

Antioxidant Activity of *Actinodaphne Hookeri* Meissn Leaves

D. Prajapati^{1*}, N. Patel¹, K. Mruthunjaya², and R. Savadi³

¹Department of Pharmacognosy and Phytochemistry, Shri B.M.Shah College of Pharmaceutical Education and Research, Modasa-383315, Gujarat, India

²Department of Pharmacognosy and Phytochemistry, J.S.S. College of Pharmacy, S.S.Nagar, Mysore-570015, Karnataka, India

³Department of Pharmacognosy and Phytochemistry, K.L.E.S' College of Pharmacy, Vidyanagar, Hubli-580031, Karnataka, India

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Abstract

Leaves of *Actinodaphne hookeri* (AH) Meissn (family, lauraceae, pisa) are used traditionally in the treatment of diabetes, disorders of the urinary tract which are more common in Chhattisgarh and eastern part of India. It contains flavonoids and quercetin-3-rhamnoside as an important constituent. The leaves were extracted with 70% hydro alcoholic solvent. Preliminary phytochemical investigation showed the presence of triterpenoids, alkaloids, tannins, flavonoids, glycosides and carbohydrates. The antioxidant activity of 70% hydro alcoholic extracts of AH was evaluated *in vitro* by various experimental parameters such as β -carotene-linoleic acid, lipid peroxidation and total antioxidant capacity. Our results showed that *Actinodaphne hookeri* leaves displayed potent antioxidant properties, supporting the ethnomedical use given to this plant to treat diabetes and urinary disorder.

Keywords: *Actinodaphne hookeri*; Antioxidant; Lipid peroxidation; Total antioxidant capacity.

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1. Introduction

Reactive oxygen species (ROS) such as superoxides, peroxides and hydroxyl radicals are known to play an important role and have been identified as major contributors to all cell and tissue damage in many disease conditions [1]. These free radicals induce damage to biomembranes, proteins and DNA. Lipid peroxidation, which involves a series of free

*Corresponding author: dharmesh09mpc@yahoo.co.in

radical mediated chain reaction processes, is also associated with several types of biological damage. Therefore, much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit ROS production and protect from damage due to ROS. The medicinal properties of plants have been investigated, in the light of recent scientific developments, through out the world due to their potent pharmacological activities and economic viability. Sources of natural antioxidants are primarily, plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks [2]. Prominent manifestation of free radical activity in biological systems is lipid peroxidation and it is involved in the development of different diseases. The primary targets of free radical attack on lipids are polyunsaturated fatty acids (PUFA). Lipid peroxidation usually precedes as a chain reaction: alkyl radicals are formed during the initiation step by the attack of lipids by free radicals or other reactive species, followed in the propagation phase by the formation of alkylperoxyl (ROO.) and alkoxy (RO.) radicals, and terminating with the formation of lipid hydroperoxides (ROOH) [3].

Antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, anti-bacterial or antiviral activities to a greater or lesser extent [4]. Antioxidant compounds are found to be useful in treatment of diabetes so the attention has been focused to natural products, especially to phenols, because of antioxidant activity of phenolic acids [5-7], tannins [8, 9], flavonoids [10].

Actinodaphne hookeri Meissn (Lauraceae family) is a moderate sized tree or some times a shrub, usually up to 6 m in height, found in the evergreen forests of eastern and Western Ghats, Karnataka, Orissa, Sikkim and North-East India [11]. Traditionally the leaves are used in the treatment of diabetes, urinary disorders [12]. The Phytoconstituents reported are quercetin-3-rhamnoside, amorphous alkaloid, β -sitosterol, hentriacontanone and hentriacontanol [13, 14]. Its traditional uses and presence of Quercetin-3-rhamnoside inspired us to carry out free radical scavenging and antioxidant activity [15].

2. Materials and Methods

2.1. Chemicals

Egg phosphatidylcholine, BHT (Butylated Hydroxy Toluene), Deoxy-d-ribose, Trichloro acetic acid (TCA) and Thiobarbituric acid (TBA) were obtained by Hi-Media Labs (Mumbai, India), 1,10-O-phenanthroline, Ferric chloride (FeCl_3), Ammonium molybdate, Sodium dithionite were obtained from Ranbaxy Fine Chemicals (New Delhi, India), Phenyl hydrazine, Folin-Ciocalteu Phenol reagent were obtained from BDH Products (UK). Silymarin was kind gift from Dr.Chidambaramurthy K.N., CFTRI, Mysore, India. All other chemicals used were of Analytical grade. The solvents used for extraction were from Ranbaxy Fine Chemicals (New Delhi, India). The UV-Visible spectrophotometric values were recorded in JASCO UV-500 Spectrophotometer.

2.2. Plant material and extraction

The leaves of *Actinodaphne hookeri* were collected from Jog Falls (near Sirsi, Karnataka) and the same was authenticated by Dr.B.D.Huddar (Head, Department of Botany, S.K.A and H.S.K Science Institute, Hubli, Karnataka) and preserved in Department of Pharmacognosy, KLES College of Pharmacy, Hubli, India.

Leaves were dried under shade, powdered and extracted with 70% aqueous ethanol by cold maceration. The extraction was done for 72 hours. After extraction, the extract was separated from marc by filtration through filter paper. The marc was pressed in muslin cloth to remove the solvent which is left in the marc after filtration. Filtrate was preserved in a well closed container. Marc left after extraction was extracted by cold maceration for 3 more days with same amount of fresh solvent and the process was repeated for one more time. i.e. the drug was extracted 3 times with a gap of 3 days each. On the 10th day, the filtrates were pooled and concentrated to a syrupy liquid under reduced pressure using Superfit Rotary vacuum evaporator, dried and stored in a dessicator. This extract AH is used for the experiment mentioned bellow.

2.3. Preliminary phytochemical investigations

The preliminary phytochemical screening of the extract was carried out to know the different constituents present in it as per the standard procedures. The extracts were tested for alkaloids [16], triterpenes, saponins [17], sterols [18], cardiac glycosides [18], flavonoids [20], tannins [21], carbohydrates [22], amino acids [23].

2.4. Antioxidant assay using β -carotene linoleate model system (B CLAMS)

The antioxidant activity of the 70% hydro alcoholic extract was evaluated by the method of Hidalgo *et al.* with slight modifications [24]. In brief, 5 mg β -carotene, 40 mg m linoleic acid and 400 mg of tween- 40 were mixed in 1 ml chloroform. Chloroform was removed under vacuum using the flash rotary evaporator at 400°C. The resulting mixture was added with 20 mL water and an emulsion was prepared. The emulsion was further diluted with 80 mL of oxygenated water (oxygen was passed into the 1L of double distilled water for 5 min at the flow rate of 10 mL/min.). 100, 200, 300, 400 and 600 μ g of BHT and 100, 200, 400, 600 and 800 μ g of AH in alcohol were added in separate test tubes and volume was made up to 0.4 mL with ethanol. 0.6 mL of water and 3 mL of emulsion was added to each test tube. Absorbances of all samples were taken at 470 nm at zero time and test tubes were placed at 500°C in water bath. Measurement of Absorbance was continued at an interval of 30 minutes, till the color of β -carotene disappeared in the control reaction ($t = 180$ min). A mixture prepared as above without β -carotene emulsion served as blank and mixture without extract served as control. BHT, instead of extract was used as positive control in the concentration of 25 g/mL to 150 μ g/mL. Dose response of antioxidant activity of AH was determined at different concentrations. The antioxidant

activity (%AA) was evaluated as the bleaching of b-Carotene with the equation: % AA = $100 \{1 - (A^o - A^t)/A^o - A^t0\}$, where % AA = antioxidant activity, A_o = absorbance of sample at zero time, A^o0 = zero time absorbance of control, A^t0 = absorbance of sample after incubation for 180 min, and A^t = Absorbance of control after incubation for 180 min. All experiments were performed in triplicate. The results are expressed as mean \pm SD.

2.5. Lipid peroxidation assay

Egg phosphatidylcholine (20 mg) in chloroform (2 ml) was dried under vacuum in a rotary evaporator to give a thin homogenous film and further dispersed in normal saline (5 ml) with a vortex mixer. The mixture was sonicated to get a homogeneous suspension of liposomes [27]. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid to a mixture containing liposome (0.1ml), 150 mM potassium chloride, 0.2 mM ferric chloride, AH (20 μ g/ml to 80 μ g/ml) or standard silymarin (1 to 10 μ g) were added separately in a total volume of 1 ml. The reaction mixture was incubated for 40 minutes at 37°C. After incubation, the reaction was terminated by adding 1 ml of ice cold 0.25 M Sodium hydroxide containing 20% w/v TCA, 0.4% w/v of TBA and 0.05% w/v BHT. After keeping in boiling water bath for 20 min, the samples were cooled. The pink chromogen was extracted with a constant amount of n-butanol, and the absorbance of the upper organic layer was measured at 532 nm. Percentage anti lipid peroxidation activity was calculated by the formula, % anti lipid peroxidation activity = $[(C - S)/C] \times 100$, where C is the absorbance of the control and S is the absorbance of the sample. Each experiment was carried out in triplicate and results were expressed as mean % anti lipid peroxidation activity \pm SD.

2.6. Total antioxidant capacity

Total antioxidant capacity was measured according to slightly modified method of Mathew [28]. 100 μ g of extracts and 100 μ g of BHT and Silymarin (as standards) were taken in 0.1 ml of alcohol, combined separately in an eppendroff tube with 1.9 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1.9 ml of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples. For samples of unknown composition, water-soluble antioxidant capacities are expressed as equivalents of ascorbic acid. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid. The experiment was conducted in triplicate and values are expressed as ascorbic acid equivalents in μ g per mg of extract (mean \pm SD).

3. Results

3.1. Preliminary phytochemical investigation

The phytochemical screening revealed presence of sterols, alkaloids, tannins, flavonoids and glycosides in AH leaves extract given Table 1.

Table 1. Phytochemical analysis of *actinodaphne hookeri* meissn leaves.

Sl.	Constituents	Present or absent
1	Alkaloids	++
2	Glycosides	++
3	Triterpenoids	++
4	Tannins	++
5	Flavonoids	++
6	Carbohydrates	++
7	Saponins	++
8	Amino acids	--
9	Sterols	--

3.2. Antioxidant assay using β -carotene linoleate model system

The antioxidant activity of AH as measured by the bleaching of M-carotene is presented in Table 2. IC₅₀ value of AH and BHT were found to be 194.16±4.04µg/ml and 84.73±2.5 µg/ml, respectively.

Table 2. Antioxidant property of AH and BHT in β - CLAMS method.

Concentration in µg/ml	% antioxidant activity of AH	% antioxidant activity of BHT
25	4.94±1.97 ^a	20.70±3.34 ^a
50	11.22±1.14	36.18±2.39
75	16.34±2.34	53.78±0.39
100	20.76±1.98	63.51±1.43
150	36.60±1.98	72.42±0.33
200	54.14±3.03	85.91±0.56
IC₅₀	194.16±4.04 ^a µg/ml	84.73±2.5 ^a µg/ml

^a Values are mean± standard deviation.

3.3. Lipid peroxidation assay

AH prevented lipid peroxidation strongly but less than the standard (Silymarin). IC₅₀ values of antilipid peroxidation of AH and Silymarin were 51.71±1.53 and 5.78±0.3 µg/ml respectively in Table 3. In biological systems, malondialdehyde (MDA) is very reactive species and takes part in the cross linking of DNA, with protein and also damaging the liver cells.

Table 3. Antilipid peroxidation activity of AH and silymarin.

Concentration in µg/ml	% Anti Peroxidation of AH	Lipid of Conc. µg/ml	in % Antilipid Peroxidation of Silymarin
20	2.85±0.31 ^a	1.0	3.12±3.65 ^a
40	33.46±0.99	2.0	28.66±1.00
60	67.21±2.08	5.0	42.34±0.48
80	86.77±1.25	7.5	58.44±1.85
---	---	10.0	88.84±1.48
IC ₅₀	51.71±1.53 ^a	IC ₅₀	5.78±0.3 ^a

^a Values are mean± standard deviation.

3.4. Total antioxidant capacity

As shown in Table 4, total antioxidant capacity of AH, silymarin and BHT were 290±18.62, 197.22±4.81 and 398.00±22.05 µg equivalent to ascorbic acid/mg of AH, silymarin and BHT, respectively.

Table 4. Total antioxidant capacity of AH, silymarin and BHT.

Extract/ Standard	Total antioxidant capacity ^b
AH	290±18.62 ^a
Silymarin	197.22±4.81
BHT	398.00±22.05

^a Values are mean± standard deviation .

^b Values are in µg equivalent to ascorbic acid per mg of extract.

4. Discussion

The mechanism of bleaching β-carotene is a free radical mediated phenomenon resulting from hydroperoxides formed from linoleic acid. B-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical, upon the abstraction of hydrogen from one of its diallylic methylene group,

attacks the highly unsaturated β -carotene molecules. As β -carotene molecule loses their double bonds by oxidation, the compound loses its chromophore and characteristic orange color, which can be monitored spectrophotometrically at 470 nm. So in presence of antioxidants, β -carotene retains its color. Because antioxidants prevent abstraction of hydrogen from linoleic acid from its diallylic methylene group by donating hydrogen from itself. Thus prevents the oxidation of β -carotene [29]. Here the IC_{50} values of BHT which is used as standard and AH are having near values. This shows that AH can donate hydrogen to linoleic acid free radicals as much as BHT can. AH can be used as natural antioxidant instead of BHT which is synthetic and use of BHT is said to be unsafe and their toxicity is a problem of concern [30].

The lipid peroxidation has been broadly defined as the oxidation deterioration of polyunsaturated lipids. The initiation of peroxidation sequence in membrane or polyunsaturated fatty acids is due to abstraction of a hydrogen atom from the double bond in the fatty acids. The free radical tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which then easily reacts with an oxygen molecule to give a peroxy radical. Peroxy radical can abstract a hydrogen atom from another molecule or they can abstract hydrogen atom to give lipid hydroperoxide, R-OOH. A probable alternative fate of peroxy radicals is to form cyclic peroxidase; these cyclic peroxidase, lipid peroxidase and cyclic endoperoxidase fragment to aldehyde including MDA and polymerization product. MDA is the major product of lipid peroxidation and is used to study the lipid peroxidation process [33]. Determination of lipid peroxidase content was carried out indirectly by means of derivatizing MDA with TBA at high temperature and acidic condition, AH has strongly inhibited the lipid peroxidation as shown in Table 5. IC_{50} value of AH is 68.13 ± 1.38 $\mu\text{g/ml}$ and that of silymarin is 6.0 ± 0.2 $\mu\text{g/ml}$. This indicates that Silymarin is more than ten times potent than AH. Silymarin is a mixture of flavonolignan and is proven potent hepatoprotective and antioxidant agent [31]. So when compared to such potent agent, the values obtained indicate that AH is a good antilipid peroxidation agent.

As phenolics are responsible for antioxidants, generally, it is expected that extract/drug which contains high TPC would show highest total antioxidant capacity. But in this case though TPC of silymarin is more than the AH and total antioxidant capacity is less than the AH. This may be due to nature of constituents of AH and silymarin. Silymarin was used as a standard because it is a mixture of flavonolignans and is a potent well known hepatoprotective and antioxidant agent. It contains only a mixture of flavonolignans [32]. AH contains constituents like carbohydrates, sterols and glycosides apart from different flavonoids, and TPC is only due to different flavonoids. This may be the reason for high TPC of silymarin and less TPC of AH. But the total antioxidant activity is due to all the components present in the extract. As stated earlier *A.hookeri* contains quercetin-3-rhamnoside, amorphous alkaloid, β -sitosterol, hentriacontanone and hentriacontanol [13]. In few experiments, silybin one of the constituents of silymarin is found to be less active than the luteolin. Because of these reasons, though the TPC of AH is less than the silymarin, AH showed more antioxidant capacity than Silymarin.

5. Conclusion

The data presented here indicate that the marked antioxidant activity of AH extracts seems to be due to presence of flavonoids like flavones, flavanes, flavonols, which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction. The qualitative chemical tests also revealed the presence the flavonoids. Further total phenolic content of extract confirmed that AH contains good amount of phenolics. There is good scope in examining the leaves for its antioxidant and free radical scavenging activity in *in vivo* models and for hepatoprotective activity, and thus establish the evidence for using this plant in treatment of diabetes and urinary disorder in folk medicine. Free radicals and reactive oxygen species are involved in a variety of pathological events such as aging, inflammation, cancer, atherosclerosis, diabetes. The plant would be useful for the treatment of various diseases mediated by free radicals. The AH found to suppress lipid peroxidation, hydroxyl radical formation, through different chemical mechanisms, including free radical quenching, electron transfer, radical addition or radical recombination. Overall, the plant would be useful as an antioxidant and free radical scavenging agent and thus help in treatment of many disease mediated by reactive oxygen species.

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