Journal of Applied Pharmaceutical Science Vol. 5 (05), pp. 018-027, May, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.50504
ISSN 2231-3354 

# Carbohydrate hydrolyzing enzyme inhibitor property, antioxidant and phytochemical analysis of *Cassia auriculata*, *Delonix regia* and *Vinca rosea* Linn: an *in vitro* study

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**ARTICLE INFO**

*Article history:*
Received on: 08/02/2015
Revised on: 12/04/2015
Accepted on: 22/04/2015
Available online: 27/05/2015

***Key words:***
*Cassia auriculata*, *Delonix reiga*, *Vinca rosea*, alpha glucosidase, alpha amylase, HPTLC, FT-IR, GC-MS.

**ABSTRACT**

The study comprises the evaluation of antidiabetic and antioxidant activities of *Cassia auriculata*, *Delonix rega* and *Vinca rosea* belonging to Fabaceae family. Initially, *In vitro* α-Glucosidase inhibitor activity was performed as a preliminary screening for petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of three collected plants. In comparison with all the extracts, methanol extracts shown promising activity with $IC_{50}$ values of 58.52µg/ml, 83.46µg/ml and 77.41µg/ml for *C. auriculata*, *D. regia* and *V. rosea* respectively. Hence, these three extracts were further selected for DPPH radical scavenging activity. *C. auriculata* resulted in 96% DPPH radical scavenging activity fallowed by *D. regia* (78%) and *V. rosea* (60%). Hence, methanol extract of *C. auriculata* was selected to evaluate α-amylase inhibitor potential, reducing power capacity and also quantified for total phenolic and total flavonoid content within them. Selected extract showed efficient α-amylase inhibitor activity, reducing power capacity and good amount of phenolics and flavonoids. HPTLC analysis identified five polyphenols and FT-IR analysis detected –OH functional groups in the extract. GC-MS analysis detected hydroquinone, myoionositol and bulletin. It can be concluded that these compounds within the methanol extract of *C. auriculata* might be accountable for antidiabetic and antioxidant nature of the plant. In future, bioactive compound could be isolated and used as a carbohydrate hydrolyzing enzyme inhibitor to treat type 2 diabetic individuals.

## INTRODUCTION

Diabetes is a chronic life threatening metabolic disorder with disturbances in carbohydrate, fat and protein metabolism. Diabetes mellitus occurs when the beta cells of pancreas fails to produce insulin or deficiency in insulin secretion, action or both (Abdel *et al*., 2014; Abdulfatai *et al*., 2012). Type 1 diabetes is due to the autoimmune destruction of beta cells of pancreas and type 2 diabetes is because of the pancreas produces insufficient amount of insulin or body cells becomes resistant towards insulin. Type 1 diabetes accounts for 5-10% whereas type 2 diabetes is much more common and accounts for 90-95% cases (Amisha *et al*., 2014). Type 2 diabetes primarily affects adults, however recently type 2 has begun developing in children. There is a strong correlation between Type 2 diabetes, physical inactivity and obesity (ADA, 2013). Postprandial hyper glycaemia is an early defect in type 2 diabetic patients that leads to severe diabetic complications (Kavitha *et al*., 2012). There are carbohydrate hydrolyzing enzyme inhibitors currently available as drugs to manage postprandial hyperglycaemia in type 2 diabetic individuals such as acarbose, voglibose and miglitol but these synthetic medicines have their own complications such as nausea, mild diarrhoea and constipation (Lustman *et al*., 1997). From a long time, medicinal plants have played their own important and effective role in preventing many kinds of failure in human body which includes diabetes too, we have many reported medicinal plants which shows anti diabetic activity and can be used as a supplement for synthetic drugs (Exarchou *et al*., 2002). Based upon the medicinal qualities of the plant and increase in the trends and research activities, their use is further expected to increase. Three plants *C. auriculata*, *D. regia* and *V. rosea* were used in the present study. They belong to the family, fabaceae and are commonly found in dry regions of India and Srilanka. *C. auriculata* is a herb and is traditionally known to be effective for diabetes, skin diseases and high blood pressure (Kumaran and Karunakaran, 2006; Jain and Sharma, 1967).

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It has been reported for antidiabetic (Pari and Latha, 2002), hepatoprotective (Jeeva and Maathangi, 2011), antibacterial (Manimegalai and Naveen, 2010), antipyretic (Vedavathy and Rao, 1991), antioxidant (Anusha *et al*., 2009), and antiulcer activites (Senthil Kumar *et al*., 2003). *D. regia* is traditionally used for arthritis, constipation, diabetes and earache (Mahafuzur *et al.*, 2011).

Scientific reports shows that it can be used for antifertility (Shambhulingaiah *et al.*, 2009), antidiarrheal (Rajabhau *et al.*, 2011), anti-inflammatory (Vaishali *et al.*, 2012), antioxidant (Mariajancyrani *et al.*, 2013), hepatoprotective and cytotoxic activities (Aly *et al*., 2011). *V. rosea* is traditionally used against asthma, cervical cancer, colon cancer and diabetes (Chattopadhyay *et al*., 1991). The scientific reports reveal that *V. rosea* is known to have anticancer and antidiabetic activities (Mohommed *et. al*., 2010). The present study comprises of phytochemical screening, evaluation of carbohydrate hydrolyzing enzyme inhibitor and antioxidant activities of *C. auriculata, D. regia* and *V. rosea*.

## MATERIALS AND METHODS

### Collection of the Plant material

The flowers of *C. auriculata*, leaves of *D. reiga* and *V. rosea* were collected from Brahmapurum, Vellore and were brought to the Molecular and Microbiology Research Laboratory, VIT University. The herbarium of the plant materials were maintained in our laboratory with accession numbers CA/VIT/MMRL/01-03-2014-01, DR/VIT/MMRL/15.03.2014-02 and VR/VIT/MMRL/14.04.2014-03 for *C. auriculata*, *V. rosea* and *D. regia* respectively.

### Processing of the Plants

The flowers of *C. auriculata* and leaves of *D. regia* and *V. rosea* were shade dried, powdered with the help of mechanical blender and was extracted with petroleum ether, chloroform, ethyl acetate, methanol and water by maceration technique. The solvent extract was kept in rotaevaporator to evaporate the solvent completely and the resultant extract yield was measured. Further, the extract was kept in air tight container, stored in refrigerator at 4$^{o}$C for further experimental use.

### Chemicals used

2, 2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). Sodium carbonate ($Na_2CO_3$), Sodium phosphate ($NaH_2PO_4$) was purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). Methanol, Ferric chloride ($FeCl_3$), Potassium Ferricyanide ($K_3Fe(CN)_6$), Trichloroacetic acid, Folin- Ciocalteau reagent, Ethanol, Ascorbic acid, Gallic acid were purchased from SRL Pvt. Ltd. (Mumbai, India). Ammonium molybdate ($(NH_4)_2MO_4$) and Aluminium chloride ($AlCl_3$) were purchased from SD Fine-Chem Chem. Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

### Phytochemical screening

Test for phenols, flavonoids, carbohydrates, fats, oils, saponins, proteins and tannins was assessed by using standard protocols of Trease and Evans, 1989.

### Carbohydrate hydrolyzing enzyme inhibitor activity

#### *α- Glucosidase inhibitor assay*

α- Glucosidase inhibitor experimental procedure was carried out by the method of Matsui *et al*., 2001. A volume of 50µl of phosphate buffer (50mM and pH6.8), 10µl of enzyme (1U/ml) and 20 µl of extracts of various extracts of three plants at different concentrations (20-100µg/ml) was pre-incubated for 5 min at 37°C. About 20 µl of the substrate, PNPG was added to the mixture and incubated at 37°C for 30min. Acarbose was used as a standard and the reaction without the addition of extract was considered as control. Enzyme inhibitor activity was analyzed at 405nm in a microtitre plate reader (Bio-TEK, USA).

% α- Glucosidase enzyme inhibition = $(A_C - A_T / A_C) \times 100$

Where, $A_C$ = Absorbance of control; $A_T$ = Absorbance of test

#### *α- Amylase inhibitor assay*

α- amylase inhibitor assay was performed using the protocol adapted from Kim *et al*., 2005. Dilutions were prepared in triplicates of different concentrations (125-1000 µg/ml). Porcine pancreatic α- amylase was used as an enzyme and starch was used as substrate. To 500µl of the extract, 500µl of sodium phosphate buffer (0.02 M) containing α- amylase was added. The mixture was incubated for 10 mints in water bath at 25°C. After incubation, 500µl of 1% starch solution was added and re-incubated at 25°C in water bath for 10 mints.

The reaction was terminated by adding 1 ml of 3, 5, dinitrosalycylic acid (DNSA) reagent and was kept in boiling water bath for 7 mints. One ml of 18.2% sodium potassium tartarate solution was added to each tube after boiling and before cooling to room temperature. Sample mixture was diluted with 10 ml of distilled water. The reaction mixture added with 500µl of sodium phosphate buffer in the place of extract was used as control. Absorbance was measured at 540nm using UV spectrophotometer. The percentage of inhibition was calculated by using following formula.

% α- Amylase enzyme inhibition = $(A_O - A_E / A_E) \times 100$

Where, $A_O$ = Absorbance without extract; $A_E$ = Absorbance with extract.

### Antioxidant activities

#### *DPPH radical scavenging activity*

The DPPH radical scavenging activity was determined by the method of Brand-Williams *et al.* 1995. Different concentrations of the extracts (20-100µg/ml) was mixed with 1 ml of DPPH solution (0.2mM/ml in methanol) and incubated at 20$^{o}$C for 40 mints in dark condition. After the incubation, absorbance was read at 517nm using UV-Vis spectrophotometer. Percentage of scavenging of DPPH by the extracts was calculated by the formula given below.

% DPPH radical Scavenging = $(A_c - A_t / A_c) \times 100$

Where, $A_c$ = Absorbance of the control; $A_t$ = Absorbance of the test sample.

**Reducing power assay**

The reducing power of extracts was determined according to the method of Oyaizu *et al.*, 1986. Extracts at different concentrations (125-1000 µg/ml) in triplicates were mixed with 1ml of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1ml of 1% potassium ferric cyanide. The mixture was incubated at 50°C in water bath for 20 mins. After incubation, 1% Trichloro acetic acid was added to the reaction mixture and centrifuged at 3000rpm for 10mints. One ml of supernatant from each test tube was taken into fresh set of test tubes and 1ml of double distilled water was added. About 0.5ml of ferric chloride was also added and incubated at 50°C in water bath for 10 minutes. Absorbance was measured at 700nm by using UV-VIS spectrophotometer at 700nm. Blank was prepared by adding double distilled water instead of extract in the reaction mixture. Absorbance is directly proportional to the reducing power capability of the extract.

**Estimation of polyphenols within the extract**

***Total phenolic content estimation***

Folin Ciocalteu reagent method was used for estimation of total phenolic content in the plant extract (Singleton and Rossi, 1965). The dilution was prepared in triplicates in various concentration (125-1000µg/ml). To 50µl of each dilution, 2.5ml of Folin ciocalteu reagent (1/10 dilution in double distilled water) and 2 ml of sodium carbonate (7.5%) were added. The mixture was kept in water bath of 45$^{o}$C for 15 mints. The absorbance reading was taken at 765 nm using sodium carbonate solution (2ml of 7.5% sodium carbonate in 2.55 ml of distilled water) as blank. The results were expressed as gallic acid equivalence in µg.

***Total flavonoid content estimation***

Aluminum chloride method was used for determination of total flavonoid content present in the extracts (Adedapo, 2009). Dilution were prepared in triplicates at different concentration (125-1000 µg/ml). To 1ml of extract from each dilution, 1ml of aluminum chloride (2g of $AlCl_3$ in 100 ml of ethanol) was added. The prepared mixture was incubated at 37°C for 1 hour. After the incubation period, the absorbance was measured at 420 nm by using UV-VIS spectrophotometer using 1ml of aluminium chloride mixed with 1ml of distilled water as blank.

**HPTLC analysis**

To 25 mg of the extract, 250 µl of methanol was added and centrifuged at 3000 rpm for 5min. This solution was used as test solution for HPTLC analysis. About 2 µl of test solution was loaded as 6mm band length in the 2 x 10 Silica gel $60F_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The sample loaded plate was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with respective mobile phase (Polyphenol) and the plate was developed in the respective mobile phase up to 90 mm. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at visible light, UV 254nm and UV366nm. The developed plate was sprayed with respective spray reagent (Polyphenol) and dried at 100°C in hot air oven. The plate was photo-documented in visible light and UV 366nm mode using Photo-documentation (CAMAG REPROSTAR 3) chamber. Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254nm. The Peak table, Peak display and Peak densitogram were noted. The software used was winCATS 1.3.4 version. The sample loaded plate was kept in TLC twin through developing chamber with mobile phase and developed up to 90 mm. Solvents from the plate was evaporated by drying in hot air. Images were captured at white light, UV 254nm by placing the plate in photo documentation chamber (CAMAG REPOSTAR 3). Finally scanning was performed at 254nm and 366nm by fixing the plate in scanner stage. The peak densotigram was noted (Shah *et al*., 2008).

**FT-IR analysis**

Translucent pellet was prepared by pressing 0.001gm of the extract mixed with 20µg of potassium bromide. The infrared spectra were performed on Mattson 1000 FT England FTIR System within the range of 500-400cm$^{-1}$ wave number (Chander *et al*., 2012).

**GC-MS analysis**

Helium was used as a carrier gas at 0.1ml/min flow rate. The initial temperature of the column was programmed at 60$^{o}$C for 2 mints ramp 10$^{6}$C/ min to 300$^{o}$C with a holding of 6 mints. The temperature of injector was about 250$^{o}$C for the analysis. Identification of the chemical constituents within the extract was based on existing mass spectral data correlation those prevailed from the Wiley 8.LIB and NIST08.LIB library spectrum provided by the software in GC-MS System (Arino *et al*., 1996).

**RESULTS AND DISCUSSION**

Medicinal plants contribute in the management of diabetes due to novel phytochemical compounds within them. Scientifically, several plants have been identified with antidiabetic properties (Sindhu and Sharma, 2013). However, there are few reports available on plants with both antioxidant and carbohydrate hydrolyzing enzyme inhibitor properties (Galvez *et al*., 2010). Recent survey report on the plants with antidiabetic activity suggests that members of Fabaceae showed good antidiabetic and antioxidant activities as well due to the rich content of polyphenols within them (Kiran *et al*., 2012; Gunjan *et al*., 2010; Jain *et al*., 2010). So, this study was undertaken by selecting three plants of fabaceae family which includes flowers of *C. auriculata*, leaves of *D. regia* and *V. rosea* based on their bioavailability, traditional uses and scientific reports.

**Yield of the extract**

10 gms of flowers of *C. auriculata*, leaves of *D. reiga* and *V. rosea* were extracted with petroleum ether, chloroform, methanol and aqueous by maceration. The filtrates were dried completely and the weight of the dried plant extract was measured and yield was calculated and given in the table 1.

**Table 1:** Yield of extract from flowers of *C. auriculata* and leaves of *D.regia and V.rosea*

| Plant | Extract weight (gm) |
|---|---|
| ***C. auriculata*** | 1.306 |
| ***D. reiga*** | 0.984 |
| ***V. rosea*** | 1.763 |

Electronic weighing machine was used, and expressed in grams.

**α- Glucosidase inhibitor assay**

Glucose is the substrate which is used as an energy source. It is produced when alpha 1, 4 linked polysaccharides are broken by α- Glucosidase. α- Glucosidase breaks PNPG to P-nitrophenolate that gives yellow color. α- Glucosidase inhibitor assay is used to estimate the action of the plant extract to stop the activity of α- Glucosidase and hence there will be reduction in the development of yellow color. Hexane, chloroform, ethyl acetate, methanol and aqueous extracts were evaluated for α- Glucosidase inhibitor assay. In comparison with all the extracts of three collected plants, methanol extracts shown promising activity with $IC_{50}$ values of 58.52µg/ml, 83.46µg/ml and 77.41µg/ml for *C. auriculata*, *D. regia* and *V. rosea* (Figure 1). Based on the result of α- Glucosidase inhibitor activity, methanol extracts of the three plants were selected to perform further experiments.

**α- Amylase inhibitory assay**

α- amylase is an enzyme which hydrolyses starch into amylase. The α-amylase inhibitor assay was performed to estimate the neutral alpha amylase present in the substrate. 3,5, Dinitrosalycylic acid is used to terminate the reaction. The positive result of the assay is based on the development of yellow color. The methanol extract of *C. auriculata*, *D. regia* and *V. rosea* has shown prominent α-amylase inhibitor activity. From the result of the experiment performed, it was observed that increase in the concentration shows increase in the inhibition of α-amylase. In comparison with all the extracts, methanol extract of *C. auriculata* has shown 90% fallowed by *D. regia* (75%) and *V. rosia* (60%) of inhibition with $IC_{50}$ values of 43.66µg/ml, 60.07µg/ml, and 86.39µg/ml for *C. auriculata*, *D. regia* and *V. rosea* (Figure 2) respectively.

**DPPH radical scavenging activity**

DPPH is a stable free radical which is used to estimate antioxidant activity of the extracts. The identification of positive results is dependent on the color reduction property of DPPH from purple to yellow and even colorless if plant extract has much of free radical scavenging activity. The result showed increase in the effect of radical scavenging with increase in concentration. The methanol extract of *C. auriculata*, *D. regia* and *V. rosea* has showed 96%, 78% and 60% of DPPH radical scavenging activity with $IC_{50}$ values 43.51µg/ml, 64.38µg/ml and 81.07µg/ml for *C. auriculata*, *D. regia* and *V. rosea* (Figure 3). Since methanol extract of *C. auriculata* was showing greater enzyme inhibitor potential and DPPH radical scavenging activity, this potent extract was selected for further antioxidant and phytochemical studies.

**Reducing power assay**

Reducing power assay is used to estimate the capacity of extract to donate hydrogen atoms. Reduction in the green color is directly proportional to the reducing power potency of the extract. Methanol extract of *C. auriculata* flowers has shown prominent reducing power property which was shown in the figure 4.

**Phytochemical Screening**

Phytochemical screening was performed for the methanol extracts of three plants that resulted in the presence and absence of various phytochemicals tested which was given in the table 2.

**Table 2:** Phytochemical screening of methanolic extract of flowers of *C. auriculata* and leaves of *V. rosaa* and *D. regia*

| Phytochemical Tests Performed | *C. auriculata* Methanolic extract | *D. regia* | *V. rosea* |
|---|---|---|---|
| **Carbohydrates** | +ve | +ve | +ve |
| **Flavanoids** | +ve | +ve | -ve |
| **Oils and Fats** | - ve | - ve | - ve |
| **Saponins** | + ve | + ve | + ve |
| **Tannins** | +ve | +ve | +ve |

+ve sign indicates the positive result for the phytochemical screening and –ve shows negative result of the phytochemical screening.

**Total phenolic content estimation**

Folin Ciocalteu reagent method is used for the estimation of total phenolic content in the plant extract where development of blue color ensures the positive result. From the experiment performed, it was observed that increase in the concentration shows positive result. The methanol extract has shown total phenolic content of 30.48 mgGAE/g of extract which was expressed as Gallic acid equivalence per gram of extract (figure 5).

**Total Flavanoid content estimation**

Flavonoids are the secondary metabolites present in the plant and they are the polyphenolic content of the plant. The presence of yellow color ensures the positive result. From the result of the experiment, it was observed that increase in the concentration shows increase in the positive result. The methanol extract of *C. auriculata* has shown total flavanoid content of 15.90mgQE/g of extract which was expressed as quercetin equivalence per gram of extract (figure 6).

**HPTLC Profile (High Performance Thin Layer Chromatography)**

HPTLC analysis resulted in the identification of five polyphenols within the extract which was shown in the table 3. TLC images, Peak densitogram and 3D view of identified polyphenols are given in the figures 7 and 8.

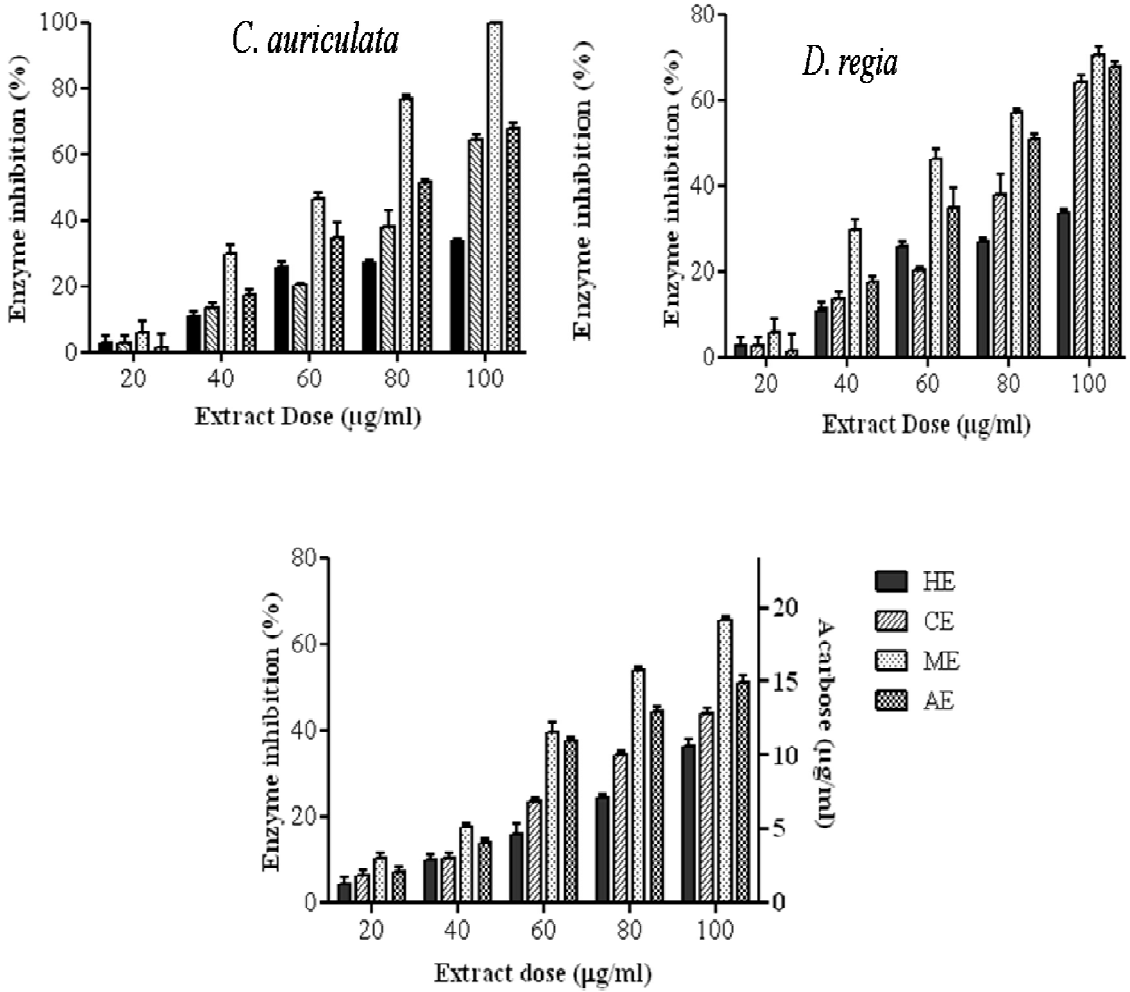


**Fig. 1:** Alpha glucosidase enzyme inhibitor activity of petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of flowers of *C. auriculata* and leaves of *D. regia* and *V. rosea*.

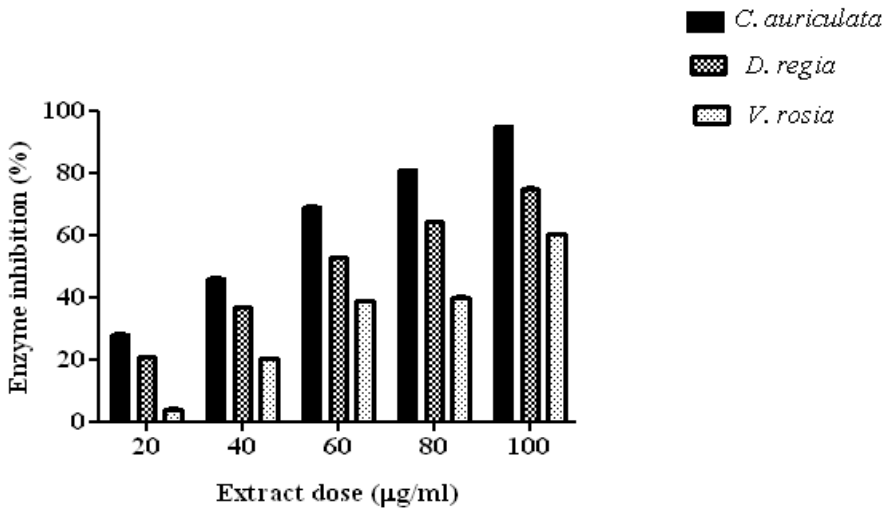


**Fig. 2:** Alpha amylase enzyme inhibitor activity of methanolic extracts of flowers of *C. auriculata* and leaves of *D. regia* and *V. rosea*

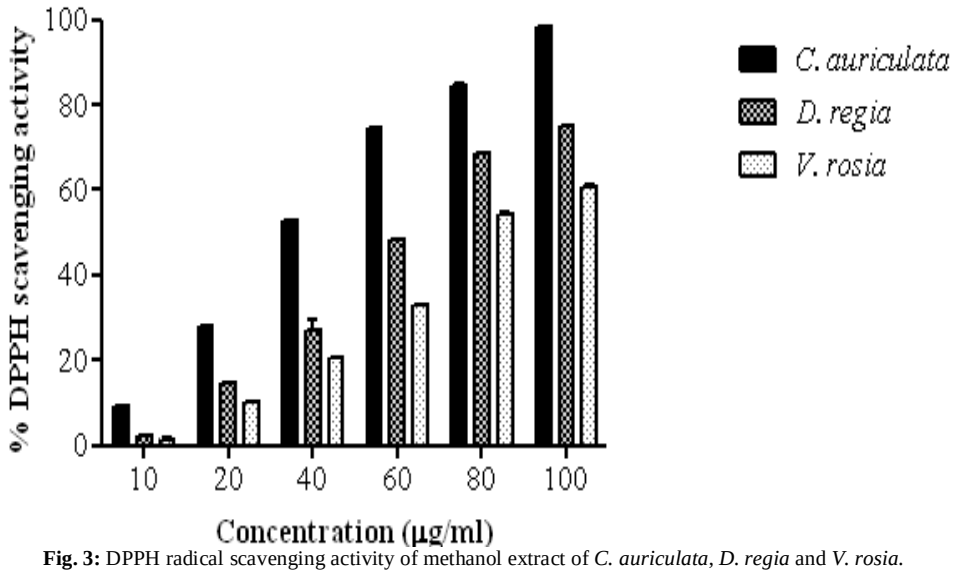


**Fig. 3:** DPPH radical scavenging activity of methanol extract of *C. auriculata*, *D. regia* and *V. rosia.*

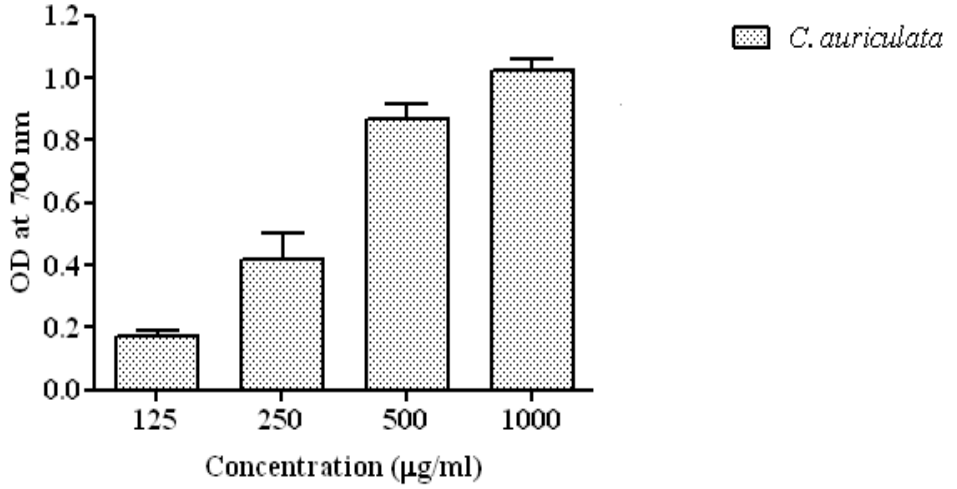


Fig. 4: Reducing power potential of methanol extract of *C. auriculata.*

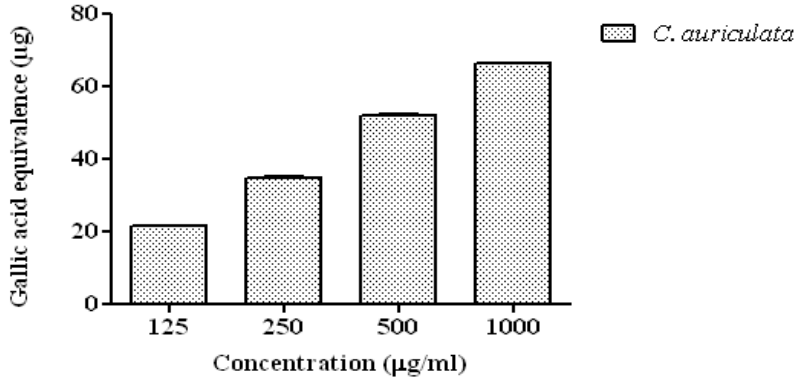


**Fig. 5:** Total phenolic content of methanol extract of *C. auriculata* flowers.

**Table 3:** HPTLC analysis of methanol extract of flowers of *C. auriculata*

| Track | Peak | Rf | Height | Area | Assigned substance |
|---|---|---|---|---|---|
| **Sample CAM** | 1 | 0.01 | 129.7 | 805.8 | Unknown |
| **Sample CAM** | 2 | 0.06 | 567.0 | 29105.3 | Unknown |
| **Sample CAM** | 3 | 0.17 | 419.9 | 18897.2 | Polyphenol 1 |
| **Sample CAM** | 4 | 0.22 | 354.5 | 16448.4 | Unknown |
| **Sample CAM** | 5 | 0.30 | 350.5 | 19347.2 | Polyphenol 2 |
| **Sample CAM** | 6 | 0.38 | 394.3 | 25976.7 | Unknown |
| **Sample CAM** | 7 | 0.49 | 392.5 | 21799.2 | Polyphenol 3 |
| **Sample CAM** | 8 | 0.55 | 300.6 | 11496.1 | Polyphenol 4 |
| **Sample CAM** | 9 | 0.60 | 335.7 | 10565.7 | Polyphenol 5 |
| **Sample CAM** | 10 | 0.67 | 348.9 | 8954.3 | Unknown |
| **Sample CAM** | 11 | 0.76 | 62.4 | 2172.6 | Unknown |
| **Sample CAM** | 12 | 0.81 | 65.4 | 1604.0 | Unknown |

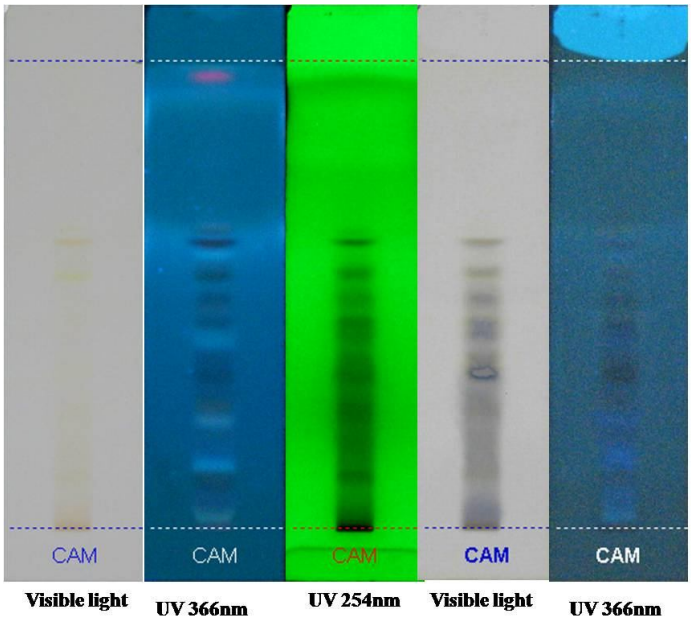


**Fig. 7:** TLC images of methanol extract of *C. auriculata* flowers.

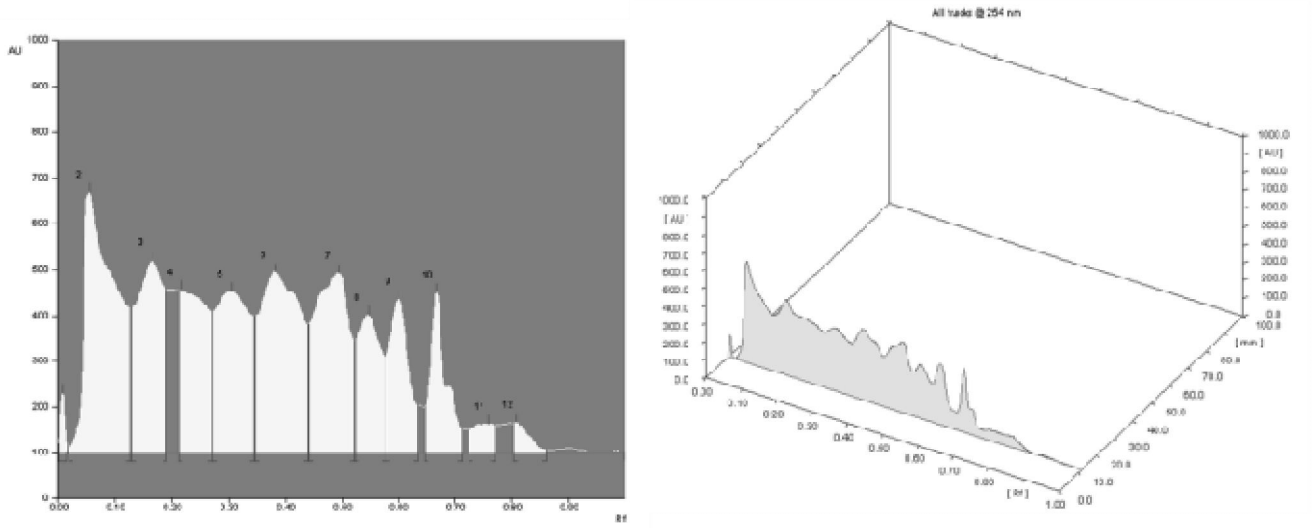

**Fig. 8:** Peak densitogram and 3D view of methanol extract of *C. auriculata* flowers.

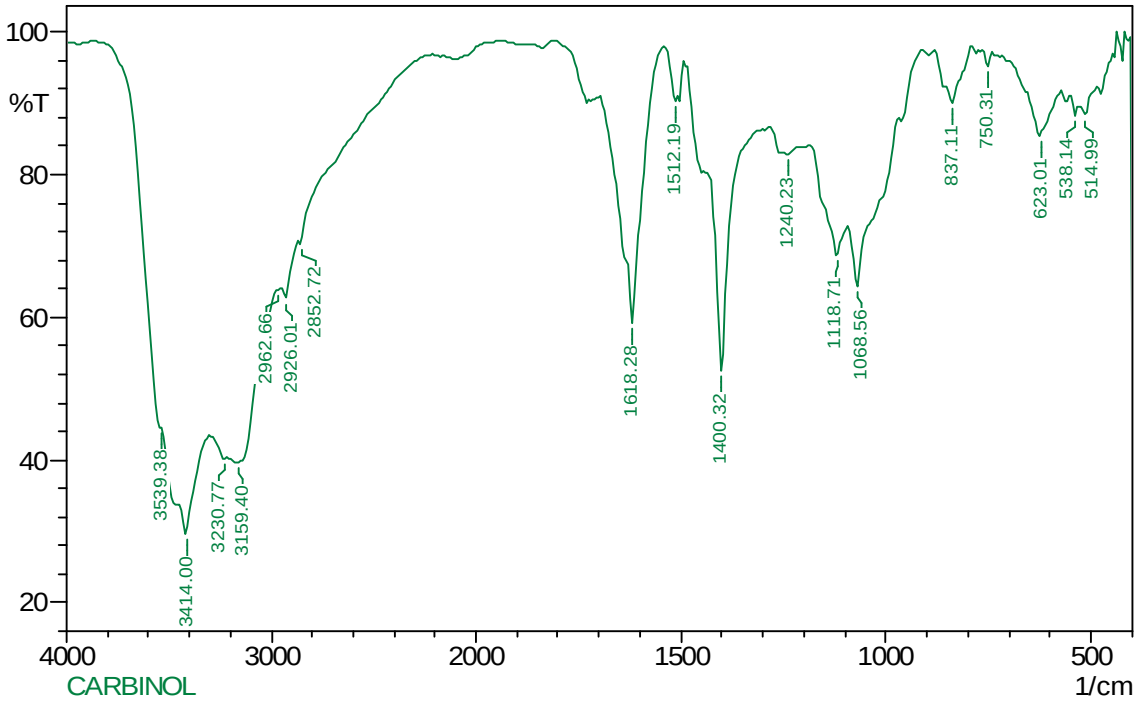


**Fig. 9:** IR spectra of the methanolic extract of *C. auriculata* flower.

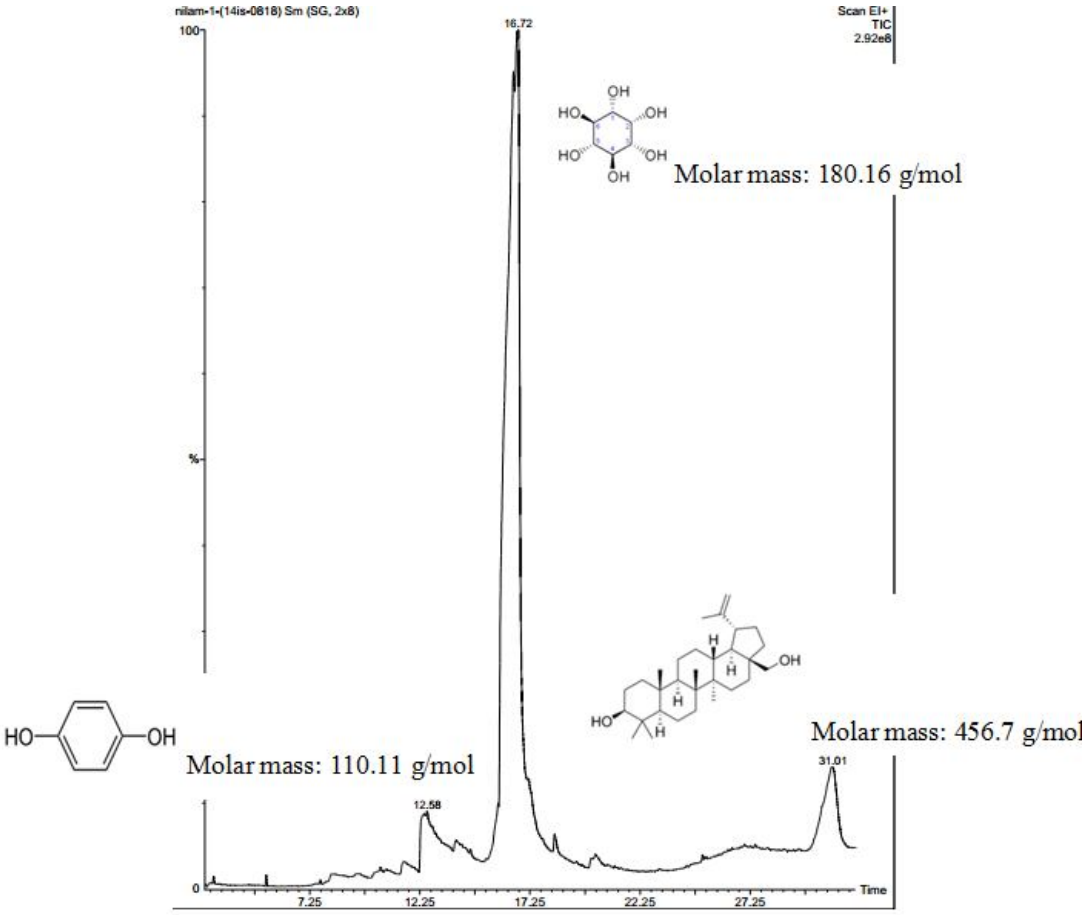


**Fig. 10:** GC-MS of the methanolic extract of *C. auriculata* flower.

**FT-IR analysis**

Analysis of IR spectra provides the result which found to have N-H bond stretch and the functional groups are primary, secondary amnies and amide group that is shown in the figure 9.

**GC-MS analysis**

The chromatograph result of GC-Ms analysis showed 3 peaks, which in library search were found to be closely related to myoionositol, 4-c-methyl of molar mass of 180.16 g/mol, Beutlin of molar mass: 456.7 g/mol, Hydroquinone molar mass: 110.11 g/mol (figure 10). So further we can relate the results as due to the presence of these compounds, methanol extract of *C. auriculata* shows antidiabetic and antioxidant property.

## CONCLUSION

The present study concludes that out of three medicinal plants, methanol extract of *C. auriculata* flowers exhibited high carbohydrate hydrolyzing enzyme inhibitor potential and promising antioxidant activities for the tests performed. It also showed greater amount of total phenolic and flavonoid content that might be because of polyphenols within the extract. HPTLC analysis identified five polyphenols in the extract. GC-MS resulted in the identification of to Myoionositol, Beutlin and Hydroquinone. These compounds within the methanol extract of *C. auriculata* flowers might be accountable for antidiabetic and antioxidant nature of the plant. In future, these compounds to be isolated and used as safe herbal medicine.

## ACKNOWLEDGEMENT

The authors wish to thank the Management and Staff of VIT University, Vellore, TN, India for providing necessary facilities to carry out this study.

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**How to cite this article:**

Nilam Yadao, Charles Lekhya Priya, Kokati Venkata Bhaskara Rao. Carbohydrate hydrolyzing enzyme inhibitor property, antioxidant and phytochemical analysis of *Cassia auriculata*, *Delonix regia* and *Vinca rosea* Linn: an in vitro study. J App Pharm Sci, 2015; 5 (05): 018-027.