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Research Article

Isolation, Characterization and Antioxidant Potential of Flavonoid Fraction from the Leaves of Plant *Ricinuscommunis*

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ABSTRACT

Antioxidants are known as the first line defense against free radical damage and play a vital role in maintaining optimum health and cell viability. Many neurogenerative diseases are associated with the increased exposure of free radicals to the cell. Synthetic antioxidant such as BHT (Butyalatedhydroxytoulene) and BHA (Butyaltedhydroxyanisole) were recently reported as a toxic compound for human cells. Thus there is a need for non-toxic herbal compounds. The plant Ricinuscommunis is widely distributed throughout Asia and has been of medical interest due to their good therapeutic value in folk medicine.The ethyl acetate extract from the leaves of the plant Ricinuscommunis was fractionated into two major fraction:flavonoid and tannin fraction .The flavonoid fraction was further purified by column chromatography and two compounds N12 and N24 were isolated and there purity was further confirmed by spectral characterization and these isolated compounds showed potent antioxidant activity when compared with the standard ascorbic acid in DPPH and FRPA assays. The Isolated compound N24 possess more potent antioxidant activity when compared with N12.

1. INTRODUCTION

Ricinusis a monotypic genus in the Euphorbiaceae family and it is widely distributed throughout Asia including India, Mauritius, China, East Africa, South Africa, America, Mexico, West Indies and Brazil. Ricinus species have been of medical interest due to their good therapeutic value in folk medicine.Ricinus species exhibit a wide range of activities ranging from antidiabetic, antioxidant, anti-inflammatory, antibacterial, antitumor, galactogogue activity[1]. Till now the methanolic extract of the plant showed significant free radical scavenging activity by inhibiting lipid peroxidation initiated by carbon tetrachloride and ferrous sulphate in liver and kidney of wistar rats[2] and the essential oil from the aerial parts was evaluated by different test systems: 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, β -carotene bleaching test and reducing power assay. The essential oil exhibited a potential antioxidant activity[3]. However no data is available regarding antioxidant activity of flavonoids in leaves extracts. In the present study an attempt has been made to isolate the compounds from the flavonoid fraction of plant R*icinuscommunis*and identify the potency of the isolated compound which posse'santioxidant activity.

2. MATERIALS AND METHODS

2.1 Plant material

Leaves of *Ricinuscommunis Linn*.

2.2 Chemicals and Reagents

1, 1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid,were purchased from Sigma Chemical Co. St.Louis, MO, USA. Potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, aluminium chloride, potassium acetate, was purchased from CDH, New Delhi. The other chemicals and solvents used in this study were freshly prepared and were of analytical grade.

2.3 Collection and identification of plant material

Air-dried leaves of *Ricinuscommunis* was collected in the month of August from Local areas

of Ludhiana (Punjab) and were authenticated by Dr. H.B. Singh Director, Department of Raw Material Herbarium & Museum, National Institute of sciences Communication and Information Resources, New Delhi under the voucher specimen number Ref. NISCAIR/RHMD/Consult/-2012-13/2039/47.

2.4 Extraction procedure

Successive solvent extraction scheme was followed for the preparation of different extracts. The leaves of plant *RicinusCommunis*(5000 g) were crushed to coarse powder and extracted with Petroleum ether (40-60ºC) using Soxhlet's extractor for 72 hrs then again the marc obtained was then air-dried and used for further extraction following the same procedure with chloroform and Ethyl acetate. The ethyl acetate extract was filtered through Whatmann filter paper and concentrated with Rota-evaporator apparatus and transferred to a tarred china dish and dried in vacuum desiccatorsuntil a constant weight of the Ethyl acetate extract (52 g) was obtained.*vacuum* in a rotary evaporator and kept in desiccators till further use.

2.5 Fractionation of ethyl acetate extract

The ethyl acetate extract (52 g) was suspended in water and extracted 2-3 times with chloroform to remove the colouring pigments and then to the aqueous layer 10% Nacl was added dropwise in order to precipitate out the tannins then the resulting solution was subjected to centrifugation .The precipitate formed are tannins (2 g) and the above supernant liquid contains flavonoids. Then the supernant liquid was partitioned with ethyl acetate and the ethyl acetate layer was evaporated to dryness to get the crude flavonoids (20 g)[4].

2.6 Isolation & characterization of active compounds present in bioactive total flavonoid fraction of ethyl acetate extract

2.6.1 Colum chromatography

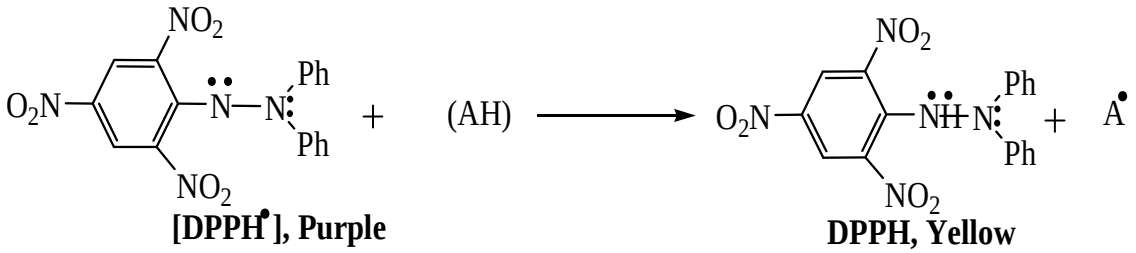
The column was packed in the form of slurry of silica gel (60-120 mesh, Merck,) using n-hexane as a solvent with total flavonoids. Gradient elution was carried out by using n-hexane and Ethyl acetate in different proportion. The elution rate was adjusted to 10-15ml/minute. Different fractions like 1-10, 11-20, 21-50, 51-75, 76-100, 101-150, and 151-200 were eluted. TLC studies were carried out using chloroform: methanol (90:10) using anisaldehydesulphuric acid as spraying agent for all the fractions. Fractions 45-50 (n-hexane-ethylacetate) were then chromatographed on a chromatotron (silica gel) again with n-hexane-ethylacetate-MeOH mixtures and from fractions 40-48 (n-hexane-ethyl acetate) Compound N12 was obtained and the other fractions 153-160 (n-hexane-ethyl acetate) were then chromatographed on a chromatotron (silica gel) with n-hexane-CHCl3-Ethylacetate mixtures and from fractions 24-32 ($CHCl_3$-ethylacetate) compound N24 was obtained. Further the spectral analysis for both the compound has been performed using UV, IR, and Mass and NMR spectroscopy.

2.7 Antioxidant activity

2.7.1 DPPH method

2.7.1.1 Principle

1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) is nitrogen centered free radical that shows strong absorbance at 517 nm. DPPH assay is based on the measurement of the scavenging ability of antioxidants (AH) towards the stable DPPH radical. The free stable radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors; this ability was evaluated by more frequently used discoloration assay, which evaluates the lowering of absorbance at 517 nm produced by the addition of the antioxidant to a DPPH solution in ethanol or methanol. This method is widely used to check the free radical scavenging antioxidants. To evaluate the antioxidant activity of specific compounds or extracts (the antioxidant) were allowed to react with a stable radical $DPPH^{\bullet}$ in a methanol solution. The extent of DPPH radical scavenged was determined by decrease in intensity of violet color in the form of IC_{50} values[5, 6].



$[DPPH^{\bullet}] + A^{\bullet} \longrightarrow DPPH - A$

$$A^{\bullet} + A^{\bullet} \longrightarrow A — A$$

In this Rxn DPPH reacts with the free radical and shows a colourchange from purple to yellow, by making a complex with free radical.In its radical form, DPPH$^{\bullet}$ absorbs at 515 nm but upon reduction, the absorption disappears or decreases. In the DPPH$^{\bullet}$ free radical method, antioxidant efficiency is measured at ambient temperature and thus eliminates the risk of thermal degradation of the molecule tested. Use of DPPH$^{\bullet}$ provides an easy and rapid way to evaluate the antiradical activity of antioxidants. It measures the hydrogen donating ability of antioxidants in a relatively short time as compared to other methods, and spectrophotometric characterization is also possible. The scavenging reaction between DPPH$^{\bullet}$and antioxidant can be expressed as shown above. In the above reaction, A$^{\bullet}$is the free radical of antioxidant which combines with another A$^{\bullet}$ to form A –A[7].

2.7.1.2 Preparation of isolated compound N12, N24, Flavonoid Fraction and ethylacetate extract solution from plant *Ricinuscommunis*

50 mg each of compound N12 and N24were weighed and dissolved in 100 ml of methanol separately to get 500 µg/ml stock solutions. Lower concentrations (5, 10, 15, 20, 25 µg/ml) were prepared by serial dilution with methanol. However, 50 mg of flavonoid fraction and ethyl acetate extract were weighed and dissolved in 100 ml of methanol separately to get 500 µg/ml stock solutions. Lower concentrations (50, 100, 150, 200, 250 µg/ml) were prepared by serial dilution with methanol.

2.7.1.3 Preparation of standard solution

10 mg of ascorbic acid was weighed separately and dissolved in 100 ml of methanol to get 100µg/ml of stock solutions. Lower concentrations of ascorbic acid (5, 10, 15, 20, 25 µg/ml) were prepared by serial dilution with methanol.

2.7.1.4 Method

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts. The 0.1 mM solution of DPPH in methanol (22.2 mg in 1000 ml) was freshly prepared. Different concentrations of the sample were added at an equal volume to methanolic solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 517 nm. Ascorbic acid was used as standard control. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals. Radical scavenging activity was calculated by the following formula:

$$\% \textbf{ Radical Scavenging power} = \frac{\textbf{Absorbance (control} - \textbf{sample)}}{\textbf{Absorbance of control}} \times \mathbf{100}$$

IC_{50}value was determined from the plotted graph of scavenging activity against the different concentrations of samples, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50 %. The measurements were carried out six times and their scavenging effect was calculated based on the percentage of DPPH scavenged[8].

2.7.2 FRPA METHOD [9, 10]

2.7.2.1 Principle: In this method, anti-oxidant compounds form a coloured complex with potassium ferric cyanide, tri-chloroaceticacid and ferric chloride that was measured at 700 nm. Increased absorbance of the reaction mixture indicates the increase in reducing power of the sample.

2.7.2.2 Preparation of test solutions

50 mg of compound N12 and N24 were dissolved in 100ml of methanol separately to obtain test solutions of 500 µg /ml concentration. The solutions were serial dilution with methanol to obtain lower concentrations (5, 10, 15, 20, 25 µg/mL). However, 50 mg of flavonoid fraction and ethyl acetate extract were weighed and dissolved in 100 ml of methanol separately to get 500 µg/ml stock solutions. Lower concentrations (50, 100, 150, 200, 250 µg/ml) were prepared by serial dilution with methanol.

2.7.2.3 Preparation of standard solution

10 mg of ascorbic acid was dissolved in 100 ml of methanol to get 100µg/ml stock solutions.Lower concentrations (5, 10, 15, 20, 25 µg/mL) were prepared by dilution prepared by serial dilution with methanol.

2.7.2.4 Procedure: 1 mL of different concentrations of test solutions (5-25 µg/mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1%

potassium ferricyanide (2.5 mL) and incubated at 50ºC for 30 min. The reaction was stopped by adding trichloroacetic acid (2.5 mL, 10%) to the mixture, which was then centrifuged at 3000rpm for 10 min. The supernatant (2.5mL) was diluted with 2.5 mL of water and shaken with 0.5 mL of freshly prepared 0.1 % ferric chloride and absorbance was measured at 700 nm. Ascorbic acid was taken as reference. All the tests were performed in triplicate and graph was plotted with the average of three determinations[11].

3. RESULTS AND DISCUSSION

3.1 Spectral Characterization data for compound N12

It was obtained as a yellowish powder and the melting point was found to be 317-319^{0}C analysis by mass spectroscopy gave base molecular peak at 302 m/z. The IR, NMR, melting point and the chemical test of suggests that the isolated compound N12 is quercetin.

3.1.1 IR Spectra

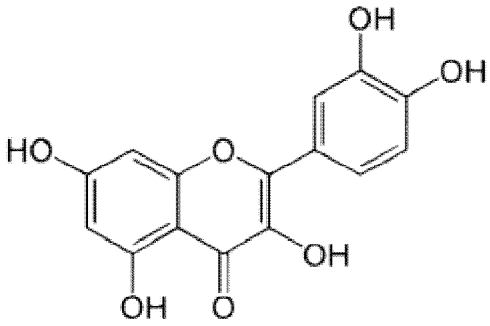
IR (Kbr cm^{-1}): 3410 cm^{-1} (OH stretch) cm^{-1}, 1659.38cm^{-1} (C=O), 1607 cm^{-1} (OH), 1517 cm^{-1} (C=C), 1258 cm^{-1}(C-O), 1161 cm^{-1} (C=C). Fig 1

1H NMR (chemical shift δ in ppm, coupling constant J in Hz) 6.19 (1H, d, J=2 Hz, C6-H), 6.39 (1H, d, J=2 Hz, C8-H),7.73 (1H, d, J=2.2 Hz,C2?-H), 6.89 (1H, d, J=8.5 Hz,C5-H),7.56 (1H, dd, J=8.5,2.2 Hz, C6-H),9.15(1H, s, C4-OH), 9.08 (1H, s, C3-OH), 8.06 (1H, s, C3-OH), 12.39 (1H, s, C5-OH), 10.46 (1H, s, C7-OH). Fig 2

3.1.3 CNMR

13C:(100MHZ,CDCl3):146.41(C2),135.64(C3),175.60(C4),163.72(C5),98.08(C6),160.67(C7), 93.18(C8),156.10(C9)102.95(C10),122.05(C11),115.31(C12),147.37(C13),144.77(C14),114.93(C15),119.87(C16). Fig 3

The structure of N12 is



3.1.2 HNMR

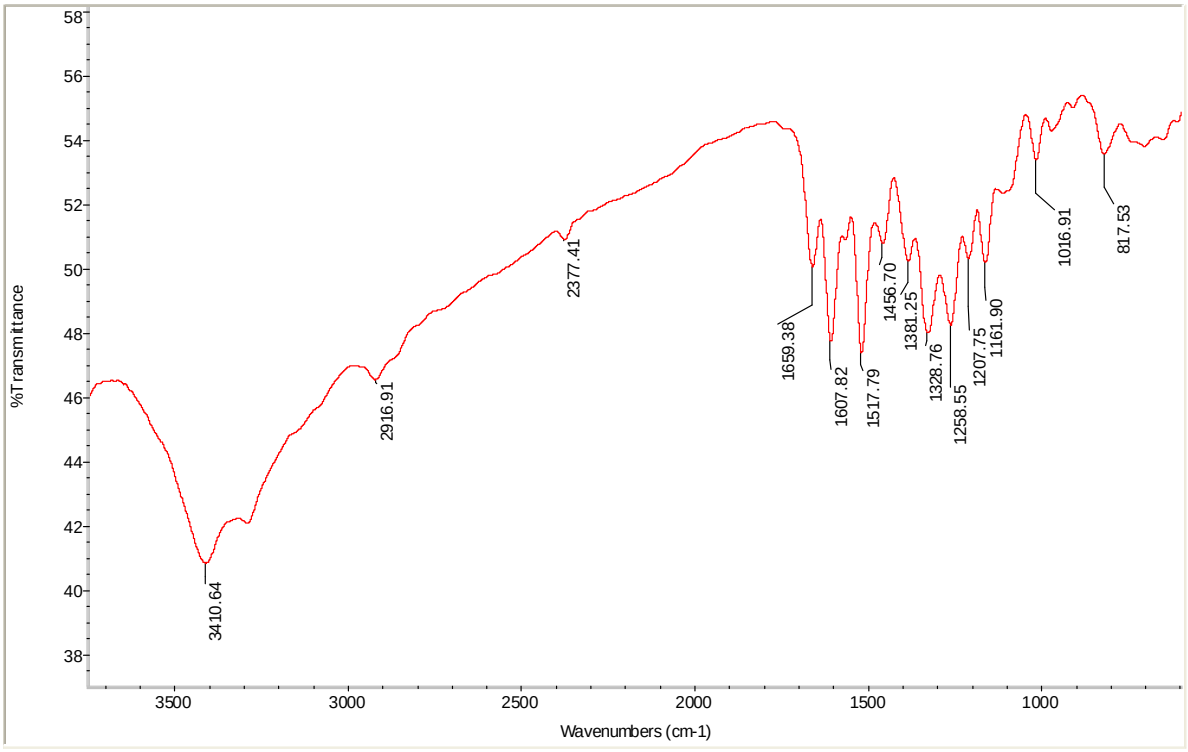


Fig.1: Represents the IR spectra of isolated compound N12

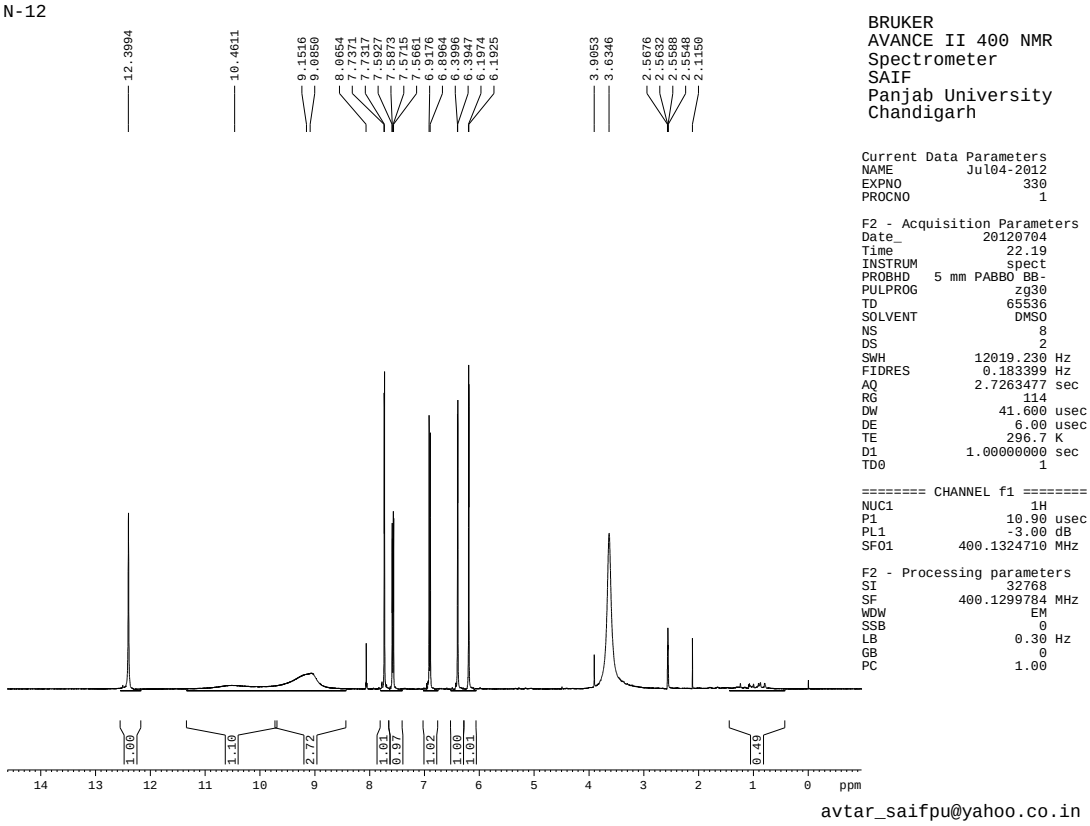


Fig.2: Represents the HNMR spectra of N12

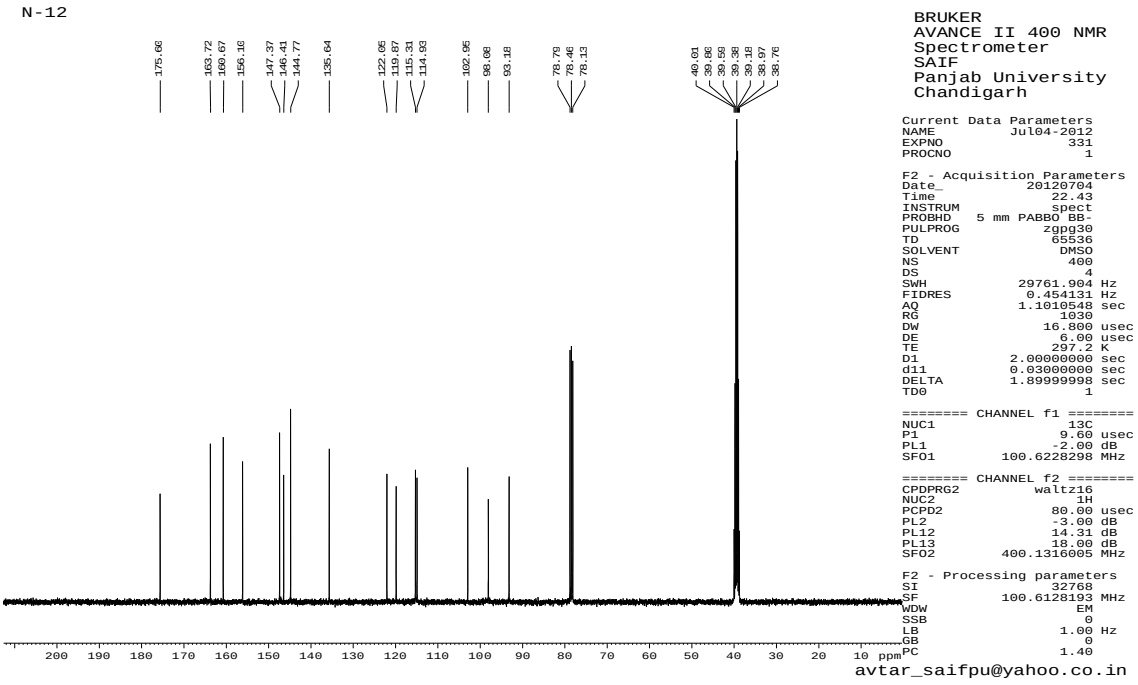


Fig. 3: Represents the 13C NMR of N12

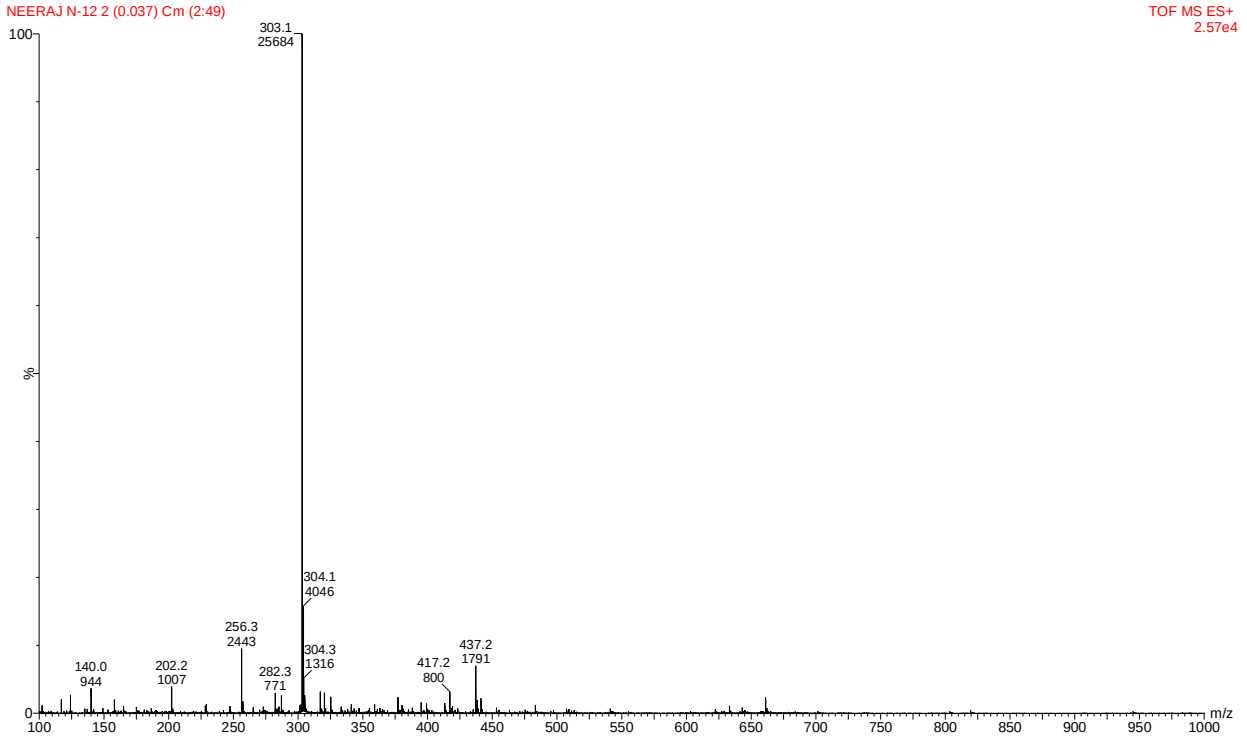


Fig.4: Represents the Mass spectra of N12

3.2 Spectral Characterization data for compound N24

3.2.1 General and Physical Property: It is pale yellow needles which gradually darken on exposure to light. It is tasteless and odorless. It is soluble in pyridine, formamide, methanol and alkaline solution. Melting point of the constituent was founded 189° C.The UV spectrum of rutin in metabolic solution shows two major absorption bands at 359 nm and 257 nm, which indicates the presence of flavonol structure.

3.2.2 IR Values: 3423 cm^{-1} (OH stretch) , 2907 cm^{-1} (CH stretch), 1646 cm^{-1} (C=O stretch), 1598cm^{-1}(C=Cstretch), 1497 cm^{-1} (C=C stretch), 1349 cm^{-1} (C-O-C), 1294 cm^{-1} (C-O-C), 1161 cm^{-1} (C-O-C), 1083 cm^{-1} (C-O-C), 805 cm^{-1} substituted aromatics other fingerprint bands characteristics to rutin are seen following 975cm^{-1}, 753cm^{-1}, and 698cm^{-1}. Fig 5

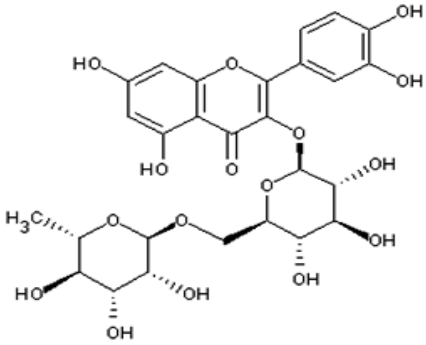
3.2.3 HNMR

^{1}H NMR (chemical shift δ in ppm, coupling constant J in Hz) 6.21(1H, d, J=2, C6-H), 6.40 (1H, d, J=2, C8-H),7.57 (1H, d, J=2.1,C2-H),6.87 (1H, d J=9,C5-H),7.60 (1H, dd, J=9,2.1, C6-H),9.15 (1H, s, C4-OH), 12.52 (1H, s, C5-OH), 10.68 (1H, s, C7-OH), 5.36 (1H, d, J=7.4, H1-G),5.30(1H, d, J=1.9, H1-R), 1.05 (3H, d, J=6.1,CH3-R). Fig 6

3.2.4 13C NMR

^{13}C NMR (chemical shift δ in ppm) 156.81 (C-2),133.40 (C-3), 177.32 (C-4), 156.40 (C-5), 98.66 (C-6), 164.02 (C-7), 93.52 (C-8), 161.16 (C-9), 103.95 (C-10), 121.13 (C-1), 115.08(C-2), 144.49 (C-3), 148.22 (C-4), 116.26 (C-5), 121.60 (C-6),101.74 (C1-G), 75.64 (C2-G), 76.46 (C3-G), 73.92 (C4-G), 78.57 (C5-G), 68.01 (C6-G), 103.95 (C1-R), 70.25 (C2-R), 70.59 (C3-R), 71.59 (C4-R), 69.69(C5-R), 17.52 (C6-R) [R and G represent signals from rhamnose and glucose moieties, respectively. Fig 7

The structure for N24 (Rutin) is-



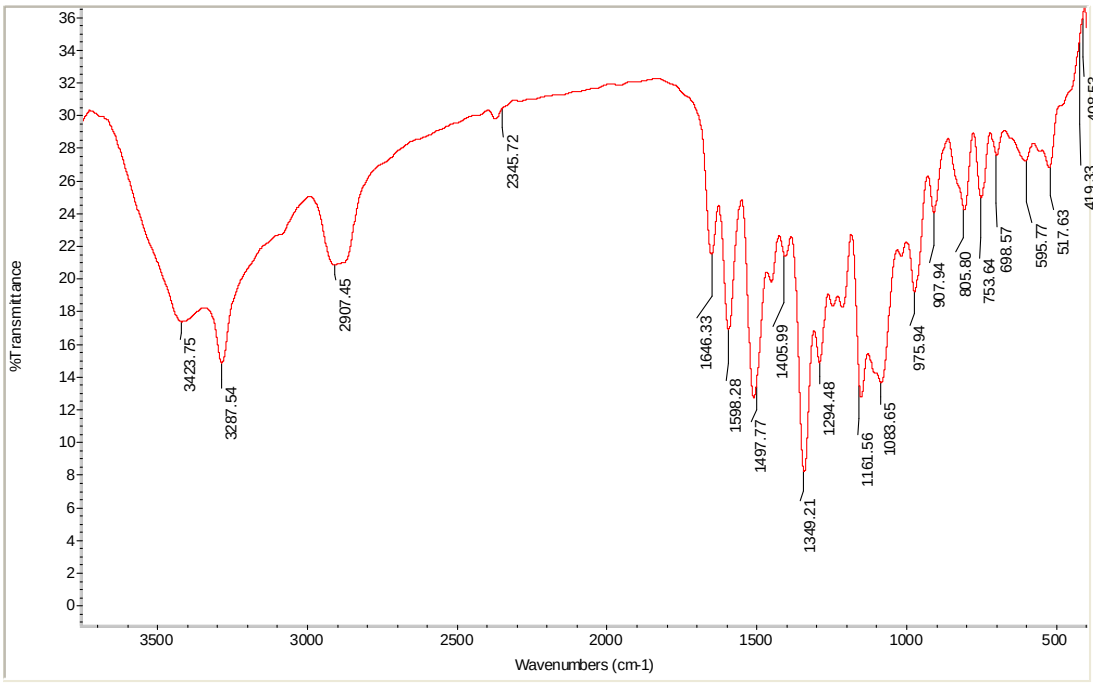


Fig. 5: Represents the IR Spectra of compound N24

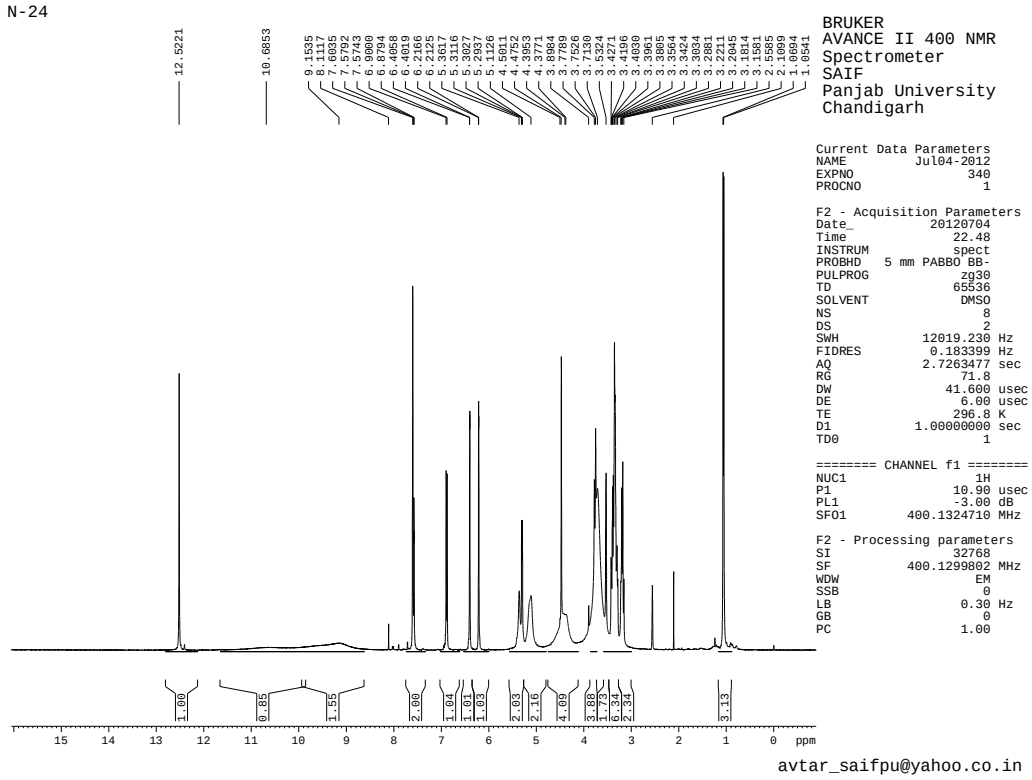


Fig. 6: Represents the HNMR Spectra of N24

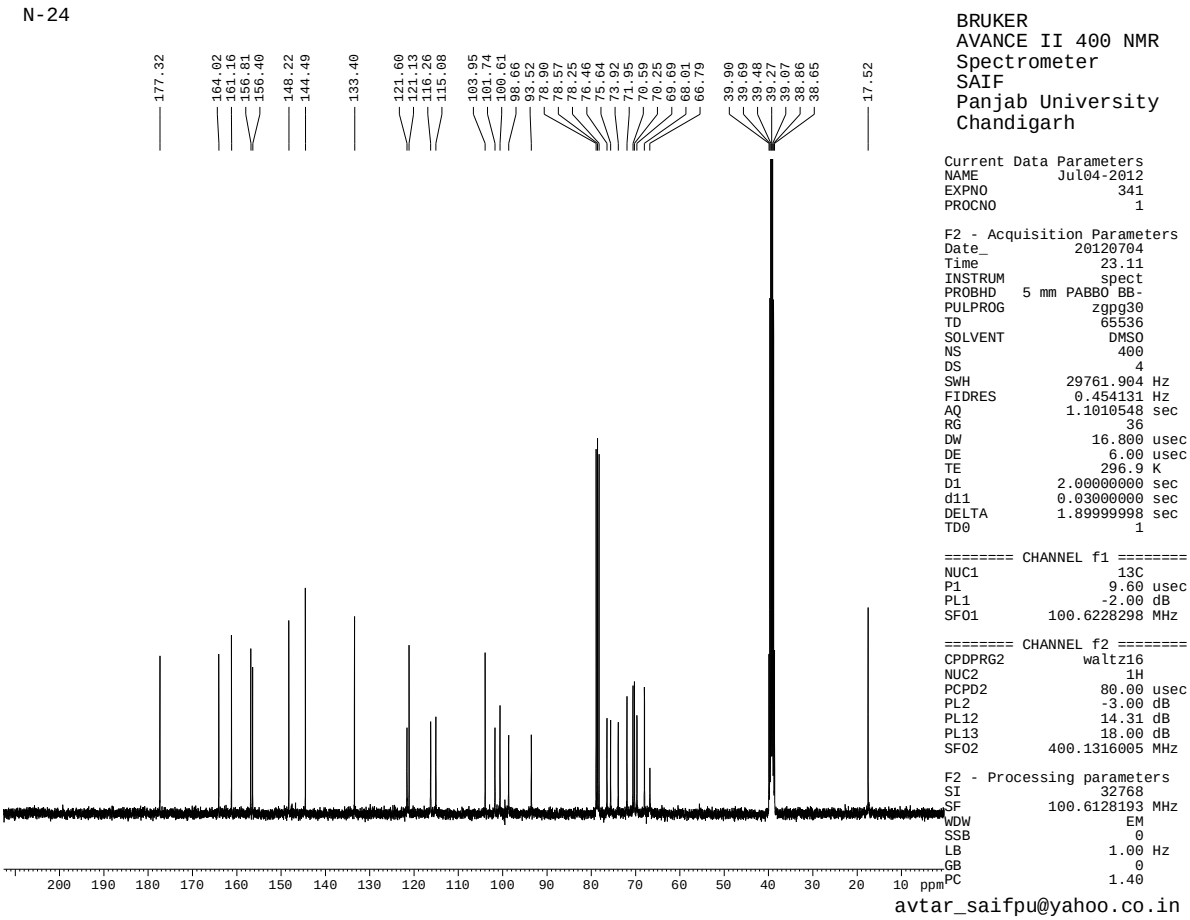


Fig. 7: Represents the 13C NMR Spectra of N24

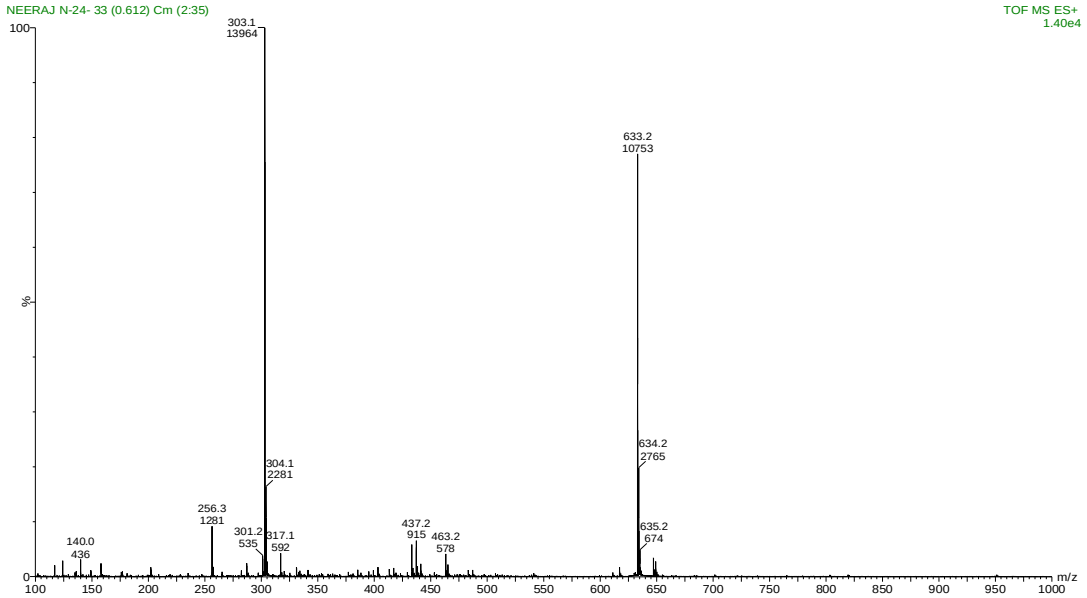


Fig. 8: Represents the Mass Spectra of N24

3.3 DPPH assay

The isolated compound N12 and N24 from flavonoid fraction from ethyl acetate extract of the *Ricinuscommunis* leavesshowed promising free radical scavenging effect of DPPH in a concentration dependent manner up to a concentration of 30 µg/ml as shown in fig 9. Whereas the flavonoid fraction and ethyl acetate extract showed free radical scavenging effect of DPPH in a concentration dependent manner up to a concentration of 250 mg/ml as shown in fig10.

Ascorbic acid was used as areference standard. The reduction of alcoholic DPPH by the flavonoid Compound N12 and N24 was high and the scavenging ability increased with increasing concentration. The results were expressed as dose required to cause 50% inhibition by samples (IC_{50}) and the results are depicted in Table1,2.

The IC_{50} values for the Compound N12 (7.21 ± 0.79), N24 (6.33 ± 0.81), flavonoid fraction (66.75 ± 1.35), ethyl acetate extract (83.46 ± 1.87) as compared with standards ascorbic acid (6.22 ± 0.73) Table 3.

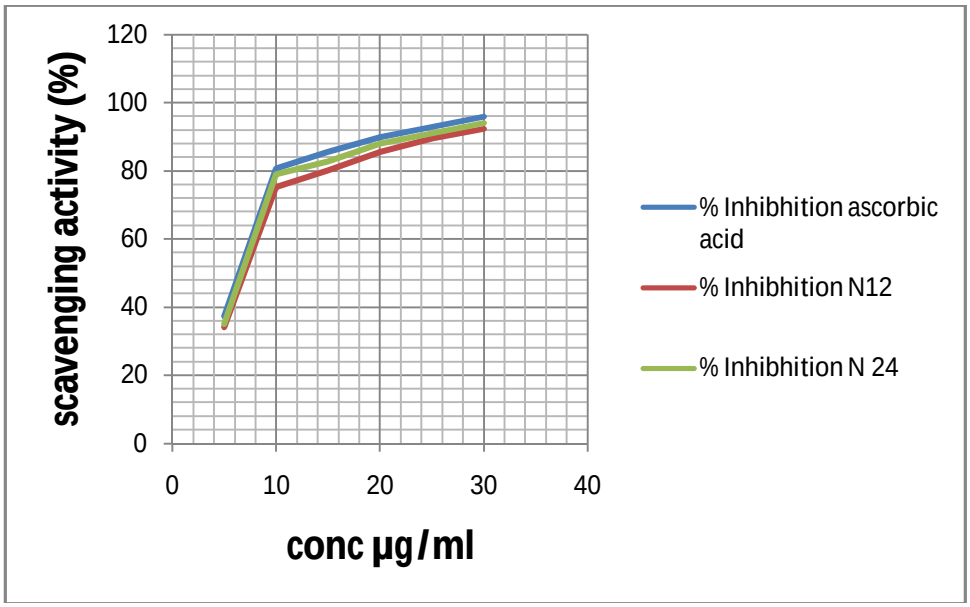


Fig. 9: Free radical scavenging effect of isolated compound N12 and N24 from flavonoid fractionfromethyl acetate extract of plant *Ricinuscommunis*by DPPH

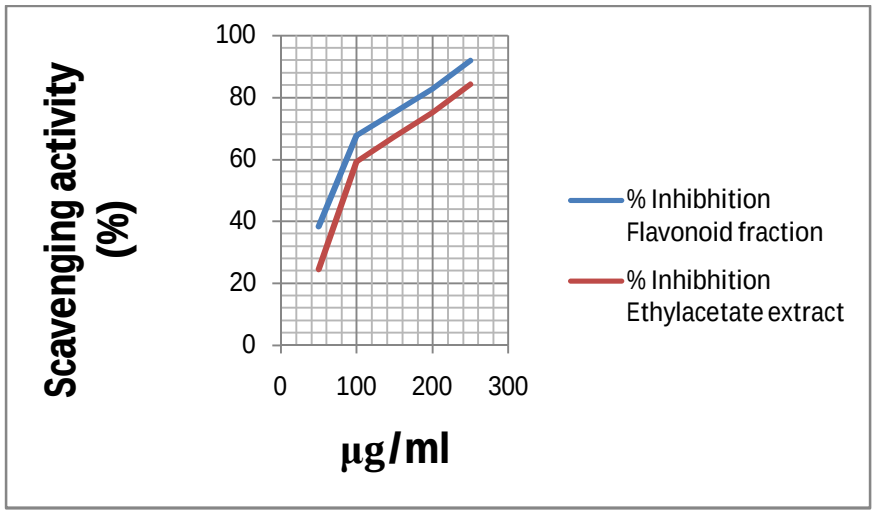


Fig.10: Free radical scavenging effect of flavonoid fraction andethyl acetate extract of plant*Ricinuscommunis* by DPPH

Table 1:Percentage inhibition of standard and the isolate compound N12 and N24 from flavonoid fractionof Ethyl acetate extract of *Ricinuscommunis* at various concentrations by DPPH

S. No.	Conc.(µg/ml)	% inhibition of Ascorbic acid	% inhibition of N12	% inhibition of N24 fraction
1	5	37.37	34.12	35.12
2	10	80.80	75.31	79.15
3	15	85.55	80.14	82.82
4	20	89.89	85.67	88.12
5	25	92.92	89.56	91.13
6	30	95.95	92.43	94.12

Table 2: Percentage inhibition of standard and the Flavonoid fraction and Ethyl acetateextractof *Ricinuscommunis*at various concentrations by DPPH

S. No	Conc.(mg/ml)	% inhibition of Flavonoid fraction	% inhibition of ethyl-acetate extract
1	50	38.37	24.56
2	100	67.80	59.17
3	150	75.12	67.34
4	200	82.89	75.12
5	250	91.92	84.21

Table 3: IC_{50} valuesof Standards and isolated compound N12, N24, flavonoid fraction andethyl acetate extract of plant *Ricinuscommunis*

S. No.	Fraction	IC_{50} Value (µg/ ml)± S.E.M
1.	Ascorbic acid	6.22 ± 0.73
2.	Compound N12	7.21 ± 0.79
3.	Compound N24	6.33 ± 0.81
4.	Flavonoid fraction	66.75 ± 1.35
5.	Ethyl acetate extract	83.46 ± 1.87

3.4 FERRIC REDUCING POWER ASSAY (FRPA)

Antioxidant studies of isolated compound N12 and N24 showed significant difference in their absorbance values as compared with standard ascorbic acid (Fig 11)whereas the flavonoid fraction and ethyl acetate doesnot show any significant difference in their absorbance values (fig 12) as compared with standard ascorbic acid .The results of FRPA reducing power are summarized inTable4 and 5.

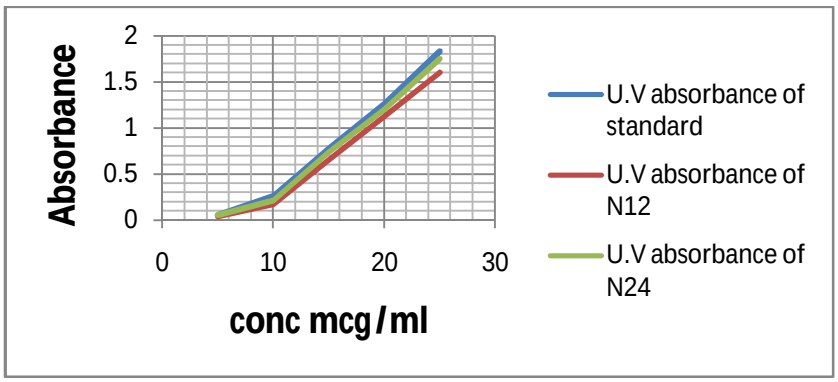


Fig. 11: Ferric Reducing power assay of Ascorbic acid (Standard), Compound N12 and N24 of plant *Ricinuscommunis*

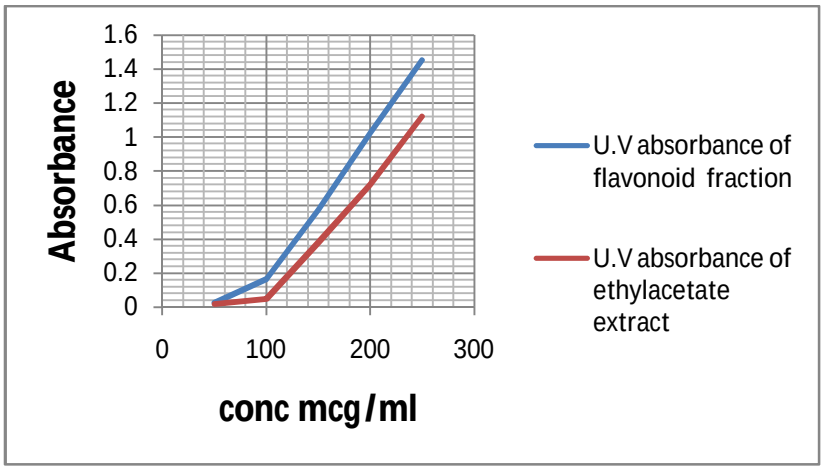


Fig.12 : Ferric Reducing power assay of Flavonoid fraction andethyl-acetate extract of plant*Ricinuscommunis*

Table 4: Absorbance of Ascorbic acid (standard), Compound N12 and N24 from Flavonoidfraction of ethyl acetate extract of plant *Ricinuscommunis* at various concentrationsin ferric reducing power assay

S. No.	Conc.(µg/ml)	Absorbance of standard (Ascorbic acid)	Absorbance of Compound N12	Absorbance of Compound N24
1.	5	0.053	0.04	0.051
2.	10	0.260	0.17	0.21
3.	15	0.773	0.65	0.73
4.	20	1.260	1.12	1.20
5.	25	1.831	1.60	1.75

Table 5 : Absorbance of Flavonoid fraction and Ethylacetate extract of plant *Ricinus communis* at various concentrations in Ferric reducing power assay

S.No.	Conc.(µg/ml)	Absorbance of Flavonoid fraction	Absorbance of Ethyl acetate Extract
1.	50	0.025	0.02
2.	100	0.163	0.05
3.	150	0.573	0.38
4.	200	1.02	0.72
5.	250	1.45	1.12

4. SUMMARY AND CONCLUSION

The fractionation of ethyl acetate leaf extract of the plant *Ricinuscommunis* has increased its antioxidant potency. The flavonoid fraction was found to poses antioxidant activity when compared with the other fraction (Tannin fraction) from the ethyl acetate extract of the plant. In addition, the flavonoid fraction was further screened and two compounds N12 and N24 were isolated by column chromatography andthere purity was further confirmed by spectral characterization (IR, Mass and NMR). The isolated compound N12 and N24 showed potent antioxidant activity when compared with the standard ascorbic acid in DPPH and FRPA assays. The IC_{50} value of ethyl acetate extract (83.46 ± 1.87) and flavonoid fraction (66.75 ± 1.35) was observed. Further, the IC_{50} value of the isolated compound N12 and N24 using DPPH was found to be comparable with standard ascorbic acid IC_{50} (6.22 ±0.73) and N12 (7.21 ± 0.79) and N24(6.33 ± 0.81). However, both the isolated compound N12 and N24 showed significant difference in their absorbance values as compared with standard ascorbic acid in Ferric reducing power assay. Hence, this suggests that isolation has increased the potency of the plant *Ricinuscommunis* andcompound N24posses more potent antioxidant activity when compared with N12.

5. REFERENCES

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