Vol 14 | Issue 1| 2023 | 25-33.

e - ISSN 2249-7544
Print ISSN 2229-7464

INTERNATIONAL JOURNAL
OF
PHYTOPHARMACY RESEARCH
www.phytopharmacyresearch.com

PRINCIPAL COMPONENT ANALYSIS AND CHROMATOGRAPHIC FINGERPRINTING OF *AZIMA TETRACANTA* SAMPLE

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ABSTRACT

Many plant species are used by traditional healers and indigenous medical systems (including Ayurveda, Siddha, and Unani) to treat a variety of human and animal illnesses. Studies on the plant's phytochemistry found that it included substances such friedelin, euphanol, gallic acid, genstisic acid, cinnamate, ferulic acid, azimine, azcarpine, and carpaine. Standard phytochemical tests, gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography, liquid chromatography–MS, and other chromatographic techniques, as well as spectral analyses such as nuclear magnetic resonance and infrared, have been utilized by a variety of researchers in order to identify chemicals or phytochemical groups that are present in different parts of the plant. Ash values, extractive values, loss on drying and foreign organic matters were determined as per the standard WHO guidelines which is very much useful in the determination of quality and purity of the crude drugs. Ethanolic extract of *Azima tetracanta* is subjected to qualitative chemical analysis. The extract was analysed using a liquid chromatography (Shimadzu, Japan). Thin Layer Chromatography of the Ethanolic extract of *Azima tetracanta* was performed and Rf values were determined. Ethanolic extract of *Azima tetracanta* was subjected to *in vitro* antioxidant studies. Ethanolic extract of *A. tetracantha* leaves exhibited a concentration dependant free radical scavenging property as observed from hydrogen peroxide assay. Antioxidant property of *A. tetracantha* may be one of the reasons for its use as a rejuvenating drug in the traditional medicinal practices.

Keywords: *A.tetracanta, Chromatographical studies, Traditional medicine, phytoconstituents, HPLC, HPTLC*

INTRODUCTION

Humans depend on plants for many aspects of daily life. Relationships and interactions between people and plants are referred to as ethno botany. Plants are used as sources of food, medicine, colours, and wood all across the world. Many plant species are used by traditional healers and indigenous medical systems (including Ayurveda, Siddha, and Unani) to treat a variety of human and animal illnesses. Secondary metabolites including alkaloids, terpenes, and polyphenol chemicals hold the medicinal potential of plants. 80% of the world's population relies on medical plants, and in India, the use of medicinal plants as therapeutic agents continues to be a significant part of the traditional system [1].

Studies on the plant's phytochemistry found that it included substances such friedelin, euphanol, gallic acid, genstisic acid, cinnamate, ferulic acid, azimine, azcarpine, and carpaine. The bark of the *A. tetracantha* root was used to cure musculoskeletal rheumatism, while the juice extracted from the leaves is used to treat tooth and ear pain. As a remedy for snake bites, the pounded root of *A. tetracantha* Lam. were topical treatment to the affected area in East Africa, and an infusion of the plant was consumed orally. As a treatment for rheumatism, the root, root bark, and leaves of the plant are frequently included in the cuisine of India and Sri Lanka. The city of Bangalore makes use of it by planting it as live fences. Standard phytochemical tests, gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography, liquid chromatography–MS, and other chromatographic techniques, as well as spectral analyses such as nuclear magnetic resonance and infrared, have been utilized by a variety of researchers in order to identify chemicals or phytochemical groups that are present in different parts of the plant. Combination of fingerprint techniques with chemometrics makes it more powerful for TMM's quality control [2]. To this day, a great number of articles introducing fingerprint or quantitation approaches targeting a variety of ATR (*A.tetracanta*) indicators have been published. However, the reasons for marker selection have not been properly stated, and more crucially, the high expense of doing quality study in comparison to the price of the treatment is another major issue. In light of the uneven quality level of ATR that is currently available, the purpose of this study was to identify rational detection indices for quality control of ATR and to develop a method that is both inexpensive and quick for assessing

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quality in order to discriminate between qualified and unqualified samples [3].

It has been demonstrated that the seed oil contains, in addition to the typical fatty acids, ricinoleic acid as well as cyclopropenoid fatty acids. It has been demonstrated that N-methoxy-3-indolylmethylglucosinolate was present in significant amounts in the plant's roots and seeds, although its presence was less prevalent in the plant's stems and younger leaves. In addition to that, the roots have another indole glucosinolate (N-hydroxy-3-indolymethyl-glucosinolate). Neoascorbigen was a condensation product that results from the reaction between N-methoxy-indole-3-carbinol and ascorbic acid. It could be found in the roots, stems, and leaves of the plant. There was a diverse combination of 26 flavonoids in the seeds, most of which are found in the form of glycosides and acyl-glycosides, with some aglycones also present. There was evidence of the presence of the dimeric piperidine alkaloids azimine, azcarpine, and carpaine in all of the tissues [4].

Methodology

Pharmacognostical Studies

Macroscopic Evaluation

Various organoleptic characters like colour, odour, taste and nature and thickness were observed.

Physiochemical Evaluation

Ash values, extractive values, loss on drying and foreign organic matters were determined as per the standard WHO guidelines which is very much useful in the determination of quality and purity of the crude drugs.

Determination of Ash Values

Ash values are helpful in determining the quality and purity of a crude drug in the powdered form. The residue remaining left after incineration of the crude drug is designated as ash. The residue obtained usually represents the inorganic salts naturally occurring in the drug and adhering to it. An ash value determination furnishes the basis for judging the identity and cleanliness of any drug and gives information relative to its adulteration/contamination with inorganic matter.

Total ash

The determination of ash is useful for detecting low grade products, exhausted drugs & excess of sandy and earthy material. Total ash is useful to exclude drugs which have been coated with chalk, lime or calcium sulphate to improve their appearance. The total ash usually consists of carbonates, phosphates, silicates and silica which include both physiological ash, which is derived from plant tissue itself and non-physiological ash which is the residue of the extraneous matter adhering to the plant surface.

Procedure

Silica crucible was heated to red hot for 30 minutes and it was allowed to cool in desiccators. About 2g of powdered sample was weighed accurately and evenly distributed in the crucible. Dried at 100 – 105ºC for 1 hour and ignited to constant weight at 600±25ºC. The crucible was allowed to cool in a desiccator. The percentage yield of ash with reference to the air-dried substance was then calculated.

$$\text{Wt. of total ash} = \frac{\text{\% Total ash value}}{\text{Wt. of crude drug taken}} \times 100$$

Water soluble ash

The ash was boiled for 5min with 25mL of water. The insoluble matter was then collected in an ash less filter paper. It was washed with hot water and ignited for 15min at a temperature not exceeding 450°C. The weight of the insoluble matter was subtracted from the weight of the ash and the difference in weight represented the water-soluble ash, the percentage of water-soluble ash with reference to the air dried substances was calculated by the formula.

$$\text{\% Water soluble ash value} = \frac{\text{Wt. of total ash} - \text{Wt . of water insoluble ash}}{\text{Wt. of crude drug taken}} \times 100$$

Acid insoluble ash

To the crucible containing total ash of the sample, 25 mL of dilute hydrochloric acid was added. The insoluble matter is collected on an ashless filter paper (Whatman 41) and washed with hot water until the filtrate is neutral. Filter paper containing the insoluble matter to the original crucible dry on hot plate and ignite to constant weight. The residue is allowed to cool in a suitable desiccator for 30 minutes and weighed without delay. Content of acid insoluble ash with reference to the air-dried drug is calculated.

$$\text{\% Acid insoluble ash value} = \frac{\text{Wt . of water insoluble ash}}{\text{Wt. of crude drug taken}} \times 100$$

Sulphated ash

About 3g of air-dried substance was ignited gently at first in a crucible, until the substance was thoroughly charred. Then the residue was cooled, moistened with 1mL of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800 ± 25ºC, until all the black particles were disappeared. The crucible was allowed to cool, a few drops of sulphuric acid was added and heated. Then it was ignited as before, cooled and weighed. The percentage of sulphated ash with reference to the air-dried substance was then calculated.

Determination of extractive values

This method is used to determine the number of active constituents in a given amount of plant material

when extracted with solvents. Extractive values are useful for the evaluation of phytoconstituents especially when the constituents of a drug cannot be readily estimated by any other means. Further these values indicate the nature of the active constituents present in a crude drug.

Determination of Water-Soluble extractive

About 5g of the powder was weighed and macerated with 100mL of chloroform water (95mL distilled water and 5mL chloroform) in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25mL of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish. 2 mL of alcohol was added to the residue and it was dried at 105ºC for 1 hour in the hot air oven and cooled in desiccators for 30min and weighed. The process was repeated till a constant weight was obtained; the percentage of water-soluble extractive value with reference to the air dried drug was calculated.

$$\text{Water soluble extractive value} = \frac{\text{weight of the dried extract}}{\text{weight of the sample taken}} \times 100$$

Determination of alcohol soluble extractive

5g of the powder was weighed and macerated with 100mL 90% ethanol in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25mL of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish. It was dried at 105ºC for 1hour in a hot air oven. The dish was cooled in desiccator and weighed. The process was repeated till the constant weight was obtained. The percentage of alcohol soluble extractive value with reference to the air-dried drug was calculated.

$$\text{Alcohol soluble extractive value} = \frac{\text{weight of the dried extract}}{\text{weight of the sample taken}} \times 100$$

Determination of ether soluble extractive

About 2g of powdered drug was accurately weighed and extracted with anhydrous diethyl ether in a continuous extractive apparatus for 20hours. The ether solution was transferred to tarred porcelain dish and evaporated spontaneously. Then it was dried over phosphorous pentoxide for 18hours and the total ether extract was weighed. The extract was heated gradually and dried at 105ºC to constant weight. The loss in weight represents the volatile portion of the extract.

Determination of moisture content (loss on drying)

Accurately weighed quantity of the substances was taken in a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle side wise shaking. The crucible with the contents was weighed accurately. The loaded crucible and the lid were placed in the drying chamber (105ºC). The substance was heated for a specified period of time to a constant weight. The crucible was covered with the lid and allowed to cool in a desiccator at room temperature before weighing. Finally, the crucible was weighed to calculate the loss on drying with reference to the air-dried substance

$$\text{\% Loss on drying} = \frac{\text{Loss in weight of the sample}}{\text{Weight of the sample}} \times 100$$

Preparation of Ethanolic Extract of *Azima tetracanta* leaves (EEATL)

The powder plant material was defatted with petroleum ether by maceration method. Defatted marc was extracted with 95% ethanol by means of maceration for a period of 72 hrs. Then the extract was filtered and evaporated under reduced pressure using Buchi rotary evaporator and the extract thus obtained is used for further experimental studies.

Qualitative Analysis

Phytochemical Investigations

Ethanolic extract of *Azima tetracanta* is subjected to qualitative chemical analysis. The various chemical tests were performed on this extract for the identification of flavonoids, phenolic compounds, alkaloids, glycosides, carbohydrates, carotenoids, proteins, tannin, aminoacids, and sterols are determined [5].

Quantitative Estimation

Determination Of Total Phenolic Content Of Eeatl Extract

Procedure

About 1 mL (1 mg/ml and 0.5 mg/mL) of ethanolic extract of *Azima tetracanta* (EEATL), 0.5 mL of Folin-ciocalteu reagent (1N) were added and allowed to stand for 15 minutes. Then 1 mL of 10% sodium carbonate solution was added to the above solution. Finally, the mixtures were made up to 10 mL with distilled water and allowed to stand for 30 minutes at room temperature and total phenolic content was determined spectro photometrically at 650nm wavelength. The calibration curve was generated by preparing gallic acid at different concentration (10, 20, 30, 40 and 50 µg/mL). The reaction mixture without sample was used as blank. Total phenolic content of HAEEA extract is expressed in terms of mg of Gallic acid equivalent per gm of extract (mg GAE/g).

Instrument:

UV visible spectrophotometer, (Shimadzu – Model 1800)

Reagents required

Folin- Ciocalteu reagent (1N), Sodium Carbonate solution (10%), Standard Gallic acid solution.

Analytical Studies

Chromatography

Chromatography methods are important analytical tool in the separation, identification and estimation of components present in the plant. Chromatographic fingerprinting has been in use for a long time for single chemical entity drug substances. Recently it has become one of the most powerful tools for quality control of herbal medicines. The use of chromatographic fingerprinting for herbal drugs tends to focus on identification and assessment of the stability of the chemical constituents observed by various chromatography techniques such as HPLC, TLC, HPTLC, GC, capillary electrophoresis [6].

Thin Layer Chromatography

TLC Plate Preparation

The plates were prepared using Stahl TLC spreader. 40g of silica gel G was mixed with 85mL of water to prepare homogenous suspension and poured in the spreader. 0.25mm thickness of plates was prepared, air dried until the transparency of the layer disappeared, then dried at 110°C for 30 minutes and kept in desiccators [7].

Selection of mobile phase

Solvent mixture was selected on the basis of the phytoconstituents present in each extract. Factors such as nature of components, stationary phase, polarity, influence the rate of separation of constituents was considered. From the vast analysis, best solvents were selected which showed good separation with maximum number of components.

$$R_f = \frac{\text{Ditance travelled by the solute from baseline}}{\text{Distance travelled by the solvent from baseline}}$$

Procedure

Thin-layer chromatography (TLC) was a chromatography technique used to separate non-volatile mixtures. After the sample has been applied on the plate, a solvent mixture (n-hexane: Ethyl acetate,) in a ratio of (9:3) used to elute a compound via drawn up the sample by capillary action 7, 8. Then the plate was kept in an oven for period of time, finally dark brown colour spot observed when sprayed with 50% Aq.H_2SO_4 as detecting agent.

HPTLC (high performance thin layer chromatography)

HPTLC is one of the versatile chromatographic methods which helps in the identification of compounds and thereby authentication of purity of herbal drugs. The time required in this method for the demonstration of most of the characteristic constituents of a drug is very quick and short. In addition to qualitative detection, HPTLC also provides semi-quantitative information on major active constituents of a drug, thus enabling an assessment of drug quality.

HPTLC severs as a convenient tool for finding the distribution pattern of phytoconstituents which is unique to each plant. The fingerprint obtained is suitable for monitoring the identity and purity of drugs and for detection adulteration and substitution. HPTLC technique is helpful in order to check the identity, purity and standardize the quantity of active principles present in the herbal extract [8].

Instrument condition

- HPTLC plate: silica gel 60 precoated plate
- Developing solvent: dichloromethane–butyl acetate–methanol–water (10:20:15:4)
- Sample application: 2 mL
- Observation: sprayed 5% aluminum chloride in ethanol
- Temperature: 90^{o}C
- Detector: UV 254 nm and Visible light

HPLC Analysis

The extract was analysed using a liquid chromatography (Shimadzu, Japan). Separation was achieved on a reverse phase C18 column, temperature at 24^{o}C. The mobile phase of the optimized chromatographic method consisted of solvent A (methanol) and solvent B (0.5% (v/v) acetic acid in water). The elution profile was as follows: 0 min 10% A in B, 28.6 min 60% A in B, 30 min 10% A in B. The flow rate was 1 mL/min, and injection volume was 20µL. Absorption was measured at 290 nm. Detection was carried out in UV-visible detector. The eluted components were identified based on the retention time by comparison with retention time of reference standard. The phenolic compounds present in the samples were characterized according to their UV-vis spectra and identified by their retention times in comparison with those of commercial standards.

Anti-oxidant Assays

Phenolic acids, polyphenols and flavonoids from plants are well known scavengers of free radicals. So it is necessary to analyse the quenching effect of ethanolic extract of *Azima tetracanta* by *in vitro* studies that relates the phenolic content with its anti-oxidant activity.

Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the methanol extract of *A. tetracantha* was determined by the method of Plant extract (4 ml) prepared in distilled water at various concentrations were mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1M, pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against the blank solution containing the plant extract without H_2O_2 [9].

RESULTS

Pharmacognostical Studies

Pharmacognostical studies play a key factor in establishing the authenticity of the plant material. The botanical identity of the plant was established by examining its anatomical features. The results of the pharmacognostical studies are as follows

Assessment of Quality of Plant Material

Physiochemical Evaluation

Physiochemical parameters are mainly used in judging the purity and quality of the powdered drug. The

physiochemical standards of *Azima tetracanta* were listed below.

The above results were given that the foreign organic matter of the crude drug material was found to be nil, the percentage of moisture was found to be 7.25±0.055%w/w, and the percentage of extractive value such as ether, ethanol, aqueous was found to be 0.98%, 1.31%, 1.24% respectively.

The determination of the ash value helps to find out where the powder material was adulterated with sand and other organic material. The percentage of ash value such as total ash, water soluble ash, acid insoluble ash and sulphated ash was found to be 7.41±0.01%w/w, 1.11±0.1%w/w, 6.78±0.15%w/w and 15.31±0.01 %w/w respectively.

Phytochemical Investigations

Preliminary Phytochemical Screening of Ethanolic Extract of *Azima tetracanta* (EEATL)

Ethanolic extract of *Azima tetracanta* was subjected to both qualitative as well as quantitative chemical analysis. The various chemical tests were performed for EEATL extract for the identification of phytoconstituent. The results were displayed in table

Determination of Total Phenolic Content of Eeatl Extract

Quantitative analysis of total phenolic contents presents in EEATL extract and their results were presented in table 6.

Analytical Studies

Thin Layer Chromatography

Thin Layer Chromatography of the Ethanolic extract of *Azima tetracanta* was performed and Rf values were determined.

The EEATL showed 3 spots 0f Rf value 0.67, 0.72 and 0.85 respectively. The standard quercetin showed Rf value 0.87. The presence of quercetin in EEATL was confirmed by thin layer chromatography.

Chromatogram was developed by HPTLC for ethanolic extract of *Azima tetracanta* (EEATL) and it showed significant separation of five phytoconstituents whose Rf values ranges from 0.241 to 0.911.

Based on HPTLC studies, it was inferred that quercetin was present in the plant extract. It was found that Rf values of a phytoconstituents (quercetin) present in HAEEA almost coincided with the Rf value of standard quercetin (0.494).

Table 1: Characteristics of leaf powder of *Azima tetracanta*

S.NO	Characteristics	Observation
1	Colour	Dark green to pale green
2	Odour	Characteristic odour
3	Taste	Tasteless
4	Margin	Simple and entire
5	Apex	Mucronate
6	Base	Acute base
7	Shape	Aristate with a spine on tip
8	Vein	Cross venulate

Table 2: Ash Values of leaf powder of *Azima tetracanta*

S.No	Particulars	Ash Value of leaves powder (%w/w)
1	Total ash	7.41±0.01
2	Acid insoluble ash	1.11±0.1
3	Water soluble ash	6.78±0.15
4	Sulphated ash	15.31±0.01

***** Values are expressed as Mean ± SD, n=3**

Table 3: Extractive Values of leaf powder of *Azima tetracanta*

S. No	Parameter	Results (%w/w)
1	Loss on drying	7.25%

Thin Layer Chromatography

Table 4: Preliminary phytochemical screening of EEATL

S. NO	TESTS	RESULTS
1	**ALKALOIDS**	+
2	**CARBOHYDRATES**	+
3	**GLYCOSIDES**	-
4	**STEROLS**	+
5	**FLAVONOIDS**	+
6	**TERPENOIDS**	-
7	**GUM**	-
8	**MUCILAGE**	-

9	**PROTEIN AND AMINO ACIDS**	+
10	**SAPONINS**	+
11	**TANNINS**	+
12	**GELATIN/FAT**	-
13	**VOLATILE OIL**	+

Table 5: Estimation of total phenolic content in EEATL

S.NO	CONCENTRATION	ABSORBANCE	
	GALLIC ACID AND EEATL µg/ml	GALLIC ACID (*MEAN +SEM)	EEATL *MEAN +SEM
1	**10**	0.067± 0.0008	0.054 ± 0.0002
2	**20**	0.109 ± 0.0008	0.085 ± 0.0001
3	**30**	0.152 ± 0.0028	0.112 ± 0.0006
4	**40**	0.192 ± 0.0029	0.141 ± 0.0003
5	**50**	0.234 ± 0.0029	0.189 ± 0.0002
		GAE	105mg/g

Table 6: Thin Layer Chromatography of Ethanolic extract of Azima tetracanta

NAME OF THE SAMPLES	MOBILE PHASE	DETECTING AGENT	RF VALUE	RESULT
STD	**n-hexane: Ethyl acetate (9:3)**	**50% Aq.H_2SO_4**	**0.87**	**Indicates the presence of Quercetin**
EXTRACT				
SPOT -1			**0.67**	
SPOT -2			**0.72**	
SPOT -3			**0.85**	

Table 7: HPTLC profile of EEATL extract and standard Quercertin

S.No	Peak	Rf of value sample	Rf value of std	UV detector (nm)
1	**Peak 1**	**0.241**		**254nm**
2	**Peak 2**	**0.391**		
3	**Peak 3**	**0.491**	**0.494**	
4	**Peak 4**	**0.501**		
5	**Peak 5**	**0.911**		

Table 8: Determination of Hydrogen Peroxide Scavenging Activity

S.NO	Conc. of ascorbic acid & EEATL	% inhibition of Ascorbic acid µg/ml	% inhibition of EEATL µg/ml
1	10	98.1551	97.1541
2	20	99.11705	97.615
3	30	99.3104	97.998
4	40	99.391	99.1254
5	50	103.011	99.1458
	IC_{50}	**2.835µg/ml**	**2.795µg/ml**

Figure 1: Leaf extract of Azima tetracanta

Figure 2: Calibration curve of Quercertin

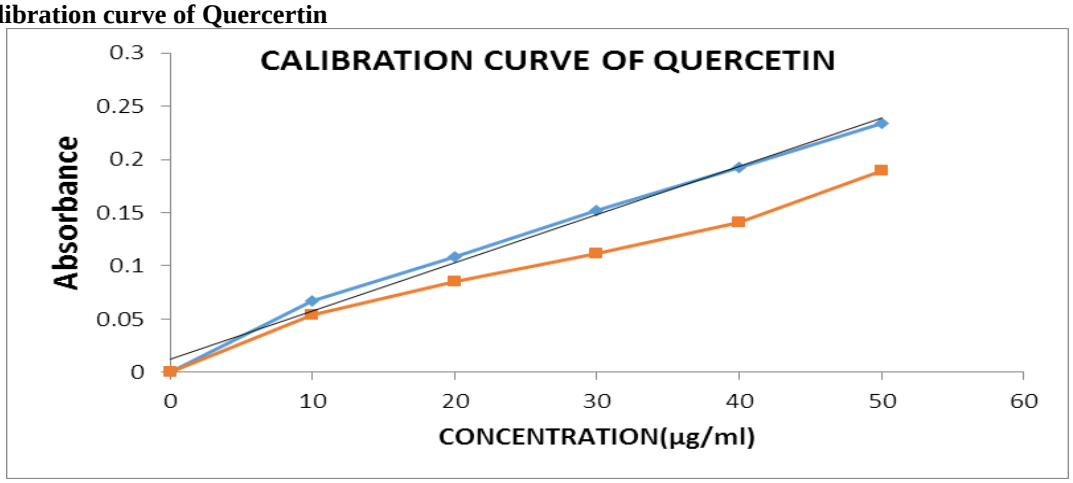


Figure 3: TLC OF QUERCETIN AND EEATL

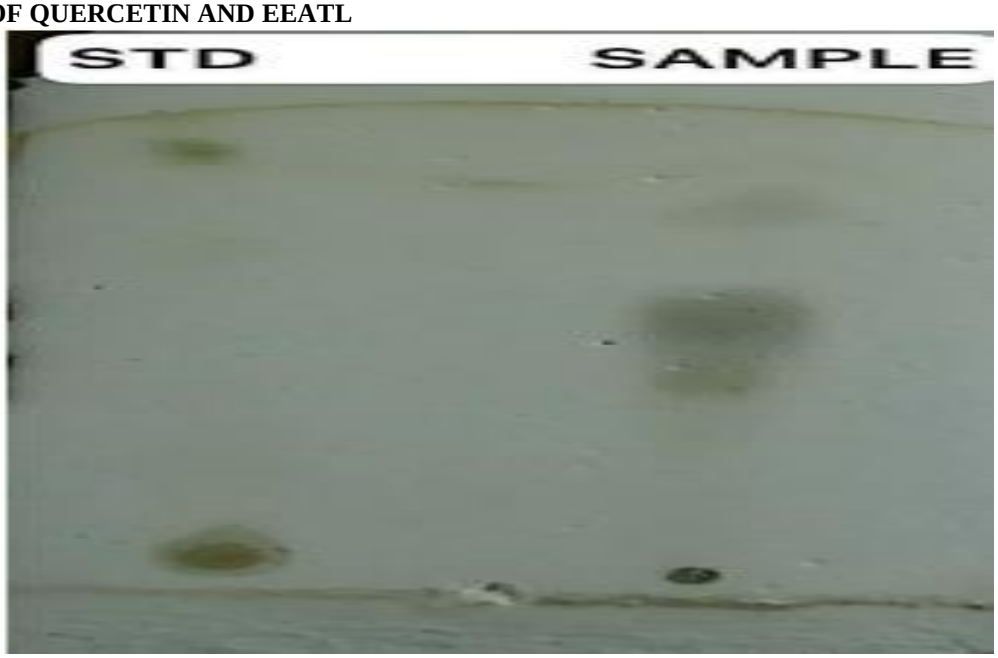


Figure 4: HPTLC CHROMATOGRAM OF EEATL

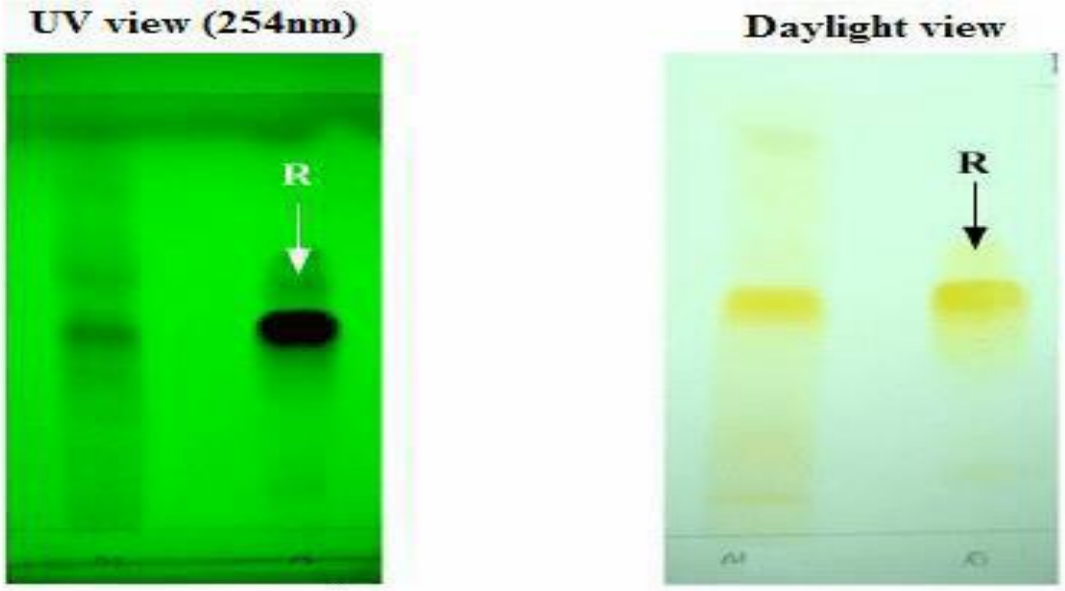


Figure 5: HPLC chromatogram of A. tetracantha extract recorded at 290 nm. Peaks related to phenolic acids are indicated, 1. Gentisic acid, 2. Benzoic acid, 3. Gallic acid, 4. Cinnamic acid, 5. Rosmarinic acid, 6. Ferulic acid, 7. Salicylic acid, 8. Vanillin

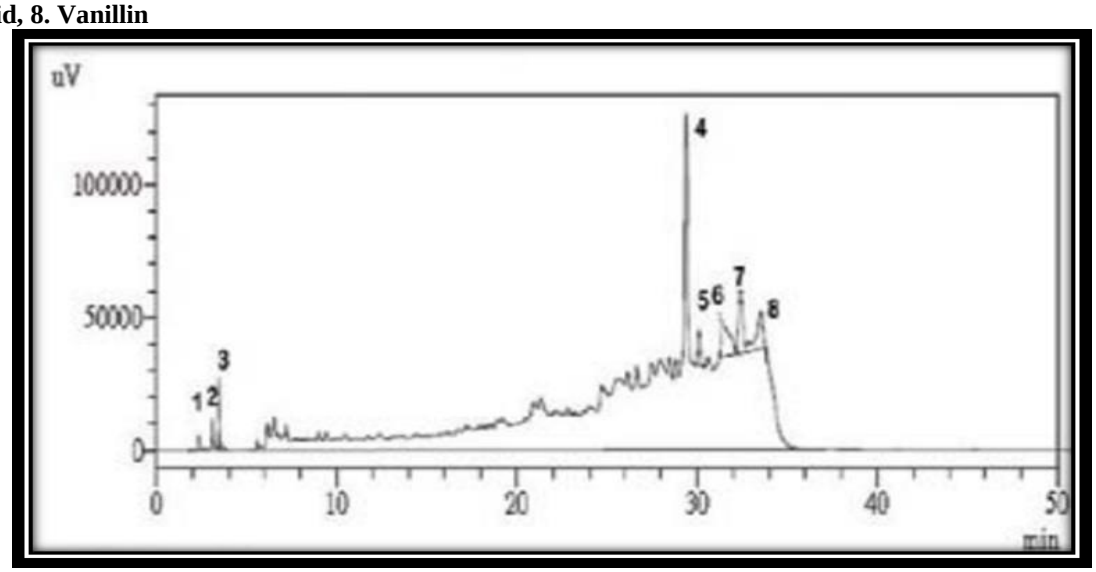
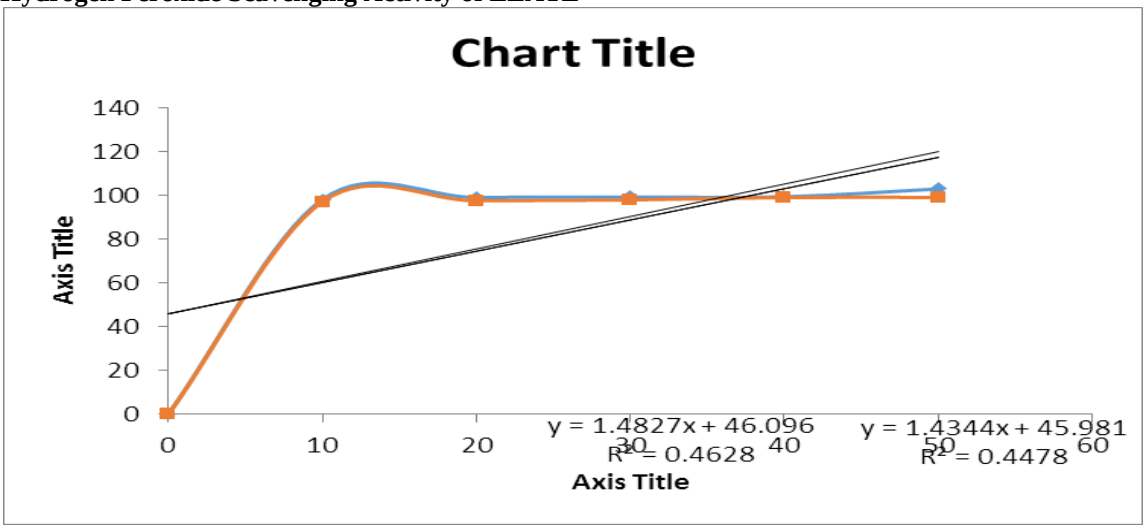


Figure 6: Hydrogen Peroxide Scavenging Activity of EEATL



HPLC ANALYSIS

The phenolic acid composition in ethanol extract was identified by HPLC analysis. In the present observation both benzoic acid as well as cinnamic acid derivatives was identified. The benzoic acid derivatives of gentisic acid (2.6 min), benzoic acid (3.6 min), gallic acid (3.7), salicylic acid (32.7) and vanillin (33.5) and cinnamic acid derivatives of cinnamate (29.2 min), rosmarinic acid (30.4), ferulic acid (31.7), were identified from ethanol extract of *A. tetracantha*, by comparison with the corresponding standard compounds shown in fig 5

Anti-Oxidant Assay

Hydrogen Peroxide scavenging assay

Ethanolic extract of *Azima tetracanta* was subjected to *in vitro* antioxidant studies, among H_2O_2 result can show below

The inhibitory concentration (IC50) of *Azima tetracanta* leaf against hydrogen peroxide scavenging effect was found to be 2.795µg/ ml in comparison with ascorbic acid 2.835µg/ml.

CONCLUSION

WHO has emphasized the need to ensure quality control of the raw materials used for Ayurvedic medicines by using modern techniques and by applying suitable parameters and standards, Science has long acknowledged the valuable medicinal shrub *Azima tetracantha*, it has wide range of application in medicinal field such as antifungal, antioxidant, antimicrobial, anticancer etc. Ethanolic extract of *A. tetracantha* leaves exhibited a concentration dependant free radical scavenging property as observed from hydrogen peroxide assay. Antioxidant property of *A. tetracantha* may be one of the reasons for its use as a rejuvenating drug in the traditional medicinal practices.

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