

Angiotensin Converting Enzyme and Trypsin Inhibition by *Garcinia Cambogia* Seeds Extract

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Abstract: In this project work, *Garcinia cambogia* seeds extract was prepared and analysed for inhibitory studies. *G. cambogia* or the Malabar tamarind, commonly used as a food preservative, flavouring agent or food-bulking agent, and as a traditional remedy to treat constipation, piles, rheumatism, edema, irregular menstruation and intestinal parasites in many Asian countries. *G. cambogia* seeds solvents (Methanol, ethylacetate, petroleum ether and acetone) extract were prepared and pharmacological activities were analyzed such as antioxidant activity, ACE inhibitory and trypsin inhibitory activities. Among the extracts tested, methanolic extract of *G. cambogia* has shown potent antioxidant, angiotensin converting enzyme and trypsin inhibitory activities.

Keywords: *Garcinia cambogia* seeds ACE, Trypsin inhibition Antioxidant activity.

1. Introduction

Studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial and antiviral activities. The ingestion of natural antioxidants will reduce the risk of cardiovascular disease, diabetes and other diseases of cancer associated with ageing. The natural plant based antioxidants play an important role in the maintenance of human health (Brannen et al., 1975). Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been used as antioxidants since the beginning of this century. But restrictions on these antioxidants have been imposed because of the carcinogenicity of these compounds. (Lo liger, 1991). Thus, the interest in natural antioxidants has increased considerably.

Garcinia gummi-gutt (L.) Roxb. or the Malabar tamarind, commonly known by its previous scientific name *Garcinia Cambogia*, is native to Southeastern Asia. The fruit rind is commonly used as a food preservative, flavouring agent or food-bulking agent, and as a traditional remedy to treat constipation, piles, rheumatism, oedema, irregular menstruation and intestinal parasites in many Asian countries. Commercial products containing *G. cambogia* catapulted onto the market and have received considerable positive and negative media attention. *Garcinia* is the largest genus of the Clusiaceae family comprising of 390 species. These polygamous trees or shrubs are mainly distributed in tropical Asia, Polynesia and Africa. *Garcinia gummi-gutta* (L.) Roxb. is

one of the most medicinally important members of the Clusiaceae family. It is a small or medium size tree of up to 12 m tall with a rounded crown and drooping branches. *Garcinia cambogia* has a limited native global distribution, being restricted to India, Nepal and Sri Lanka, but it has been introduced elsewhere where it is distributed in the subtropical region of Asia including China, Malaysia and the Philipines. These trees are found mainly in the semi-evergreen to evergreen forests of Southwest India, predominantly in the Western Ghats (Maharashtra, Karnataka, Kerala and Tamil Nadu). *G. cambogia* is an economically important spice tree valued for the sun-dried smoked rind which is widely used as a flavour condiment, especially in fish curries. It is also used as a substitute of Kokum butter and as a common additive to make meals more filling. The fruit rind is used medicinally to treat rheumatism and bowel complaints and it is employed as a purgative, hydragogue, anthelmintic and emetic. It is also used in veterinary medicine where a rinse is used to treat mouth diseases in cattle. The fruits are edible, but very acidic and are not generally eaten raw.

Garcinia Cambogia

Kingdom	: Plantae
Class	: Angiosperms
Order	: Malpighiales
Family	: Clusiaceae
Genus	: <i>Garcinia</i>
Species	: <i>Cambogia</i>
Binomial name	: <i>Garcinia cambogia</i>



A tonic prepared from the fruit, which contains a high concentration of vitamin C, is used in India to treat various heart diseases. *G. cambogia* is not only used medicinally; the rind is used to polish gold and silver ornaments and as a substitute for acetic acid for the coagulation of rubber latex, the gum is used

as a varnish and the resin is used as a pigment in miniature paintings and water colors. In background of the above mentioned properties associated with *G. cambogia* seeds, this study has been taken up to evaluate scientifically the pharmacological properties of study the effect of solvent extract on antioxidant, ACE and trypsin activities.

2. Methodology

A. Chemicals

Porcine kidney used as angiotensin converting enzyme (ACE) source in this project was obtained from the local pork stall, Tumkur. Hippuryl-histidyl-leucine (HHL) acetate salt was purchased from Sigma-Aldrich Chemical Laboratories. All the other chemicals and reagents used in this study were of analytical grade.

B. Partial purification of ACE

1) Preparation of porcine kidney acetone extract

The porcine kidney acetone powder was prepared according to the method of Vermeirssen et al., (2002) with some modifications. The porcine kidney cortex was chopped into small pieces and suspended in chilled acetone for 30 min. The cortex was homogenized with chilled acetone using conventional mixer grinder. Thus obtained homogenate was air dried and powdered. The acetone powder was dissolved in 100 mM phosphate buffer (pH 8.3) in the ratio of 1:10, stirred using magnetic stirrer overnight at 4°C, centrifuged at 8000 rpm for 20 min. The supernatant obtained served as crude enzyme source of ACE and subjected to protein estimation.

2) Ammonium sulphate precipitation of crude enzyme

The crude enzyme extract (45mL) was subjected to ammonium sulphate precipitation (20% saturation), using magnetic stirrer, kept it in refrigerator overnight. Then centrifuged at 8000 rpm for 20 min. Precipitated protein is dissolved in 2ml of 10 mM sodium phosphate buffer (pH 8.3). Then the precipitated protein is dialysed using 12 kDa cut off dialysis membrane against distilled water. Dialyzed sample was subjected to protein estimation and evaluated for the ACE activity.

C. ACE assay

ACE activity was measured by the spectrophotometric assay of Cushman & Cheung (1971), with some modifications. This method is based on liberation of Hippuric acid (HA) from hippuryl -L- histidyl-L-leucine (HHL) catalysed by the ACE. (Chaudhary et al.,2013). The crude enzyme extract at different concentrations (50, 100 µl) with 150µl HHL substrate (8.3mM) in 50 mM sodium borate buffer (pH 8.3) containing 300mM NaCl incubated at 37 °C for 30 minutes. The reaction was terminated by the addition of 250µL of 1M HCL. To the resulting solution 1 ml of ethyl acetate was added and centrifuged at 3000 rpm for 10 minutes. HA, produced in the reaction mixture due to the hydrolysis of substrate catalysed by ACE is in the ethyl acetate upper layer. After that 0.5 ml of ethyl

acetate was taken out by a micropipette and transferred to a new test tube and heat evaporated to dryness by keeping in boiling water bath. HA precipitated in the test tube was dissolved in 1 ml of distilled water. Absorbance of this solution was measured at 228 nm using an UV-Spectrophotometer.

The concentration of hippuric acid in the test reaction compared to control reaction was expressed as percentage of ACE activity. The activity of enzyme is expressed as nmoles of hippuric acid released/30 min/mg protein at 37 °C and the specific activity of enzyme is expressed as nmoles of hippuric acid released/min/mg protein at 37°C. For inhibition studies, ACE is pre incubated with extracts for 30 min and assayed as mentioned above.

D. Collection of the seeds

The seeds of *G. cambogia* were purchased from local market and were grinded to fine powder form using mixer. The powder was used for further solvent extraction purpose.

E. Solvent extraction

G. cambogia seeds powder was extracted with petroleum ether, ethyl acetate, acetone and methanol using Soxhlet apparatus. Each extract was concentrated in rotor evaporator. The extracts were dried and stored at -20 °C until further use.

F. Trypsin inhibition study

Trypsin inhibition was assayed in accordance with the method of Satake et al., 1963 by using casein (2% in 200 mM Tris-HCl buffer; pH 7.4) as the substrate. Briefly, different concentrations of plant extracts (50-500µg) are preincubated with trypsin at 37 °C for 30 minutes. Then all the reaction mixtures were incubated with 0.4 mL of casein solution at 37 °C for 2 h. The reaction was stopped by adding 1.5 mL of 0.44 M Trichloroacetic acid and allowing the mixture to stand for 30 min. The mixture was then centrifuged at 3,000 rpm for 5 min. An aliquot (1 mL) of the supernatant was mixed with 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of 1:2 diluted FC reagent and incubated for 20 min at room temperature. The intensity of color developed in the reaction mixture was measured at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm in 1 min. Appropriate controls were maintained in all cases, and the results were expressed as percentage of inhibition.

Percentage of trypsin inhibition is calculated using following equation.

Percentage of trypsin inhibition

$$= \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance} \times 100}$$

G. Antioxidant activity

DPPH assay was carried out according to Sanja et al., 2009. In this method, different concentrations of test samples were diluted with 50% Methanol and the volume was made upto 1ml.

Table 1
 Partial purification of angiotensin converting enzyme

	Protein concentration (µg)	ACE activity*	Fold purity	% yield
Crude extract	400	335	-	-
Ammonium sulphate precipitation	50	670	200	25

*nmoles of hippuric acid released/30 min/mg protein.

Then 140µL of 1 mM DPPH solution was added to each test tube. DPPH diluted with methanol was taken as control. All the test tubes were incubated at room temperature for 30 minutes. Absorbance was taken at 520 nm in UV-visible spectrophotometer. The results are expressed as mean % antiradical activity. The free radical scavenging activity was calculated using the following equation:

Percentage of antiradical activity

$$= \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance} \times 100}$$

H. Protein estimation

The protein content of test samples was estimated according to the method of Lowry et al., (1951) using bovine serum albumin (BSA) as standard.

3. Results

A. Partial Purification of ACE

In this project, ACE is partially purified by ammonium sulphate precipitation method. The kidney acetone powder was prepared by homogenizing kidney cortex using chilled acetone. The kidney acetone powder was dissolved in 300 mM sodium borate buffer (pH 8.3) in the ratio of 1:10, centrifuged and supernatant served as crude enzyme. Further, the crude enzyme sample was subjected to ammonium sulphate precipitation (20%). The precipitate was dialyzed to remove ammonium sulphate. The dialyzed fraction was subjected to protein estimation and evaluated for ACE activity. The dialyzed sample is considered as partially purified enzyme and used for inhibition studies. Crude sample of ACE had a protein concentration of 8mg/mL. Ammonium sulphate precipitate had a concentration of 1mg/mL. The specific activity, fold purity and yield are tabulated in table 1.

B. ACE assay

ACE activity was assayed by using HHL as a substrate. Crude extract of porcine kidney has shown activity of 335 nmoles/30 minute/mg protein. Ammonium sulphate precipitate has shown a activity of 670 nmoles/30 min/mg protein.

C. Antioxidant activity

The solvent extracts were evaluated for their antioxidant activity in vitro. The extracts showed differential antioxidant activity with varied IC₅₀ values. Among the extracts tested, Methanol extract of *G.cambogia* exhibited a potent antioxidant activity with IC₅₀ value of 20µg/mL followed by ethyl acetate extract (Fig. 1).

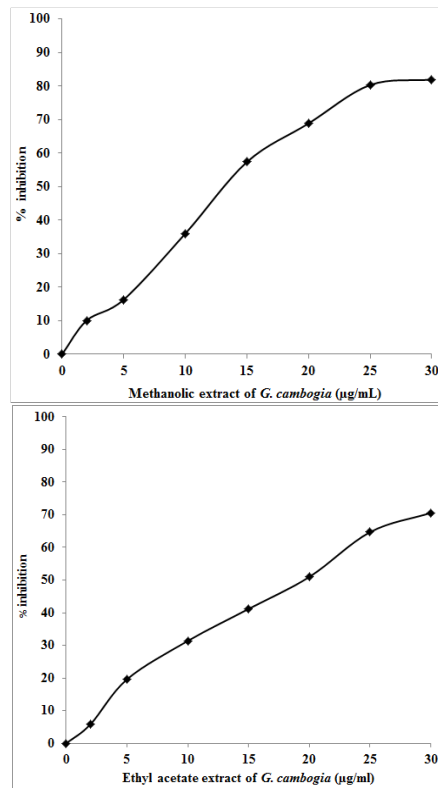


Fig. 1. DPPH radical scavenging activity of *G. cambogia* seeds extract. Values are average of three independent experiments. A) Methanolic extract B) Ethyl acetate extract

D. ACE inhibition study

ACE inhibition was done for all the solvent extract of *G. cambogia* seeds. But only methanolic extract showed inhibition of ACE activity. The inhibition was found to be dose dependent. The maximum inhibition of upto 76% was observed at a concentration of 500 µg (Fig. 2).

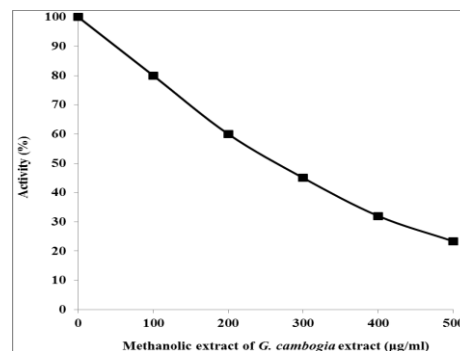


Fig. 2. Inhibition of angiotensin converting enzyme by methanolic extract of *G. cambogia* seeds. For inhibition study the ACE was preincubated with the extract for 30 min and analysed for activity using HHL as substrate. Values are average of three independent experiments. Inhibition was expressed as activity in percentage

E. Trypsin inhibition assay

Trypsin inhibition was done for all the extracts of *G. cambogia*. Among the four extracts, methanolic and ethylacetate extracts showed inhibitory property. Methanolic extract has shown potent trypsin inhibitory activity of 80%, while the ethyl acetate extract showed 72% of inhibition (Fig. 3).

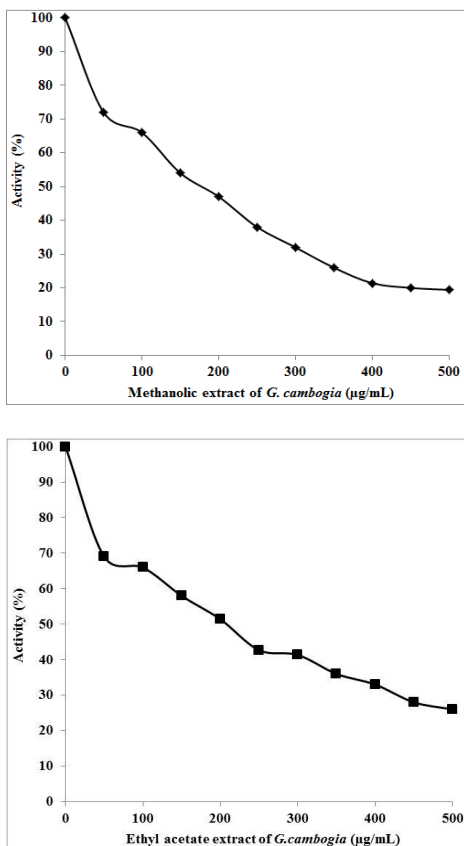


Fig. 3. Trypsin inhibitory activity of *G. cambogia* seeds extract. For inhibition studies trypsin is pre-incubated with extract for 30 min. Then analysed for proteolytic activity as mentioned in the methodology section. A) Methanolic extract B) Ethyl acetate extract. The inhibition is expressed as % activity

4. Discussion and Conclusion

In our project work ACE was partially purified from porcine kidney by employing ammonium sulphate precipitation technique. *G. cambogia* solvent extract have been prepared by Soxhlet extraction method. Later the samples were analyzed for antioxidant activity by DPPH method. Antioxidants are compounds which inhibit the oxidation by removing the free radical intermediates. Antioxidants are effective because they are willing to give up their own electrons to free radicals. After donating an electron an antioxidant becomes a free radical, in this state antioxidants are not harm full because they have the ability to accommodate the change in electrons without becoming reactive. The reduction in absorbance of DPPH because of decrease in its absorbance at 520 nm is induced by antioxidants. The decrease in absorbance of DPPH free radical

is observed as the concentration of the sample increases. In this study the both the methanol and ethyl acetate extracts of *G. cambogia* showed prominent antioxidant activity. Further, *G. cambogia* was screened for trypsin inhibitory activity. The extracts were evaluated for their trypsin-inhibitory activity in vitro. Among the extracts tested, methanol extract has shown potent trypsin inhibitory activity (80%) followed by ethyl acetate extract (61%). Protease inhibitors, including trypsin inhibitors (TIs), are found in numerous organisms. Protease inhibitors isolated from plants have activities against insect larval growth, suggesting these proteins may play a role in defense. In humans, protease inhibitors are known to be essential for physiological regulation and have been associated with a range of diseases including neuropathic pain and skin desquamation. The extracts showed significant antioxidant activity and ACE and trypsin inhibitory activities *in vitro*. Among the four extracts, the methanol extract of the seeds showed the highest anti-oxidant activity with good ACE and trypsin inhibitory activities. Therefore, the methanolic extract of seeds has potent inhibitory molecule. Further isolation and characterization of the bioactive molecule will have great importance for pharmacological studies.

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