature. The skin contains secretions that can kill bacteria and the pigment melanin provides a chemical pigment defense against ultraviolet light that can damage skin cells.[2] The primary function of the skin is to serve as a protective barrier against the environment. Loss of the integrity of large portions of this barrier as a result of injury or disease- may lead to major complications or even death.[3] The ability of the skin to repair any wound is therefore a main skin "duty" in order to keep a proper homeostasis. Wound healing represents thereforea major organ functional ability of the skin in humans as well as in mammals. Skin repair is a multistep complex biological process that requires a close interaction of multiple cell types in a highly coordinated program.[4] It involves hemostasis, inflammation, angiogenesis, migration and proliferation of progenitor cells, as well as production and remodeling of the extracellular matrix (ECM). As a peculiar example recent studies have allowed a better understanding of the intervention of distant stem cells derived from distant tissues such as bone marrow in order to allow cutaneous healing.[5]

MATERIAL AND METHODS

Glassware and Chemicals

Glassware of good quality were uses and prior work all the glassware was thoroughly washed, rinsed with distilled water and soaked in solution of chromic acid. Petroleum ether, ethyl acetate, methanol, sodium carbonate, potassium ferricyanide, DMSO, NaOH, ferric chloride, trichloroacetic acid were procured from SD fine chemicals Pvt. Ltd. Mumbai, India. Monosodium iodoacetate, complete freund's adjuvant (CFA), gallic acid, rutin, folin-ciocalteu reagent, 1,1- diphenyl-2-picrylhydrazyl (DPPH), Nitro blue tetrazolium (NBT) and ascorbic acid was procured from Sigma Aldrich chemicals Pvt. Ltd, Hyderabad, India. Indomethacin was obtained from Akums Drugs and Pharmaceuticals, India. All other chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Lobo Chem, Ltd. (Mumbai, India), SRL Pvt. Ltd. (Mumbai, India) and Merck Life Sci. Private Ltd. (Mumbai, India). Kits for biochemical estimation were procured from Beacon Diagnostics, Pvt. Ltd. Navsari, Gujarat, India. All other chemicals used in this study were obtained commercially and were of analytical grade and triple distilled water was used for whole experiment was generated

in house.

COLLECTION AND AUTHENTICATION OF PLANT MATERIAL

Plant *Phragmites karka* was collected from local area of Gondia (M.H.) India. Herbarium of plant was prepared graciously and submitted to Department of Botany, Manoharbhai Patel College of Agriculture, Gondia, India, for authentication.[6]

Table 1: Plant part used for present investigation

S. No.	Botanical name	Part used
1.	*Phragmites karka*	leaf

Processing of plant

Collected plant material washed under running tap water and kept in shade for drying. Dried plant materials were then powdered using blender and further observed for color, odor, and texture then placed in packed labeled air tight container for further use.[7]

Extraction

In present study, plant material was extracted by continuous hot percolation method using Soxhlet apparatus. Powdered material of *Phragmites karka* was placed in thimble of soxhlet apparatus. Soxlation was performed at 60°C using petroleum ether as non- polar solvent. Exhausted plant material (marc) was dried and afterward re-extracted with ethyl acetate and methanol solvent. For each solvent, soxhlation was continued till no visual colour change was observed in siphon tube and completion of extraction was confirmed by absence of any residual solvent, when evaporated. Obtained extracts was evaporated using rotary vacuum evaporator (Buchi type) at 40°C. Dried extract was weighed and percentage yield for each extract was determined using formula[8]

$$\%yield = \frac{\text{weight of extract}}{2aweight\ of\ plant\ material} \times 100$$

Prepared extracts were observed for organoleptic characters (percentage yield, color and odor) and were packed in air tight container and labeled till further use.

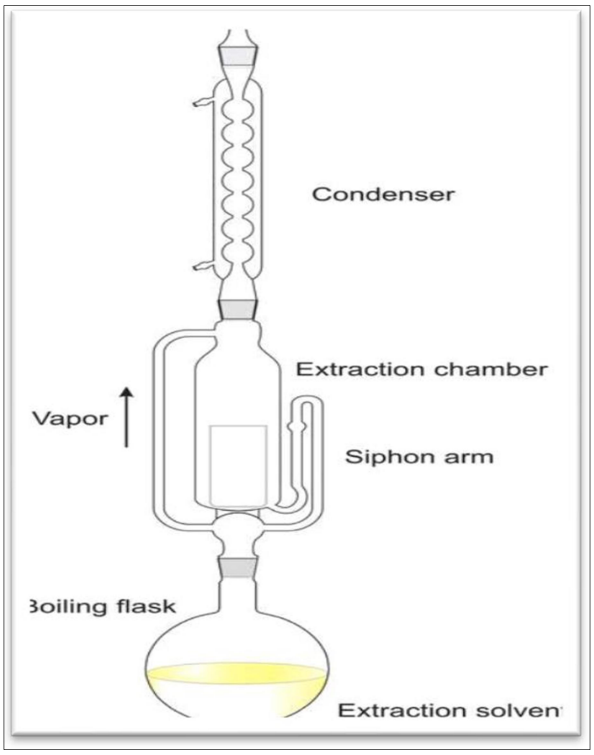

Figure. No. 1: Continuous Hot Extraction

QUALITATIVE PHYTOCHEMICAL ESTIMATION OF EXTRACTS

The plant drugs show miraculous cure and prevention over many diseases due to the presence of phytoconstituents. Many of the phytoconstituents at minute quantities itself shows an effective therapeutic activity.[9] It is perhaps obvious that different species of plants would havedifferent chemical profiles. However, these differences can extent to different varieties or even the same variety grown in a different location or harvested at a different time of the year. Different plant parts, such as roots, leaves, flowers and seeds can also have strikingly differentprofiles.[10] The renaissance of herbal medicine in primary health care creates a demand for studies in the science of pharmacognosy. From the practical perspective this includes quality control (identity, purity and consistency), efficacy and safety. In indigenous traditional system of medicine, the drugs are primarily dispensed as water decoction or ethanol extract. Fresh plant parts, juice or crude powder are rarity than a rule.[11] Thus, whenever medicinal plant parts are subjected to chemical and pharmacological screening it becomes an essential prerequisite to establish the botanical identity of the plant material and it should be free from harmful materials like pesticides, heavy metals, microbial or radioactive contamination. In order to identify various phyto-constituents namely alkaloids, terpenoids,

glycosides, steroids, triterpenoids, flavonoids, carbohydrates, saponins and tannins in different extracts, phytochemical screening was performed using standard procedures.[12]

TESTS FOR CARBOHYDRATES

A. **Molisch test:** To 1ml of test sample, 2-3 drops of α- naphthol was added. Conc. sulphuric acid was added along the side of the test tube. The appearance of purple ring at the junction of two liquids was observed, which confirms the presence of carbohydrates in the test samples.

B. **Fehling's test:** To 1 ml of test sample, similar quantity of Fehling's solution A and B was added and heated on a water bath for few minutes. The development of brick red precipitate was observed.

C. **Benedict's test:** To 1 ml of test sample, equal quantity of Benedict's reagent wasadded, boiled. Development of red colour precipitate confirmed the presence of carbohydrates.

TEST FOR ALKALOIDS

All the test extracts were first treated with dil. hydrochloric acid separately and filtered. The filtrate of all the test extracts were exposed to following tests:

A. **Mayer's test:** 1 ml of the test extract filtrate was treated with 1ml of Mayer`s reagent and formation of cream precipitate was observed.

B. **Hager's test:** 1 ml of the test extract filtrate was treated with 1mL of Hager`s reagent and formation of yellow precipitate was observed.

C. **Wagner's test:** 1ml of the test extract filtrate was added to 1ml of Wagner's reagent and formation of reddish-brown precipitate was observed.

TESTS FOR TERPENOIDS

A. **Salkowski test:** Chloroform solution of test sample was treated with equal amount of conc. sulphuric acid. The presence of steroid components in the test extracts was observed by the appearance of red color in chloroform layer and green fluorescence in acid layer.

B. **Libermann - Burchard test:** To 2ml of test sample, chloroform was added followed with 2-3 drops of acetic anhydride and conc. sulphuric acid. The test solution was observed for colour change from red to blue and then finally to bluish green colour which confirms the presence of steroids in the test extracts.[13]

TEST FOR FLAVONOIDS

A. **Shinoda test:** The test extract when treated with magnesium turnings and 2-3 drops of conc. hydrochloric acid, pink colour appeared was noted.

B. **Alkaline reagent test:** 1mL of test sample was dissolved in dilute sodium hydroxide solution, yellow colour appeared was noted.

TESTS FOR TANNINS AND PHENOLIC COMPOUNDS

A. **5% $FeCl_3$ solution**: To 2-3 ml of extract, few drops of 5% $FeCl_3$ solution was added, deep blue-black colour was observed.

B. **10% lead acetate solution:** To 2-3 ml of extract, few drops of 10% lead acetate solution was added, white precipitate was observed.

C. **Gelatin test:** After dissolving some quantity of extract in distilled water, 2 ml of 1% gelatin solution containing 10% sodium chloride was added which led to the formation of white precipitate indicating presence of phenolic compounds.[14]

TESTS FOR SAPONINS

A. Foam test*:* 1ml of test sample was dissolved in 20 ml of distilled water and shaked for 15 min in a graduated cylinder. Formation of persistent foam around 1cm layer was observed.

TEST FOR PROTEINS AND AMINO ACIDS

A. **Ninhydrin test:** 3 ml of the test solution was heated with 3 drops of 5% ninhydrin solution, placed a test-tube in a boiling water bath for 10 min and then the colour change was observed.

B. **Biuret test:** Test sample was treated with same volume of 1% copper sulphate and 4% sodium hydroxide solution and appearance of violet or pink color was observed.

TEST FOR GLYCOSIDE

A. **Legal test:** 1 ml of sodium nitroprusside was added in 1 ml of pyridine solution containing test sample and colour change was observed.

B. **Keller killani test**: To 2 ml of extract, 3 ml of glacial acetic acid and 1 drop of 5%

ferric chloride were added. This solution was carefully transferred to the surface of 2 ml concentrated H_2SO_4 and the observation was noted down.

TEST FOR FATS AND LIPIDS

A. Spot test: One drop of extract was placed on filter paper and solvent was allowed to evaporate. An oily stain on filter paper indicates the presence of fixed oil.

QUANTITATIVE PHYTOCHEMICAL ESTIMATION

Total Phenolic Content Estimation

Prepare different concentration of Gallic acid (20 to 100µg/ml) in methanol. Prepare test sample in methanol or, solvent of near about same polarity (100µg/ml). Add 0.5 ml of different concentrations of Gallic acid/ test sample with 2 ml Folin-Ciocalteu Reagent (1:10 in deionized water) Add 4 ml of sodium carbonate solution. Incubate at room temperature for 30 min with intermittent shaking. Take absorbance at 765 nm (due to developed blue colour) using methanol as blank. Prepare standard curve for different concentration of Gallic acid, and find out line of regression. Put absorbance of test sample in line of regression of standard curve of gallic acid. Calculate total phenolic content. Express as mg/gm or, µg/mg galic acid equivalent.

TOTAL FLAVONOID CONTENT ESTIMATION

Prepare different concentration of Rutin (20to100µg/ml) in methanol. Prepare test sample in methanol or, solvent of near about same polarity (100µg/ml). Mix 0.5-mL aliquot of appropriately diluted sample solution with 2 ml of distilled water and subsequently with 0.15 mL of a 5% $NaNO_2$ solution. After 6 min, add 0.15 ml of a 10% $AlCl_3$ solution and allow standing for 6 min, and then adding 2 ml of 4% NaOH solution to the mixture. Immediately, add water to bring the final volume to 5 ml, and then mix the mixture thoroughly and allowed to stand for another 15 min. Take absorbance of the mixture at 510 nm versus a prepared water blank. Prepare standard curve for different concentration of Rutin, and find out line of regression. Put absorbance of test sample in line of regression of standard curve of Rutin. Calculate total flavonoid content. Express as mg/gm or, µg/mg Rutin equivalent.

EVALUATION OF *IN-VITRO* ANTIOXIDANT ACTIVITY

Antioxidants are known to prevent or delay the free radical mediated oxidation of substrates and thus help the body to protect from the oxidative stress and associated degenerative diseases Hence, in order to strengthen the natural antioxidant defense system, antioxidants may be supplied externally. The antioxidants may act by different action of mechanism and at different level of oxidation. They may reduce oxidative damages by decreasing oxygen concentrations, prevent the initiation of chain reaction by scavenging free radicals such as hydroxyl radicals, or may bind to free metal ions to prevent metal induced free radical generation and thus the oxidative damage. Natural antioxidants are a rich source of phenolic and polyphenolic compounds. The phytochemical composition of herbal plants was evaluated for antioxidant potential so that they can be further exploited for the conditions associated with the disease. The aim of the present study was to compare the antioxidant potential as well as free radical scavenging activity of the extract with the synthetic compound vitamin C (Ascorbic acid) towards the reactive oxygen species.

1,1- diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The DPPH (1,1- diphenyl-2-picrylhydrazyl) assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). When antioxidants react with DPPH, which is a stable free radical, becomes paired off in the presence of a hydrogen donor (e.g., a free radical scavenging antioxidant) and is reduced to the DPPHH and as consequence the absorbance's decreases from the DPPH radical to the DPPH-H form, results in decolorization (yellow color) with respect to the number of electrons captured. More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of new drug. DPPH radical scavenging activity was measured using the method of a solution of 0.1mM DPPH (4mg/100ml) in methanol was prepared and 1 ml of this solution was mixed with 1 ml of different concentrations of the different extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Ascorbic acid was used as reference standard while methanol was used as control. Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of extracts. The change in color was measured at 517 nm wavelength using spectrophotometer (UV-MINI-1240, SHIMADZU) with methanolic solution as a reference solution. This was related to the absorbance of the control without the plant extracts. All the tests were carried out in triplicates. Though the

activity is expressed as 50% inhibitory concentration (IC_{50}), IC_{50} was calculated based on the percentage of DPPH radicals scavenged. The lower the IC_{50} value, the higher is the antioxidant activity. The percentage inhibition of free radical DPPH was calculated from the following equation:

% inhibition = [(absorbance of control- absorbance of sample)/absorbance of control] ×100%

(DPPH) + H-A→DPPH-H + A

(Purple) (Yellow)

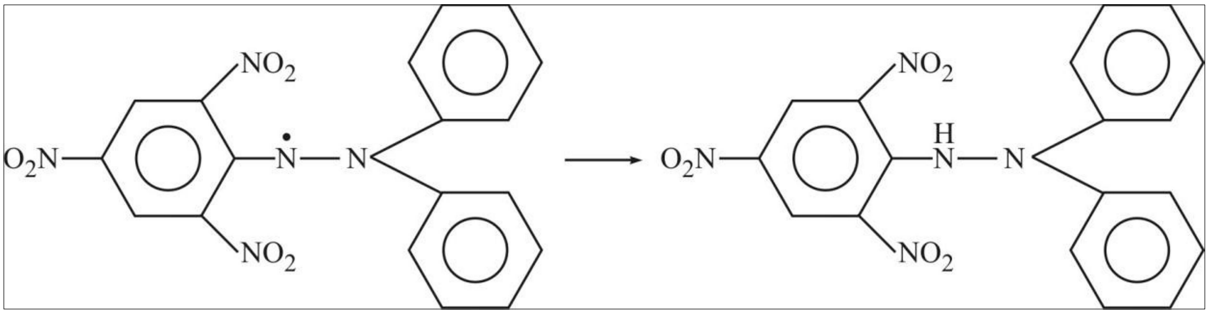

Figure No.2: Reduction of DPPH

HPTLC Analysis

Instrument: CAMAG Automatic TLC Sampler, with win CATS software.

Stationary phase: TLC plates silica gel 60 F 254 pre coated layer (20 cm X 10 cm), thickness 0.2 mm.

No. of tracks: 8, band length: 8 mm.

Mobile phase: Toluene: Ethyl acetate: Formic (8:2:0.5)

Standard: Light yellow powder
Sample: Brown powder
Solubility: Methanol

Standard concentration: 50µg/ml

Standard Injection volumes (µl): 2, 4, 6, 8, 10µl

Sample concentration: 1 mg/ml

Sample application volumes (µl): 2 µl

Development chamber: Twin trough chamber (20 X 10)

Development mode: Ascending mode

Distance run: 75 mm

Scanning wavelength: 260 nm

PREPARATION OF THE PLATES

The plates used for HPTLC was silica gel 60 F 254 (E. MERCK KGaA). 100µg/ml of the standard was applied in the form of bands using LINOMAT IV applicator. The volumes applied were 2, 4, 6, 8 and 10 µl. The sample concentration was 10mg/ml and the different volumes were 2, 4, 8, 12 and 16 µl. The mobile phase used was Toluene: Ethyl acetate: Formic (8:2:0.5). The chromatograph was developed for15 minutes, dried at room temperature and scanned at 260 nm. Calculated the average peak area of the standard. Regression equation was obtained, via the Win Cats software using the calibration curve of the standard drug concentration (X-axis) over the average peak height / area (Y-axis).

IN VIVO WOUND HEALING ACTIVITY

Formulation of ointment (British pharmacopoeia, 1996)

(a) Preparation of 20g simple ointment (B.P.) base.

Wool fat (1g), hard paraffin (1g), cetostearyl alcohol (1g) and white soft paraffin (17g) was mixed and heated gently with stirring then cooled.

(b) 1 gm 50% methanolic extract of *Phragmites karka* was added separately to 20gm of base (5% ointment).

(c) 2 gm 50% methanolic extract of *Phragmites karka* was added separately to 20gm of base (10% ointment).

Animals

Protocol

Animal used	*Wistar* Rats
Weight	100±150 gm
Sex	Either
Housing Condition	Animals were housed in a group of six in separate cages under controlled conditions of temperature (22 ± 2°C). All

animals were given standard diet (golden feed, New Delhi) and water regularly.

Wound healing activity

Excision wound model

The surgical materials were sterilized and dorsal fur of the animals was shaved with an electric clipper. The rats were anesthetized with (Xylocaine®) 2% Jelly, Cadila (Zydus) Healthcare India ltd and anticipated area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless-steel stencil. A full thickness of the excision wound of circular area of 500mm^2 and 2mm depth was created along the markings using toothed forceps, scalpel and pointed scissors. In this model, wound contraction was monitored by measuring wound area, plan metrically, on alternate days till the wounds were completely healed.

ANIMAL PROTOCOL

24 animals were divided into groups of four and treated as follows:

Group 1: Simple ointment base was applied once daily and served as vehicle control.

Group 2: Standard drug nitrofurazone ointment (0.2% w/w) was applied once daily served as positive control.

Group 3: *Phragmites karka* 50% methanolic extract ointment (5% w/w) was applied once daily.

Group 4: *Phragmites karka* 50% methanolic extract ointment (10% w/w) was applied once daily.

All the above mentioned treatments were started from the day of wound creation and continued till 20th day of healing.The wound closure rate was assessed by tracing the wounds on day 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 16th, 18th and 20th. Post wounding using transparency paper and a permanent marker. The progressive changes in wound area were measured planemetrically by tracing the wound margin on a graph paper on every alternate day. The changes in healing of wound i.e., measurement of wound on graph paper was expressed as unit (mm^2). Wound contraction was expressed as percentage reduction of original wound size.

$$\%wound\ Contraction = Healed\ area/Total\ area \times 100$$

([a]Healed area = original wound area-present wound area).

Statistical analysis

Results obtained from wound models have been expressed as mean±SEM. The data was evaluated by one way ANOVA followed by Dunnett's t-test, $P < 0.05$ was considered as significant.

Conclusion

Extraction of *Phragmites karka* was done by soxhlation method. Maximum yield was achieved in methanol extracts of *Phragmites karka* (6.21%). Ethyl acetate extract of *Phragmites karka* shows yield of 3.74 %, whereas minimum yield was achieved in petroleum ether extracts of *Phragmites karka* as 1.86%. All the extracts of *Phragmites karka* had characteristic odour and appeared greenish brown in colour.

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