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Bioactive phytoconstituents in methanolic extract and ethyl acetate fraction of methanolic extract of coriander (*Coriandrum sativum* L.) seeds

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Abstract

Identifying bioactive compounds and establishing their health effects are active areas of scientific inquiry. The discovery of novel health effects of bioactive compounds will provide the scientific basis for future efforts to use biotechnology to modify/fortify foods and food components as a means to improve public health. Coriander is one of the oldest spices, possessing multiple traditional health benefits. The present investigation was aimed to identify some of the phenolic compounds present in the methanolic extract of coriander seeds using various analytical techniques. The RP-HPLC analysis revealed chlorogenic acid to be predominant in the extract, followed by rutin, caffeic acid and quercetin. High performance thin layer chromatography (HPTLC) of ethyl acetate fraction revealed the presence of many phenolic compounds, out of which quercetin and rutin could be identified and quantified; rutin being predominant in the fraction, followed by quercetin. Analysis using nuclear magnetic resonance (NMR) spectroscopy through ^{1}H, ^{13}C, and IR spectra indicated the E2 compound (obtained from ethyl acetate fraction) to be hydrocarbon in nature. Thus, coriander (*Coriandrum sativum* L.) seeds are a promising source of phytochemicals with wide applications in the prevention and treatment of diseases induced by free radicals.

Key words: *Coriandrum sativum* L., HPTLC, RP-HPLC, flavonoid, caffeic acid, chlorogenic acid, quercetin, rutin

1. Introduction

India is one of the 12 mega biodiversity centers, having over 45,000 plant species. About 1500 plants with medicinal uses are mentioned in ancient texts and around 800 plants have been used in traditional medicine. Of these, only about 6% have been screened for biologic activity, and a reported 15% have been evaluated phytochemically (Fabricant and Farnsworth, 2001). Plants constitute an important source of natural products which differ widely in their structures, biological properties and mechanism of action. The medicinal value of plants lies in some chemical substances that produce a definite physiological action in the human body. The most important of these bioactive constituents of plants are phenolic compounds, *viz*., tannins, flavonoids which are responsible for their antioxidant activity (Rajeshwari and Andallu, 2014). However, very little information is available on such activity of medicinal plants and, of the 400,000 plant species on Earth, only a small percentage has been systematically investigated for their biological activities. Additionally, there is a rich local ethnobotanical bibliography, describing the species most frequently used by the population to cure various diseases. The importance of a plant lies in its biologically active principles. There are two types of plant chemicals, primary metabolites such as sugars, proteins, amino acids and chlorophylls and secondary metabolites, which includes alkaloids, terpenoids, saponins and phenolic compounds. These chemicals exert a significant physiological effect on the mammalian system (Shaheen *et al*., 2009). Some of these phytochemicals are known as nutraceuticals because of their biological activities (Dillard and German, 2000).

Identifying bioactive compounds and establishing their health effects are active areas of scientific inquiry. In recent years, these secondary metabolites are being used, either directly as precursors or as lead compounds, in the pharmaceutical industry and it is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant pathogens. Research on phytochemicals has been driven in recent years by their beneficial health effects, including antioxidant, anticarcinogenic, and antimutagenic activities (Huang *et al*., 1992) and their ability to reduce the risk of coronary heart disease and other degenerative diseases (Hertog *et al*., 1993). The screening of the plants for their biological activity is done on the basis of either their chemotaxonomic investigation or ethnobotanical knowledge for a particular disease. Identification of a particular compound against a specific disease is a challenging long process. Although the screening of Indian medicinal plants has revealed varying degrees of biological activity, there is still a lack of experimental scientific studies and antioxidant properties of a great number of these remedies. Thus, it is considered worthwhile to screen the medicinal plants that have been used in traditional medicine (Shokeen *et al*., 2009).

The spectrometric method is used traditionally for quantification of phenolic compounds in plant extracts. However, due to complexity of the phenolic compounds, quantification of individual phenolic compound cannot be determined by spectrometric method.

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Modern chromatographic techniques are successfully used for the individual phenolic compound quantification. Chromatographic methods combined with instrumentation analysis, used for the profiling and quantification of phenolic compounds. Gas chromatography (GC) is widely used for phenolic and flavonoid compounds identification based on volatile and non-volatile nature of compounds (Stalikas, 2007). High-performance liquid chromatography (HPLC) is employed to obtain an accurate elucidation and quantification of individual polyphenolic compounds, found in plant-based materials as it provides the most successful instrumentation by which phenolic compounds including anthocyanins, tannins, flavonols, flavan-3-ols, flavanones, flavones, and phenolic acids in different plant extract and food samples (Pawlowska *et al*., 2008) could be qualitatively and quantitatively analysed. The reversed-phase (RP) columns have considerably enhanced HPLC separation of different classes of phenolic compounds and RP C18 columns are almost exclusively employed. It was found that column temperature may affect the separation of phenolics such as individual anthocyanin. Acetonitrile and methanol are the most commonly used organic solvents as mobile phase. In many cases, the mobile phase was acidified with a modifier such as acetic, formic and phosphoric acid for better phenolic compounds separation and to minimize peak tailing. Both isocratic and gradient elution are applied to separate phenolic compounds. The choice depends on the number and type of the analyte and the nature of the matrix. Previously several reviews have been published on the application of HPLC methodologies for the analysis of phenolics (Dai and Mumper, 2010).

Identification of plant constituents can also be achieved by colour test, solubility, spectral characteristics and spot formation in thin layer chromatography (TLC) and paper chromatography. Now-a-days, pre-coated plates of commercial manufacture are utilized since these have additional uniformity and supply good results. Recent advanced techniques like high performance thin layer chromatography (HPTLC) offers speedy separations in shorter time with higher resolution. The fundamental distinction between TLC and HPTLC lies in particle and pore size of the adsorbents (Harbone, 1998). Due to the complexity of the natural mixtures of phenolic compounds of various plants, it is rather difficult to elucidate their structure and assess the antioxidant and biological potentials. Indeed, the determination of individual flavonoid glycosides form plant extracts, could prove to be a difficult task. Coriander is one of a few savory plants, a potential source of phenolic compounds having biological activities. Coriander has been documented as a traditional treatment for diabetes, indigestion, flatulence, insomnia, renal disorders and loss of appetite, and as a diuretic and all parts of the plant are edible, but the fresh leaves and the dried seeds are the most common parts used in cooking (Aissaoui *et al*., 2008).

Due to the complexity of the natural mixtures of phenolic compounds of various plants, it is rather difficult to elucidate their structure and assess the antioxidant and biological potentials. Indeed, the determination of individual flavonoid glycosides form plant extract could prove to be a difficult task. Coriander contains active phenolic acids, including caffeic acid and chlorogenic acid. Most of these compounds (flavonoids, polyphenols) are also known to inhibit free radicals generated in the cellular system, when they are consumed through the diet (Rajeshwari *et al*., 2012). The study on antioxidants that are ubiquitously present in spices is gaining momentum in human health, as these are easily absorbable in human system (Rajeshwari and Andallu, 2011). Hence, it was aimed in this work to identify some of the phenolic compounds present in the methanolic extract of coriander (*C. sativum* L.) seeds by using analytical techniques.

2. Materials and Methods

2.1 Procurement of sample

A sample of coriander seeds was taken from the whole lot purchased from wholesale spice supplier of Anantapur. The plant grown from these seeds in our garden was identified by Dr. Ravi Prasad and a voucher specimen (SKU 45778) was stored in the herbarium, Department of Botany, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India. The remaining lot of coriander seeds was cleaned; shade dried and powdered finely using a blender.

2.2 Chemicals

All the chemicals and solvents were of analytical grade obtained from Sisco Research Laboratories (SRL), E-Merck and Sigma-Aldrich (Germany).

2.3 Preparation of methanolic extract and its fractions from coriander seeds

The powdered coriander seeds were extracted with 80% methanol (Me), thrice (1:1, w/v), at room temperature (Petra *et al*., 1999). The combined extract was concentrated in a vacuum evaporator and the residue was dissolved in water and fractionated successively with the solvents in the increasing order of polarity, *viz.*, hexane (He), benzene (Be), ethyl acetate (Ea), n-butanol (nBu) and water (Aq) and each extract was evaporated to dryness. A small amount of each fraction was redissolved in various solvents as required at a concentration of 1mg/ml (Hashim *et al*., 2005) and used for the qualitative analysis to test the fractions of methanolic extract for the presence of various phytochemicals.

2.4 Identification of phytochemicals in methanolic extract of coriander (*C. sativum* L.) seeds using reverse phase high performance liquid chromatography (RP-HPLC) technique (pilot study)

2.4.1 Chemicals

Formic acid, acetonitrile, methanol, water and all the other solvents were of HPLC grade, obtained from SRL and E-Merck and standards were from Sigma-Aldrich (Germany).

2.4.2 Reverse phase high performance liquid chromatography (RP-HPLC) technique

2.4.2.1 *Preparation of extract*

The extraction was carried out by mixing 50 g of seed powder in 250 ml of methanol. The solution was stirred regularly for 15 d and filtered. The filtrate was subjected to evaporation, using rotary vacuum evaporator and the residue (1.75 g) was dissolved in10 ml methanol. The extract was filtered and dried, using flash evaporator at room temperature. Sample cleanup was done to remove the impurities using a C18 Sep-Pak cartridge and 20 µl aliquots were analyzed by HPLC.

2.4.2.2 *Preparation of standard solutions*

Standard solutions of caffeic acid and chlorogenic acid were prepared by dissolving 10 mg of the standards in methanol in 25 ml volumetric flasks, sonicated and volume was made up to 25 ml with respective solvents to give concentration of 400 ppm.

Standard stock solutions of two flavonoids, rutin and quercetin were prepared in methanol, at concentrations of 100 ppm (10 mg of the standards were dissolved in 25 ml methanol in volumetric flasks, sonicated and volume made up to 25 ml with methanol to give 400 ppm, 2.5 ml of stock solution was taken and made up to 10 ml with methanol to give concentrations of 100 ppm).

2.4.2.3 *Apparatus*

The chromatography separation was performed using a Shimadzu LC-20AD with a quaternary pump system. Auto injector or auto sampler (SIL-20ACHT) was used for 20 µl of sample injection. Separation was carried out at ambient temperature with a column (VARIAN Pursuit XPs- C_{18}) dimension 250 × 4.6 mm, S/N 436, protected by a guard column. The detector signal was recorded with a UV/VIS detector (SPD-20A).

2.4.2.4 *Procedure*

Reverse phase high-performance liquid chromatography with C_{18} columns is the most popular technique for the analysis of polyphenols of the different foods. A UV-vis multiwavelength detector (SPD-20A) was used because all phenolic compounds show intense absorption in the UV region of the spectrum. This method used for the separation of caffeic acid and chlorogenic acid (320 nm), rutin and quercetin (370 nm) included mobile phase 0.5% formic acid: acetonitrile (ACN)(70:30) at a flow rate 0.9 ml/min; column (VARIAN Pursuit XPs- C_{18} dimension 250 × 4.6 mm) at 40°C temperature. The identification of polyphenols was based on the comparison of their retention times with those of the standards.

2.5 High performance thin layer chromatography (HPTLC)

High performance thin layer chromatography studies were carried out for methanolic extract and ethyl acetate fraction of methanolic extract of coriander seeds to confirm the presence of different flavonoids.

2.5.1 Procedure

The sample was applied on (E. Merck) aluminium plate pre-coated with Silica gel 60 F254 of 0.2 mm thickness. An amount of 5 µL up to 30 µL of the sample was applied on aluminium plate pre-coated with silica gel (60 F254 of 0.2 mm thickness) in a band-shaped of 1cm and then was air dried and run for 8 cm height, using a standardized solvent (5% methanol in chloroform) in a glass chamber. After air drying, the plate was visualized in UV 254 and 366 nm and comparison was done using different standards (apigenin, quercetin, rutin, chlorogenic acid and caffeic acid) with respect to the number of spots and the length of elution (Rf).

Relative front (Rf)

$$= \frac{\text{Distance travelled by the solute from the origin}}{\text{Distance travelled by the solvent from the origin}}$$

2.5.2 Sub-fractionation of ethylacetate fraction of methanolic extract of coriander seeds

2.5.2.1 *Column and thin layer chromatography techniques*

The ethyl acetate fraction of methanolic extract of coriander seeds was subjected to sub-fractionation by column chromatography, using various eluting systems as mentioned in the flowchart given below:

The dark brown solid (1.5 g) was adsorbed on silica gel (20 g) and transferred to a column of silica gel (150 g) equilibrated with hexane

↓

Elution was performed with I) hexane, II) hexane:chloroform (3:1), III) hexane:chloroform (1:1), IV) hexane:chloroform (1:3), V) chloroform, chloroform: ethyl acetate (3:1), VI) chloroform:ethyl acetate (1:1), VII) chloroform:ethyl acetate (1:3), VIII) ethyl acetate, ethylacetate:methanol (3:1), IX) ethyl acetate:methanol (1:1), X) ethyl acetate:methanol (1:3) and XI) methanol

↓

Fractions were collected in 20 ml portions and monitored on TLC and the fractions showing similar spots were combined

For thin layer chromatography, the solvent systems tried for best performance [a) 30% ethyl acetate in hexane, b) 20% ethyl acetate in chloroform, c) 5% methanol in chloroform] revealed c) 5% methanol in chloroform to be the best solvent system for the separation of compounds in ethyl acetate fraction.

2.6 Nuclear magnetic resonance (NMR)

2.6.1 Preparation of sample

The methanolic and ethyl acetate sub-fractions (M1-M4 and E1-E4, respectively) were concentrated in a vacuum evaporator and dissolved in dimethyl sulphoxide and used for analysis.

2.6.1.1 *Procedure*

Nuclear magnetic resonance spectroscopy is a tool to rapidly detect the isomeric composition of natural flavanone glycosides present in food. Standard ^{1}H, ^{13}C NMR spectra can give a wealth of chemical information on liquid foodstuff and even semi-solid foods. NMR was performed on a Varian NMR instrument using a Pro Star pump system, a Pro Star UV detector, an Unity INOVA 300 MHz NMR spectrometer and a micro flow NMR probe. The probe has 1H {13C} channels (1H observed with 13C decoupling) with pulsed-field gradient along *z* axis. Approximately 60 µL of sample (dissolved in DMSO-d6) was used and the transfer time from the UV cell to the active volume was calibrated to be 21s at a flow rate of 1.0 ml/min. Proton NMR experiments were performed in 'stop-flow' mode, where the HPLC flow was halted after the sample elution fraction was transferred to the NMR probe which was equilibrated at 25°C.

2.7 Statistical analysis

The results obtained were subjected to two-way analysis of variance (ANOVA) and the significance of the difference between means was calculated. Values expressed are means of three independent samples analyzed in triplicate ± standard error of means (SEM) (SPSS version 15).

3. Results and Discussion

3.1 Reverse phase high performance liquid chromatography (RP-HPLC)

In the course of optimization of the methods for the separation and analysis of the flavonoid aglycones in the methanolic extract of the seeds of *C. sativum* L. through reversed-phase high performance

liquid chromatography (RP-HPLC) with UV detection, good resolution of the flavonoids was achieved using different combinations of isocratic and gradient techniques. The retention time (Rt) of the standards, *viz*., caffeic acid, chlorogenic acid, quercetin and rutin were 4.033, 3.605, 11.638 and 3.711 min. (Table 1 and Figures 1-4), respectively represent the chromatograms of caffeic acid, chlorogenic acid, quercetin and rutin. By comparing with the retention times of the standards, the compounds in the methanolic extract were identified to be caffeic acid, chlorogenic acid, quercetin and rutin. Figure 5 represents HPLC chromatograms of caffeic acid and chlorogenic acid and Figure 6 represents that of rutin and quercetin in the methanolic extract of coriander seeds. This method gave a quick analysis of the flavonoids present in the methanolic extract of *C sativum* L. seeds.

The concentration of the compounds, *viz*., caffeic acid, chlorogenic acid, quercetin and rutin present in the multicomponent extract was calculated using peak area of the standard and that of the compound. Therefore, RP-HPLC with UV/VIS detection revealed the presence of caffeic acid (11.48%), chlorogenic acid (1.89), quercetin (0.11%), rutin (28.01%) in the methanolic extract of coriander seeds.

Table 1: Retention time, peak area and concentration of the compounds in methanolic extract of coriander seeds

Compounds	Retention time (min.)	Peak area of the standard	Peak area of the compound in the sample	Concentration (%)
Caffeic acid	4.033	30271994	2537878	1.89
Chlorogenic acid	3.605	11466523	14420915	28.01
Quercetin	11.638	9280414	182395	0.11
Rutin	3.711	2307114	4960987	11.48

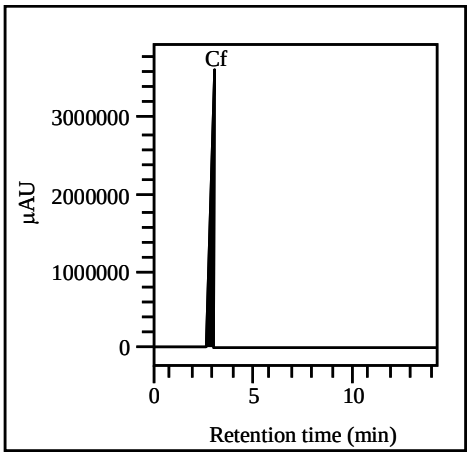

Figure 1: HPLC chromatogram of of standard caffeic acid

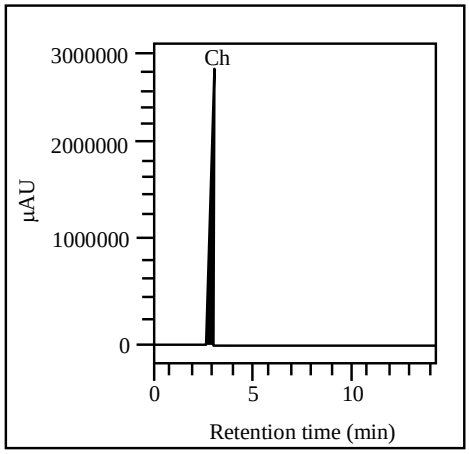

Figure 2: HPLC chromatogram of standard chlorogenic acid

(Detection at 320 nm, peaks : Ch-chlorogenic acid, Cf-caffeic acid)

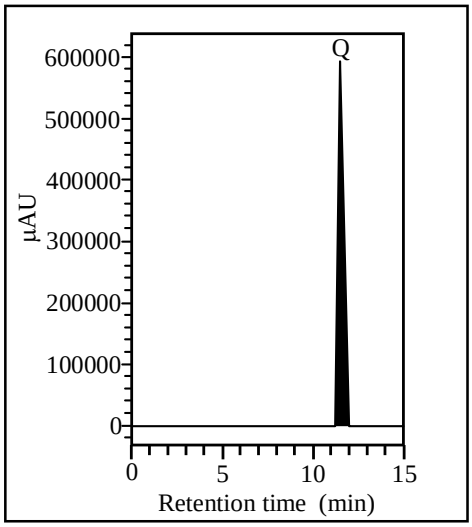

Figure 3: HPLC chromatogram of standard quercetin

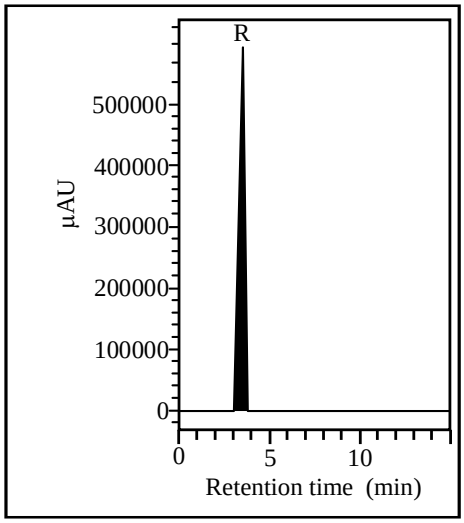

Figure 4: HPLC chromatogram of standard rutin

(Detection at 370 nm, peaks: R-rutin, Q-quercetin)

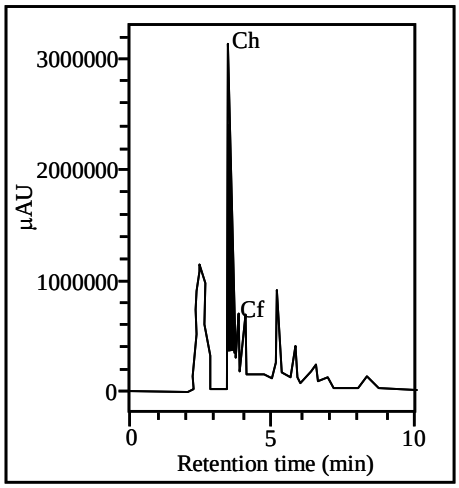

Figure 5: HPLC chromatogram of methanolic extract showing chlorogenic acid and caffeic acid

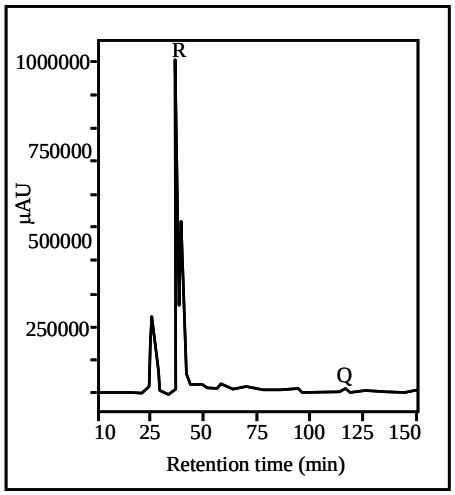

Figure 6: HPLC chromatogram of methanolic extract showing rutin and quercetin

3.2 High performance thin layer chromatography (HPTLC)

Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired separation was achieved, using ethyl acetate:methanol:water (EMW), (7:15:0.8, v/v/v) as the mobile phase. HPTLC profile of *C. sativum* L. was recorded at 254 nm. The results of HPTLC confirmed the presence of various phenolic compounds in the extract. Fractionation of methanolic extract resulted in separation of compounds according to their polarity.

High performance thin layer chromatography (HPTLC) carried out for ethyl acetate fraction of methanolic extract of coriander seeds indicated very interesting information revealing the presence of 5 phenolic compounds, out of which rutin and quercetin could be identified and quantified, rutin (0.0066%) being predominant in the fraction, followed by quercetin (0.0051%) (Figures 7 and 8).

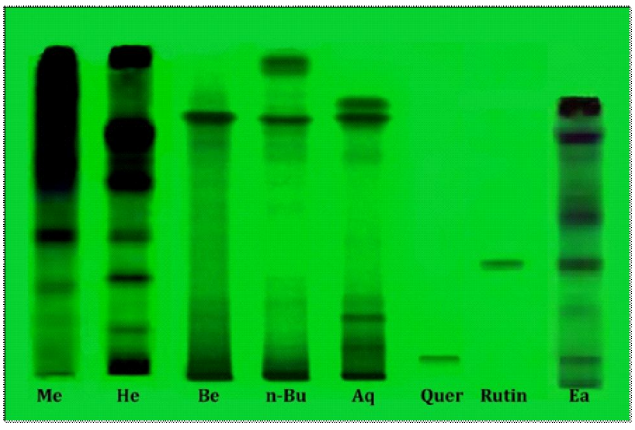

Figure 7: HPTLC finger print profile of methanolic extract and fractions of coriander seeds and the standards-quercetin and rutin (solvent system ethylacetate: methanol: water - 77:15:8)

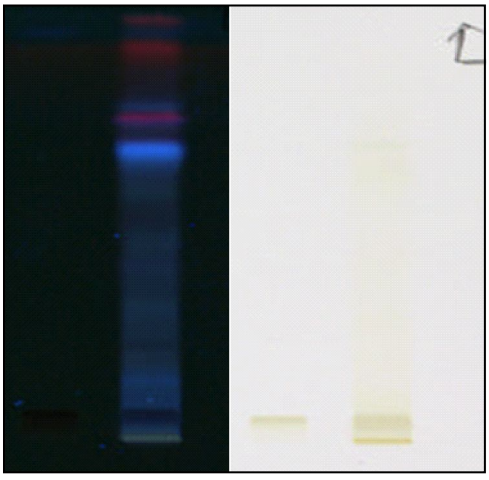

Figure 8: HPTLC finger print profile of standard quercetin and ethyl acetate fraction of coriander seeds under fluorescent and visible light

3.3 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance spectroscopy gives molar response that allows structure elucidation and quantification simultaneously. The information provided by NMR spectroscopy regarding the structure of compounds has made this technique a great tool for the identification and characterization of novel products. When compared to other analytical techniques, NMR spectroscopy is one of the most sensitive techniques. The presence of impurities can create practical problems in identifying the compound and these impurities may originate from various sources, like residual solvents, synthetic intermediates, by-products, formulation related impurities, degradation impurities and impurities formed during storage. When the impurities are above 0.1% level then identification is not considered to be necessary (Andersen and Fossen, 2003).

E2 obtained from ethyl acetate:methanol (3:1) solvent system of column chromatography, was found to be comparatively pure and was detected by TLC using methanol:chloroform (5%) as mobile

phase. E2 was brown in colour and was found to be soluble in chloroform, ethyl acetate and methanol. E2 compound was isolated in workable quantities from the ethyl acetate fraction of methanolic extract of coriander seeds. E2 compound was analysed using Nuclear Magnetic Resonance (NMR) spectroscopy through ^{1}H (Figure 9), ^{13}C (Figure 10), and IR (Figure 11). Preliminary analysis with ^{1}H, ^{13}C, and IR spectra confirmed the compound in E2 subfraction to be hydrocarbon in nature.

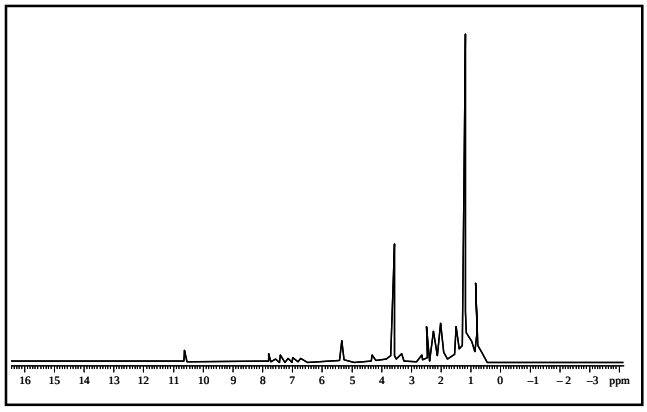

Figure 9: ^{1}H spectra of E2 compound

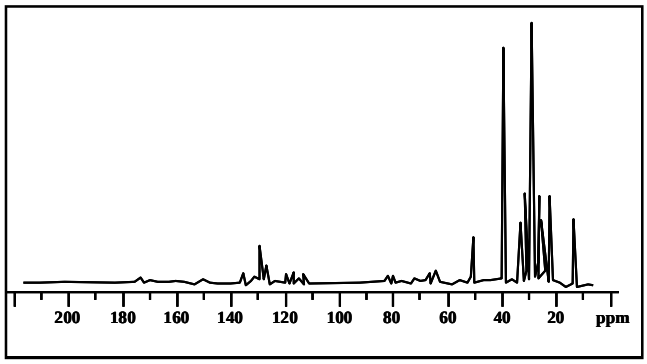

Figure 10: ^{13}C spectra of E2 compound

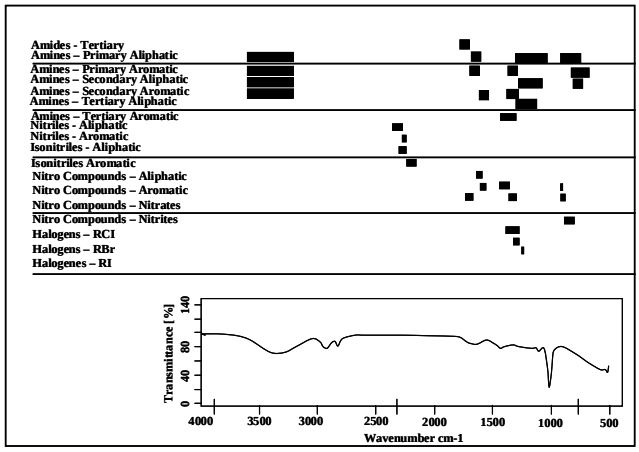

Figure 11: Infrared spectra of E2 compound

4. Conclusion

The RP-HPLC analysis revealed chlorogenic acid to be predominant in the extract followed by rutin, caffeic acid and quercetin. The method is simple sensitive, reproducible and very suitable for the determination of flavonoids, *viz*., caffeic acid, chlorogenic acid, quercetin and rutin in a multicomponent extract. High performance thin layer chromatography (HPTLC) of ethyl acetate fraction revealed the presence of many phenolic compounds, out of which quercetin and rutin could be identified and quantified; rutin being predominant in the fraction, followed by quercetin. The Infra red, ^{1}H, ^{13}C NMR spectrums of the E2 compound revealed itself as a hydrocarbon compound present in the ethyl acetate fraction of methanolic extract of coriander seeds.

Conflict of interest

We declare that we have no conflict of interest.

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