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ANALYSIS OF ANTIOXIDANT AND LIPID PEROXIDATION EFFECT OF ORGANIC EXTRACTS OF *CALYCOPTERIS FLORIBUNDA* (LINN) IN RAT FED WITH HIGH FAT DIET

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ABSTRACT

The aim of this study was to investigate effect of various extract of *Calycopteris floribunda* on the lipid peroxidation and invivo antioxidant systems in tissue (Liver, Heart and Aorta) of high fat fed rats. High fat diet rats showed significantly ($P < 0.001$) reduced the levels of tissue enzymatic antioxidant and non enzymatic antioxidant (Glutathione). The level of Thiobarbituric reactive substances (TBARS) are elevated in HFD rats (group II) When compared with control rats (Group I). After administration of ethyl acetate extract of *Calycopteris floribunda* in high fat diet rats showed significantly ($P < 0.001$) increased the levels of antioxidant enzymes (CAT, SOD, GPx, GR) and non enzymatic antioxidant glutathione (GSH, CAT, SOD, GPx, GR) and lowered the concentration of TBARS when compared with other two extracts. The ethyl acetate extract of *Calycopteris floribunda* in high fat diet rats were found reduced the concentration of TBARS (a measure of lipid peroxidation) than that of HFD rats (Group II). In conclusion, ethyl acetate extract of *Calycopteris floribunda* could be an option to enhance the supply of antioxidants and to safeguard against oxidative stress and thereby preventing the formation of atherosclerotic plaques.

KEYWORDS: *Calycopteris floribunda*, Antioxidant, High Fat diet, Rats, Lipid peroxidation.

1. INTRODUCTION

Currently, the investigation for natural sources of antioxidants from plants is a strong propensity.[1] An imbalance between protector system and production of free radicals causes the oxidative stress.[2] Biomolecules are oxidized by the high levels of free radicals in living systems leading to tissue damage, cell death or various diseases such as cancer, cardiovascular diseases arteriosclerosis, neural disorders, skin irritations and inflammation.[3,4] Reactive oxygen and nitrogen species participate in normal physiological process, including cellulose life / death process, production from pathogens, various cellular signaling pathways and regulation of vascular tone.[5] Pathogenesis of atherosclerosis has been implied by the free radical induced lipid peroxidation and reactive oxygen sepsis (ROS) are known to be the initiators of lipid peroxidation.[6] The organisms use endogenous and exogenous antioxidant defenses to protect against harms of oxygen and nitrogen reactive species. They are classified in enzymatic: catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD); and non-enzymatic systems; thiol reduced (GSH), vitamins, minerals and polyphenols.[7] Antioxidant substances and enzymes in the body are not wholly effective in preventing oxidative damage especially in conditions like hyperlipidemia and diabetes mellitus where free radicals are produced in excess. Currently available hypolipidemic drugs have been associated with a number of side effects.[8] However, most people cannot successfully control their blood cholesterol because of the modern life style. Therefore, a medication is considered the lost option for these individuals. Antioxidant compounds can deactivate and scavenge the free radicals. Recently, investigation of new sources of natural antioxidant became very important for human health. Natural antioxidants commonly exist on plants which contain polyphenolic compounds.[9-11] In recent times, antioxidants from plant sources have received a lot of attention and are preferred to synthetic ones. There is a plethora of plants that have been found to possess strong antioxidant activity.[12]

Calycopteris floribunda Lam. (Combertaceae) commonly known as Kokkarai in Hindi, Minnarakoti in

Tamil, a scadent woody and climbing shrub which is 5-10 cm long with slender brown streaked branches with vine storing water abundantly. So it is referred as a life saver by the forest dwellers during summer when streams dry up, people quench their thirst by using this plant.[13-15] The leaves have reported to possess anti-diabetic activity.[16] The hepato productive activity of various stem and leave extracts have been reported[17,18] and even fruits claimed to treat jaundice. Calycopterone, isocalycopterone and 4-dimethyl-calycopterone showed a wide range activity against solid cell lines/.[19] The leaves are reported to have medicinal uses as a laxative and anti-helmintic while the juice derived from the young twigs is used for the treatment of diarrhea, dysentery and malaria.[20] Volatile oil extracted from the leaves of *C. floribunda* was reported to exhibit high antimicrobial activity.[21] Previous phytochemical studies have reported on the isolation of the flavonoids, calycopterin, quercetin and five bi flavonoids.[22, 23] An ethnomedicinal survey conducted in Uttara Kannada District; evidence the wound healing activity.[24] The calycopterin is used to synthesize many flavones displaying high anti-proliferative activity.[25] Toxicity studies of *C. floribunda* reported in calf, rabbit and rats.[26]

This research work tries to shed some light on the functional properties of various extracts of whole plant of *Calycopteris floribunda.* To the best of our knowledge, this is the first report about the invivo antioxidant and lipid peroxidation effect of various extracts of whole plant of *Calycopteris floribunda* (Lam.) in rat fed with high fat diet.

2. MATERIAL AND METHODS

2.1. Collection and identification of plant materials

The whole plant of *C. floribunda* (Lam) was collected from Pulliyankudi, Nellai District of Tamil Nadu, India. Taxonomic identification was made from Botanical Survey of Medical Plants Unit Siddha, Government of India, Palayamkottai. The whole plant material of *C. floribunda* (Lam) was dried under shade, segregated, pulverized by a mechanical grinder and passed through a 40 mesh sieve. All the three extracts worth stored in screw cap vial at 4°C until further use.

2.2. Preparation of extracts

The above powered materials were successively extracted with petroleum ether (40-60°C) by hot continuous percolation method in soxhlet apparatus[27] for 24 hrs. Then the marc was subjected to ethyl acetate (76-78°C) for 24 hrs and then marc was subjected to methanol for 24 hrs. The extracts were concentrated by using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained. The extracts were suspended in 2% tween 80.[28]

2.3. Animals and treatment

Male Wistar rats of 16-19 weeks age, weighing 150-175g were procured from the Central Animal House, Sankaralingam Bhuvaneswari College of Pharmacy, Sivakasi. The rats were kept in cages, 2 per cage, with 12:12 hr light and dark cycle at 25°±2°C. The rats were maintained on their respective diets and water *ad libitum.* Animal Ethical Committee's clearance was obtained for the study. Animals were given enough care as per the Animal Ethical Committee's recommendations. The experiment were carried out as per the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethics Committee (IAEC) Sankaralingam Bhuvaneswari College of Pharmacy, Sivakasi, (Approved number: SBCP / 2012 – 2013/ CPCSEA / IAEC – III / 03)

2.4. Experimental Design

Rats were divided into following six groups of six rats each:

Group I : Standard chow diet
Group II : High Fat Diet
Group III : High fat diet + Petroleum ether extract of *Calycopteris floribunda* (200mg/kg B.wt)
Group IV : High fat diet +Ethyl acetate extract of *Calycopteris floribunda* (200mg/kg B.wt)
Group V : High fat diet + Methanol extract of *Calycopteris floribunda* (200mg/kg B.wt)
Group VI : High fat diet + Standard drug atorvastatin (1.2 mg/kg B.wt)

2.4.1. Animal diet

The compositions of the two diets were as follows.[29]

2.4.1.1. ontrol diet: Wheat flour 22.5%, roasted bengal gram powder 60%, skimmed milk powder 5%, casein 4%, refined oil 4%, salt mixture with starch 4% and vitamin & choline mixture 0.5%.

2.4.1.2. High fat diet: Wheat flour 20.5%, roasted bengal gram 52.6%, skimmed milk powder 5%, casein 4%, refined oil 4%, coconut oil 9%, salt mixture with starch 4% and vitamin and choline mixture 0.5%, cholesterol 0.4%.

2.5. Testing of *in vivo* antioxidant and lipid peroxidation

Rats of group III, IV and V were orally fed with the various extracts of *Calycopteris floribunda* and rats of group IV were fed with standard drug atorvastatin. Both the extracts and atorvastatin were suspended in 2% tween 80 separately and fed to the respective rats by oral intubation. At the end of 9 weeks all the animals were sacrificed by cervical dislocation after overnight fasting. Liver, heart and aorta were cleared of adhering fat, weighed accurately and used for the preparation of homogenate. Animals were given enough care as per the Animal Ethical Committee's recommendations. Portions of the tissues from liver, heart and aorta were blotted, weighed and homogenized with methanol (3 volumes). The lipid extract obtained by the method.[30] It was used for the estimation of thiobarbituric acid reactive

substances[31] (TBARS). Another portion of the tissues was homogenized with phosphate buffer saline and used for the estimation of reduced Glutathione[32] (GSH), Superoxide dismutase[33] (SOD), Catalase[34] (CAT), and Glutathione peroxidase[35] (GPx), Glutathione reductase[36] (GR).

2.6. Statistical analysis

Results were expressed as mean ± SE of 6 rats in each group. The statistical significance between the groups was analyzed by using one way analysis of variance (ANOVA), followed by Dunnet's multiple comparison tests. A difference in the mean values of P<0.05 was considered to be significant.

3. RESULTS AND DISCUSSION

High fat diet can cause the formation of toxic intermediates that can inhibit the activity of antioxidant enzymes[37] and the accumulation of O_2 and H_2O_2 which in turn forms hydroxyl radicals.[38] The variable effect of a high cholesterol diet on antioxidant enzymes may be due to the severity and / or duration of hypercholesterolemia.[39] The Presence of Lipid peroxides in tissues and cell lipids has long been assumed to be a sign of atherosclerosis.[40] Hydrogen peroxide is highly important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radical in the cell by reacting with Fe^{2+} and possibly cu^{2+} ions.[41] On this account, there are various reports indicating the beneficial effects of antioxidant supplementation in preventing dyslipidemia and cardiovascular disease.[42-44] TBARS (Thiobarbituric acid reactive substances) are produced in the body as a result of lipid peroxidation. Hence they are measured in order to determine the extent of oxidative stress occurring inside the body. 99% of TBARS is a compound known as malondialdehyde. Reactive oxygen species (ROS) degrade polyunsaturated lipids, forming malondialdehyde.[45]

Table 1. Effect of various extracts of *Calycopteris floribunda* on tissue TBARS and Glutathione (GSH) in rats fed HFD

Groups	TBARS(n mol of MDA formed/g tissue)				GSH(mg/g tissue)	
Group I	26.44 ±1.12 b*	40.87 ± 2.49 b*	19.42 ± 1.26b**	4.76 ± 0.24b**	8.10 ± 0.39 b*	5.76 ± 0.42 b*
Group II	79.67 ± 4.45 a*	83.87 ± 4.37 a*	67.45 ± 3.77a*	1.76 ± 0.45a**	4.40 ± 0.46 a*	2.68 ± 0.15 a*
Group III	62.73 ± 2.56 a**,b**	71.83 ± 3.16 a*,b*	50.15 ± 2.92 a**,b*	1.98 ± 0.36a**,b*	4.90 ± 0.56a**,b**	2.96 ± 0.36 a*,b*
Group IV	35.45 ± 3.63 a**,b*	45.78 ± 3.24 a*,b*	29.56 ± 2.24 a*,b*	3.98 ± 0.10a*,b*	7.18 ± 0.22 a*,b*	5.15 ± 0.15 a*,b**
Group V	47.68 ± 3.56 a*,b*	58.76 ± 3.67 a*,b*	38.45 ± 3.12 a*,b*	2.24 ± 0.34 b**	5.01 ± 0.45 b*	3.78 ± 0.14 b**
Group VI	27.87 ± 1.56 a*,b*	42.98 ± 3.66 a*,b**	19.17 ± 1.69 a*,b**	4.25 ± 0.48 b*	7.34 ± 0.82 b*	5.65 ± 0.34 b*

Values are mean ± SE of 6 rats
P values : *<0.001, **<0.05
NS : Non significant

a → group I compared with groups II, III, IV, V, VI.
b → group II compared with groups III, IV, V, VI.

Group I : standard chow diet. (Control)
Group II : High Fat Diet.
Group III : High fat diet + Pet.ether extract of *Calycopteris floribunda* (200mg/kg B.wt)
Group IV : High fat diet + Ethyl acetate extract of *Calycopteris floribunda* (200mg/kg B.wt)
Group V : High fat diet + Methanol extract of *Calycopteris floribunda* (200mg/kg B.wt)
Group VI : High fat diet + standard drug atorvastatin (1.2 mg/kg B.wt)

As shown in Table 1, The TBARS levels in liver, heart and aorta were found to be significantly increased in HFD group compared to control group indicating an increase in oxidative stress and thus indicative of enhanced lipid peroxidation and failure of the antioxidant defense mechanism to inhibit free radical generation. However, the ethylacetate extract of *Calycopteris floribunda* significantly (P<0.001) decrease tissues (liver, heart and aorta) TBARS level in ethyl acetate extract plus HFD groups compared to HFD rats (Group II). The similar result was found in other two extract treatment groups. This may be due to the free radical scavenging activity of the ethyl acetate of *Calycopteris floribunda.*

GSH is the most important biomolecule against chemically induced toxicity and can participate in the elimination of reactive intermediates by reducing hydroperoxides in the presence of GSH-Px.[46-48] As shown in Table 1, a significant (P<0.001) decrease in liver, heart and aorta GSH levels in HFD rats Compared to control rats (Group I), While increase in liver, Heart, aorta GSH levels in ethyl acetate extract plus HFD group compared to HFD (Group II) and other extracts treatment groups (III &IV). In HFD group, significant raise in lipid peroxidation and concomitant GSH activity may be a consequence of depleted glutathione stores.

Table 2. Effect of various extracts of *Calycopteris floribunda* on tissue Superoxide dismutase (SOD) and Catalase (CAT) in rats fed HFD

Groups	SOD (unit min/mg/protein)			CAT (µ moles of H_2O_2 consumed min/mg/protein)		
	Liver	Heart	Aorta	Liver	Heart	Aorta
Group I	3.97 ±0.45 b*	1.96 ±0.12 b*	2.97 ±0.17 b*	30.22 ±1.45 b*	48.24 ±2.62 b*	32.34 ±1.54 b*
Group II	1.58 ±0.54 a*	0.74 ±0.05 a*	1.89 ±0.14 a*	17.19 ±1.44 a*	27.34 ±1.65 a*	20.22 ±1.69 a*
Group III	1.94 ± 0.56 a**,b*	0.93 ± 0.11 a*,b**	2.05 ± 0.24 a*,b*	19.54 ±1.72 a*,b*	29.18 ±1.36 a*,b*	21.78 ±1.74 a**,b**
Group IV	3.63 ± 0.45 a**,b*	1.67 ± 0.16 a*,b*	2.69 ± 0.22 a**,b*	28.86 ±1.43 a*,b*	45.78 ±1.46 a**,b*	28.98 ±1.56 a**,b*
Group V	2.24 ± 0.26 a*,b*	1.09 ± 0.22 a*,b*	2.13 ± 0.25 a*,b*	21.43 ±1.54 a*,b*	33.48 ±1.79 a*,b*	22.58 ±1.57 a*,b*
Group VI	3.82 ± 0.43 a*,b*	1.71 ± 0.14 a*,b*	2.89 ± 0.22 a*,b**	28.86 ±1.87 a*,b**	47.18 ±1.74 a*,b*	31.56 ±1.67 a*,b*

Values are expressed as mean ± SE (n=6 rats)
P values : * < 0.001, ** < 0.05
NS : Non Significant
a →group I compared with groups II, III, IV, V, VI.
b →group II compared with groups III, IV, V, VI.
Details of group I-VI are same as in Table 1.

SOD is the first enzyme in antioxidant defense that protects tissues against oxygen free radicals by catalyzing the removal of superoxide radical (O^{2-}) which damages the membrane and biological structures.[49] Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it function to catalyze the decomposition of hydrogen peroxide to water and oxygen. Catalase has one of the highest turnover numbers of all enzymes; One catalase enzyme can convert 40 million molecules of hydrogen peroxide to water and oxygen each second.[50] As shown in Table 2, the activities of SOD and CAT in the tissue like liver, heart and aorta were significantly (P<0.001) lowered in rats fed with high fat diet (Group II) than control group animals. The ethyl acetate extract of *Calycopteris floribunda a*lso restored the levels of antioxidant enzymes such as SOD and CAT almost back to the normal levels. SOD and catalase activity diminishes in disease conditions involving oxidative stress like hyperlipidemia.

Further more, it is well reported that the cholesterol-enriched diet would appear to induce free radical production, followed by oxidative stress and hypercholesterolemia[51] such as decreasing the activities of catalase and SOD and thereby elevating the lipid peroxide contents resulting in the production of toxic intermediates.

Table 3. Effect of various extract of *Calycopteris floribunda* on tissue Glutathione peroxidise(GPx) and Glutathione reductase (GR) in rats fed HFD

Groups	GPx (mg of GSH consumed/min/mg protein)			GR (mg of GSH consumed/min/mg protein)		
	Liver	Heart	Aorta	Liver	Heart	Aorta
Group I	9.28 ± 0.14 b*	15.34 ± 0.26 b*	14.45 ± 0.15 b*	1.78 ± 0.14 b*	3.08 ± 0.12 b*	1.92 ± 0.24 b*
Group II	5.08 ±0.17 a*	7.15 ± 0.17 a*	6.90 ± 0.46 a*	0.78 ± 0.01 a*	1.35 ± 0.16 a*	0.88 ± 0.06 a*
Group III	5.32 ± 0.55 a*,b*	8.56 ± 0.42 a*,b**	7.60 ± 0.15 a*,b*	0.93 ± 0.08 a*,b*	1.71 ± 0.03 a*,b*	1.06 ± 0.16 a*, b*
Group IV	8.78 ± 0.13 a*,b*	13.82 ±0.26 a*,b*	12.16± 0.18 a*,b*	1.65 ± 0.54 a*,b*	2.91 ± 0.17 a*,b*	1.74 ± 0.12 a*,b**
Group V	6.45 ± 0.32 a*,b*	10.80 ± 0.42 a*,b*	9.46 ± 0.12 a**,b*	1.19 ± 0.08 a*,b*	2.02 ± 0.23 a*,b*	1.22 ± 0.24 a**,b**
Group VI	8.67 ± 0.16 a*,b**	14.16 ± 0.14 a*,b*	13.56 ± 0.16 a**,b*	1.56 ± 0.12 a*,b*	2.92 ± 0.44 a*,b*	1.81 ± 0.12 a*,b*

Values are expressed as mean ± SE (n=6 rats)
P values : * < 0.001, ** < 0.05
NS : Non Significant
a →group I compared with groups II, III, IV, V, VI.
b →group II compared with groups III, IV, V, VI.
Details of group I-VI are same as in Table 1.

The increased oxidative stress is related to an over production of free radicals or deficiency in the antioxidant defense system glutathione is a major sourse

of reducing power and it is maintained in the reduced form by GR, acting together with NADPH.[52]

Glutathione peroxidase (GPx) is more important than catalase for detoxification of hydrogen peroxide in brain, because the brain contains small amounts of catalase and GPx can also interact directly with lipid peroxides.[53, 54]

As shown in Table 3, tissue Glutathione peroxidase and reductase levels were significantly ($P<0.001$) decreased in rats fed with HFD (group II) as compared to the control rats (Group I). Administration of ethyl acetate of *Calycopteris floribunda* along with HFD significantly ($P<0.001$) enhanced the levels of glutathione peroxidase and glutathione reductase in all the tissues as compared with HFD rats. Similar result was not observed in other two extract treatment groups. A standard drug Atorvastatin administered also showed elevated level of glutathione peroxidase and glutathione reductase. Therefore, the increment or maintenance of total glutathione and rise in GR and GPx could be an indication of antioxidant status improvement after ethyl acetate extract of *Calycopteris floribunda* intake.

4. CONCLUSION

The present study demonstrated that the attenuation of oxidative stress by ethyl acetate extract of *Calycopteris floribunda* supplementation, resulting from the enhanced antioxidant effect in the organs, might be one of the mechanisms contributing to the amelioration of atherosclerosis in hyperlipidemic rats. Further detailed studies need to be carried out using the fractions containing the active constituents responsible for particular activities to exactly pinpoint on the active principle responsible for antihyperlipidemic activity and to check the in depth mechanism hypocholesterolemic activity of the ethyl acetate extract of *Calycopteris floribunda* at molecular level.

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6. Conflict of Interest: None.

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