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"PHYTOCHEMICAL INVESTIGATION, HPTLC METHOD DEVELOPMENT AND PHARMACOLOGICAL SCREENING OF EXTRACT LEAVES OF CASSIA SENNA SOPHERA LINN"

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ABSTRACT

HPTLC is a perfect analytical tool which has been used for qualitative and quantitative estimation of active markers. HPTLC fingerprint is the applicable and critical parameter associated with the standardization of crude drugs. Densitometric HPTLC has been widely used for the phytochemical evaluation of the herbal drugs, due to its simplicity and minimum sample clean up requirement. Hence, a densitometric HPTLC method has been developed in the present work for quantitation of gallic acid from hydroalcoholic extract of dried leaves of Cassia sennasopheralinn. The phytochemical analysis of ethanolic, aqueous and hydro alcoholic extract suggests the presence of flavonoids the prominent compound was developed by HPTLC method using nonpolar and polar solvent systems in the form of different density gradients. Finally the Quercetin is subjected to various spectral analyses such as UV, IR and H1 NMR. The spectral data was characterized for the structural elucidation of the molecule. Ethanolic, aqueous and hydro alcoholic extract were used for the evaluation for the anti-bacterial activity. In the present study Gentamycin used as standard and gram positive & gram negative bacteria used.

KEYWORDS: HPTLC, Phytochemical Evaluation, Cassia SennaSophera Linn. Quercetin, Anti-Bacterial Activity.

INTRODUCTION

The World Health Organization in 1980 has recommended the evaluation of the effectiveness of plants in conditions where there is lack of safe synthetic drugs. Today we are witnessing a great deal of public interest in the use of herbal remedies. There are many herbs, which are predominantly used to treat cardiovascular diseases, liver disorders, central nervous system, digestive and metabolic diseases. Ethanopharmacological studies on such medicinally important plants are an area of interest for the investigators throughout the world. One such species, *Cassia* invites attention of researcher's world-wide for its pharmacological activities ranging from anti-diabetic to antiviral. The ICH has given some guidelines for standardizing the Ayurvedic and herbal formulations. These can be a very effective means for evaluating and standardizing our traditional medicine bank. The bioactive extract should be standardized on the basis of active principles or major compounds along with the chromatographic fingerprints (TLC, HPTLC, HPLC and GC). Though the microscopy and macroscopic characters, chemical assays and test are used as the diagnostic tools for herbal drugs, the chromatographic fingerprint gives the accurate and reliable results for standardization of herbal drug. HPTLC is a perfect analytical tool which has been used for qualitative and quantitative estimation of active markers. No any reports are also available on HPTLC method development of *cassia sennasopheralinn*by fingerprint analysis of quantitative estimation of this crude drug.

OBJECTIVES

In recent years, tremendous work is going on for isolating active principles from plant sources their pharmacological and biochemical estimations and their preclinical, clinical studies for new drug development.

The crude drug was subjected for following studies

- Collection and authentication of leaves of *Cassia sennasopheralinn*.
- Extraction of leaves of *Cassia sennasopheralinn*.
- Pharmacognostical Studies: Macroscopy
- Preliminary Phytochemical Screening
- Isolation of major compound.
- HPTLC method development of Quercetin.
- Structural elucidation of isolated compound
- Pharmacological screening

***Cassia sennasophera*Linn. Var. purpurea Roxb**

1. Plant Profile
2. Scientific name- *Cassia sennasopheralinn*.
3. Family- Caesalpiniaceae
4. Synonyms

Taxonomical classification of the genus Cassia

KINGDOM	**PLANTAE**
Division	Magnoliophyta
Class	Magnoliopsida
Subclass (Unranked)	Rosidae
Order	Eurosids I
Family	FabalesLeguminosae
Subfamily	Caesalpinioideae
Tribe	Cassieae
Subtribe	Cassinae*Cassia* L.

Reported Phytoconstituents

Sr.No.	Phytoconstituents	Part	References
1.	Flavone glycoside- 3,5,3,4, Pentahydroxy-7-methoxy flanove-8rhamnopyranoside.	Leaves	Anonymous., Wealth of India., 1992
2.	Anthraquinone	Root bark	Dass et al., 1984
3.	Anthraquinone	Heart wood	MalhotraandMishra, 1982
4.	Sterols	Flowers	Ghosh andThakur, 1982
5.	Anthraquinone 3-neohesperidoside	Root	Shukla et al., 1985
6.	Flavonol-8-C-glycoside	Leaves	Tiwari et al., 1980
7.	Sopheranin, β sistosterol	Leaves	Rastogy., 2001

METHODOLOGY

Experimental work

1. Plant material procurement
2. Identification and Authentication of plant
3. Macroscopic Study of plant material
4. Drying of plant material
5. Determination of physicochemical constants of powder plant material

- Determination of ash value
- Determination of extractive value
- Determination of leaf constant

6. Extraction of plant material
7. Phytochemical screening
8. Steps involved in High performance thin layer chromatography
9. Method of Extraction for Flavonoids
10. Development of Quercetin by HPTLC
11. Structure elucidation of isolated compound

- Infra red spectroscopy
- Nuclear Magnetic Resonance of isolated compound

12. Instruments Used:

A) Soxhletcontinous extraction apparatus (Borosil, Mumbai)

B) HPTLC (CAMAG Linomat,)

C) IR spectra (Jasco)

D) ^{1}H-NMR (BRUKER)

EXPERIMENTAL WORK

1. Plant Material Procurement

Leaves of *Cassia sennasopheralinn*. (CS) were collected from Sangli (M.S).

2. Identification and Authentication

The plant was identified and authenticated from Vivekananda College, Kolhapur.

3. Macroscopic study of plant material

Morphological study was carried out for organoleptic evaluation. The color, structure, shape and size were visually observed.

4. Drying of plant material

The crude drug was shade dried for 72 hrs and powdered by using industrial grinder.

5. Determination of Physicochemical Constants of Powdered Plant Material (The Ayurvedic Pharmacopoeia, 1990)/ Proximate Analysis

Ash values

The total ash, acid insoluble ash and water soluble ash were determined by using procedures given in the Ayurvedic Pharmacopoeia.

Determination of total Ash value Procedure

1. A crucible dish was weighed and ignited.
2. About 2 gm of powder was weighed and added to crucible.
3. The crucible was kept about 7 cm away from flame till the vapors cease.
4. Then it is heated strongly to burn off all the carbon, and crucible was cooled inDesiccators.
5. The ash was weighed and percentage of total ash was calculated with reference to air dried powder.

Determination of acid-insoluble ash value

Procedure

1. The ash obtained from above procedure and it was washed by 25 ml of dilute Hydrochloric acid in 100 ml beaker.
2. The above solution was boiled for 5 minutes.
3. The solution was filtered through ash less filter paper and residue was washed twice with Water, residue was taken into pre-ignited, cooled and weighed crucible.

4. The filter paper with residue was heated gently until vapors cease and then Heated strongly until whole carbon was removed. Crucible was cooled in a desiccator.
5. The ash was weighed and percentage of acid insoluble ash was calculated with reference of air dried Powder.

Extractive value

Using procedures given in the Ayurvedic Pharmacopoeia, determination of alcohol soluble extractive value and water soluble extractive value were carried out.

Procedure

5 gm of powder was dissolved in 20 mL ethanol and water separately and sonicated for 15 minute. The solution was filtered through Whatman No. 41 filter paper. The residue was dried and weighed. The percentage yield of residue was calculated.

Determination of leaf constant

The leaf constants like vein islet no, stomatal index, stomatal no and palisade ratio was calculated as per procedure by using Camera Lucida.

Extraction of plant material

The powder of plant material obtained were passed through sieve no. 85, weighed & then used for extraction. Drug extracts preparations obtained by extracting herbal drug at certain particle size with suitable extraction medium.

Ethanolic extract

In the present study, the plant was carefully selected and shade dried. The dried material was reduced to powder in the mechanical grinder and passed through a sieve no. 40 to obtain powder of desired size.

About 125 gms of powdered material was subjected to exhaustive extraction with 95% alcohol in a soxhlet extractor at a temperature of 45-50 ^{0}C. The extraction was continued until the solvent in the thimble became clear, then few drops of solvent were collected in the test tube during the completion of the cycle and chemical test of the solvent was performed.

After each extraction the solvent was distilled off and the extract was concentrated at low temperature. Some part of the total extract was reserved for phytochemical investigation and rest of the extract was used for evaluation of various activities.

Aqueous Extraction

About 500 gm of fresh powder was used. It was then subjected to cold maceration with chloroform: water (1.0 %) I. P. in a 2 liters round bottom flask for about 7 days at room temperature. The flask was securely plugged with absorbent cotton and was shaken periodically till complete maceration. After maceration, the marc was pressed in a muslin cloth and the filtrate was concentrated to residue at low temperature.

Phytochemical Screening

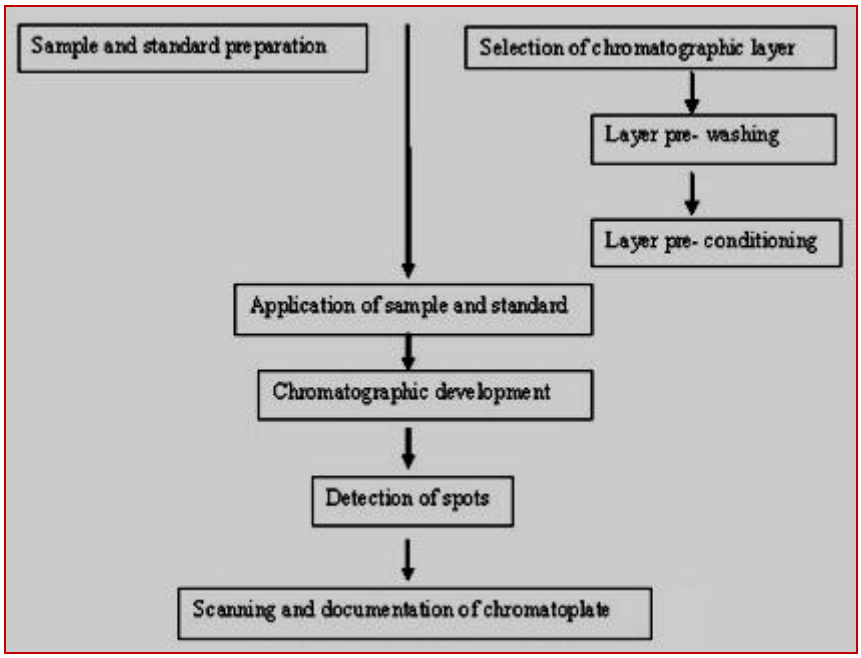
Preliminary Phytochemical Tests

1. **Test for Alkaloids**
   - Dragendroff's test
   - Mayer's test
   - Hager's test
   - Wagner's test
2. **Test for Amino acids**
   - Ninhydrin test
3. **Test for Anthraquinone glycosides**
   - Borntragger's test
4. **Test for Cardiac glycoside**
   - Keller's killani test
   - Legal's test
5. **Test for Cyanogenic glycoside**
6. **Test for Coumarin**
7. **Test for Flavonoids**
8. **Test for Mucilage**
9. **Test for Proteins**
10. **Test for Saponins**
11. **Test for Tannins**
12. **Test for Sugars**

Steps involved in High Performance thin Layer Chromatography

- Selection of TLC/ HPTLC plates and sorbent
- Sample preparation
- Application of sample

- Detection including post- chromatographic derivatization
- Quantitation



QUANTITATION (EVALUATION)

Requirements for various steps in TLC/ HPTLC are more stringent for quantitative analysis. Accurate and precise application of samples is the most critical. Further, the chromatographic development should clearly and completely separate all the compounds of interest with no loss by decomposition, evaporation or irreversible adsorption during application or development. Sample and standard as a rule should be chromatographed on the same plate under similar conditions. Earlier, a typical approach was scrapping the separated analyte zone from support material and exctracting with a suitable solvent. Compounds thus eluted could be analyze by any convenient analytical method; Spectrophotometric, flurometric or by suitable colour develop method. To compensate for any interference from the sorbent usually a blank area is also eluted simultaneously and used as a blank for final analysis. Such blanks volumes can be lowered by prewashing of TLC layers methanol, chloroform (1:1) or methylene chloride prior to chromatographic procedure. However, this method of separating and elution has limited application as the compound under analysis may be irreversibly bound to the TLC/ HPTLC supports or elution / isolation steps may causethe some chemical transformation or there is likely hood of a analytes during extraction. In spite of these limitations, spot/ band elution method is still widely used because it is simple and inexpensive. Layers containing gypsum as binder are considerably softer and speciously

suited for preparative chromatography scrapping for and subsequent elution and estimation. The simplest method in situ quantitative TLC is based on visual comparison of size and/ or intensity of coloured, fluorescent or UV (254 nm) absorbing compounds. The method is simple, quick and needs no elaborate instrumentation and laboratory facilities; however, the results are semi quantitative (10%). Accuracy can be improved when standard and sample concentrations are bracketed. Such visual comparisons are more meaningful on TLC plates than HPTLC plates because of wider size of the sorbent in the former. Basically, this technique involves bracketing the concentration of standard at 90 and 110% of the claim in respect of components present in the sample under analysis. Both samples (presumed to be 100%) and standards are chromatographed on the same plate under similar conditions. Comparison of the intensity and the spot size is used for estimation. For such visual comparison of size of zones of sample and standard, it is important that Rf values are same. However, due to matrix effects, Rf values with the sample may not exactly compare with the standard, thus zone size may differ due to different diffusion pattern of the substance on the chromatogram. This procedure though semi-quantitative is extremely useful to establish whether analytical values so obtained are above or below pre-set limiting values.

METHOD OF EXTRACTION OF FLAVONOIDS

Process of Extraction of Flavonoids

Extraction was done in soxhlet apparatus; 930 gm of powder was used for extraction. Petroleum ether was used for removal of fatty material from powder material. After treatment of petroleum ether marc was treated with chloroform, followed by ethanol, Water, Hydroalcohol. Fractions of ethanol water and Hydroalcohol extracts were kept for seven days. The presences of flavonoids were confirmed by taking chemical tests.

Isolation and Development of Quercetin

Preparation of Sample Solution

An accurately weighed 100 mg of flavonoids fraction was transferred to 20 ml of volumetric flask and about 15 ml of methanol was added to the flask. The flasks was sonicated for 20 minutes in an ultrasonic water bath and diluted up to the mark with methanol. The solution was filtered through Whatman no. 41 filter paper and used for further chromatographic analysis. The TLC plate was allowed to run up to 80 mm from the point of application. TLC plate was dried in hot air oven at 60^0C. Densitometric scanning was performed using CAMAG TLC scanner 3 in the absorbance mode at 280 nm and operated by winCATS

software (V 1.4.3.6336). The slit dimension was 5.0 × 0.45 mm with the scanning speed of 20 mm s^{-1}. Evaluation was done via peak area with linearregression.

Development of the optimum mobile phase

The TLC procedure was optimized with a view to quantify the herbal extract. Initially chloroform: ethyl acetate: formic acid in varying ratios was tried. The mobile phase Toluene : Ethyl Acetate : Acetonitrile : Formic Acid (10:1:1:1, v/v/v/v) gave good resolution with Rf = 0.40 for gallic acid but peak shape was missing. Finally, the mobile phase consisting of Toluene : Ethyl Acetate : Acetonitrile : Formic Acid (10:2.5:1:1, v/v/v/v) gave a sharp and well-defined peak at Rf = 0.45 in fig. Welldefined spots were obtained after the chamber was saturated with mobile phase for 15 min at room temperature. The TLC plate was visualized under UV light at 282 nm, without derivatization. A photograph of a TLC plate after chromatography of Quercetine standard and a hydroalcoholic extract of the leaves of *Cassia sennasopheralinn.* The identity of the Quercetin bands in sample chromatograms was confirmed by comparison of the chromatogram obtained from the sample with that obtained from the reference standard solution and by comparing retention factors of Quercetin from sample and standard solutions. The peak corresponding to Quercetin from the sample solution had same retention factor as that of the retention factor from the Quercetin standard (Rf 0.45).

Preparation of Quercetin standard solution and Calibration curve

A stock solution of standard Quercetin (40 µg/mL) was prepared by transferring 4 mg of Quercetin, accurately weighed, into a 100 mL volumetric flask, dissolving in 50 mL methanol. It was then sonicated for 15 minutes and the final volume of the solutions was made up to 100 mL with methanol to get stock solutions containing 40µg/mL. The calibration curve from 60-180 ng/ spot was prepared and checked for Reproducibility.

Preparation of sample solution

Accurately weighed 100 mg of dried hydroalcoholic extract of *C. Sophera L.* was transferred to a 100 mL volumetric flask dissolving in 80 mL of methanol. It was then sonicated for 15 minutes and the contents of the flask were filtered through Whatman No. 1 paper (Merck, Mumbai, India). The final volume of the solution was made up to100 mL with methanol to get stock solution containing 10 µg/ mL.

Chromatographic conditions

HPTLC was performed on 20 cm × 10 cm aluminum backed plates coated with silica gel 60F254. Standard solution of gallic acid and sample solution were applied to the plates as bands 6.0 mm wide, 30.0 mm apart, and 10.0 mm from the bottom edge of the same chromatographic plate by use of a CamagLinomat V sample applicatorequipped with a 100-µL Hamilton syringe. Ascending development to a distance of 80 mm was performed at room temperature (28°C), with Toluene : Ethyl Acetate : Acetonitrile : Formic Acid (10:1:1:1, v/v/v/v), as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapor for 15 min. After development, the plates were dried and then scanned at 280 nm with a Camag TLC Scanner with WINCAT software, using the deuterium lamp.

Structure Elucidation of Isolated Compound

Spectroscopy

UV Visible spectra ofisolated compound were monitored byUVVisibleSpectroscopy (Jasco duel beam UV Visible spectrophotometer, Model V-570, Japan).

Infra red spectroscopy

The isolated compound was characterized by using infra red spectroscopy (Jasco Japan). The isolated compound was grounded with KBr powder and pressed into pellets for IR spectra measurement in the frequency range of 400-4000 cm^{-1}.

Nuclear Magnetic Resonance of isolated compound.

^{1} HNMR was performed in $CDCl_3$ solvent.

PHARMACOLOGICAL EVALUATION

ANTI-BACTERIAL ACTIVITY

An antimicrobial is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi, or protozoans. Antimicrobial drugs either kill microbes (microbiocidal) or prevent the growth of microbes (microbiostatic). Disinfectants are antimicrobial substances used on non-living objects or outside the body. The discovery of antimicrobials like Penicillin, Gentamycin and tetracycline paved the way for better health for millions around the world. Before penicillin became a viable medical treatment in the early 1940s, no true cure for gonorrhea, strep throat, or pneumonia existed. Patients with infected wounds often had to have a wounded limb removed, or face death from infection. Now, most of these infections can be cured easily with a short course of antimicrobials. However, the last few

years have seen a major increase in their use in the developed world. Nowadays multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment.

Organism

*Bacillus subtilis, Bacilluaureus*and *Staphylococcus aureus* (Gram positive)

Escherichia coli, K *pneumonia* and *Pseudomonas aeruginosa* (Gram negative)

Culture Media

The media used in Pour Plate Method was sterile nutrient agar.

Materials used

- Nutrient agar
- Distilled water
- Beef extract
- Peptone
- Sodium chloride

Apparatuts

Conical flask, Graduated volumetric flask, Glass rod, Burner,Wireguage, Chemicals, Autoclave, Sterile petridishes, Sterile pipettes, Sterile cotton swabs, Sterile cork borer.

Preparation of Nutrient Agar

1. Weigh all ingredients separately by physical balance.
2. Add weighed ingradients in suitable container.
3. Heat on water bath with stirring till agar completely dissolved.
4. Most of the agar dissolves to give a clear liquid. If necessary, filter it with funnel in warm conditions.
5. Adjust the pH to 7.2 to 7.4 by adding just enough Hcl or NaOHdropwise.

Procedure for Autoclaving

1. Your instructor will demonstrate the use of the autoclave.
2. Load the autoclave with the freshly prepared culture media.
3. Close and lock the autoclave door.
4. Set the autoclave time for 15 minutes or longer and select a slow rate of exhaust.
5. Make certain that the autoclave temperature is set to 121°C &15 p.s.i. for 15 min.

6. Start the autoclave by pushing the start button or twisting the knob to the start position.
7. When the period of sterilization is completed and the pressure in the chamber reads 0, carefully open the door and remove the containers, using heat-proof gloves.

Preparation of Bacterial Suspension

The bacterial suspension was prepared by transferring a loopful of inoculum into 1ml sterile saline solution from the stock culture maintained at 4^0C in 10 ml nutrient broth.

Preparations of Plates

Nutrient agar medium was sterilized at 15lb/cm^2 pressure for 20 min in an autoclave about 15 ml of medium was poured in each Petri plates under sterile conditions and keep for solidification in freeze for 20 mins.

Preparation of test Samples

Alcoholic and aqueous bark extracts of *Pongamiapinnata*was prepared in sterile distilled water (1mg/ml). Further test dilutions were made ranging from 10 µg/ml to 100µg/mlin sterile distilled water.

Test Bacteria

The Bacterial culture employed in this study are *Bacillus subtilis, Bacilluaureus, Staphylococcus aureus, Escherichia coli*, K *pneumoniae and Pseudomonas aeruginosa*obtained from the department of Microbiology, Gulbarga University, Gulbarga. (Karnataka) INDIA.

RESULTS

Macroscopic Study

Powder, seeds, leaves, stems and roots of *Cassia sennasopheralinn*

Total ash values

Sl. No	Particulars	Observed Values (% w/w)
1	Total Ash	12.2
2	Acid insoluble Ash	2.7

Extractive values

Sl. No	Particulars	Observed Values (% w/w)
1	Ethanol soluble extractive value	19.6
2	Water soluble extractive value	15.7

Leaf constants

Sl. No	Particulars	Values
1	Palisade ratio (Upper epidermis) (lower epidermis)	Upper: 7.8 Lower: 9.2
2	Vein-islet no	38-44
3	Vein termination no	12-20
4	Stomatal no	32-45
5	Stomatal index	12.9

Extraction

1. The leaves were dried in shadow and powered. The powders obtained were passed through sieve no. 85, weighed and then used for extraction.
2. The weighed powder was placed in thimble made up of filter paper and was continuously extracted for 18-20 hours using ethanol (95%) as a solvent.
3. The resulting extracts were concentrated under reduced pressure using rotary vacuum evaporator to get the semisolid mass. This mass was transferred in petridish and allowed to dry in an oven for about 2 to 3 hours.
4. This ethanol extract was subjected for further sequential fractionation.
5. The ethanol extract was adsorbed on silica gel (60-120), dried and placed in thimble and then used for sequential fractionation with chloroform and ethyl acetate by using soxhlet extraction method. The remaining residue was washed with the ethanol and considered to be ethanol fraction.

Various parameters of parent ethanol and Aqueous extract of Cassia SennaSopheralinn.

Extract	Color	Odor	Taste	Yield
Ethanolic Dar19.6% extract Green sweet smell				
Aqueous Extract	Brown	Characteristic	Characteristic	24.3%

Preliminary phytochemical analysis

Sr. No	Chemical tests	Aqueous Extract	Ethanolic Extract
1	**ALKALOIDS**		
	Dragendroff's test	-	-
	Mayer's test	-	-
	Hager's test	-	-
	Wagner's test	-	-
2	**GLYCOSIDE**		
	Anthraquinone glycosides	+	+
	Cynogenic glycoside	-	-
	Cardiac glycoside	-	-
3	**FLAVONOIDS**	+	+
4	**MUCILAGE**	+	+
5	**COUMARIN**	-	-
6	**TANNINS**	+	+
7	**SAPONINS**	+	-

HPTLC Method Development for Quantitative Determination of Quercetin in Extract of *Cassia sennasopheralinn*.

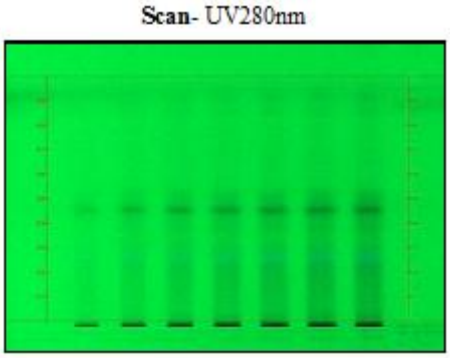
Chromatographic conditions

Mobile Phase- Toluene: Ethyl acetate: Acetonitrile: Formic acid (10: 2.5: 1: 1)

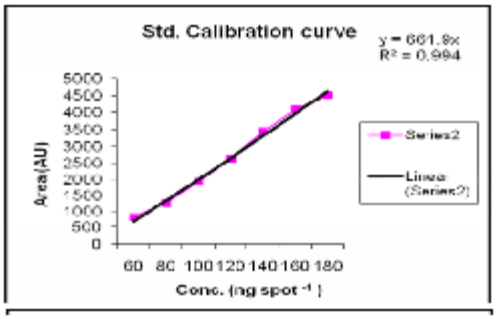
Sample Preparation- Take a 50 mg of extract sample in 10 mL mL Volumetric Flask Add 8 mL Methanol sonicate for 15 min. and dilute upto mark with Methanol. Filter solution with whatman 41 No. filter paper and use filtered solution.

Injection- Inject 20 µL solution on HPTLC

Saturation Time -15min



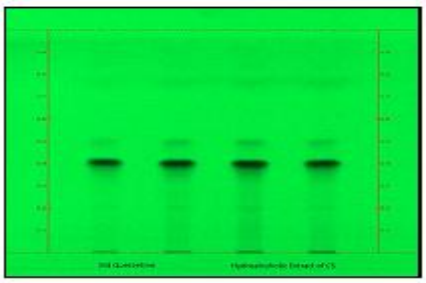
HPTLC of Calibration of Standard Quercetin



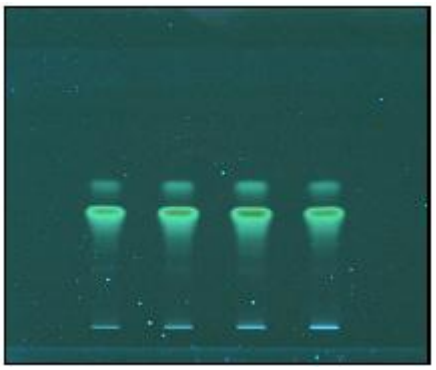
Calibration curve of standard Quercetin

Conc.	Area
60	803
80	1250
100	1915
120	2632
140	3419
160	4080
180	4503

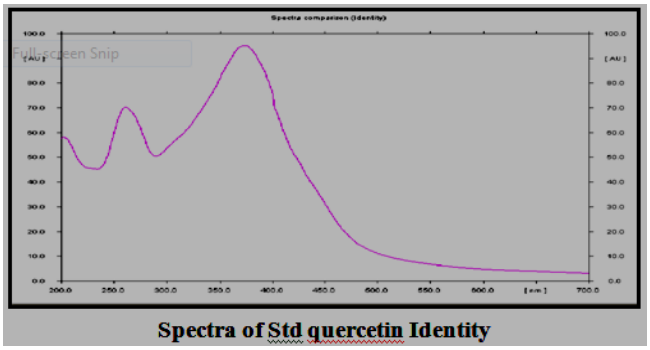
Calibration of STD Quercetin



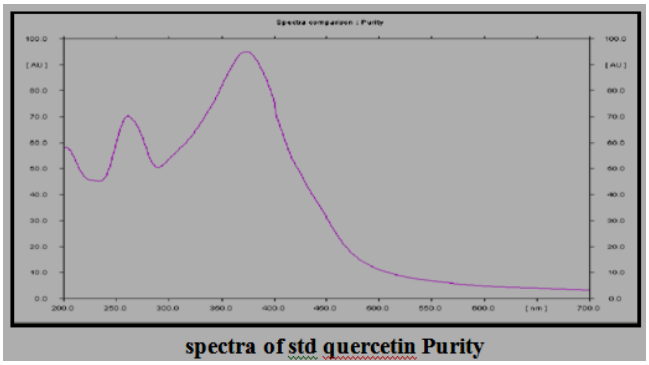
Comparison of STD and Hydroalcoholic Extract of *Cassia sennasopheralinn* (Visual mode)



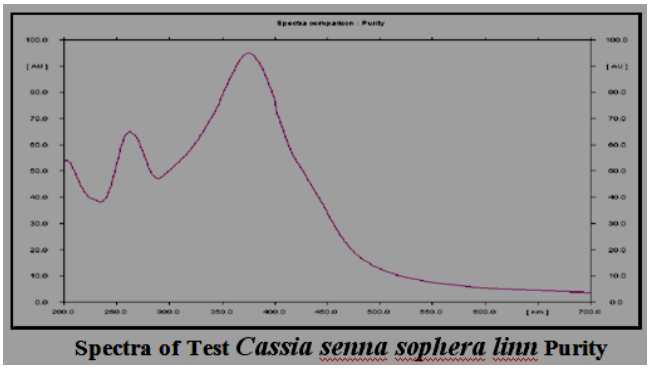
Comparison of STD and Hydroalcoholic Extract of *Cassia sennasopheralinn* (UV mode)



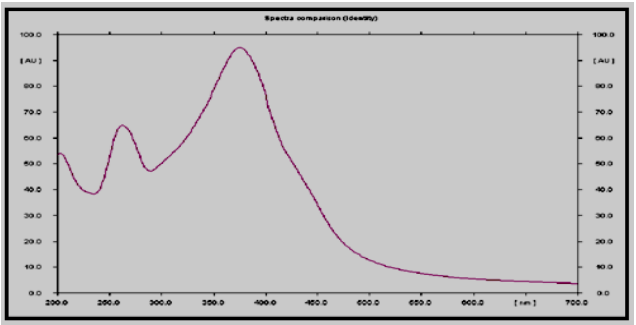
Spectra of Std quercetin Identity



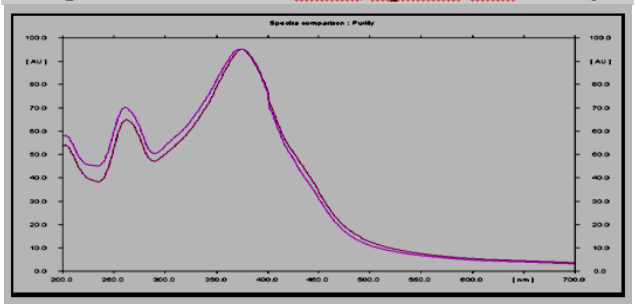
spectra of std quercetin Purity



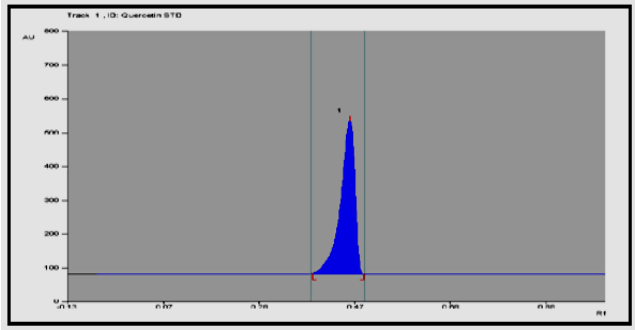
Spectra of Test *Cassia senna sophera linn* Purity



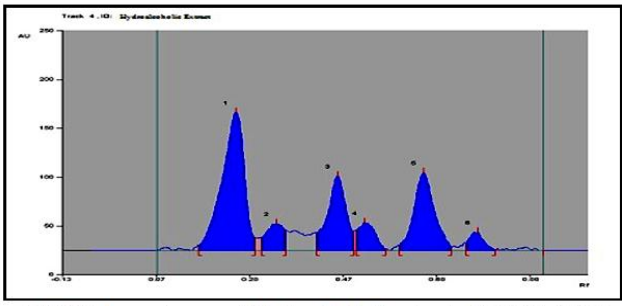
Spectra of Test *Cassia senna sophera linn* Identity



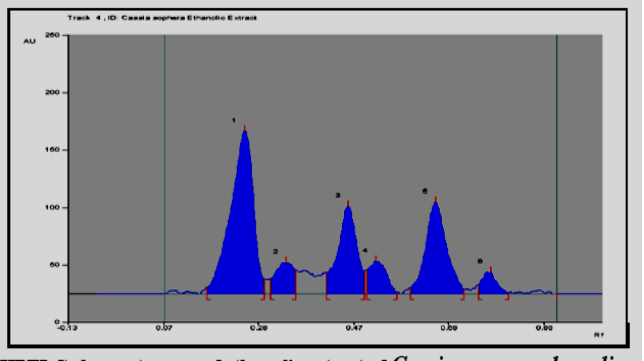
Spectra of std quercetin and Test *Cassia senna sophera linn*



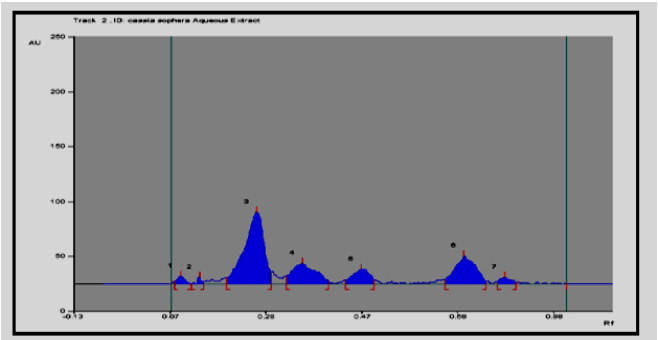
HPTLC chromatogram of Std Quercetin



HPTLC chromatogram of Hydro alcoholic extract of *Cassia senna sophera linn*



HPTLC chromatogram of ethanolic extract of *Cassia senna sophera linn*

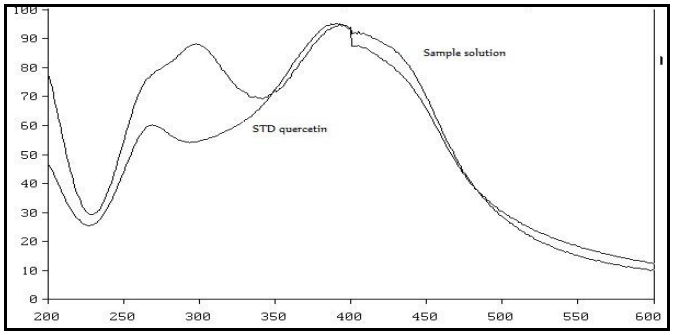


HPTLC chromatogram of Aqueous extract of *Cassia senna sophera linn*

From the above observation Rf value of TLC profile 4 was found 0.45 and having very good separation of Quercetin so, we finally decided to choose mobile phase Toluene: Ethyl acetate: Acetonitrile: Formic acid (10: 2.5: 1: 1) for HPTLC method development othanolic, Aqueous andHydroalcoholic extract of ***Cassia sennasopheralinn*** for Quercetin.

Structure Elucidation of Isolated Compound

UV Spectrophotometry



Overlay spectra of standard Quercetin and Quercetin

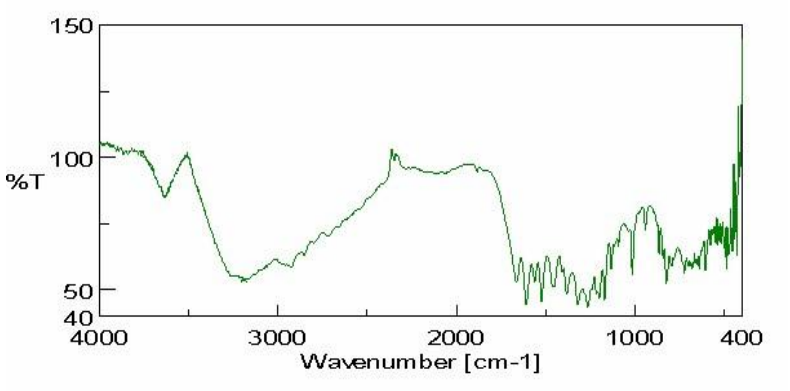
From ethanolic extract.

Wave number(cm^{-1})	Functional group
3628.4	O-H stretching
2923.56	Aromatic C-H stretching
1665.23	C=O Stretching
1610.27	Aromatic C=C stretching
1381.7	C-O Stretching of Phenol
1320.04	C-O Stretching of ether

Wave number and functional group of isolated compound

Infra-red spectroscopy

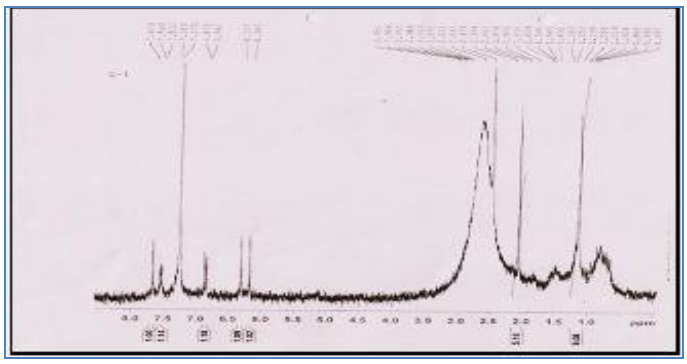
Infrared spectroscopy was one of the most powerful analytical techniques which offer the possibility of chemical identification. One of the most advantage of IR was that provides useful information about the structure of molecule quickly. The fact that many functional groups were identified by their characteristic vibration frequencies makes the IR spectrum the simplest and often the most reliable method of assigning a compound to its class. IR spectroscopy was usefully contributed to structural elucidation, when new compounds were encountered in plants. The wave number, assigned functional group and % transmittance of isolated compound was reported.



IR spectra of isolated compound

Nuclear Magnetic Resonance of isolated compound

^{1}HNMR study was performed in the $CDCl_3$. According to the position of different protons in $CDCl_3$ should be in range of 5.5 to 8 ppm.

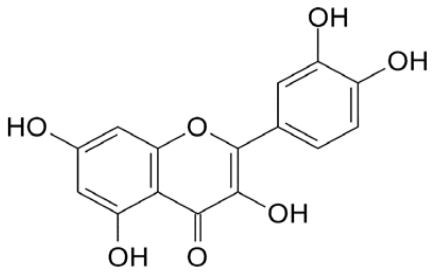


Interpretation of H-NMR spectra

△	Multiplicity	No. of protons	Group
7.67	S	1	-OH of coumarin ring
7.31	S	1	H of aromatic ring
6.84	S	1	H of aromatic ring
6.31	S	1	H of aromatic ring
6.18	S	2	-H of coumarin ring
2.41	S	2	–OH of coumarin ring
1.008	S	2	–OH of aromatic ring

Prediction of compound isolated from flavonoids fraction of leaves of *Cassia sennasopheralinn.*

By observing the results of UV, IR and H^1NMR spectra it was predicted that the isolated compound was found probably to be 2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4*H*-chromen-4-one.



2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4*H*-chromen-4-one

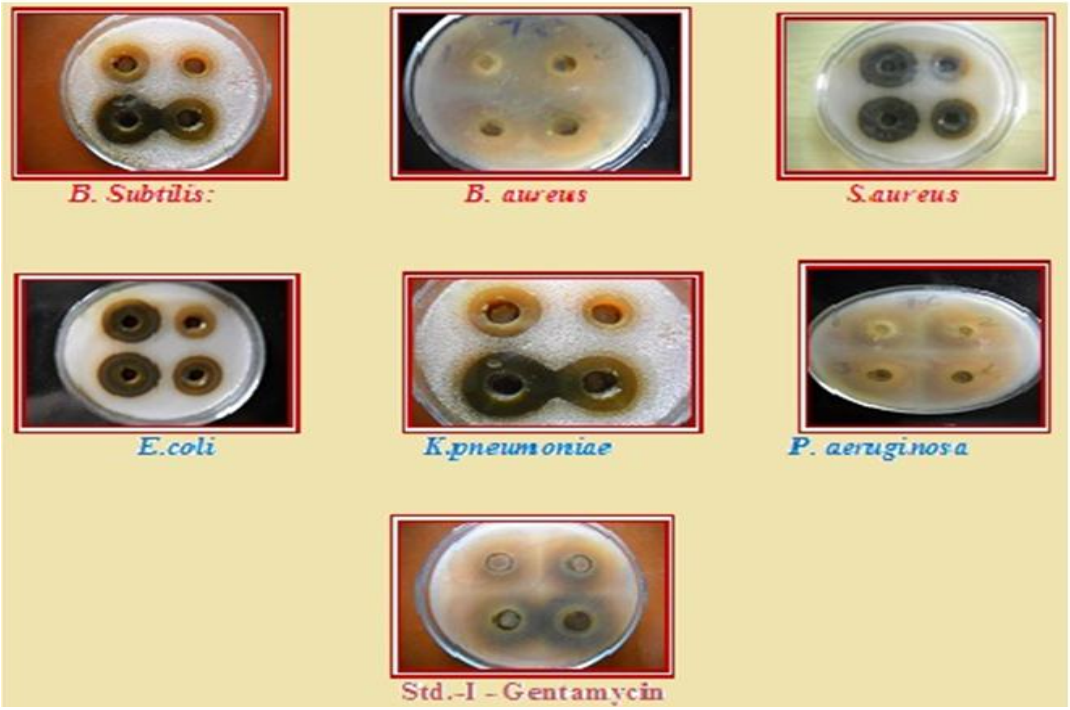
Antibacterial Assay

Each extracts was tested against three Gram-positive bacteria (*Bacillus subtilis, Bacilluaureus*and *Staphylococcus aureus*) and three Gram-negative bacteria (*Escherichia coli*, K *pneumoniae*., *Pseudomonas aeruginosa*). Antibacterial activity was determined by Pour plate method in sterile nutrient agar medium plate. The 6.0 mm wells were made each Petri plate. Plate were allowed to stand for 1hr and inoculated with 1ml extract. Respective dilutions ranging from 10 µg/ml to 100 µg/ml was prepared. The applied sample extracts were allowed to diffuse properly by keeping the Petri plates in refrigerators at 4^0C for 4 hrs. Then the Petri plates were transferred to incubation chamber for 24 hrs at 37^0C. After incubation check the diameter zone of inhibition in mm was measured. Greater the diameter more active is plants extracts tasted on the colony of the organisms.

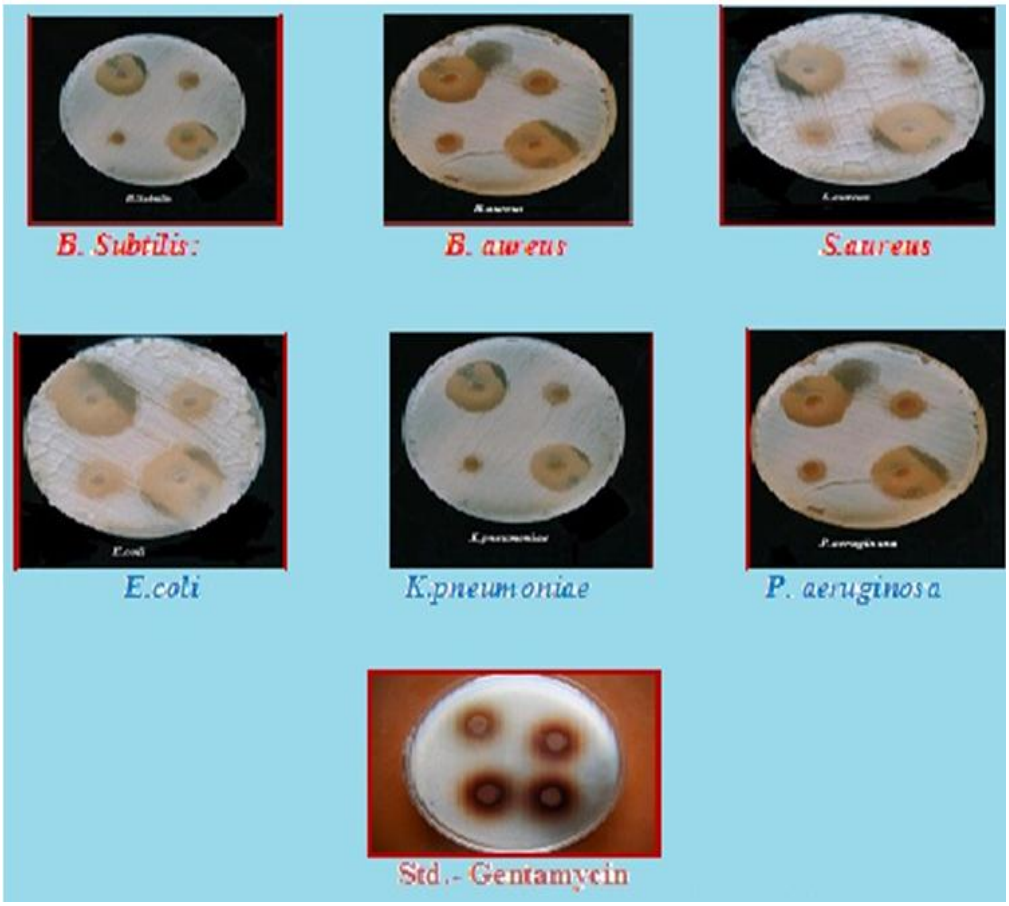
Preliminary screening for antibacterial activity of leaves extract of Cassia *senna sopheraL*

Test Samples	Conc. (µg/ml)	Zone of Inhibition (mm)					
		Gram positive			Gram negative		
		B. Subtilis	*B.aureus*	*S.aureus*	*E.coli*	*K.pneumoniae*	*P. aeruginosa*
ALECSS (Alcoholic)	25	9	10	11	13	12	11
	50	15	17	19	15	14	18
	75	20	21	26	27	27	20
	100	25	27	34	30	31	25
AQECSS (Aqueous)	25	7	9	13	12	11	10
	50	10	13	20	14	13	17
	75	18	19	21	20	18	21
	100	20	20	29	27	25	24
Gentamycin	25	15	14	11	16	14	13
	50	22	16	14	20	17	20
	75	24	30	19	24	19	24
	100	30	34	23	27	29	28

Preliminary screening for antibacterial activity of aqueous extract of Cassia*sennasopheralinn*



Preliminary screening for antibacterial activity of alcoholic leaf extract of Cassia*sennasopheralinn*



DISCUSSION

Macroscopical study of each part of Cassia*sennasophera* gives details about physicochemical constants which help in standardization of crude drugs. Microscopical inspection of crude drugs from plant origin is essential for the identification of the grounded or powdered materials. The quality and purity of crude drugs depends upon ash values. The total ash usually consists of inorganic radicals like carbonates, phosphates, silicates and silica of sodium, potassium, magnesium and calcium. Inorganic parts like calcium oxalate, silica, carbonate content of crude drug affects "total ash" values, such variables are then removed by treating with acid (Hydrochloric acid) and then acid-insoluble ash value is determined. The results for total ash, acid-insoluble ash have been reported first time in present work. The extractive value of drug reported is directly related to amount of a constituent or complex of constituents present in the drug. In some cases the amount of drug soluble in a given solvent is also represents its purity. Extractive values are useful for evaluation of crude drugs and gives an idea about the nature of chemical constituents present in them. All standardization

parameter of plant has been reported first time in present work. The qualitative chemical tests were carried out for the identification of the nature of phytoconstituents present in all fractions of Cassia*sennasophera*. Results obtained from all phytochemical screening showed mainly the presence glycosides and flavonoids.

Calibration curves

The calibration plot indicates the response is a linear function of concentration in the range 60 to 180 µg Quercetin. The correlation coefficient, intercept and the slope were 0.994, 661.9x respectively The chromatographic study showed the presence is Quercetin in hydro alcoholic, ethanolic and aqueous extract After structural elucidation it was found that the isolated single compound was probably found to be 2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4H-chromen-4-one. 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one it is also known as Quercetin is a flavonol type of flavonoids.

The ^{1}HNMR of the isolated compound showed the peak at ɗ 7.67 due to the presence of –OH of coumarin ring. The peak observed at ɗ 7.31, ɗ 6.84, ɗ 6.31 due to single proton of the aromatic ring, while the peak observed at ɗ 6.18 showed the two protons of coumarin ring. Other peak at ɗ 2.41 and ɗ 1.008 due to two –OH group of aromatic ring.

Anti-bacterial activity

All the extracts were tested for their anti-bacterial activity by cup-plate method against *S.aureus*(gram positive) and *E.coli*(gram negative) organisms at the concentration of 50µgm/ml and 100µgm/ml. All the compounds showed good anti-bacterial activity of alcoholic and aqueous bark extracts of Cassia*sennasophera* used for antibacterial agents compared to **Gentamycin** as a standard drug. The result of antibacterial activity of alcoholic and aqueous extracts against gram positive and gram negative bacteria.

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CONCLUSIONS

In the present study, the phytochemical screening suggests the presence of flavonoids in the Hydro alcoholic, aqueous and ethanolic extracts. A rapid, simple, accurate and specific HPTLC method for quantitative estimation of Quercetin present in the extract of leaves of Cassia*ennasophera* L. has been developed. The data could be used as a QC standard. The method used in this work resulted in good peak shape and enabled good resolution of Quercetin from other constituents of the hydroalcoholic, ethanolic and Aqueous extract of leaves of Cassia*ennasophera* L. There were no interferences with the Queretin peak from other constituents present in the extract of leaves of Cassia*ennasophera*L. The aqueous bark extract of Cassia*ennasophera* shownless significant activity against six organisms tested but the aqueous extract of Cassia*ennasophera* less activity against ***B. Subtilis*** organism as compared to the standards drugs. The aqueous bark extract ofCassia*ennasophera* shown most significant against ***S.aureus*** as compared to standards drug. The aqueous extractat 100 µg/ml concentration was significant as compared to the standard drugs 25 µg/ml Gentamycin and 25µg/ml against all organisms. The alcoholic bark extract of Cassia*ennasophera shown* mostsignificantactivity against six organisms tested but the alcoholic extract of Cassia*ennasophera* low activity against ***B. Subtilis*** organism as compared to the standards drugs. The alcoholic bark extract of Cassia*ennasophera* shown most significant against *S.aureus* as compared to standards drug. The alcoholic extractat 100 µg/ml concentration was significant as compared to the standard drugs 25 µg/ml Gentamycin and 25µg/ml against all organisms.

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