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"Isolation, Identification and Characterization of Flavonoids from leaves of *Annoma Squomosa*"

(Sagar Institute of Research and Technology –Pharmacy, Sage University, Bhopal (M.P)

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Abstract: Flavonoid is major phenolic compounds are becoming the major subject of medical research. They have been reported to possess many useful properties, including estrogenic activity, anti inûammatory activity, enzyme inhibition, antimicrobial activity. For centuries preparations that contain flavonoids as the principal physiologically active constituents have been used by physicians and lay healers in attempts to treat human diseases. Various medicinal importance of rutin are anti-hyperglycemic and antioxidant activity. Isolation of flavonoid from leaves of *Annoma Squomosa* by using TLC, Column Chromatography. Characterization by UV & FTIR.

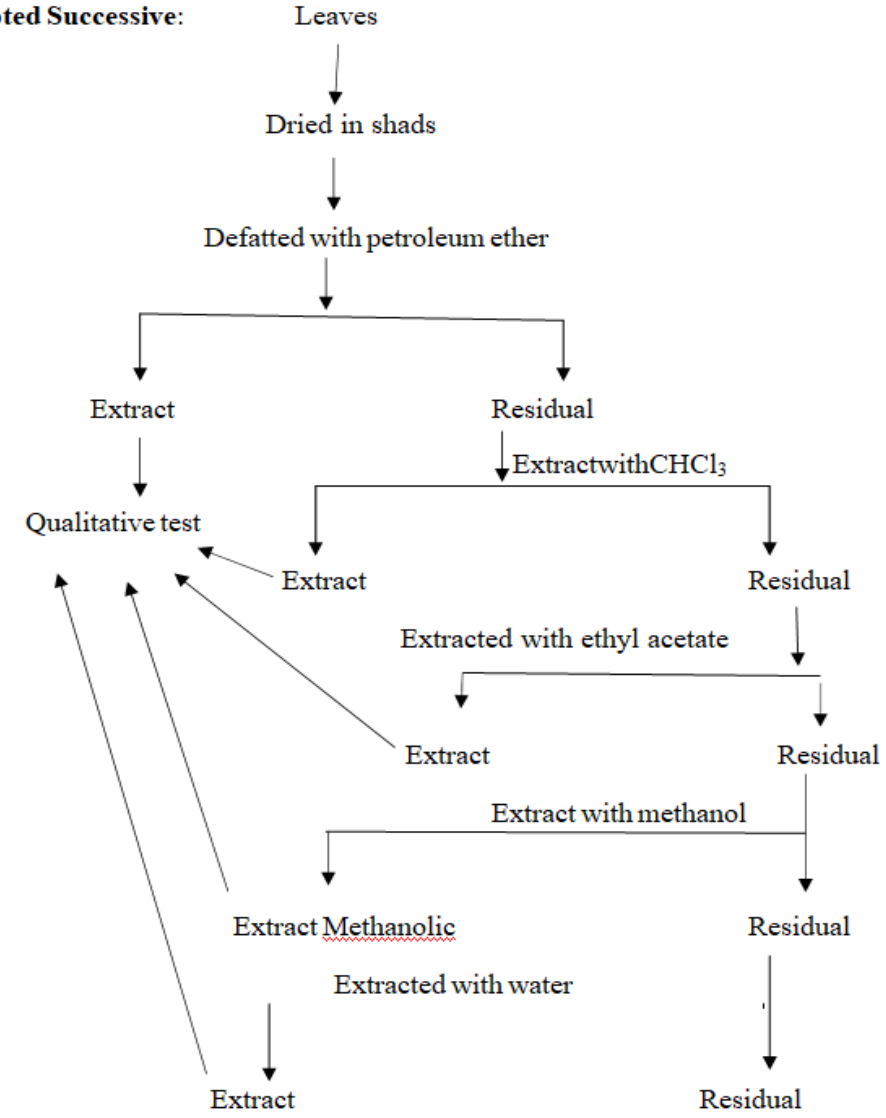
Key Word: Isolation Methods for Flavonoids, Phytochemical Investigation, TLC, HPLC, UV, FTIR etc.

Introduction: *Annona squamosal* L. (Annonaceae), also known as "custard apple," is a tropical, endemic species of the West Indies, South and Central America, Ecuador, Peru, Brazil, India, Mexico, Bahamas, Bermuda, In India, as reported by the Indian Council of Agricultural Research (ICAR), *Annona squamosal* is extensively cultivated in various states (Maharashtra, Gujarat, Madhya Pradesh, Chhattisgarh, Assam, Uttar Pradesh, Bihar, Rajasthan, Andhra Pradesh, and Tamil Nadu) with a total area of 40,000 ha. *Annona squamosa* has been widely grown in India for its fruits, apart from its leaves, stem, and roots, which are also important because they possess nutraceutical and pharmaceutical value. *Annona squamosa* is known for its edible fruits, and the tree grows as a small sapling, rising from 3 m and reaching up to 8 m, with large, randomly spread branches having brownish or light brownish bark with thin leaves.[1] *Annona squamosa* has been utilized as a natural medicine and in various other food applications, e.g., its pulp is utilized as a flavoring agent in ice cream, and 50–80% of custard apple fruit is edible and can be pulped as juice. It contains appreciable vitamin C in the range of 35–42 mg per 100 g, and dietary fiber, vitamin B1 (thiamine).

Material Methods:

Plant materials: Leaves of *Annona squamosa*, Family **Annonaceae**.The aerial part (leaves) of ***Annona squamosa*** (Custard Apple) were collected in month of April (01/04/2022) from MP .They were authenticated from Botanical survey of India .Leaves of ***Annona squamosa*** were collect and kept for Shade drying at a room temperature. Dried leaves convert into course powdered with the help of mixer.

Extraction adopted Successive:



Preparation of extract of leaves of *Annona squamosa* by using extraction method

The coarse powder of leaves (100g) was extracted with Petroleum ether (400ml) for 48hrs. at 60 to70°C for defatting of leaves powder of *Annona squamosa*

⇕ Petroleum ether extract

And then defatted extract of leaves of *Annona squamosa* were extracted with Chloroform (400ml) for 24 hrs at 70°C to 80°C

⇕ Chloroform extract

Then extracted with (400ml) ethyl acetate for 24 hrs at 70°C to 80C° then

⇕ Ethyl acetate extract

extract with (400ml) methanol at 80°C to 85°C for 24 hrs.

⇕ Methanolic extract

then further extracted with water for 2 hrs

⇩

Then all extract was evaporated under vacuum in a Rotator evaporator upto50ml.

Specific method for isolation of rutin: 4gm of methenolic extract was dissolved in methanol (20ml), then added with 30 ml petroleum ether in a separating funnel, and kept for some time.

⇩

Then discard petroleum layer and this process are repeat three times. Then alcoholic layer was evaporated toup10ml

⇩

Then added 20ml of chloroform with alcoholic extract in a separating funnel, then remove chloroform layer and methanolic layer kept in a refrigerator for overnight.

⇩

Solid yellowish–Brown crystal was appeared

⇩

Phytochemical screening

⇩

TLC & Characterization by UV, IR, LC -MS, MS, NMR

Physicochemical evaluation of crud drugs

Extractive value: The extract obtained by exhausting crud drug with different solvents measure soft heir chemical constituents.

Determination of solvent extractive value.

The extraction of any crude drugs with the amount a particular solvent yield a solution containing various Physicochemical constituents .The composition of chemical constituents in that particular solvent depend on the nature of drug and solvent.

- Water soluble extractive value.
- Alcohol soluble extractive value.

Swelling index:

Swelling index is defined as the volume of ml taken up by the swelling of 1 g of plant materials under specific condition.

Determination of swelling index:-

Swelling index of plant materials were determined using the modified method which was reported by Gauthami and Bhat**.**1 g coarse powder leaves of *Annona squamosa* was accurately weighed, then transferred to measuring cylinder (100ml). The volume occupied by coarse powder of *Annona squamosa was* to be noted .Then poured 60 ml distilled water in a measuring cylinder and cylinder was closed by suitable closure, continuously shaking then allow stand for 24 hrs. The volume occupied by swollen powder leaves of *Annona squamosa* was to be noted after 24 hrs. Swelling index is expressed in percentage and was calculated by following equation.

Swelling index % = V_f-V_0/V_0x100 Calculated value Shaw in result & discussion.

Physicochemical characterization and preliminary qualitative investigation

Photochemical screening: - Photochemical investigations were conducted employing different phytochemical test and the test phytochemical constituents were detected as elaborated by Khandelwal (2001) and Kokate, (2001). Phytochemical screening is done by following procedure which shows in table.

S. No.	Reagent	Method	Expected Observation
1.	Shinoda Test	2 ml of filtrate few drops of cone. HCl and magnesium turnings were added.	Mag Pink Red enta
2.	Alkaline Reagent Test	2 ml of filtrate + NaOH solution were added and few drops of dil. HCl.	Intense yellow color Which turns to color less when add HCL.
3.	Lead Acetate Test	2 ml of filtrate + 10% Lead acetate Solution was added.	White precipitate.

Qualitative characterization of isolated compound

Qualitative characterization of isolated compound by various analytical techniques HPLC, IR Spectroscopy, Rutin was dissolved in 2 ml methanol in a volumetric flask (10 ml) then volume make up to mark, then prepared sub-stock solution (100 mcg/ml) and show absorbance in Shimadzu UV- Spectrometer in between range 200 to 500 nm.

Preparation of sample:-

Dry isolated methenolic extract (isolate from plant) was dissolved methanol (volumetric flask).Methanol was taken as reference solvent. Then volume makeup to the mark and show absorb Shimadzu UV Spectrometer

Characterization by High Performance Liquid Chromatography:

High-performance liquid chromatography is a separation technique which is used for separation, isolation and identification of mixture of compound. HPLC analysis was performed by using Shimadzu HPLC system, LC solution software.

Preparation of mobile phase:-

The mobile phase prepared by mixing of acetonitrile and water with 0.5% phosphoric acid (70: 30) and maintains pH 3.2. The mobile phase was degassed by sonication with the help of sonicator and they were filtered through vacuums filtration before HPLC analysis.

Preparation of standards:-

We prepared stock solution (1000 mcg/ml), by 10 mg standard rutin was accurately weighed and dissolved into 2 to 3 ml methanol in a volumetric flask (10 ml) and volume make by methanol up to the mark. The methanol is a reference solvent. Then sub stock solution (100 mcg/ml) was prepared from previously prepared fresh stock solution. Then prepared further dilution 10 mcg/ml solution was prepared from sub-stock solution, then it was filtered through 0.4 µm membrane filter. The samples were analyzed by RP-HPLC for qualitative estimation of standard rutin by Shimadzu model HPLC equipped with, O.D.S. Hypersil C18 Column (250 mm × 4.6 mm, 5 µm particle size), flow rate 1 ml/min. and wavelength (270nm and 340nm) programmable UV/VIS detector and built-in L.C. solution software was used for drug analysis.

Preparation of Sample:

Take 1mg of isolated compound dissolved in 2 to 3 ml of methanol in a volumetric flask (10 ml) and make up the volume by methanol up to mark. Than it was filtered through 0.4 µm membrane filter. The samples were analyzed by RP-HPLC for qualitative estimation of rutin by Shimadzu model HPLC equipped with, O.D.S. Hypersil C18 Column (250 mm × 4.6 mm, 5 µm particle size), flow rate 1 ml/min. and wavelength (270 nm and 340 nm) programmable UV/VIS detector and built-in L.C. solution software was used for drug analysis. Then compared chromatogram of standard rutin and isolated rutin. And compared RT value of both standards rutin and isolated compound.

Characterization by IR: -

IR spectroscopy is the most convenient technique which is used for identification functional group of the compound. The bond between the vibrate atom and vibration motion- Stretching and bending. In stretching, change the bond length and the in bending change the bond angle

Bonding divided in two different type Wagging, Scissoring, Rocking and Twisting. The infra-red portion of the electromagnetic spectrum is generally classified into three regions, near, mid and far-infrared. Visible reason of IR spectra is approximately 1400 to 4000.

The IR spectrum of isolated constituents was determined by Jasco Japan spectrometer. We prepared sample for IR characterization few amounts of isolated compound (1 to 2 mg) mixed with KBR (2mg to 4mg) prepared sample. And Sample take in a sample holder, then placed into spectrometer and Shaw spectra. IR spectra of isolated compound were compared with IR spectra of standard rutin.

Result & Discussion

Swelling index: -Swelling index of leaves of *Annoma Squmosa* was to be calculated.

Initial volume occupied by leaves powder = 15

finally volume occupied by leaves powder = 25

SI %= 25 -15 /15 x100

SI % = 66.66%

Phytochemical screening.

The result of different type of qualitative test performed in the laboratory which is shaw table. The presence various phytochemical constituents like Alkaloids, Flavonoids, Phenoliccompound, Steroids' and Amino acid. Positive (+) sign is indicating presence and Negative (-) sign is indicating absence of phytochemical constituent.

S. No.	Reagents & Test	Sample extract	Pt. Ether Extract	Chloroform Extract	Et. Acetate Extract	Methanol Extract	Water Extract
1.	Shinoda Test		-	+	+	+	-
2.	Alkaline Reagent Test		-	-	-	+	+
3.	Lead Acetate Test		+	+	+	+	+
4.	Zinc Hydrochloride Test		-	-	+	+	-

Qualitative characterization of isolated compound

Characterization by UV-Visible spectroscopy:

UV Spectra: - The UV spectra of rutin in a methanolic solution show two absorbance bands at257 and 360 nm

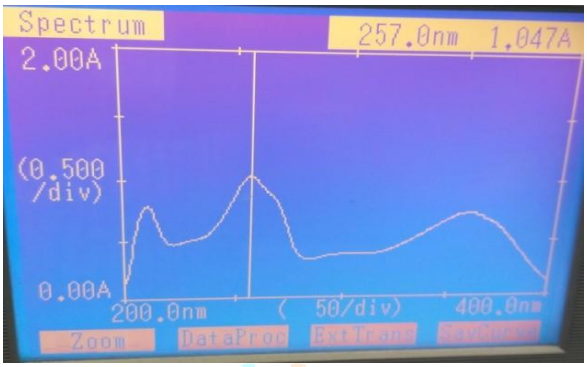


Fig. UV Spectra of standard rutin.

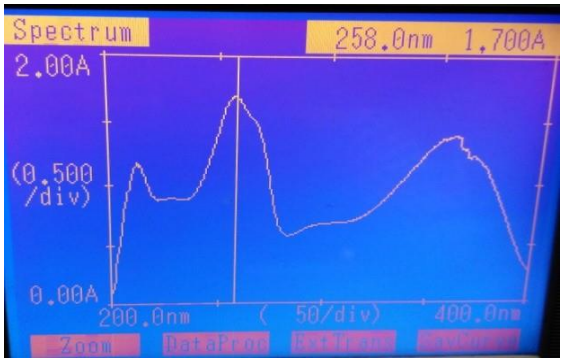


Fig. UV Spectra of isolated compound.

OBSERVATION

S.no.	Solvent	Λ max of standard rutin	λ max of isolated compound
1.	Methanol	257 nm	258 nm

Characterization by HPLC:

Isolated compound was characterized by most convenient technique HPLC. Compared chromatogram of standard rutin and isolated rutin. And compared RT value of both standardsrutin and isolated compound. Which show in Fig no.

Fig.Chromatograph of standard rutin

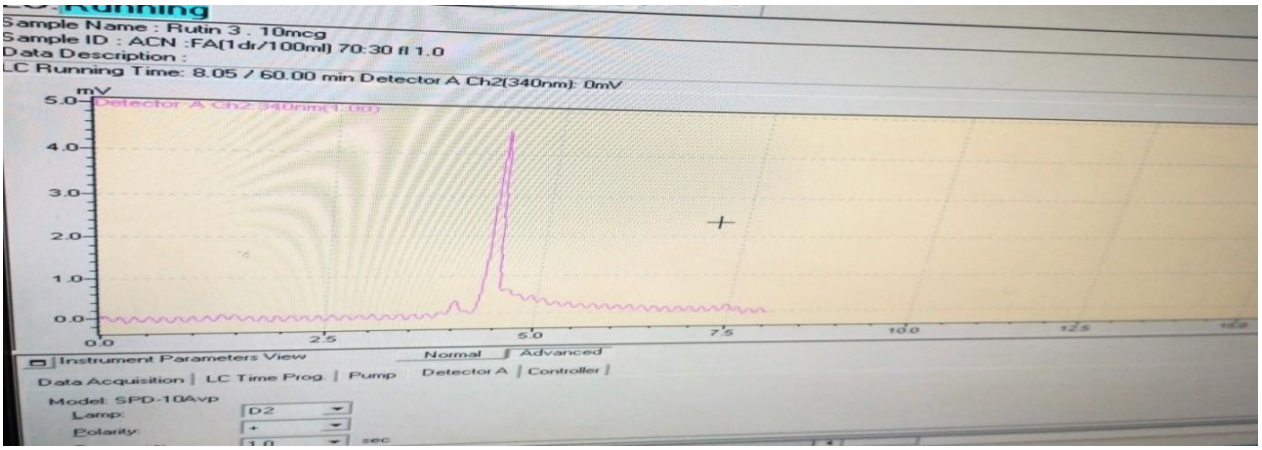


Fig.Chromatograph of standard rutin

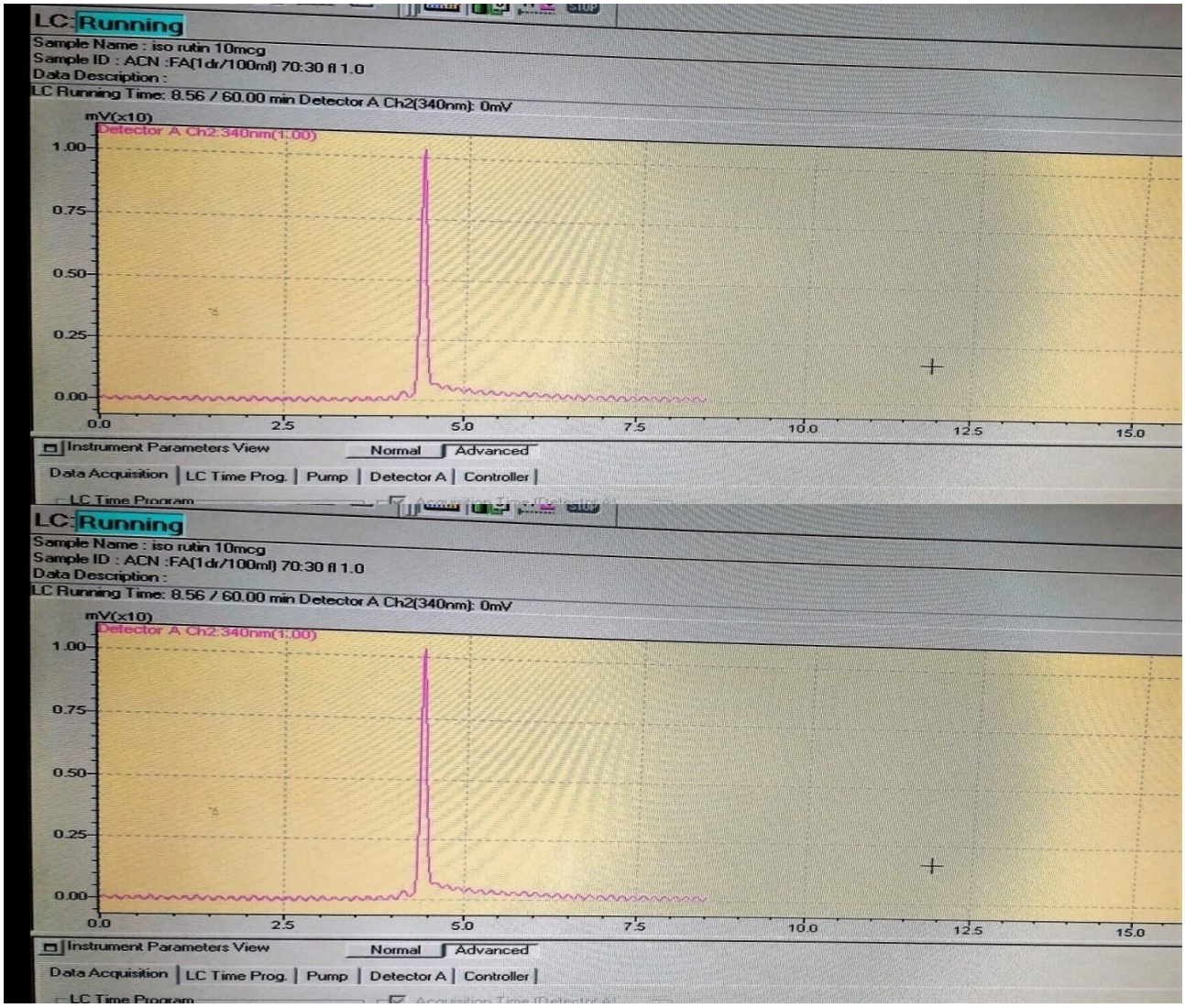


Fig. Chromatogram of Isolated compound

Observation Table.

S. No.	Compound	Mobile phase & ratio	λ max	RT value
1.	Standard	ACN: water with formic acid (70:30)	272	4.43
2.	Isolated compound	ACN: water with formic acid (70: 30)	272	4.41

Characterization by IR:

It's a most convenient technique for identification of functional group Identification of isolated compound by using IR spectroscopy. Compare IR spectra of standard rutin, and IR spectra of isolated compound which is show in given spectra.

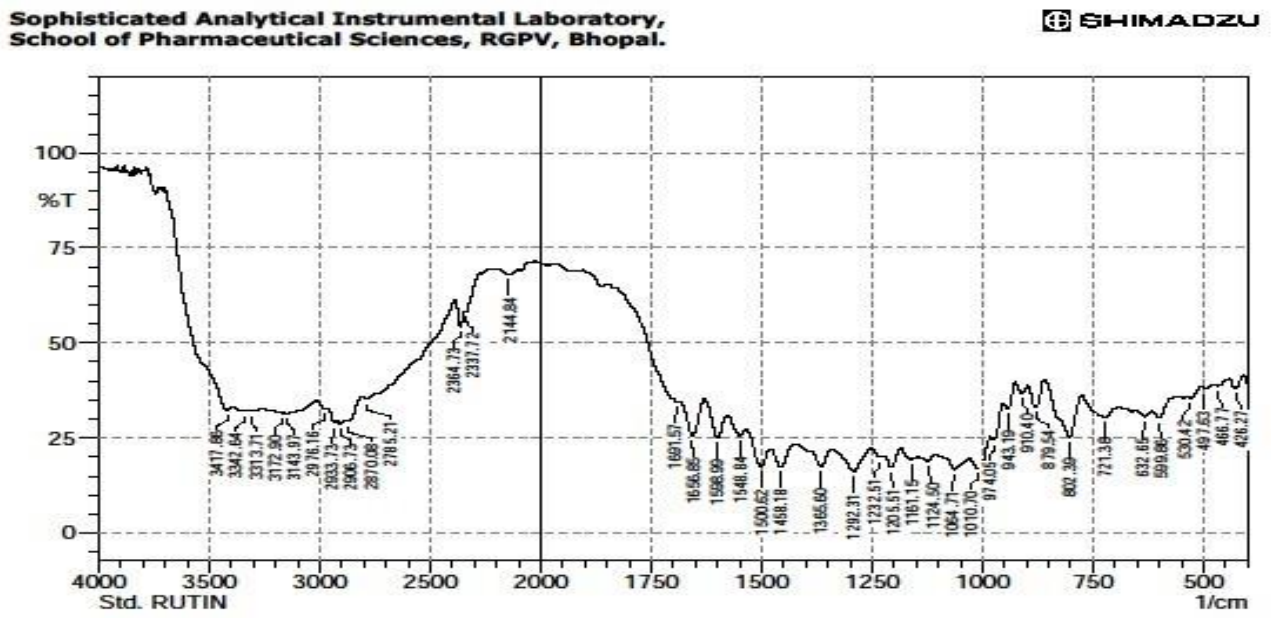


Fig 6.8 IR Spectra of Standard rutin

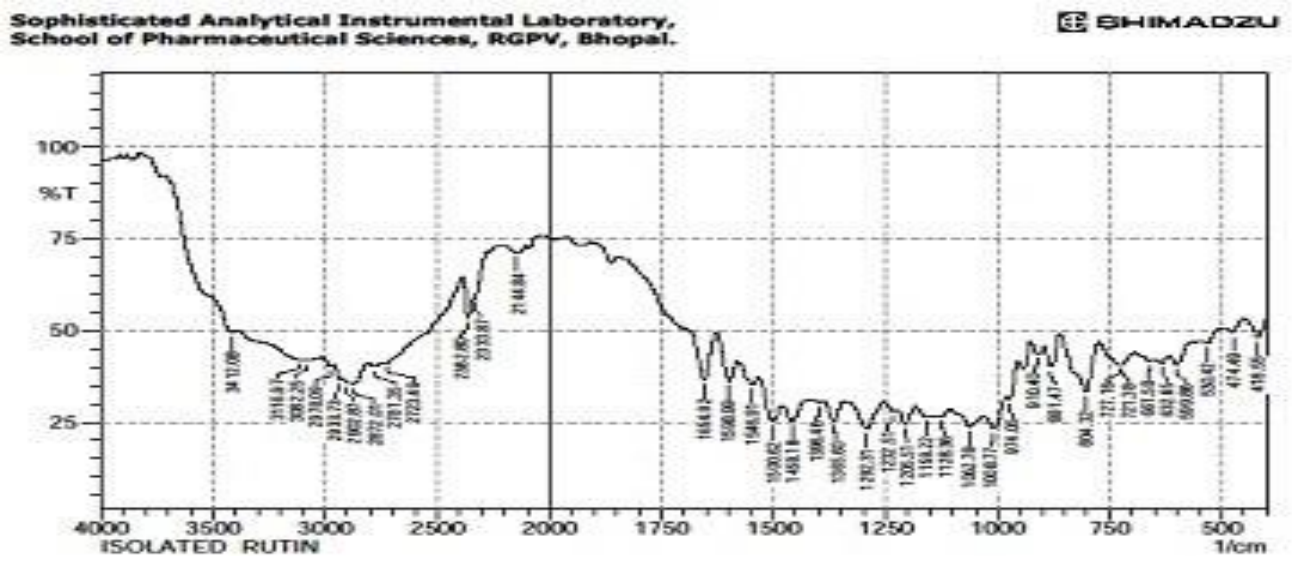


Fig. 6.9 IR Spectra of Isolated Compou

Observation table of interpitation of IR spectra

S. No.	Functional group	Interpitate value
1	-OH	3412.08
2	-OH Str.	3082.25
3	Phenolic -OH	3412.08
4	C-H starching	2978.09 , 2933.02
5	C=O aromatic	1654.98
6	C=C aromatic	1558.99
7	CH	2872.01

IR Spectrum of isolated compound Fig .6.9. IR Value: 3412.08 due to phenolic compound, 2978.08 Value is due to C-H Starching, 1654.98 value due to aromatic.

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