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Research Paper

ANGIOGENIC TOXICITY STUDY AND CHROMATOGRAPHIC ESTIMATION FOR METHANOLIC EXTRACT OF HELICONIA PSITTACORUM

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ABSTRACT

Ethnobotanically, *Heliconia psittacorum* is used for the treatment of ulcers in the scalp in South America. This study aims at standardisation and quality evaluation of the isolated quercetin from the leaf methanolic extract (LME) of *H. psittacorum*. Methanolic extracts of dried leaves were prepared and was subjected to phytoconstituents screening. The extract was then subjected to different chromatographic techniques such as thin layer chromatography (TLC), column chromatography and High Performance Liquid Chromatography (HPLC) for quality evaluation of the isolated quercetin. The extract was also subjected to antioxidant assay and anti-bacterial assay and also determination of anti-angiogenic activity of the extract using Chorioallantoic membrane assay (CAM). The method proved to be accurate, sensitive, and precise with the recovery values within the acceptable ranges. The extract showed good inhibition towards E. coli and showed acceptable result in the antioxidant activity showing the IC_{50} value at minimal concentration. On subjecting the CAM to different concentrations of LME the average score determined showed strong anti-angiogenic activity at 100-150µg/ml. So LME can be used for further investigation for its different medicinal values.

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INTRODUCTION

Medicinal plants have always paved the way for the new drug discovery since they have many active constituents which can be used for curing many human ailments.

Heliconia psittacorum belongs to the family Heliconiaceae, is a herbaceous perennial found in Caribbean and North South America in the Amazon rain forest. The plant apart from being used as an ornamental plant also has incredible capacity to prevent and cure diseases which has emerged its use in the medicinal world. It is used for ulcers in the scalp in South America (1).

Flavonoids are a particular chemical found in plants which have a wide range of health nutrients. As per previous report it was found that flavonoid was an active phytoconstituents in *Heliconia rostrata* so it can be expected that *Heliconia psittacorum* belonging to the same class may also contain flavonoid as a bioactive compound and since quercetin is a most powerful flavonoid found in plants and also helps to reduce inflammation, eliminate pain, acts as an anti-cancer, boost immune systems and many more, so quercetin was chosen as a targeted compound in the leaf methanolic extract of *Heliconia psittacorum* and since there is not much data about the medicinal use of the plant *H. psittacorum*, so our study aims for the standardisation and quality evaluation of isolated quercetin from the leaf methanolic extract using an HPLC method (2).

There are some conjectures that the generation of free radicals inside the body may lead to cellular changes and development of cancer which can be neutralised by the antioxidants from different medicinal plants. Previous research reports have suggested that the higher intake of antioxidant rich food is associated with rick of degenerative diseases and cancer. So antioxidant study was performed using DPPH and ABTS radical scavenging assay (3).

The failure of existing antimicrobials in the treatment of infectious diseases is being increased continuously. So the researchers are now focussing on the development of new drug molecules for controlling the microbial infections (4).

The chorioallantoic membrane assay (CAM) is an in vivo assay method which is used to study angiogenesis or anti-angiogenesis or both. Semi-quantitative scoring system was used to find the anti-angiogenic activity of plant extract (5).

MATERIALS AND METHODS

Collection of leaves of *Heliconia psittacorum*

Leaves of *Heliconia psittacorum* were collected from Regional Plant Research Centre, Bhubaneswar. The leaves were cleaned, cut into small pieces and then dried at room temperature for 7 days before being grinded and sieved and then further processed for extraction.

Percentage yield (%) = [Weight of Extract (g)/ Weight of dried matter (g)] X 100

Preparation of the extract

About 120gm of powdered sample was taken for extraction by soxhlet extraction procedure by taking 400 ml of methanol for 48 hrs and then filtered. The filtrate was concentrated by evaporating the solvent to obtain the methanolic extract of *H. Psittacorum* which was then refrigerated.

After extraction of plant material, the extract was subjected to phytochemical screening for the presence of alkaloids, flavonoids, proteins, carbohydrates, glycosides etc.

Thin layer chromatography

By TLC, the crude extract obtained was analysed visually. TLC analysis was carried on glass plate using silica gel 60GF. Extracts were spotted manually using capillary tube. A mixture of Hexane: Ethyl acetate: Chloroform (7:1.5:1.5) was used as an eluent for sample separation. The eluent moves through the plate and goes up by capillary action carrying the compounds which separates and appears as spot. The distance travelled by the solvent was marked as the solvent font. Then the retention factor was calculated by the formula:

$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Column chromatography

The TLC plate showed 4 spots when viewed in a UV chamber. So for better separation and identification of compounds column chromatography was used. In column chromatography the stationary phase was a glass column filled with silica gel. The column packing was done with silica gel 100-200 mesh size which was suspended in Hexane and then poured in the glass column to form the stationary phase. The mobile phase was allowed to flow under gravity for separation of phytoconstituents. The solvent was collected at different intervals and analysed. For the separation of sample through the column the same mobile phase was used in TLC. The methanolic extract was

dissolved in methanol and then poured into the column. The different fraction that was further obtained was further identified by TLC using a standard sample.

Identification of compounds

For identification the different fractions were subjected to TLC analysis. In this study flavonoid was targeted and thus quercetin was taken as the standard. The mobile phase that was used for the identification of quercetin was Ethyl acetate: toluene: formic acid (4: 3.5: 0.5) the fraction that showed the same RF value as that of standard quercetin was taken into consideration for further study by HPLC.

UV spectroscopy

The standard quercetin was dissolved in methanol to make a concentration of 1mg/ml which was observed in UV at 256nm. The fraction that showed the similar Rf value as that of quercetin was also observed in UV spectroscopy and the absorbance was measured at 256 nm.

Preparation of standard stock solution

Stock standard solution of quercetin was prepared by dissolving accurate amount of quercetin in HPLC grade methanol to obtain a concentration of 1000 ppm with the help of sonication. Working standard solution were prepared by diluting the standard stock solution with methanol.

Preparation of sample

The fraction after column chromatography was further diluted to different concentrations which was filtered through Millipore filter paper before being injected in the HPLC system.

HPLC analysis

All analyses were performed using JASCO HPLC system equipped with a photodiode array detector. A reversed phase C18 analytical column was used with a mobile phase consisting of a mixture of solvent A (acetonitrile) and B (methanol) in the ratio of 90:10. An isocratic elution at a flow rate of 1.0 mL/min was used for efficient separation and quantification.

Quantitative analysis

External standard method was performed using Quercetin as the standard solution for the quantitative determination of quercetin in the methanolic extract of *H. Psittacorum*.

Method validation

The validation of the method was performed as defined by ICH guidelines (International Conference on Harmonisation).

Validation of the method was performed on the basis of robustness, linearity, limit of detection, limit of quantification and accuracy in terms of recovery, repeatability, precision (6).

Linearity was established by triplicate injections of solutions containing standard quercetin. Linear calibration curves in the range from 10 to 100 µg/ mL were constructed by plotting the peak area against the concentration. The precision of the method was indicated by examining six sample solutions of different concentration containing isolated quercetin and from the peak area obtained the concentration was calculated and the results were expressed as %RSD. The ruggedness of the method was calculated by carrying out the experiment on different days, by different analysts. By varying the chromatographic condition, the robustness of the method was evaluated The flow rate of the mobile phase was changed from 0.8 to 1.0mL/min and from 1.0 to 1.1 mL/min. Using the standard addition method, the accuracy of the method was studied at three different levels that is 80, 100,120%. Accuracy of the method was studied by recovery experiments using the standard addition method at three different levels (80, 100, and 120%). Known amounts of standard solutions to reach different levels known amount of standard solution of quercetin was added to sample solutions. The sample were analysed in triplicate and the difference between the spiked and unspiked sample was determined for different recovery levels. The LOD and LOQ values were calculated from the calibration curves

$$LOD = 3.3\sigma/s$$

Where, σ = the standard deviation of the response S = the slope of the calibration curve. The slope may be estimated from the calibration curve of the analytes. The σ can be estimated as the standard deviation of the blank. The value of σ can also be estimated based on the calibration curve.

$$LOQ = 10\sigma/s$$

Where, σ = the standard deviation of the response S = the slope of the calibration curves. The value of S and σ are estimated as for the detection limit. For system suitability six replicates of injection of standard solution was injected and retention time, asymmetric factor, resolution, capacity factor, and the number of theoretical plates were determined for different concentrations to check the stability of the instrument (7).

ANTIOXIDANT STUDY (8)

Antioxidant activity of the plant was performed in methanolic extract.

Scavenging of 1, 1-Dipheny-2-Picrylhydrazyl (DPPH) (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol.

DPPH assay was performed using stock solution of the sample with a concentration of 1mg/ml which was further diluted to different concentration that is 5, 10, 25, 50, 125, 200µg/ml in methanol. 1ml of the sample from different dilutions was taken separately in test tubes to which 3ml of DPPH solution was added. The mixture was shaken and was kept in dark in room temperature. Ascorbic acid was taken as the positive control. After 30 min absorbance was measured at 517 nm against the blank solution. Assays were performed in triplicate. The percentage inhibition of DPPH was calculated using the following equation;

$I\% = (A_0\text{-}A/A_0) * 100$

Where A_0 is the absorbance of the blank

A is the absorbance of the sample.

The concentration at which 50% inhibition is observed (IC_{50}) is calculated in µg/ml. ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)

Another method for determining the antioxidant activity of plant extract is ABTS radical scavenging assay. Stock solution of the sample with a concentration of 1mg/ml which was further diluted to different concentration that is 5, 10, 25, 50, 125, 200µg/ml in methanol. ABTS stock solution was prepared using 7Mm ABTS and 140Mm potassium persulphate which was mixed and kept in dark for 16 hrs at room temperature. To get an absorbance of 0.700 ± 0.05 at 734nm. 1ml of the sample from different dilutions was taken separately in test tubes to which 3ml of ABTS solution was added. The absorbance was measured at 734nm after 30 min. the ABTS scavenging activity was calculated by the following equation;

ABTS scavenging activity (%) = $(A_0\text{-}A/A_0) * 100$

Where, A_0 is the absorbance of the blank solution and A is the absorbance of the sample solutions.

The concentration at which 50% inhibition is observed (IC_{50}) is calculated in µg/ml.

ANTIBACTERIAL STUDY

BACTERIAL STAINS

The antibacterial activity of each plant extracts was evaluated using five bacterial strains causing food poisoning diseases. Four

strains of gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salomonella typhi, Shigella flexneri*) and one gram positive bacteria (*Straphylococcus aureus*). The bacterial strains were provided by RMRC (Regional Medical Research Centre), Bhubaneswar.

INOCULUMS PREPARATION

Each bacterial strains were subcultured overnight at 37°C in Nutrient Agar media or Lysogeny agar media. The bacterial growth was harvested using 1ml of sterile saline water.

WELL DIFFUSION METHOD FOR THE DETERMINATION OF ZONE OF INHIBITION

The antibacterial activity evaluation of LME was performed using the well diffusion method. The inoculums of the microorganism was prepared from bacterial cultures. Fifteen millilitres of Mueller-Hilton agar medium was poured in clean sterilized Petri-plates and allowed to cool and solidify. Hundred microliters of broth of bacterial stain was pipetted out and spread over the medium evenly with a swab and dried properly. Wells of 6 mm in diameter were bored using a sterile cork borer. Solutions the extract (10 mg/ml) in dimethyl sulphoxide (DMSO) were prepared. Hundred microlitres of plant extract solutions was added to the wells. The Petriplates were incubated at 37° for 24 h. Antibacterial activity was evaluated by measuring the diameters of the zones of inhibition (ZI). All the determinations were performed in triplicate.

Chorioallantoic Membrane Assay

5 days old fertile chicken eggs were collected from Central Poultry Development Organization (CPDO), Bhubaneswar. Eggs were cleaned with 70% ethanol to remove dirt and contaminants. The whole assay method was conducted under laminar flow.

Assay method (9)

Candling experiment was done before the experiment to check for the viability of the eggs. DMSO was used as a negative control. 1 mg/ml of standard stock solution of leaf methanolic extract was prepared using DMSO. A 3mm diameter filter paper was sterilized by dipping in ethanol and then subjected to UV rays and laminar flow to dry. The filter paper was dosed with different concentrations of leaf methanolic extract. A window in the egg shell about 1x1 cm was made to expose the Chorioallantoic membrane to implant the filter papers carrying the drug. Photograph of the embryos were captured before and while dosing. The eggs were closed with the cellophane tape

and returned to the incubator at 37˚C. The filter paper disc was gently discarded from CAM after 48 h and examined for anti-angiogenic effect at site of sample application. A third photograph was taken to obtain the image of the CAM after treatment with various extracts. Images of the surface of the same CAM within the same test sample (before and after treatment) were compared and the anti-angiogenic effect was quantified solely in the area of the CAM covered by the filter paper disc. A modified semi-quantitative score system with a scale of 0–2 was used for grading according to Table 8. The degree of anti-angiogenic effect was recorded blindly by ten observers. For every test sample, the average score was calculated and the interpretation of anti-angiogenic effect was quantified according to Table 9.

RESULTS AND DISCUSSIONS

For the qualitative analysis of different extracts of the leaves of selected medicinal plants, the presence or absence of alkaloids, carbohydrates, flavonoids, glycosides, phytosterols and phenols was investigated. The results of this study were shown in the following Table 1.

Table 1: Result of phytochemical screening of plant extract

Phytoconstituents	Result
Alkaloid	Absent
Saponin	Absent
Glycosides	Absent
Tannins	Present
Flavonoids	Present
Steroids	Present
Carbohydrates	Present
Fixed oil and fats	Present
Protein test	Absent

The result of these analysis indicated that carbohydrates, tannins, steroids, fats, oils and flavonoids were present in leaves of *Heliconia psittacorum* which indicates a good therapeutic activity may be present in our selected plant. The Figure 3.1 of the thin layer chromatography plate shows 3 spots of the methanolic extract of the leaves with a good separation from the first spot having a retention factor from 0.5-0.7. The methanolic extract of the leaves were further subjected to column chromatography for better purification. 3 different fractions were obtained out of which fraction 1&2 showed more than one spot on when TLC analysis was carried out and fraction 3 showed single spot which when compared with standard quercetin showed the same retention factor value as shown in the Figure 2 which was further subjected for HPLC analysis. A simple high performance liquid

chromatography method was developed to identify and quantify the flavonoid, quercetin in crude *H. psittacorum* extract. The method was optimized for good appearance of extract components with sufficient sensitivity and appropriate peak symmetry. The chromatographic separation was achieved using reverse phase (C18 column) and it was possible to analyse the presence of quercetin with adequate resolution and forming symmetrical peaks. Optimization of the chromatographic conditions led to a more rapid and efficient separation of quercetin from other extract components. The chromatograms from the plant sample extract confirmed that the retention times of flavonoid compound in the sample was comparable to retention time of standard (quercetin) (Figure 3). Calibration graphs were constructed using concentrations in the range of 10-100µg/ml as shown in Figure 4, obtaining an equation y = 0.2394x + 12.393 and R^2 = 0.9992 showing a good regression correlation. The assay value gave good recovery % as shown in Table 2.

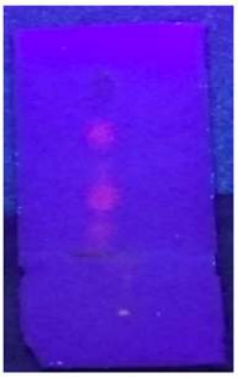

Figure 1: Thin Layer Chromatographic separation of methanolic extracts of the plant. 3 spots were identified with good separation

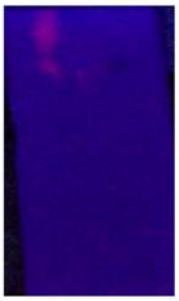

Figure 2: Column chromatography and thin layer chromatographic plate showing same Rf value (rf= 0.78) for both standard quercetin and the isolated fraction after column chromatography

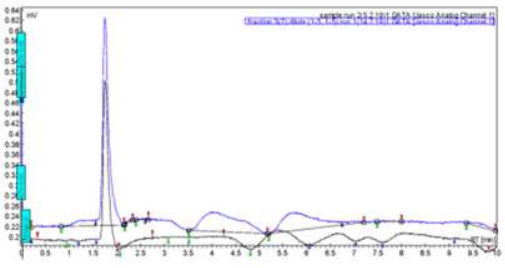

Figure 3: Overlay chromatogram of the standard quercetin and the isolated fraction showing the same retention time i.e. 1.758 min.

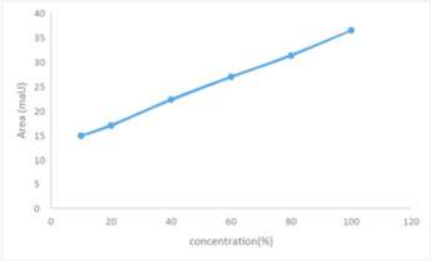

Figure 4: Calibration curve for the linearity test result for quercetin. Concentration versus peak area scatter graph of LME, the r^2 indicated good regression correlation

Table 2: Assay of methanolic extract of the leaf of H. psittacorum

Compound Name	Amount Injected (mg/ml)	amount (mg) found by the proposed method	Mean (±S. D) amount (mg) found by the proposed method	% Mean (S.D) amount injected
Isolated fraction of the methanolic extract	0.5	0.491027	0.4903±0.001	98.06%±0.19
	0.5	0.489275		
	0.5	0.490887		
	0.3	0.29001	0.28676±0.004	95.58%±0.17
	0.3	0.28924		
	0.3	0.28103		

Accuracy was determined from three concentrations (10, 20, 30µg/ml), with three replicates each taking into consideration the levels of the method's linear range, through the standard addition test and expressed through the recovery percentage of the quercetin. It can be observed in Table 3 and from Figure 5 that all the results of the accuracy test showed a concordance between the results found in the analysis of the extract, proving that the method is accurate.

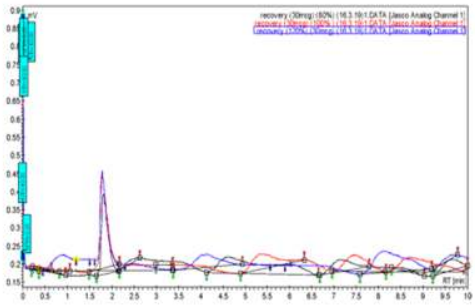

Figure 5: Overlay of the chromatographs for recovery study at 30µg/ml proving the method to be accurate.

Table 3: Result for the accuracy study of the plant extract. According to the result the standard deviation was found to be within the acceptable limit.

Conc. (µg/m)	Amount (%)	Amount added (µg/ml)	% Recovery	±S.D	%RSD
10	80	8	100.55	0.19	0.18
	100	10	102.77	0.43	0.44
	120	12	99.44	0.24	0.25
20	80	16	98.3	0.78	0.8
	100	20	99.16	0.58	0.61
	120	24	101.38	1.02	1.01
30	80	24	101.11	0.3	0.32
	100	30	99.5	0.35	0.38
	120	36	100.28	0.7	0.71

Table 4: LOD and LOQ obtained from the extract indicating minimal concentration is needed for detection and quantification

Conc. (µg/ml)	LOD	LOQ
20	0.65	1.21
30	0.98	0.92

Table 5: Results obtained in the four tests performed to evaluate the quercetin's robustness showing little changes but the values of standard deviation was within the acceptable range.

ANALYST-1				ANALYST-2			
Conc. (µg/ml)	Peak area	Calc. amt (µg/ ml)	Statistical analysis	Conc. (µg/ml)	Peak area	Calc. amt (µg/ ml)	Statistical analysis
20	44.82	19.8	Mean= 19.45	20	40.26	20.51	Mean= 19.7
20	45.10	19.6	SD= 0.33	20	39.82	18.25	SD= 0.84
20	43.25	19.2	%RSD = 1.69	20	41.55	20.42	%RSD = 0.04
20	43.22	18.9		20	40.20	19.31	

Table 6: Results obtained to evaluate the quercetin's ruggedness on varying the chromatographic conditions which showed the method to be sensitive on varying the chromatographic conditions.

Sl. No.	Chromatographic changes (factors)	Retention time (R.T) in min.
10	Acetonitrile : Methanol	
	60:40	1.51
	80:20	1.6
	90:10	1.7
20	Flow rate (ml/min)	
	0.5	1.9
	0.8	2.0
	1.0	1.7

Table 7: System suitability test values of HPLC method

Conc.(µg/ ml)	R.T	Asymmetric value	Capacity factor	Resolution	No. of theoretical plate
20	0.083	1.64	0.00	0.00	0.00
20	1.083	1.09	0.00	2.19	2224.7
20	1.775	2.64	0.00	1.15	1623.08
20	2.667	1.62	0.00	2.81	1928.80
20	3.917	1.43	0.00	1.00	1295.20
20	5.133	1.83	0.00	1.24	2387.23
30	0.342	2.58	0.00	0.00	1356.65
30	1.358	0.69	0.00	8.88	0.00
30	1.775	0.50	0.00	2.79	1564.14
30	3.251	2.58	0.00	1.62	940.89
30	5.125	1.46	0.00	2.10	1423.6

Table 8: Semi quantitative scoring system of quantitative estimation of damage/destruction in CAM assay after treatment [10]

Scale	Effects observed on CAM after treatment
0	No change
0.5	Minor change in the blood vessels
1	Small capillary free area below the area of treatment; a few micro vessels congregate or the growth of blood vessels marginally reduced.
2	Capillary free area below the area of treatment; micro vessels no longer visible and large vessel merging.

Table 9: Interpretation of anti-angiogenic effect to be drawn from the average scores of semi-quantitative scoring systems

Average score	Conclusions
< 0.5	No anti-angiogenic effect
≥ 0.5 and ≤ 1	Weak anti-angiogenic effect
> 1 and < 1.5	Good anti-angiogenic effect
≥ 1.5	strong anti-angiogenic effect

Table 10: Anti-angiogenic effects of LME of *Heliconia psittacorum* by CAM assay showing with increase in concentration anti-angiogenic activity increases.

Extracts	Dose (µg)	Average score	Anti-angiogenic effect
DMSO	0 mg	0.10 ± 1.50	No
LME	0.10 mg	1.65 ± 0.43	Strong
	0.15 mg	1.71 ± 0.53	Strong

The precision of the method as shown in Figure 6. Showed that there were no significant differences between the results of the test, indicating that the precision of the proposed method was satisfactory. The robustness test (Table 5) presented variation in the retention time of quercetin showing that the method is sensitive to small variations of flow rate and mobile phase

composition. Flow rate of 0.5, 0.8 and 1.0 mL/min resulted in variation of retention time.

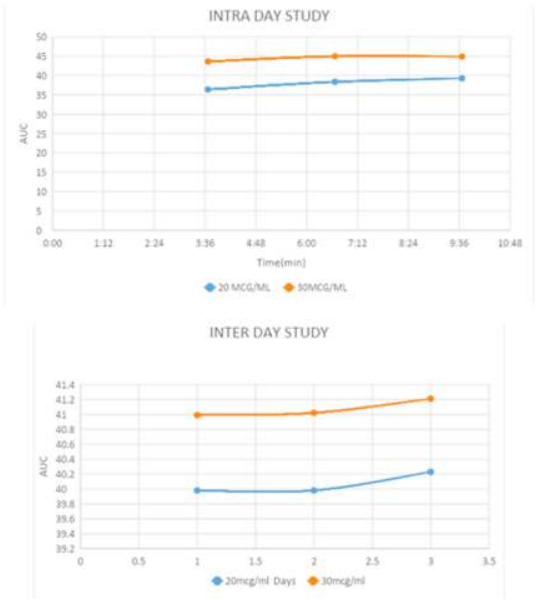

Figure 6: Intra and inter-day precision for the quantitative determination of standard phenolic compounds by HPLC indicating its stability for longer durations.

The antioxidant study showed IC50 value at lower concentration that is 0.115µg/ml for DPPH radical scavenging assay and 0.455µg/ml for ABTS radical scavenging assay Figure 7 and Figure 8.

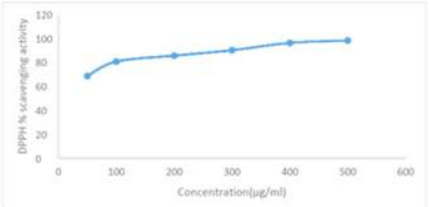

Figure 7: Antioxidant activity study by DPPH radical scavenging assay and the IC50 value was found at 0.115µg/ml.

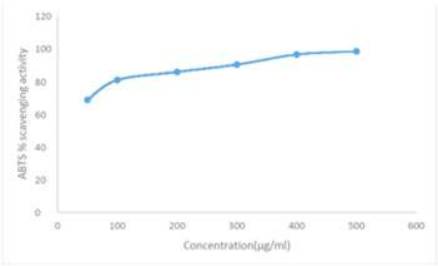

Figure 8: Antioxidant activity study by ABTS^{+} radical scavenging assay and IC50 value was found to be 0.455µg/ml

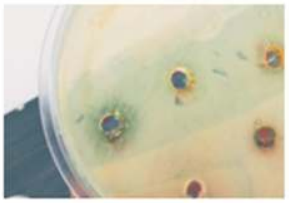

Figure 9: Antibacterial assay result showing the inhibition of E. coli

As per the bioassay result Figure 9 it was found that to some extent the methanolic extract of the leaves are capable of inhibiting E. coli (Escherichia coli) the organism that cause opportunistic infections and are found in the intestinal tract, causing urinary tract

infection, infection of wound, sepsis, diarrhoea disease, dysentery, abdominal wound than the petroleum ether extract which did not show any activity. CAM has natural fibroblast growth factor (bfgf), a protein which plays an important role in the development of blood vessels in the chick embryo. [11] The filter paper carrying the drug was implanted in the CAM. The different changes in the blood vessels were recorded and anti-angiogenic activity was found out using a semi quantitative method of estimation done blindly by 10 different observers. The average score of the estimation was done. It was found that LME at a concentration 100µg/ml proved to show better inhibitory activity than at 150µg/ml. The CAM with negative control dosing did not show anti – angiogenic activity. (Figure 10, Table- 10).

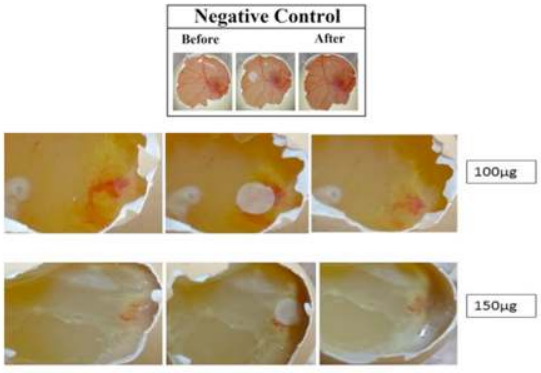

Figure 10: Images of live CAM implanted with LME at different doses. DMSO was used as negative control in which no significant inhibition of angiogenesis was observed. The concentration of 100µg/ml and 150µg/ml showed good angiogenesis inhibition in LME

CONCLUSION

Heliconia psittacorum, the plant which is basically used as an ornamental plant and was not used for medicinal plant. But earlier literature and our study showed the presence of medicinal value on a major content as can be used for many serious diseases. The phytochemical analysis showed the presence of many active phytoconstituents which indicates its therapeutic value. When the methanolic extract of the plant was subjected to Thin Layer Chromatography and column Chromatography it showed well differentiated spots and also showed similarity with standard quercetin in every aspect. Moreover, the method development results proved that the method used for the isolation and quantification of quercetin was found to be accurate, precise and sensitive. The plant not only gave good results in chemical aspects but also showed an acceptable result in biological field also. The antioxidant study that was based on % scavenging activity showed IC50 at minimal concentration. The antimicrobial activity proved the ability of the plant in inhibiting harmful microorganisms that are responsible

for food poisoning. The CAM assay done to find out the toxicological value of the plant extract showed maximum inhibition at 100-150µg/ml.

Hence we can conclude that Heliconia psittacorum can be used for further investigation in the field medicines considering its toxicological and other chemical values.

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