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COMPARATIVE QUANTITATIVE ESTIMATION OF SECONDARY METABOLITES AND HPLC ANALYSIS IN DIFFERENT PLANT PARTS OF *TRIGONELLA FOENUM GRACEUM* (L.)

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ABSTRACT

Medicinal plants used in folk medicine may be an interesting and largely unexplored source for the development of potential new compounds. But it is necessary to isolate the active principles and characterize their constituents for the benefit of human being. Trigonella foenum graceum is one of the most promising medicinal herbs since ancient times. Its seeds and green leaves are used for multipurpose. The fenugreek seed contains a number of important compounds such as volatile oils, flavonoidssaponins, fatty acids and a rich source of polysaccharide galactomannan. In the present study, qualitative &quantitative estimation of Trigonellafoenum-graecum plant extracts like Mature plant leaf and stem, Immature plant (cotyledonary stage) leaf and stem, seeds(Green and brown) and Kasoorimethi(dried plant) were carried out. The comparative study showed that phenolic content was found the maximum in the kasoorimethi. Using TLC presence of flavonoids in mature plant leaf and Kasoorimethi were identified.HPLC analysis detected the presence of Quercetin, rutin, luteolin, and kaempferol. The presence of various secondary metabolites justifies the use of this plant in the medicinal system of healthcare.

KEYWORDS: *Trigonellafoenum-Graecum, Phenolic Content, Flavonoids, TLC, HPLC*

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum*) is an annual herb that belongs to the family Leguminosae commonly grown in India, Pakistan, and some Middle Eastern countries, which has many beneficial medicinal effects [1] Both the leaves and seeds of the fenugreek plants were widely consumed as food and medicine in Indo-Pak subcontinent and also in other countries. Fenugreek is rich in the source of vitamins, iron, β-carotene, etc [2]. It also has been reported to exhibit pharmacological properties such as antimicrobial, antiviral, antitumor, anti-inflammatory and antioxidant activity [3-5].

The fenugreek plant was included in normal diet generally because it has haematinic value [6]. The fenugreek has medicinal properties such as hypoglycemic, anticancer, and gastroprotective effects [7-9]. Despite the multiple bioactive properties that have been attributed to *Trigonella foenum-graecum*, the effects of this plant were widely studied. It was reported that the phytochemical analysis of fenugreek seeds contain a variety of alkaloids, flavonoids, saponins, and carbohydrates [10].

The fenugreek seeds are rich in dietary fiber,that it can lower blood sugar levels in diabetes. Fenugreek seeds are widely used as a galactagogue that is often used to increase milk supply in lactating women and cure breast cancer [11]. Fenugreek seeds is useful for tuberculosis, diabetes, atherosclerosis, constipation, high cholesterol, hypertriglyceridemia and externally is used as the poultice for abscesses, boils, carbuncles etc [12].

Fenugreek contains different alkaloids, flavonoids, and saponins, but all of these compounds contain maximum concentratrion in fenugreek.[13-15]. Alkaloids are natural bases containing at least one nitrogen atom in its heterocyclic ring and are found in plants. The alkaloids, flavonoids, and saponins of fenugreek have a pharmacological effect. They act as antilipidemic, hypoglyceaemic and cholagogic agent and their use should be promoted to manage diabetes, mellitus, hypercholesterolemia because clinical evidence shows promising results in reducing serum cholesterol level [16].

The plants form secondary metabolites for various purposes i.e. for the protection against pests, for fragrance and as the plant's own hormones. Secondary metabolites have been primarily used in the pharmaceutical industry, food, perfumery and growth and morphological differentiation of cells [17]. Some secondary compounds produced in plants are important to protect these plants against microorganism and other plant species for ecological habits.

The term secondary metabolite introduced by A.Kossel in 1981 implies that while primary metabolites are present in every living cell capable of dividing, the secondary metabolites are present only incidentally and are not of paramount significance for plant life.

The phytochemical analysis of the leaves revealed the presence of two major flavonoids namely quercetin and kaempferol, either in the free form or as glucosides found in the aerial part of *Trigonella foenum-graecum* [18]. Moreover identification of an essential compound from the methanolic and acetonic extract of leaf, stem, seeds of both stages of the plant by HPLC analysis was carried out.

As there is a lot of potential of this plant for its pharmacological activity, in present study various secondarymetabolite for *Trigonellafoenum graceum* were extracted and characterized qualitatively and quantitatively. Moreover, identification of essential compounds from methanolic and acetone extract of leaf, stem, seeds of both stags of the plant by HPLC analysis was carried out.

MATERIALS AND METHODS

Sample Collections and Preparation

Healthy plant (Both Mature & Immature Plant), dried fenugreek (Kasoori Methi) and seeds (both type) (*Trigonella foenum graceum. L*) were collected from the local market of Surat, Gujarat. The plant was taxonomically identified was authenticated by herbarium of Veer Narmad South Gujarat University, Surat.

The leaf & stem of the plant was kept for the sun drying. After 10 days the leaf and stem were dried and crushed into powdered form and used for the analysis. The seeds were also crushed into powdered form and used for the analysis.

Phenol Content

- 80% Methanol
- 7.5% Sodium carbonate(Na_2Co_3)

- Folin–Ciocalteu reagent

The phenolic content of the extract was determined by the Folin–Ciocalteumethod[19]. Briefly, 200 µL of crude extract (1 mg/mL) were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Flavonoids Content

Materials & Methods

- Sodium nitrite ($NaNo_2$) (5%)
- Aluminum chloride ($AlCl_3$) (10%)
- Sodium hydroxide(NaOH)
- Methanol (80%)
- Sulphuric acid (7%)
- Ethyl acetate
- Petroleum ether
- Diethyl ether

The flavonoids content of the crude extract was determined by the aluminum chloride colorimetric method [20]. In brief, 50 µL of crude extract (1 mg/mL ethanol) was made up to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$solution; 0.3 mL of 10% $AlCl_3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution was added, and the final volume of the mixture was brought to 10 mL with double-distilled water. The mixture was allowed to stand for 15 min, and the absorbance was measured at 510 nm. The total flavonoids content was calculated from a calibration curve, and the result was expressed as mg Quercetin equivalent per g dry weight.

Flavonoids Extraction

Different plant parts Mature plant Leaf and Kasoori Methi were air dried and powdered, separately. Each of these extracted separately with 80% methanol on water bath [21] (Subramanian &Nagarajan, 1969) for 24 h. The methanol soluble fractions were filtered, concentrated *in vacuo* and aqueous fractions were fractioned by sequential extraction with petroleum ether (Fr1), diethyl ether (FrII) and ethyl acetate (FrIII) separately. Each step was repeated thrice for complete extraction, fraction 1 was discarded in each case because it contained a fatty substance, whereas fraction II and fraction III were concentrated and used for determining flavonoids.

Fraction III was further hydrolyzed by refluxing with 7% sulphuric acid (10mLg^{-1} plant material for 2 h), filtered and the filtrate was extracted thrice with ethyl acetate. All ethyl acetate layers were pooled separately, neutralized by distilled water with repeated washings and concentrated *in vacuo.* Both fraction II and III were taken up in a small volume of ethanol (2-5mL) before the chromatographic examination.

Qualitative

Thin Layer Chromatography (TLC)

Thin glass plates (20x20 cm) were coated with Silica gel G (250mμ thick). The freshly prepared plates were air dried at room temperature; thereafter these were kept at 100 ^{0} C for 30 minutes to activate and then cooled at room temperature. The freshly prepared and activated plates were used for analysis. Each of the extracts was co-chromate graphed with authentic flavonoid as a marker. (quercetin mainly). These plates were developed in an air-tight chromatographic chamber saturated with the solvent mixture (Benzene: Acetic Acid: Water:: 125:72:3; Wong & Francis, 1968). The developed plates were air dried and visualized under UV light by exposure to ammonia fumes. The mouth of a 100 mL containing concentrated NH_4OH was held in contact with each spot for about 5-10 seconds and fluorescent spots corresponding to that of standard markers were marked. The developed plates were also sprayed with 5% $FeCl_3$, 0.1% alcoholic $AlCl_3$ and kept in I_2 chamber separately. The colored spots thus developed were noted and the Rf value of each spot was calculated. Several others solvent systems such as n- butanol, acetic acid, water (4:1:5), tertiary butanol, acetic acid, water (3:1:1) were also tested, but the solvent system containing benzene, acetic acid, water (125:72:3) gave better results.

The identity of isolated flavonoids were confirmed by mp,mmp performed in capillaries (Toshniwal melting point apparatus), IR(Infra red spectrophotometer; Perkin, Elmer337, Grating Infra red spectrophotometer),UV (Ultraviolet and visible spectrophotometer; Carl Zeiss,VSU-2P spectrophotometer)analysis along with their respective authentic samples.

HPLC Analysis of Flavonoids

The HPLC system (Varian) consisted of a pump (9012Q), a diode array detector (9065) and a Raininautosampler (AI200). Methanol was from Merck (Art. MX0488-1). Water was HPLC grade and acidified to pH 3.0 with phosphoric acid. Quercetin was isolated from the different plant parts and characterized by spectroscopic methods. Quercetinwere purchased from Merck. Qualitative analysis was made with one of the samples, in step gradient mode, with methanol/water in the ratio of 7:3 (10-20 min) at a flowrate of 1 mL min-1. The injection volume was 20 µL and the eluate was monitored at 339 nm. The filtered methanolic extract was injected under these conditions as well as a mixture of authentic samples of Quercetin. The purity of each identified peak was determined by comparison of the reported data.

Results

Qualitative Analysis

TLC RESULTS

Based on results, TLC analysis was employed to define the qualitative content of flavonoids in fenugreek extract of different plant parts. Quercetin was identified in the investigated extracts by comparing spectra with those of standards. The result of the qualitative analysis showed that mature plant leaf contains Quercetin shown in figure.3, while in dried plant part Kasoorimethi also contains Quercetin and its derivatives shown in figure.4.

Quantitative Analysis

The phytochemicals such as phenolic and flavonoids content were determined in different plant parts of *Trigonellafoenumgraceum* and quantified by standard procedures.

The phenolic content was found more in kassoorimethi than any other plant material in both Methanolic and Acetone extract. Inmethanolic extract the maximum quantity of phenol was found in Kasoorimethi and minimum content was found in the small green stem, while in Acetone extract maximum quantity of phenolic quantity was found in Kasoorimethi and minimum was found in green seeds(Table:1). The comparative graph of phenol content is shown in figure1.

Total flavonoids content was found more in Kasoorimethias compared to mature and immature plant leaf and stems. The maximum content of flavonoids was found in the Kasoorimethi and minimum was found in the brown seeds in the methanolic extract (Table:2). The comparative graph of phenol content is shown in figure 2.

HPLC

Based on experimental results, HPLC analysis was employed to define the qualitative content of flavonoids in fenugreek extracts. Chromatograms of standard and different plant parts are represented. Quercetin was identified in the investigated extracts by comparing spectra with those of standards. The standard results are shown in figure.5 The result of the qualitative analysis showed that mature plant leaf contains Quercetin & its derivatives shown in figure.6, while in dried plant part (Kasoorimethi) along with Quercetin, two other flavonoids are identified that is rutin and kaempferol shown in figure 6.

DISCUSSIONS

Plants are regarded as molecular factory, as they have the capacity to synthesize the enormous diversity of byproducts termed as "*Bioactive compounds*". The plants contain these bioactives were known to ancient civilizations for various healing properties. These bioactive are extra nutritional constituents occur in small quantities, and vary widely in chemical structure and function and are grouped such as phenols, alkaloids, steroids, lignin’s, tannins etc.

Kaur C, Kapoor H C(2002) has found the highest phenolic content in methanolic extract of cotyledons derived callus (412.087 mg/l) followed by 211.1937 mg/l in methanolic extract of hypocotyls derived callus and 124.84 mg/l in methanolic extract of seeds.[22]whereas, in the present study, the highest phenolic content was observed in methanolic extract of Kasoorimethi followed by seeds of the mature plant. Instead, Naidu MM, Shyamala BN, Naik JP, Sulochanamma G, Srinivas P(2011) has observed that the seed extract exhibited the higher phenol content (150.8±0.33 mg GAE/g) than the extract of aerial parts of the plant.[23]

Kaur C, Kapoor H C(2002)has observed the highest level of flavonoid content were detected in methanolic extract of seeds 424.951 mg/l followed by 217.285 mg/l in methanolic extract of cotyledons derived callus and 95.92 mg/l in methanolic extract of hypocotyls derived callus.[22], while in comparison with the above data our in our present study we found highest flavonoid content in methanolic extract of kasoorimethi.

CONCLUSIONS

The present research work was carried out on *Trigonella* L. for their metabolite profiling and to establish its bioefficacy. These plant species was also evaluated for the presence of different bioactive secondary metabolites through different plant parts i.e. seeds, leaves, the stem of a mature and cotyledonary stage of plant and dried plant (Kasoorimethi). During the present research, the leaves; stem was subjected to secondary metabolite analysis. They were evaluated by their Rf value, TLC behavior, color, melting point, UV, IR and NMR spectral studies.

Isolation and characterization of flavonoids were done using the method of Subramanian and Nagaranjan (1969). All the experimental plant parts showed the presence of rutin and quercetin. Total flavonoid content was found to be maximum in Kasoori Methi. Mature Plant Leaf & Kasoorimethi of the plant were screened further for biochemical analysis of HPLC technique. Quercitin and kaempferol. The need to know theidentify of the flavonoids individual compounds in the sample requires the replacement of traditional technique. HPLC is probably the most widely used analytical technique for characterizing the polyphenolic compounds. Quercetin was identified in mature plant leaf by comparing retention time, absorption and mass spectroscopy with that of standards.

Finally, it can be concluded that the active chemical compounds present in fenugreek should certainly find a place in the treatment of various bacterial infections. The result from the present research work indicates that this herb should be studied more extensively to explore its potential in the treatment of many infectious diseases.

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APPENDICES

Phenol Content

Table 1

Names	Methanol Extract(mg/gdw)	Acetone Extract(mg/gdw)
Brown seeds	7	7.5
Green seeds	1.25	1
Big green leaf	2.8	3.9
Small green leaf	2.7	1.7
Big green stem	1.15	1.9
Small green stem	1.4	1.6
Kasoori Methi	8.6	7.9

Favonoids Content

Table 2

Names	Methanol Extract(mg/gdw)	Acetone Extract(mg/gdw)
Brown seeds	0.1	-
Green seeds	0.20	-
Mature Plant Leaf	1.15	-
Immature Plant Leaf	0.5	-
Mature Plant Stem	0.2	-
Immature Plant Stem	0.2	-
Kasoori Methi	2.2	-

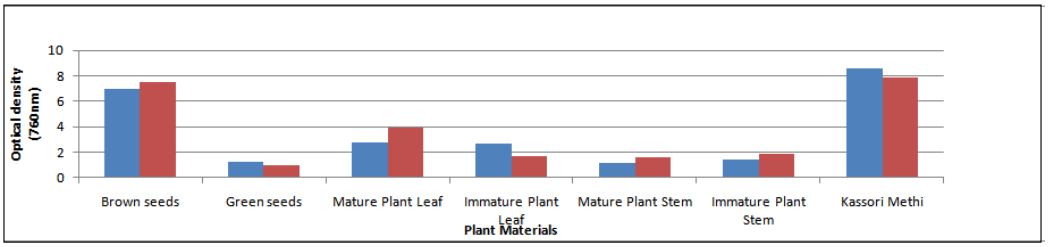


Figure 1: (Comparative Graph of Phenol Content)

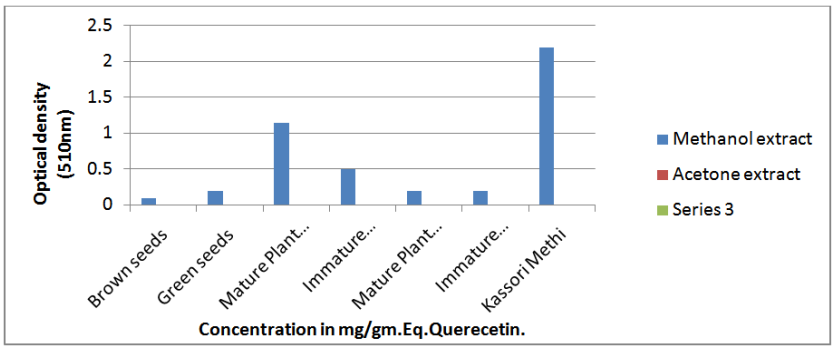
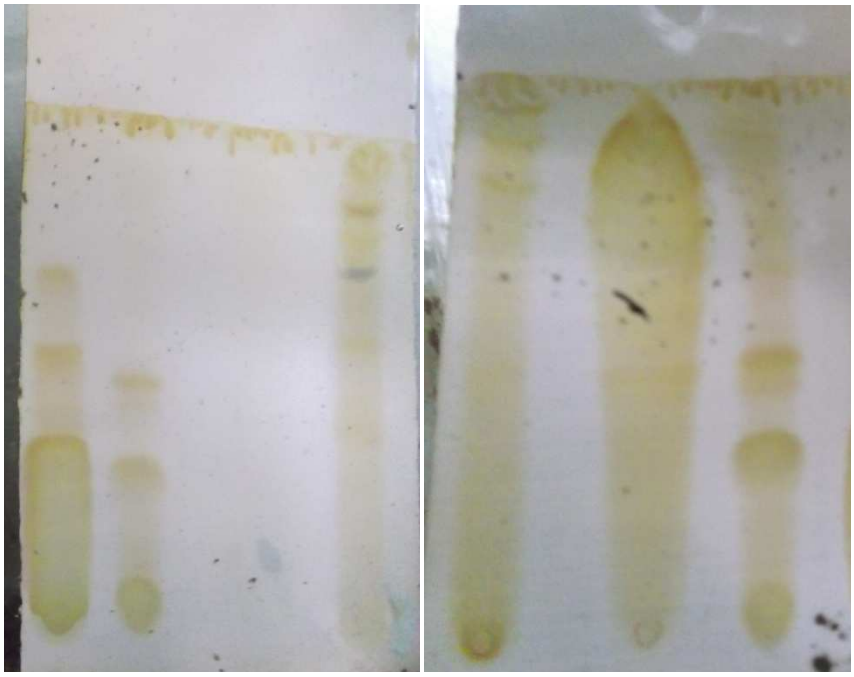


Figure 2: (Comparative Graph of Flavonoids Content)



Standard Fr 2 Fr 3

Figure 3: TLC Showing Flavonoids in Mature Plant Leaf

Standard Fr 2 Fr 3

Figure 4: TLC Showing Flavonoids in Kasoorix

Standard=Querecetin, Fr2= Bound Flavonoids, Fr3=Free Flavonoids

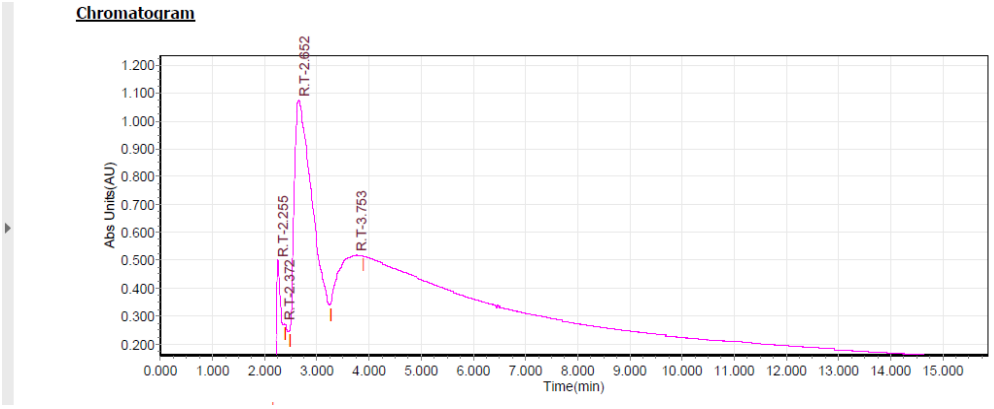


Figure 5: HPLC Profiling of Standard Graph of Querecetin

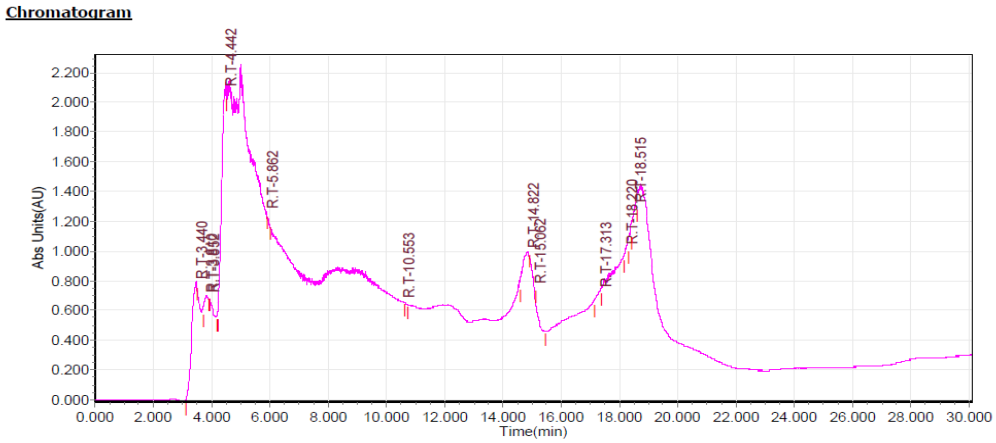


Figure 6: HPLC Profiling of Mature Plant Leaf

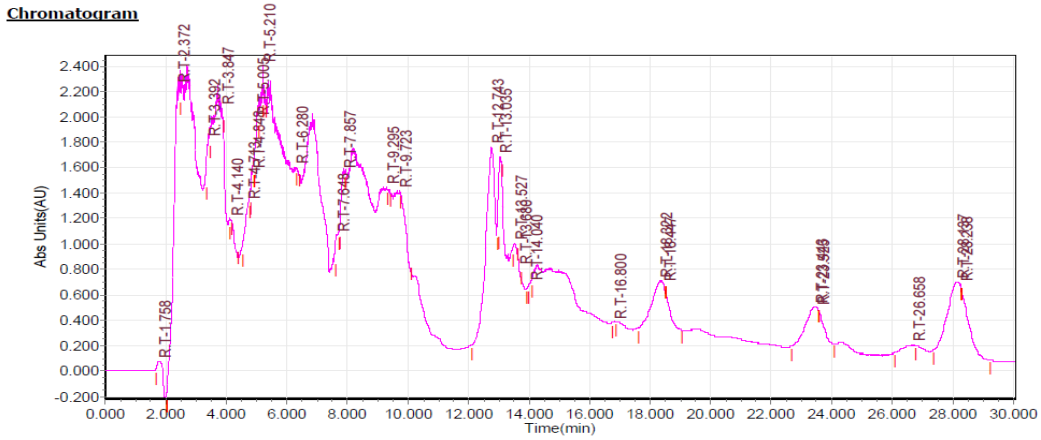


Figure 7: HPLC Profiling of Kasoori Methi