Research Article

Antioxidant Activity of Combined Ethanolic Extract of *Pisonia grandis* and *Cardiospermum halicacabum*

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ABSTRACT

The *in-vitro* free radical scavenging efficacy of the combined ethanolic Biherbal extract (BHE) from equal quantities of the leaves of *Pisonia grandis* and *Cardiospermum halicacabum* was investigated. This was compared with its individual preparation of ethanolic extract of *Pisonia grandis* (EPG) and ethanolic extract of *Cardiospermum halicacabum* (ECH). The hydroxyl, hydrogen peroxide and super oxide radical scavenging activity from BHE, EPG, and ECH were investigated employing various established *in vitro* systems. Total phenolic and flavonoid content were also determined. The results revealed that BHE has notable activity in quenching of hydroxyl, hydrogen peroxide and super oxide radicals when compared to its individual preparation of EPG and ECH. The BHE at 1000µg/ml showed maximum scavenging of hydroxyl (77.63%) hydrogen peroxide (61.76%) and superoxide (65.26%) against the scavenging of EPG and ECH which showed hydroxyl (51.58%, 66.09%) hydrogen peroxide (44.02%,45.00%) and superoxide (51.95 %, 48.54 %) radicals respectively at the same concentration. The quantitative estimation of the extracts revealed the considerable amount of phenols and flavonoids. The results of this study strongly indicate that the BHE has more potent antioxidant potential action than its individual preparation EPG or ECH.

Keywords: BHE, *Cardiospermum halicacabum* , free radical scavenging activity *Pisonia grandis*.

INTRODUCTION

In human a given amount of oxygen taken in by the body is always converted to Reactive oxygen species (ROS) such as O_2^-, H_2O_2 and hydroxyl radical (OH) by various enzymatic metabolism system.[1] ROS affects various molecular components of the cell, like fatty acids, proteins and DNA and an excess production of ROS leads to cell degeneration and death.[2] When the amount of ROS exceeds the limit of defense mechanism of the body, many serious diseases may induced such as cancer, arteriosclerosis, gout, alzheimer's disease and various age–related diseases.

Endogenous antioxidant chemicals present in plants may play an important role in anti-oxidative defense, thereby protecting the biological functions of cells against the oxidative stress.[3] Plants are endowed with free radical scavenging molecules such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains and other metabolites, which are rich in antioxidant activity and offer protection against harmful diseases.[4]

Pisonia grandis R.Br (*Nyctaginaceae* family) commonly known as 'Leechai kottai keerai[5] is distributed throughout India from the Himalayas down to Ceylon. The plant is used traditionally as an antifungal and anti-rheumatic.[6] Leaves are useful in chronic rheumatism, wound healing and also used as vegetable.[7-9] *Cardiospermum halicacabum* (Sapindaceae family) known as the Balloon Plant or Love in a puff, are widely distributed in Asia and Africa. The whole plant of *Cardiospermum halicacabum* is diaphoretic, diuretic, emetic, emmenagogue, laxative, refrigerant, rubefacient and stomachic.[10] It is used in the treatment of rheumatism, nervous diseases, stiffness of the limbs and snakebite.[11] With the above scenario, the Biherbal extract (BHE) made up equal quantities of leaves of *Pisonia grandis* and *Cardiospermum halicacabum* and their individual preparations EPG and ECH were subjected to various assays in order to evaluate their antioxidative and free radical scavenging capabilities.

MATERIALS AND METHODS

Chemicals

All routine chemicals such as Ascorbic acid, Gallic acid, Catechin, were obtained from SD Fine Chemicals Ltd., India. Acetyl Salicylic Acid, nitro blue tetrazolium chloride were purchased from Lab Chemicals Ltd, India. All other chemicals and reagent used were of analytical grade.

Collection of Plant Material

The leaves of *Pisonia grandis* and *Cardiospermum halicacabum were* collected from the National Siddha Medical College and Research center India and were authenticated by Dr. Sankaranarayanan, Assistant Director, Dept of Research and Development, of the same college. The voucher specimen is also available in herbarium file of the same center.

Preparation of Plant Extract

The leaves of plants *Pisonia Grandis* (100gms) and *Cardiospermum halicacabum* (100gms) separately and a synergy mix of 100gms of *P.grandis* and 100 gms of *C.halicacabum* were shade dried and pulverized to a coarse powder. The powder was passed through 40-

mesh sieve and exhaustively extracted with 90% (v/v) (200ml) ethanol by cold maceration process at room temperature for three days. The extract was filtered and the filtrate was evaporated until all the solvent had been removed and further removal of water was carried out by freeze drying to give an extract sample with the yield of 19.6% (w/w). Similarly the EPG and ECH were also prepared separately. The EPG extract yield was 9.6% (w/w) and the ECH sample yield was 8.6% (w/w). The extracts were stored in refrigerator and used for the future investigation.

Antioxidant Activity Determination

The antioxidant activity of the ethanolic extracts of EPG, ECH and BHE was determined using the scavenging activities of hydroxyl, hydrogen peroxide and super oxide radicals. Total phenolic and flavonoid content, the chemicals responsible for the antioxidant activity of these extracts were determined quantitatively.

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity was determined according to the method of Halliwell.[12] Briefly for the non site-specific hydroxyl radical system, the reaction mixture containing 0.1ml deoxyribose, 0.1ml $FeCl_3$, 0.1ml ascorbic acid, 0.1ml EDTA, and 0.1ml H_2O_2 were mixed with or without various concentrations of the three extracts in 1ml of final volume made with KH_2PO_4–KOH buffer pH 7.4 and was incubated in a water bath at 37°C for 1hr. The extent of deoxyribose degradation was measured by thiobarbituric acid (TBA) method. 1ml of TBA and 1 ml trichloroacetic acid were added to the mixture and heated at 100°C for 20 min. After cooling to room temperature the absorbance was measured at 540 nm. Mannitol, a classical hydroxyl radical scavenger was used as positive control. The hydroxyl radical scavenging activity was calculated using the following formula: Hydroxyl radical scavenging activity (%) = $[(A_0 - A_1/A_0)$ x100], where A_0 is the absorbance of the control and A_1 is the absorbance of plant extract or the standard sample.

Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide scavenging activity was determined according to a ferrous ion oxidation - xylenol orange (FOX assay) by Long LH,[13] with minor changes. FOX reagent was prepared by adding nine volumes of reagent 1 to one volume of reagent 2, where reagent 1 was 4.4 mM butylated hydroxytoluene (BHT) in methanol and reagent 2 was 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 250 µM H_2SO_4. Plant extracts of different concentrations were incubated with 10 µL of 40 mM H_2O_2 for 10 min at room temperature in dark and 0.2 mL of FOX reagent was added and the volume was made upto1 ml with distilled water. The reaction mixture was then vortexed and incubated at room temperature for 30 min. Development of violet colour indicates control reaction and discoloration was considered as scavenging activity after the addition of plant extracts or standard (Vitamin E). The FOX reagent without extracts/H_2O_2 served as blank and with H_2O_2 served as control. The absorbance of the ferric-xylenol orange complex was measured at 540 nm. The Hydrogen peroxide radical scavenging activity was calculated using the following formula, Hydrogen peroxide radical scavenging activity (%) = $[(A_0 - A_1/A_0)$ x100], where A_0 is the absorbance of the control and A_1 is the absorbance of plant extract or the standard sample.

Superoxide Scavenging Activity

Superoxide anion scavenging activity was measured based on the described method by Robak and Gryglewski (1988).[14] Superoxide radicals were generated in nicotinenamide adenine dinucleotide, phenazine methosulphate (PMS–NADH) system by the oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In this experiment, the superoxide radical was generated in 3 ml of sodium phosphate buffer containing 1ml of 50Mm NBT solution, 1 ml of 0.1mM NADH solution, and different concentrations of the plant extracts (100 to 1000µg) in water. The reaction was started by adding 1 ml of 50mM PMS solution to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance was measured against the corresponding blank solution. L-Ascorbic acid was used as the positive control. The decrease of NBT reduction measured by the absorbance of the reaction mixture correlates with the superoxide radical scavenging activity of the plant extrats. The superoxide radical scavenging activity was calculated using the following formula: Superoxide radical scavenging activity (%) = $[(A_0 - A_1/A_0)$ x100], where A_0 is the absorbance of the control and A_1 is the absorbance of plant extract or the standard sample.

Determination of Total Phenol Content

Total phenolic content in the lyophilized extract was determined with the Folin–Ciocalteu's reagent (FCR) according to a published method of Slinkard and Singleton (1977).[15] 100mg of the sample was dissolved in 0.5ml 0f water, mixed with 2.5 ml Folin–Ciocalteu's reagent (diluted 1:10, v/v) followed by 2 ml of sodium carbonate solution. The absorbance was then measured at 765 nm after incubation at 30°C for 90 min. Results were expressed as gallic acid equivalents (mg gallic acid/g dried extract).

Determination of Total Flavonoid Content

The total flavonoid content of the extracts were determined by a colorimetric method as described in the literature of Zhishen[16]. An aliquots of sample containing 100mg of test drugs was mixed with 2 ml of distilled water and subsequently with 0.15 ml of sodium nitrite solution was added. After 6 min, 0.15 ml of aluminium chloride solution was added and allowed to stand for 6 min, then 2 ml of NaOH solution (4%) was added to the mixture. Immediately, water was added to bring the final volume to 5 ml and the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was then determined at 520 nm versus

prepared water blank. Results were expressed as catechin equivalents (mg catechin/g dried extract).

Statistical Analysis

All data was analyzed statistically with Statistica/Macsoftware (Prism, USA).

The experimental results were mean ± SEM of three parallel measurements. Mean differences were analyzed statistically by running one-way analysis of variance test (ANOVA). $P < 0.05$ was considered statistically significant when compared to relevant controls.

RESULTS AND DISCUSSION

Free radicals have been implicated in many disease conditions, the important ones being, hydroxy radical, hydrogn peroxyl radical and superoxide radical. Herbal drugs containing radical scavengers are gaining importance in treating such diseases. It has long been recognized that naturally occurring substances in higher plants have antioxidant activity. In the present study all the three plant extracts showed free radical scavenging activities. On comparison it was found BHE has the highest antioxidant activity than its individual preparation EPG and ECH.

Because of the complex nature of phytochemicals the antioxidant activities of plant extracts cannot be evaluated by only a single method. Therefore, commonly accepted assays were employed to evaluate the antioxidant effects of the BHE.

Effect of Plant Extracts on Hydroxyl Radical Scavenging Activity

Table-1 explains the hydroxyl radical scavenging activity of plant extracts which is compared with standard ascorbic acid.

There was a marked reduction in the deoxyribose cleavage induced by hydroxyl radicals by the plant extract in the present study. All the three extracts scavenged the hydroxyl radicals in a concentration dependent manner. The hydroxyl radical scavenging activity was more for the BHE ($p<0.1$) when comparable with EPG and ECH.

Table 1: Hydroxyl radical scavenging activity of BHE, ECH and EPG extracts

Concentration (µg/ml)	Inhibitory activity of ECH (%)	Inhibitory activity of EPG (%)	Inhibitory activity of BHE (%)	Inhibitory activity of Mannitol (%)
50	13.79 ± 0.44a**	6.29 ± 0.26b**	19.62 ± 0.60 c^{ns}	21.30±0.72
100	15.82 ± 1.18a**	11.35 ± 0.96b**	25.35 ± 0.63c^{ns}	24.75 ± 0.77
200	19.88 ± 0.21a**	16.00 ± 1.20b**	27.28 ± 2.43c^{ns}	28.52 ± 2.53
250	26.76 ± 2.32a**	19.58 ± 1.42b**	31.00 ± 0.79c^{ns}	36.36 ± 0.70
500	46.37 ± 1.56a**	26.90 ± 1.82b**	55.46 ± 2.07c^{ns}	66.72 ± 0.88
1000	66.09 ± 0.83a**	51.58 ± 0.37b**	77.63 ± 1.02c^{ns}	81.75 ± 0.56

Values are expressed in mean ± SEM (n = 3), statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test Comparison between: a: Mannitol vs ECH; b: Mannitol vs EPG and c Mannitol vs BHE. *p<0.05, **p<0.1 and NS: Non–Significant.

Table 2: Super oxide radical scavenging activity of BHE, ECH and EPG extracts

Concentration (µg/ml)	Inhibitory activity of ECH (%)	Inhibitory activity of EPG (%)	Inhibitory activity of BHE (%)	Inhibitory activity of Ascorbic acid (%)
50	13.79 ± 0.44a**	6.29 ± 0.26b**	19.62 ± 0.60 c^{ns}	21.30 ± 0.72
100	15.82 ± 1.18a**	11.35 ± 0.96b**	25.35 ± 0.63c^{ns}	24.75 ± 0.77
200	19.88 ± 0.21a**	16.00 ± 1.20b**	27.28 ± 2.43c^{ns}	28.52 ± 2.53
250	26.76 ± 2.32a**	19.58 ± 1.42b**	31.00 ± 0.79c^{ns}	36.36 ± 0.70
500	46.37 ± 1.56a**	26.90 ± 1.82b**	55.46 ± 2.07c^{ns}	66.72 ± 0.88
1000	66.09 ± 0.83a**	51.58 ± 0.37b**	77.63 ± 1.02c^{ns}	81.75 ± 0.56

Values are expressed in mean ± SEM (n = 3), statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test Comparison between: a: Ascorbic acid vs ECH; b: Ascorbic acid vs EPG and c: Ascorbic acid vs BHE. *p<0.05, **p<0.1 and NS: Non–Significant.

In the present investigation the free radicals such as hydroxyl, hydrogen peroxide and superoxide radical scavenging activities of the plant extract were studied.

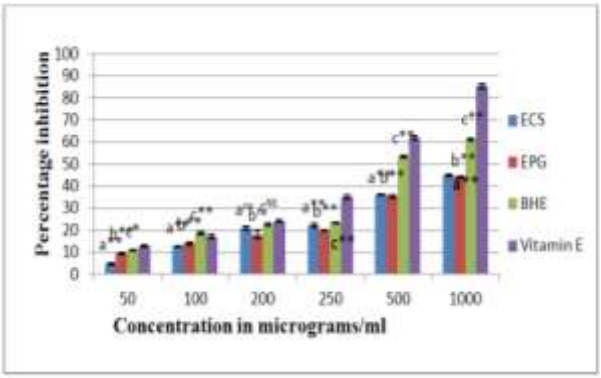
We examined the inhibitory action of these plant extracts on deoxyribose degradation which gives an indication of hydroxyl radical scavenging activity.[17] Our results clearly demonstrated the capacity of these extracts to quench hydroxyl radicals with BHE exhibiting higher scavenging activity. Moreover, hydroxyl radicals are capable of the quick initiation of lipid peroxidation process as by abstracting hydrogen atoms from unsaturated fatty acids.

BHE was more effective in scavenging the hydroxyl radicals when compared with Individual extracts which seems to be directly related to the prevention of

propagation of lipid peroxidation. The phytochemicals present in the plants *Pisonia grandis* and *Cardiospermum halicacabum* synergically interact in scavenging the hydroxyl radicals. Rao and Chandra, 2006 in their Phytochemical screening of *Cardiospermum halicacabum* confirms the presence of flavones, aglycones, triterpenoids, glycosides and a variety of fatty acids and volatile esters.[18] The Other secondary metabolites reported by Deepan, include alkaloids, carbohydrates, proteins, saponins lignin, steroids, cardiac glycosides found in small quantities in the extracts.[19] The phytochemical study of *Pisonia grandis* reveals that the presence of steroids likes octocosanol, betositosterol, alphaspinosterol, dulcitol and flavonoids in the leaves of the plant.[20]

Effect of Plant Extracts on Hydrogen Peroxide Radical Scavenging Activity

In the present investigation significant results were obtained from EPG, ECH and BHE on hydrogen peroxide radical scavenging activity. Figure-1 shows the scavenging ability of the different extracts on hydrogen peroxide radicals that increases in a dose dependent manner when compared with the control. In correlation with hydroxyl radical scavenging activity the BHE effectively scavenged the hydrogen peroxide radicals than EPG, ECH extracts. At the concentration of 1mg/ml the percentage inhibition of hydrogen peroxide radicals by BHE was 61.16% that of EPG was 44.02% and ECH was 45.00% against the positive control vitamin E which showed about 85.26% inhibition of hydrogen peroxide radicals at the same concentration.



Values are expressed in mean ± SEM (n = 3), statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test Comparison between: a: Vitamin E vs ECH; b: Vitamin E vs EPG and c: Vitamin E vs BHE. *p<0.05, **p<0.1 and NS: Non–Significant.

Hydrogen peroxide is formed by two electron reduction of O_3 which is not a free radical, but an oxidizing agent. In the presence of O_3 and transition metal ions, the H_2O_2 can generate OH radical via Fenton reaction. Mallakckakron reported, that in addition, H_2O_2 can easily cross the cell membrane and exerts an injurious effect on tissues through a number of different mechanisms such as, perturbing intracellular Ca^{2+} homeostasis, increasing intracellular ATP, inducing DNA damage, and cell apoptosis.[21] So the removal of H_2O_2 is important for the antioxidant defense mechanism. In Comparing EPG and ECH, BHE effectively scavenged the hydrogen peroxide radicals. Babu and Krishnakumari, stated that to manage the oxidative stress produced by sunrays and oxygen, *Cardiospermum halicacabum* has several small molecular mass and oxidative constituents hence; it has been effectively employed in phytotherapy.[22]

A flavonoid glycoside, 4-0-methyl-5'-0-actylmyricelin 3-o-gluco(2 to 1) rhamnoside have been isolated from methanolic extract of the leaves of plant *Pisonia grandis*[23].

These phyto constitutents are responsible for quenching the hydrogen peroxide radicals.

Effect of plant extracts on Superoxide radical scavenging activity

Table-2 depicts Super oxide scavenging activity of different plant extracts.

The extracts like EPG, ECH, BHE and the positive control ascorbic acid demonstrated a concentration-dependent scavenging activity of super oxide radicals.

The inhibitory activity was minimum in low concentration of plant extract and increases with increase in the concentration.

The BHE demonstrated a maximum scavenging activity among all the three extracts.

Superoxide, the one-electron reduced form of molecular oxygen, is a precursor of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen that have the potential of reacting with biological macromolecules, there by inducing tissue damages, and also it has been implicated in initiating oxidation reactions associated with aging.[24]

Results on inhibition of the superoxide (ROS) *in vitro* showed that the BHE is more potent when compared with its individual preparations like EPG and ECH extracts. In the GC-MS examination, Senthilkumar and Vijayakumari,[25] have reported 15 bio active phytochemical components for example, acetic acid, 1,6,10-dodecatriene, 7,11, dimethyl-3-methylene-(E)-, phenol, 2,6-bis (1,1-dimethylethyl)-4-methylmethylcarbamate, 3-O-methyl-d-glucose, 1,14-tetradecanediol, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, phytol, pseudoephedrine, 2-propenamide, N-[2-(dimethylamino)ethyl], E-2-octadecadecen-1-ol etc. in *Cardiospermum halicacabum* and *Pisonia grandis* have the bio active compounds includes Pinnatol, Allantoin, β-Sitosterol, α-Spinasterol, β-Sitosterol glucoside, Octocosanal, Dulcitol, Flavonoids and Quercetin.[26]

The synergistic effect of the active principles present in *Pisonia grandis* and *Cardiospermum halicacabum* s are responsible for the protective and antioxidant activity of the BHE.

Determination of Total Phenol and Flavonoid Content

Table-3 shows the total phenolic and flavonoid contents of EPG, ECH, BHE which were determined and expressed in terms of gallic acid and catechin equivalents.

Total phenolic and Flavonoid contents of each gram of dried extract were estimated to be equivalent to 77.33 mg gallic acid and 75.66 mg catechin.

The antioxidant activity of BHE is probably due to its phenolic content and the secondary plant phenolics the flavonoids.

It is well known that phenolic compounds are constituents of many plants, and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants**.**

Table 3: Total Phenolic and flavanoid content of BHE, ECH and EPG extracts extract

Extracts	Total phenolic content (mg/g)	Total flavonoid content (mg/g)
ECH	47.66±2.60	65.30±2.02
EPG	57.00±1.00	71.00±1.55
BHE,	77.33±0.88	75.66±1.45

Values are expressed in mean ± SEM (n = 3)

Total phenolic content was expressed as mg gallic acid equivalents/g dried extract.

Total flavonoid content was expressed as mg catechin equivalent/g dried extract.

Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties. Therefore, it would be valuable to determine the total phenolic and flavonoid content of the plant extracts.

The presence of significant quantities of phenol and flavonoid in the plant extracts are considered as the major contributors towards the antioxidant activity in our present study.

CONCLUSION

The obtained results suggest that the combined ethanolic Biherbal extract (BHE) can form a good source of effective inhibitors of free radicals. Further investigations regarding their phytoactive compounds are needed.

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