PREPARATION, CHARACTERIZATION, BIOLOGICAL EVALUATION AND QUANTIFICATION OF PLANT EXTRACT FROM *MIMOSA HAMATA* LEAF BY HPTLC METHOD

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Abstract:

The method is humble, suitable and eco-friendly. On the basis of researcher observations, it was observed that plant bodies rich in Gallic acid or proteins, a reducing carbohydrate, potency to reduce metal nanoparticle preparation. Hence a few locally available plant leaves including Mimosa Hamata which are known to contain relatively high amount of Gallic acid were selected for extraction. The study related to quantification with HPTLC also performed which shows very accurate results. The Biological study also shows the less growth of microorganisms in this extract.

Keywords- Mimosa hamata, HPTLC, Antibacterial.

Introduction:

M. hamata commonly known as Jinjani belongs to family Mimosaceae which is a much branched, armed shrub, commonly distributed along the open sandy places, often gregarious and abundant throughout the arid zone of Rajasthan, Punjab. M. hamata is one of the essential medicinal plants used in the conventional system of medicine for the treatment of assorted diseases such as jaundice, diarrhea, coagulant, fever, dysentery, blood-purifier, wounds, tonic in urinary complaints, piles. Paste of leaves is applied to burn, over glandular swelling and also used in dressing for sinus, sores and piles.[1]

Chromatography is a method in which components of a mixture are separated on an adsorbent column in a flowing system. Adsorbent are thin layers of solids attached to glass plates, immobilized liquids, gels and solid particles packed in columns. The flowing component of a system or mobile phase is either liquid or gas. Concurrent with development of methods more specific to particular classes of analytes in general, however the trend in development of chromatography has been toward faster, more efficient systems. Liquid chromatography, which is one of the form of the chromatography, is an analytical technique that is used to separate mixture in solution into its individual components. In this method, the separation relies on the use of two different "phases" or "immiscible layers", one of which is held stationary while the other moves over it. Liquid chromatography is the generic name used to describe in chromatographic procedure in which the mobile phase is a liquid. [2] The separation occurs

because, under an optimum set of conditions, each component in a mixture will interact with the two phases differently related to the other components in a mixture.

High Performance Thin Layer Chromatography (HPTLC):

HPTLC is the most simple separation technique today available to the analyst. It can be considered as time machine that can speed your work and allows you to do many things at a time usually not possible with other analytical techniques. It can simultaneously handle several samples even of divergent nature and composition supporting several analysts at a given time. A major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase; this reduces the time and cost of analysis. In addition, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thereby reducing possibilities of environment pollution. HPTLC also facilitates repeated detection of chromatogram with the same or different parameters. Furthermore, in case of HPTLC, there are no restrictions on the choice of solvents and mobile phases; drug and lipophilic excipients can be dissolved in a suitable solvent that would evaporate during spotting on HPTLC plate leaving behind analytes as a thin band. Therefore, for such methods, extraction procedure is not required always and could be developed for analyzing drug without any interference from excipients. [3]

Quantitation:

The chromatographic development should clearly and completely separate all the compounds of interest with no loss by decomposition, evaporation or irreversible adsorption during application or development. Sample and standard as a rule should be chromatographed on the same plate under similar conditions.

Materials and methods:

Plant Sample:

- Dried leaves powder of Mimosa Hamata
- De-ionized water

Instrument required:

- HPTLC method for quantitative determination of Gallic acid in ethanolic extract of dried leaves Mimosa Hamata[4]
- Hydrogen peroxide sensing capability

Antibacterial activity Bacterial strains:

- Bacillus subtilis
- E.coli

Experimental Data:

- **METHODS:**
- **Collection of plant materials:**
  The leaves of M. hamata were collected from Marathwada region.
- **Extraction of Medicinal Plant:**
  Fresh leaves were collected from Marathwada region and washed with water numerous times to remove the dust particles and in order to remove the residual moisture, leaves are sun dried and grinded to form powder. Then plant extract was prepared by using soxhlet apparatus. Dry powder of leaves extracted with ethanol (1:3) for 6 hours at temperature 40˚c. resultant extract is dried to get powder form of extract. Powdered extract is kept in glass container at 4˚c for further use.

PHYTOCHEMICAL PROFILING:	
TEST FOR CARBOHYDRATES:	
Molisch's test	200 mg of extract were dissolved separately in 5ml of water and filtered. 2ml of the above sample solution is placed in a test tube. Two drops of the Molisch's reagent is added. The solution is then poured slowly into a tube containing 2ml of conc. Sulfuric acid and observed.
Fehling's test	1ml of Fehling's solution A and 1ml of Fehling's solution B were added to 100mg of extracts separately. They were heated on a boiling water bath for 5 min and observed.
Benedict's test	To the 150mg of each extract, 2ml of Barfoed's reagent was added. Then the mixture was heated on a boiling water bath for 5min, cooled and observed.
TEST FOR ALKALOIDS:	
Wagner's test	2ml of Wagner's reagent was added to the above filtrate solution and observed.
Hager's test	To the 2ml of above filtrate solution, 2ml of picric acid was added and observed.
TEST FOR GLYCOSIDES:	
Foam test	To 200mg of each extract, 15ml of distilled water

	was added, shake it well and observed.
Legal's test	To 50mg of each extracts, 1ml of pyridine, 1ml of sodium nitro prusside solution were added and observed.
Keller-Kiliani test	To 50mg of each extract, 2ml of glacial acetic acid, 1ml of FeCl3 solution were added, heated and then cooled. This was transferred to test tube containing 2ml conc. H2SO4 and observed
Borntrager's test	To 200mg of each extract, dil. H2SO4 was added and boiled. Then it was filtered and cooled. To the cold filtrate, 3ml of benzene was added and mixed. The benzene layer was separated and to it, ammonia (2ml) was added and ammonical layer was observed.
TEST FOR FLAVANOIDS:	
Lead acetate test	To the 100mg of each extracts, lead acetate (5ml) was added and observed.

ANTIBACTERIAL ACTIVITY OF MIMOSA HAMATA LEAF EXTRACT

Culturing and preservation of bacteria:

The conventional cup-plate method was used to determine the antibacterial activity of biologically prepared Sample. It is a screening test generally done for verifying antimicrobial activity of samples. For performing the experiment, cultures of test bacterial strains-staph. aureus & Bacillus subtilis(a gram-positive bacterium) and Phytopthara (a gram –negative bacterium) were collected from microbiology department, science college of pharmacy, Nanded. The strains were grown and preserved in the culture media following standard procedures. [5]

Preparing an enriched medium:

The medium for selective growth of a bacterial strain may differ for various bacteria but each medium must contain such components which should support growth by providing the basic elements needed for growth and the source of energy. In this study, nutrient agar medium was used which support growth of a wide range of bacteria including Bacillus subtilis and Phytopthara. For 100ml nutrient agar medium, 2.8g nutrient agar powder was added in 100ml distilled water; for the cup-plate method, 0.5g of agar powder was added in addition to the medium. The medium kept in cotton-plugged glass container was sterilized in an autoclave at 121˚C for 15 min. it was distributed inside a laminar hood either in culture tubes of on Petri dishes when hot (about 45˚C) and allowed to solidify.

Serial subculture:

Micro-organism should be preserved in a manner that will allow their long-term survival and genetic stability. The method of preservation depends on the organism. Sub culturing in a suitable gelled medium after intervals is a popular method for preservation of bacteria. Serial subculture in liquid medium is also a simple method where cultures are transferred to fresh medium of same type and allowed to grow in an incubator at 37˚C. We mentioned the bacterial strains in both liquid and gelled medium. [6]

Evaluation of antibacterial activity of Sample by cup-plate method:

Nutrient agar medium (25ml) was taken into two 100ml Erlenmeyer flasks and sterilized. After cooling the medium at about 45˚C, freshly grown liquid culture (0.25ml) of Bacillus subtilis and Phytopthara was added in either of the flask, mixed quickly and poured in four (two for each bacterium) Petri dishes equally. The dishes were kept at room temperature in a laminar hood. After medium was solidified, three cups of ~0.5cm diameter were made by a cork-borer at three corners of each Petri dish at about 1.5cm away from the disk-wall. Occasionally some liquid may come out from the gelled medium in the cups, which is removed by aspiration by a Pasteur pipette or a micro-pipette. Thereafter samples were added in each well of each Petri dish. The Petri dish were marked as 1,2,A and B; the cups were also marked as well. 1 and 2 were seeded with staph. Aureus and B. subtilis respectively whereas A and B with Phytopthara .As control, 0.1ml of sterile leaf extract was added in one cup in 1; in two other cups of 1, 0.1ml of silver nitrate and leaf extract mixture with 10mM and 3.33 mM concentrations respectively were added. In 2, suspension of AgNPs was added in one cup and the mixture of silver nitrate and fruit extract with 10mM and 3.33 mM respectively were added in two other cups. The same combination of samples was applied in A and B where Phytopthara was seeded. The suspension of Sample was prepared by ultracentrifugation of the clear supernatant derived from the mixture of 10mM $AgNO_3$ solution with the grape extract after 48 hours incubation as described before. After adding the samples in the cups, the dishes were kept in a refrigerator for an hour for proper absorption of the samples into the surrounding medium from the well. The plates were transferred into an incubator set at 37 ˚C to allow bacterial growth on the medium. After 24hrs the plates were taken out of incubator and observed for zone of bacterial growth inhibition around the cups.

Formation of 'zone of inhibition':

After 24hrs of incubation at 37˚C, distinct zone of bacterial growth inhibition was observed around all the experimental and positive control cups whereas no such zone was formed around the cups containing grape juice only. The results strongly suggest that silver nanoparticles possess antibacterial activity similar to Sample of extract. The particles show activity against both gram-positive and gram-negative bacterial strains used in this study through the inhibition zone appears better in case of the gram-negative bacterium, Phytopthara. [7]

HPTLC method for quantitative determination of Gallic acid in ethanolic extract of dried leaves Mimosa Hamata:

Instrumentation and Chromatographic Conditions:

A HPTLC (Camag) system including Camag Linomat V sample applicator, Hamilton syringe (100 μL), Camag TLC Scanner-3 with win CAT software version 1.4.2, Camag twin- trough chamber (20×20 cm) (CAMAG, Muttenz, Switzerland) were used for the present work. Chromatographic separation was established by use of precoated silica gel 60 F_{254} aluminium plates (20 cm×20 cm×250 μm) as stationary phase and Toluene: Ethyl acetate: Formic acid (3: 6: 1, v/v/v) as mobile phase. Other optimized chromatographic parameters were: Chamber saturation time; 15min, detection wavelength: 280 nm. After development, the plates were dried in current of air and densitometric scanning of separated bands was carried out at 280 nm with the help of CAMAG TLC Scanner III operated by winCATs software version 1.4.2.

Preparation of Gallic acid standard solution:

Standard stock solution of Gallic acid was prepared by taking accurately weighed 10 mg and transferred 10 ml volumetric flask and then the volume was made up to 10 ml with ethanol to get concentration of 1mg mL^{-1}. From the above stock solution, 1ml of the solution was pipetted out and further diluted upto 10 ml to obtain the final concentration of 100 μg mL^{-1}.

Calibration Curve for Standard Gallic acid:

Volumes 1, 2, 3, 4 and 5 μL from standard solution (100 ng μL^{-1}) were applied on the TLC plates with Linomat V sample applicator in nitrogen stream. These deposits are done on bands of 6 mm with an interval of 8 mm between them. This plate was then dried at room temperature for 10 min and then was developed on 80 mm in a mobile phase previously saturated during 15 min and composed of Toluene: Ethyl acetate: Formic acid (3: 6: 1, v/v/v) contained in a tank of a thin layer chromatograph (TLC). The separated spots were analyzed densitometrically at 280 nm using Camag TLC Scanner 3 operated by the WinCATs software version 1.4.2. Regression data obtained from calibration curve demonstrated excellent linear relationship over 100-500 ng $band^{-1}$ concentration range. [8]

Analysis of Extract:

10 mg of Dry extract was dissolved in ethanol, filtered through a Millipore filter and volume was made with the solvent. The above stock solution was further diluted to produce concentration of 100 ng μl^{-1}. Two microlitre volumes were spotted on precoated silica gel 60 F_{254} aluminium plates using Camag Linomat V sample applicator. The sample spotted plate was developed upto 80 mm in a mobile phase composing of Toluene: Ethyl acetate: Formic acid (3: 6: 1, v/v/v). The separated spots were analyzed densitometrically at 280 nm using Camag TLC Scanner 3 operated by the WinCATs software version 1.4.2. The content of Gallic acid was calculated using an external standard (calibration curve). The content (%) of Gallic acid was found to be 93.66 (Area: 2724)

Results and Discussion:

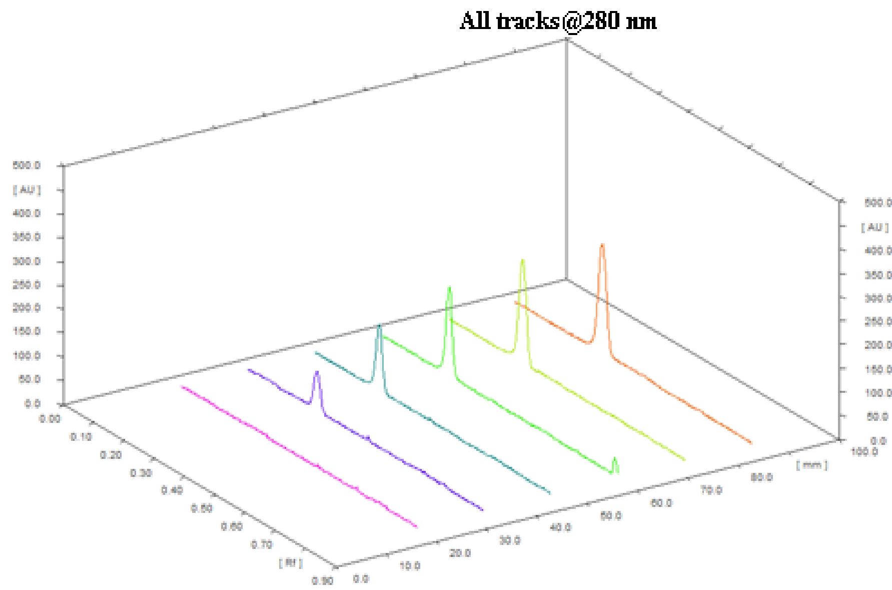
1. Physicochemical evaluation:

The physicochemical constituents present in plant Mimosa hamata have shown in following table-1.

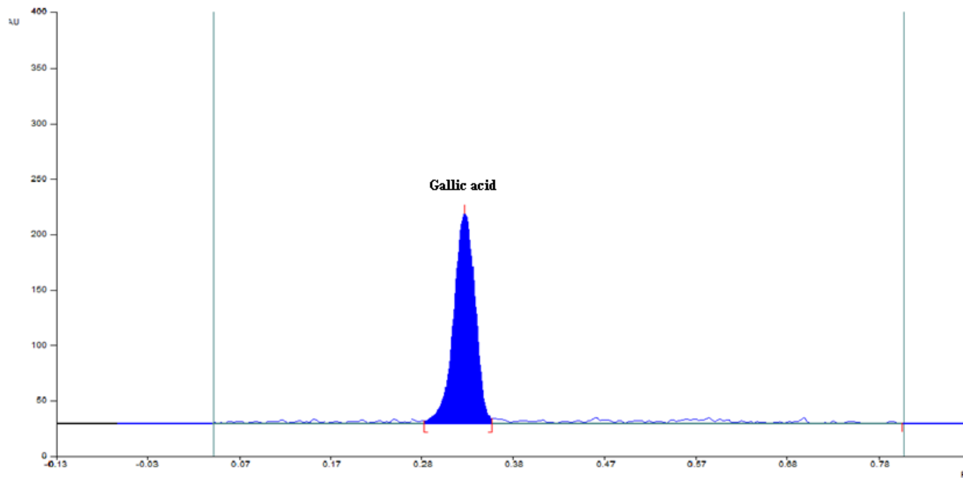
Sr.No.	Chemical tests	Results
1	Test for Carbohydrates:	
	Molisch's test	Positive
	Fehling's test	Positive
	Benedict's test	Positive
2	Proteins test	
	Biuret's test	Positive
	Million's test	Positive
3	Test for Glycosides	
	Legal's test	Positive
4	Test for Alkaloids	
	Mayer's test	Positive
	Wagner's test	Positive
	Hager's test	Positive
5	Test for Amino acids:	
	Ninhydrin test	Positive
	Tyrosine test	Positive
6	Test for Tannins & Phenolic compounds	
	Lead acetate test	Positive
	Iodine test	Positive

HPTLC method for quantitative determination of Gallic acid in ethanolic extract of dried leaves Mimosa Hamata:

Calibration Curve for Standard Gallic acid

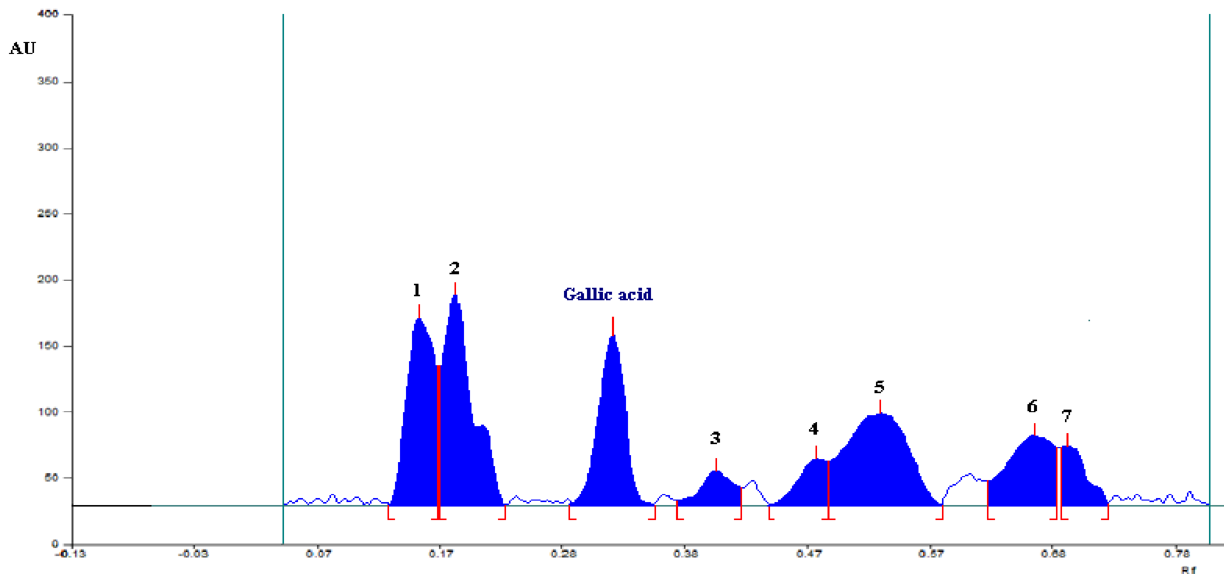


Graph for Calibration curve of Gallic acid



Representative densitogram of Standard Gallic acid (Rf = 0.30)

Analysis of Extract:



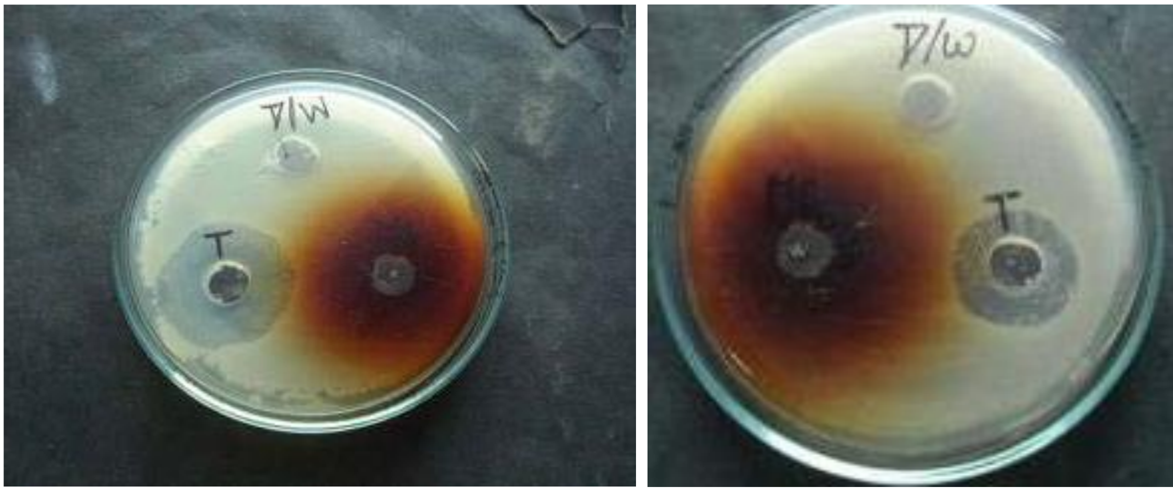
Representative densitogram of ethanolic extract of Mimosa Hamata (200 ng band^{-1}) showing the Gallic acid at Rf = 0.30

After analysis of extract, spots of gallic acid were found at same Rf value. The extract of **Mimosa Hamata** was analyzed by TLC using gallic acid as a marker because of its greater commercial availability and lower cost. In that way, gallic acid, the major active principle in the extract was identified.

Determination of Antibacterial activity:

Method: analysis was performed to reveal the antimicrobial activity of the samples with the help of agar well diffusion method. Muller-Hinton agar plates were prepared to evaluate the antimicrobial activity of the mimosa hemata extract with the solvent extracts, against selected human pathogens viz., E.coli, B.subtils, P. aureginosa, S. aureus and a fungus A.niger. 100µl inoculum of each selected pathogen which was grown overnight in nutrient broth was uniformly spread on Muller-hinton agar plates with the help of a swab. After 5 minutes of incubation, 6 mm diameter well was punched in the plates with the help of sterile cork borer. 100 µl of the concentrated sample was added into the well. The plates were incubated at 37ºC for 24hrs and after incubation plates were observed for zone of inhibition

Streptomycin (5µg/ml) antibiotic used as standerd and Distillled water as control.



Sr no	microbial strain	*Mimosa hemata* extract(Zone of inhibition in mm)	Streptomycin(Zone of inhibition in mm)	Distiled water(zone of inhibition)
1	*S. aureus*	22.80	24.50	00
2	*E. coli*	22.50	22.30	00
3	*B.subtilus*	21.30	22.50	00
4	*P.aeruginosa*	20.20	22.20	00
5	*A. niger*	18.10	20.20	00

Conclusion: The extract from *Mimosa hamata* leaf was Prepared with standard methods and Characterization was done by HPTLC method which gives very accurate results. The Biological study also shows the less growth of microorganisms in zone of Inhibition of this extract.

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