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ISOLATION AND CHARACTERIZATION OF MAJOR PHYTOCHEMICALS FROM THE LEAVES OF *PIPER BETLE*. LINN

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ABSTRACT

Diabetes mellitus (DM) is a chronic metabolic disorder arises from deficiency (T1DM) and/or efficiency (T2DM) of insulin. T2DM accounts for more than 90% of all diabetics and its prevalence is increasing alarmingly worldwide. It is intimately associated with improper utilization of insulin by target cells and tissues. Insulin, a polypeptide hormone synthesized by the pancreatic beta cells, is responsible for the maintenance of glucose homeostasis in human and other mammals. It is essential for the entry of glucose across the muscle and adipocyte cell membranes for energy production, hepatic glycogen synthesis, protein and nucleic acid synthesis and inhibition of gluconeogenesis, glycogenolysis and lipolysis. Thus, T2DM is a multifactorial, multisystemic endocrine disorder for which monotherapy often fails as the disease progress to later stages. Traditional medicinal plants used for the treatment of DM contain various biologically active ingredients which act in a synergistic way in maintaining normal glycemia. However, only few of them have been subjected to scientific validation. One such medicinal plant which lacks scientific scrutiny is *Piper betle* leaves. Hence, in the present study an attempt has been made to extract and identify the chemical nature of biologically active phytochemicals present in the distinct variety of *Piper betle* leaves of South India. The leaves cultivated in Kumbakonam are known for their taste, quality and medicinal properties. The data obtained by HPLC analysis and spectral studies such as FTIR, Mass, ^{1}H NMR, ^{13}C NMR evidenced that the ethanolic extract of *Piper betle* leaves contains Caffeic acid, p-Coumaric acid, Eugenol, Rutin and Hydroxychavicol as major secondary metabolites which are known for their beneficial and pharmacological properties. The results of the present study suggest that the betel leaves are the rich source of pharmacologically important lead molecules and also provide the scientific rationale for the use of *Piper betle* leaves in the traditional medicine.

Keywords: *Piper betle*, Spectral studies, HPLC, Rutin, Hydroxychavicol.

INTRODUCTION

Piper betle Linn. (PBL) is the leaf of betel vine popularly known as "Paan" and "Betel" in English. It belongs to the dicotyledonous black pepper family "*Piperaceae*". [1] *Piper betle* vine is a glabrous, evergreen, slender, shade loving, perennial root climber. [2, 3] It has an alternate, heart shaped, smooth, shiny and long stalked green leaves with pointed apex. The most probable place of origin of betel vine is Malasyia but today the plants are cultivated in India, Srilanka, Burma, Bangladesh and Nepal. [4] The plant is much more popular in India than any other country in the World since the antiquity which is evident from the numerous citations laid down in the ancient literature, particularly, the Indian scriptures. [5]

The betel vine growers invariably name their cultivars with local or vernacular names and are often named after their localities, villages or town where they are grown. There are about 100 varieties of betel vine. [6] Due to familiarity with grapes, many writers projected the similarity between the two and described that it was grown like grape vine. The significance of betel leaves has been explained in

relation to every sphere of human life including medicinal, social, cultural, religious and even day-to-day life, which is very much relevant even these days. [5] It is customary to serve betel leaves along with areca nut and/or sugar on various ceremonial occasions and also offered while inviting the guests as a mark of respect as well as recognition and for such traditional use of betel leaves really stands alone without any parallel even today. [7]

The habitual use of betel leaves can be traced as far back as two thousand years. The betel leaves are chewed together in a wrapped package along with the areca nut and slaked lime and other flavoring substances such as fennel seeds, cardamom combinations and spices to improve the taste and to prevent halitosis. This combination is traditionally known as "betel quid" or "paan" and nearly 600 million people consume it daily in one form or the other globally as a mouth refresher and masticator. [8] The stimulating effect coupled with strong flavors in addition to low cost and availability results in addiction among users who consume betel quid alone or in combination with smokeless tobacco. The consumption and method of chewing can vary widely from country to country. [9]

Betel quid chewing ranks second to coffee and tea in terms of daily consumptions and it is known to be the fourth most widely used additive substance in the World after nicotine, alcohol and caffeine. [10] The present day consumption pattern of betel leaves does not show any major change from the past except for the fact that it has become more of an addiction due to its association with tobacco and its consumption for positive reasons has gone down. [11]

Cardiovascular response of *Piper betle* acquires a great significance by the fact that it is consumed globally, making it a feasible substitute for *Digitalis purpurea.* [12, 13] Saini et al., in 2009 [14] reported that the microbial count of human saliva is more than 1 Lakh colony forming unit (CFU) per ml and there was a significant reduction in total microbial count of the mouth after masticating the *Piper betle* leaves. [15-17] Thus, it can be said that chewing of *Piper betle* leaves after every meal is good for oral as well as enteric health. [8] In fact, *Piper betle* leaves are a customary post prandrial offering in Indian Sub-continent. [18]

Piper betle leaves has a significant effect on various metabolic activities of the liver such as detoxification, protein synthesis and production of substances necessary for digestion which is evidenced from several *in vivo* studies. [19-20] *Piper betle* leaves offers a possibility for use in drug delivery through buccal mucosa bypassing the gastric route, where the drug has to endure gastric juices and acidic pH. [21] The collective antioxidant activity of *Piper betle* leaves was relatively superior to tea. [22] Several biologically active phytochemicals such as Hydroxychavicol, Apigenin and Luteolin have been reported to be present in the leaves of *Piper betle*. [23] Irrespective of the traditional uses, betel vine is arguably the most maligned plant whose regular consumption is believed to cause cancer of the oral cavity. This infamous accreditation is principally due to the fact that habitual chewing of betel quid which consisting of areca nut, slaked lime, smokeless tobacco in addition to betel leaves. [24] In numerable studies have been conducted with the individual constituents of the betel quid and the observations have conclusively evidenced that tobacco and areca nut are carcinogenic and slaked lime to promote carcinogenesis. [25, 26] Furthermore, contrary to the accepted belief, scientific studies have conclusively shown that the betel leaves are devoid of mutagenic and carcinogenic effects. Recently, we have reported the antidiabetic properties of betel leaves in alloxan induced experimental type 2 diabetes in rats. [27]

In spite of the above claims, relevant data from a complete chemical analysis and their biological properties were not available from any single source. In view of the above, the present study was aimed to analyze the presence of various phytochemicals present in the distinct variety of betel leaves cultivated in the district of Kumbakonam, Tamilnadu, India which is traditionally known for their quality, taste and medicinal properties.

METHODS

Plant material: Fresh, green and matured *Piper betle* leaves were collected from the healthy betel vines cultivated near Cauvery basin at Kumbakonam, Tamil Nadu and identified by a plant taxonomist in CAS in Botany, University of Madras where a voucher specimen was deposited in the herbarium.

Preparation of leaves extract: The *Piper betle* leaves were washed, dried in a hot air oven at 40°C and subsequently ground in to powder in an electrical grinder, which was stored in an airtight brown container at 5°C until further use. The powdered leaves were delipidated with petroleum ether (60-80°C) for overnight. It was then filtered and soxhalation was performed with 95% ethanol. Ethanol was evaporated in a rotary evaporator at 40-50°C under reduced pressure and the resultant product was lyophilized. The yield was around 9 % of dry weight.

Preliminary phytochemicals screening: The ethanolic extract of *Piper betle* leaves were subjected to phytochemical screening for the qualitative analysis of various phytoconsituents such as Alkaloids , Flavonoids, Glycosides Saponins, Tannins, Phytosterols, Triterpenoids, Anthraquinones, Phenols. [28, 29]

High performance liquid chromatography (HPLC)–DAD system for analysis of phenolic compounds: HPLC analysis was performed using Shimadzu HPLC system equipped with a diode array detector. The chromatographic separations were performed on an Inertsil C18 analytical column (4.6 mm × 250 mm i.d., 5 µm). The composition of solvents and the gradient elution conditions used were based on prescribed methods by Bengoechea et al., (1997), Schieber et al., (2001) and Butsat et al., (2009), [30-32] with slight modifications. The mobile phase consisted of purified water with acetic acid (pH 2.74) (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 ml/minute. Gradient elution was performed as follows: From 0 to 5 minutes, linear gradient from 5% to 9% solvent B; from 5 to 15 minutes, 9% solvent B; from 15 to 22 minutes, linear gradient from 9% to 11% solvent B; from 22 to 38 minutes, linear gradient from 11% to 18% solvent B; from 38 to 43 minutes, from 18% to 23% solvent B; from 43 to 44 minutes, from 23% to 90% solvent B; from 44 to 45 minutes, linear gradient from 90% to 80% solvent B; from 45 to 55 minutes, isocratic at 80% solvent B; from 55 to 60 minutes, linear gradient from 80% to 5% solvent B and a re-equilibration period of 5 minutes with 5% solvent B used between individual runs. Operating conditions were as follows: Column temperature, 38°C, injection volume, 20 µl, and ultraviolet (UV)-diode array detection at 280 nm and 370 nm at a flow-rate of 0.8 ml/minutes. Spectra were recorded from 200 to 600 nm. Phenolic compounds in the samples were identified by comparing their relative retention times and UV spectra with those of authentic compounds and were detected using an external standard method. Infrared (IR) spectral studies were carried out in the solid state as pressed KBr pellets using Perkin Elmer Fourier transform (FT)-IR spectrophotometer in the range of 400-4000/cm. The mass spectrum of the complex was obtained using Jeol Gcmate. The ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR at 500 and 125 MHz were carried out respectively. The spectra were recorded without any correction for instrumental characteristics.

RESULTS AND DISCUSSION

The phytochemical screening of ethanolic extract of betel leaves showed the presence of biologically active secondary metabolites such as alkaloids, flavonoids, glycosides, saponins, phytosterols, tannins and phenols. Five isolated compounds were identified in the ethanolic extract of *Piper betle* leaves by HPLC (Figures 1-5). HPLC analysis of the purified fraction showed that the isolated components (Figure 6) have retention times similar to that of Caffeic acid, p-Coumaric acid, Rutin, Eugenol and Hydroxychavicol standards respectively **(**Figure 7).

Caffeic acid: Caffeic acid (3, 4-dihydroxycinnamic) is one of the hydroxycinnamate and phenylpropanoid metabolite widely distributed in coffee drinks, blueberries, apples and cider. [33] Besides acting as a carcinogenic inhibitor, it is also reported to possess antioxidant and antibacterial properties. [34] It is reported to contribute to the prevention of atherosclerosis and other cardiovascular diseases. [35] The IR spectrum of the isolated caffeic acid is shown in Figure 8. The IR spectrum of the isolated compound exhibited a broad absorption band around 3396 cm^{-1} for OH group of the carboxylic acid, an absorption band at 1672 cm^{-1} for –C=O of carboxylic acid and an absorption band near 1610 cm^{-1} for –C=C- stretching. The strong peak at 1379 cm^{-1} was characteristic of O-H bending vibration and the normal peak at 1201 cm^{-1} was attributed to C–OH stretching vibration of phenol. The above observations were in consistent with an earlier report. [36]

Mass spectral analysis of caffeic acid is illustrated in Figure 9. It exhibited a molecular ion peak at m/z 180.84. Other fragments around 161.7 and 135.8 are due to the loss of water and carbon dioxide molecules respectively from the precursor ion. These observations are in accordance with the data of previous report. [37]

^{1}H NMR spectrum of the isolated caffeic acid is presented in Figure 10. The singlet at δ 4.862 (2H) is attributed to the presence of aromatic – OH. The doublet in the regions of δ 6.3 (1H, J = 6.44) and 7.3(1H, J = 7.5) corresponds to the presence of ethylene hydrogen. The presence of quadrant in the region of δ 6.6 (3H) is due to the presence of protons in the benzene ring. Moreover, the singlet in the region of δ 10 (1H) corresponds to the presence of carboxylic acid hydrogen. The ^{13}C NMR spectrum of the compound showed a total of 9 signals for 9 carbons as presented in Figure 11. The signal observed at δ 188.4 was allocated to carboxylic acid carbon. The ethylene carbon peaks were observed in the region of 117.5 and 151.07. The other peaks appeared in the spectrum are attributed to the carbons in the benzene ring. Both the ^{1}H and ^{13}C NMR

spectral data are in accordance with earlier reports. [38, 39]

p-Coumaric acid: p-Coumaric acid (CA) is a phenylpropanoid widely distributed in the fruits such as apples, pears and green vegetables. It has been reported to have significant antidiabetic and antioxidant properties. [40, 41] The IR spectrum of the p-coumaric acid is shown in Figure 12. The IR spectrum showed a strong absorption band for the existence of hydroxyl group at 3392 cm^{-1}. The band in the 1544 cm^{-1} region is assigned to υ (-C=C-) stretching vibration. The band around 841cm^{-1} corresponds to the benzene ring moiety. The observed bands are comparable with earlier reports. [42]

The mass spectrum of p-coumaric acid is depicted in Figure 13. The mass spectra showed a peak at 164 corresponding to $C_9H_8O_3$. Other fragments are also found around 162, 127, 118 and 64.

The proton NMR of p-coumaric acid is shown in Figure 14. The ^{1}H NMR spectrum of the compound exhibited a signal at δ 10.23 (1H) which is attributed to a carboxyl proton group. Further, doublet signals are observed around 6.27(1H, J = 6.2) and 7.81(1H, J = 7.9) as doublet for ethylene protons. The additional protons observed are assigned for the benzene ring protons. [43] The ^{13}C NMR spectrum of p-coumaric acid exhibited 8 signals for 9 carbons as shown in Figure 15. The signal at δ 178 corresponds to the presence of carboxylic carbon. The signals observed around 114 and 148 ppm are owing to the presence of ethylene carbons. The other signals are due to the presence of carbons in the benzene ring. These observations are in accordance with previously reported data. [44]

Rutin: Rutin (3, 3′, 4′, 5, 7-pentahydroxyflavone-3-rhamnoglucoside) is a bioflavonoid mostly found in edible plants such as buckwheat, onions, apple, berries, tea and wine. [45] Till date, over 130 registered therapeutic medicinal preparations are containing rutin in their formulations. [46] Rutin exerts its multispectrum pharmacological benefits for the treatment of various chronic diseases such as cancer, diabetes, hypertension and atherosclerosis. [47]

IR spectrum of rutin is shown in Figure 16. The –OH groups are observed in the range of 3398 cm^{-1}. The –C-H stretching was observed in the range of 3052 cm^{-1}. The carbonyl group C=O was observed in the range of 1668 cm^{-1}. The band at 1621 cm^{-1} is owing to the presence of -C=C group. The aromatic –C=C group was observed in the region of 1571 cm^{-1}. The other bands at 1373, 1219 and 1081 cm^{-1} are due to the presence of -C-O-C groups. These findings are in accordance with an earlier report. [48]

The Mass spectrum of rutin is shown in Figure 17. The negative-ion electrospray ionization (ESI) mass spectrum of the isolated compound showed a quasi-molecular ion [M+H]- at m/z 611, indicating a relative molecular weight (M) of 610. The negative-ion ESI-MS also exhibit a prominent fragment ion at m/z 303 [M-H].

The ^{1}H NMR of rutin is represented in Figure 18. In the ^{1}H NMR spectra of the rutin, the singlet in the region of 1.106 ppm (3H) is due to the presence of a methyl group in the sugar moiety. The peak at 2.192 ppm (5H) as singlet is owing to the presence of –OH group in the sugar moiety. Furthermore, the C-H protons in the sugar moiety were observed as multiplets in region of 2.9 – 4.0 ppm (13H). The peak at 4.8 ppm (4H) as singlet is assigned for the presence of aromatic –OH group. The presence of protons in the benzene ring was confirmed by a peak as multiplet in the region of 5.8 – 6.0 ppm (5H).

The ^{13}C-NMR showed the presence of 30 carbon environments as represented in Figure 19. The peak at 18.255 ppm was assigned for the presence of methyl carbon present in the sugar moieties. The carbons present in the aliphatic moiety (sugar) are observed in the region of 59 – 96 ppm. The aromatic carbons present in the benzene ring are assigned in the range of 98 – 165 ppm. The most important –C=O peak was obtained in the range of 179 ppm. The results obtained are comparable with earlier reports. [49, 50]

Eugenol: Eugenol (4-allyl-2-methoxyphenol), with a molecular formula of $C_{10}H_{12}O_2$ and molecular weight of 164.21, mainly exists in clove oil, basil and cinnamon leaf oil. [51] It is a remarkably versatile phenylpropanoid incorporated as a functional ingredient with various pharmacological, agricultural and culinary properties. [52] Both the FAO and WHO, have proclaimed eugenol as non-toxic and recommended an acceptable daily intake of eugenol of 2.5 mg/kg. b.w. for humans. [53-55] The FT-IR of the isolated active compound eugenol (Figure 20) showed a band at 3488 cm^{-1} assigned for Phenolic OH stretching. The band around 2934 cm^{-1} corresponds to stretching in =C-H. The bands around 2822 and 2841 cm^{-1} are due to the presence of aliphatic C-H stretching. The C=C in allyl group was observed around 1632 cm^{-1}. The sharp band at 1606 cm^{-1} with shoulder peak at 1441 cm^{-1} was assigned for the C=C in aromatic ring. The strong and sharp band at 1368 cm^{-1} is owing to the presence of CH_3 group in the parent compound. The C-O-C

asymmetric stretching was observed around 1256 cm^{-1}. The band at 1041 cm^{-1} corresponds to the presence of C-O-C symmetric stretching. These data are in accordance with the previous reported findings. [56]

Mass spectrum of eugenol is presented in Figure 21. The mass spectrum obtained showed the m/z 164 corresponding to M^+. The peak at 149 is due to the loss of methyl group. The peak at 137 is due to loss of $CH=CH_2$). Other important peaks are observed at m/z 131, 103, 91 and 77.

^1H NMR of eugenol (DMSO d_6, 500 MHz) is represented in Figure 22. The ^1H NMR spectra of the obtained compound showed a peak at δ 3.43 as a doublet for the presence of methylene CH_2 group. The singlet around δ 3.96 corresponds to the presence of methyl group in the compound. The peaks around δ 5.08 and δ 6.20 are due to the presence of ethylene protons. The singlet around δ 5.48 was attributed to the presence of aromatic hydroxyl proton. Other peaks correspond to the presence of protons in the benzene ring.

The ^{13}C-NMR of eugenol showed the presence of ten carbon environments as represented in Figure 23. The peak at 41.33 ppm was attributed to the presence of -CH_2 carbon. The strong peak at 54.93 was due to the presence of methyl carbon. The peaks around 116.83 and 137.69 are owing to the presence ethylene carbons in the isolated compound. Moreover, the peaks at δ: 143.64, 145.88, 110.73, 131.65, 121.42, 115.07 indicate the presence of six aromatic carbons, confirming the existence of aromatic ring in the molecule. The NMR spectral assignments are in accordance with the previously reported findings. [57]

Hydroxychavicol: Hydroxychavicol (3, 4 dihydroxyallybenzene), a major phenolic compound was originally isolated from *Piper betle* leaves. Previous studies have suggested that Hydroxychavicol exerts significant antioxidant, antimutagenic and anticarcinogenic properties and most of the beneficial and pharmacological properties of *Piper betle* leaves are attributed to the presence of hydroxychavicol. [58-62]

IR spectrum of hydroxychavicol is shown in Figure 24. The FT-IR of the isolated hydroxychavicol showed a band at 3415 cm^{-1} corresponds to Phenolic OH stretching. The band at 1682 cm^{-1} corresponds to -(C=O) group. The aromatic functional groups are observed around 1622, 1540, 1392 and 1318 cm^{-1}.

The mass spectral analysis of the hydroxychavicol is represented in Figure 25. Mass spectrum of active compound showed m/z 149 [M-H, 100%], other important peaks are observed at m/z 131, 123, 103, 77 and 51.

The ^1H NMR spectrum of the compound is depicted as Figure 26. The doublet at peak around δ 3.18 (2H) corresponds to the presence of CH_2 group in the isolated compound. The peak observed at δ 4.69 (2H) as singlet was assigned for the aromatic hydroxyl protons. The multiplet around δ 5.1(2H) and δ 5.9 (1H) are attributed to the presence of ethylene protons. The other peaks are assigned for the aromatic protons.

The ^{13}C-NMR showed 9 signals for 9 carbons as shown in Figure 27. The peak at 42.34 ppm was due to the presence of aliphatic CH_2 group carbon. The ethylene group carbons are observed around 118.56 and 138.74 ppm. The peaks around 113.82, 121.45, 134.11, 143.94 and 146.53 ppm are attributed to the presence of carbons in the benzene ring. The spectral data and assignments are in accordance with the previously reported findings. [63]

CONCLUSION

The results of the present study clearly established that the betel leaves cultivated in the study area contain ecologically derived phytoingredients such as Caffeic acid, p-Coumaric acid, Rutin, Eugenol and Hydroxychavicol. These secondary metabolites are reported to possess a wide spectrum of medicinal properties. Thus, the results provide a systematic study for the presence of pharmacologically important phytochemicals and the scientific rationale for the use of *Piper betle* leaves in the traditional system of medicine. Further, studies are in progress to evaluate the antidiabetic properties of individual active ingredients present in the betel leaves in alleviating both the primary and secondary complications of diabetes mellitus.

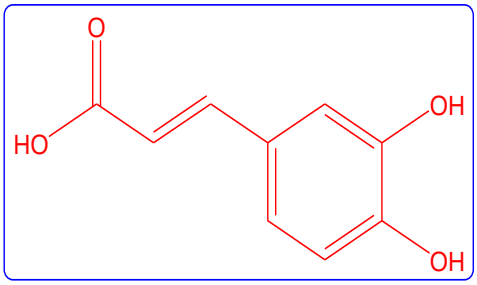

Figure 1: Caffeic acid.

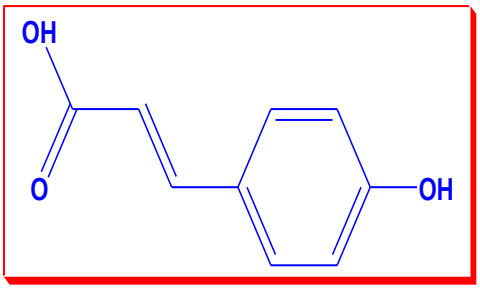

Figure 2: p-Coumaric acid.

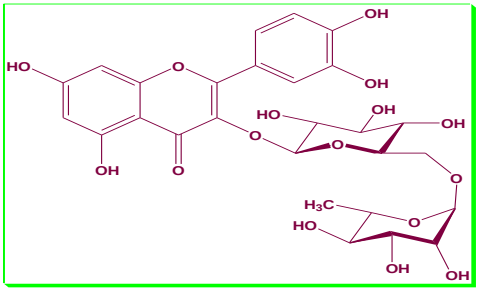

Figure 3: Rutin.

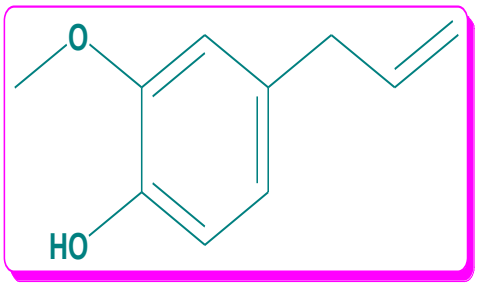

Figure 4: Eugenol.

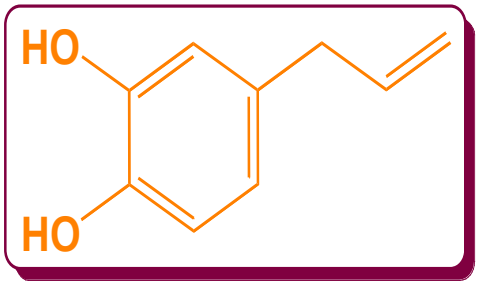

Figure 5: Hydroxychavicol.

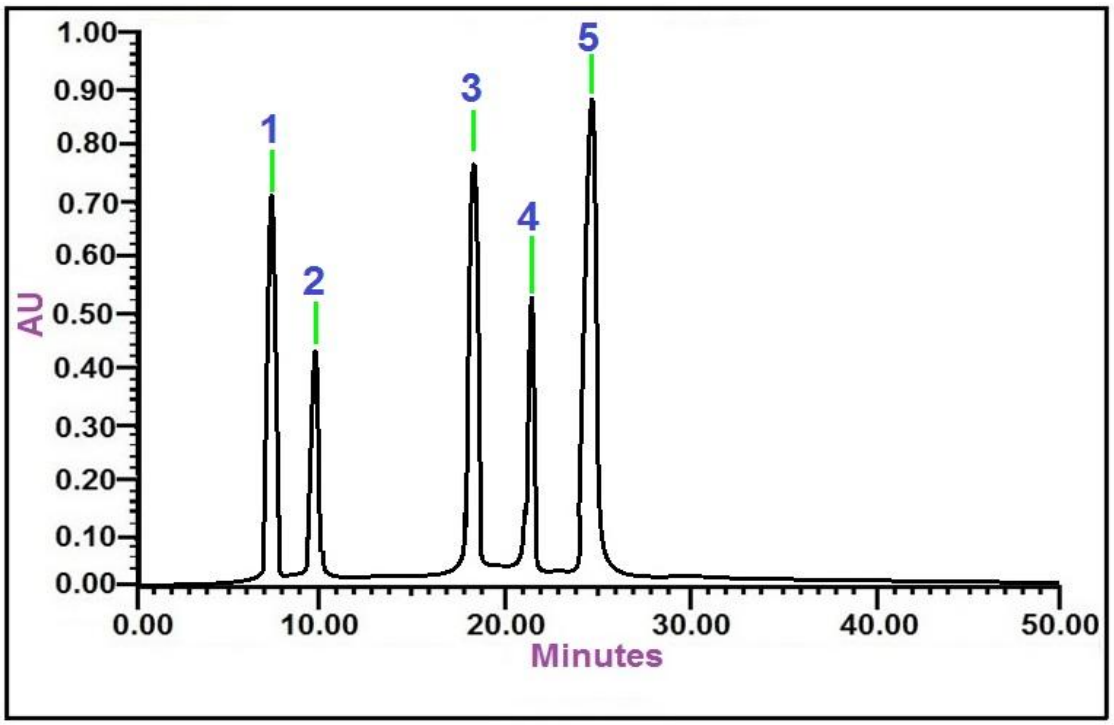

Figure: 6 HPLC spectrum of isolated compounds.

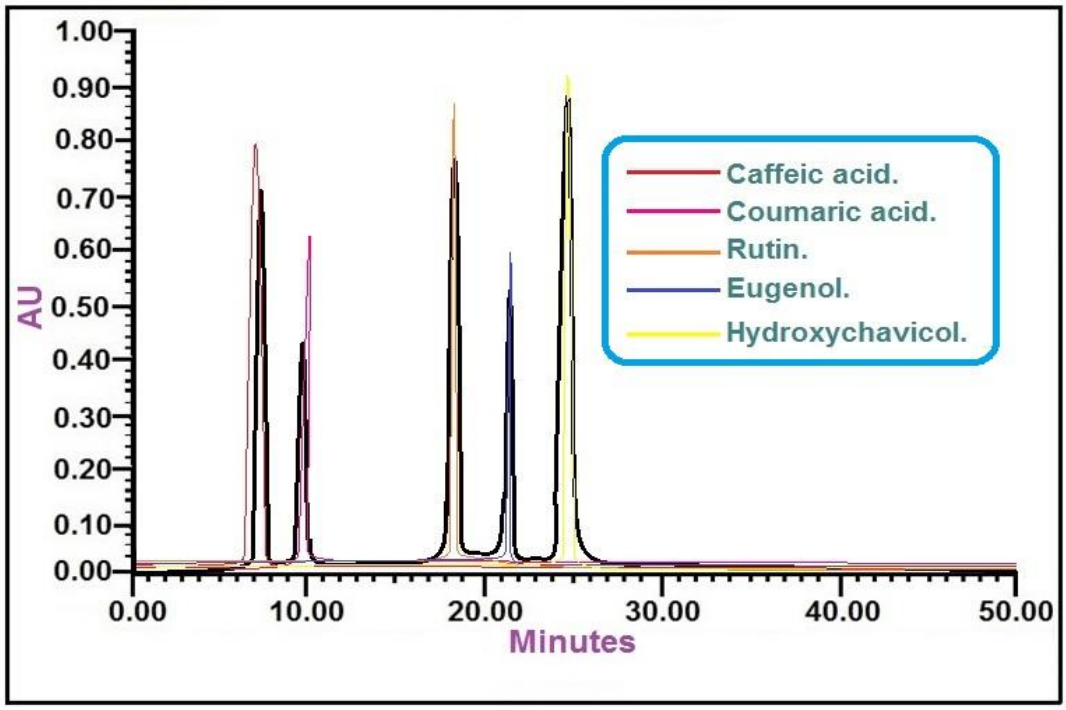

Figure 7: HPLC Spectrum of standard compounds.

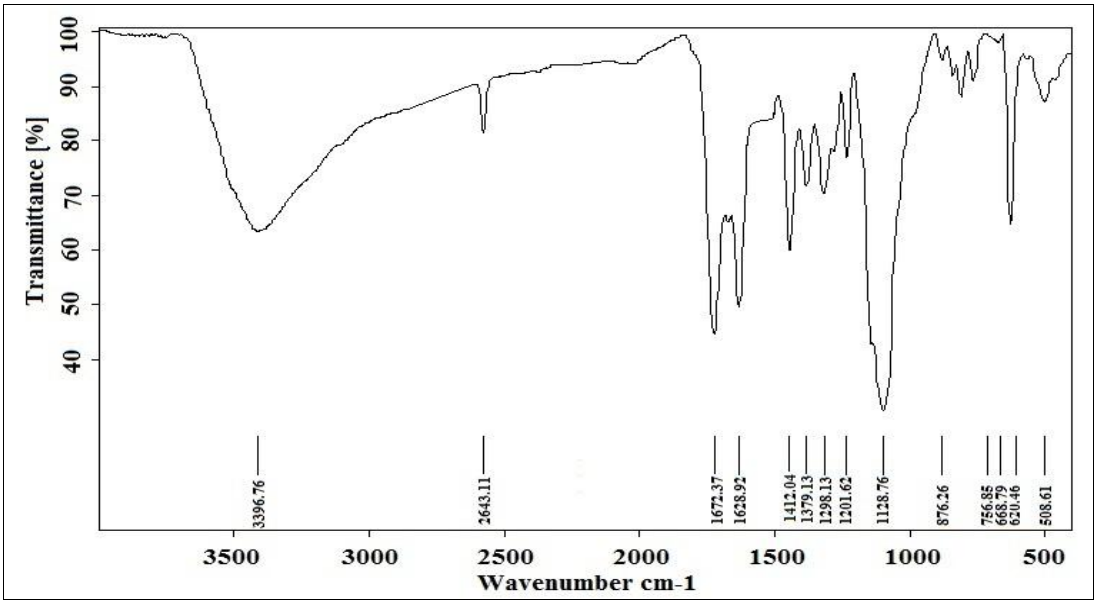

Figure 8: Infrared spectrum of Caffeic acid

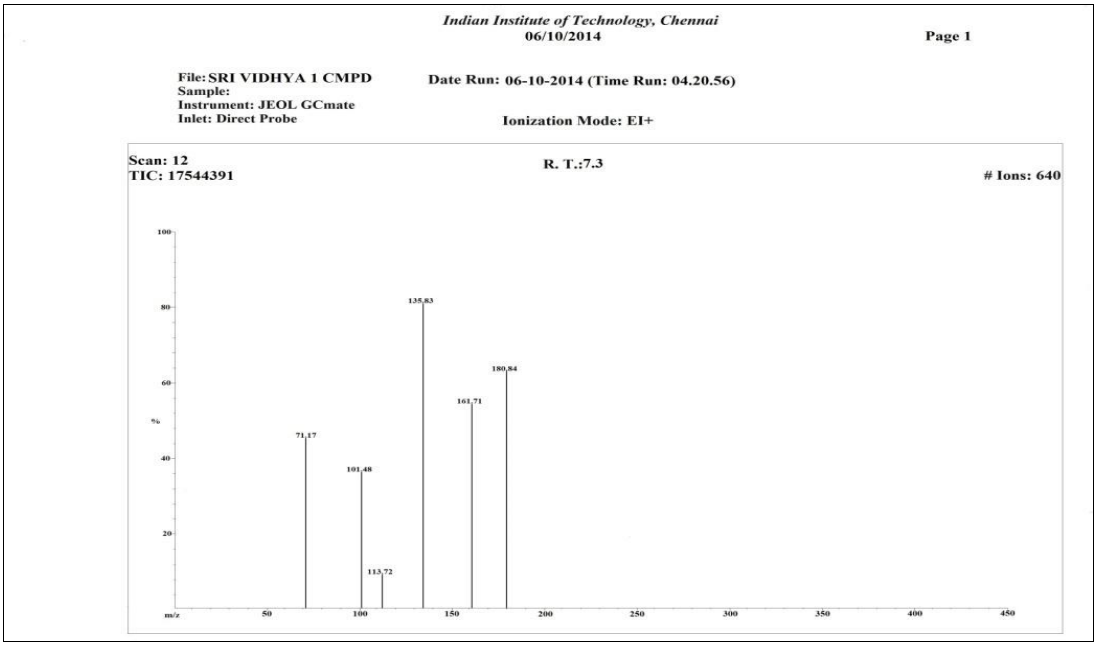

Figure 9: Mass spectrum of Caffeic acid

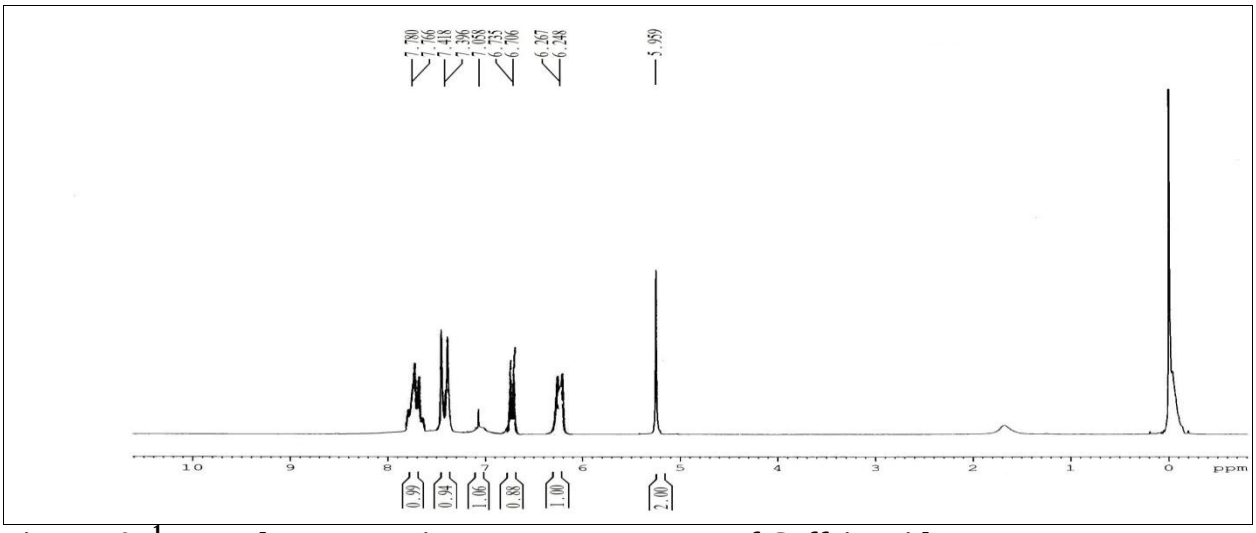

Figure 10: [1] H Nuclear magnetic resonance spectrum of Caffeic acid

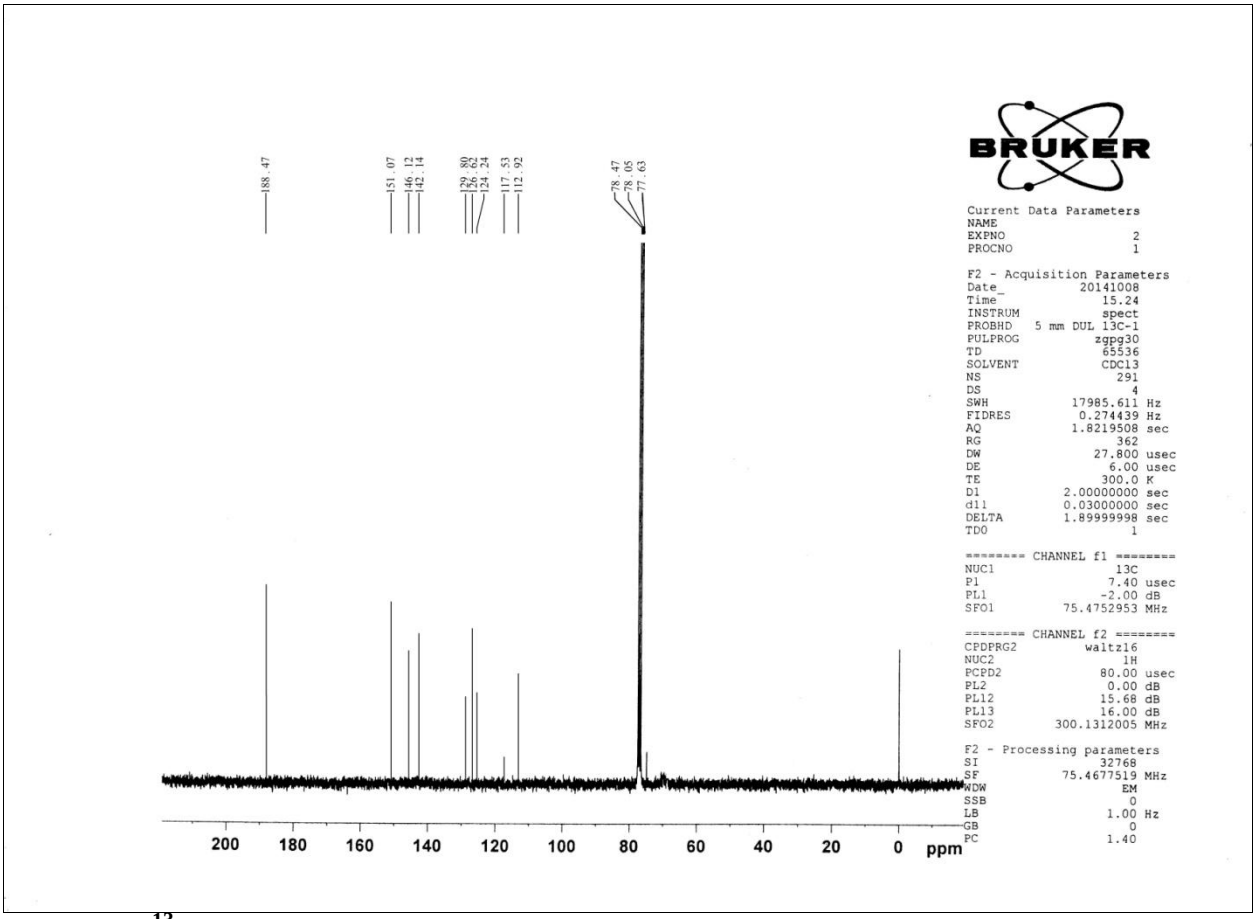

Figure 11: [13] C Nuclear magnetic resonance spectrum of caffeic acid

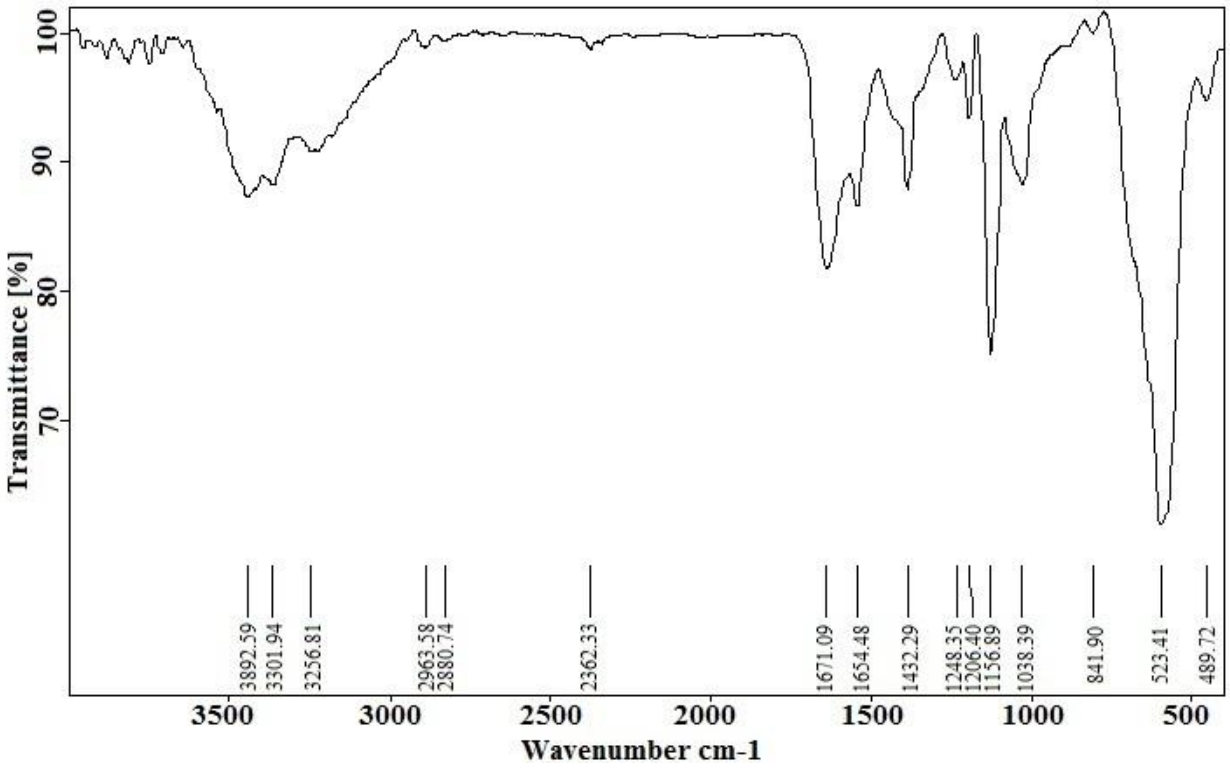

Figure 12: Infrared spectrum of p-Coumaric acid

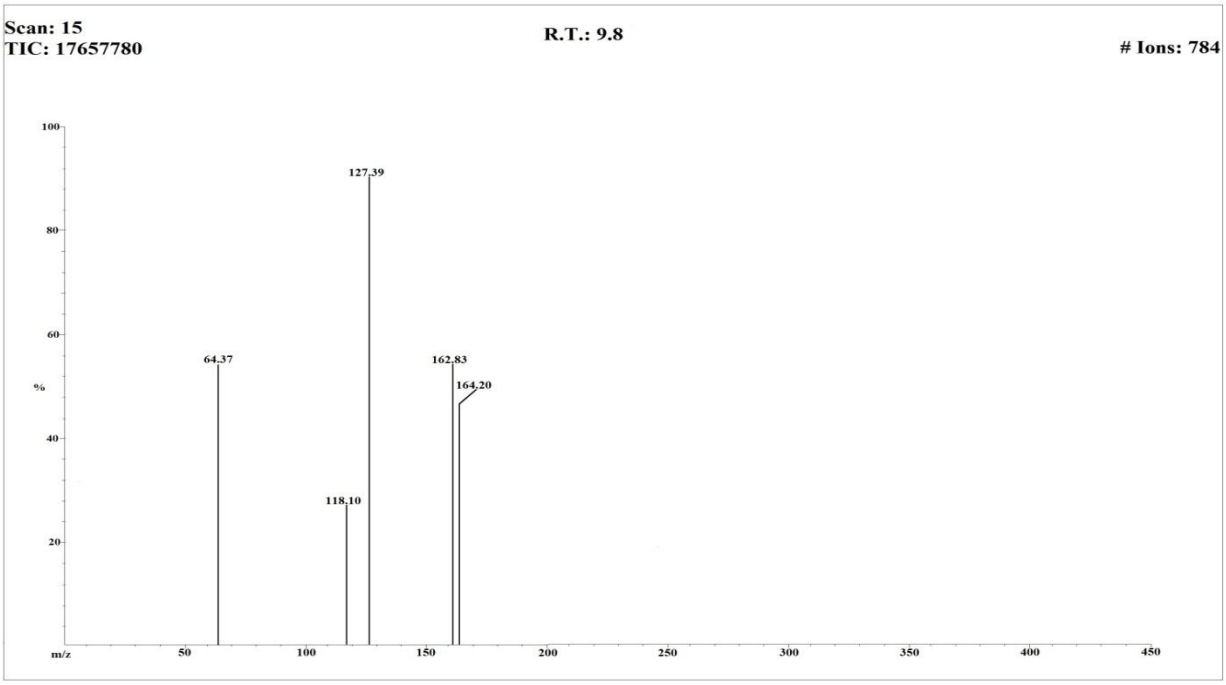

Figure 13: Mass spectrum of p-Coumaric acid

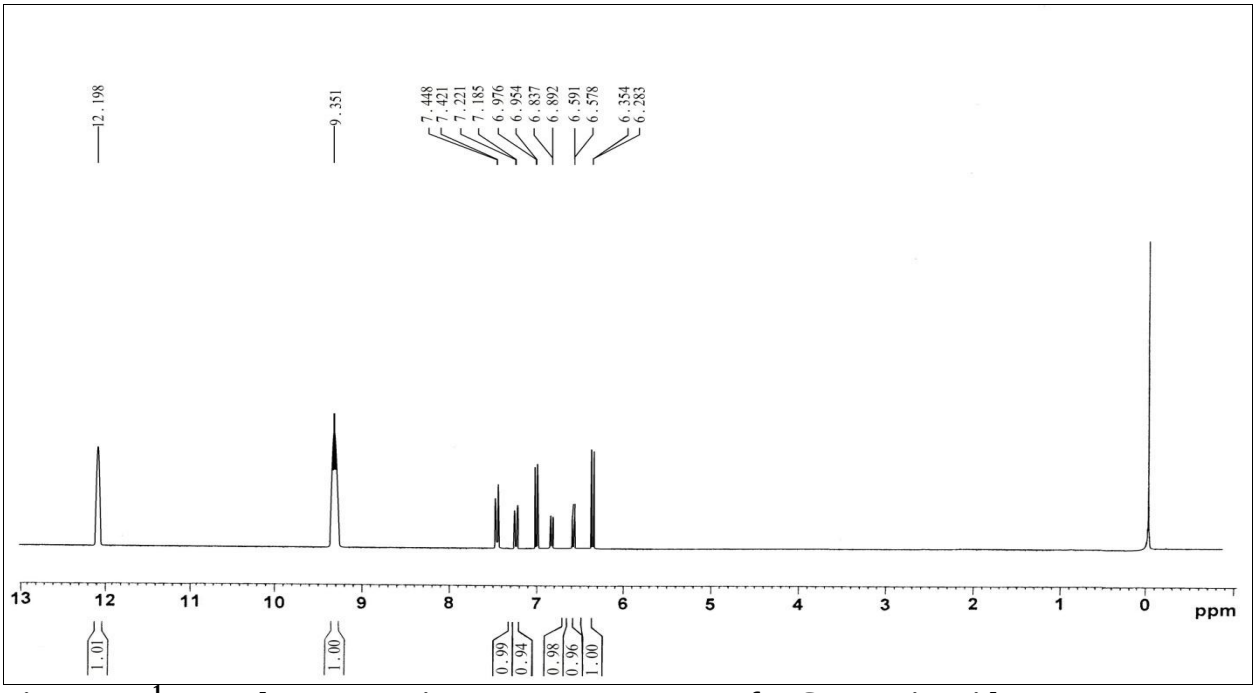

Figure 14: [1] H Nuclear magnetic resonance spectrum of p-Coumaric acid

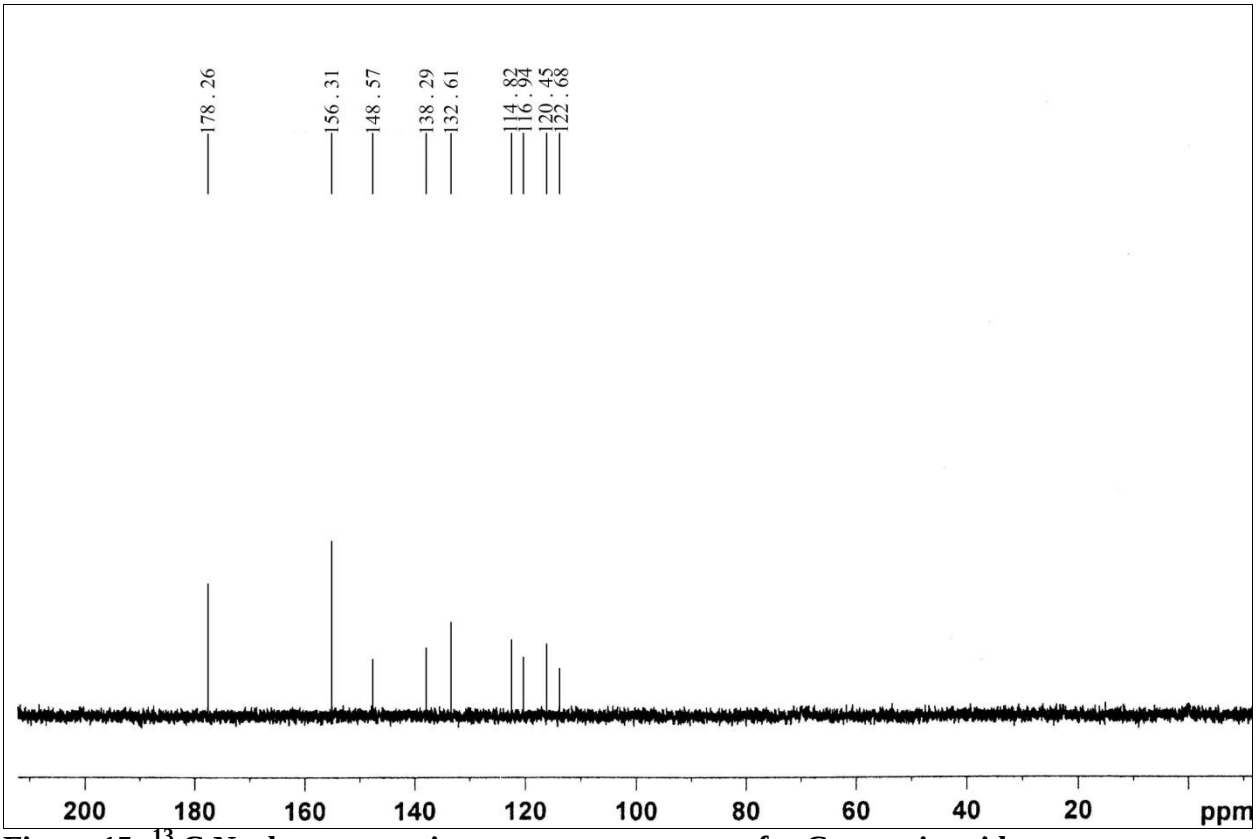

Figure 15: [13] C Nuclear magnetic resonance spectrum of p-Coumaric acid

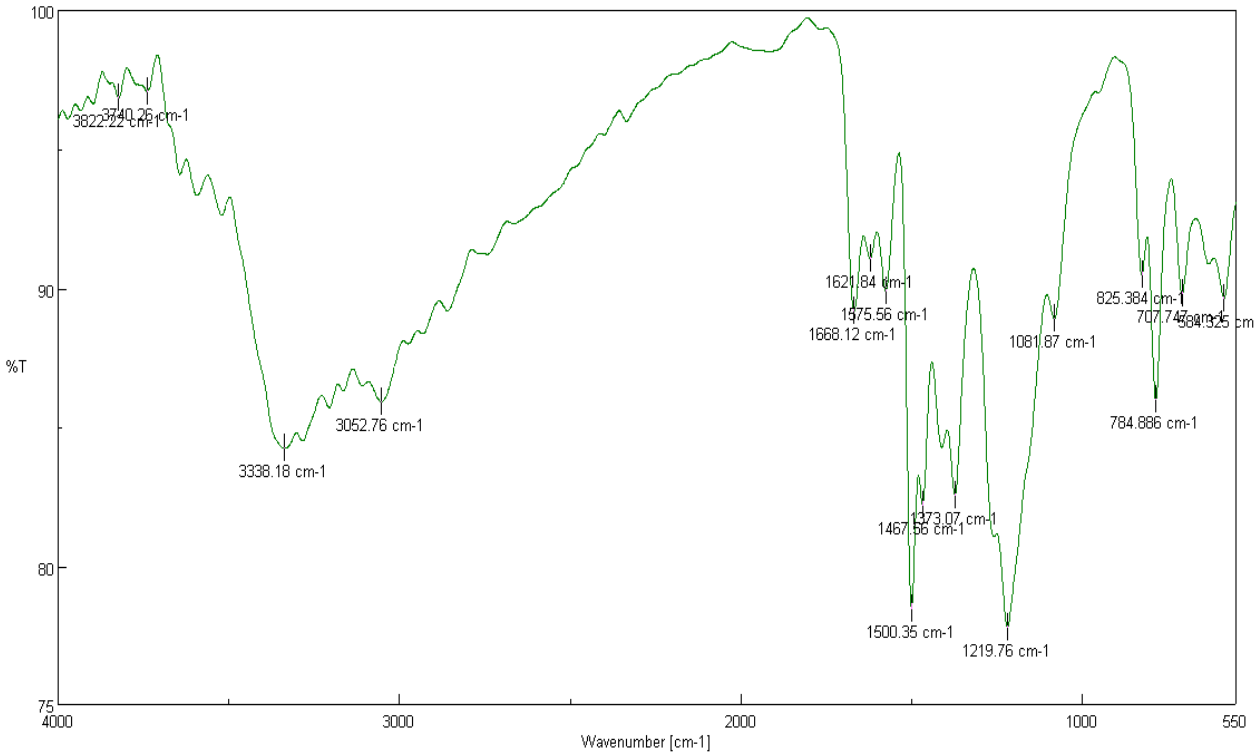

Figure 16: Infrared spectrum of Rutin

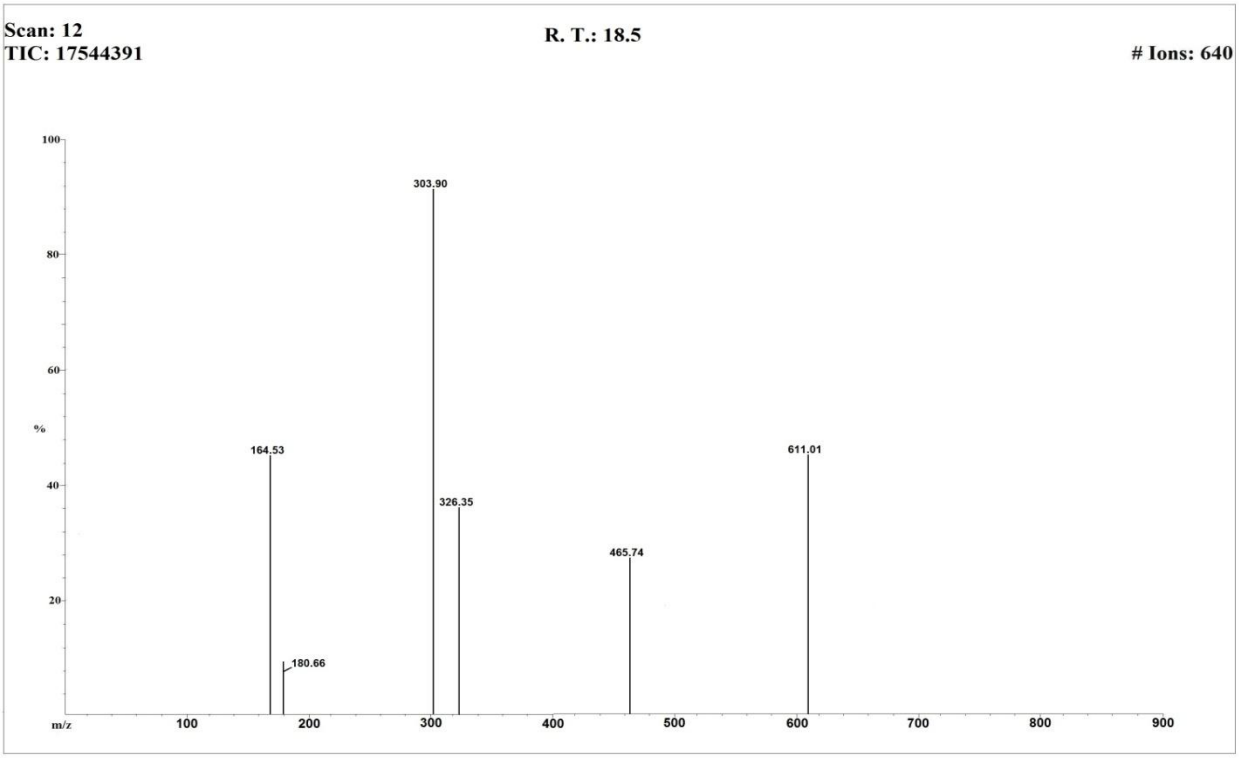

Figure 17: Mass spectrum of Rutin

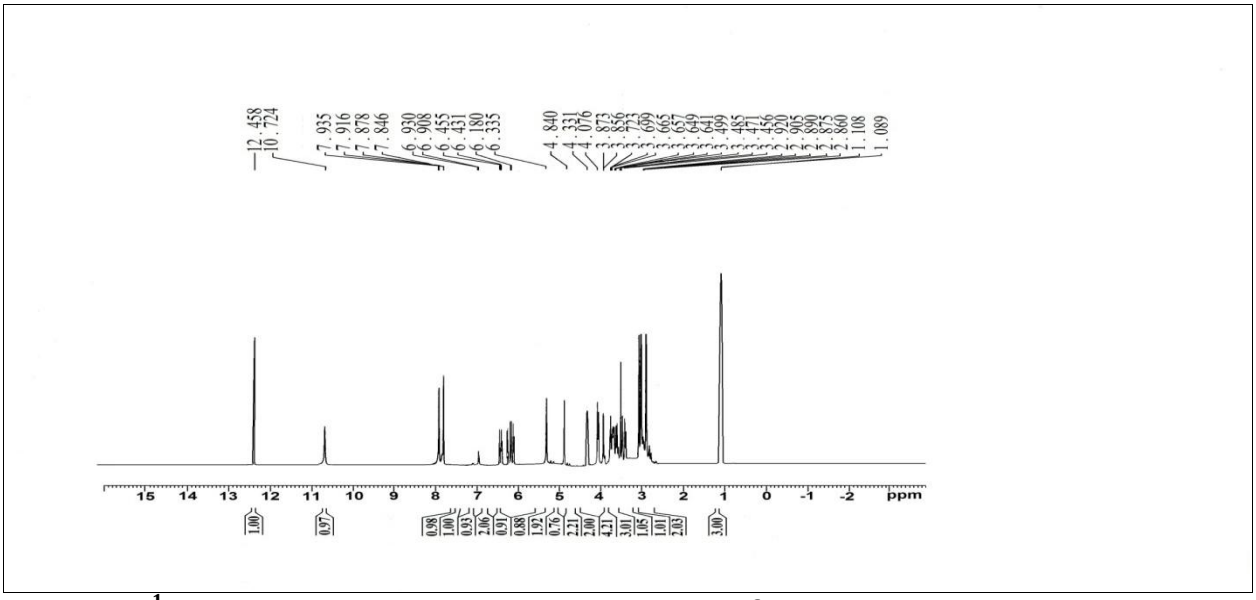

Figure 18: ^{1} H Nuclear magnetic resonance spectrum of Rutin

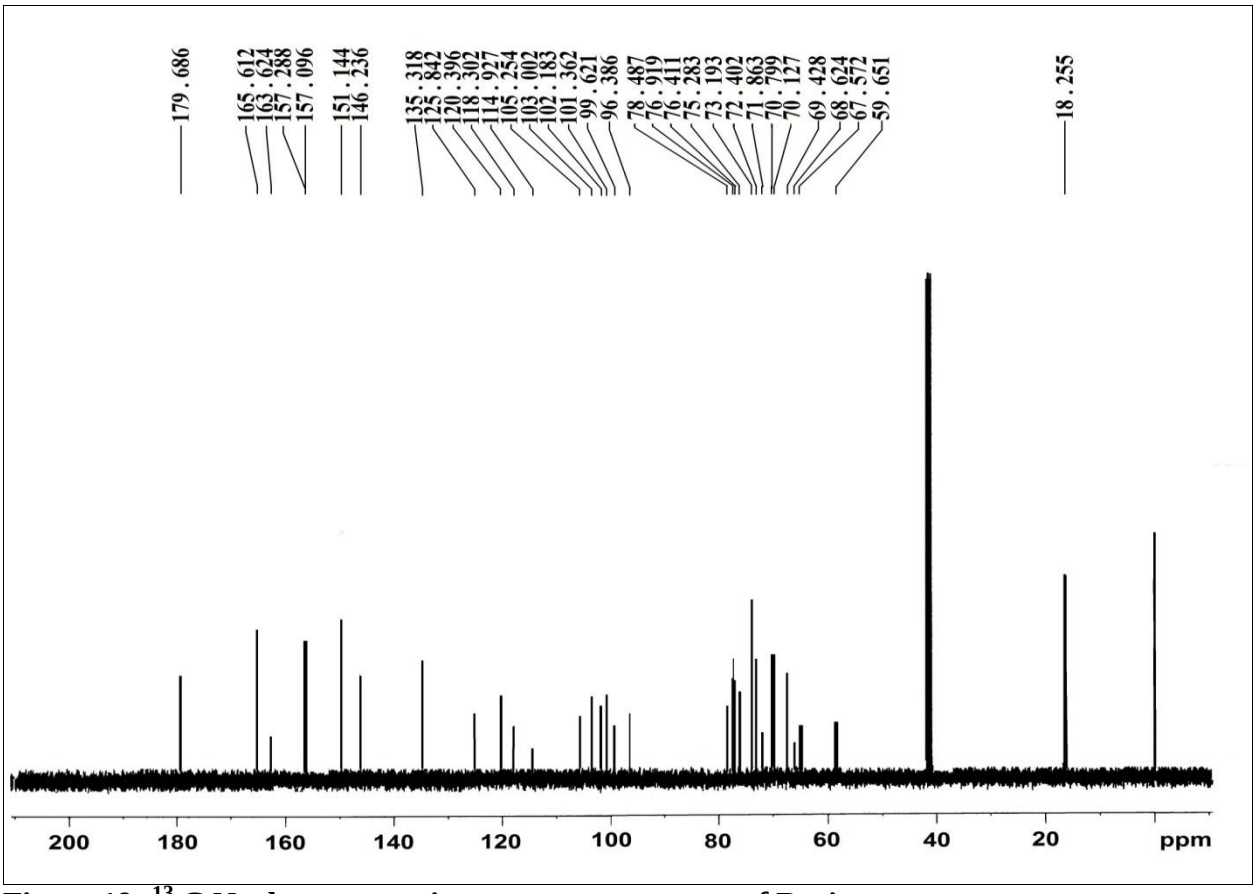

Figure 19: ^{13} C Nuclear magnetic resonance spectrum of Rutin

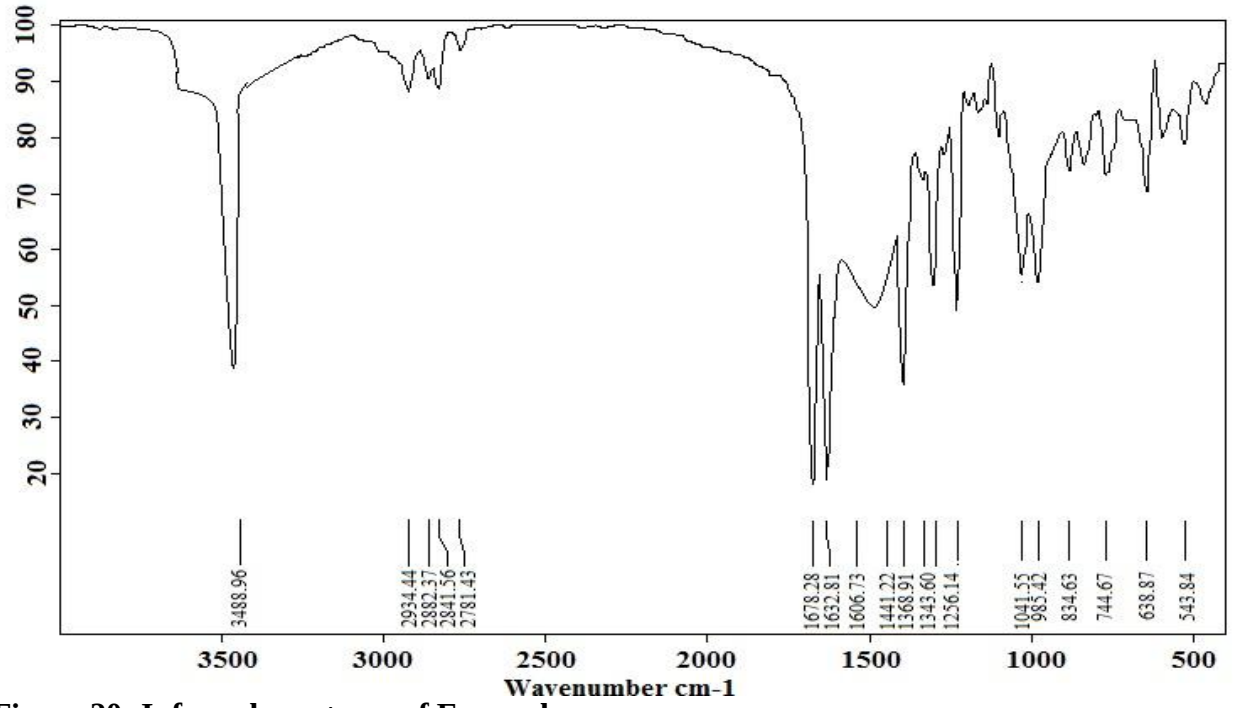

Figure 20: Infrared spectrum of Eugenol

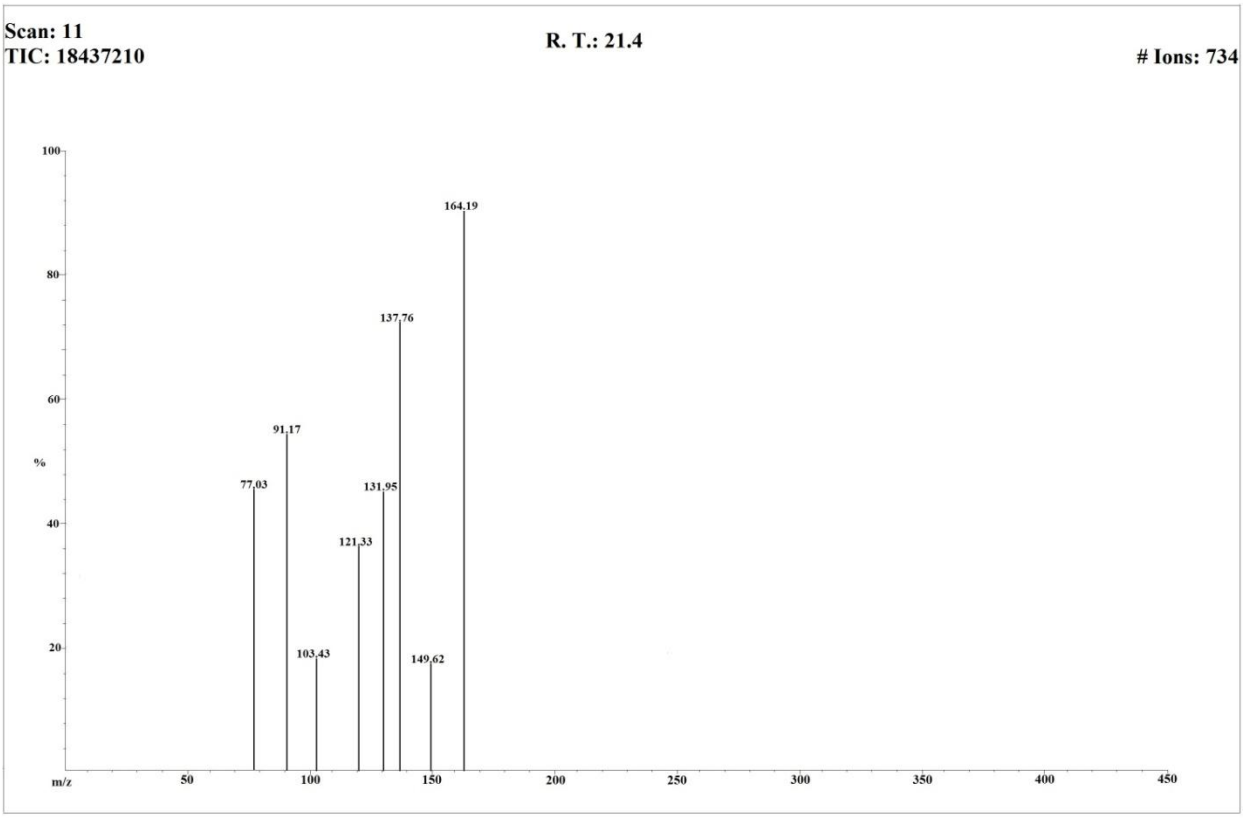

Figure 21: Mass spectrum of Eugenol

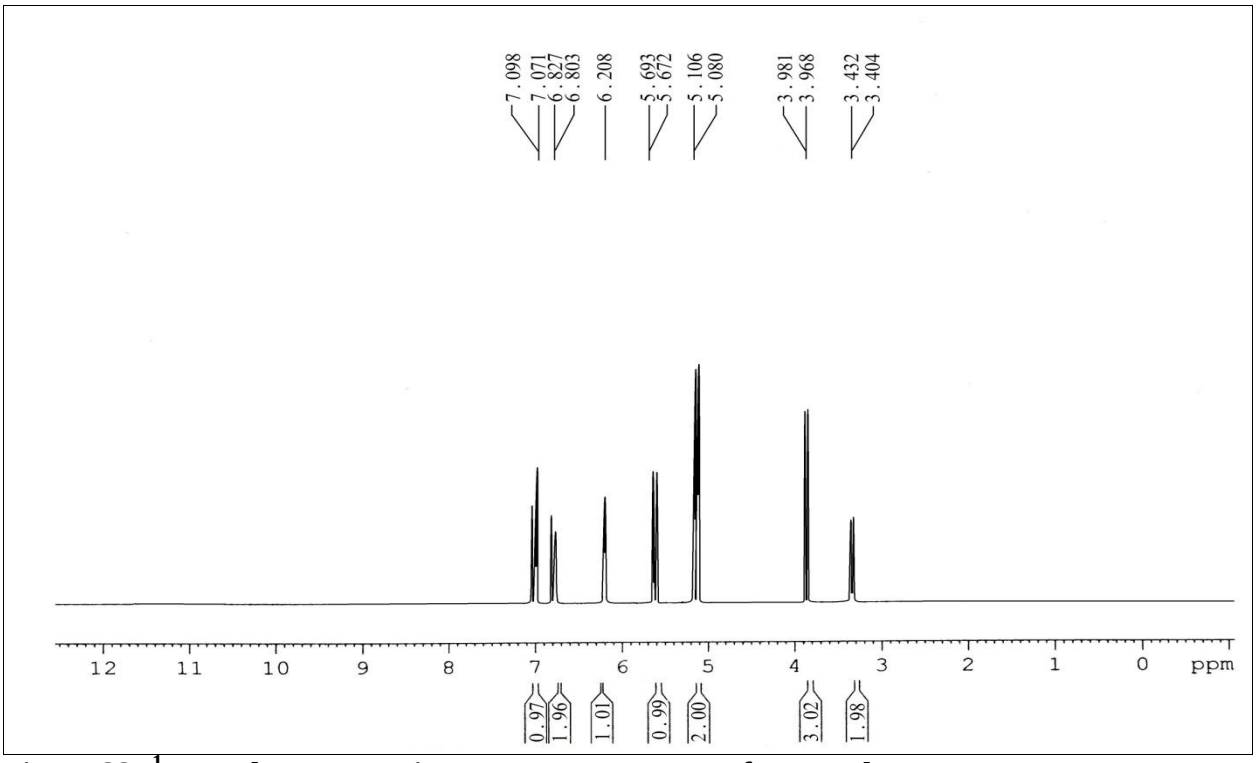

Figure 22: [1]H Nuclear magnetic resonance spectrum of Eugenol

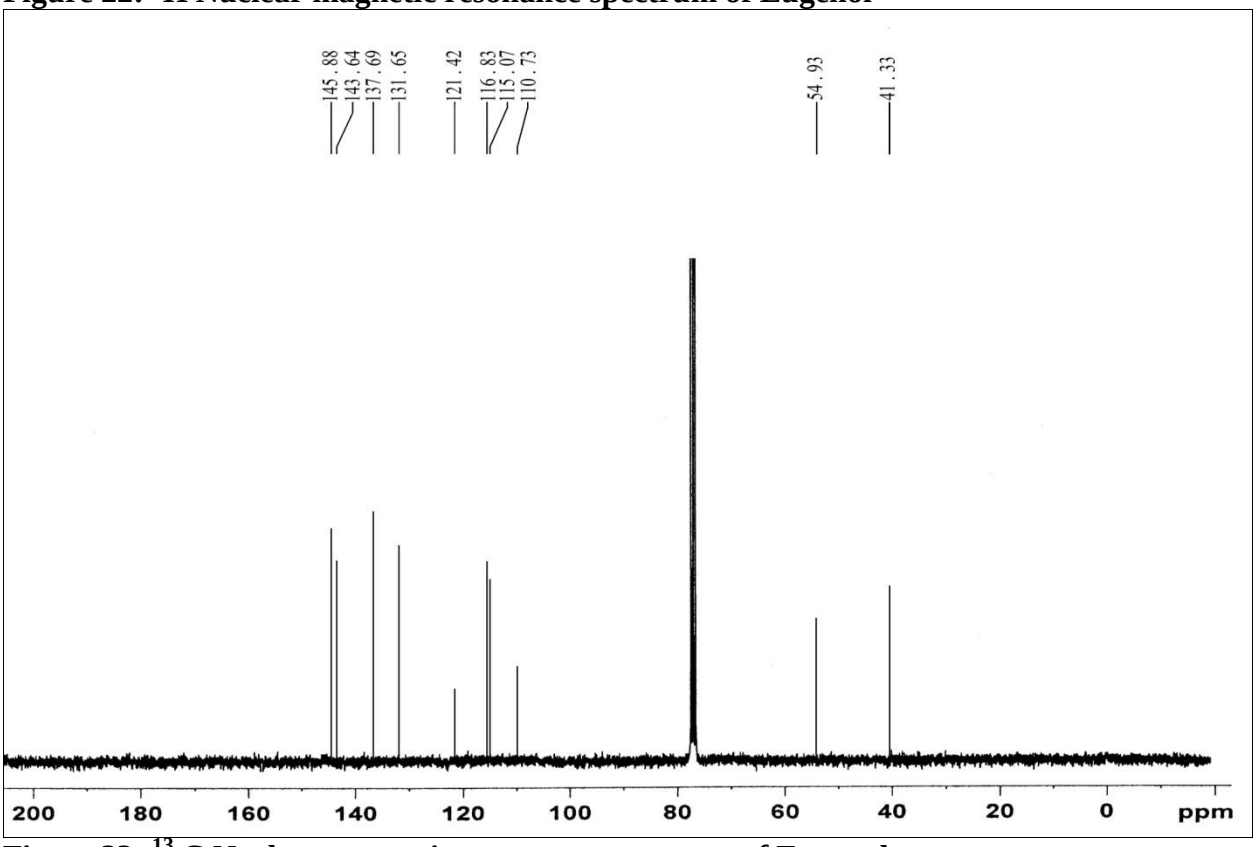

Figure 23: [13] C Nuclear magnetic resonance spectrum of Eugenol

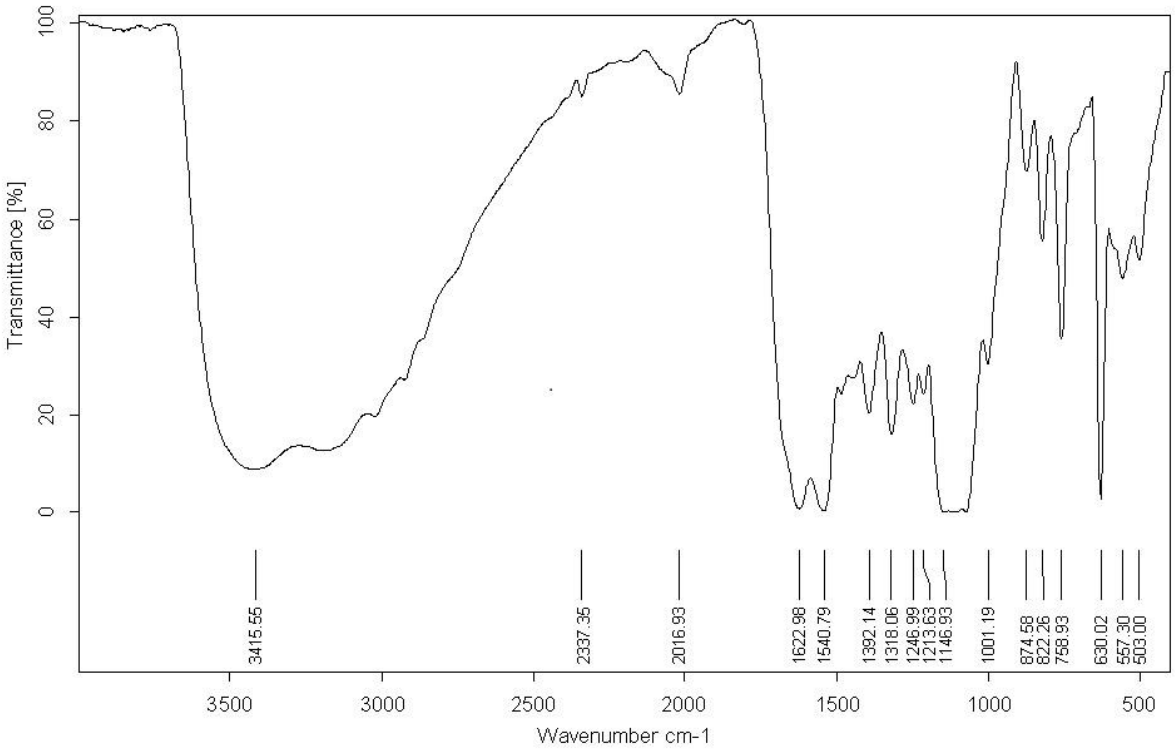

Figure 24: Infrared spectrum of Hydroxychavicol

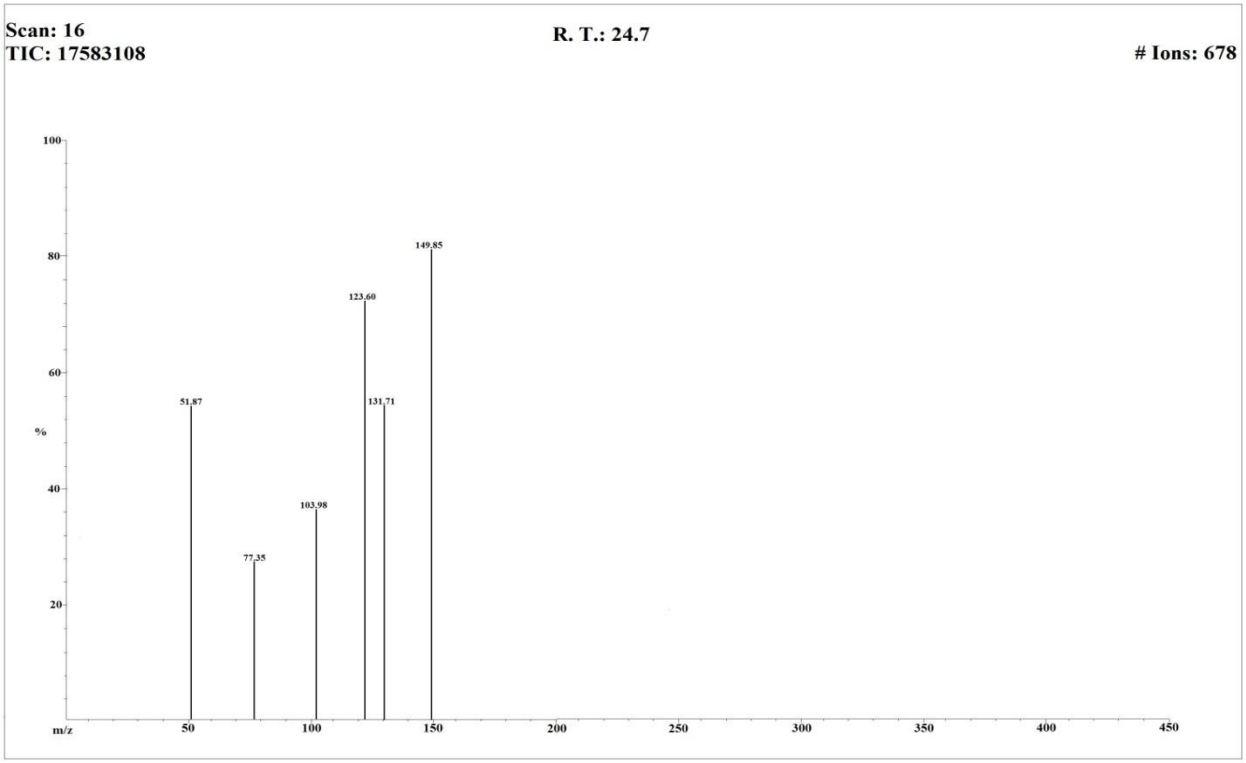

Figure 25: Mass spectrum of Hydroxychavicol

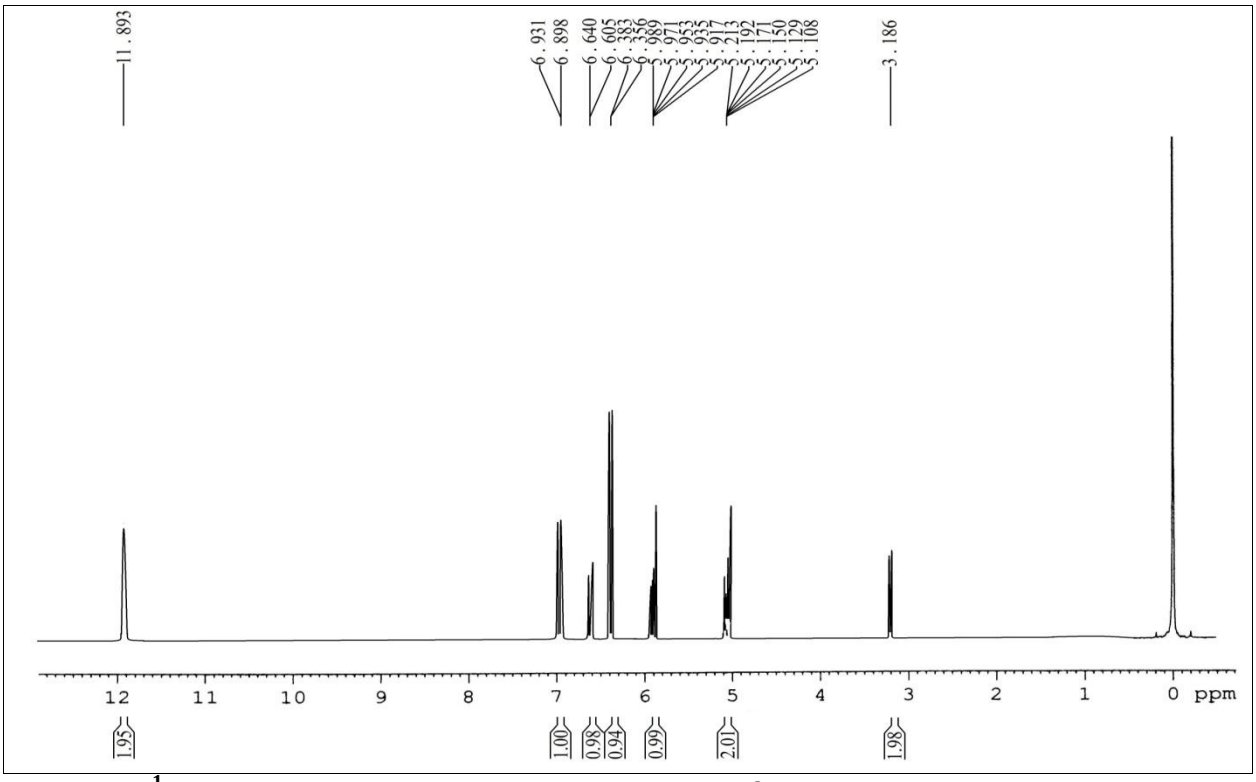

Figure 26: [1] H Nuclear magnetic resonance spectrum of Hydroxychavicol

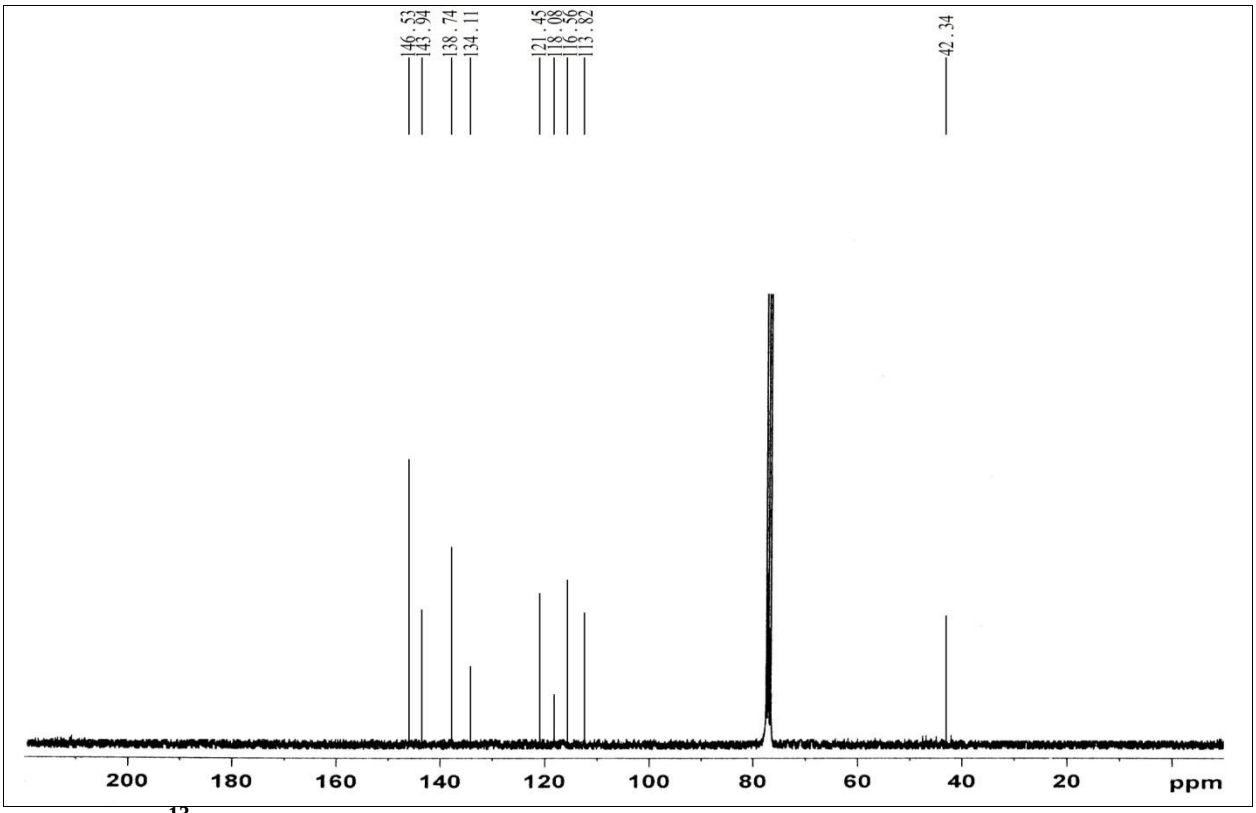

Figure 27: [13]C Nuclear magnetic resonance spectrum of Hydroxychavicol

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