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IDENTIFICATION OF QUERCETIN PRESENT IN ETHANOL LEAF EXTRACT OF *AMARANTHUS SPINOSUS* BY CHROMATOGRAPHIC TECHNIQUES

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ABSTRACT
To establish the analytical issues for simultaneous determination of different phytochemicals present in the *Amaranthus spinosus*. Phytochemical tests were carried out in the present investigation. Further, a rapid and simple thin-layer chromatography, high-performance thin-layer chromatographic (HPTLC) and high pressure liquid chromatography method was developed for the qualitative and quantitative analysis. Preliminary phytochemical analysis revealed the presence of carbohydrates, protein, glycosides, flavonoids, phenol, steroids, alkaloids and absence of saponin and tannin. Toluene: ethyl acetate: formic acid (5:4:0.2) was used as a mobile phase for the identification of quercetin in the *Amaranthus spinosus* extract. HPLC analysis revealed 0.002% of quercetin in the ethanol extract. These results may be useful in search of plant – based pharmaceutical preparation to treat various disorders.

KEYWORDS: TLC, HPTLC, HPLC, Quercetin.

INTRODUCTION

Millennium, plants have been used as a medicine. World Health organization (WHO) conducted a survey. The survey conveyed, all over the world around 2,50,000 plant species exist on the earth, out of which about 20,000 plant species were used as medicine. About 6000 plants were used in traditional folk and herbal medicine. The world health organization (WHO) also estimated that approximately a total population of about 80% of world people population mainly in developing countries use exclusively traditional medicines to treat their health problems (WHO IUCN/WWF, 1993). More recently, developed countries has risen interest in the value of plants as source of new drug and herbal medicines for healthy life style. In India, between 2500 and 500 B.C., the concept of Ayurveda appeared, developed, and remains as an important system of medicine and drug therapy. In Sanskrit, Ayurveda cover all aspects of diseases, pharmacy and therapeutics. Totally, Ayurveda consists of 8 sections. 8 sections are divided into 180 chapters with 314 medicinal plants (Subhose et al., 2005). Around 20,000 medicinal plants were recorded in India **(**Dev 1997). In the indigenous system of medicine, the medicinal plants listed in siddha (600), Ayurveda (700), Amchi (600), Unani (700) and Allopathy (30) (Rabe and Taden 1997). All over the world the Ayurvedic system of medicine is widely accepted and practised. Among them Indian Ayurvedic medicine, Chinese traditional medicine and Tibetan *so-wa-sig*-Pa medicine are the most popular traditional medicinal system practiced in the world wide.

METHODS AND MATERIAL

COLLECTION OF PLANT MATERIAL AND EXTRACTION

The fresh leaves of *Amaranthus spinosus* Linn were collected from in and around Vellore District and were authenticated by Dr. P. Jayaraman, Retd. Professor, Presidency College, Chennai. The voucher specimen was deposited in college Herbarium. The leaves of *Amaranthus spinosus* were shade dried and coarsely powdered. The course powder was subjected to extraction with ethanol by cold maceration method. The extracts were filtered by using muslin cloth and whatmann No: 1 filter paper. The filtrates were concentrated by using rotary evaporator under reduced pressure of 40^0c. Then the extract was stored in a refrigerator at 4^oc until use.

PRELIMINARY PHYTOCHEMICAL SCREENING

The ethanolic extracts of *Amaranthus spinosus* were screened for the presence of various phytoconstituents **(Harborne 2005).**

1. Test for carbohydrates

a. Molisch's test

To 2-3 ml of filtrate, two drops of alcoholic solution of α - napthol was added. The mixture was shaken well. Then 1ml of conc. H_2SO_4 was added slowly along the sides of the test tube. Allow the test tubes to stand. A violet ring at the junction of two liquids indicates the presence of carbohydrates.

b. Fehling's test

1ml of filtrate was boiled on water bath. To this 1ml of Fehling's solution A and 1ml of Fehling's solution B were added and mixed. It was heated in a boiling water bath for 5-10 min. Blue colour changes to yellow, then brick red colour precipitate was observed. A brick red precipitate indicated the presence of sugar.

c. Benedict's test

To 0.5 ml of filtrate, 1ml of Benedict's reagent was added in test tube. Mixed well. The mixture was heated on a boiling water bath for 5 minutes. Solution appears green, yellow or red colour depending on amount of reducing sugar present in the filtrate. A characteristic brick red precipitate indicated the presence of reducing sugar.

2. Test for Proteins and Aminoacid

a. Biuret test (General test)

To 3ml of filtrate, few drops of Biuret reagent was added. Violet or pink coloration denoted the presence of proteins.

b. Millon's test

To 2 ml of filtrate, few drops of millon's reagent was added. A white precipitate confirmed the presence of proteins. Precipitate warmed turns brick red or precipitate dissolves giving red colour was observed.

c. Ninhydrin test: (General test)

2 ml of filtrate, 2 drops of 5% Ninhydrin solution was added and heated in a boiling water bath for 10 minutes. A characteristic purple or bluish colouration indicated the presence of amino acids.

3. Test for Alkaloids

a. Mayer's test

To 2 ml of filtrate, few drops of mayer's reagent was added along the sides of the test tube. The precipitate was observed. If the test gives white creamy precipitate, it indicates the positive result to alkaloids.

b. Wagner's test

2-3 ml of filtrate, few drops of wagner's reagent was added slowly along the sides of the test tube. The precipitate colour was observed. Formation of reddish brown precipitate confirmed the test as positive.

c. Hager's test

To 2-3 ml of filtrate, 1 or 2 ml of Hager's reagent was added. The positive test indicated by a prominent yellow precipitate.

4. Test for Flavanoid

a. Shinoda test or Magnesium – hydrochloric acid reduction

A little quantity of extract was dissolved in 5 ml of 95% alcohol and few fragments (0.5g) of mg turnings and conc. HCl (dropwise) was added. Pink or crimson red colour was noted down.

b. Alkaline reagent test (or) sodium hydroxide test

To the extract, few drops of NaOH solution was added. An intense yellow colour was formed which turned to colourless or decolorization on addition of few drops of dilute acid. This colour changes observed for the presence of flavonoids.

5. Test for Saponins

a. Foam or froth test

The extract (50mg) was diluted with distilled water and made up to 20ml. The suspension was shaken in a graduated cylinder for 15 minutes. A two centimeter layer of foam or froth, stable for 10 minutes indicated the presence of saponins.

b. Haemolytic test

0.2 ml of extract was added to 1 drop of blood and placed on a glass side and observed for haemolytic zone formation.

6. Test for Tannins and Phenolic compounds

a. Ferric chloride test

50 mg of extract was dissolved in 5 ml of distilled water. To this few drops of neutral 5% ferric chloride solution was added and heated. A change in colour from blue, green, violet colour signified the phenolic compound presence.

b. lead acetate test

50mg of extract dissolved in distilled water. To this 3 ml of 10% lead acetate solution was added. Bulky white precipitate confirmed the presence of both phenolic and tannin compounds.

7. Test for Steroids and Terpenoids

a. Salkowski's reaction

To 2 ml of extract, 2 ml of chloroform and 2 ml of concentrated H_2So_4 was added. Shake well. Allow the tubes to stand. Red colour in the lower layer indicated the presence of steroids and yellow colour in upper layer confirmed the presence of triterpenoids.

b. Libermann-Burchard's test

To 2 ml of extract, few ml of chloroform, 1-2 ml of acetic anhydride was added, boiled and cooled. Then add conc. H_2SO_4 along the sides of the test tubes. A brown ring formed at the junction of two layers. Green colour in

the upper layer showed the presence of steroids and deep red colour in lower layer indicated the triterpenoids presence.

8. Test for Glycosides

a. Borntrager's test

2ml of filtrate, 3 ml of chloroform mixed and shaken well. Chloroform layer was separated. Add 10% ammonia solution and followed by shaken. A rose pink to red colour changes in ammonical layer indicated the presence of anthraquinone glycosides.

THIN LAYER CHROMATOGRAPHY

Ethanol extract of *Amaranthus spinosus* spotted on precoated (silica gel F254) aluminium plates using 1.0 ʎ micro pipette, about 1.0 cm from the edge along with standard Quercetin compound. It was dried using hot air dryer. The strip was lowered into a small chromatographic jar containing the solvent system. The jar was covered with a glass lid. The solvent system Toluene: ethylacetate: methanol (4.4: 5:0.6) was allowed to ascend until the solvent front was about ¾ of the length of the strip. The strip was removed and dried by a hot air dyer and viewed under UV lamp at 254 nm to identify the spot. The strip also visualized by spraying 5% $Fecl_3$ as spray reagent. The colour reaction was recorded and the relative Retardation factor (R_f) value was calculated based on the formula described below. **(Wagner *et al.*, 1996; Male *et al.*, 2004).**

$$R_f = \frac{\text{Distance traveled by the spot from the starting point}}{\text{Distance traveled by the solvent from the starting point to the solvent front.}}$$

PREPARATIVE THIN LAYER CHROMATOGRAPHY (PTLC)

Glass plates (20 × 20 cm) thickly coated with silica gel (0.4 – 0.5 nm) and activated at 100^0c for 30 minutes and cooled at room temperature were used for preparative thin layer chromatography (PTLC). The extract applied on separate plates and developed plates were air dried and visualized under UV light. Spots coinciding with those of standard reference compound were marked and scrapped and collected separately along with silica gel 'G'. The elution was dissolved with methanol. The purified compounds were subjected to HPTLC and HPLC.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Standard stock solution quercetin was prepared by dissolving 10mg of quercetin in 10ml of methanol. From this 10µl of solutions was applied on pre-activated (at 110^0c) silica gel 60 F 254 (Merck, Germany) plates. Samples were applied to the plates and the plates were developed in an automatic developing chamber using toluene: ethyl acetate: formic acid 5:4:0.2 as mobile phase. After development, the plate was air – dried.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS (HPLC)

Quercetin was purchased from Q4951, HPLC grade, sigma Aldrich and methanol (HPLC grade, Merk) for chromatography. Quercetin is soluble in methanol hence methanol selected as mobile phase. 10 µl solution was injected into chromatographic instrument through syringe. Recorded the chromatogram at wavelength 280 nm and calculated the area of chromatogram. Calculate the percentage of quercetin content from the peak areas.

RESULT

Table 1 shows the preliminary phytochemical analysis of *Amaranthus spinosus* ethanol leaf extract. In this study, the results of the present preliminary investigation showed that the ethanol extract of plant material (*Amaranthus spinosus*) possess the metabolites like carbohydrates, protein, glycosides, flavonoids, alkaloids, steroids, oils and fat and gums and mucilages and absence of saponin and tannin etc. Chromatogram of Thin layer chromatography in the solvent system Toluene: ethylacetate: methanol (4.4: 5:0.6) produced a single spot. The developed plates of Thin layer chromatography were sprayed with 5% ethanolic ferric chloride solution, the ethanol extract showed a bluish grey spots which coincided with that of the reference quercetin. Similar results were found when plates were placed in a chamber, saturated with ammonia vapours, it also showed deep yellow colour of R_f value 0.85 coinceded with standard quercetin. The plates developed under UV light also showed same results. Figure 1 and Figure 2 shows the thin layer chromatography of standard quercetin and ethanol extract visualized using 5% ferric chloride and UV light at 254nm. HPTLC in recent time acts as a screening technique for qualitative and quantitative phytochemical analysis of phytomolecules due to its low cost, simple methodology and high sample input (Srivastava *et al*., 2008; Yadav *et al*., 2008). The developed plates of HPTLC analysed at 254nm. By HPTLC method chromatogram peak, it was found that ethanol extract peak and standard quercetin peak were similar. Figure 3 shows the HPTLC analysis of standard quercetin and ethanol extract. HPLC analysis also confirmed the presence of quercetin with concentration of 0.002% in ethanol leaf extract which got matched with standard at retention time 8.936. Figure 4a, b shows theHPLC chromatogram of standard and ethanol extract.

In conclusion, the present study was undertaken with an aim of preliminary phytochemical evaluation, identification of an active quercetin compound present in the extract of *Amaranthus spinosus*. TLC, HPTLC and HPLC confirmed that phenolic compound querectin present in *Amaranthus spinosus* crude extract compared to standard quercetin.

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Table 1: Preliminary phytochemical analysis of ethanol leaf extract of *Amaranthus spinosus*

S.NO	Preliminary Analysis	Ethanol
1.	**Carbohydrates** a. Molisch test b. Fehlings test c. Benedicts test	 + + +
2.	**Proteins and Amino Acids** a. Biuret test b. Millon's test **c.** Ninhydrin test	 + + +
3.	**Alkaloids** a. Mayer's test b. Wagner's test c. Hager's test	 + + +
4.	**Flavanoid** a. Shinoda test b. Alkaline test c. Ferric chloride test	 + + +
5.	**Saponins** a. Foam or froth test b. Haemolytic test	 - -
6.	**Tannins & Phenolic compounds** a. Ferric chloride test b. Lead acetate test	 -/+ -/+
8.	**Steroids and Terpenoid** a. Salkowski's reaction b. Libermann-Burchard's test	 + +
9.	**Glycosides** **1. Anthraquinone glycosides** Borntrager's test	 +
10.	**Fat & oils** a. Soap test b. Spot test c. Saponification test	 + + +
11.	**Gums and Mucilages**	+

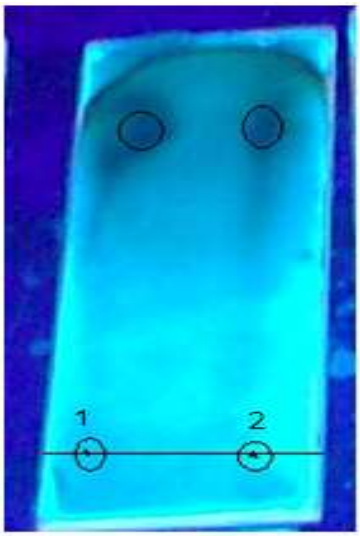

Figure 1: Thin layer chromatogram of standard quercetin and Amaranthus spinosus ethanol extract at 254nm. 1- standard Quercetin. 2- ethanol extract.

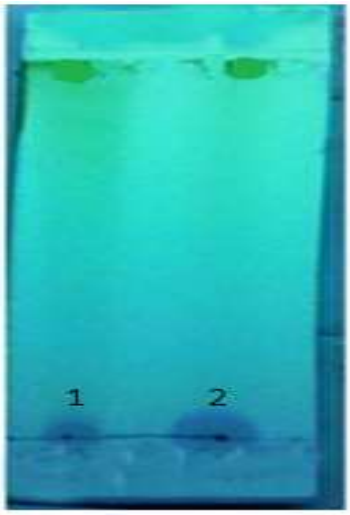

Figure 2: Thin layer chromatography of standard quercetin and Amaranthus spinosus ethanol extract with 5% ferric chloride visualizing agent. 1- standard Quercetin. 2- ethanol extract.

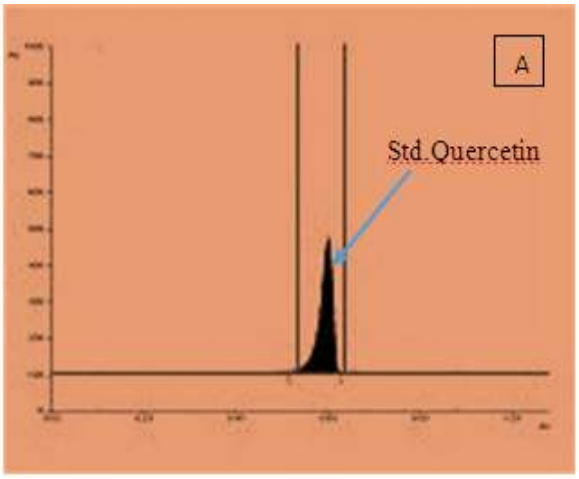

Figure 3A. HPTLC chromatogram of standard quercetin.

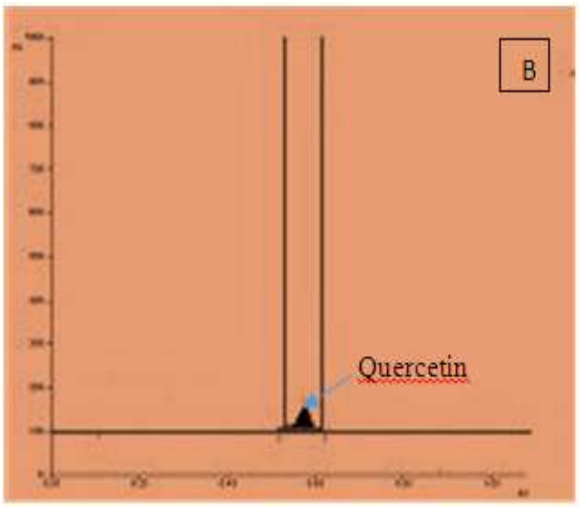

Figure 3B. HPTLC chromatogram of ethanol extract of *Amaranthus spinosus* leaves.

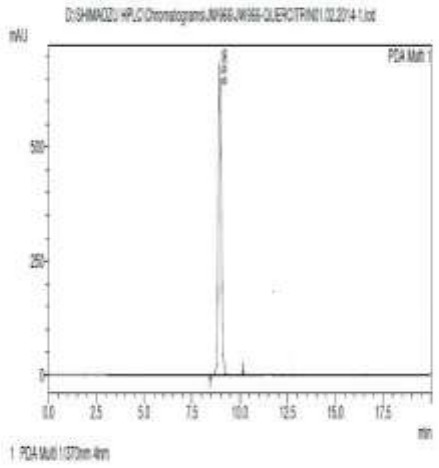

Figure 4 A- HPLC chromatogram of standard quercetin.

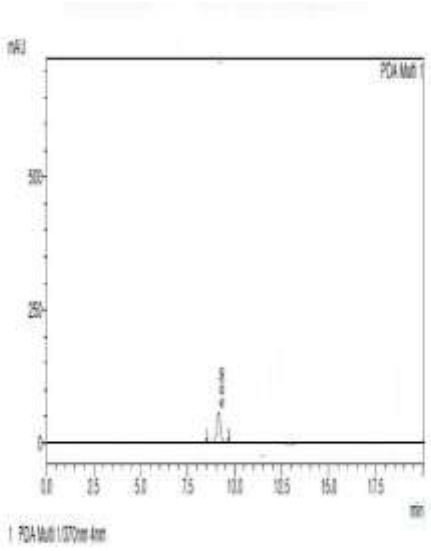

Figure 4 B- HPLC chromatogram of ethanolic extract of Amaranthus spinosus leaf.

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