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Phytochemical Standardization Of Aegle Marmelos Leaves Extract By Using High Performance Thin Layer Chromatography Fingerprinting (Hptlc)

Sushma Rathod[1*], Dr. Mohini Sihare[2]

[1]Reasearch scholar, Institute of Pharmacy, Oriental University Indore MP*
[2]Professor, Faculty of Pharmacy, Oriental University Indore MP

***Corresponding Author:** Sushma Rathod*
**Reasearch scholar, Institute of Pharmacy, Oriental University Indore MP*

Abstract:

Objective: *Aegle marmelos* is well known for its therapeutic properties in the Indian traditional medicinal system. The presence of important bioactive compounds in plants makes them pharmacologically valuable. Therefore, in the present study, the high-performance thin layer chromatography (HPTLC) analysis of hydroalcoholic extract of *Aegle marmelos* was performed for its phytochemical profiling.
Methods: Preliminary phytochemical screening was done. HPTLC studies were carried out. CAMAG make HPTLC system equipped with Linomat 5 applicator, TLC scanner 3, Reprostar 3 and WIN CATS were used. The HPTLC densitometric analysis of the hydroalcoholic extract of *Aegle marmelos* was carried out using CAMAG HPTLC system, and the results were obtained in the form of chromatograms (scanned at the wavelength of 254 nm and 366 nm) representing several peaks.
Results: Preliminary phytochemical screening of the extract showed the presence of alkaloids, flavonoids, glycosides, saponins, tannins, terpenoids, phenolic compounds, proteins, fats and oils. TLC resulted in identification of three spots found in the hydroalcoholic extract. The phytochemical profile of the plant was determined and presented in the tables showing the total number of peaks, peak heights, peak area, percent area, and Rf values. HPTLC finger printing of hydroalcoholic extract of *Aegle marmelos* under white light reveal presence of five components 0.04, 0.03, 0.29, 0.42, 0.49. Component number 2,3 and 4 at Rf 0.03, 0.29 and 0.42 showed maximum concentration.
Conclusion: The study concluded that hydroalcoholic extract of *Aegle marmelos* contains a rich variety of phytochemicals which might be accountable for its therapeutic value and thus justifies its traditional use in India.

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Keywords: Aegle marmelos, HPTLC, Alkaloids, hydroalcoholic extract

Introduction:

Medicinal plants, due to the presence of bioactive phytochemicals, play a very important role in human life for maintaining good health. The use of medicinal herbs in the treatment of infection is an age-old practice, and

several natural products are used as phytotherapy for the treatment of many diseases.[1] The search for a newer source of antibiotics is a global challenge, since many infectious agents are becoming resistant to synthetic drugs.[2] There are thousands of medicinal plants known to have a long history of usage for their curative properties against various diseases and ailments.[3] The use of herbal drugs is once more escalating in the form of Complementary and Alternative Medicine (CAM).[4,5]

A. marmelos (L) Correa belonging to family Rutaceae and commonly known as Bael has been used as a folklore medicine since ancient time to cure various human diseases. Aegle marmelos is an important medicinal plant with wide ethno-medicinal applications in traditional and folk medicinal systems. A. marmelos plant has been blessed with seven major phytochemicals such as alkaloids, cardiac glycosides, terpenoids, saponins, tannins, flavonoids and steroids which are biologically active and have been the major source in curing different diseases.[6] All most all parts are used in preparing medicine. The leaves of the plant are reported to possess anticancer, anti-hyperglycaemic, anti-inflammatory, antipyretic, and analgesic, anti-diabetic, anti-spermatogenic, anti-bacterial, anti-diarrhoeal and chemo preventive activities.[7]

The World Health Organization (WHO) has stressed on the need for scientific validity of herbal drugs and ensuring, devising, and implementing sound science.[8] Several techniques are available for the qualitative and quantitative estimation of phytochemicals present in plants. Nowadays, new technology has made it possible to identify, screen, and isolate these active compounds.[9] The HPTLC (high-performance thin layer chromatography) is an advanced form of TLC as it provides high resolution and much accurate data. It is accepted all over the world as one of the most powerful analytical techniques used for phytochemical and biomedical analysis. It is an inexpensive, simple, and rapid method for the estimation of chemical components present in test sample and therefore most widely used by pharmaceutical industries for new drug discovery. The present study was performed for the phytochemical profiling of plant extracts by the HPTLC technique.

The present study was, therefore, aimed at evaluating the phytochemical potential and A. marmelos hydroalcoholic leaf extract. The aim of the present paper is to determine the bioactive substances contributing to the medicinal value of the leaves of A. marmelos using suitable solvent systems.

Material and Methods

Collection of plant material: The plant material was collected from local market, Akola. The plant was authenticated from Dr. P. P. Umale (Kokate) Professor and head Department of Botany, Shri Shivaji College of Arts, Commerce and science, Akola Maharashtra India.

Extraction: The plant material was washed and then kept for shade drying for 7 days. The dried plant sample was powdered by mechanical grinder into a fine powder. The air-dried powdered material of all plants was extracted with hydroalcoholic solvent [ethanol and water solvent (60:40)] using the Soxhlation process with the help of a Soxhlet apparatus. Excess solvent was then evaporated in a water bath at 50–100°C to obtain the crude and stored in airtight containers.

Chemicals and solvents: All the solvents used were of chromatography grade, and all the chemicals used were of analytical reagent grade.

Phytochemical methods: Preliminary phytochemical screening for alkaloids, steroids, carbohydrates, tannins, fixed oils, proteins, terpenoids, flavonoids and glycosides were carried out according to the official procedures to know the presence of different phytoconstituents in the plant extracts.[10,11,12,13,14]

THIN LAYER CHROMATOGRAPHY [15,16,17,18]

Thin Layer Chromatography (TLC): Thin layer chromatography was carried out to know the chemical profile of hydroalcoholic extract of A. marmelos leaves.

Preparation of TLC plates: The TLC plates were prepared. Briefly, 25g of silica gel-G (Hi media, Manufactured, India) was uniformly spread over TLC plates with a thickness of 0.25 mm using the spreader. The plates were allowed to dry at room temperature and heated in an oven at 100°C for 2 h.

Standardisation of solvent system: Each sample of the crude extract of A. marmelos leaves was diluted in ethanol. The prepared TLC plates were marked 1 cm from bottom and 10 µl each sample was applied on TLC plates at equal distance with the help of capillary tubes. For separation of maximum bands on TLC plates different solvent systems were used according to polarity and Toluene: Ethyl acetate: Formic acid: Methanol

(3:3:0.3:0.2) was selected as standard solvent system. A. marmelose extract was screened for preliminary phytochemical analysis. Observations were recorded for presence or absence of phytochemicals, number and Rf values of bands (compounds) present in extract.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY [19,20,21,22]
HPTLC is semiautomatic instrumental TLC. HPTLC is different from conventional TLC due to smaller particles (<10 µm) of adsorbent and less thickness of applied layer (<150 µm). The automatic sample applicators of HPTLC applies sample in the form of bands and hence it gives better separation and resolution in close Rf value components. Simple spot application in TLC causes mixing of close Rf value components and look like tailing. HPTLC enables simultaneous analysis of many samples in less time with better analytical accuracy and precision. There is no need of filtration and degassing for solvents as required in HPLC. HPTLC plates give the faster development and less mobile phase is required per sample. There are no chances of contamination from previous analysis as fresh stationary and a mobile phase has to use for each sample. Separated samples can be analysed visually by simple UV detector. By pre- or post-chromatographic derivatization even non-UV absorbing compounds can be detected.
Following are the general techniques involved in HPTLC.

SELECTION OF STATIONARY PHASE: According to nature of components to be separated stationary phase can be polar, nonpolar or less polar. It can be organic (charcoal, cellulose) or Inorganic (silica gel, alumina, kishelghur, diatomus earth, magnesia). According to active sites (functional groups present in stationary phase) the adsorbent can be Strong active (alumina), medium active (silica gel) and less active (magnesia)

SELECTION OF MOBILE PHASE: According to nature of components to be separated Based on principle of "Like dissolves like". Instead of one solvent always try to select composition of more than one solvent. Selection is also based on technique of elution like isocratic, fractional, gradient. Generally volatile solvents should be selected. Highly polar solvents should be avoided

PREPARATION OF PAPER/PLATE: The readymade plates like Silica Gel F254 from Merck Company should be cut in to desired size and shape

ACTIVATION OF PRE-COATED PLATES: Manually prepared as well as readymade plates activated prior to spotting by placing in an oven at 110-120ºc for 30 min. This removes the water absorbed and makes available maximum active sites which should be reacted with sample to be separated. Hence better resolution and separation can be possible. Aluminium sheets should be kept in between two glass plates and placing in oven at 110-120ºc for 15 minutes.

PRE-CONDITIONING (CHAMBER SATURATION): Automatic multiple development chambers (AMD), Automatic development chamber (ADC) or Twin trough chambers are commonly preferred chambers in HPTLC. Un- saturated chamber causes high Rf values and dispersion of spots. Saturated chamber by lining with filter paper for 30 minutes prior to development causes uniform distribution of solvent vapours and thus less solvent required for the sample to travel as well as with better Rf values.

APPLICATION OF SAMPLE: Automatic sample applicator like Linomat 5 is used to apply sample automatically as per software command.

DEVELOPMENT OF CHROMATOGRAM: As per the software the automatic chamber develops the plate to given height otherwise put plate/paper in saturated chamber until plate gets developed for 3/4th of length. Method use for development can be ascending, descending, circular, two dimensional, repeated or preparative.

DETECTION AND VISUALIZATION/LOCATION OF SPOTS: Detection under UV light is first method of choice; spots of fluorescent compounds can be observed at 254 nm or at 366 nm. Spots of non-fluorescent compounds can be seen on fluorescent stationary phase (e.g. silica gel G F254). Non-UV absorbing compounds can be observed by dipping the plates in 0.1% iodine solution. Derivatisation by spraying reagents is necessary when individual component does not respond to UV.

EVALUATION OF CHROMATOGRAM: HPTLC measures the chromatographic separation by densitometer also called as scanner. Determination of optical density of separated spot with that of empty area and same for standard spot gives the results in the form of graph which explains area, height, Rf value of peaks etc.

Result

Preliminary Phytochemical Screening: Observations on presence or absence of phytochemicals namely, fixed oils and fats, alkaloids, steroids and flavonoids, tannins and phenolic compounds were noted as + for presence and – sign for absence and are presented in Table 1.
The early phytochemical screening revealed the presence of a variety of phytochemicals as shown in the Table No. (+ Present, - Absent).

Table No.1: Phytochemical screening of extract

Test	Aegle
Alkaloids	+
Glycosides	+
Flavonoids	+
Tannins	+
Proteins	+
Fixed oils	+
Steroids	+
Terpenoids	+
Carbohydrates	+
Cardiac Glycosides	-

Results presented in Table 1 revealed that, from all the tested phytochemicals, alkaloids, steroids and fixed oils and fats, flavonoids, tannins and phenolic compounds were observed in A. marmelos extract.
TLC: TLC is a fundamental technique for separating and identifying several classes of natural products. This method allows the identification of distinct components by differential solute migration amongst two phases, one stationary and one migratory. The adsorbent (Silica) functions as a stationary phase while the solvent system function as mobile phase. Polar compounds have lower affinity for the solute, adhere to it and gradually ascends as a mobile phase advances. Compounds will travel relatively fast up the plates, resulting in increased Rf values. Mixture of substances will be separated according to their polarities.

Table No.2: Thin Layer Chromatography of extract

Plant	Extract	Solvent system	Rf value
Aegle mamelos	Hydroalcoholic extract	Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.3:0.2)	0.03, 0.28, 0.40

The Rf values of extract of A. marmelos run under Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.3:0.2) solvent system were 0.03, 0.28, 0.40

High Performance Thin Layer Chromatography: HPTLC analysis of Aegle mamelos extract was performed using a particular solvent system Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.3:0.2) which was detected under UV 254. HPTLC finger printing of hydroalcoholic extract of Aegle marmelos under white light reveal presence of five components 0.04, 0.03, 0.29, 0.42, 0.49. Component number 2,3 and 4 at Rf 0.03, 0.29 and 0.42 showed maximum concentration.

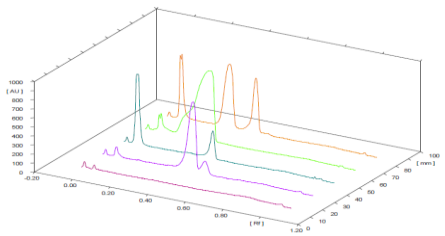


Fig No.1: 3D Overlay of HPTLC chromatogram of all tracts, at all wavelengths

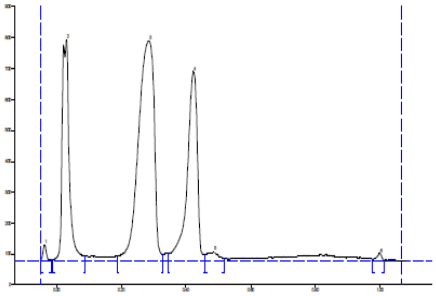
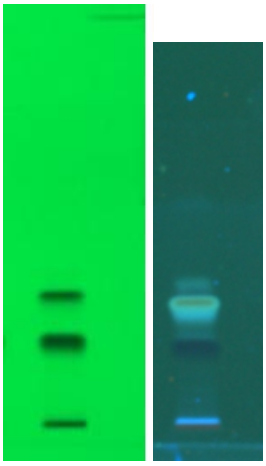


Fig No. 2: HPTLC chromatograms



(a) **(b)**

Fig. No.3: HPTLC chromatograms visualized under **a.** UV 254 nm, and **b.** UV 366 nm

Table No. 3 HPTLC peak table of hydroalcoholic extract of Aegle mamelos

Peak	tart Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.05	2.2	0.04	51.1	2.39	0.02	1.7	380.9	0.74
2	0.01	3.0	0.03	715.2	33.40	0.09	14.9	11202.2	21.82
3	0.19	15.3	0.29	710.9	33.20	0.33	20.4	24924.5	48.55
4	0.35	22.3	0.42	612.4	28.60	0.46	20.9	13840.5	26.96
5	0.46	20.9	0.49	28.5	1.33	0.52	6.8	724.3	1.41

Discussion: Herbal medicines are composed of many constituents and are therefore very capable of variation. Hence it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic components of the herbal medicine. HPTLC fingerprinting profile is very important parameter of herbal drug standardization for the proper identification of the medicinal plants.
The HPTLC performed on the hydroalcoholic extract of Aegle marmelos showed the presence of various phytoconstituents in different concentrations as illustrated in figures and tables. The number of peaks indicates the presence of different phytoconstituents present in the sample. The Rf values (Tables no. 3) calculated for the phytoconstituents present in the tested sample would be helpful in the identification of the unknown compounds by comparing them with the reference standards, and from the values of peak area, the concentration of the compounds can be determined.

Conclusion: The present study revealed the presence of several phytochemicals in hydroalcoholic extract of Aegle marmelos which might be the cause for its healing properties and thus justifies its usage as a remedy in various ailments. New drug formulations require the isolation and identification of important Phyto-compounds possessing pharmacological properties. The HPTLC study carried out for hydroalcoholic extract of Aegle marmelos chemical profiling will be helpful in the identification of bioactive compounds and markers, by comparing the Rf values of the compounds with the reference standards.

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