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Original Article

DETERMINATION OF PHYTOCOMPOUNDS FROM *TERMINALIA CHEBULA RETZ* BY HPTLC DENSITOMETRIC METHOD

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ABSTRACT

Objective: The present investigation has been focused on the detection of antibacterial activity of methanolic extract by disc diffusion method and the quantitative estimation of phyto constituents from *Terminalia chebula*, (King of Medicine) by High Performance Thin Layer Chromatography (HPTLC) method.

Methods: An in vitro study on the efficacy of methanol extract of *T.chebula* was carried out. For this analysis, Tannic acid (TA), Gallic acid (GA), Ellagic acid (EA) were as used as standard markers by using toluene: ethyl acetate: formic acid: methanol (4.3:4.3:1:1.2:0.3, V/V/V/V) was used as a mobile phase. Detection and quantification were performed densitometrically at Lambda 254 nm.

Results: The methanol extract has shown best activity against test strains. The Rf values of standards were 0.78 for TA, 0.74 for GA and 0.63 for EA. The total peak areas of the standards and the corresponding peak areas of extracts were composed and the statistical analysis was carried out.

Conclusions: Based on the present findings, there is a wide opportunity for the development of new drug formulations for the effective treatment against multiple drug resistant micro organisms with no side effects with lesser costs.

Keywords: HPTLC, Densitometry, Ellagic acid, Tannic acid.

INTRODUCTION

For Past few decades compounds from natural sources have been gaining importance because of the vast chemical diversity that they offer. This has led to phenomenal increase in the demand for herbal medicines in the last two decades and a need has been felt for ensuring the quality, safety and efficacy of herbal drugs. Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening; chemo profiling and marker compound analysis using modern analytical techniques. In the last two decades (HPTLC) has emerged as an important tool for the qualitative semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of mobile phase [1]. In India, medicinal plants have an important role as a therapeutic agent and an effective antimicrobial agent in various ailments. Today there is a wide spread interest in drugs derived from plants, which leads to the screening of several medicinal plants for their potential activities [2]. Dried fruits of *Terminalia chebula* (Combretaceae), commonly known as (Haritaki) black Myrobalan, indigenous in India, is s popular folk medicine in many Asian and African countries and has been well known for numerous pharmacological activities [3]. The fruits of Haritaki are one of the main ingredients in many Ayurvedic formulations. Eg: The popular 'Triphala'. Haritaki fruits are highly nutritious for human health as they contain various vitamins, minerals and proteins. They are an excellent source of vitamin C. These fruits are also rich in several minerals including selenium, potassium, manganese, iron and copper.

According to Lewis.and Ansubel, 2004 [4] stated that, plants are rich in wide variety of secondary metabolites such as tannins, terpenoids. Alkaloids and flavanoids were found to have antimicrobial properties in vitro. Cowan, 1999 [5] explained that, tannin is a general descriptive name for a group of phyto constituents of polymeric phenolic substances. Many physiological activities such as stimulation of phagocytic cells, host mediated tumor activity and wide range of anti – infective actions have been assigned to tannins. This mode of antimicrobial action may be related to their ability to inactivate microbial enzymes and transport proteins. Tannic acid is commercial form of tannins, a type of poly phenol. It has been described as having antimutagenic, anticarcinogenic and antioxidant activities. It may also found on fruits of many plants [6].Gallic acid is phenyl propanoid, chemically it is 3, 4, 5, - Trihydroxybenzoic acid, and possess astringent activity [7]. It is commonly used in pharmaceutical industry. It seems to have antifungal, antiviral and antioxidant activities. Yoshimura et al., 2005, [8] has stated that, ellagic acid is a natural phenol found in numerous fruits. It is a di lactone of hexahydroxydiphanic acid. It has been found to have anticarcinogenic, anti fibrosis and antioxidant activities. It has a high affinity for copper at the active site of tyrosinase and inhibits its activity.

Despite of many reports on the pharmacological activity of phyto constituents only limited numbers of papers have been published on method of quantification of *T.chebula* and its composition by using TA, GA and EA as standards. In general, two or more markers are pharmacologically active components in herbs and herbal mixtures were currently employed for evaluating the quality and authentity of herbal medicines and in assessing the quantitative herbal composition of a herbal formulation [9].

Hence the present research has been focused to standardize and quantitatively determine the bioactive principles from *Terminalia chebula*, for its various pharmacological activities. This may lead researchers to turn their new dimension of work for establishment of new drug formulation against mild to dreadful multiple drug resistant micro organisms in an effective way.

MATERIALS AND METHODS

Collection of plant material

Terminalia chebula (Haritaki) dried fruits were collected from the local siddha centre, at Erode and it was authenticated at Botanical Survey of India (BSI), Tamil Nadu Agricultural University (TNAU) campus, Coimbatore, Tamil Nadu. A voucher specimen was

preserved in our laboratory for future reference. From the dried fruits (figure - 1), seeds from the individual fruit were removed and the dried fruit pulp was crushed in to coarse powder (figure - 2) by using grinder. The powder was stored in a closed vessel for future use.

Fig. 1: shows the dried fruit of *T.chebula*

Fig. 2: shows the pulp powder of *T.chebula*

Preparation of plant extracts: [10]

Methanol Extract preparation

500 gm of coarsely powdered dried fruit pulp material was soaked in methanol and it was extracted by using soxhlet apparatus 1liter of 70 % methanol over 48 hours of extraction. The homogenate was continuously stirred for 1 hour and centrifuged at 1500 rpm for 10 minutes. The pellet was extracted twice and the extract was evaporated to dryness under reduced pressure by rotary evaporator at 35^{0}C. The dried extract was freeze dried and stored at 4^{0}C until it has utilized for experimental purpose. This methanol extracts was reconstituted in 5% Dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/ml.

Micro organisms used

To carry out antibacterial activity assay, various strains of Multiple Drug Resistant (MDR) Uropathogenic *Escherichia coli* (UPEC) were selected based on their commercial drug resistant profile.

Antibacterial screening of methanol extract by using Disc Diffusion method

Antibacterial activities of methanol extracts were determined by using agar disc diffusion method. The microbial cultures were adjusted in accordance with McFarland's turbidity standards. 20 µl of the culture was spread on the Mueller Hinton agar (MHA) plates. Various concentrations (10, 20, 30, 40 and 50µg) of methanol extracts were added to the sterile discs, used for the determination of zone of inhibition. Then the discs were placed on the surface of the MHA plate with the help of sterile forceps. DMSO and gentamycin was used as a negative control and reference antibiotic (positive control) respectively. All the plates were kept for incubation at 37^{0}C for 24 hours. Following incubation, zone of inhibition was measured by using standard chart. The test was performed in triplicates and the final results were presented as the zone of inhibition.

Estimation phytocompounds by using HPTLC method [11]

Instrumentation

Analysis was performed on a Camag HPTLC (High Performance Thin Layer Chromatography) system equipped with a sample applicator Linomat V, twin trough development chamber (10x10) size, TLC Scanner III, Wincats integration software was used.

Reagents and Chemicals

Analytical grade Toluene, Ethyl acetate, Methanol, Formic acid, was obtained from SD fine chem. Ltd, Mumbai. Pure Tannic acid (TA), Gallic acid (GA), and Ellagic acid (EA) were obtained from Sigma Aldrich Pvt Ltd, Chennai. Pre coated TLC aluminium sheets silica gel F_{254} (10 x10 cm, 0.2 mm thick) plates were obtained from Hi Media, Mumbai.

Preparation of Standards- Tannic acid, Gallic acid, Ellagic acid Solution

10 mg of Tannic acid, Gallic acid, Ellagic acid were accurately weighed and dissolved into 10 ml volumetric flask by means of ultrasonication for 15 minutes. The solution was diluted up to 10ml with methanol (1mg/ ml). From the Stock solution, 1ml was pipette out and further diluted up to 10 ml to obtain the final concentration (100 µg/ml) [12].

Preparation of sample (methanolic extract)

T.chebula dried fruit methanolic extract was accurately weighed (1gm) in to 10 ml volumetric flask, dissolved in 5 ml methanol and then it was getting filtered through Whatmann No 1 filter paper and then it was made up to the mark with methanol.

Development of HPTLC technique

The Samples of methanolic extracts of *T.chebula* and standards, TA, GA and EA were spotted in the form of bands by using automatic TLC applicator Linomat V, 10mm from the bottom on a Precoated TLC aluminium sheets silica gel F_{254} (10x10cm, 0.2mm thickness) plates. The mobile phase used was Toluene: Ethyl acetate: Formic acid: Methanol (4.3:4.3:1.2:0.3 v/v/v/v). Following saturation of the solvent system, the plates were kept in Camag twin trough chamber for 15min. After development the plates were dried in hot air oven at 60^{0}C for 5 minutes and scanned by using CAMAG TLC Scanner III with absorbance at 254nm and operated by Win CATS software 4.03 versions.

Summary of Estimation of TA, GA and EA in *T.chebula*

Stationary Phase: Silica gel F_{254} plates

Mobile Phase: Toluene: Ethyl acetate: Formic acid: Methanol

(4.3:4.3:1.2:0.3 v/v/v/v).

Standard 1: TA (1mg/ml) – 5 µl

Standard 2: GA (1mg/ml) – 5 µl

Standard 1: EA (1mg/ml) – 5 µl

Sample 1: *T.chebula* methanolic extract (100mg/ml) – 5 µl

Sample 2: *T.chebula* methanolic extract (100mg/ml) – 10 µl

Migration distance: 80 mm

Scanning Wavelength: 254 nm

Mode of scanning: Absorption (Deuterium).

Quantification

Sample solutions (5µl) and standard solution (2.5µl, 5µl, 7.5µ) were spotted on HPTLC plate (E.Merck). The percentage of GA, EA and TA present in methanolic extract of *T.chebula* was calculated by comparison of the areas measured for standard solution.

Linearity

The linearity of GA,EA and TA was determined by applying standard solution of different concentrations ranging from 2.5-7.5 µg/ml spot on 20x20 cm HPTLC plates, precoated with silica gel GF 254 (E.Merck) in the form of sharp 6 mm bands, the distance between 2 adjacent bands was 9.5 mm.The plate was developed in a solvent system of Toluene: Ethyl acetate: Formic acid: Methanol (4.3:4.3:1.2:0.3 v/v/v/v) up to a distance of 80 mm, at room temperature. The plate was dried in hot air oven. The detector response for GA, EA and TA was measured for each band at

wavelength of 254 nm, using Camag TLC scanner & win CAT software. The peak area of above mentioned phytocostituents was obtained by plotting a graph of peak vs. applied concentration (in µg).

RESULT AND DISCUSSION

Medicinal plants are important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for synthesis of drugs or as models for pharmacologically active compounds [9]. Identification and quality evaluation of crude drugs is a fundamental requirement of industry and other organizations dealing with natural health products (NHP).The fact must be taken in to consideration that the plant material to be examined has a complex and inconsistent compositions based on its contents of secondary metabolites [13].

Antibacterial activity of methanol extract of *Terminalia chebula*

Among bacterial pathogens, gram positive bacterial strains were found to be more susceptible than gram negative bacterial strains. This may be attributed to the fact that cell wall in gram positive bacteria consist of a single layer, whereas, gram negative cell wall is multilayered structure bounded by an outer cell membrane [14]. The inhibitory effect of the extracts may be attributed to the presence of bioactive metabolites. Several reports have shown that bioactive compounds isolated from plant extract have growth inhibitory effect on pathogenic strains [15, 16, and 17]. Antibacterial activity of methanolic extract of *T.chebula* was presented in table – 1 and figure – 3.

Table 1: shows antibacterial activity of methanolic extract of *T.chebula*

S. No.	Strains used	Zone of inhibition (in mm)					Gentamycin (20 µl)
		Concentration (in µl)					
		10	20	30	40	50	
1	*E.coli* 30	21	24	26	27	25	
2	*E.coli* 62	15	16	16	15	15	20
3	*E.coli* 117	14	14	15	16	15	
4	*E.coli* 129	15	16	17	17	16	
5	*E.coli* 168	14	15	16	16	17	

a) Control plate (DMSO)

b) *E.coli* strain no 30

c) ***E.coli* strain no 117**

d) *E.coli* strain no 62

e) ***E.coli* strain no 129**

f) *E.coli* strain no 168

Fig. 3: shows Antibacterial Activity of Methanolic extract of *T.chebula* against MDRUPEC strains by disc diffusion method

All the strains shows zone of inhibition against the methanolic extract in which the greater activity was observed in *E.coli* 30, has exhibited the zone of inhibition as 21nm (10µl), 24 nm (20 µl), 26 nm (30µl), 27 nm (40 µl) and 25 nm (50µl) as compared to the control.

In HPTLC analysis, the Rf values of standards such as Tannic Acid is found to be 0.78 (Figure – 4), Gallic Acid is found to be 0.74 (Figure-5) and Ellagic Acid is found to be 0.63 (Figure - 6) and the peak areas covered are represented in graph, Tannic Acid (20 µg) – 5250.32; Gallic Acid (1µg) – 17258.09; Ellagic Acid (20µg) – 17109.96. Our sample *T.chebula* methanolic extract has shown the amount of TA, GA and EA was found to be 5.3 % (w/w), >0.1 % (w/w), 7.3 % (w/w) respectively, shown in table – 2.

Table 2: shows the recovery of phytocompunds from the methanol extract of *T.chebula*

S. No.	Name of the compounds	Rf values	Amount of sample loaded	Area	Recovery	Yield % (w/w)
1	Tannic Acid	0.78	3 µl	5103.82	16.13 µg	5.3
2	Gallic Acid	0.74	3 µl	5953.52	279.42 ng	>1%
3	Ellagic Acid	0.65	3 µl	23818.41	22.00 µg	7.3

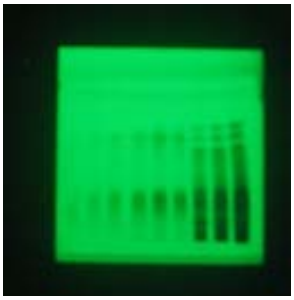

Fig. 4: shows the detection of Tannic Acid @ 254nm

Picture shows that, lane 1 to lane 5 denotes increasing concentration (1, 3, 6, 9 and12µl) of standard Tannic acid; lane 6 to lane 8 denotes different concentration (4, 8 and 12 µl) of *T.chebula*

Fig. 5: shows the detection of Gallic Acid @ 254nm

Picture shows that, lane 1 to lane 5 denotes increasing concentration (1, 3, 6, 9 and12µl) of standard Gallic acid; lane 6 to lane 8 denotes different concentration (4, 8 and 12 µl) of *T.chebula*

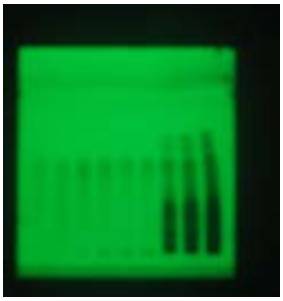

Fig. 6: shows the detection of Ellagic Acid @ 254nm

Picture shows that, lane 1 to lane 5 denotes increasing concentration (1, 3, 6,9 and12µl) of standard Ellagic acid; lane 6 to lane 8 denotes different concentration (4,8 and 12 µl) of *T.chebula*.

Gallic acid, ellagic acid, tannic acid, ethyl gallate, chebulic acid, chebulagic acid, corilagin, mannitol, ascorbic acid and other compounds had been reported from *T. chebula* [18]. The method of sample preparation and the development of a suitable mobile phase are two important steps in devising an analytical procedure. With respect to the phytomedicines, these steps ae more significant because of the complexity of the chemical composition and the affinities of the components towards various solvents. By analysing different mobile phases for the effective separation of extract of *T.chebula* and its constituents by HPTLC, the desired resolution of TA,GA and EA with symmetrical and reproducible peaks were achieved by using Toluene: Ethyl acetate: Formic acid: Methanol (4.3:4.3:1.2:0.3 v/v/v/v) as suitable mobile phase which was supported by many researchers. Thus, HPTLC method is more rapid, very precise, more sensitive, and simple. Low cost, accurate and reproducible, which can be utilized for quantitative monitoring of phyto constituents of *T.chebula* by using markers.

CONCLUSION

The developed HPTLC method was simple, accurate, precise and cost-effective and can be utilized for the routine analysis of quantitative determination of plant sample. The present findings indicate that *T.chebula* contains a number of markers of varying proportions that may be responsible for its therapeutic activity. Hence this study forms a platform for researchers to undertake an effective drug formulation of various phyto constituents from *T.chebula* using this more precisemethod of quantification by HPTLC method against the emerging multiple drug resistant Micro organisms towards the human population. The findings of the present investigation validate their traditional use and suggests that *T. chebula* extracts has better efficacy and can be a source for natural antimicrobial agent.

CONFLICT OF INTERESTS

Declared None

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