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Research Article

# *IN VITRO* ANTIOXIDANT AND ANTICHOLINESTERASE ACTIVITIES OF *GARCINIA COMBOGIA*

**N.SUBHASHINI,*[1] G.NAGARAJAN[1], S.KAVIMANI[2]**

**[1] Department of Pharmacology, Faculty of Pharmacy, PRIST University, Thanjavur, Tamilnadu, India-614904, [2] Department of Pharmacology, College of Pharmacy, Mother Teresa Institute of Pharmaceutical Sciences, Puducherry, Tamilnadu, India-605006. Email:drsubhas2006@rediffmail.com**

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**ABSTRACT**

The present study evaluated the free radical scavenging and anticholinesterase activity of the water extracts of *Garcinia combogia* (WEGC) fruit rinds *in vitro.* The antioxidant activity of WEGC was evaluated by using the free radical scavenging activity assay (DPPH method), hydroxyl radical scavenging activity, ferric reducing antioxidant potential, total peroxy radical trapping potential and lipid peroxidation activity. In addition, total phenolic (TP) and total flavonoid was determined by using pyrocatechol and quercetin as a standard. The anticholinesterase activity of WEGC was significantly lower than neostigmine, a standard drug. Furthermore, a significant correlation between the total phenolic content, antioxidant and anticholinesterase activities of both the extracts indicating that total phenolics might be responsible for the observed antioxidant and anticholinesterase activities.This is the first report in this direction.

**Keywords**: Acteylcholinesterase, antioxidant, *Garcinia combogia,* Phenolics.

## INTRODUCTION

Free radicals such as superoxide anion, and hydroxyl and peroxyl radicals, which are produced in biological systems and foods, are responsible for oxidation of cell lipids and DNA damage, and they may cause serious diseases (e.g. cancer, coronary arteriosclerosis, diabetes mellitus)[1]. Dietary antioxidants may be effective in prevention of oxidative damage. Many scientists have focussed on medicinal and edible plants to discover natural antioxidants since some synthetic antioxidants have toxic effects. In addition, natural antioxidants may have an important role in protecting human health[2, 3]. Studies performed to find natural antioxidants indicated that many *Garcinia* species and some of their constituents have shown significant antioxidant activity. Some *Garcinia* species have been used commercially in the food industry to prevent or delay spoilage of foods.

The acteylcholinesterase (AChE) is a biologically important enzyme that hydrolyzes acetylcholine (ACh), a neurotransmitter considered to play role in the pathology of Alzhiemer's disease[4]. One of the most important approaches for treatment of this disease involves the enhancement of acetylcholine level in brain using AChE inhibitors. Several studies have reported anti-cholinesterase activity of the plant extracts and drugs. Certain reports have claimed that, a few herbal extracts can act on the central nervous system, thereby enhancing the facuties of learning and memory. One of the richest resources for new anticholinesterase drugs are natural products[5]. The fruit rinds of *Garcinia combogia*, commonly known as kodampuli have been used in the Indian systems of the medicine for hundreds of years for their beneficial role in hypolipidemic effect. The fruit rinds of *Garcinia combogia*, (Cluciaceae) mentioned in sidha, have been used to treat central nervous system dis orders. The same plant has also been used as herbal combinations with other herbs to evaluate depressant activity. Recent studies show that, the extracts of the fruit rinds of *Garcinia combogia*, also contain hepatoprotective compounds. Hence, the present study was planned to explore the antioxidative activity and anticholinesterase activity of the indigenous medicinal plants namely, *Garcinia combogia.*

## MATERIALS AND METHODS

### Chemicals:

2,2 Diphenyl –1-picryl hydrazyl hydrate (DPPH), were purchased from Himedia, Mumbai, 2deoxy 2-ribose, xanthine oxidase, quercetin, hypoxanthine, pyrocatechol were purchased from SRL, Mumbai, thiobarbituric acid, trichloroacetic acid, were purchased from SD Fine Ltd, Mumbai, (2'-2' azobis (2-amidinopropane) dihydrochloride), Trolox from Sigma Aldrich, USA, 2,7 Dichloro flurescein diacetate from Fluka and Butylated hydroxyl toluene from Loba cheme. All other chemicals used in the study were of analytical grade procured from local suppliers.

### Plant material

The fruit rinds of *Garcinia combogia* collected from Nagarkovil district, Nagarkovil, Tamilnadu, India. The plant material were identified and authenticated by Dr. M. Jagadeesan, Professor and Head, Department of Environmental & Herbal Sciences, Tamil University, Thanjavur, India (Ref.No142136 dated on 13.6.2008). The Voucher specimen is available in the herbarium file of our department.

### Extraction

The fruit rinds of the *Garcinia combogia* were dried under shade separately and stored in air tight container for further use. Extraction was carried out using water by a simple maceration technique. Seven hundred and fifty milliliter (750 ml) of solvent was added to 75 g of powder and kept on mechanical shaker for 4 h and filtered through Whatmann No.1 filter paper. The filtrate was concentrated to a small volume using flash evaporator and further evaporated to dryness in a vacuum desiccator. The extracts obtained were in the form of thick paste due to the presence of resinous matter. The percentage yield of water extract is 4.5 g.

### Animals

*Wistar* albino male rats of 150-200 g were used for the study. The inbred colonies of rats were purchased from Venkateshwara enterprises, Bangalore. They were acclimatized to controlled conditions of temperature (23±2ºC), humidity (50±5%) and 12 h light-dark cycles. The animals were randomized into experimental and control groups and housed two each in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free assessed to standard pellets as basal diet and water ad libitum. All the studies conducted were approved by the Institutional Ethical Committee, Faculty of Pharmacy, PRIST University, Thanjavur, according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA/5/10), Government of India. We selected male rats for our studies, since females are shown to be protected from cardiovascular complication.

### Phytochemical screening

Preliminary phytochemical screening of the powdered seed was performed for the presence of alkaloids, phenolics, flavonoids, saponins, tannins, carbohydrates and proteins.

### In vitro antioxidant activity

### DPPH radical scavenging assay[6]

The hydrogen donating ability of extracts was examined in the presence of DPPH stable radical. One millilitre of 0.3 mM DPPH

ethanol solution was added to 2.5 ml of sample solution of different concentration and allowed to react at room temperature. After 30 minutes the absorbance values were measured at 517 nm. Ethanol (1.0 ml) plus plant extract solution (2.5ml) was used as a blank, DPPH solution (1.0ml, 0.3 mM) plus ethanol (2.5ml) served as negative control. The positive controls were those using the standard (Ascorbic acid) solutions.

**Deoxyribose degradation assay[7]**

The decomposing effect of WEGC on hydroxyl radicals was determined by the assay of malondialdehyde chromogen formation due to 2-deoxy 2-ribose degradation. The assay mixture contained in a final volume of 1ml. 100 µl of 28 mM 2-deoxy 2-ribose dissolved in phosphate buffer, pH 7.4, 500 µl of the plant extract of various concentrations in buffer, 200 µl of 200 mM ferric chloride (1:1 v/v) and 1.04 mM EDTA and 100µl of 1.0 mM hydrogen peroxide and 100 µl of 1.0 µM ascorbic acid. After incubation of the test sample at 37°C for one hour the extent of free radical damage imposed on the substrate deoxyribose was measured using thiobarbituric acid (TBA) test. Percentage inhibition of deoxyribose degradation was calculated. Quercetin was used as standard.

**Estimation of total phenolic component[8]**

Total soluble phenolics of the extract were determined with Folin-ciocalteu reagent using pyrocatechol as a standard following the method. One millilitre (1.0 ml) of extract solution in a test tube was added to 0.2 ml of Folin Ciocalteu reagent (1:2 in distilled water) and after 20 min 2.0 ml of purified water and 1.0 ml of sodium carbonate (15%) was added. Allowed to react for 30 min and then absorbance was measured at 765 nm. The concentration of total phenolic component in the extract was determined as microgram of pyrocatechol equivalent.

**Total flavonoid content[9]**

Total soluble flavonoid of the extract was determined with aluminium nitrate using quercetin as a standard. Plant extracts (1000 µg/ml) added to 1ml of 80 % ethanol. An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10 % aluminium nitrate, 0.1 ml of 1 M potassium acetate and 4.3 ml of 80 % ethanol. The absorbance of the supernatant was measured at 415 nm after 40 min at room temperature. Total flavonoid concentration was calculated using quercetin as standard.

**Total peroxy radical trapping potential (TRAP) [10]**

A water soluble azo initiator 2, 2' azo bis (2-amidino propane) dihydrochloride (AAPH) produced the peroxyl radicals while a spectrophotometric analysis of 2, 7 dichlorofluresecin - diacetate (DCF) monitored the scavenging activity of the plant extracts. A 350 µl of 1 mM stock of DCF in ethanol was mixed with 1.75 ml of 0.01 N sodium hydroxide and allowed to stand for 20 min before the addition of 17.9 ml of 25 mM sodium phosphate buffer (pH 7.2). The reaction mixture contained 0.5 ml of various concentration of plant extract in methanol, 150 µl of activated DCF solution and 25 µl of AAPH (56 mM). The reaction was initiated with the addition of the AAPH. Absorbance was read at 490 nm. Trolox (6-hydroxy 2, 5, 7-8 tetra methyl chroman 2 carboxylic acids) was used as standard and all the determination was done in triplicate.

**Ferric reducing antioxidant power assay (FRAP)**

The iron-reducing power of the samples was tested using the assay by Huang and Kuo[11]. Briefly, 1 ml of samples was added to 2.5 ml of phosphate buffer (0.1 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%, w/v) and was incubated at 50° C for 20 min. Then 2.5 ml of 10% trichloroacetic acid (TCA) were added. 2.5 ml of this solution were mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%, w/v). After 30 min incubation, the absorbance was read at 700 nm. Analyses were triplicated. Gallic acid and BHA were used as the references tested at 0.125, 0.25, and 0.50 mg/ ml. Increase in absorbance is commented as indicative of increased reducing power.

**Assay of lipid peroxidation method[12]**

Lipid peroxidation induced by $Fe^{2+}$- ascorbate system in rat liver homogenate was estimated by TBA reaction method. The reaction mixture consisted of rat liver homogenate 0.1 ml (25% w/v) in Tris –HCL buffer (20 mM, pH 7.0), potassium chloride (30 mM), ferrous ammonium sulphate (0.16 mM), ascorbate (0.06 mM), and various concentrations of the WEGC in a final volume of 0.5 ml.

The reaction mixture was incubated for 1 h at 37 °C. After the incubation time, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS) (8.1%), 1.5ml TBA (0.8 %), and 1.5 ml glacial acetic acid (20%, pH 3.5). The Total volume was made upto 4 ml of distilled water and then kept in a water bath at 95-100° C for 1 h. after cooling, 1.0 ml of distilled water and 0.5 ml of n- butanol and pyridine mixtute (15: 1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4,000 g for 10 min. the organic layer was removed and its absorbance at 532 nm was measured. Inhibition of lipid peroxidation was determined by comparing the OD of the treatments with that of control. Ascorbic acid was used as standard.

**Calculation of 50% inhibitory concentration ($IC_{50}$)**

The Concentration (mg/ ml) of the extract required to scavenge 50% of the radicals was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated using the formula.

$$I\ \% = \left[\frac{(A_c - A_s)}{Ac}\right] 100$$

Where $A_c$ is the absorbance of the control and $A_s$ is the absorbance of the sample.

***In vitro* acteylcholinesterase inhibition assay**

AChE inhibition activities of selected extracts were measured by slightly modifying the spectrophotometric method developed by Ellman[13].Acetylthiocholine iodide was used as substrate and 5,5 -Dithiobis [2-nitrobenzoic acid] was used for the measurement of cholinesterase activity and rat heart homogenate was used as source of acteylcholinesterase enzyme.

**Preparation of the enzyme**

Male rat of *Wistar* strain weighing 150 g was sacrificed by cervical dislocation; the heart was immediately excised and homogenized with 0.1mM sodium phosphate buffer (pH 7.0) in cold condition. The homogenate was stored at -80°C till use.

**Assay procedure**

Various concentrations of the extracts in 2.6 ml of 0.1mM sodium phosphate buffer (pH 8.0) were added to 100 µl of DTNB (0.75 mM) and 5 µl heart homogenate (Crude enzyme) and incubated for 5 min at 25°C. The reaction was then initiated by the addition of 20 µl of acetylthiocholine. The hydrolysis of acetylthiocholine was monitored by the formation of yellow 2-nitro-5-sulfidobenzene-carboxylate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine for 10 min, at a wavelength of 412 nm. The percentage Inhibition of cholinesterase activity was calculated using the following formula:

***% inhibition=*** E – S / E x 100

Where E is the absorbance of the control reaction (containing all reagents except the test compound), and S is the absorbance of the test compound. Neostigmine bromide was used as positive control and all tests were carried out in triplicate.

**Statistical analysis**

Tests were carried out in triplicate for 3-5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, $IC_{50}$, was graphically determined by a linear regression method using Ms Windows based graph pad instat (version 3) software. Results were expressed as graphically mean ± standard deviation.

## RESULTS

### Phytochemical screening

Phytochemical screening of the plant extract revealed the presence of alkaloids, saponins, tannins, carbohydrates and protein.

### Hydrogen donating assay

The radical scavenging activity of WEGC was determined from the reduction in the optical absorbance at 517 nm due to scavenging of stable DPPH free radical. Positive DPPH test suggests that the extract is a potential free radical scavenger. WEGC showed strong activity compared with the standard, ascorbic acid. Table 1 shows the $IC_{50}$ values of the sample and the standard.

### Deoxyribose degradation

The scavenging effect of WEGC on hydroxyl (OH) was quantified by measuring the effect on the 2- deoxy –ribose degradation produced by reacting $Fe^{3+}$ with ascorbate, in the presence of EDTA. The $IC_{50}$ value of WEGC was 0.56mg /ml and that of standard, quercetin was 0.112 mg / ml (Table 1).

### Total phenolic & flavonoid content

The content of total phenolics in WEGC was determined using folin ciocalteau assay, calculated from regression equation of calibration curve of pyrocatechol. Phenolic content of WEGC was found to be 75g pyrocatechol equivalent / mg. the total flavonoid content of WEGC was found to be 30 g quercetin equivalent / mg (Table 3).

### Total radical antioxidant potential (TRAP)

The peroxyl radical scavenging activity was determined for WEGC and the results were compared with trolox (Table 1). Addition of increasing concentration of WEGC to solution containing AAPH-derived peroxyl radical decreased the luminescence produced by DCF and the absorbance decreased in a linear fashion. WEGC and Trolox exhibited $IC_{50}$ values of 0.262 and 0.099 mg/ml respectively.

### Ferrous reduced antioxidant power (FRAP)

The reducing ability of the extract served as a significant indicator of its potential antioxidant activity. WEGC and standard (BHT) were used at dose range of 50- 800 µg/ ml. the reducing power of WEGC increased concentration dependently. All concentration of the extract offered higher activities than control (Table 2).

### Lipid peroxidation

WEGC was effective in inhibiting the lipid per oxidation induced by $Fe^{2+}$- ascorbate system in rat liver homogenate. The MDA generated as a result of lipid peroxidation reacts with thiobarbitutric acid and was found to be inhibited in the presence of the extract. The $IC_{50}$ value was found to be 0.241 mg/ml for WEGC while for standard ascorbic acid the $IC_{50}$ was found to be 0.081 mg/ml (Table 1).

### Cholinesterase activity

In the present study, a dose dependent inhibition of AChE was found and the results are presented in table 4. Neostigmine inhibited AChE to an extent of 92% which was significantly higher ($p$ =0.05) than *Garcinia combogia*. WEGC was found to inhibit AChE to an extent of 67.3% at 1 mg/ml concentration.

### Discussion

Antioxidants exert their mode of action by suppressing the formation of reactive oxygen species either by inhibition of enzymes or by chelating trace elements. DPPH is widely used to evaluate the free radical scavenging effect of natural antioxidant. DPPH is a stable free radical at room temperature, which produces a violet solution in ethanol. DPPH shows a strong absorption band at 517 nm in visible spectrum (deep violet color). As the electron became paired in the presence of free radical scavenging the absorption vanishes and the resulting discoloration stochiometrically coincides with the number of electrons taken up. The bleaching of DPPH absorption is representative of the capacity of the test drugs to scavenge free radicals independently. The bleaching of DPPH molecules can be correlated with the number of available hydroxyl groups. We can infer that, the very good activity of the extract may be probably due to the presence of substance with an available hydroxyl group.

Hydroxyl radical is highly reactive oxygen centered radical formed from the reaction of various hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecule it contacts and is known to be capable of abstracting hydrogen atoms from membrane lipids and brings about peroxidic reaction of lipids. WEGC exhibited concentration dependent scavenging activity against hydroxyl radical generated in a Fenton reaction system [14].

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen, flavonoids are wide spread in all natural compounds and posses a broad spectrum of biological activities. The chemical composition of *Garcinia combogia* indicates the presence of phenolic compounds including tannins and flavonoids, which are known to possess antioxidant activities. The high phenolic and flavonoid content in the WEGC may be responsible for its free radical scavenging activity.

TRAP assay is based upon the potential of antioxidants in extract to scavenge peroxyl radicals generated by thermal decomposition of a water soluble azo initiator AAPH. WEGC decreased the absorbance upon increasing concentrations of the sample, which is similar to that of the standard, Trolox.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing ability is generally associated with the presence of reductones, which breaks the free radical chain by donating a hydrogen atom. The extract had reductive ability which increased with increasing concentrations of the extract[15].

Oxidative stress can lead to peroxidatin of cellular lipids and can be measured by the determining the levels of thiobarbituric acid reactive substances. Quantification of MDA, one of the products of lipid peroxidation, with TBA at low pH and high temperature (100$^{o}$ C) resulted in the formation of a red complex, which is measured at 532 nm. WEGC inhibits the rate of lipid peroxidation by a reduction in the red color complex formed reflecting its anti- lipid per oxidative potential[16].

AChE is found among neurofibrillary tangles and neuritic plaques [17] and its inhibition is an effective tool for the treatment of Alzheimer's disease and related Dementia[18]. Tacrine, a standard drug, exerts its pharmacological effect by increasing the acetylcholine level in the mouse brain[19]. Hence, the AChE inhibitory effects of plant extracts indicate their potential in the development of natural therapeutics for Alzheimer's disease and related problems.

Based on the various *in vitro* and *ex vivo* assays, it can be concluded that the WEGC posses strong antioxidant activity and anticholinesterase, evidenced by the free radical scavenging property, iron chelating, and reducing power property, which may be due to the presence of phenolic components in the extract. Overall, the plant extract is a source of natural antioxidant that can be important in disease prevention, health preservation and promotion of longevity promoter.

**Table 1: Antioxidant activity of *Garcinia combogia* by different models**

| ***In vitro* methods - $IC_{50}$ (µg/ml)** | | | | |
|---|---|---|---|---|
| **Drugs** | **DPPH** | **OH·** | **TRAP Assay** | **Lipid Peroxi Dation** |
| WEGC | 36.20 ±3.04 | 50.5±2.22 | 44.46 ±2.44 | 62.16± 0.16 |
| Ascorbic acid | 10.24 ±4.02 | | | 24.22± 1.04 |
| Quercetin | | 36.4±0.46 | | |
| TROLOX | | | 18.24 ± 62 | |

Values are mean ± SD (n = 3)

**Table 2: Reducing power ability of *Garcinia combogia***

| Drug | Absorbance at 700 nm | | | | |
|---|---|---|---|---|---|
| | 25 µg/ ml | 50 µg/ ml | 100 µg/ ml | 200 µg/ ml | 400 µg/ ml |
| WEGC | 0.2576±0.002 | 0.2969±0.003 | 0.3576±0.004 | 0.5041±0.005 | 0.6242±0.002 |
| BHT | 0.092 ± 0.002 | 0.214 ± 0.004 | 0.314 ± 0.004 | 0.640 ± 0.001 | 1.092 ± 0.008 |

Values are mean ± SD (n = 3)

**Table 3: Total phenolic and flavonoid contents of the water extract of *Garcinia combogia***

| Sample | Phenolic content (*µg* PEs/mg extract)[1] | Flavonoid content (*µg* QEs/mg extract)[2] |
|---|---|---|
| *WEGC* | 75.56 ± *2.*20 | 30.28 ± *1.*14 |

Values are mean ± SD (n = 3); [1]PEs, Pyrocatechol Equivalents; [2]QEs, Quercetin Equivalents

**Table 4: Anticholinesterase activity of the water extract of *Garcinia combogia***

| Concentration (µg / ml) | % Inhibition WEGC | Concentration (µg / ml) | % Inhibition neostigmine |
|---|---|---|---|
| 125 | 6.88 ± 1.14 | 1.25 | 32.16 ±4.44 |
| 250 | 14.28± 2.22 | 2.5 | 55.62 ±2.20 |
| 500 | 30.14 ± 1.10 | 5 | 78.80 ± 7.22 |
| 1000 | 67.3 ±1.08 | 10 | 92.04 ±4.86 |

Values are mean ± SD (n = 3)

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