Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2022; 11(2): 222-228
Received: 14-01-2022
Accepted: 18-02-2022

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Isolation, characterization and radical scavenging activities of rutin isolated from leaves of *Annona squamosa*

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Abstract
Flavonoids are universally in photosynthesizing cells and are commonly found in various parts of nuts, fruit, vegetables, seeds, stems and flowers. Flavonoids are principal active constituents have been used to treatment of various human diseases. Rutin (quercetin-3- rhamnosyl glucoside) as the main flavonoid reported in leaves of *Annona squamosa*. It is a multipurpose tree with eatable fruits & is a source one of the cosmetic products. *Annona squamosa* Linn is used in the treatment of diabetes, hepatotoxicity and in cancer cell line, It has been recognized that flavonoids display anticancer, antiviral, anti-inflammatory, and heart disease protective activities. The present study was carried out to isolation, characterization and antioxidant activity of rutin from leaves of *Annona Squamosa* from 80% ethanolic extract. The chemical compound isolated was analyzed by TLC, HPLC & IR. Several studies had been done for the isolation of rutin by different chromatographic method. In this study rutin was isolated from leaves of *Annona squamosa* by precipitation and fractional solubilization without the use of any chromatographic technique. The radical scavenging activities were carried out by various *In-vitro and In-vivo* methods. Rutin showed the most potent DPPH scavenging activity (IC_{50} = 3.17 µg/ml) slightly higher than ascorbic acid (IC_{50}=4.92 µg/ml). The IC_{50} value of lipid peroxidation assay for isolated rutin and BHT were 18.27 µg/ml and 46.79 µg/ml respectively.

Keywords: *A.squamosa*, rutin, HPLC, IR, Butylhydroxytoluene (BHT)

Introduction
In plants, Flavonoids are widely distributed as a naturally occurring polyphenols. Being both dietary and biologically active compounds, flavonoids have attracted much attention of investigators as potent species capable of affecting various biological processes in living organisms. They are able to modulate various enzymes present in biological system. (Alexander *et al*., 2007) [1]. The family (Annonaceae), is a large family which comprising about 130 genera over 2000 species; the most important genera having a largest number of species are Annona, with 120 species, from genera (Yoganarasiman *et al*., 2000) [19], *Annona squamosa* also known as custard apple which is cultivated throughout India, mainly dietary fruit. *Annona squamosa syn*. Arabic (gishta); Bengali (ata);German (Rahm Annone, Rahmapfel, Zimtapfel, Süßsack); Hindi (sitaphal, ata, sharifa); Lao (Sino-Tibetan) (khièb); Malay (nona sri kaya,sri kaya,buah nona); Mandarin (fan-li-chi); Portuguese (atta,fructa do conde); Sanskrit (sitaphal); Spanish (candongo, chirimoya, fructo do conde, anón, anona blanca, pinha, saramuya, anona).The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problem, worm infection, constipation, hemorrhage, antibacterial infection, dysuria, fever, and ulcer. It also has anti fertility, anti tumor and abortifacient properties (Soni *et al*., 2012) [15]. *Annona* saponins, tannins, carbohydrates, flavonoids, proteins, phenolic compounds, phytosterols, amino acids. The various chemical constituents isolated from *Annona squamosa* including aporphine, coryeline, anonaine, isocorydine, glaucine. Leaves contains Stigmasterol, 4-(2-nitro-ethyl 1)-1-6-((6-o-β- Dxylopyranosy1- β -D-glucopyranosyl)- oxy)benzene, Borneol, Camphene, Camphor, car-3- ene, Anonaine, β-Sitosterol, Benzyltetrahydroisoquinoline, Carvone, β- Caryphyllene, Farnesol, Geraniol, 16-Hetriacontanone, Hexacontanol, Higemamine, Isocorydine, Limonine, Linalool acetate, Menthone, Methyl anthranilate, Methylsalicylate, Methylheptenone, p-(hydroxybenzyl)-6,7-(2- hydroxy,4-hydro) isoquinoline, n-Octacosanol, a- Pinene, b-Pinene, Eugenol Rutin, Thymol and n- Triacontanol. Alkaloids, proteins & amino acids are vanished in the leaf extract (Patel *et al*., 2008) [12]. Among these constituents, Rutin most widely used natural products present in leaves of *A.squamosa*. Rutin is the rhamnoglucoside of the flavonoid quercetin, and found in many plants and used for treatment of various diseases related to the vascular

(Toker *et al*., 1998). It is quercetin-3-rutinoside or 3,3',4', 5,7-pentahydroxy flavones-3-rutinoside, and has a chemical formula $C_{27}H_{30}O_{16}$.Rutin by acting as antioxidants exhibited several beneficial effects, such as anti-inflammatory, anti-allergic, antiviral as well as an anticancer activity. They have also been suggested to play a protective role in liver diseases, cataracts, and cardiovascular diseases (Parabathina *et al*., 2010) [11].

Pharmacological action of Rutin

Rutin showed the most potent intrinsic activity, and produced the strongest inotropic responses among the different flavonoids. It exhibits antiulcer activity. Rutin and venorutin showed regenerative and hepato-protective effects in experimental cirrhosis. Quercetin and rutin have been used as effective constituents of several pharmaceuticals used for treatment of capillary fragility and phlebosclerosis. Flavonoids and esters of phenolic acids were investigated for their antibacterial, antifungal and antiviral activities. Rutin active against *Bacillus anthracis*, *Para influenza virus* & *Influenza virus* (Narayana & Krishna, 2001) [6].

Fig 1: *Annona squamosa*

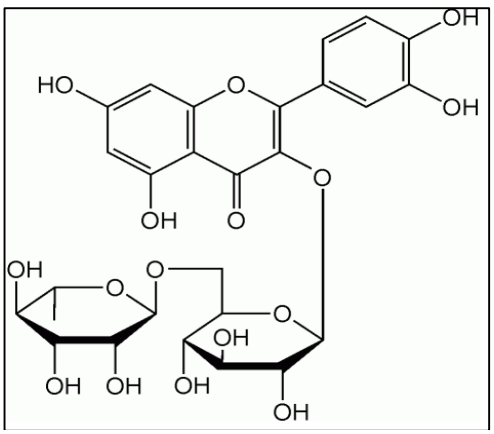

Fig 2: Structure of Rutin

Free radicals are atoms or groups of atoms that have at least one unpaired electron, which make them highly unstable and reactive. Living organisms accumulate free radicals through both normal metabolic processes and exogenous sources. Although radicals have beneficial effect during energy production and as antibacterial, excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death (Asres *et al*., 2006) [3]. Antioxidant compounds in food play an important role as a health protecting factor. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydro- peroxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Jacobs & Joanne, 2000) [4]. Rutin act as an inhibitor of iron-ion-dependent lipid peroxidation system because it forms chelation of iron ions. The chelating mechanism of rutin blockage was more potent than quarcetin (Igor *et al*., 2002) [5].

Material and Method

Plant material collection

The leaves were collected from botanical garden L.N.C.P.Bhopal (M.P.) and authenticated (Voucher No. 004/bot/LNCP/10 & 004/bot/LNCP/11). They were dried in shade for several days at room temperature and then grinded as powder.The standard rutin was obtained as a gift sample from Jamia hamdard, Delhi.

Extraction and isolation

Twenty grams of the powdered leaf was extracted by Soxhlet apparatus with 250 ml of 80% ethanol till exhaustion. The extract was filtered and concentrated by evaporation under vacuum to about 10ml then mixed with 25ml distilled water, and extracted with petroleum ether (50ml x3), then with chloroform (50 ml x 3). After extraction, the aqueous layer was collected and left to stand in a cold place for 72 hours; a yellow precipitate separated out of the solution. The precipitate was filtered and washed with a mixture of chloroform: ethyl acetate: ethanol (50:25:25). The un-dissolved part of the precipitate was dissolved in hot methanol and filtered; the filtrate was evaporated to dryness to give 110 mg yellow powder (rutin).

General and Physical Properties

Appearance, color, solubility and melting point of the isolated constituents will be determined.

Chemical identification of Constituents

Little amount of the isolated constituent are dissolve in methanol and perform the following test (Kokate, 2003) [7].

Shinoda Test (Magnesium Hydrochloride reduction test)

To the methanolic solution of rutin add few drops of Con. HCl and 0.5 gm magnesium turning.

Zinc Hydrochloride Reduction Test

To the methanolic solution of rutin add a mixture of Zinc dust and conc. Hydrochloric acid. Heat the solution and observe the color.

Alkaline Reagent Test

To the methanolic solution of rutin add few drops of sodium hydroxide solution and observe the colour formation.

TLC and paper chromatography

Isolated rutin was compared with standard rutin using TLC method; a pre-coated aluminum sheet with silica gel GF_{254}

with the following mobile phases: ethyl acetate: butanone: formic acid: water (50:30:10:10), ethyl acetate: formic acid: acetic acid: water (100: 11: 11:27). In paper chromatography, Whatmann No.1 filter paper was used as a stationary phase and mobile phases of: Acetic acid: water (15:85) and isopropyl alcohol: water (60:40) (Lederer, 1957) [8].

Spectrophotometric analysis

The isolated rutin was dissolved in methanol and its UV absorption peaks were determined and compared with standard rutin. Spectrophotometric analysis was carried out in Shimadzu 1700 UV spectrophotometer.

HPLC analysis

The HPLC analysis was carried out using a LC-100, Cyberlab TM, Salo Torrace, Millburry, MAO 1527, USA with LC-UV-100 UV detector. A CAPCELL (C-18) HPLC-packed column (4.6 mm I.D.X 250 mm), type MG 5 µm, number AKAD/05245 was used for the chromatographic separations. The mobile phase contain methanol: water (70:30). The flow rate was 0 .5 mL/min, and a column temperature of 25°C. The injection volume was 25µl, and UV detection was achieved at 280 nm.

Antioxidant activity

DPPH Radical Scavenging Activity

Ascorbic acid and rutin were weighed (25 mg each) and dissolved in 250 ml of methanol to get 500µg/ml stock solutions separately. Lower concentrations of ascorbic acid and rutin (2, 4, 6, 8, 10 µg/ml and 2, 4, 6, 8, 10 µg/ml respectively) were prepared by serially diluting stock solutions. The stable DPPH radical 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 1000ml) was freshly prepared. Different concentrations of rutin were added at an equal volume (2ml) to methanolic solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 517 nm. 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.

% Radical Scavenging Activity = $A_{control}$- A_{sample} /$A_{control}$ * 100
Where, $A_{control}$ = Absorbance of control A_{sample} = Absorbance of sample

The inhibition curve was plotted for duplicate experiments and represented as % of mean

Inhibition ± standard deviation.

Ferric Reducing Antioxidant Power (FRAP) Assay

3mg of isolated rutin was dissolved in 30 ml of methanol to get 100 µg/ml stock solutions separately. Lower concentrations of rutin (20, 40, 60, 80, 100µg/ml) were prepared by serially diluting stock solution. Ascorbic acid was weighed (5 mg) and dissolved in 50 ml of methanol to get 500 µg/ml stock solutions Lower concentrations of ascorbic acid (20, 40, 60, 80, 100 µg/ml) were prepared by serially diluting stock solution. Various concentrations of sample and standard solutions (1ml each), 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were mixed separately and allowed to incubate at 50 º C for 30 min and 2.5 ml of 10% TCA was added to the mixtures and centrifuged for 10 min at 3000 rpm. About 2.5 ml of the supernatant was diluted with 2.5 ml water and is shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. All tests were performed in triplicate and the graph was plotted with the average of the three determinations (Poonia *et al*., 2011) [13].

Thin Layer chromatography analysis of antioxidant constituents

TLC analysis of antioxidant constituents the antioxidant constituents were analyzed using thin layer chromatography (TLC) followed by DPPH (2, 2- Diphenyl-1-picrylhydrazyl) technique (Moore J., Yin J. and Yu L 2006). About 100 µg of sample was applied on TLC plate (Merck, 10x 10 cm^2). The plate was developed in 10% chloroform in methanol and Methanol: Chloroform: Hexane (7:2:1) . Then developed plate was air dried and observed under visible and UV light (240 and 300 nm). The R_f values was calculated. The intensity of spot was observed after treatment with 0.05% of DPPH solution in methanol. The active antioxidant constituents were detected as yellowish white spots produced by bleaching of DPPH by resolved bands on the TLC plates. Ascorbic acid was used as positive control.

***In-Vitro* Lipid Peroxidation Method**

Lower concentrations (10, 20, 30, 40, 50 µg/ml) of isolated rutin sample were prepared by serially diluting stock solutions. Similarly BHT stock solution (500 µg/ml) was prepared (50 mg BHT in 500 ml methanol). Lower concentrations (25, 50, 100, 200, 400 µg/ml) of BHT were prepared by serially diluting stock dilution. Normal male rats (250 g) were used for the preparation of liver homogenate. The perfused liver was isolated, and 10% (w/v) homogenate was prepared with homogenizer at 0-4°C with 0.15M KCl. The homogenate was centrifuged at 8,000 rpm for 15 min, and clear cell-free supernatant was used for the study with *in vitro* lipid peroxidation assay. Different concentrations sample were added in test tubes and 1ml of 0.15M KCl and 0.5ml of rat liver homogenates were added. Peroxidation was initiated by adding 100 µl of 0.2 mM ferric chloride. After incubation at 37 °C for 30 min, the reaction was stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 15% TCA, 0.38% TBA, and 0.5% BHT. The reaction mixtures were heated at 80 °C for 60 min. The samples were cooled and centrifuged, and the absorbance of the supernatants was measured at 532 nm. The percentage inhibition of lipid peroxidation is calculated by the formula:

Inhibition of lipid peroxidation (%) =1– (sample OD/blank OD) × 100

IC_{50} value was determined from the plotted graph of scavenging activity against the different concentrations of sample, which is defined as the total antioxidant necessary to inhibit lipid peroxidation by 50 % (Arora & Singh, 2009) [2].

Results and Discussion

Rutin is a flavonol glycoside comprised of Quercetin and rutinose. Rutin was isolated from leaves of *Annona squamosa* by precipitation and fractional solubilization without the use of any chromatographic technique. Isolated rutin showed a melting point at 196°C which is identical with that reported for rutin (Merck index, 2006) [17].

The general physical properties observed in isolated rutin were tabulated in table 1.Chemical identification of rutin showed presence of flavonol moiety (table 2).Qualitative

analysis of isolated rutin were carried out by TLC and paper chromatography and the results revealed that R_f value were more or less similar to standard rutin (table 3). The UV spectrum of rutin in methanolic solution shows three major absorption bands at 236, 257 & 358.5nm, which indicates the presence of flavonol structure (fig. 3) IR analysis, were tabulated in table 5. IR Spectrum comparison of Rutin (standard) with sample showed in fig.4. The construction of chromatographic fingerprints plays an important role in the quality control of complex herbal medicines. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines. HPLC separations of isolated sample with reference to standard were performed on a Cyber Lab C-18 column (250 x 4.0 mm, 5µ). A chromatographic fingerprint plays an important role in the quality control of herbal medicines. HPLC separations of isolated sample with reference to standard were performed on a Cyber Lab C-18 column (250 x 4.0 mm, 5µ). Thus chromatographic fingerprint should be considered as a tool to evaluate the quality of herbal medicines. (Soni *et al*., 2011) [16]. The HPLC chromatogram of standard and isolated rutin showed 2.55 & 2.375 respectively (fig 5 & 6). The HPLC analysis was tabulated in table 4. DPPH is stable nitrogen centered free radical which can be effectively scavenged by antioxidants and shows strong absorbance at 517 nm. The change in absorbance of DPPH radical caused by the sample was due to the reaction between the antioxidant molecules and the sample, which resulted in the scavenging of the radical by hydrogen donation. It was visually noticeable as a discoloration from purple to yellow. Extent of DPPH radical scavenged was determined by the decrease in intensity of violet colour in the form of IC_{50} values. The antioxidant potential rutin was tabulated in table 6 & fig. 9. Ferric Reducing Antioxidant Power Assay The reductive capability of the isolated rutin was measured with reference to ascorbic acid. For the measurement of the reductive ability, we investigated the ferric (Fe) - ferrous (Fe^{2+}) transformation in the presence of the rutin was investigated. In this method, the rutin form a colored complex with potassium ferricyanide, trichloroacetic acid and ferric chloride that was measured at 700 nm. The reducing capacity of the rutin may serve as the antioxidant activity, the reducing power of the rutin increased with increasing the concentration. Increase in absorbance of the reaction mixture indicates the increase in the reducing power of the sample. The antioxidant activity can be attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and free radical scavenging (Roshan *et al*., 2010) [14]. The result of Ferric reducing power assay was showed in fig. 7 & 8. The absorbance of the sample and standard were tabulated in table 6. The TLC analysis of antioxidant constituent revealed the intense yellow spot after treated with DPPH (table 8). In-Vitro Lipid Peroxidation Method, the decrease in the MDA levels in the presence of increased concentration of sample (rutin) indicates their role as antioxidants. TBARS assay was used to determine the anti lipid peroxidation properties of the rutin isolated from leaves of *A.squamosa*. Thus, rutin inhibit the initiation of lipid peroxidation by scavenging the free radicals that form alkyl peroxyl and alkoxyl radicals or can donate hydrogen atom to alkyl peroxyl and alkoxyl radicals and thus stop chain propagation (Mruthunjaya & Hukkeri, 2010 [10]. These observations also support our finding that rutin have more antioxidant potential in comparison to with standard antioxidants. The result was tabulated in table 9.

Conclusion

The results of this study, it is clearly indicate that isolated rutin from leaves of *Annona squamosa* have high radical scavenging activity against various antioxidant systems *in vitro* & *in vivo*. These assays have important appositeness for the food and pharmaceutical industry. Moreover rutin can be used as an easily attainable source of natural antioxidants and as a possible food supplement.

Fig 2: Isolated from leaves of *A.squamosa*

Table 1: General Physical Properties of isolated Rutin

S. No.	Physical properties	Inference
1.	Appearance	Powder
2.	Color	Pale Yellow
3.	Solubility	Pyridine, methanol
4.	Melting point	196^0C

Table 2: Chemical identification of Constituents

S. No.	Test	Observation	Inference
1.	Shinoda Test	Green to blue color appears after few minutes	Flavonol are present
2.	Zinc Hydrochloride reduction test	It gives red color after few minutes	Flavonol are present
3.	Alkaline reagent test	Formation of an intense yellow color, which turns to Colorless on addition of few drops of dil. Acid.	Flavonol are present

Table 3: TLC and Paper chromatography Analysis

TLC			Paper Chromatography		
Solvent System	R_f(standard)	R_f(isolated)	Solvent System	R_f(standard)	R_f(isolated)
Ethyl acetate: butanone: formic acid: water ((50:30:10:10)	0.29	0.30	Acetic acid: water (15:85	0.57	0.61
Ethyl acetate: formic acid: acetic acid:water(100: 11: 11:27)	0.38	0.34	Isopropyl alcohol: water(60:40)	0.79	0.81

Table 4: HPLC Analysis of Rutin

S. No	Sample	Height	Area	Conc.	RT	Inference
1.	Standard Rutin	59566	1014993	97.4524	2.55	Rutin
2.	Isolated Rutin	14631	152866	94.4032	2.375	Rutin

Table 5: IR Analysis of rutin

IR Values	Inference
3330	OH (bonded)
2920	CH stretch
1660	C=O
1620	C=C
1600	Aromatic structure
1510	C=C aromatic
1360	C-O-C
1295	C-O-C
1200	C-O-C
1060	C-O-C
810	Substituted aromatics
Other fingerprint bands characteristic to rutin are seen following 970, 880, 730 and 700	

Table 6: 50% inhibition (IC_{50}) for Rutin by DPPH method

S. No	Sample	IC_{50} (µg/ml)
1.	Ascorbic acid	3.17
2.	Rutin	4.92

Table 7: Total reducing power of isolated rutin

Concentration(µg/ml)	Absorbance	
	Ascorbic acid(standard)	Isolated rutin
20	0.130	0.245
40	0.221	0.268
50	0.302	0.614
80	0.745	0.702
100	0.850	0.908

Table 8: Thin Layer chromatography analysis of antioxidant constituents

Solvent system	R_f value		Colour intensity of spot	
	Isolated rutin	Ascorbic acid	Isolated rutin	Ascorbic acid
Chloroform : methanol(10:90)	0.62	0.60	yellow	pale yellow
Methanol: Chloroform: Hexane(7:2:1)	0.63	0.57	yellow	yellowish white

Table 9: 50% inhibition (IC_{50}) for Rutin by TBARS method

S. No	Sample	IC_{50} (µg/ml)
1.	BHT	46.79
2.	Rutin	18.27

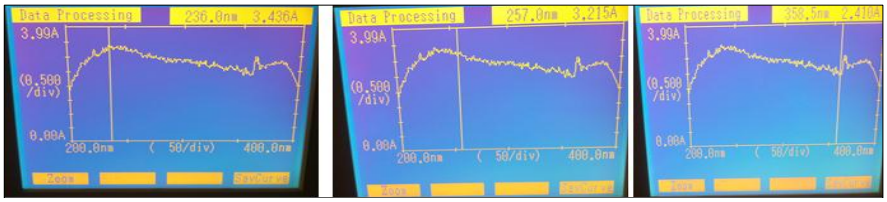

Fig 3: UV Spectrum

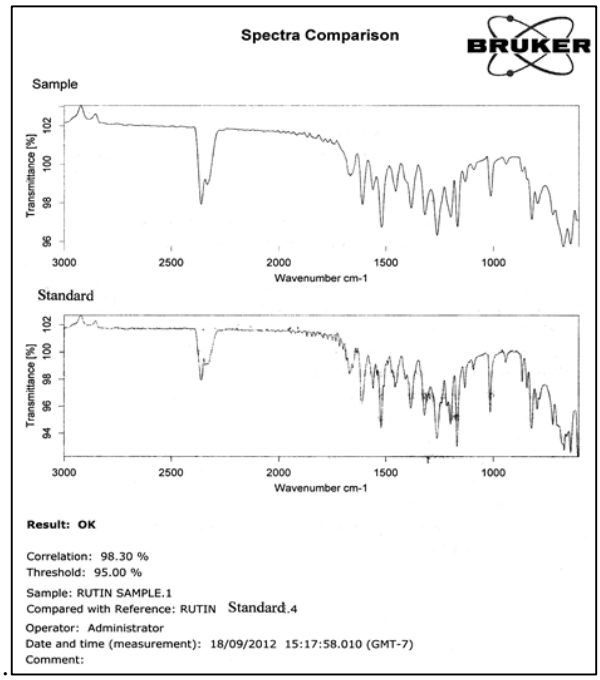

Fig 4: IR Spectrum comparsion of Rutin (standard) with sample.

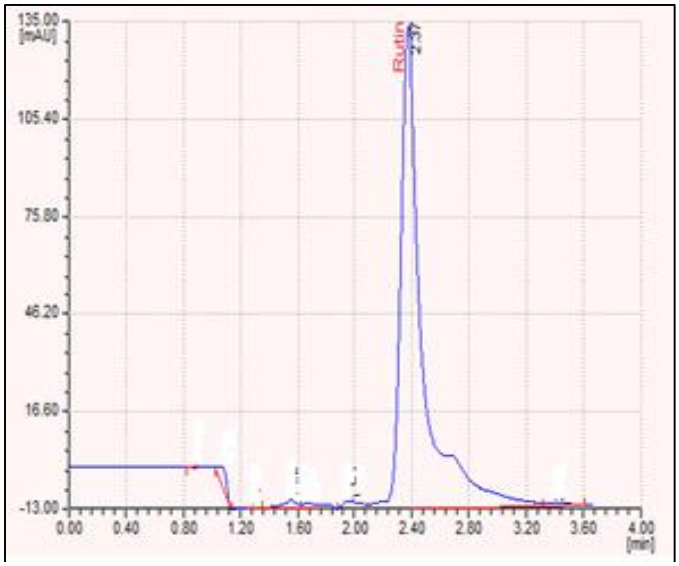

Fig 5: HPLC Chromatogram of isolated rutin

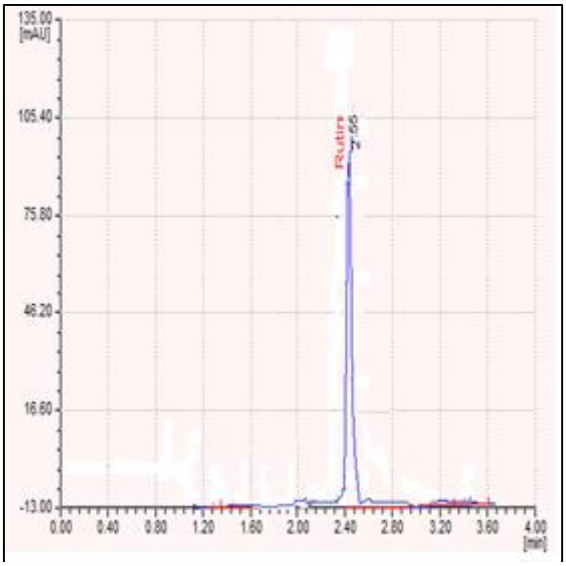

Fig 6: HPLC Chromatogram of standard rutin

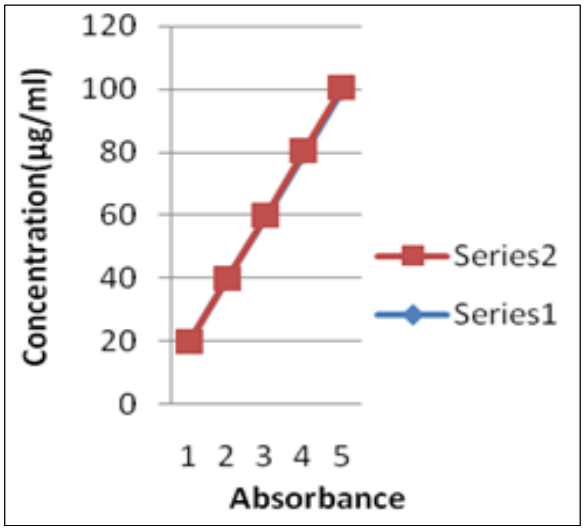

Fig 7: Reducing Power of Ascorbic acid (standard)

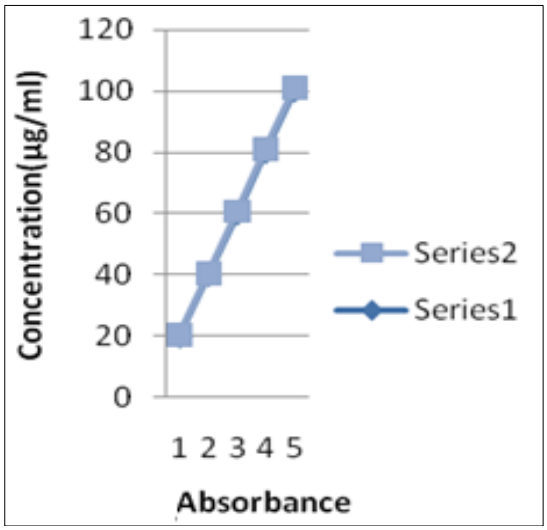

Fig 8: Reducing Power of isolated Rutin

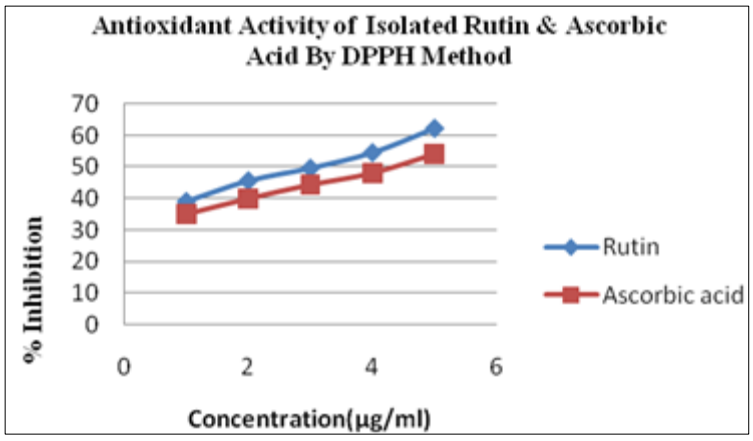

Fig 9: Antioxidant activity of Isolated Rutin & Ascorbic acid by DPPH Method

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