Research Article

Antidiabetic and Antioxidant Properties of *Cassia auriculata* Flower Extract: An *in vitro* Study

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ABSTRACT

Cassia auriculata Linn. (Family: *Caesalpiniaceae),* known as Avaram in Tamil possess a number of medicinal properties. The present study was aimed in evaluating the free radical scavenging as well as glucose uptake potential of *Cassia auriculata* flower extract. *Cassia auriculata* flower extract was found to contain phytochemicals such as alkaloids, flavonoids, saponins, tannins, glycosides, anthraquinones and phenols. The amount of phenolic and flavonoid content in the flower extract were found to be 262.31± 3.01mg Gallic acid equivalents and 61.33 ± 3.05 mg quercetin equivalents respectively. *Cassia auriculata* flower extract (1000µg/ml) significantly scavenged 82 % of DPPH radicals with an IC_{50} of 724.44µg/ml and 83.5% ABTS radicals with an IC_{50} of 700µg/ml. There was 76% inhibition of hydroxyl radicals (IC_{50} = 711.90µg), and 79% hydrogen peroxide radicals (IC_{50}: 735.17 µg) indicating the antioxidant nature of *Cassia auriculala* flower extract. *C. auriculata* flower extract exhibited 66.0 ± 1.79% glucose uptakes over control. *C. auriculata* flower extract increased the expression of GLUT4 in rat L6 myotubes.

Keywords: *C. auriculata* flower extract; Antioxidant; antidiabetic; GLUT4.

INTRODUCTION

With Advances in the field of medicinal technology for eradicating the communicable disease, keen awareness is focused to ease the burden of non-communicable disease [1]. It is predicted that, by 2020, non-communicable diseases such as diabetes mellitus and cancer will cause seven out of every ten deaths in developing countries. An increase in the diabetic individuals or with a longer duration of diabetes alters the disease profile globally, which is due to a prevalence of diabetes associated secondary complications, such as nephropathy and peripheral arterial disease [2]. Medicinal plants are known to exert their pharmacological properties through the synergistic action of various biologically active ingredients present in them. The secondary metabolites synthesized by the serve as an invaluable chemical library for drug discovery and current medicinal chemistry [3].

Cassia auriculata Linn belonging to family Caesalpiniaceae, known as Avaram in Tamil language [4] has been reported to possess a number of therapeutic activities against leprosy, asthma, gout, rheumatism [5] and diabetes [6]. They possess antipyretic, antiulcer, possess antiperoxidative , antihyperglycemic and antimicrobial activity [7-10]. *Cassia auriculata* is used in Ayurvedic medicine as "Avarai Panchaga choornam" (mixture of five parts of the shrub i.e. roots, leaves, flowers, bark and unripe fruits).

***Cassia auriculata* flowers**

Having known the pharmacological significance *Cassia auriculata*, an earnest attempt has been made to evaluate the free radical scavenging as well as glucose uptake potential of *Cassia auriculata flower extract.*

MATERIALS AND METHODS

Plant Material

Fresh flowers of *Cassia auriculata* were collected from local areas of Thiruvottiyur, Chennai and were identified by a botanist.

Preparation of Extract

The flowers of *Cassia auriculata* were dried at room temperature and powdered in an electrical grinder. The powdered flowers were delipidated with petroleum ether (60 - 80° C) for the removal of lipids and were extracted with ethanol in soxhlet apparatus. The extract obtained was evaporated in rotary evaporator. The yield of extract was around 13.7g.

Preliminary Phytochemical Screening

The phytochemicals present in *Cassia auriculata flowers* were identified by standard established procedures [11, 12].

***In vitro* Antioxidant Assays**

The free radical scavenging capacity of the ethanolic extract of *C.auriculata flower extract* was determined using DPPH [13]. ABTS radical scavenging activity of ethanolic extract of *C.auriculata flower extract* was determined according to the method of Re et al., 1999 [14]. Hydroxyl Radical Scavenging Assay was performed according to the method of Smirnoff and Cumbes (1989) [15]. Hydrogen Peroxide Scavenging Activity was performed according to the method of Ruch et al., (1989)[16].

Evaluation of role of *C.auriculata* flower extract on glucose uptake in rat L6 skeletal muscle cells

Culture of rat L6 myoblast

The rat L6 myoblast cell line was procured from NCCS, Pune, India and were maintained in DMEM supplemented with 4.5 g/L glucose, 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin-B (250 ng/ml). Cultures were maintained at 37ºC with 5% CO_2 in an incubator. After, the cells had reached 60–70% confluence; Cell differentiation was induced by replacing the growth medium with DMEM supplemented with 2% fetal bovine serum instead of 10% fetal bovine serum [17]. Myotubes formation was achieved after 6-7 days with subsequent media change for every 48 h. The experiments were performed in differentiated L6 myotubes.

MTT assay

The effect of *C. auriculata* flower extract on the viability of the L6 myotubes was analyzed by colorimetric MTT assay [18]. After overnight fasting with serum free DMEM, the cells were treated with various concentrations (62.5, 125, 250, 500, 1000 µg) of *C. auriculata* flower extract at 37ºC with 5% CO_2 in an incubator for 24 h. After treatment, culture medium was removed from the wells, and 100 µl of MTT (5 mg/ml in D-PBS) was added to each well. After 4 h incubation at 37ºC, MTT in D-PBS was removed and then the formazan crystals were solubilised in 100 µl of 2-propanol. The absorbance of dye was measured at a wavelength of 570 nm. The results were expressed as percentage of control cell viability.

Determination of glucose uptake by cultured rat L6 myotubes

L6 myoblasts (5 x 10^4 cells/well) were seeded in 24-well tissue culture plates and for differentiation, cells were grown in DMEM with 2% fetal bovine serum for 6-7 days with subsequent change in media for every 48 h [17]. After differentiation, the cells were fasted overnight with serum-free DMEM containing low-glucose and then treated with insulin (100 nM) for 1 h as well as *C. auriculata* flower extract in fresh serum-free DMEM, for 3 h. After treatment, glucose concentration in medium was determined by glucose oxidase method. The glucose of the wells with cells was subtracted from the glucose of the blank wells to calculate the glucose uptake [19].

Western blot analysis of GLUT 4

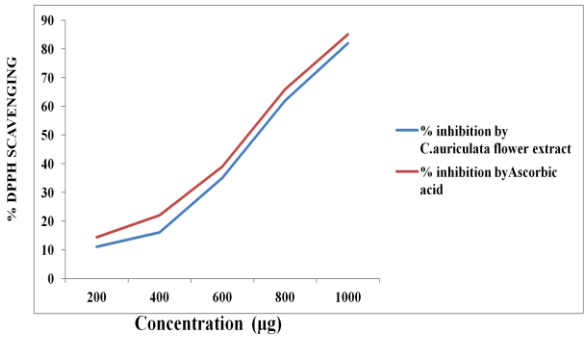
The translocation of GLUT4 was determined by western blot method. Myotubes were serum starved overnight and then incubated with *C.auriculata* flower extract with and without wortmannin, Insulin for 3 h. After treatment, cells were harvested, homogenized and the translocation of GLUT4 was analyzed by western blot analysis. The plate 1 shows the representative western blotting analysis of GLUT4 protein translocation. The intensity of protein bands was quantified using image analysis software and expressed as relative intensity units.

RESULTS

The flower extract is found to contain alkaloids, flavonoids, saponins, tannins, phytosterol, triterpenoids, glycosides, anthraquinones, and phenols. The total phenolic and flavonoid content were found to be 262.31± 3.01 mg Gallic acid equivalent and 61.33 ± 3.05 mg quercetin equivalent respectively.

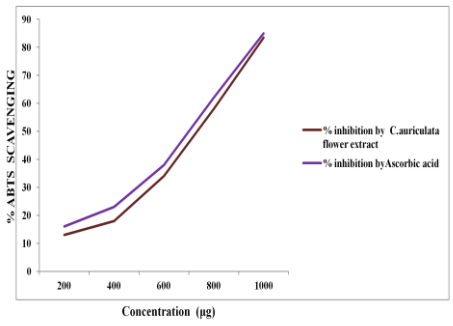
The DPPH and ABTS radicals scavenging activity of *C.auriculata* flower extarct is depicted in Figure 1 and 2 respectively. The extract showed 82.1% in DPPH assay and 83.5% inhibition in ABTS radical assay reflecting its significant radical scavenging capacity.

Figure 1: DPPH radical scavenging potential of *C.auriculata* flower extract



At a concentration of 1000µg/ml significantly scavenged 82 % of DPPH radicals
IC50=724.44µg

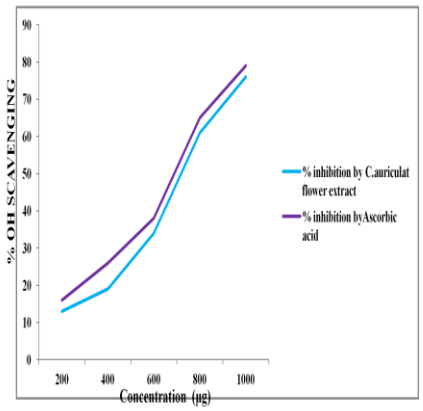
Figure 2:ABTS radical scavenging potential of *C.auriculata* flower extract



At a concentration of 1000µg/ml, the extract significantly scavenged 83.5% ABTS radicals IC50=700µg

Hydroxyl radical scavenging activity of *Cassia auriculata* flower extract is graphically represented as Figure 3. At a concentration of 1000µg/ml, *Cassia auriculata* flower extract showed 76% hydroxy radical scavenging potential.

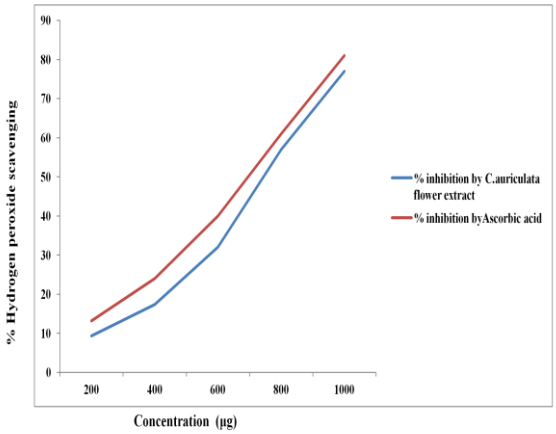
Figure 3: Hydroxyl radical scavenging potential of *C.auriculata* flower extract



At a concentration of 1000µg/ml, the extract significantly scavenged 76% radicals
IC50: 711.90µg

Hydrogen peroxide radical scavenging activity of *Cassia auriculata* flower extract is graphically represented as Figure 4. At a concentration of 1000µg/ml, Cassia auriculata flower exhibited 79% hydrogen peroxide radical scavenging potential .

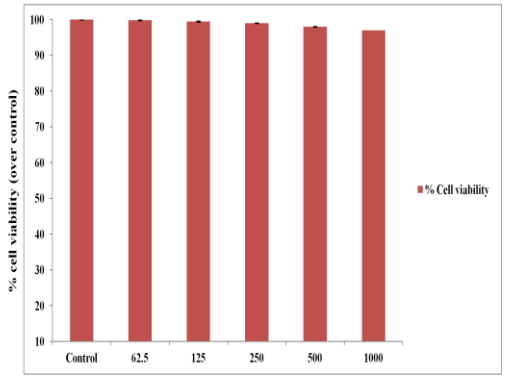
Figure 4: Hydrogen peroxide radical scavenging potential of *C.auriculata* flower extract



A concentration of 1000µg/ml, the extract significantly scavenged 79% hydrogen peroxide radicals
IC50: 735.17 µg

The effect of *C.auriculata* flower extract on the viability of L6 myotubes was evaluated by MTT assay (Figure 5) at different concentrations of extract (62.5, 125, 250, 500, 1000µg). There was more than 95% viability at concentrations of the flower extract up to 1000µg, when compared with control survival. Hence the minimum dosage of 100µg/ml was fixed as the dosage for further experimentation.

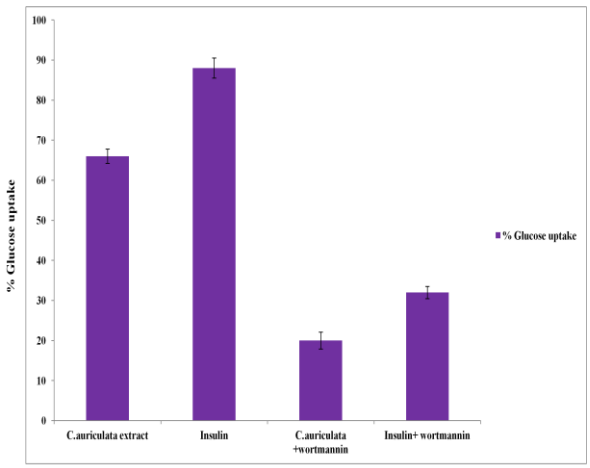
Figure 5: Effect of *C.auriculata* flower extract on cell viability in L6 myotubes



The effect of C.auriculata flower extract on the viability of L6 myotubes was evaluated by MTT assay at different concentrations of extract (62.5, 125, 250, 500, 1000µg).
There was more than 90% viability at concentrations of the flower extract up to 1000µg, when compared with control survival.
Hence the minimum dosage of 100µg/ml was fixed as the dosage for further experimentation.

In Glucose uptake assay (Figure 6), *C.auriculata* showed 66.0± 1.79% glucose uptake over control compared with the standard insulin (1 IU/mL) which showed 89± 2.5% glucose uptake over control. In the presence of wortmannin (PI3K inhibitor), *C.auriculata* exhibited 22 ± 2.1% glucose uptake over control and the standard insulin (1 IU/mL) showed 32 ± 1.55% glucose uptake over control.

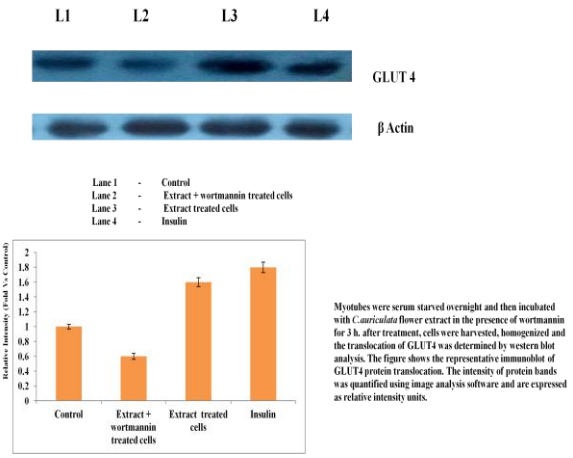
Figure 6: Effect of *Cassia auriculata* and insulin on glucose uptake in L6 muscle cell lines in the absence and presence of wortmannin (100 nM).



In Glucose uptake assay, *C.auriculata* showed 66.0± 1.79% glucose uptake over control compared with the standard insulin (1 IU/mL) which showed 89± 2.5% glucose uptake over control. In the presence of PI3K inhibitor wortmannin, *C.auriculata* showed 22 ± 2.1% glucose uptake over control and the standard insulin (1 IU/mL) showed 32 ± 1.55% glucose uptake over control

The distribution of membrane GLUT4 in the extract (Plate 1) treated myotubes was increased in extract treated cells whereas the membrane GLUT4 was reduced in the presence of wortmannin.

PLATE 1. IMMUNO BLOTTING ANALYSIS OF GLUT 4 PROTEIN IN L6 MYOTUBES



Myotubes were serum starved overnight and then incubated with *C.auriculata* flower extract in the presence of wortmannin for 3 h. after treatment, cells were harvested, homogenized and the translocation of GLUT4 was determined by western blot analysis. The figure shows the representative immunoblot of GLUT4 protein translocation. The intensity of protein bands was quantified using image analysis software and are expressed as relative intensity units.

DISCUSSION

Natural products are widely used in the prevention and treatment of various human ailments. Over 60% of the currently available drugs are originally derived in one way or another from the natural sources. Herbal medicines are comparatively safe and highly efficacious when compared to their synthetic counterparts. The potential importance of secondary metabolites produced by the plants with diverse therapeutic properties of preparations obtainable from its seeds, leaves and other parts has been the subject of detailed study in recent years.

Flavonoids, widely distributed in fruits, vegetables and certain beverages, possess a polyphenolic structure. They exert antioxidant effects associated with cancer, Alzheimer's disease, diabetes, atherosclerosis, etc. [20-22]. Flavonoids display health-promoting effects and are an important component in various nutraceutical, pharmaceutical and medicinal applications in routine life which may be due to their antioxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties [23-24].

Phenolic compounds are of considerable importance, particularly due to their antioxidant activity[25]. Phenolic compounds possess anti-aging, antiinflammatory, antioxidant and antiproliferative properties[26-27]. Dietary plant polyphenols and polyphenol-rich products modulate carbohydrate and lipid metabolism, ameliorate hyperglycemia, dyslipidemia and insulin resistance, improve β-cell function, stimulate insulin secretion, improve adipose tissue metabolism and alleviate oxidative stress, stress-sensitive signaling pathways and inflammatory processes. In the present study, the flower extract contains significant amounts of flavonoids and phenols which may be attributed to their strongest antioxidant potential.

Reactive oxygen species damage the biological molecules such as carbohydrates, nucleic acids, lipids, and proteins and modulate their functions. The shift in the balance between oxidants and antioxidants in favor of oxidants is termed "oxidative stress [28, 29]. The free radical scavenging potential of extract was determined using in vitro antioxidant assays. The extract showed 82% inhibition at a concentration of 1000 µg in DPPH assay and 83.5% inhibition at a concentration of 1000 µg in ABTS radical assay reflecting its significant radical scavenging capacity. *Cassia auriculata* flower extract possess significant free radical scavenging potential against DPPH and ABTS radicals [30- 31].

Hydroxyl radicals (˙OH) are generated in the reaction of metal ions with hydrogen peroxide in biological systems and foods [32]. At a concentration of 1000µg/ml, *Cassia auriculata* flower extract significantly scavenged76% radicals indicating hydroxy radical scavenging potential of the flower extract. H_2O_2 is highly important because of its ability to penetrate into biological membranes and may give rise to hydroxyl radicals in the cells [33]. The H_2O_2 scavenging may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water [34]. At a concentration of 1000µg/ml, Cassia auriculata flower extract significantly scavenged 79% radicals indicating hydroxy radical scavenging potential of the flower extract.

The observed antioxidant activity of ethanolic extarct of *Cassia auriculata* flower may be related to the presence of total phenolics and flavonoids. It indicates the direct correlation between antioxidant potential and total phenolics and flavonoids content. Various parts of *Cassia auriculata* (Linn.) could serve as natural sources of antioxidants and could be used in the treatment of free radical mediated diseases.

The skeletal muscles which account for the majority (~75%) of insulin-mediated glucose uptake in the post-prandial state play a chief role in maintaining glucose homeostasis. In skeletal muscles, insulin facilitates the uptake of glucose by activating phosphatidylinositol-3 kinase (PI3K) and Akt, leading to increased translocation of glucose transporter 4 (GLUT4) to the plasma membrane [35]. Another GLUT4 translocation promoter is 5 adenosine monophosphate- activated protein kinase (AMPK) which is composed of three subunits [36]. The effect of flower extract on the glucose uptake was evaluated in L6 muscle cell lines. The mechanism of action of the flower extract in the uptake of glucose is studied by analyzing the expression patterns of Glucose transport protein-4.

The effect of *C.auriculata* flower extract on the viability of L6 myotubes was evaluated by MTT assay at different concentrations of extract (62.5, 125, 250, 500, 1000µg). There was more than 95% viability at concentrations of the flower extract up to 1000µg, when compared with control survival. Hence the minimum dosage of 100µg/ml was fixed as the dosage for further experimentation.

In Glucose uptake assay, *C.auriculata* showed 66.0± 1.79% glucose uptake over control compared with the standard insulin (1 IU/mL) which showed 89± 2.5% glucose uptake over control. In the presence of PI3K

inhibitor wortmannin, *C.auriculata* showed 22 ± 2.1% glucose uptake over control and the standard insulin (1 IU/mL) showed 32 ± 1.55% glucose uptakes over control. Wortmannin is a fungal metabolite that was identified as a potent and selective inhibitor for phosphoinositide 3-kinases (PI3Ks) and PI3K-related enzymes. The glucose uptake is reduced in the presence of wortmannin, which evidence the fact *C. auriculata* flower extract may facilitate the translocation of GLUT4 via PI3 kinase mediated pathway.

In order to determine whether *Cassia auriculata* flower extract stimulates skeletal muscle glucose uptake by increasing the translocation of GLUT4 to the membrane either via insulin mediated PI-3kinase or AMPK, myotubes were treated with *Cassia auriculata* flower extract with and without Wortmannin (PI-3 kinase inhibitor). Plate 1 showed the distribution of membrane GLUT4 in control as well as *Cassia auriculata* flower extract treated myotubes. The distribution of membrane GLUT4 in the extract treated myotubes was increased in extract treated cells whereas the membrane GLUT4 was reduced in the presence of wortmannin indicating the Cassia auriculata flower extract mediates glucose uptake via PI3 kinase pathway.

CONCLUSION

The flowers of *Cassia auriculata* showed significant antioxidant as well as glucose uptake potential *in vitro*. *C. auriculata* flower extract facilitates the translocation of GLUT4 via PI3 kinase mediated pathway which is evident from glucose uptake assay and western blot analysis. The observed effect may be due to the presence of biologically active ingredients in the flower extract. Hence, from the results obtained it can be concluded that *C.auriculata* flower extract can be used for the treatment of diabetes mellitus.

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