In vitro anti-inflammatory, anti-diabetic and antioxidant potential of *Cissus quadrangularis* along with its orexigenic activity in *Drosophila melanogaster*

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**Article Info**  
https://doi.org/10.31018/jans.v13i3.2835  
Received: July 3, 2021  
Revised: August 23, 2021  
Accepted: August 28, 2021

**How to Cite**  
Zaki, S. et al. (2021). In vitro anti-inflammatory, anti-diabetic and antioxidant potential of *Cissus quadrangularis* along with its orexigenic activity in *Drosophila melanogaster*. *Journal of Applied and Natural Science*, 13(3), 962 - 969. https://doi.org/10.31018/jans.v13i3.2835

**Abstract**  
Plants with diverse pharmacological activities are actively being explored for human health. *Cissus quadrangularis* (L) has been reported to possess numerous phytochemicals and is used to relieve various disorders. The aim of the present study was to provide evidence of the diverse pharmacological activities in terms of orexigenic, anti-inflammatory, anti-diabetic and antioxidant activities of *C. quadrangularis* for further application in clinical development. The results revealed that inhibition of hemolysis was within the range of 8-9-25.6% at concentrations of 12.5-200 µg/ml. Methanol extract of the stems exhibited porcine pancreatic α-amylase (PPA) inhibition ($p \leq 0.05$) at concentrations of 0.25 and 0.30 mg/ml. The glucose adsorption capacity of the plant was observed to be inversely proportional to the molar concentration of glucose. The higher food intake by *Drosophila* in food medium with plant extract was presumably related to the orexigenic property of *C. quadrangularis*. Protease activity of the stem extract revealed total activity 975 U/ml and specific activity as 3768 U/mg. The absorbance of the plant in reducing power assay was between 0.91and 1.85. The highest total antioxidant activity of 67.2 µg TE/g was observed and the hydroxyl radicals scavenging activity was observed in a dose-dependent manner. The results provided supporting data that *C. quadrangularis* may contain active compounds useful in treating anti-inflammatory, anti-diabetic disorders.

**Keywords:** Antioxidant, Appetite stimulant, *Cissus quadrangularis*, Orexigenic

**INTRODUCTION**  
Poor appetite is associated with essential nutrient deficiencies, and appetite stimulants’ use is limited due to their side effects (Howard et al., 2019; Harrison et al., 2019; Levitt and O’Neil, 2018 and Homnick et al., 2004). Bioactive compounds from plants are used in treating digestive system illnesses. Hormones like
ghrelin and some phytochemicals like gentiopicroside, tetrahydrocannabinol, linalool promote appetite, thus increase food intake (Kola et al., 2008; Nematy et al., 2013 and Azadabkht et al., 2020). However, understanding the dosage and side effects is crucial to provide safe and effective treatment. Further, treatments targeting appetite regulation have thus far limited clinical success.

The production of inflammatory mediators is a defense mechanism of the host, but its excessive production may lead to chronic diseases (Oguntibeju, 2018). Suppression of inflammatory reactions using steroids, non-steroid and immunosuppressants are associated with adverse effects (Ghasemian et al., 2016). Plants with anti-inflammatory effects are reported to exhibit low or no side effects. Uncontrolled diabetes leads to morbidity and /or mortality and the use of synthetic drugs alongside insulin fail to reverse the course of diabetic complications though they are main route for controlling diabetes (Rao et al., 2010). Plant-derived active principles have established their role in treating diabetes, thus serving as an alternative source of anti-diabetic agents (Rizvi and Mishra, 2013; Salehi et al., 2019). Further, plants with antioxidant potential can be utilized for lowering blood glucose levels (Sekhon-loodu and Rupasinghe, 2019).

It is worth noting that plants have significantly fuelled drug development, and identifying potential plants with active principles for multiple disorders is much needed. Cissus quadrangularis is a tropical climbing shrub of Vitaceae family used in many parts of the world. This plant is rich in flavonoids, triterpenoids, stilbene derivatives and phytosterols with antioxidant potential, but more studies are needed to clarify pharmacological mechanisms of action. Bioactive components of C. quadrangularis have been used for cancer, obesity, antioxidant, anti-inflammatory disorders (Chatree et al., 2021; Siddiqui et al., 2021; Sundaran et al., 2020; Zaki et al., 2020; Stohs and Ray, 2013; Sriskok et al., 2011). The plant also has hypoglycemic and anti-glycation activity (Chaudhari et al., 2013; Lekshmi and Mini, 2013). Phytochemical analysis of the plant revealed the presence of quercetin and resveratrol (Syed et al., 2021). Based on the aforementioned pharmacological activities of this plant, the present study investigated the effect of C. quadrangularis on food intake in Drosophila melanogaster. In addition to its orexigenic activity, anti-inflammatory, anti-diabetic and antioxidant assays were performed to explore its collective pharmacological activities.

**MATERIALS AND METHODS**

**Preparation of plant extract**

Fresh stems of C. quadrangularis were air-dried in blotting paper and defatted with petroleum ether. The defatted powder was extracted with 80% methanol in a soxhlet apparatus for 48 hrs. The obtained extract was concentrated under reduced pressure and used for antioxidant assays.

**Antioxidant assays**

**Measurement of reducing Power**

The Fe	extsuperscript{3+}-reducing power of the extract was determined by a standard method (Fejes et al., 2000). Different concentrations (0-1.0 mg/ml) of the extract were mixed with equal volume of 0.2 M phosphate buffer (pH 6.6) and 0.1% potassium hexacyanoferrate, followed by incubation for 20 min at 50°C. After incubation, the reaction was terminated with 0.5 ml 10% TCA. Then, 1 ml reaction mixture was diluted with 1 ml distilled water followed by the addition of 0.1 ml FeCl	extsubscript{3} solution (0.01%). The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. Butylated hydroxy toluene (BHT) was used as a standard.

**Total antioxidant activity**

The total antioxidant activity of plant extract was estimated by phosphomolybdenum assay (Jayaprakasha et al., 2002). Methanolic extract of C. quadrangularis in different concentrations ranging from 100-500 μg ml	extsuperscript{−1} were added to each test tube individually containing 3 ml of distilled water and 1 ml of molybdate reagent solution. The tubes were incubated at 95°C for 90 min followed by normalization to room temperature for 20-30 min and the absorbance of the reaction mixture was measured at 695 nm using a UV–Vis spectrophotometer. The total antioxidant activity was as expressed as μg equivalents of α-tocopherol (μg TE/g).

**Hydroxyl radicals scavenging assay**

The hydroxyl radical scavenging assay was performed using a standard protocol method (Ilavarasan et al., 2005). In a final volume of 1 ml, various concentrations of the test sample or reference compound was mixed with 2-deoxy-2-ribose (2.8 mM); KH	extsubscript{2}PO	extsubscript{4}-KOH buffer (20 mM, pH 7.4); FeCl	extsubscript{3} (100 μM); EDTA (100 μM); H	extsubscript{2}O	extsubscript{2} (1.0 mM); ascorbic acid (100 μM) and incubated for 1 h at 37°C. 0.5 ml of the reaction mixture was added to 2.8% TCA, followed by 1% TBA and incubated at 90°C for colour development and the absorbance was measured at 532 nm against an appropriate blank solution. The scavenging activity on hydroxyl radical was calculated as follows.

\[
\text{Hydroxyl Radical Scavenging activity (%) } = \frac{(1-A_{532}\text{Sample})/A_{532\text{Control}}) \times 100}{..\text{Eq. 1}}
\]

**Anti-inflammatory activity**

**Inhibition of protein denaturation method**

Protein denaturation assay was done according to the method of Mizushima et al. (1968). Different concentra-
tions of the stem extract were added to 1% bovine se-
rum albumin solution. The mixture was incubated in a
water bath (37°C for 20 min) and the reaction mixture
was heated at 57°C for 20 min and was allowed to cool.
The turbidity of the samples was measured at 660 nm
and percent inhibition of protein denaturation was
calculated as follows:

\[
\text{Protein denaturation inhibition} = \left(1-\frac{A_{660\text{Sample}}}{A_{660\text{Control}}}\right) \times 100 \quad \text{ Eq.2}
\]

Red blood cell membrane stabilization test
Anti-inflammatory activity of C. quadrangularis stem extract was evaluated by red blood cells membrane
stabilization test. Blood sample collected from ICAR-
Indian Veterinary Research Institute, Bengaluru was
diluted to 10% v/v with saline. Plant extract (1 ml) was
added to 1 ml of red blood cell suspension and incubated
at 56°C for 30 min. The reacted mixture was centri-
fuged at 2500 rpm for 5 min and the absorbance of the
supernatant was measured at 560 nm. Saline was used
as a control for the experiment and the membrane sta-
ibility percentage was calculated using the following
formula (Sadique et al., 1989).

\[
\text{Membrane stabilization} = \left(1-\frac{A_{560\text{Sample}}}{A_{560\text{Control}}}\right) \times 100 \quad \text{ Eq.3}
\]

α-amylase inhibitory assay
In vitro α-amylase of C. quadrangularis was determined
by preparing various concentrations of the extract in
DMSO. Substrate was prepared by adding 0.5 M tris-
HCl buffer (pH 6.9), 0.01 M CaCl₂ (0.2 ml) and starch
(2 mg). The substrate mixture was boiled for 5 mins and
different concentrations of the plant extract (0.2 ml)
was added to each tube followed by porcine pancreatic
amylase (0.1 ml in Tris–HCl buffer (2 units/ ml). The
reaction mixture was incubated at 37°C for 10 min and
the reaction was stopped by adding 50% acetic acid
(0.5 ml) followed by centrifugation at 3000 rpm for 5
mins. Absorbance of the supernatant was measured
at 595 nm using a spectrophotometer. Acarbose was
used as positive control and the α-amylase inhibitory
activity was calculated using the following formula
(Tamil et al., 2010).

\[
\% \text{ α-amylase inhibition} = \left(1-\frac{A_{595\text{Control}}-A_{595\text{Sample}}}{A_{595\text{Control}}}\right) \times 100 \quad \text{ Eq.4}
\]

Glucose uptake by yeast cells
Suspension of commercial Baker’s yeast was prepared
in distilled water (10% w/v), centrifuged and the super-
natant was used for glucose uptake studies (Cirillo,
1962). Various concentrations of C. quadrangularis
extract were added with 1 ml of glucose solution (5-25
mM) in test tubes and incubated at 37°C for 10 mins.
This was followed by the addition of 100 μl of yeast
suspension into each tube and further incubated at 37°
C for 60 min. After incubation, the tubes were centri-
fuged at 5000 rpm for 5 mins and the glucose was esti-
mated in the supernatant using a Spectrophotometer at
520 nm with metronidazole as standard. The following
formula was used to calculate the percent increase in
glucose uptake by yeast cells.

\[
\% \text{ increase in glucose uptake} = \left(\frac{A_{520\text{Sample}}-A_{520\text{Control}}}{A_{520\text{Control}}}\right) \times 100 \quad \text{ Eq.5}
\]

Orexigenic studies
Fly husbandry and culture media
Wild type Drosophila melanogaster were housed and
maintained at 25°C, 65% humidity, on a 12h: 12h light:
dark cycle in culture bottles. The standard growth me-
dium consisted of yeast (2 g), sucrose (10 g), cornmeal
(3.3 g), propionic acid (1g), agar (1 g), ampicillin (0.01
g), chloramphenicol (0.002 g), autolyzed yeast powder
(20 g) and 2.5% (w/v) blue food dye (F D & C blue dye
no.1) in distilled water (100 ml). For the orexigenic ex-
periments, C. quadrangularis extract was added to the
growth medium at 0.5% (w/v).

Food intake assay
For the quantification of food intake, 10 flies were col-
lected from control and experimental bottles using light
chloroform anaesthesia, homogenized in 1 ml of phos-
phate buffer (0.1 M) and diluted to 1 ml. Homogeniza-
tion was performed quickly to prevent the interference
of eye pigments. The homogenized suspension was
centrifuged at 10000 rpm for 10 mins. The absorbance
of the supernatant was then measured at 625 nm in a
UV-Vis Spectrophotometer (Edgecomb et al., 1994).
Food-dye medium without plant extract was used as
control and absorbance values were converted to vol-
umes of medium consumed by interpolation from
standard curves of pure dyes (Shell et al., 2018).

Extraction of protease
Freshly collected stems of C. quadrangularis were
rinsed with distilled water and air-dried in blotting pa-
paper. Ten grams of the sample was extracted with Tris–
HCl buffer (50 mM; pH 7.2) containing ascorbic acid
(0.01% w/v), poly(Vinylpyrrolidone) (0.1% w/v) in
the ratio of 1:3. The mixture was filtered, centrifuged at
10,000 rpm for 15 min at 4°C and the supernatant was
used as source of protease enzyme.

Estimation of protease activity
The amount of protease present in the C. quadrangu-
laris extract was determined by mixing the extract with
0.5 ml of tris-HCl buffer (50 mM; pH 7.2) and 0.5 ml of
casein (2% w/v) dissolved in citrate phosphate buffer
(50 mM; pH 6.8). The reaction mixture was incubated at
37°C for 1 h and the reaction was terminated by with
1 ml of 10% (w/v) ice-cold TCA. Centrifugation of the
mixture was done at 5000 rpm for 5 mins to remove
unhydrolyzed casein substrate and the supernatant
was mixed with 2.5 ml of the reagent containing 2.9% Na$_2$CO$_3$ and 0.3 N NaOH was added followed by the addition of 0.75 ml of Folin Ciocalteu's phenol reagent (Folin phenol: distilled water ; 1:3). The samples were incubated at 37°C for 20 min and read at 650 nm using a spectrophotometer. A standard curve constructed with tyrosine was used to construct the standard curve. One unit of protease activity was defined as the amount of enzyme that liberates 1 µmol of tyrosine equivalent per minute under the assay conditions (McDonald and Chen, 1965).

RESULTS AND DISCUSSION

The present study observed that reducing power ability of the methanolic stem extract of $C$. quadrangularis was comparable to that of BHT. At 50-300 µg/ml concentration, the absorbance of the extract and ascorbic acid were 0.76-1.63 and 0.91-1.85 respectively as shown in Fig. 1. Reducing power of $C$. quadrangularis is associated with its phytochemicals (Vijayalakshmi et al., 2013). Nabavi et al., (2009) indicated that Fe$^{3+}$-reducing power is mainly associated with the phenolic antioxidant activity of plants while investigating Pyrus boissieriana and Diospyros lotus extracts.

Total antioxidant activity of $C$. quadrangularis extracts expressed as the number of equivalents of α-tocopherol was obtained from the calibration curve. The phosphomolybdate method is a quantitative method to express the total antioxidant activity. At 300 µg/ml concentration, the highest total antioxidant activity (67.2 µg TE/g) was observed. However, the antioxidant potential was lower than the standard BHT (82.3 µg TE/g) (Fig. 2).

Hydroxyl radicals produced from Fenton reaction degrade the deoxyribose. Measuring the inhibition of deoxyribose degradation indicated the hydroxyl radical scavenging activity of plant extract. $C$. quadrangularis extract, when added to the reaction mixture, scavenged hydroxyl radicals in a dose-dependent manner. The scavenging activity was comparable with the standard, quercetin and the activity is shown in Fig. 3. When the concentration of plant extract was more than 200 µg/ml, the scavenging effect was more than 60%. Similar hydroxyl radical scavenging inhibition activities were observed at 200 and 400 µg/ml concentrations in an earlier study using aerial parts of $C$. quadrangularis extracted in ethanol and methanol (Dhanasekaran, 2020). In the present study, 80% methanol was used to prepare stem extracts of $C$. quadrangularis and the scavenging of hydroxyl radicals was in line with antioxidant activity of aerial parts of the same plant indicating that antioxidant potential of stems. Therefore, selection of the most appropriate plant part is required while investigating its bioactivity instead of the whole plant as it was proven in this study that stem of the same plant had exhibited similar free radical scavenging activity when compared to the aerial parts extracted with the same solvent. Based on the results obtained in this study, $C$. quadrangularis stem extract is a good source of antioxidants as determined through different assays. The antioxidant activities were comparable to the standard drugs such as BHT and quercetin in terms of inhibition and free radical scavenging activities.

$C$. quadrangularis stem extract was able to inhibit RBC hemolysis and protein denaturation in a concentration dependent manner. Inhibition of hemolysis was within the range of 8-9-25.6% at concentrations of 12.5-200 µg/ml (Fig. 4). Protein denaturation of the extract was within the range of 28.2 – 61.2% and the ability of the extract to prevent protein denaturation is attributed to its anti-inflammatory properties. Membrane destabilization property of the extract could be associated with interference of release of inflammatory mediators and prevention of hypotonicity induced lyses of RC membrane as well as lysosomal constituents (Iwueke et al., 2006; Chou., 1997). Anti-inflammatory activities of roots and stem extracts of $C$. quadrangularis is reported by Shadmani et al., (2018) and the activity is involved with in-

**Fig. 1.** Reducing power activity of $C$. quadrangularis.

**Fig. 2.** Total antioxidant activity of $C$. quadrangularis.
duction of heme oxygenase enzyme (Srisook et al., 2011). Alpha-amylase inhibitors prevent the hydrolysis of starch thereby reducing the postprandial blood glucose levels (Gulati et al., 2012). Alpha amylase inhibition potential of *C. quadrangularis* is shown in Fig. 5. The inhibition percentage of the enzyme was based on the concentration of the extract in a dose-dependent manner. Methanol extract of *C. quadrangularis* stems exhibited moderate porcine pancreatic α-amylase (PPA) inhibition (p≤0.05) at concentrations of 0.25 and 0.30 mg/ml. Fig. 6 highlights the glucose adsorption capacity directly proportional to the glucose concentration observed in this study. The plant extract has promoted the uptake of glucose across the plasma membrane of yeast cells. The glucose adsorption capacity of the *C. quadrangularis* was observed to be inversely proportional to the molar concentration of glucose. Glucose adsorption by both plant extract and standard drug was reduced with an increased glucose concentration. The results showed that *C. quadrangularis* stem extract could bind glucose effectively, suggesting its contribution in weakening the postprandial hyperglycemia. Salehi et al., (2019) reported that the anti-diabetic effect of medicinal plants native to Asian countries is attributed to the phytochemical components of the plant extracts as observed in animal (alloxan/streptozotocin induced diabetic rats) and human (diabetic, prediabetic patients, type 2 diabetes mellitus subjects) models. *Drosophila* exhibits similarities with mammals, which is suitable for nutritional studies (Staats et al., 2018). The goal of this present study was to develop and validate a simple, inexpensive method for determining appetite inducing activity of *C. quadrangularis* through food intake studies by *Drosophila*. Estimating food uptake by dye usage is easy, inexpensive and can be rapidly assessed by visual inspection. Food dyes added up to 2.5% concentrations do not affect the food intake by *Drosophila* (Deshpande et al., 2014). Blue dye remains within the digestive tract and passes out of the fly unaffected by gut pH and enzymes. Fig 7a and 7b indicate *Drosophila* fed in food media with and without *C. quadrangularis* stem extract. Flies fed with *C. quadrangularis* extract exhibited more blue dye in their abdomen region revealing the more food intake influenced by orexigenic property of the plant. Similarly, flies fed food media without *C. quadrangularis* stem extract shown lesser blue dye uptake. The amount of food intake was

![Fig. 3. Hydroxyl radical scavenging activity of C. quadrangularis.](image1)

![Fig. 4. Hemolysis inhibition activity of C. quadrangularis.](image2)

![Fig. 5. Alpha-amylase inhibition of C. quadrangularis stem extract.](image3)

![Fig. 6. Glucose adsorption assay using C. quadrangularis stem extract.](image4)
measured by quantitating the dye via spectrophotometry (Fig. 8). Proteases are present in plant tissues that hydrolyze protein into peptides and amino acids. Proteases from *Pongamia pinnata*, *Wrightia tinctoria*, *Acalypha indica*, *Adhatoda vasica*, *Curcuma longa*, *Carica candalmarcensis* and *Ananas comosus* are used in pharmaceutical applications (Chinnadurai *et al*., 2018; Mello *et al*., 2008; Kelly, 1996). In this study, protease activity of *C. quadrangularis* stem extract revealed total activity as 975 U/ml and specific activity as 3768 U/mg. Plant extracts with higher proteolytic activity (>3000U/mg) have been used in traditional medicine for treating various disorders (Otsuki *et al*., 2010; Mello *et al*., 2008) and the presence of higher protease activity in *C. quadrangularis* stems as determined in this study reveals its pharmacological applications. In general, proteases from medicinal plants are preferred for industrial application as they do not require any co-factors (Asif-Ullah *et al*., 2006). Cysteine proteases were purified from *C. quadrangularis* by Muthu *et al.* (2017), and the safety of this plant in bone fracture treatment, including enhanced bone growth and anti-osteoporotic activities, were reported by Sawangjit *et al.* (2017).

**Conclusion**

This study indicated that *Cissus quadrangularis* had a positive effect on the food intake of *Drosophila melanogaster*. Future studies evaluating the effects of this plant on human appetite are warranted. The results provide supporting data that *C. quadrangularis* contain proteases and phenolic compounds and α-amylase inhibitors that are useful in treating anti-inflammatory, anti-diabetic disorders. However, purification of specific bioactive compounds and clarifying their pharmacological mechanisms of action is needed to treat various disorders by using this plant.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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