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In Vitro Antioxidant and Antimicrobial Activity of Isolated Flavonoid from the Methanolic Extract of *Spermacoce hispida* Leaves

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Abstract: The purpose of this research was to separate the flavonoid component of the *Spermacoce hispida* plant that is responsible for its medicinal effects. A methanolic extract of *Spermacoce hispida* leaves was used to study the separation of flavonoids from a plant component. Phytochemical analysis of methanol extract confirmed the presence of flavonoid compounds. The extracted flavonoid component was refined using thin-layer chromatography and column chromatography. The structures of the isolated compounds were characterised using UV-Visible, FT-IR, ^{13}C, ^{1}H NMR, and Mass spectroscopy. Therefore, 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[-L-rhamnopyranosyl-(16)—D-glucopyranosyloxy] was given as the name for the isolated molecule. There has to be further biological investigation of this plant's isolated flavonoid. Flavonoids are a distinct class of plant secondary metabolites having pharmacological benefits that have been used for centuries in traditional medicine. They support a diverse array of medical procedures that may provide novel and therapeutic medication discoveries. Flavonoids' potential as antioxidants in the face of oxidative stress has been the subject of a lot of recent research. To what extent flavonoids may serve as antioxidants and as antimicrobials is explored in this study.

Keywords: *Spermacoce hispida,* separation of flavonoids, Rutin, Antioxidant activity

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Introduction

One of the largest angiosperm groupings, the Rubiaceae has 650 genera and 13,000 species, mostly found in tropical and subtropical temperatures but also in temperate and northern parts of Europe and Northern Canada (Harborne 1984; Evans, 1997). There are around 130 genera and 1500 species of this family in Brazil, where it is found mostly in the Atlantic Forest (but it is also found in other types of vegetation). The primary purpose of this study was to separate and analyze

the active components of the *Spermacoce hispida* plant utilizing spectroscopic techniques including UV-Visible, FT-IR, ^{13}C and ^{1}H NMR, and Mass Spectrometry.

Substances known as antioxidants are able to neutralize free radicals by either accepting or giving up an electron, so halting the oxidation process (Abirami *et al*., 2014). Numerous studies showed that natural antioxidants may lead to lipid peroxidation in addition to protecting against ROS (Tang *et al*., 2013; Saravanan *et al*., 2014; Wu *et al*., 2015). An antioxidant is a substance that inhibits oxidation. Antioxidants found in nature have been shown to shield cells from free radical damage. Food and pharmaceutical antioxidants lower lipid oxidation activity and delay the development of many chronic illnesses. Secondary metabolites found in plants, such as phenolic and flavonoid compounds, have been demonstrated to be powerful free radical scavengers. Leaves, fruits, seeds, roots, and bark are all potential sources (Matthew *et al*., 2006)

There is clinical evidence that shows traditional medicine based on plant extracts is effective and safer than standard medications (Awaad *et al*., 2011). Determining physiologically active compounds from plant material is highly dependent on the solvent type utilized in the extraction procedure (Govindarajan *et al*., 2005). Proteins, lipids, DNA, carbohydrates, and more are all susceptible to be damaged from free radicals. Further, free radicals cause cellular damage and may disrupt homeostasis. The biological effects of these compounds have been connected to their antioxidant capability. Diabetic problems and other brain illnesses like Parkinson's disease are linked to reactive oxygen species (Oyedemi *et al*., 2010). It has been discovered that phenolic molecules are far more powerful antioxidants than any other naturally occurring compounds (Yingming *et al*., 2004). The goal of this research was to examine the antibacterial and antioxidant properties of the *Spermacoce hispida* leaf.

Materials and Methods

Collection of Plant material:

Spermacoce hispida (Linn) was used in this study was collected from Archampatty, Karur District, Tamil Nadu, India. Dr. Arulanandam, Director, Rapinat Herbarium, St. Joseph College, Tiruchirappalli, Tamil Nadu, validated and confirmed the identification of *Spermacoce hispida* leaves. SDD002 is the voucher number for specimens. The leaves of *Spermacoce hispida* (Linn) plant was sun-dried, sorted, crushed in a grinder, and sieved through a 40-mesh screen.

Extraction:

Hexane (35-65°C) was used in a Soxhlet apparatus for a hot continuous percolation extraction of the powdered plant materials over the course of 48 h. In order to purify and concentrate the extract, a rotary evaporator was utilized. Following 48 h incubation in Chloroform, the remaining marc was subjected to hexane extraction. In a similar fashion, we collected and concentrated extracts in ethyl acetate and methanol. Each extract was freeze-dried in a lyophilizer to a dry powder and then kept in screw cap vials at 4°C.

Separation of flavonoid from the methanolic extract of Spermacoce hispida:

The methanol extract passed the flavonoid test, thus further separation was performed on it. A few spots of a methanol extract were seen on preparative TLC (Thin Layer Chromatography). The mobile phase consists of methanol, glacial acetic acid, and water (90:5:5), whereas the stationary phase is composed of benzene, acetic acid, and water (60:30:10). TLC was inspected in a UV-Chamber, and the Rf value of the spots was recorded.

Isolation of flavonoid by using the Column Chromatography:

20 mg of methanolic extract of *Spermacoce hispida* leaves were combined with 20 mg of silica gel (60/120 meshes) to obtain uniform mixing. After carefully packing 200 g of silica gel (70/325 meshes) onto a suitable column, we prepared a

slurry out of the gel using petroleum ether. The column was taken out of commission for an hour so that it could be packed securely. The compounds were separated by eluting with the different solvent mixtures shown on the TLC, after which the admixture was added to the top of the stationary phase. Each portion of the column was extracted and concentrated independently at atmospheric pressure. The concentrated portion was analyzed structurally.

Chemical identification of flavonoids:

The following tests were conducted to verify the existence of flavonoid fraction (Tanaka *et al*., 2013; Narendhiran *et al*., 2014; Arun *et al*., 2014):

Shinoda Test:

A few drops of concentrated hydrochloric acid were added to a little quantity of alcohol containing a test solution; the resulting pink colour indicated the presence of flavonoids, which were detected by adding a magnesium ribbon to the mixture.

Zn- Hydrochloride Reduction Test:

The solution included a combination of zinc dust and strong hydrochloric acid. After a few min, the solution's hue became red at high heat.

Aluminum Chloride Test:

Small amounts of test solutions containing flavonoid were coloured yellow by adding two drops of 1 per cent aluminium chloride.

Characterization of Flavonoid:

The isolated substance was first found using a phytochemical screening test, and subsequently characterised using spectrum analyses. Methanol was employed as a reference solvent when recording the UV-visible Absorption spectra of CD-1. A little quantity of CD-1 was combined with spectroscopic grade KBr, and the resulting pellet was finely powdered and analysed using FT-IR to detect functional groups. Proton NMR (H1-NMR) and carbon-13 NMR spectroscopy was performed using a JEOL NMR 200MHz spectrophotometer (C13-NMR). DMSO and TMS were used as the solvent and internal standard, respectively. The extract underwent mass spectral analysis on a JEOL GC MATE-11 HR Mass spectrometer (Suthar *et al*., 2001; Jain *et al*., 2010; Sarfaraj *et al*., 2014).

Antioxidant activity (*DPPH free radical scavenging activity*) *determination*:

The scavenging ability of DPPH free radicals was used to examine rutin's antioxidant activity after it was isolated as a compound. A 300 µl ethanolic solution of DPPH (0.05 mM) was added to 40 µl of isolated compound rutin at various concentrations (20 - 100 µg/ml). We made the DPPH solution from scratch and kept it in the fridge at 4 °C in the dark. After adding 96 per cent ethanol (2.7 ml), the liquid was agitated violently. The absorbance was measured spectrophotometrically at 517 nm after the sample had stood for 5 min. Use of ethanol led to a zero absorption reading. Another sample was made with the same amounts of ethanol and DPPH as a control. There were three sets of each measurement (Shimada *et al*., 1992). Using the following formula, we were able to determine the radical scavenging activities of the examined samples as a percentage of inhibition:

$$\text{DPPH activity inhibition percentage (\%)} = [(A - B) / A] \times 100 \quad (1)$$

Where (B, A) stands for the absorbance of the test sample and (A, B), correspondingly, the absorbance of the blank sample. The sample concentration (IC_{50} value) that resulted in 50% inhibition was calculated by plotting a per cent inhibition versus concentration curve for each of the test solutions.

Antimicrobial assay:

We used the disc diffusion approach to create the antibiogram (Awoyinka *et al*., 2007). Thirty millilitres of NA/PDA medium were poured onto petri dishes. Micropipette inoculations were made onto a plate of solidified agar, and the plate was then distributed and left to dry for 10 min. Surfaces of the medium were injected with pathogens grown in a broth culture. To inoculate the Nutrient agar /PDA plates, a sterile cotton swab is dipped into a standardized

microorganisms test mixture. Quickly, microbial strains were inoculated into plates of Nutrient agar/PDA. We used sterile forceps to place six-millimeter-diameter filter sheets containing 50 µl, 100 µl, and 150 µl of samples, 30 µl each of standard solution as Chloramphenicol and Fluconazole, and 30 µl of control on top of the infected agar plate. Both the bacterial and yeast strains were allowed to grow on plates for 24 and 48 h, respectively, at 37°C. Triple tests were performed on every sample.

Values expressed as Mean ± SD for triplicate. Statistically data were followed by correlation coefficient analysis. R^2 value was calculated as concentration vs % of inhibitions, they are relationship between the concentration and % of inhibitions using MS-excel.

Results and Discussion

Separation of Flavonoids:

Solvent solutions of methanol: glacial acetic acid: water (90:5:5) and benzene: acetic acid: water (90:5:5) both yielded yellow bands with Rf values of 0.43 and 0.32 on TLC (60:30:10). By employing column chromatography, the flavonoid was separated from the crude methanol extract. Images of thin-layer chromatography (TLC) and column chromatography are shown in Figures 1 and 2. Phytochemical and spectroscopic analyses were used to determine the compound's identity and structure. A summary of the findings is shown graphically.

Characterization of Flavonoid:

A very light yellow powder was the isolated substance. The isolated substance melted at a temperature of 190 °C. The UV spectrum (Fig. 3) of this chemical revealed the existence of a flavonoid nucleus structure, with strong absorption peaks at 354 nm and 298 nm.

With FT-IR, we noticed that the OH group was stretching at 3426.21 cm^{-1}, the CH2 group was stretching at 2931.75 cm^{-1}, the CH bonding group was at 2082.53 cm^{-1}, the CO group was at 1458 cm^{-1}, and the C-OH vibration group was vibrating at 1363.07 cm^{-1}. Figure 4 shows the FT-IR analysis result for flavonoids.

The 1H-NMR report revealed the peak with different values and its possible notations, which are (300 MHz, CD1) ppm = 6.84 (d, J=2.0 HZ, 1H,H-6), 6.39 (d, J=2.0Hz,1H,H-8), 7.55 (d, J=2.05Hz,1H,H-2'), 6.88 (d, J=8Hz,1H,H-5'), 7.62 (dd, J=8.5 (m). Figure 5 depicts the H1-NMR of a flavonoid.

In 13C-NMR reports and interpretations are given below (9) and are (75.5 MHz, CD3OD)δppm =158.6 (C-2), 135.8 (C-3), 177.8 (C-4), 161.64 (C-5,) 101.4 (C-6), 164.5 (C-7), 94.1(C-8), 157.0 (C-9,) 104.4(C-10,) 121.8(C-1'), 116.7 (C-2'), 145.1 (C-3'), 148.8 (C-4') 116.7 (C-5'), 123.4 (C-6'), 104.4 (C-1'') 76.5(C-2''), 77.3 (C-3''), 70.9 (C-4''), 78.1 (C-5'') 68.7 (C-6''), 102.5 (C1'''), 72.3 (C-2'''), 72.2 (C-3''') 73.9 (C-4'''), 68.7 (C-5'''), 18.1 (C-6''').The result of ^{13}C –NMR is shown in Figure 6.

Mass spectrum of Flavonoid:

The mass spectra showed that the compounds were identified as Rutin because of a base peak [M] + for compound at m/z 609.80. (Gupta *et al*., 2015). $C_{27}H_{30}O_{16}$ was used to get the molecular formula (Fig. 7)

The isolated compound was confirmed to be 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl 6-O-(6-deoxy-α-Lmannopyranosyl)-β-D-glucopyranoside based on physical, chemical, and spectral properties. Figure 8 depicts the structure, i.e., Rutin. The isolated compound had a molecular weight of 609.80 and a molecular formula of $C_{27}H_{30}O_{16}$.

Antioxidant activity:

DPPH radical scavenging activity:

Stable free radical molecules make up the dark-colored chemical DPPH, or 2, 2-diphenyl-1-picrylhydrasyl. In the presence of antioxidants, DPPH loses its purple hue. The antioxidant potential of a substance may be calculated from its change in absorbance at 517 nm in the presence of antioxidants (Table 1). The scavenging of DPPH

Fig. 1: TLC. Fig. 2: column chromatography.

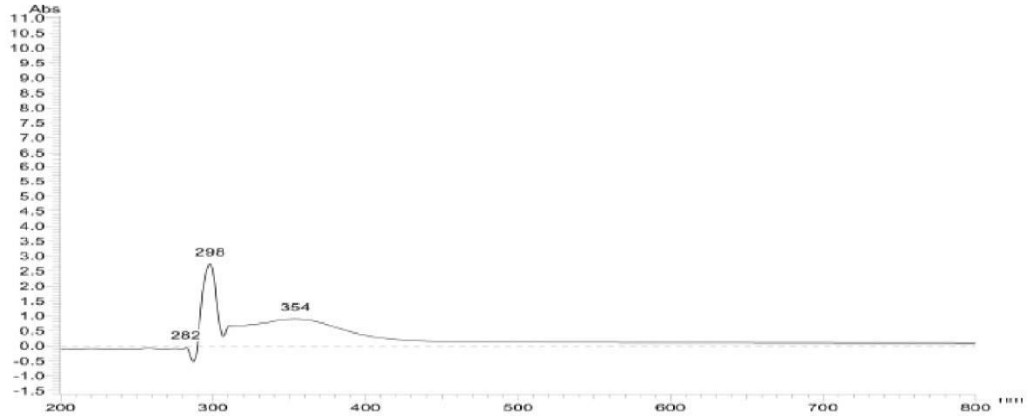


Fig. 3: UV - Visible spectrum of Flavonoid.

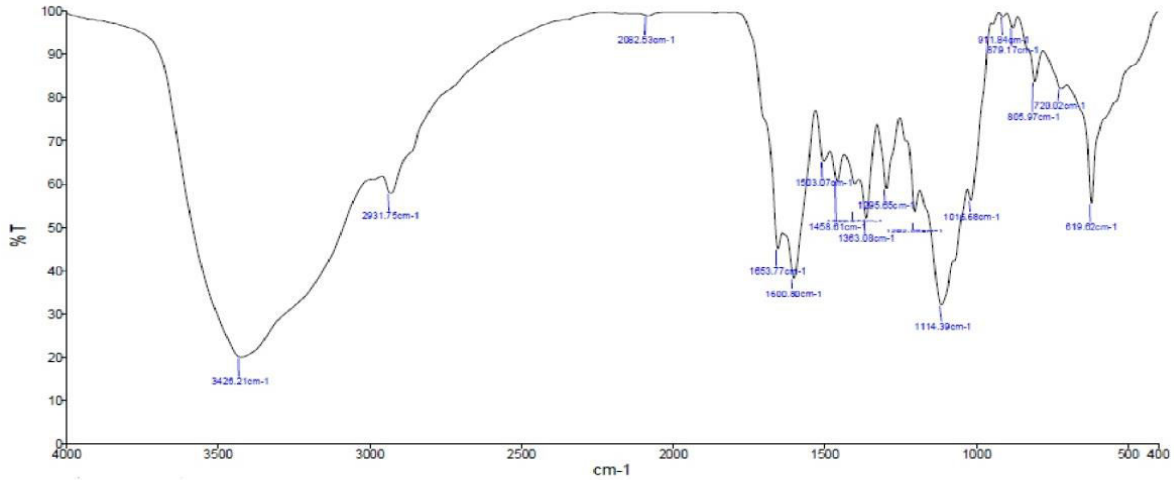


Fig. 4: FT-IR spectrum of flavonoid.

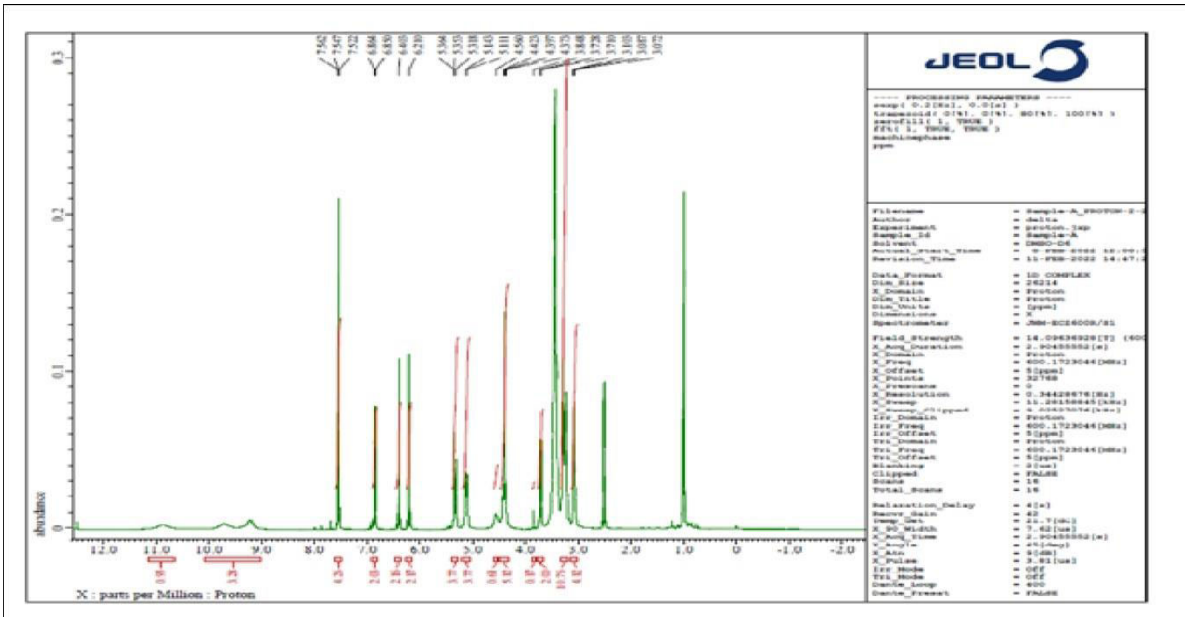


Fig. 5: H^1 - NMR spectrum of Flavonoid.

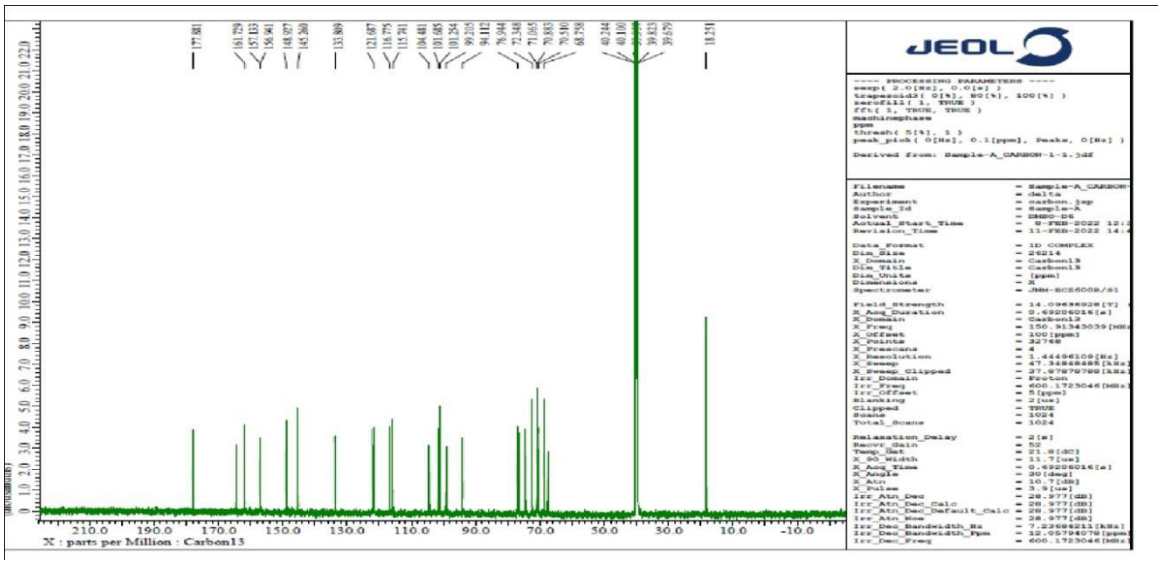


Fig .6: C^{13} - NMR spectrum of Flavonoid

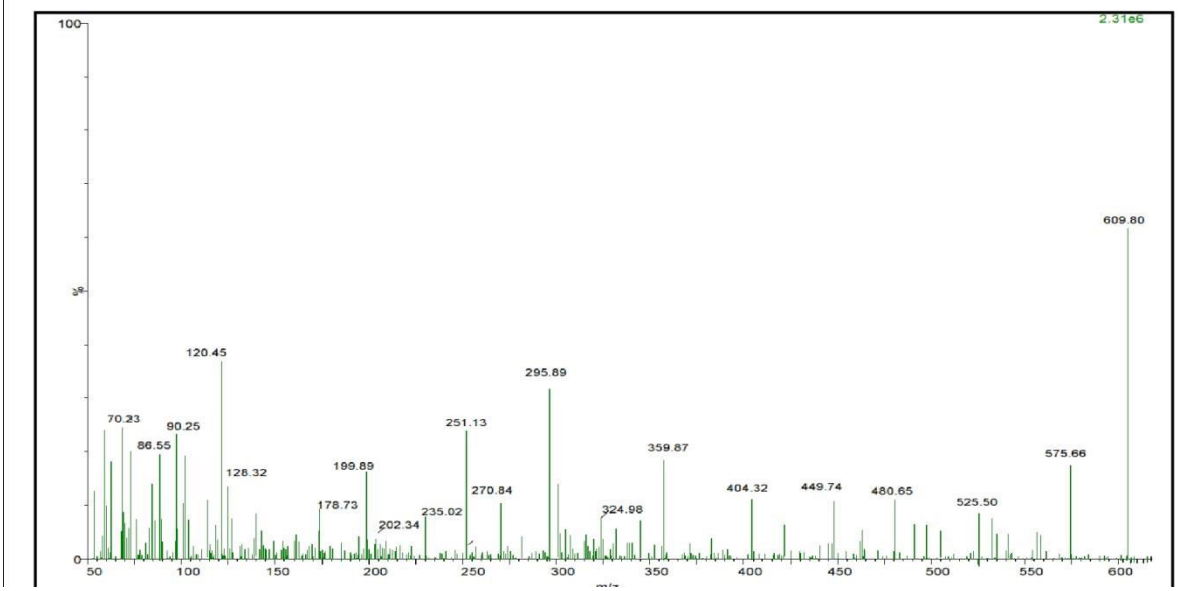


Fig. 7: Mass spectrum of Rutin.

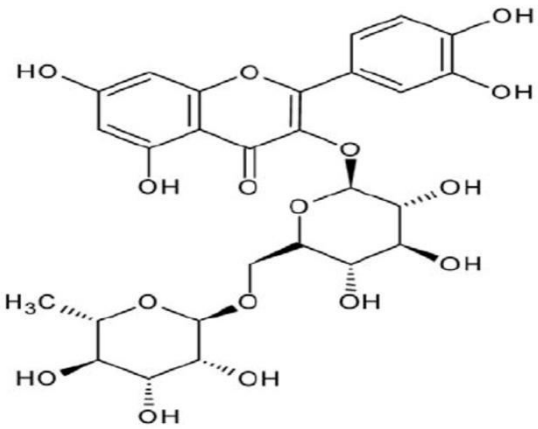


Fig. 8: Structure of Rutin.

Table 1: *In vitro* DPPH radical scavenging activity of rutin and standard ascorbic acid

Concentration (µg/ml)	**% of inhibition**	
	Rutin	**Std. (Ascorbic acid)**
20	18.50±0.76	20.80±0.51
40	37.23±0.65	41.65±0.63
60	55.98±0.98	59.47±0.74
80	72.15±1.32	77.45±0.82
100	94.56±1.65	95.78±0.61
IC_{50} value (µg/ml)	50.27	52.90

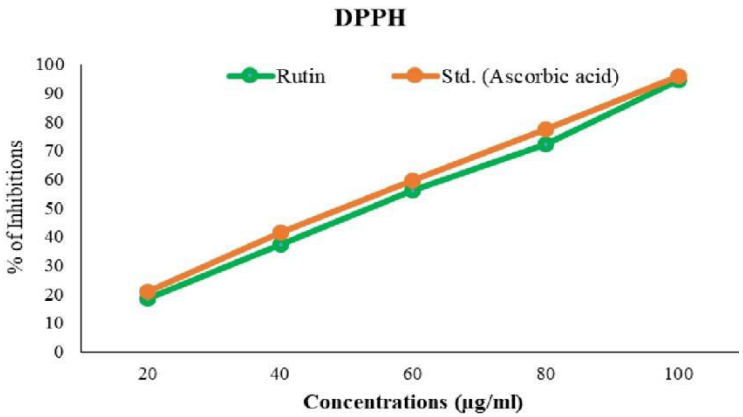
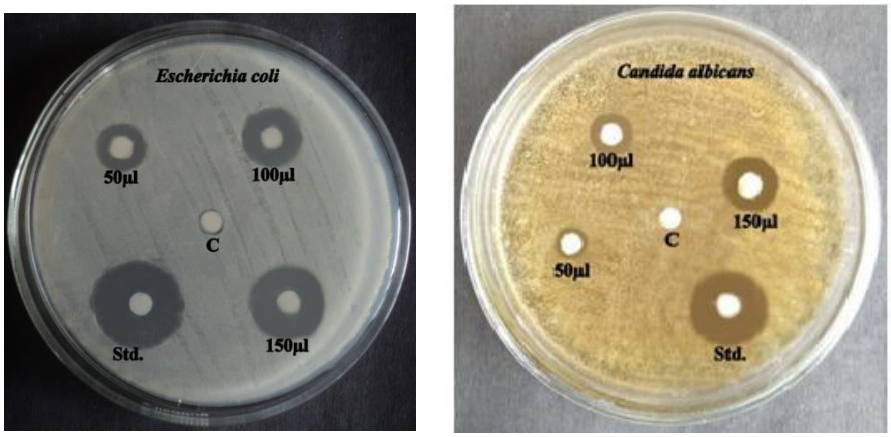


Fig. 9: *In vitro* DPPH radical scavenging activity of rutin.

Table 2: Anti-microbial activity of Rutin

Microbial Strains	Control	50 µl	100 µl	150 µl	Std. 30µl
Bacterial strain					
Escherichia coli (mm)	Nil	3.40±0.23	6.80±0.46	9.50±0.60	11.20±0.82
Fungal strain					
Candida albicans (mm)	Nil	1.80±0.12	4.90±0.36	6.90±0.40	10.50±0.73

Values expressed as Mean ± SD for triplicates.
Bacterial standard: Chloramphenicol; Fungal standard: Fluconazole; Control: Distilled Water



Escherichia coli *Candida albicans*

Fig. 10: Anti-microbial activity of Rutin.

radicals is measured by observing the solution's colour shift from purple to yellow after the DPPH molecule has been reduced by an antioxidant that donates hydrogen. Half-inhibition concentrations (IC50s) for DPPH test activity inhibition were determined to be 52.90 µg/ml for ascorbic acid and 50.27 µg/ml for rutin, respectively (Fig. 9). Antioxidant activity is greater with the lower IC50 value (Thippeswamy *et al*., 2013).

Antimicrobial activity:

From the Table 2 and Figure 10, it is evident that Rutin has remarkable antimicrobial activity against *Escherichia coli* and *Candida albicans.*

Conclusion

This study revealed that methanol solvent was an effective solvent for extracting flavonoids from plant sources. Flavonoids may be extracted using thin-layer chromatography (TLC) or column chromatography (CC). The presence of rutin in this plant is the focus of the study. Because of its antioxidant and antibacterial characteristics, rutin is utilised in the treatment of many different ailments. Therefore, flavonoids found in plants are promising antioxidant and antibacterial material sources due to their broad-spectrum actions in inhibiting the development of all microbes studied.

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