Research Article

Quality Standardization and Characterization of Rutin Fraction and Protein Estimation with Antihelminthic Property from the Methanolic Plant Extract of *Olax Psittacorum*

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ABSTRACT

From ancient times, herbal plants are used for the treatment of various diseases due to its minimal or no side effect. The present study aims at isolation of flavonoid, rutin from the methanolic leaf extract of *Olax psittacorum*. Protein estimation and anti-helminthic activity of the methanolic plant extract was carried out. Fractionation was carried out using column chromatography method as the separation technique. The fractions were analyzed and compared by TLC (thin layer chromatography) using the standard. The solvent system selected was ethyl acetate: chloroform: butanol (4:2:4). The Rf value on TLC was observed 0.52. Antioxidant radical scavenging assay like DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid)diammonium salt) radical scavenging assay, Hydrogen peroxide (H_2O_2) assay, by taking ascorbic acid as standard. The regression value of standard was observed 0.938, 0.939 and 0.961 for DPPH, ABTS and H2O2 assay respectively and values observed for fraction was 0.936, 0.983 and 0.924 for DPPH, ABTS and H_2O_2 assay. Anti-microbial activity was carried out under gram negative and gram positive. For protein estimation modify lowery method was carried out. Anti-helminthic study was carried out using earthworms and piperazine (10mg/kg) was taken as standard. The paralysis time and death time was recorded and the result found to be effective on methanolic extract of the plant.

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INTRODUCTION

Tribal in India usually live on hill and is surrounded by forest areas. Besides the crops grown on the field, they mainly depend on the wild fruit and flower and leaves for them to supplement the diet and medicinal purpose. The forest fruits, leave, barks provide them resisting power to fight against diseases and provide energy. Green leaves and vegetables are mainly shows antioxidant properties[1]. Green leaves are very rich in vitamins, protein, minerals their extract were used in health benefits they are particularly rich in antioxidant and contain very rich amount of Vitamin-C, Flavonoids, and Carotenoids. Antioxidant properties that are present in leaves showing oxidative damage from oxygen process caused by free radical like O_2, OH, H_2O_2 is associated with cellular injury, inflammation. Herbal drugs are being used cost-effective and show minimum side effects.

The extraction procedure involves soaking the crude drug for a specified period. It is the separation of a medicinally active portion of plant tissue using selective solvent through the standard procedure. Like Soxhlet extraction, aqueous, counter-current extraction, microwave extract. The choice of solvent for extraction should be low toxicity, easy of the evaporator at low heat, preservative action.

Chromatography is a laboratory separation technique for the separation of a mixture that dissolved in the Fluid is a mobile phase and passes through another material called the stationary phase. In this method various constituents of the mixture travel on different speed through the stationary phase.

Infrared spectroscopy involves the interaction of infrared radiation with matter these spectroscopy techniques are used to identified and study the chemical composition of the compound. The sample may be solid; liquid on gas can be used for the study.[2]

Phytochemical analysis indicated the presence of various phytoconstituents like Flavonoid, Phenolic compound, tannins, Carbohydrate, Proteins, Amino acid, fats are should be analyzed by different chromatographic procedures.[3]

A thin layer of silica gel, Alumina on a slide flat inert substrate TLC is very versatile multiple samples can be separated frequently on the same layer. This process is very useful for screening of component the possibilities of cross-contamination is less [4].

Column chromatography is a separation technique in which the stationary phase inside the tube. The solid stationary phase coated with the liquid stationary phase whole of the tube. The mobile phase moves through the sample inside the tube

along with the stationary phase. The solvent is driven through the tube by applying positive pressure. Different rates of movement through the medium are calculated to the different retention time of the sample.

Ultraviolet-visible Spectrophotometry works on the principle of reflection on the absorption of light through the sample. UV/VIS Spectroscopy is mainly used in analytical chemistry for the quantitative determination of analyte like organic compound and biological micro molecules. The maximum absorbance is denoted as λmax [5].

IR spectroscopy is used for forensic analysis in criminal and civil cases. It can be used for the chemical identification of the compound. It is used for measuring the concentration of various compounds in the different food products [6].

EXPERIMENTAL METHOD

COLLECTION AND EXTRACTION

Leaves of *Olax psittacorum* were collected from local area of BHUBANESWAR, ODISHA, during rainy season of in the 1st week of August 2019. The chemical and solvents are collected from Merck Specialties private limited.

EXTRACTION

Fresh leaves of *Olax psittacorum*

were washed through tap water to remove the dirty particles and dried under shadow at room temperature for 1month. After complete drying, the leaves were crushed into powder. The powder was subjected to methanol, ethanol, pet-ether, water extraction by using a Soxhlet extractor apparatus. After extraction, the solvent vaporizes to produce sticky material.

% of yield = total amount of powder taken / total amount of sticky material×100.

PHYTOCHEMICAL SCREENING

Leaves of *Olax psittacorum* were subjected to various chemical procedures to ensure the percentage of Protein, Flavonoids, Quercetin, Reducing Sugar, Tannin, total phenolic contents present in them, using standard method.

QUALITATIVE ESTIMATION OF PROTEIN

MILON'S TEST

Small quantity of leaf extract when added with few drops of millions reagent, formation of white precipitate was observed that confirmed the presence of protein.

BIURET TEST

When 2ml of protein solution was mixed with 5-6 drops of dil. $CuSO_{6T}$ (1/10th dil. Fehling's solution with water). After that further add 3ml. 40% NaOH. Green colour appeared that indicates positive result for protein.

ESTIMATION OF TOTAL PROTEIN

The total protein estimation was carried out according to the modified lowery method. The assay was carried out by diluting some extract with 1ml. of water. After that Solution A- 2 g/L potassium sodium tatrate ($KNaC_4H_4O_6.4H_2O$) was mixed with 100g/L Sodiumcarbonate (Na_2CO_3) in 0.5M NaOH. And Solution B- 0.2g/l $KNaC_4H_4O_6.4H_2O$ and 0.1 g/L copper sulfate pent hydrate ($CuSO_4.5H_2O$) in 0.1M NaOH and then add 0.9 ml of solution, A was mixed with the extract. Then the solution was incubated for 10 min of time period at 50ºC and the solution was settled to cool down to normal room temperature40. Next 1 ml of solution B was added and left for 10 min. Again 3 mL of solution C (Folin–Ciocalteu phenol reagent in H_2O (1:16 v/v) ratio was added.Then the solution was incubated for 10 min time period at 50ºC. Then the solution was scanned under the wavelength of 200-800 nm to fix the maximum absorbance. A standard curve made by BSA difference. The dilution were made from the stock BSA solution in 5 test tubes ranging from 0.5 to 1 mg/ ml. and read the absorbance under 409 nm.[(7)]

ISOLATION OF FLAVONOIDS

Flavonoids were isolated from the extract by column chromatography along with their respective solvent system. The silica gel (60-180) was packed in a glass column and tapped for a better settlement of silica. Then extract was triturated with silica gel and the solvent system started to flow through the extract along with the stationary phase. The separation occurs on the column as a color band. And the adsorption of the solute molecule to the column occurs reversibly

TLC (THIN LAYER CHROMATOGRAPHY)

The fraction was collected from the column chromatography. Then the fraction was confirmed the compound by comparing it with the reference compound. The TLC plate was prepared by silica gel and was mixed with water and makes a layer on the TLC plate and dry under room tempreature and later in the oven for better dry. The fraction was collected from the column were run on the TLC with their respective solvent. The movement of fraction confirmed by the RF value as compared to reference compound Rutin[(8)]

UV -VIS SPECTROSCOPY

UV – Vis analysis was performed for qualitative analysis of the compound, where fraction was checked for the qualitative of flavonoids from the fraction to the column and compared with pure compound rutin. Then some amount of dry fraction was taken and the standard

rutin was powdered and made a solution with the same solvent system and taken for scanning of UV range from 200 -800 nm.

IR (INFRARED SPECTROMETER)

IR scan confirmed the chemical composition of the drug by using the IR light. The appropriate amount of fraction was collected from column chromatography. Same quantity of standard crude compound rutin was taken and made a solution with methanol solvent adjusting the pH 4.5 to 7.5. 0 then the compound was under IR individually and overlapping the two graphs for the confirmation of flavonoids.

IN VITRO ANTI-OXIDANT ACTIVITY

The most prevalent anti-oxidant assay are DPPH free radical, ABTS free radical and hydrogen peroxide assay and were performed as follows.

DPPH RADICAL SCAVENGING ASSAY

DPPH assay was carried out, preparing 0.24 mg/mL solution labelled as stock solution. By utilizing the methanol solvent the stock solution was diluted and spectrophotometrically checked at 517nm to get 0.980 ±0.020 absorbance. Here ascorbic acid was taken as the standard. 100 µl fraction was taken and added with three ml of prepared stock solution. Concentration of 50-250 µg/ml both the fraction and the standard was prepared for measurement of % scavenging of the DPPH.

% of yield = Control absorbance at 15mins – sample absorbance at 15mins/ control absorbance × 100.

ABTS RADICAL SCAVENGING ASSAY

To carry out the study mixed the total amount of 4.9mm/litre of potassium persulphate and 14 mm/litre solution. The solution was shaken properly and kept the solution of mixture at normal temperature for 16 hour time in the dark condition. The solution was kept for 16 hours to producing cation radicals and labelled it as stock solution. Here ascorbic acid was taken for the standard. By utilizing the ethanol solvent the solution was diluted and spectrophotometrically checked at 745 nm to get 0.700±0.020 absorbance. 100 µl fraction was taken and added the fraction with three mL of ABTS stock solution. The solution was shaken properly and kept it in normal temperature for 5minute period of time. The solution was checked under spectrophotometrically at 745 nm and the absorbance was noted. Concentration of 50-250 µg/ml both the fraction and the standard was prepared for

measurement of % scavenging of the ABTS.

% scavenging of the ABTS= control absorbance at 5mins – sample absorbance at 5mins/ control absorbance at 5 mins × 100.

HYDROGEN PEROXIDE (H_2O_2) ASSAY

The assay was carried out by preparing a solution of 40 mM of H_2O_2 and mixed it with 0.2 mole/liter phosphate buffer having pH 7.4. 100µl of Concentration and 50-250 µg/ml concentration both the fraction and the standard were taken and mixed it with the stock solution of H_2O_2. Then the solution was shaken properly and kept the solution at room temperature for 10 minutes. After 10 minutes period of time, the solution was spectrometrically checked at 230nm.

% hydrogen peroxide Assay= control absorbance at 10mins – sample absorbance at 10mins / control absorbance at 10mins × 100.

ANTIBACTERIAL STUDY

The antibacterial activity study was tested against 3gm positive bacteria. Escherichia coli, pseudomonas aeruginosa, vibrio cholera, and acinetobacter baumannii bacterial was taken. All the microorganisms were obtained from the laboratory of microbiology RMRC (regional medical research center), Bhubaneswar. The antibacterial study of the flavonoids obtained from the *Olax psittacorum* leaves using a well definition method preparing the mha plate with dissolve 3.8 gm/ medium in 100ml of water and autoclave for 15 min at 121ºC and cooled down and poured into sterilized petridish. After the plating, the prepared bacterial strain wish saline water as comparing with macfarland standard 100ml of bacterial strain pipetted out and spread over the plate by using spreading rod till dried properly. Deep well on the plate 6mm diameter by the sterile cork borers. The solution of the 500mg/ml. Rutin isolates with methanol, ethanol, n-hexane, chloroform was prepared. From these stock solutions one hundred microliters were diffused in well and the petridish were kept in the incubator at 37ºc for 24 hours.

ACUTE ORAL TOXICITY STUDIES[9]

Toxicity study in the animal model was done to determine the dose level recognition for the treatment of disease as a drug. This dose level of characterization and the advice effect can be calculated by doing the following test. OECD guideline 420 was followed to carry out the toxicity study. The animals were 1st goes for fixed-dose for sighting study. In this, the

test substance is given to animals orally determine the fixed-dose for the main study, the starting dose for sighting study fixed dose level 5, 50, 300, 2000(mg/kg), and observed the animal for 24 hours of dosing of each animal.45 According to the guideline 5mg/kg bodyweigt,50 mg/kg bodyweight, 300 mg/kg bodyweight ,2000 mg/kg bodyweight mentioned. the study was started from lower dose to higher. if the rat was survived by given first dose5mg/kg body weight then moved towards second dose 50 mg/kg bodyweight. if the rat was survived by given second dose then moved towards 3rd dose 300 mg/kg bodyweight. If the rat still survived by 3rd dose then increased dose level to 2000 mg/kg bodyweight After the sighting study, the dose level in shifting study that dose level is not accepted for the main study. For the main study, the fixed-dose is given to the group of animals and observes for 14 days and changed behavior observed.

ANTI-HELMINTHIC ACTIVITY [10-16]

The anti-helminthic activity of *olax psittacorum* leaf extract was tested against the adult earthworm (phenctima posthuman) to evaluate. The earthworms are collected from the moist soil and washed with saline water to remove all the contaminant particles from it. The test solution prepared in the concertation of 10,20,30,40, 50 mg/ml. with methanolic extract with saline water and poured into the Petridish up to 25ml. Then all the earthworms were dipped into the different concentration of test solution. After that Piperazine was taken as a standard drug in the 10, 20, 30, 40, 50mg/ml concentration. After Deeping of all the worms in both test and standard solution at room temperature and observe the time of paralysis and no movement of worms and the death time should also in terms of the minute.

RESULTS AND DISCUSSION

EXTRACTION

The percentage yield of leaf extract by taking 700gm of O. *psittacorum* leaves powder were extracted using methanol is 47.2%.

Table 1: yield of extraction of powder leaves

	Amount of powder leaves	Amount of concentrate obtain	%of yield
L.M.E	700gm	330.4	47.2%

QUALITATIVE TEST OF PROTEIN

TOTAL PROTEIN ESTIMATION

The total protein estimation of plant extract was carried out by modified Lowry method scanning value of dilute extract at 409nm is maximum with absorbance 0.832. And this concentration is apply over the BSA calibration standard curve at 409nm.

Table 2: Calibration curve for BSA solution

Concentration	Absorbance
0.625	0.172
1.5	0.29
2.5	0.415
5	0.63
10	0.087

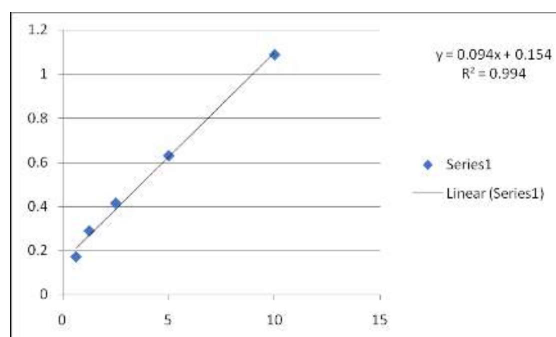


Figure 1: calibration curve of BSA for total protein estimation

$Y=0.094x+0.154$

⇨ $0.832=0.094x+0.154$

$X= (0.832-0.154)/0.094=7.22$mg/ml

The total assay of protein in olax psittacorum leaves 7.22 mg/ml.

From the protein test perform the plant contents 7.22 mg/ml amount of protein in the extract which is present in the extract according to our study which is very nominal value. Further protein isolation can be performed and characterized to achieve some potent functional application

ISOLATION OF FLAVONOIDS

Column chromatography-column chromatography was used for the isolation of rutin by using the solvent system N-hexane: ethylacetate: chloroform: butanol(0.1:2:1:2)

Figure 2: fraction was separated and collected by column chromatography

TLC

By using ethyl acetate:chloroform: butanol (4:2:4) solvent system yellow spot was appeared with the reference rutin.

Isolate rutin Standard rutin

Figure 3: Thin layer chromatography for fraction was observed Identification of rutin by spectroscopy methodUV-Visible spectroscopy

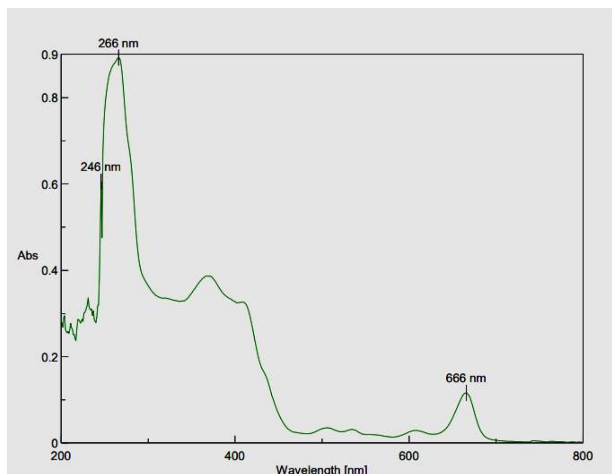


Figure 4: UV- vis spectra of isolate rutin

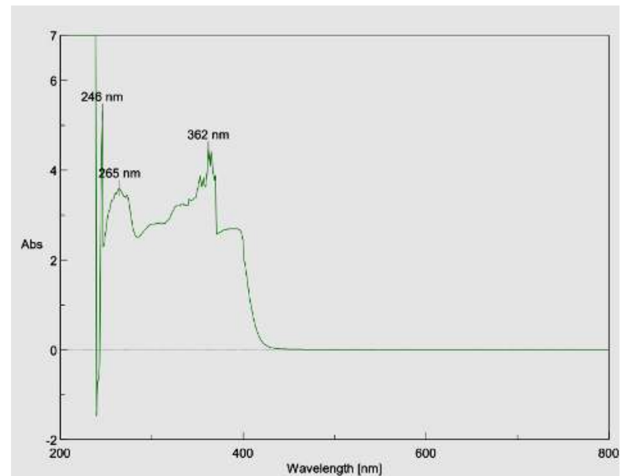


Figure 5: UV – Vis spectra of standard rutin

FTIR SPECTROSCOPY

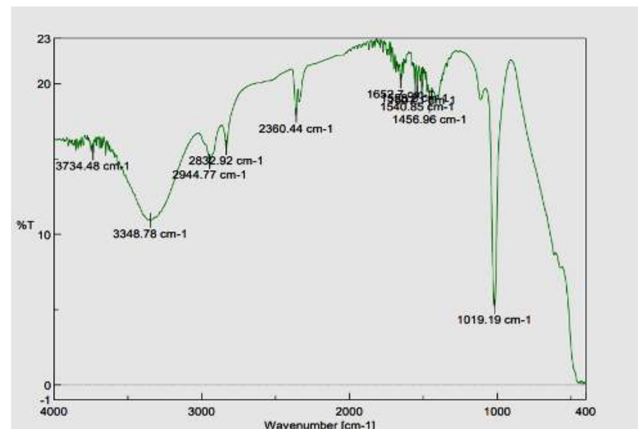


Figure 6: FTIR spectra of standard rutin

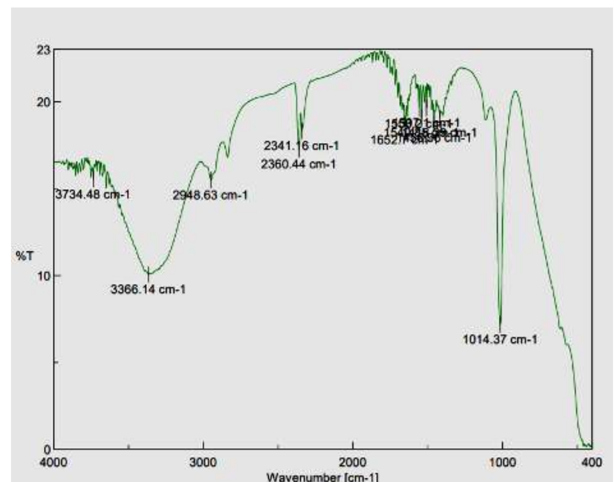


Figure 7: FTIR spectra of isolate rutin

Above spectrum is isolate rutin which was mixed with the methanol specific quantity and compare with the standard one.

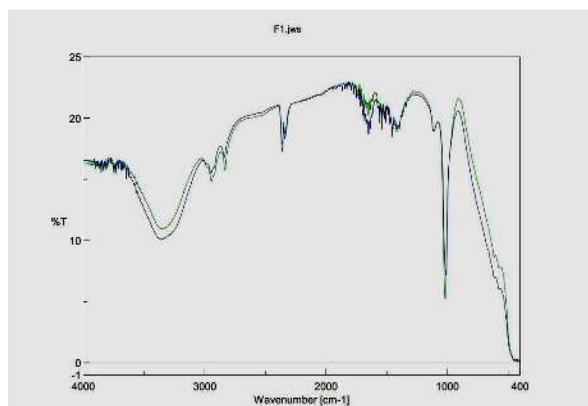


Figure 8: FTIR overlay spectra of fraction sample and standard

Above diagram show the comparative spectra of rutin and standard rutin. The Flavonoid isolation study was carried out by column chromatography by using solvent system N-hexane, ethyl acetate, chloroform, Butanol (0.1:2:2:1) ratio and sufficient amount of flavonoid was collected by appearance of hinoda test for identification of flavonoids further to identifying the particular flavonoid using preliminary test like TLC and spectroscopic methods were performed. From the TLC study using solvent system Ethyl acetate , chloroform , Butanol (4:2:4) yellow spot was observed on the TLC plate along with standard rutin and the Rf value was found to 0.17 (Rf value=distance travel by sample / distance travel by solvent) Uv visible spectroscopic method was performed for peak identification of isolated rutin was compared with standard rutin from fig no 7 and 8 it was found that maximum 57 wavelength 246nm, 265nm, 362nm found in standard and 246nm, 266nm, 666nm found in isolate shown in fig 7 and 8 Further FTIR study was carried out , as shown in figure 10 the overlay spectra of fraction sample and standard reveals the probable presence of rutin in the isolate.

ANTI-OXIDANT ACTIVITY

DPPH (2, 2- diphenyl-1-picrylhydrazyl) radical scavenging assay

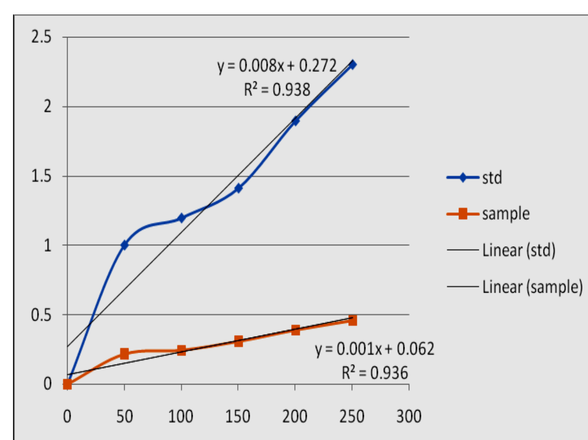


Figure 9: DPPH (2, 2- diphenyl-1-picrylhydrazyl) radical scavenging assay)

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical scavenging assay

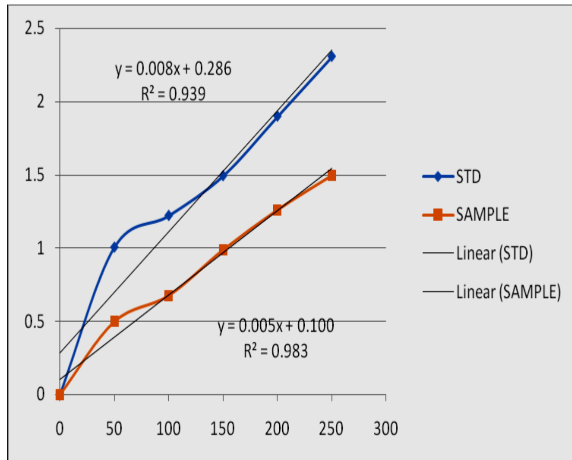


Figure-10: ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical scavenging assay

HYDROGEN PEROXIDE (H_2O_2) ASSAY

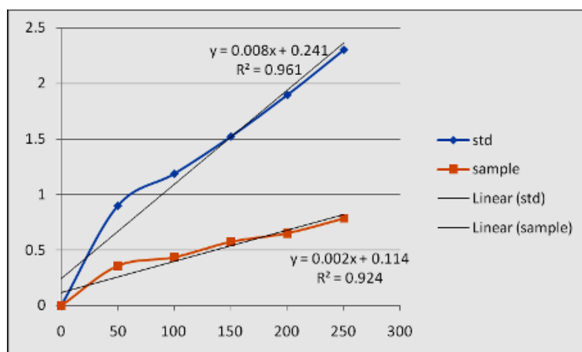


Figure11: Hydrogen peroxide (H_2O_2) assay

Rutin is a flavonoid and has good antioxidant properties from the methanolic extract of *olax psittacorum,* rutin fraction was collected using solvent system. N-hexane, ethyleacetate, chloroform, butanol (0.1:2:1:2) ratio The radical scavenging assay was perform using DPPH, ABTS, H_2O_2, and the assay value was found to be 0.936µg/ml, 0983µg/ml ,0.924µg/ml with the standard value 0.938µg/ml, 0.939µg/ml, 0.961µg/ml respectively as shown in figure 9, 10, 11.

TOXICITY STUDIES

The animals were introduced for fixed-dose for sighting study. In this, the test substance was given to animals orally determine the fixed-dose for the main study, the starting dose for sighting study fixed dose level 5, 50,300,2000(mg/kg), and observed the animal in 4hr and 24 hours after dosing of each animal. After the sighting study, the dose level in sighting study that dose level was not accepted for the main study. For the main study, the fixed-dose was given to the group of animals and observed for 14 days. The study rutin was not shown any side effect in both sighting study and main study.

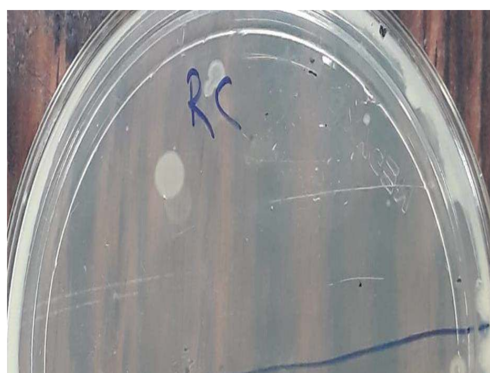
TABLE NO-3 Orally toxicity study table

RAT	DOSE mg/kg	OBSERVATION		
		4hr	24hrs	14days
1	5	Not showed any effect	Not showed any effect	
2	50	Not showed any effect	Not showed any effect	
3	300	Not showed any effect	Not showed any effect	
4	2000	Not showed any effect	Not showed any effect	Not showed any effect

By following the OECD guideline 420 after dosing of all dose level orally there is no showed any side effect on any dose level and there is no sacrifice of any rat model.

ANTI-BACTERIAL ACTIVITY

500mg/ml solution was prepared using methanol, ethanol, chloroform and n-hexane for this activity. It was shown the activity on Pseudomonas aeruginosa in chloroform solution.



No bacterial growth seen on the plate on chloroform solution of isolate

Figure 12: Anti microbial activity of isolate rutin in chloroform solution

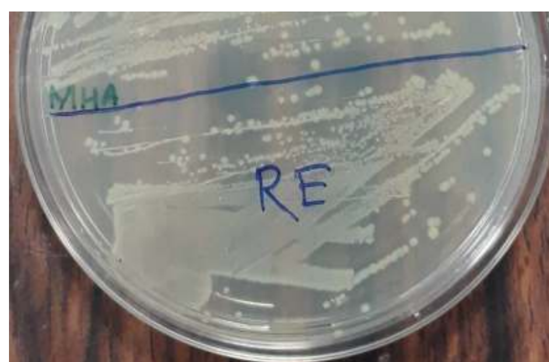


Fig13:Antimicrobial activity of isolate in ethanol solution

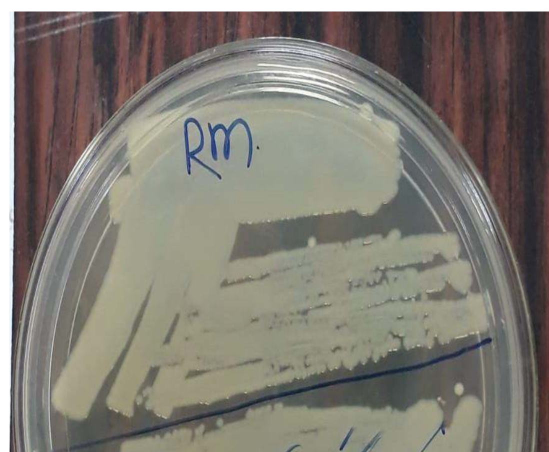


Figure 14: Antimicrobial activity of isolate in methanol solution

Figure 15: Antimicrobial activity of isolate in N-hexane solution

From the above anti-microbial study on our isolate was soluble in methanol, ethanol, chloroform, N-hexane solvents and test against pseudomonas aeruginosa gram negative bacteria and seen that only in the chloroform solution of isolate show clear zone of inhibition which clear seen in the figure 14.

ANTIHELMINTIC ACTIVITY

The in vitro and the paralysis time and time were recorded. The results show that the (10mg/ml) concentration, piperazine show best activity for death time (130.63±6.9 min) and the methanolic extract of *olax psittacorum* leaves (146±6.9 min), for the 30 mg/ml of piperazine show high activity against the worms (83±2.0 min) and methanolic extract show death time (96±4.1 min), for 50 mg/ml conc. Piperazine least death time (54± 3.8 min) and the methanolic extract show death time (69±6.5 min). The time of paralysis time given in table-2, and table-3.the study concluded that the methanolic extract of plant leaves show maximum activity at higher conc. (50 mg/ml) compare with the standard piperazine 50mg/ml.

Figure 16: Earth worm treat with std(piperazine)

Figure 17: Earthworm treat with methanolic extract

TABLE 4: **Treatment of standard against helminthiasis**

Treatment	Concentration(mg/ml)	Time of paralyze(min)	Time of death (min)
	10	67.3±2.3	130.63±6.9
Pirperazine Std.	20	55.46 ±4.30	102±2.4
	30	43.30±1.6	83±2.0
	40	34.54±0.6	66±1.7
	50	30.46±2.3	54±3.8

TABLE 5:Treatment of methanolic extract against helminthiasis

Treatment	Concentration(mg/ml)	Time of paralyze (min)	Time of death(min)
	10	82±3.21	146±6.9
Olax psittacorum leaves	20	67±3.6	137±4.8
	30	54±5.6	96±4.1
	40	42±2.14	81±3.2
	50	36±1.32	69±6.5

Figure15 shows that earthworm treat with standard piperazine and figure16 shows that earthworm treat with methanolic extract of *Olax psittacorum* leaves, from which time of paralyze and the time of death was noted down on above table no6 and 7 the methanolic extract was subjected to antihelmintic activity which shows good results when compared with the standard drug.

CONCLUSION

From the above experiment on the methanolic extract of *olax psittacorum* leaves it was concluded that collected fraction may be rutin which was confirmed by using TLC, UV-Visible, and FTIR. The study shows good antioxidant activity and antibacterial activity against. *Pseudomonas aeruginosa* the methanolic extract was subjected to antihelmintic activity which shows good results when compared with the standard drug. Toxicity study was performed on the isolate rutin

following OECT guideline 420. This isolate can be further studied for mouth ulcer activity and quantification in plasma and so on.

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