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Isolation, Phytochemical Investigation and Biological Screening of Flowers of *AVERRHOA BILIMBI*

Akshay C*, D.Visagaperumal and Vineeth Chandy

Department of Pharmaceutical Chemistry, T. John College of Pharmacy
Bangalore, Karnataka

ABSTRACT

The present study comprises phytochemical evaluation of flowers of *Averrhoa bilimbi* in different extracts by using standard methods. The phytochemical evaluation was carried out for the screening of bioactive compounds. The herb possesses many phytochemicals, marked for its remarkable properties such as antioxidant, anticancer, antibacterial and anti-inflammatory activity. The study was subjected to investigate antidiabetic activity of ethyl acetate extract of flowers of *Averrhoa bilimbi*, which was assessed by α-amylsae inhibitory action. HPLC analysis was carried out for estimation of standard compound in ethyl acetate extract of flowers. The study confirmed that ethyl acetate extract of flowers of *Averrhoa bilimbi* showed significant antidiabetic activity.

Keywords: *Averrhoa bilimbi,* bilimbi, phytochemicals, antidiabetic, α-amylase.

Corresponding Author :Akshay C, Department of Pharmaceutical Chemistry,T.John College of Pharmacy,Bangalore, Karnataka 560083, India
Email:akshaybkn88@gmail.com

1. INTRODUCTION:

Averrhoa bilimbi has been recognized as an important herb for treating various physiological disorders worldwide. *Averrhoa bilimbi* commonly known as bilimbi. It has been reported that *Averrhoa bilimbi* provides key nutrients, good sources fiber, improve insulin sensitivity, and boost immune system. It exhibits a remarkably high therapeutic and safety profile that makes it popular as a health enhancer and food supplement worldwide. *Averrhoa bilimbi* parts including fruits, bark, seeds, leaves, and flowers are utilized on their own for individual nutritional and therapeutical values, however, the leaves is considered to contain the most valuable chemical compounds. *Averrhoa bilimbi* (Bilimbi) is medicinally used as a folk remedy for many symptoms. It is used as antibacterial, antiscorbutic, astringent; post–partum protective medicine. It is also used for the treatment of fever, mumps, pimples, inflammation of the rectum and diabetes, itches, boils, rheumatism, syphilis, bilious colic, whooping cough, hypertension, stomach ache, aphthous ulcer and as a cooling drink[1].

2. MATERIALS AND METHODS

2.1 PLANT MATERIAL IDENTIFICATION AND COLLECTION:

Collection of bilimbi flower

*Averrhoa bilimbi*flowers were handpicked. Flowers were collected in months of September–October from Calicut Kerala(India). The flowers were collected for further use. The plant material was then washed with running tap water and finally washed with distilled water to remove the dirt.

Authentication of bilimbi flower

Flowers of *Averrhoa bilimbi*has to be authenticated by an expert botanist, a good specimen from the collected plant material, which is having flower, stem, leaves etc., were washed with water, then dried, then washed with alcohol, and kept for drying by pressing method and prepared the herbarium. Plant materials was identified and authenticated by Department of Botony, Calicut university India.

2.2 PREPARATION OF PLANT MATERIAL:

All flowers were handpicked and washed with tap water. Cleaned flowers were allowed for air dry.

2.3 PREPARATION OF EXTRACTS

The appropriate dried powdered plant material (20 g) was successively extracted using a soxhlet apparatus with ethyl acetate, methanol and water

(200ml each). The extracts were filtered through Whatmann No: 1 filter paper.The filtrate werethen evaporated to dryness with a rotary evaporator, under reduced pressure at 40°C.

2.4 ISOLATION OF ACTIVE CONSTITUENTS FROM THE EXTRACT:

Detection of mobile phase:

The ethyl acetate extract was introduced to activated TLC plates using a capillary tube at 1/2 inch besides the lower edge of the TLC plate, and thus the plate was left in a developing chamber bearing a proper solvent system for a specified time. Once the developing solvent reached the top of the top edge of the TLC plate, the plate has been removed from the chamber; the solvent front was marked with lead pencil and dried. Visual detection of compound bands / spots has been carried out on TLC chromatoplate which detected under UV light (254 nm) for the presence of specific compounds. The spots of the components in the TLC plate were marked and the *Rf*valueof each spot was calculated by the formula:

$$Rf \text{ value} = \frac{Distancetraveledbyt\quad hesolute}{Distancetraveledbyt\quad hesolvent}$$

Column Chromatography:

Partial purification of ethyl acetate extract was carried out using silica gel column chromatography. Glass column was packed by wet method. The adsorbent slurry (silica gel; 60-120 mesh) was prepared by stirring the adsorbent with the same mobile phase and used as a stationary phase. Then, it was dripped into the glass column (43 cm x 3.5 cm) (sintered glass disk at the bottom) and allowed to remain and settle. The air entrapped was removed by tapping the column with rubber tube. A small amount of sand and cotton was kept at top of the column to provide the latter a flat base. Excess solvent was run off once the mobile phase level dropped to 1 cm just above the upper edge of the sand and cotton layer. 6 g of ethyl acetate extract was mixed with 3g of silica gel as stationary phase loaded onto the column.Flow rate was set to 10 ml/min. The column was eluted with toluene, ethyl acetate, formic acid (7:3:0.1) mobile phase.

Separation of bioactive constituents from first mobile phase was carried out by eluting the column at uniform interval (10 drops per minute), the eluents (each of five ml) which were collected in test tube and the progress of separation was monitored by thin layer chromatography (TLC) (silica gel G 60 F254 TLC plates of E. Merck, layer thickness 0.2mm) using the same solvent system,) for ethyl acetate extract.

2.5 PHYSICOCHEMICAL ANALYSIS:

Physicochemical constants such as the foreign matter, fluorescence analysis, percentage of total ash content were calculated based upon standard procedures prescribed by Kokate as follows[2]. The other physicochemical parameters that were determined as per The Unani Pharmacopoeia of India include the following.

Extractive Values

3g of concentrated residue was taken in 250 ml conical flask with stopper and 30 ml of solvent was added -polar to non-polar (hexane, acetone, ethanol, water, petroleum ether, ethyl acetate, methanol). Cold maceration technique was used for extraction. The solvent with powder were kept for 24 h at room temperature, with occasional shaking. The mixture were filtered through Whatmann No:1 filter paper. Filtrate was then transferred to a weighed petri plates and was allowed for evaporation of solvents. Different extracts were weighed quantitatively and percentage with respect to the weight of the plant material taken was calculated.

The extractive value in percentage was calculated by using

$$\text{Extractive value} = \frac{Weight\ of\ the\ dried\ extrct}{Weight\ of\ the\ drug} \times 100$$

2.6 PRELIMINARY PHYTOCHEMICAL ANALYSIS

Qualitative analysis of aqueous, ethyl acetate and methanolic extracts flowers of *Averrhoa bilimbi*was performed for the identification of various classes of active chemical constituents like alkaloids, carbohydrates, glycosides, proteins, amino acids, steroids etc. using different methods of Harborne[3] and using standard procedures[4-6].

2.7 IDENTIFICATION AND DETECTION OF SUBSTANCES WITH IR SPECTROSCOPY

IR spectroscopy is used to establish whether a given sample of an organic substance is identical with another or not. This is because large number of absorption bands is observed in the IR spectra of organic molecules and the probability that any two compounds if produce identical spectra is almost zero.Soif two compounds have identical IR spectra then both of them must be samples of the same substances[7].

2.8 α- AMYLASE INHIBITORY ACTIVITY

Reagents

- 0.02 M Sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride.
  3.561 g of $Na_2HPO_4.2H_2O$ and 2.76g $NaH_2PO_4.H_2O$ were dissolved separately in H_2O. The volume of each solution was adjusted to 1000 ml. For 1000ml buffer 770 mL of $Na_2HPO_4.2H_2O$ and 230 mL of $NaH_2PO_4.H_2O$ mixed together. And adjusted the pH with 0.006M sodium chloride (0.350g in 1000ml of water)
- 2 N Sodium hydroxide
  8 g of sodium hydroxide in 100 ml of water.
- Dinitrosalicylic acid color reagent.
  Prepared by dissolving 1.0 gm of 3,5-dinitro salicylic acid in 50 ml of reagent grade water. 30.0 g sodium potassium tartrate tetrahydrate slowly added to the above solution. 20 ml of 2 N NaOH was added. Final volume was made upto 100 ml with reagent grade water.
- 1% Starch was prepared by dissolving 1.0 g soluble starch, (Merck) in 100 ml 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride. Gently boil the solution to dissolve. Cooled and the volume was made to 100 ml, with water, Incubated at25℃ for 4-5min priorto assay.

Assay

A starch solution (1% w/v) was prepared by stirring 1g starch in 100 ml of 20 mM of phosphate buffer (pH 6.9) containing 6.7mM of sodium chloride. The enzyme solution was prepared by mixing 27.5 mg of porcine pancreatic amylase α-amylase (PPA) in 100 ml of 20 mM of phosphate buffer (PBS, pH 6.9) containing 6.7mM of sodium chloride. To 100 µl of (2, 4, 8, 10,15µg/ml) plant extracts, 200µl porcine pancreatic amylase was added and the mixture was incubated at 37 ºC for 20 min. To the reaction mixture 100 µl (1%) starch solution was added and incubated at 37 ºC for 10 min. The reaction was stopped by adding 200 µl DNSA (1 g of 3,5-dinitro salicylic acid, 30g of sodium potassium tartrate and 20 ml of 2N sodium hydroxide was added and made up to a final volume of 100 ml with distilled water) and kept it in a boiling water bath for 5 minutes. The reaction mixture diluted with 2.2 ml of water and absorbance was read at 540 nm. For each concentration, blank tubes were prepared by replacing the enzyme solution with 200 µL in distilled water.

Control, representing 100% enzyme activity was prepared in a similar manner, without extract. The experiments were repeated thrice using the same protocol[8].

$$I\% = \frac{(AC - AS)}{AC \times 100}$$

Were, I% = percentage inhibition

AC = absorbance of control

AS = absorbance of sample

2.9 HPLC ANALYSIS

Chromatographic estimation of quercetin inethyl acetate extracts of flowers of *Averrhoa bilimbi.*

It is routinely used in phytochemistry for analytical as well as preparative separation purposes. For chemotaxonomic purposes, the botanical relationships between different species can be shown by chromatographic comparison of their chemical composition. Comparison of chromatograms, used as fingerprints, between authentic samples and unknowns permits identification of drugs and/or search for adulteration. HPLC is thus, very effective technique for an efficient separation of crude plant extracts. Analysis of components present in flowers ethyl acetate extracts of*Averrhoa bilimbi,* was done by selecting a standard compound. quercetin was found to be one of an activecompound in*Averrhoa bilimbi*.Studies reported that quercetinexerts antidiabetic properties by enhancing pancreatic secretion of insulin from β-cells in STZ induced models[9,10]. HPLC analysis of flowers ethyl acetate extracts of *Averrhoa bilimbi*was made by identifying the quercetin present in the sample.

3. RESULTS AND DISCUSSIONS

3.1 EXTRACTIVE VALUES:

Extractive values obtained from *Averrhoa bilimbi*using different solvents were recorded and the values are shown in fig1. It is useful for the evaluation of a crude drug as it gives idea about the nature of chemical constituents present in it and is useful for estimation of chemical constituents,soluble in that particular solvent used for extraction[11]. Watersoluble extractive value was indicating the presence of sugar, acids and inorganic compounds and alcohol soluble extractive values indicated the presence of polar constituents like phenols, alkaloids, steroids, glycosides, flavonoids and secondary metabolites present in the plant sample[12]. The ethyl acetate extract gives better extractive values.

SOLVENT	EXTRACT(mg)	PERCENTAGE (%)
Methanol	0.51	17
Ethyl acetate	0.60	20
Aqueous	0.45	15

Table 1: Extractive values

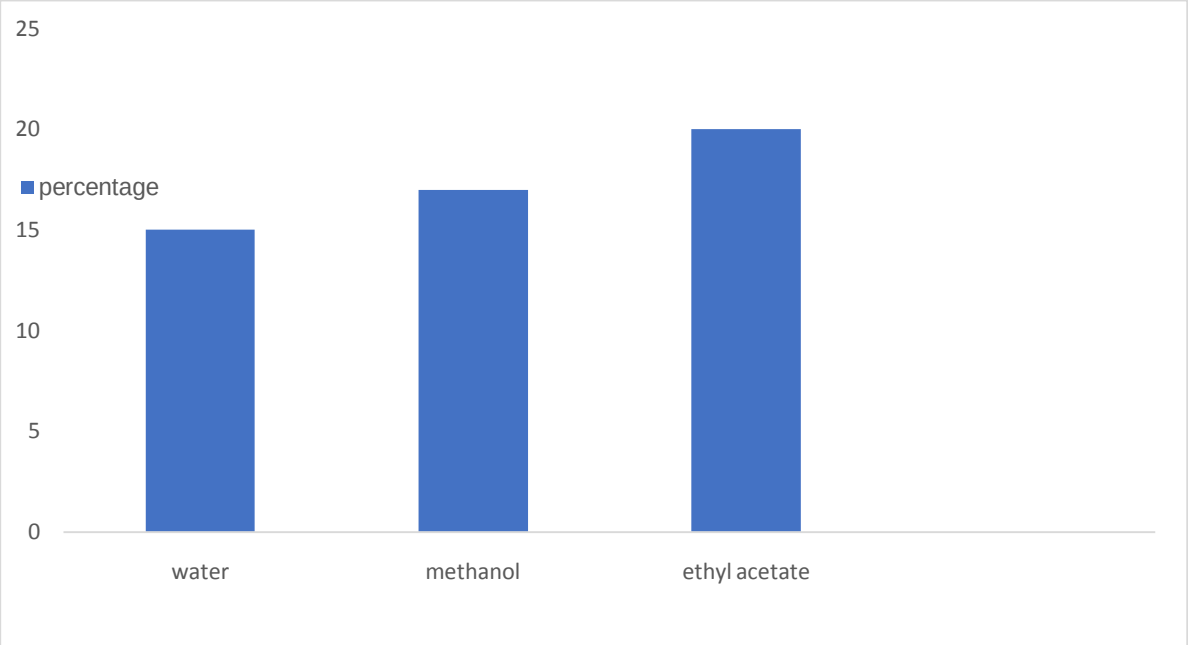


Figure1: Extractive Values

3.2 PHYSICOCHEMICAL PARAMETER:

The determination of physicochemical parameter was important in determination of adulterants and improper handling of drugs. Theextract was analyzed for physicochemical characteristics. The observed parameters were recorded as shown in table 2. Different ash value was also determined on the residue of *Averrhoa bilimbi*and reported in table 2. Ash values were important quantitative standards[13] and criterion to judge the identity and purity of crude drugs especially in the powder form[14].Moreover the total ash of a crude drug also reflects the care taken in drug preservation, and the purity of crude and the prepared drug[15].

SL.NO	TESTS	OBSERVATIONS
1.	Nature	Sticky

2.	Colour	Red
3.	Odour	Characteristic.
4.	Taste	Sour- sweet
5.	Foreign matters	Nil
6.	Fluorescence analysis	orange–Fluorescence
7.	Loss on drying	0.25
8.	Totalashvalue(%w/w)	2.95
9.	Acid insoluble ash value	1.3
10.	Water soluble ash valve	0.78
11.	Sulphated ash valve	1.0
12.	pH	5.0

Table 2: Physicochemical parameters for flower extract of *Averrhoa bilimbi*

3.3 PHYTOCHEMICAL SCREENING:

The results of phytochemical screening of different extracts of *Averrhoa bilimbi*plant were reported in table3. The phytochemical study revealed the presence of various phyto-compounds in different solvents solvent extracts.

Sl No	**NameOf Phytochemical Costituents**	**Methanolic Extract**	**Ethyl acetate extract**	**Aqueous Extract**
1.	Carbohydrate	+	+	+

2.	Proteins	+	+	-
3.	Alkaloids	+	+	+
4.	Phenolic Compounds	+	+	+
5.	Tannins	+	+	+
6.	Saponin	-	+	+
7.	Phlobatannins	-	-	-
8.	Flvanoids	+	+	+
9.	Terpenoids	+	+	+
10.	Glycosides	+	+	+
11.	Steroids	+	+	-
12.	Volatile Compounds	+	+	-
13.	Anthraquinone	-	-	-
14.	Reducing Sugar	+	+	+
15.	Anthocyanin	-	+	+
16.	Acidic Compounds	+	+	+
17.	Resins	+	+	+
18.	Fats And Lipids	+	+	-

Table 3: Preliminary Phytochemical Screening of *Averrhoa bilimbi*Flower Extract

Phytochemical screening of various flowers extracts of *Averrhoa bilimbi* showed the presence of carbohydrate, flavonoids, alkaloids, phenolic compounds, tannins, glycosides, reducing sugar, acidic compounds, resins and saponis while, it gave negative results for phlobatannins and anthraquinones. However, the aqueous extract showed negative results for proteins, phlobatannins, steroids, volatile compounds, fats and lipids were found to be absent. In ethyl acetate extract except phlobatannins and anthraquinone other compounds were found to be present. And in methanolic extract carbohydrate, alkaloids, proteins, tannins, glycosides, reducing sugar, phenolic compounds, flavonoids, acidic compounds, resins, volatile compounds, steroids, terpenoids fats and lipids were tested positive.

3.4 ISOLATION OF ACTIVE CONSTITUENTS FROM THE EXTRACT:

3.4.1. Detection of mobile phase:

About 50 TLC plates were eluted using different solvent in which the TLC plates showing number of bands (chemical compounds) for each fraction, that can be further isolated and purified using Column Chromatography (CC). Out of 50 TLC plates using different solvents (single solvent and in combination), best TLC plates with good separation were selected for carrying out the Column Chromatography. The selected mobile phase with good separationcompare to other mobile phase are found to be TLC plate toluene: ethyl acetate: formic acid in the ratio of 7:3:0.1 and for TLC plate methanol: acetonitrile in the ratio of 1:1.

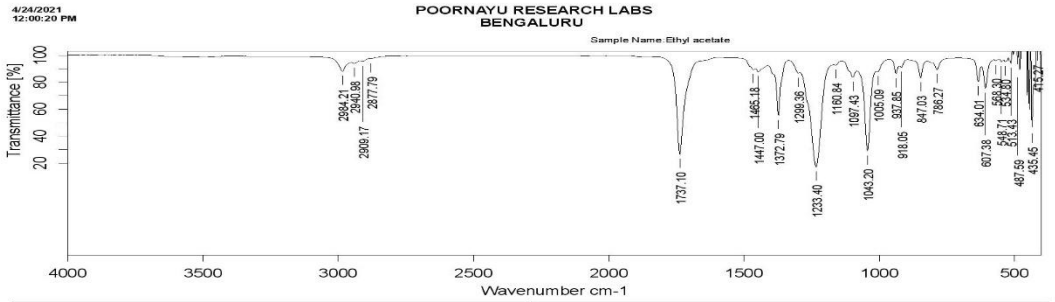
3.4.2.Column chromatography:

The same solvent system which found by TLC chromatography, toluene: ethyl acetate: formic acidwere used for column chromatography. After running the column the fractions were collected and checked for its*Rf* value by TLC. Fractions CCF01-10(0.564 g) was showed similar *Rf* value and CCF11-17 were shown similar *Rf* value (0.055 g) even fractions CCF18-21(0.144 gm) also shown similar *Rf*value. Fractions CCF22-26(0.467 gm) were not showed any spots after checking with TLC, after evaporating the solvent yielded less quantity.

3.5 IDENTIFICATION AND DETECTION OF SUBSTANCES WITH FT-IR SPECTROSCOPY:

IR spectroscopy is used to establish whether a given sample of an organic substance is identical with another or not. This is because large number of absorption bands is observed in the IR spectra of organic molecules and the probability that any two compounds will produce identical spectra is almost zero. So if two compounds have identical IR spectra then both of them must be samples of the same substances.

IR spectra of all fractions were checked, and the result shows that many fractions are having similar IR peaks.



Item	Value
Sample name	Ethyl acetate
Sample ID	Liquid
Date of Measurement	4/24/2021
Intensity mode	Transmittance
Apodization	ÂPF
No. of Scans	16
Resolution	4

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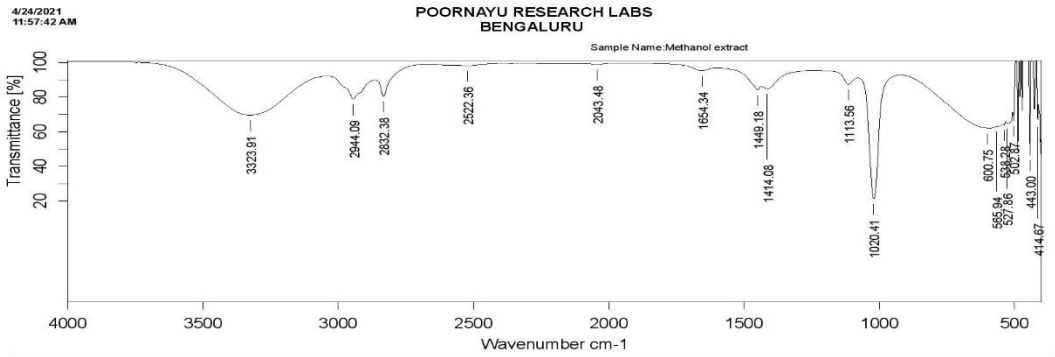
Figure 2:IR spectra of ethyl acetate



Item	Value
Sample name	Methanol extract
Sample ID	Liquid
Date of Measurement	4/24/2021
Intensity mode	Transmittance
Apodization	ÂPF
No. of Scans	16
Resolution	4

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Figure 3: IR spectra of methanol extract

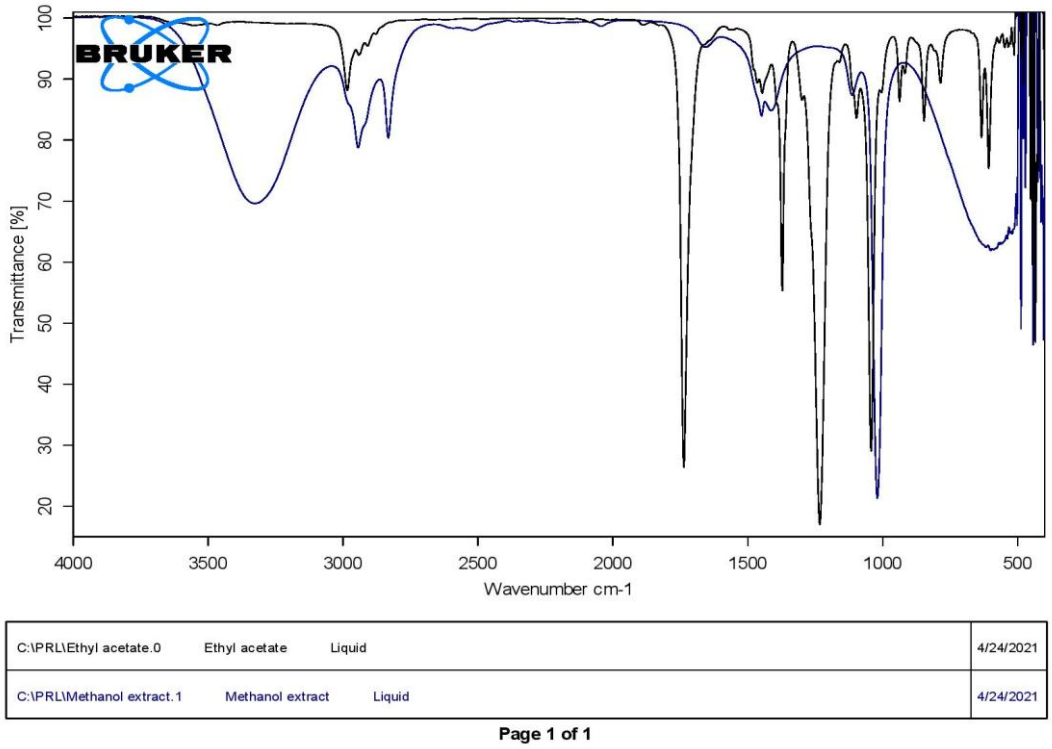


Figure 4: IR spectra methanol and ethyl acetate

3.6α- AMYLASE INHIBITORY ACTIVITY

After analyzing the IR spectra, the fractions were divided into 3 groups, sample 1 (CCF 1-10), sample 2 (CCF11-17,) and sample 3 (CCF 18-21,21-27), and their enzyme inhibitory activity has been checked.

And found that sample 2 was having the enzyme inhibitory activity. Other two samples (1&3) didn't have inhibitory action. Inhibition rate was increased with the increase of concentration.

CONCENTRATION	SAMPLE 1		SAMPLE 2		SAMPLE 3		CONTROL
	Abs. (nm)	I%	Abs. (nm)	I%	Abs. (nm)	I%	Abs.(nm)
2	0.095	-13.0	0.074	11.90	0.104	-23.8	0.084
4	0.092	-9.52	0.066	21.42	0.100	-19.0	
6	0.090	-7.14	0.058	30.95	0.096	-14.2	
8	0.087	-3.5	0.047	44.04	0.094	-11.9	
10	0.085	-1.19	0.030	64.28	0.091	-8.33	

Table 4: α- amylase inhibitory activity

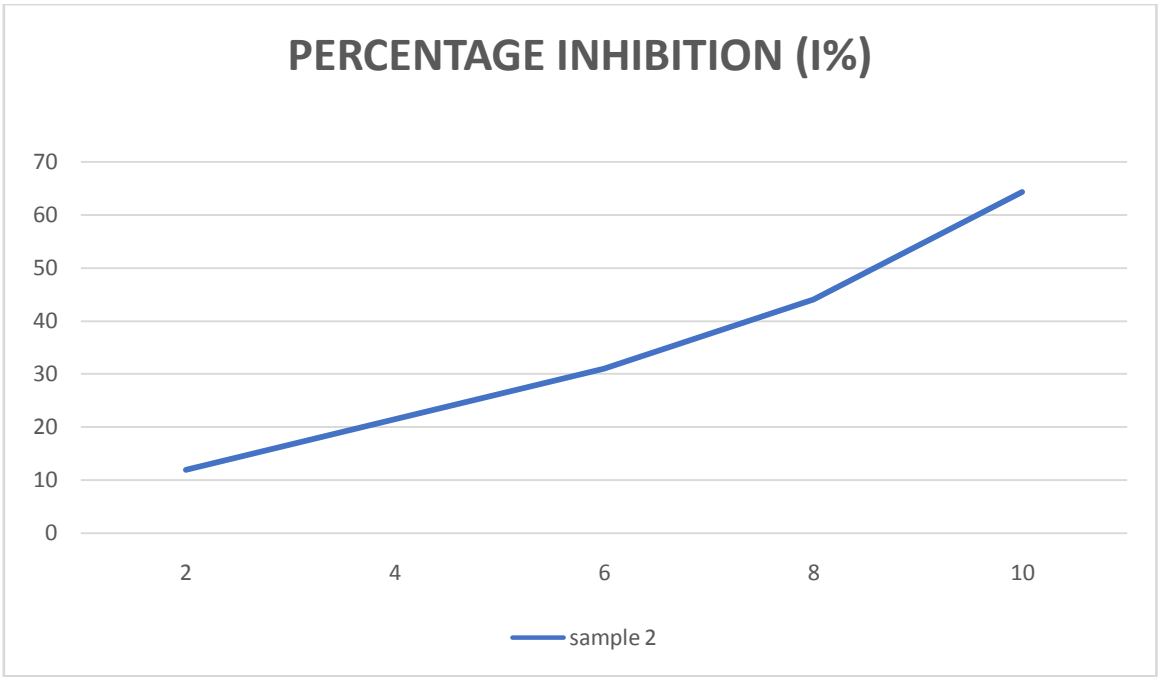


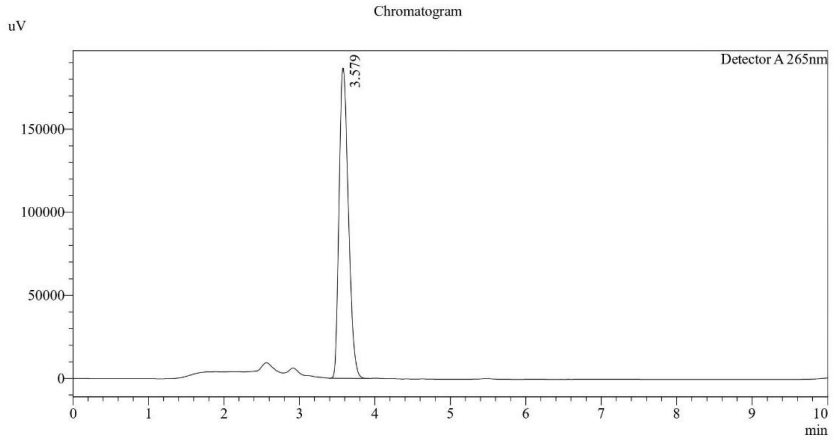
Figure 5: Percentage Inhibition

3.7 HPLC ANALYSIS

6.5.1. Estimation of Quercetin

Quercetin is a naturally occurring flavonoid.The flavonoids are polyphenolic compounds found as integral components of the human diet. They are universally present as constituents of flowering plants, particularly of food plants. The flavonoids are phenyl substituted chromones (benzopyran derivatives) consisting of a 15-carbon basic skeleton (C6-C3-C6), composed of a chroman (C6-C3) nucleus (the benzo ring A and the heterocyclic ring C), also shared by the tocopherols, with a phenyl (the aromatic ring B) substitution usually at the 2-position. Different substitutions can typically occur in the rings, A and B.

Quercetin was found to be one of an activecompound in*Averrhoa bilimbi*.Studies reported that quercetinexerts antidiabetic properties by enhancing pancreatic secretion of insulin from β-cells in STZ induced models[9,10]. HPLC analysis of the sample which is ethyl acetate extract of *Averrhoa bilimbi* flowers, has shown the presence of quercetin. Comparing the standard HPLC graph ofquercetin in the same condition with the sample.



Peak Table

Detector A 265nm

Peak#	Name	Ret. Time	Area	Area%	Tailing Factor	Number of Theoretical Plate(USP)
1	QUERCETIN	3.579	1604634	100.000	1.296	3682
Total			1604634	100.000		

Figure 6: HPLC of bilimbi

CONCLUSION

The present work entitled with isolation, phytochemical investigation and biological screening of flowersof *Averrhoa bilimbi* plant belonging to Oxalidaceae family for its antidiabetic activity was carried out.From, the present work the chemicals compounds from the flowers of *Averrhoa bilimbi* has been extracted successfully. After extraction the extract was concentrated using rotatory evaporator and the resultant residue was used for the further use. The solvents for extraction were selected by checking the solubility of the residue. Ethyl acetate, methanol, waterwere used for extraction. And it is found that ethyl acetate shows highest rate of extraction.Various chromatographic techniques have been used for successful fractionation and purification of biologically active compounds from variety of sample. Column chromatography is one of the most popular and widely used separation techniques to characterize both organic and inorganic materials suggesting the potential usefulness in chemical analysis of complex extract material. This research visualized successful application of column-chromatographic techniques for the isolation of biologically active secondary metabolites from plant sample. In this study a new and novel method using TLC was developed to find the best mobile phase and also the use of column chromatography for isolation of individual component present in the plant extract. After separating the fraction using column chromatography all the fractions were checked, using thin layer chromatography as well as subjected to FT-IR for identification of the similar compound. It was thoroughly, investigated for its physicochemical characters and phytochemical investigation of flowers of plant*Averrhoa bilimbi.*The biological activity was screened by identifying it antidiabetic activity. The screening of antidiabetic activity was carried out by performing α-amylase inhibition. Finally the chromatographic estimation (HPLC) was performed. By this study we can conclude that flowers of *Averrhoa bilimbi*have α-amylase inhibitory activity.

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