3, 5, 7, 3', 4'-PENTA HYDROXY FLAVONES - ISOLATED FROM IPOMOEA PES-CAPRAE BY NORMAL PHASE COLUMN CHROMATOGRAPHY

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Abstract: It deals with the isolation of compound from most active extracts of Ipomoea pes-caprae by using column chromatographic techniques. Active compounds of aerial part of Ipomoea pes-caprae, gradually extracted by solvents and purified by silica gel column, resulted in a compound has also been discussed. The structures of isolated compound is characterised by using different spectrometric techniques like UV, IR, 1H NMR, 13C NMR and mass spectrometry. The compound is 3, 5, 7, 3', 4'- Pentahydroxy flavones (IUPAC name is 2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4H-chromen-4- one.) with molecular formula C15H10O7 and molar mass 302.04. EC50 value and in-vitro cytotoxic activities were analysed for the purified compound with suitable assay method and results were discussed. The biological study of the tested compound shows very effective anti-cancer activityagainst cell lines MCF-7. It can even function as starter material in cancer drug.

Keywords: Keywords: Ipomoea, TLC, silica gel, column, FT-IR, NMR, Mass, cytotoxic activity

Introduction

Natural products also play an important role in the health care system in developed countries. Natural products can also be used as starting materials for semisynthetic drugs. The main examples are plant steroids, which led to the manufacture of oral contraceptives and other steroidal hormones. Today, almost every pharmacological class of drugs contains a natural product or natural product analog. The investigation of higher plants has led the discovery of many new drugs. So far only a small portion of higher plants has been investigated. Consequently, they still remain big reservoir of useful chemical compounds not only as drugs, but also as templates for synthetic analogues.

Different chromatographic techniques and spectrometric methods are generally used for the extraction and identification of compounds of interest. Chromatography is a technique which is used to separate different components of a mixture. Adsorbent material known as stationary phase is packed in the column and it provides support. Flowing system is normally known as mobile phase [1]. Chromatographic techniques have a wide range of applications especially in the field of pharmaceutical biology and drug analysis and for the analysis of other biologically active compounds. Column chromatography technique is used to purify compounds of interest from a mixture by using glass tube of varying length and diameter. In this chromatographic technique stationary phase is packed in a glass column of varying diameter depending on the requirement. Stationary phase is solid and is mostly silica gel.[2]

It is a slow process and its slightly modified form known as flash column chromatography is used in which slight air pressure is applied to accelerate the movement of solvent [3]. In Normal phase column chromatography is a stationary phase and mobile phase is applied in raising order of polarity that is starting from the least polar one and ending at the most polar one. The technique in which reverse phase silica is used as stationary phase and mobile phase is also applied in the reverse order that is starting from the most polar solvent and ending at the least polar is known as reverse phase column chromatography.

Liquid chromatography mass spectrometry (LC-MS) is a technique in which separation abilities of liquid chromatography are combined with mass analysis ability of mass spectroscopy. This technique is used where high selectivity and sensitivity is demanded. Usually its application is concerned with detection and identification of chemicals of interest present in the complex mixtures. It has an advantage over the GC-MS that highly polar and less volatile compounds can also be detected. Fourier transform infrared (FTIR) spectroscopy is used to identify chemical bonds in a compound by production of an infrared absorption spectrum. The FTIR spectrum is comparable to the reference catalogues and in this way different bonds can be identified. Nuclear magnetic resonance (NMR) spectroscopy is used to identify the structure of compounds in detail. Nuclear magnetic resonance (NMR) is a property that nuclei have in a magnetic field and applied

electromagnetic pulse. Different NMR experiments are conducted to fully explore the structure of unknown sample these include Proton NMR, carbon NMR, carbon DEPT, HETCORE, HMBC and HSQC.

Ipomoea pes-caprae (L.) R. Br.- bayhops is a pan tropical, trailing vine that routinely colonises on sand dunes. It grows just above the high tide line along coastal beaches, forming large mats that assist in stabilising sands. This is an evergreen perennial with a large, thick rootthat can be 10 ft long and 2 inch in diameter [4]. Traditionally *Ipomoea pes-caprae* is used in different ways like; the juice from the succulent leaves has been used as a first aid to treat jellyfish stings. Some Indians use it in ritual baths to alleviate evil spells. Leaves are used in rheumatism, and as stomachic and tonic. The extract of the leaves have the astringent, diuretic and laxative properties [5]. It has biological activity like anti-oxidant, analgesic and anti-inflammatory, antispasmodic, anti-cancer, antinociceptive, antihistaminic, insulogenic and hypoglycemic. It is also used in inhibition of platelet aggregation, diarrhoea, vomiting, and piles [6]

The present investigation deals with the isolation of compounds by using different chromatographic techniques from the most active extract of aerial parts of *I. pes-caprae* [7] and characterising the structure of isolated compounds by using different spectrometric methods.

Material and Methods

Thin Layer Chromatography (TLC)

TLC have been performed on a pre-coated silica gel TLC plates grade F_{254} (E-Merck, Darmstadt, Germany) to determine the number of compounds present in the given sample. The R_f values of the compounds were calculated using the following formula.

$$R_f = \frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent front}}$$

Column chromatography

Based upon the anti-microbial efficacy; the most active methanol extract of the sample of *I. pes-caprae* purified through Silica gel column chromatography. The concentrated crude metabolites were mixed with methanol–silica gel slurry and loaded into a silica gel 100–200

mesh (E-Merck, Darmstadt, Germany) column, packed in hexane: (the dimension of column was 450 30 mm). The column was eluted with stepwise gradient of chloroform/methanol (100:0; 90:10; 80:20; 70:30; 50:50; 30:70; 10:90, v/v) solvents. Each fraction have been concentrated and checked for its anti-oxidant activity [8].

The separation was done by gradient elution with low polar/high polar (gradient from 100 % low polar/0 % high polar to 0 % low polar/100 % high polar) using the flow rate of 2 ml/min. One hundred tubes of10 ml each were collected and then analysed by TLC. Fractions showing similar spots with same R_f values were pooled and concentrated by a speed-vac under low pressure with evaporating temperature of 40°C. All the fractions were tested for their anti-oxidant activity by DPPH assay. The active compounds were checked for their purity by TLC.

Characterisation of purified molecules

Physical properties: The physical appearance of the purified compounds was determined visually. Solubility was checked with methanol, ethyl acetate, chloroform, hexane, DMSO and water.

UV–vis spectra: The purified compounds were dissolved separately in methanol at 2–10 µg/ml concentrations and their UV–vis spectra were recorded using a UV-vis spectrophotometer (Shimadzu, Japan) between 200 and 800 nm. Methanol was used as blank.

FTIR: IR spectra for the purified compounds were recorded on a Perkin–Elmer 1600 series. FTIR spectrometer using KBr pellets.

NMR: ^{1}H NMR and ^{13}C NMR of the purified compounds were recorded in deuterated DMSO with tetramethylsilane (TMS) as internal standard solution using 400 MHz Bruker machine.

Mass: The ESI-MS was recorded using the Thermo Finnigan LCQ Advantage MAX 6000 ESI mass spectrometer with nano-ESI-API-ion source (Finnigan, MAT, San Jose, CA). Isolated compounds were also subjected to biological activity.

Results and discussion

Isolation of Bio active compound from most active extract of aerial parts of *I.pes caprae*

For isolation of the active principal, air-dried and finely ground aerial parts of *Ipomoea pes- caprae* was extracted sequentially with solvents and most active methanol extract was assayed for anti-microbial efficacy. The crude anti-microbial principals were loaded to silica gel (100–200 mesh) column purification. The aerial crude sample of 5 g was fractioned through low resolution silica gel column chromatography using chloroform/methanol as eluting solvent. About 13 fractions were obtained and single spotted fractions on TLC were pooled together and concentrated for further studies (Fr.1–Fr.9). Among these, seventh fraction [Fr. 7] showed 51. 8% DPPH scavenging activity, while, Fr. 1 to 3 showed no activity while Fr. 4, 5, 6, 8 and 9 showed 18.6 %, 17.4 %, 21 %, 16.9 % and 9.8 % DPPH scavenging activity respectively. Further purification of the seventh fraction (Fr.7) by using high resolution silica gel (230–400 mesh) chromatography yielded about 8 fractions. The fractions having same number of spots with similar R_f values on TLC plate were pooled in sub five fractions Fr.7.1-7.5. Among these, fourth fraction [Fr. 7.4] showed 74.8 % DPPH scavenging activity, while, Fr. 7.1, 7.2, 7.3 and 7.5 showed 20.6 %, 34.5 %, 18.03 % and 39 % DPPH scavenging activity respectively. Fr. 7.4 was further purified using high resolution column chromatography resulted 17 fractions, of which fractions 3–7 showed significant radical scavenging activity (78.8 % - Fr. 7.4.2) and a single spot on TLC. Among these, Fr. 7.4.1, Fr. 7.4.3 and Fr. 7.4.4 exhibited 29 %, 38 % and 42%. Isolatedcompound of aerial was designated as Compound-1 (49 mg). Physical characteristics of fractions of active compounds were given, and they responded to specific tests for flavonoids compounds as recommended by Stahl 1969.The isolated purified compound was subjected to various spectrometric methods viz, IR, NMR and LC-MS to elucidate the structure.

Spectral analysis of Compound

Physical properties: A yellow amorphous powder compound with no UV absorbing band on TLC with the R_f value of 0.45 (9:1, chloroform: methanol) was purified from aerial parts. This anti-oxidant

compound was fully soluble in organic solvents such as methanol, ethanol and ethylacetate. Compound was purified from most active methanol extracts of aerial parts of *Ipomoea pes-caprae* by chromatographic techniques. TLC of compound shows single spot with good R_f values. It reveals the isolated compound was the pure compound without any mixtures. It is presented in figure 1. So, the isolated compound moved for further confirmation studies via UV, IR, ^{1}HNMR, ^{13}CNMR and mass spectrometric methods.

UV–vis spectrum: The spectrum of the purified compound solution in methanol exhibited absorption maximum at 254 nm and 364 nm. Figure 2 shows the UV-Vis absorption spectrum of Quercetin, indicating this compound display maximum absorption in the vicinities of 250 – 260 nm and 370nm.

FT-IR: The IR spectrum of the isolated compound was shown in Figure 3. The IR spectrum of compound shows a broad band at 3413 cm^{-1}corresponding to the phenolic-OH group and carbonyl stretching vibrations in region 1617-1663 cm^{-1}. The peaks at 2871 cm^{-1}corresponds to C-H stretching frequency of alkyl group.The carbonyl peak of the ketone group has shown absorption at longer wavelength. O-C stretching band was seen at 1024 cm^{-1}. Aromatic C=C stretching bands were observed in the range of 1600-1400 cm^{-1}. The compound showed carbonyl stretching vibrations at 1617 cm^{-1}.

^{1}H NMR: The ^{1}H NMR shift of the purified compound showed the aromatic proton appears as multiples in range 6.17 to 7.76. The hydroxyl OH proton appears as broad singlet 3.

^{1}H NMR spectrum of the isolated compound was shown in Figure 4. The ^{1}H NMR spectrum was recorded using DMSO-d6 as the solvent. Spectrum for purified compound was identical and showed the following details. Theresonance peaks for aromatic protons and phenolic protons were found in the region of δ 6.0–8.0 respectively. In addition, most of the peaks were in the low field region except for peak at δ 3.49 for H_2O and peak at δ 2.513 for dimethyl sulfoxide (DMSO). The ^{1}H-NMR spectrum showed 2 peaks at δ 6.17 (1H, d, *J* = 2.0 Hz) and 6.35 ppm (1H, d, *J* = 2.0 Hz) consistent with the meta protons H-6 and H-8. Another peak at 7.64 (1H, d, *J* = 2.2 Hz, H-2'), 7.76 (1H, dd, *J*= 2.0 Hz,8.4 Hz, H-6'), and 6.85 (1H, d, *J* = 8.4 Hz, H-5').

^{13}C NMR: The ^{13}C NMR spectrum showed thearomatic carbon appeared at aromatic region at 93.56, 103.21, 116.93, 121.07, 137.06, 145.67,149.94, 158.96, 161.12, 165.58 ppm. The carbonyl carbon appears at 177.44 ppm. Figure 5 shows the ^{13}C-NMR spectrum indicated the presence of 15 carbon atoms, the signal at δ 177.4 was attributed to a carbonyl carbon placed at C-4, and the other signals were the aromatic carbon appeared at aromatic region at 93.56, 103.21, 116.93, 121.07, 137.06, 145.67, 149.94, 158.96,161.12, 165.58 ppm.

Mass: The ESI–MS spectrum of the purified compound of aerial parts of*Ipomoea pes-caprae* showed a molecular ion peak at *m*/*z* 303.07 (100 % pure, M+) indicating the molecular mass of peak of the purified compound. A full scan electrospray ionisation (ESI) mass spectrum was performed. The mass scanning range (*m*/*z*) was from 200 to 800. The massspectrum of isolated compound has been depicted in figure 6. The spectra of the purified compound exactly match with the structure of the purified compound. It is observed from the spectra that the mass peak of 303.07 corresponding to M+1 is found to be Quercetin. Molecular weight of Quercetin is 302.24 g/mol. The molecular weight was determined using LC-MS. Mass spectrum showed the parent molecular ion at 302.24 g/mol. The molecular formula of the compound was found to be $C_{15}H_{10}O_7$. Thus it is confirmed that the extracted sample was Quercetin.

The molecular weight search between 301 and 305 in the Novel Antibiotic database, PubChem, NIST and SDBS databases revealed that a most similar compound with molecular mass of 302.04 g/mol. Based on the physico–chemical a properties, the purified compound was identified as **3, 5, 7, 3', 4'-Pentahydroxy flavone** and its structure is depicted. Its molecular formula is $C_{15}H_{10}O_7$ and the IUPAC name is 2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4H- chromen-4-one. Its calculated molecular weight is 302.24 g/mol and the exact mass is 303.4 g/mol. **PubChem** *CID*: 5280961, **Quercetin**.

Bioactive properties of isolated compound

EC_{50} Determination

Effective concentration 50 was done by DPPH assay to determine DPPH radical scavenging activity of isolated compound. The result is presented in Figure 8. Compound (Quercetin) was isolated from most active methanol extracts of aerial parts of *I. pes-caprae* shows DPPH activity with an EC_{50} value is found to be 110 µg/ml. The anti-oxidant activity of this compound may be attributed to the presence of –OH and C=O groups, as reported in structurally similar compounds. It confirms the isolated compound could be a good anti-oxidantagent.

Anti-cancer activity [9]

The in-vitro anti-proliferative activities of this compound proved that cancer cell lines inhibited their proliferation significantly with the increase in drug concentration. It was observed that MCF-7 cell line more cytotoxicity effect was observed in drug in 24 hours treatment, it also revealed the increased concentration of drugs shown good toxicity over the cancer cell. Isolated compounds show more potent activity. The results are presented in Figure 9. The results showed that the isolated compound exhibited a potent cytotoxic activity against the MCF-7 cell lines with IC_{50} value 150 g/ml. So, it could be a good anti-cancer agent.

Conclusions

Ipomoea pes-caprae is an effective plant source for traditional drug preparations. One of the purified compound and crude extracts showed good anti-microbial activity [Ethalsha and Malar, 2014]. These findings may be a lead for further ethno-pharmacognostic studies to identify new compounds with therapeutic interest. Many solvent extracts of *Ipomoea pes-caprae* showed biological activity. Furthermore, some solvent extracts were only preliminary studies for their in- vitro activities, so, the advance clinical trial of them deserves to be further investigated. For the first time quercetin has been isolated successfully from the medicinal plant *Ipomoea pes-caprae* under present study. The isolation of the characterised flavanoids

would be useful to prepare plant based pharmaceutical preparation to treat various complications linked withhumandiseases.

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Figure

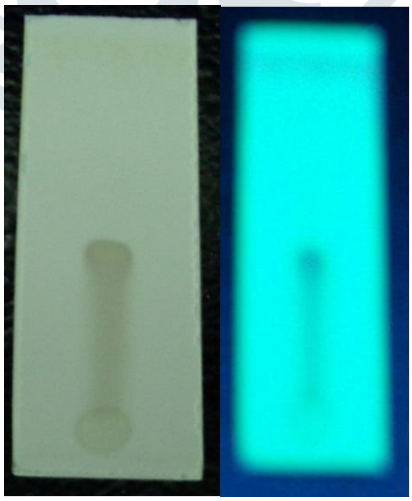


Figure 1 TLC of compound

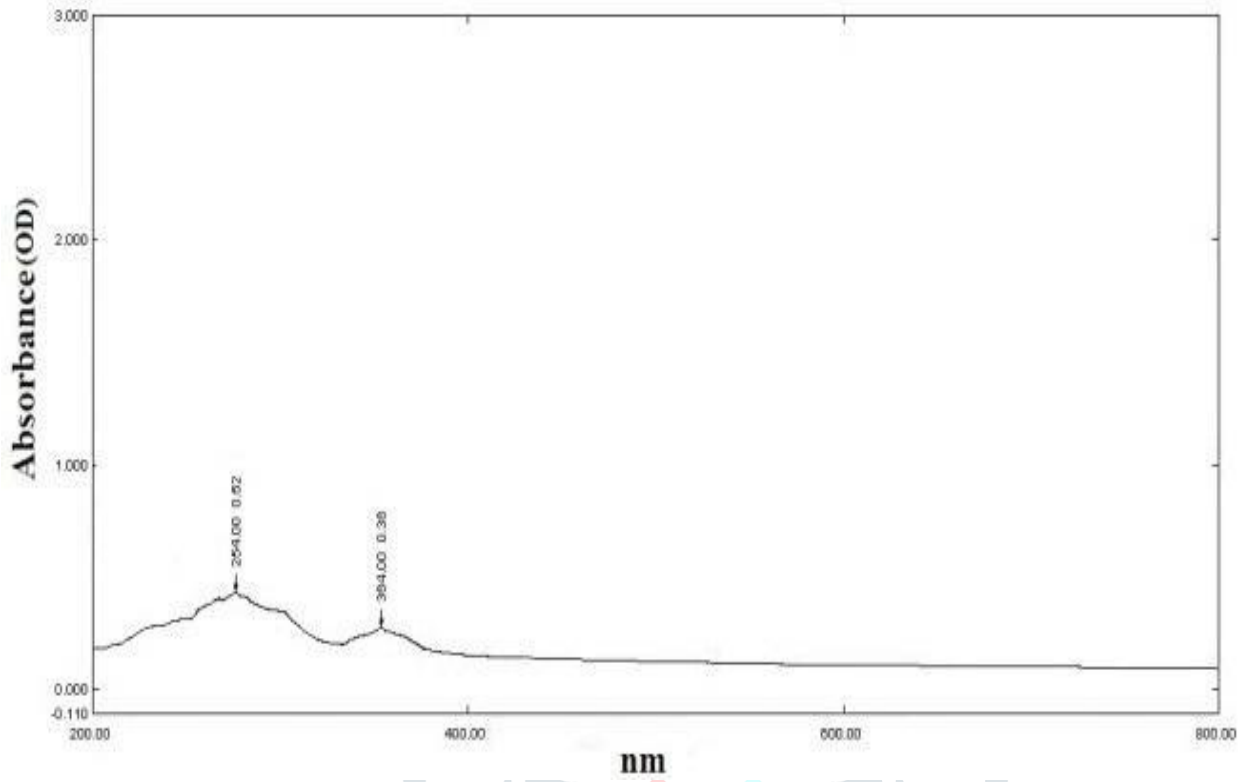


Figure 2 UV-vis Spectrum of compound

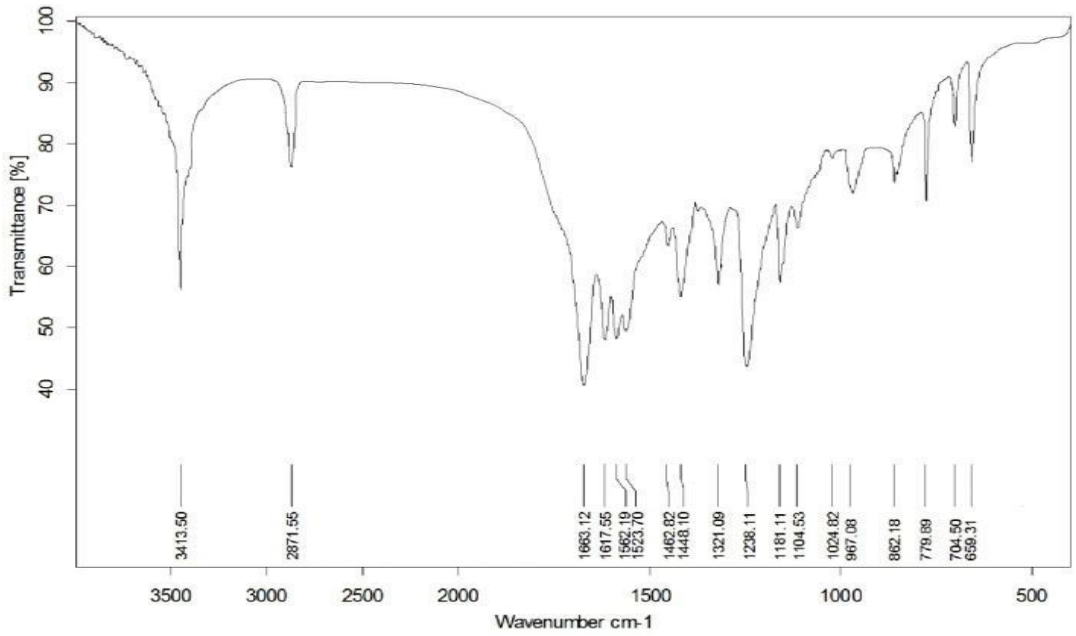


Figure 3 FT-IR spectrum of purified compound

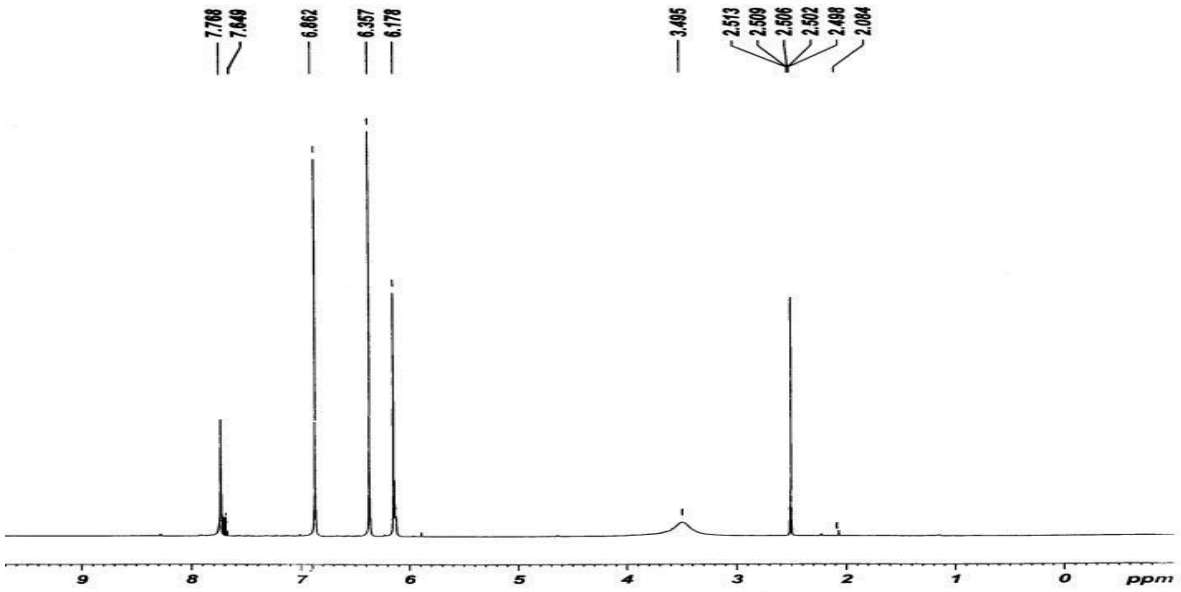


Figure 4 ^{1}H NMR spectrum of purified compound

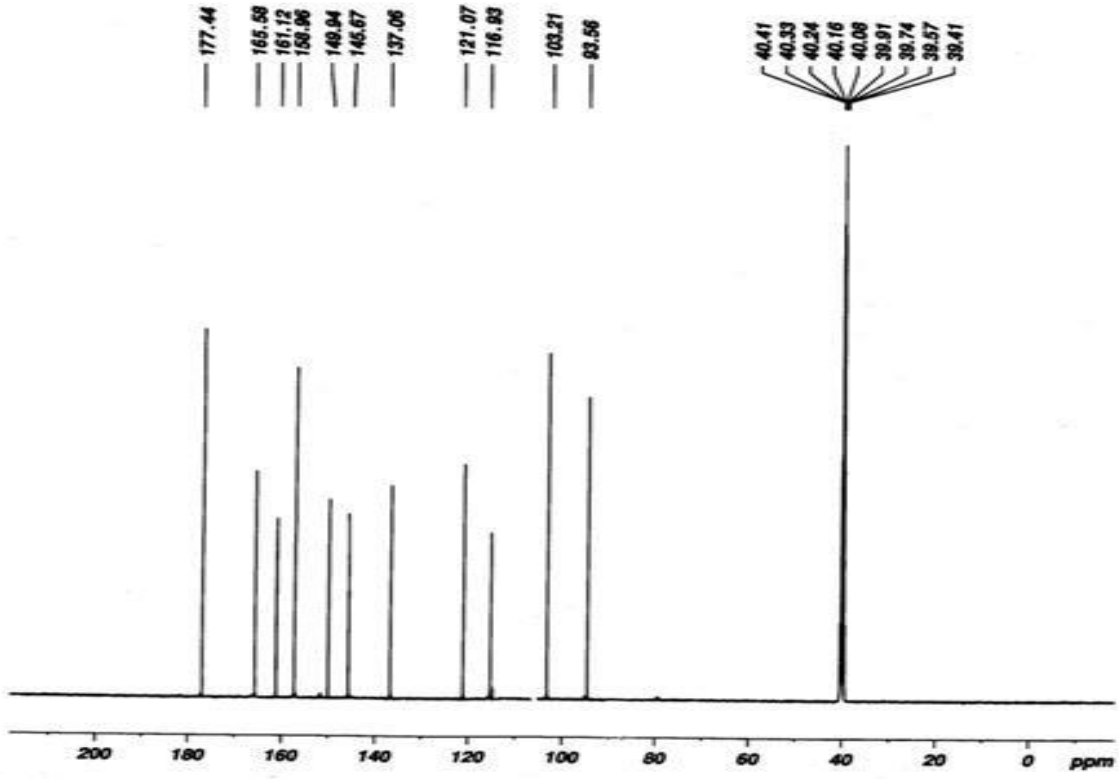


Figure 5 ^{13}C NMR spectrum of purified compound

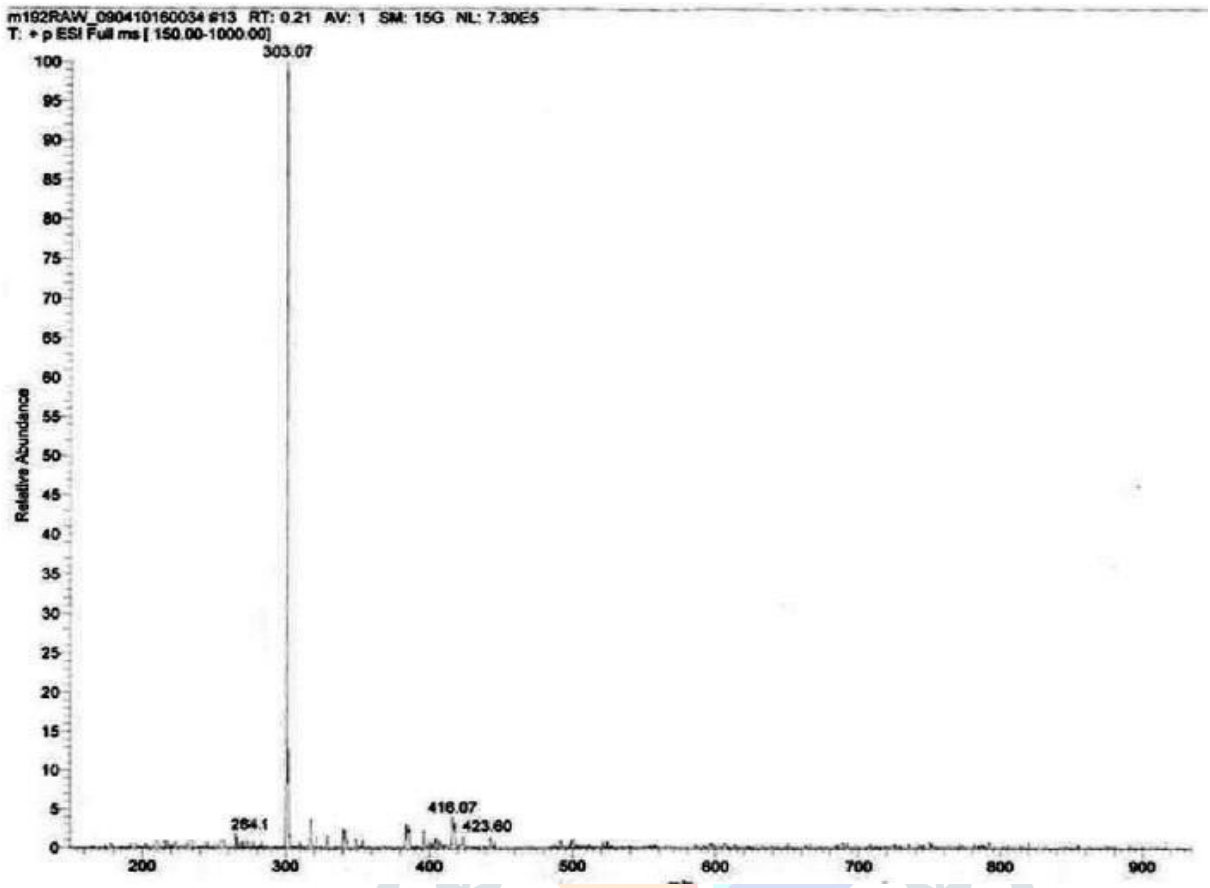


Figure 6 Mass spectrum of purified compound

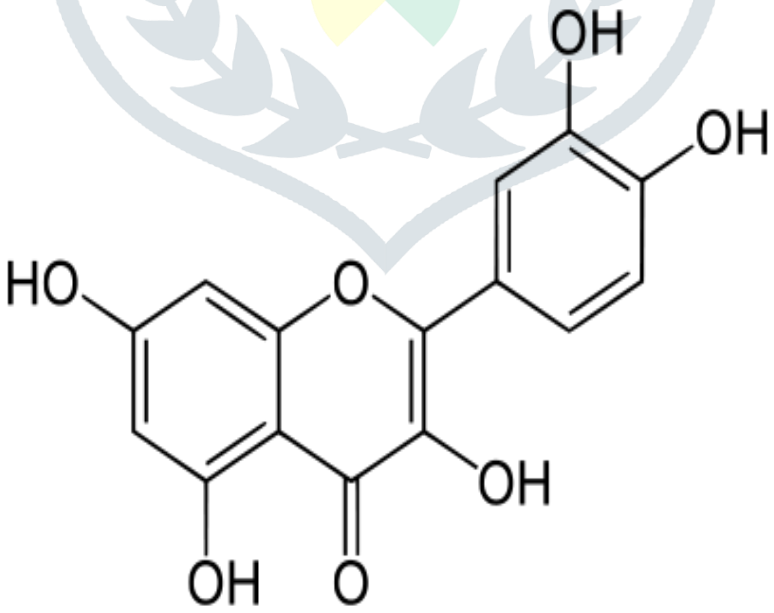


Figure 7 Molecular structure of purified 3, 5, 7, 3', 4'-Penta hydroxy flavone

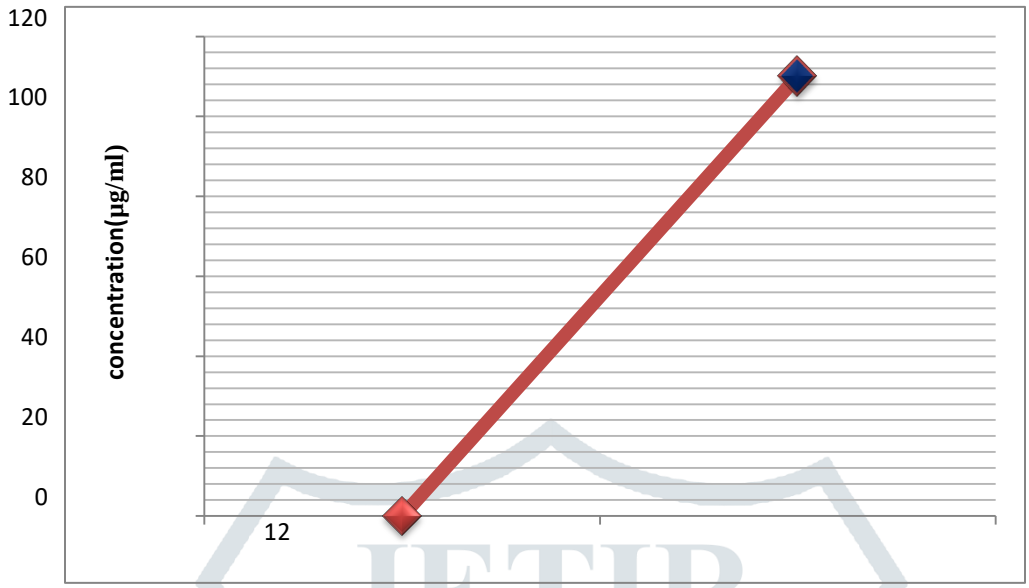
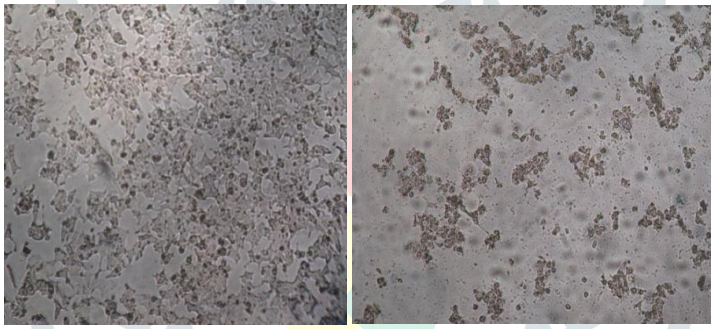


Figure 8 EC_{50} value of Quercetin (CM-1)



Without Compound IC50

Figure 9 MCF-7 Cell line against the isolated compound