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EVALUATION OF PHYTOCONSTITUENTS, FT-IR ANALYSIS, TOTAL PHENOLIC, FLAVONOID CONTENTS, *IN-VITRO* ANTIBACTERIAL AND ANTIOXIDANT STUDIES OF ETHANOLIC ROOT EXTRACT OF *MILLETTIA PINNATA*

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ABSTRACT: This research work presents the phytochemical and FTIR analysis, total phenolic, and flavonoid quantification, and evaluation of *in-vitro* antibacterial and antioxidant activity of ethanolic root extract of *Millettia pinnata*. Ethanolic root extract was subjected to the phytochemical and FTIR spectrum analysis. Total phenolic and flavonoid contents were quantitatively determined by Colorimeter. The antibacterial activity against Gram-positive, Gram-negative, and *in-vitro* antioxidant activity of ethanolic root extract of *Millettia pinnata* was determined by using agar well diffusion method and DPPH radical scavenging method. Phytochemical screening revealed carbohydrates, glycosides, alkaloids, steroid and triterpenoids, tannins, polyphenols, and phenolic compounds. FTIR spectrum of ethanolic root extract was obtained and confirmed the presence of functional groups. The intense bands indicate the presence of N-H₂, O-H stretch, C-H stretch, C=O, C=C, C-H Benzene, C-O stretch, and C-Cl. The total phenolic and flavonoid contents of the ethanolic root extract were found to be 8.976 ± 0.0833 GAE mg/g and 4.9 ± 0.2516 RUE mg/g of dry extract. The extract showed bactericidal activity, zone of inhibition was determined against *Escherichia coli* and *Pseudomonas aeruginosa* found as 17.3 and 18.3mm and antioxidant activity was expressed in IC₅₀ value and it was found to be 26 µg/mL for ascorbic acid and 40 µg/mL for ethanolic root extract of *Millettia pinnata*.

INTRODUCTION: Many medicinal plants are used as an alternative medicine for diseases, most of them are without side effects when compares with synthetic drugs. Identification of the chemical nature of phytoconstituents present in the medicinal plants will provide some information on the different functional groups responsible for their medicinal properties¹. The phytochemical analysis is a process of tracing phytoconstituents.

Hence, the plant authentication and phytochemical evaluation of an herbal drug is an essential criterion, before proceeding for its pharmacological and toxicological studies. Plants generally contain phytoconstituents like phenolics, flavonoids, glycosides, coumarins, saponins, terpenoids, alkaloids, which reveal their specific characteristic properties and attribute to their pharmacological properties². FTIR (Fourier Transform Infrared) spectroscopy has played a remarkable role in the field of medicinal plant analysis. FTIR is one of the most widely used methods to identify the chemical constituents, functional groups, and the chemical structure of the constituents. The measurements made by FTIR were extremely accurate and reproducible³.

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According to the World Health Organization, medicinal plants are the best source to obtain a variety of medicinal drugs. Natural systems of treatment played significant protection and management against pathogens. Plant extracts highly involved in the inhibition of pathogens and improvement of quality and yield of health⁴. The activities of free radicals have been implicated in aging, destruction of DNA, obstruction of arteries, cancer, strokes, cardiac and central nervous system (CNS) disorders which have led to an increase in the investigation of substances that can protect against these reactive oxygen species and thus may play a role in disease prevention⁵. Oxidative stress is caused by the imbalance in the production of ROS, and the biological systems are damaged. High levels of oxidative stress can cause ATP depletion, necrosis, and apoptosis. Excess free radicals need to be either scavenged/neutralized or quenched. The most common naturally occurring antioxidants are Vitamin A (retinol), C (Ascorbic acid) and E (Tocopherol) and also polyphenols, flavonoids⁶. Catalase, glutathione peroxidase, aldehydes dehydrogenases, and sulfiredoxin belong to the enzymatic antioxidants⁷.

The plant *Millettia pinnata* belongs to the family Fabaceae. It contains several phytoconstituents belonging to category flavonoids and fixed oils. In traditional systems of medicine the *Millettia pinnata* plant is used for tumors, Piles, skin diseases, wounds, as anti-hepatoprotective^{8, 9}, antifungal¹⁰, anti-hyperglycemia^{11,12}, anti-arthritis activity¹³, antioxidant¹⁴, anti-ulcer¹⁵, antifilarial¹⁶, anti-nociceptive and antipyretic¹⁷, anti-inflammatory¹⁸, anti-plasmodial¹⁹, anti-bacterial²⁰, analgesic²¹. Roots and barks of *Millettia pinnata* are used externally for joint pain.

The aim of the present study was to evaluate phytochemical and FTIR spectrum analysis, total phenolic, and flavonoid quantification, *in-vitro* antibacterial and antioxidant activities of ethanolic root extract of *Millettia pinnata*.

MATERIALS AND METHODS:

Collection of Plant Materials: The roots of *Millettia pinnata* were collected from Agricultural fields, Mangalam, Tirupati. The collected plants were identified by Dr. Madhava Chetty, Botanist, Department of Botany, Sri Venkateswara

University, Tirupati, deposited in herbarium with voucher specimen number 0127.

Preparation of Ethanolic Root Extract of *Millettia pinnata*: The dried roots were coarsely powdered, the root powder was extracted with ethanol in a Soxhlet apparatus for 6 h, the ethanolic root extract was concentrated under reduced pressure to yield a dark brown viscous mass (50g; 7.2% yield) was calculated²².

Phytochemical Screening: The ethanolic root extract was subjected to various phytochemical tests to detect the presence of different phytoconstituents like alkaloids, glycosides, carbohydrates, proteins, amino acids, polyphenols, tannins, steroids, terpenoids, and saponins.

FTIR Spectrum Analysis: FTIR spectrum of ethanolic root extract of *Millettia pinnata* was recorded in KBr by a sophisticated computer-controlled FTIR BRUKER spectrometer with He-Ne Laser as reference²³ FTIR spectrum helps to identify the functional groups present in *Millettia pinnata*. The extract of *Millettia pinnata* was scanned at the spectral range of 4000-400 cm⁻¹.

Determination of Total Phenolic Content: The total phenolic content of ethanolic root extract was determined using the Folin-ciocalteu colorimetric method²⁴. The extract (10-100 µg/mL) or standard solution of gallic acid (10-100 µg/mL) was added to 25 mL volumetric flask containing 9 mL of distilled deionized water. A reagent blank was prepared using distilled water instead of a sample. 1 mL of Folin-Ciocalteu phenol reagent was added to the mixture and shaken well. After 5 min, 10 mL of 7% aqueous sodium carbonate was added to the mixture.

The solution was diluted to 25 mL with double distilled water. After incubation for 90 min at room temperature, the absorption against prepared reagent blank was determined at 760 nm using a colorimeter. Quantification was done with respect to the standard gallic acid and expressed as gallic acid equivalents (GAE) in mg per gram of extract.

Total Flavonoid Content: The total flavonoid content of ethanolic root extract was measured using the aluminum chloride colorimetric method²⁵.

The extract (10-100 µg/mL) or standard solution of Rutin (10-100 µg/mL) was added to 10 mL volumetric flask containing 4 mL of double-distilled water. To the flask, 0.3 mL of 5% sodium nitrite solution was added. After 5 min, 0.3 mL of 10% aluminum chloride solution was added. At the 6th minute, 2 mL of 1M NaOH was added, and the total volume was made up to 10 mL with Double distilled water. The solution mixture was mixed well and absorbance was measured at 510 nm against blank in colorimeter. The total flavonoid content was expressed as Rutin equivalents in mg per gram of extract.

Antibacterial Activity: Antibacterial activity was examined for the ethanolic root extract of *Millettia pinnata*. Determination of antibacterial activity (zone of inhibition) of the extract against two pathogenic bacteria (gram-positive and gram-negative) was investigated by the agar disc diffusion method; streptomycin was taken as a standard antibiotic for comparison of the results²⁸.

The extract was screened for their antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*. The sets of six dilutions (10, 20, 40, 60, 80, 100 µg/mL) of *Millettia pinnata* root extract and standard drug (streptomycin) were prepared with distilled water. The sensitivities of the microorganism species to the plant extract were determined by measuring the sizes of inhibitory zones (including the diameter of the disc) on the agar surface around the discs.

In-vitro Antioxidant Activity:

DPPH Free Radical Scavenging Assay:

Antioxidant activity of ethanolic root extract was determined using DPPH free radical scavenging activity²⁶. 1 mL of 0.1 mM solution of DPPH in methanol was added to 2.5mL of the test extract in methanol (10-100 µg/mL). The reaction mixture was then allowed to stand for 30 min at room temperature in a dark place. After 30 min, absorbance was measured at 517 nm using a colorimeter. Ascorbic acid was used as a standard. The scavenging activity of DPPH radical (%) was calculated using the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

A₀ - absorbance of control

A₁ - absorbance of the test sample

RESULTS AND DISCUSSION:

Phytochemical Screening: The ethanolic root extract of *Millettia pinnata* was subjected to the qualitative chemical analysis for the identification of various plant constituents. The results were given in **Table 1**. The phytochemical evaluation of ethanolic root extract of *Millettia pinnata* was revealed different phytoconstituents like carbohydrates, glycosides, alkaloids, steroids and triterpenoids, tannins, proteins, amino acids, polyphenols, and phenolic compounds were present whereas saponins were absent.

TABLE 1: PHYTOCHEMICAL EVALUATION OF ETHANOLIC ROOT EXTRACT OF MILLETTIA PINNATA

S. no.	Name of the test	Result
1	Carbohydrates	+ ve
2	Glycosides	+ ve
3	Alkaloids	+ ve
4	Steroids	+ ve
5	Triterpenoids	+ ve
6	Tannins	+ ve
7	Polyphenols and phenolic	+ ve
8	Amino acids	+ ve
9	Proteins	+ ve
10	Saponins	- ve

The phytochemical evaluation of ethanolic root extract of *Millettia pinnata* was revealed different phytoconstituents; they were denoted by + ve = present, -ve = absent.

FTIR Spectrum Analysis: The ethanolic root extract of *Millettia pinnata* was subjected to the FTIR spectrum analysis, and data of the FTIR spectrum revealed the presence of various characteristic functional groups in the ethanolic root extract of *Millettia pinnata* was obtained and depicted in **Fig. 1**, which confirmed the presence of functional groups, the frequency ranges, and functional groups obtained from absorption spectra are presented in **Table 2**. The intense bands occurring at 3772.49 cm⁻¹, 3525.94 cm⁻¹, 2939 cm⁻¹, 1619.63 cm⁻¹, 1450.14 cm⁻¹, 1253.01 cm⁻¹, 1036.89 cm⁻¹ and 781.87 cm⁻¹ corresponding to N-H₂, O-H stretch, asymmetric C-H₂ stretching, C=O, C=C, C-H Benzene, C-O stretch and C-Cl. This confirms the presence of functional groups in *Millettia pinnata* like amines, amides, hydroxyl, carbonyl, Benzene, ether, and Halogen. These are active secondary metabolites of the plant. The functional groups that appeared in FTIR were correlated to a qualitative phytochemical screening of the ethanolic root extract of *Millettia pinnata*.

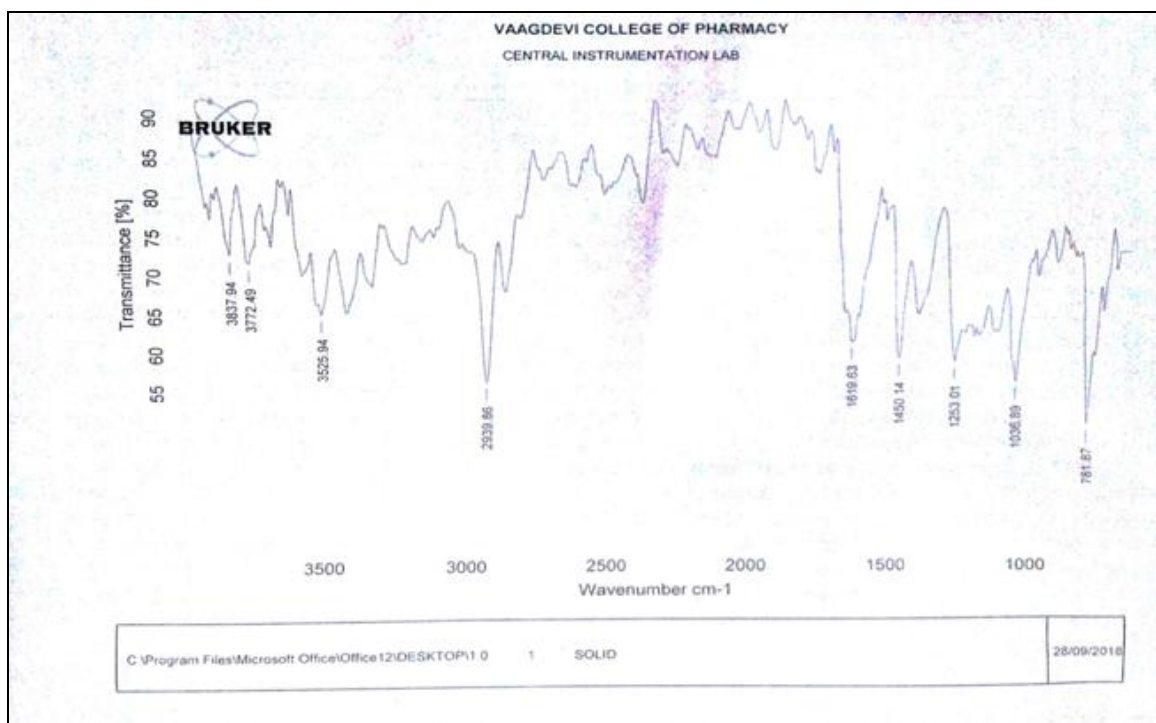


FIG. 1: FTIR SPECTRUM OF ETHANOLIC ROOT EXTRACT OF *MILLETTIA PINNATA*

FTIR spectrum revealed the presence of various characteristic functional groups in ethanolic root extract of *Millettia pinnata*.

TABLE 2: FTIR FREQUENCY RANGE AND FUNCTIONAL GROUPS PRESENT IN THE ETHANOLIC ROOT EXTRACT OF *MILLETTIA PINNATA*

S. no.	Frequency range (cm ⁻¹)	Functional group
1	3772.49 cm ⁻¹	N-H ₂ stretching of amines and amides
2	3525.94 cm ⁻¹	O-H stretching vibration
3	2939 cm ⁻¹	Asymmetric C-H ₂ stretching vibration
4	1619.63cm ⁻¹	C=O
5	1450.14cm ⁻¹	C=C
6	1253.01cm ⁻¹	C-H Benzene
7	1036.89cm ⁻¹	C-O stretch

Quantification of Total Phenolic Content:

Quantification of total phenolic content of ethanolic root extract of *Millettia pinnata* was estimated by Folin ciocalteu’s method using gallic acid as standard. The reagent is formed after oxidation of the phenols, mixture of phosphotungstic acid and phosphomolybdic acid, which after oxidation of the phenols, is reduced to tungsten and molybdenum. The tungsten and molybdenum are reduced, then

blue coloration produced. The blue color has maximum absorption in the region of 760 nm, and it is directly proportional to the total quantity of phenolic compounds originally present. The gallic acid solution of concentration conformed with a regression coefficient (R²) = 0.991. The plot has a slope (m) = 0.012 and intercept = 0.0925. The equation of the standard curve is y = mx+c **Fig. 2**. Total phenolic content for ethanolic root extract of *Millettia pinnata* was estimated, and the quantity was found to be GAE 8.976 ± 0.0833 mg/g of dry extract **Table 3**.

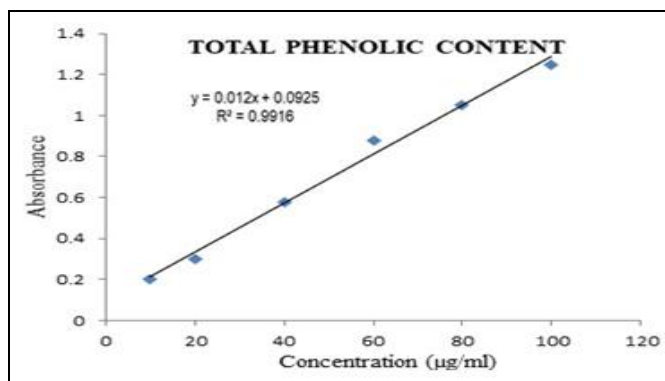


FIG. 2: STANDARD GRAPH OF GALLIC ACID

TABLE 3: TOTAL PHENOLIC CONTENT OF ETHANOLIC ROOT EXTRACT OF *MILLETTIA PINNATA*

S. no.	Sample solution µg/ml	Weight of dry extract per ml. M (gms)	Absorbance	GAE conc. µg/ml	GAE conc. mg/ml	TPC = c x v/m mg/g	Mean ± SEM
1	100	0.01	1.15	88.1	0.0881	8.81	8.976 ±
2	100	0.01	1.18	90.6	0.090	9.06	0.0833
3	100	0.01	1.18	90.6	0.090	9.06	

The ethanolic root extract of *Millettia pinnata* was subjected to FTIR analysis. The intense bands occurring at 3772.49 cm^{-1} , 3525.94 cm^{-1} , 2939 cm^{-1} , 1619.63 cm^{-1} , 1450.14 cm^{-1} , 1253.01 cm^{-1} , 1036.89 cm^{-1} and 781.87 cm^{-1} corresponding to N-H₂, O-H stretch, asymmetric C-H₂ stretching, C=O, C=C, C-H Benzene, C-O stretch and C-Cl. cm⁻¹ (centimeter) (Parameter for frequency).

Quantification of Total Flavonoid Content: The total flavonoid content for the ethanolic root extract of *Millettia pinnata* was measured by using aluminum chloride colorimetric assay using rutin as standard. Aluminum chloride forms acid-stable complexes with the flavones and flavonols. In addition, it also forms liable complexes with ortho dihydroxide groups in A/B rings of flavonoids. The rutin solution of concentration (10-100 ppm) conformed to Beer's Law at 510 nm with a

regression coefficient (R^2) = 0.997. The plot has a slope (m) = 0.001 and intercept = 0.00444. The equation of standard curve is $y = 0.001x + 0.0444$
Fig. 3. Total flavonoid content for ethanolic extract of *Millettia pinnata* was estimated, and the quantity was found to be RUE 4.9 ± 0.2516 mg/g of dry extract **Table 4.**

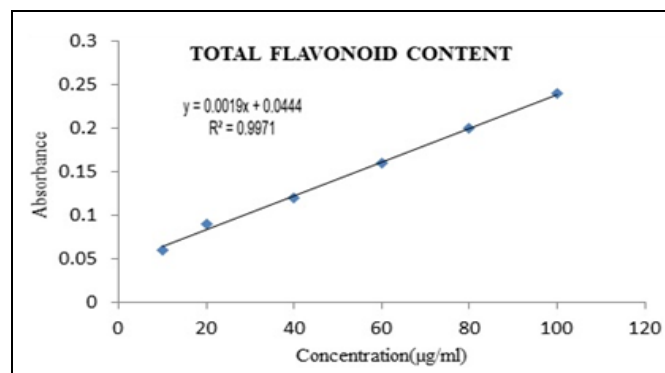


FIG. 3: STANDARD GRAPH OF RUTIN

TABLE 4: TOTAL FLAVONOID CONTENT OF ETHANOLIC ROOT EXTRACT OF MILLETTIA PINNATA

S. no.	Sample solution µg/ml	Weight of dry extract per ml. M (gms)	Absorbance	RUE conc. µg/ml	RUE conc. mg/ml	TPC = c x v/m mg/g	Mean ± SEM
1	100	0.01	0.088	44	0.044	4.4	4.9 ±
2	100	0.01	0.096	52	0.052	5.2	0.2516
3	100	0.01	0.095	51	0.051	5.1	

Antibacterial Activity: Antibacterial activity was carried out by measuring the zone of inhibition of different concentrations of ethanolic root extract of *Millettia pinnata*. All the concentrations showed good and more activity as standard ascorbic acid (21.1 mm and 18.6 mm shown in **Table 5**).

The extract showed bactericidal activity, the zone of inhibition was determined against *Escherichia coli*, and *Pseudomonas aeruginosa* found as 17.3 mm and 18.3 mm at 100 µg/mL concentration. The results were given in **Table 6**.

TABLE 5: ANTIBACTERIAL ACTIVITY OF STREPTOMYCIN (STANDARD)

Compound	Antibacterial activity (Zone of inhibition) of Streptomycin (Standard)		
	Concentration µg/ml	Zone of inhibition in mm	
		<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>
Streptomycin (Standard)	10	8.1	6.3
	20	11.1	8.3
	40	14.1	15.3
	60	16.5	16.4
	80	18	17.3
	100	21.1	18.6

TABLE 6: ANTIBACTERIAL ACTIVITY OF ETHANOLIC ROOT EXTRACT OF MILLETTIA PINNATA

Compound	Antibacterial activity (Zone of inhibition) of extract		
	Concentration µg/ml	Zone of inhibition in mm	
		<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>
<i>Millettia pinnata</i> ethanolic root extract	10	3.6	3.3
	20	6.3	6.6
	40	13.6	12.3
	60	15.3	13.6
	80	16.3	15.6
	100	18.3	17.3

DPPH Radical Scavenging Assay: Antioxidant activity was carried out in DPPH radical scavenging assay. The ethanolic root extract of *Millettia pinnata* showed good and more activity as standard. Both the ascorbic acid and extract exhibited a concentration-dependent DPPH radical scavenging activity. The IC₅₀ concentration for the standard ascorbic acid and ethanolic root extract of

Millettia pinnata was found to be 26 µg/ml and 40 µg/ml, respectively **Table 7** and **Fig. 4, 5**. The reducing power activity showed an increase with an increase in the concentration of extract. The antioxidant potential directly linked to the phenolic and flavonoid contents present in the ethanolic root extract of *Millettia pinnata*.

TABLE 7: DPPH RADICAL SCAVENGING ACTIVITY OF ASCORBIC ACID AND ETHANOLIC ROOT EXTRACT OF *MILLETTIA PINNATA*

Groups	Concentration (µg/ml)	Absorbance	% Inhibition	IC ₅₀ Value (µg/ml)
Ascorbic acid	10	0.421	37.3	26µg/ml
	20	0.341	49.2	
	40	0.292	56.5	
	60	0.210	68.7	
	80	0.169	74.8	
	100	0.014	85.26	
The ethanolic root extract of <i>Millettia pinnata</i>	10	0.059	37.3	40µg/ml
	20	0.050	47.36	
	40	0.047	50.52	
	60	0.044	56.84	
	80	0.039	58.94	
Control	100	0.033	65.26	
		0.095		

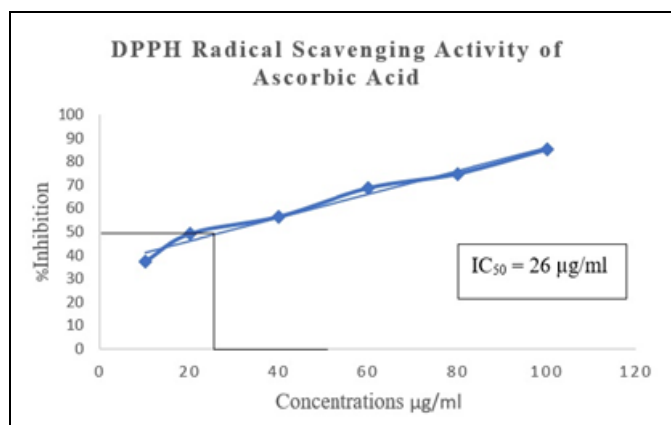


FIG. 4: EFFECT OF ASCORBIC ACID ON DPPH SCAVENGING ACTIVITY

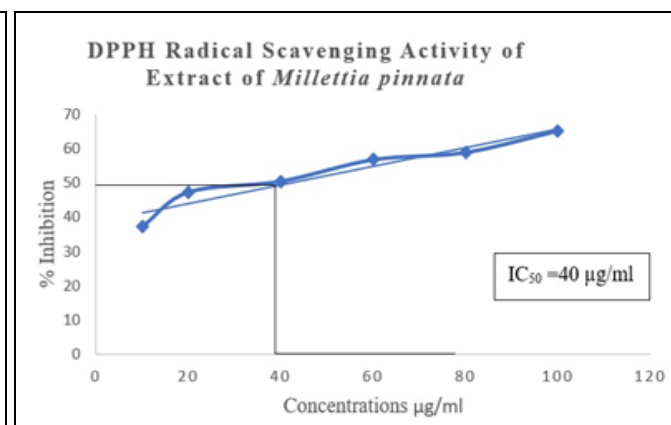


FIG. 5: EFFECT OF ETHANOLIC ROOT EXTRACT OF *MILLETTIA PINNATA* ON DPPH SCAVENGING ACTIVITY

CONCLUSION: From this study, it's concluded that the presence of phytoconstituents in the ethanolic root extract of *Millettia pinnata* suggests that the contribution of these compounds in the antibacterial and antioxidant activities should be evaluated. However, further studies will need to be undertaken to isolate and screening of bioactive compounds from the ethanolic root extract of *Millettia pinnata* and find out its biological activity.

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