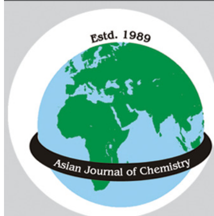

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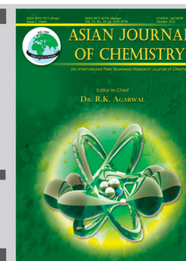

Acetylcholine Esterase Inhibitory Potential and Antioxidant Activity of Various Extracts of *Leucas cephalotes* and *Juglans regia* L.

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The aerial parts of *Leucas cephalotes* were extracted in *n*-hexane, chloroform and ethyl acetate successively, while seeds of *Juglans regia* L. were extracted in *n*-hexane and ethyl acetate. Column chromatography of the *n*-hexane fraction of *J. regia* yielded eight fractions. Total phenolic contents were calculated using Folin-Ciocalteu (FC) reagents while, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, ferric reducing antioxidant power (FRAP) and acetylcholine esterase (AChE) inhibition potential of all extracts/fraction was evaluated according to their respective *in-vitro* models. Ethyl acetate extract of *L. cephalotes* contained highest total phenols (864.5 ± 1.2 mg GAE/g of extract) with 83.3 ± 1.1 % DPPH activity and IC_{50} value 271 ± 6 µg/mL. The column fractions of *J. regia* (JRH-8) demonstrated significant antiradical activity (80.8 ± 1.1 %, IC_{50} = 283 ± 4 µg/mL). All extracts/fractions showed remarkable FRAP activity. The *n*-hexane extracts of both plants exhibited maximum enzyme inhibition activity 78.7 ± 1.1 % (IC_{50} = 175 ± 4 µg) and 73.5 ± 1.5 % (IC_{50} = 108 ± 3 µg) for *J. regia* and *L. cephalotes* respectively.

Key Words: Acetylcholine esterase, Antioxidant activities, *Leucas cephalotes*, *Juglans regia* L.

INTRODUCTION

Leucas cephalotes Roth (Family; Labiatae), a rainy season weed, mainly found in North India, commonly known as 'Kubo or Kubi'[1]. The genus *Leucas* includes about 100 Asiatic and African species. The plant is useful in inflammation, bronchitis, dyspepsia, asthma, paralysis, jaundice, urinary discharge and leucoma[2]. According to Ayurveda, *L. cephalotes* is mild stimulant, diaphoretic and used in fever and also used in liver disorder. Flowers mixed in honey are used as domestic remedy of cough, colds and has antidiabetic activity[3]. Phytochemical studies showed that this plant contains triterpenes, oleanolic acid, sterols and flavones[4]. Several compounds have been identified from the methanolic extract of *L. cephalotes* such as 7-oxostigmasterol, oleanolic acid, 7-oxysitisterol, 7α-hydroxysitosterol, 7α-hydroxystigmasterol, stigmasterol, 5-hydroxy-7, 41-dimeathoxyflavone, gonzalitosin, tricin and apigenin 7-O-β-D-(6-O-p-coumaroyl) glucopyranoside[5]. Other constituents such as lauric acid, tridecanoic acid, adipic acid, glutaric acid and labellenic acid were also reported from seed oil[6].

Juglans regia L. (Family; Juglandaceae) commonly known as walnuts can be found all around the world, either as a wild or cultivated tree. It has been used as a medicinal plant to treat many different ailments including 'tisis' and 'scrofula', which are synonyms of tuberculosis and tuberculosis of the cervical glands[7]. The health benefits of walnut are usually attributed to its chemical composition[8]. Its seeds contain 25-70 % oil, polysaturated oil in excess amount[9]. Walnut is a good source of essential fatty acids and tocopherols and contain sufficient amount of proteins (24 % by weight), fibre (1.5-2 %), carbohydrates (12-16 %) and minerals (1.7-2 %)[10]. Phenolic compounds in walnut seeds give astringent flavour. Linoleic acid is the major fatty acid, followed by oleic, linolenic, palmitic and stearic acid[11].

For the treatment of neurological disorders such as Alzhemier's disease and Parkinson's disease, inhibition of acetylcholine esterase is an effective strategy. Currently several natural and synthetic medicines *e.g.* tacrine, donepzil, galathamine and rivastigmine are available for the treatment of Alzhemier's disease. Problems associated with these compounds are reduced bioavailability and gastrointestinal

TABLE-1
TOTAL PHENOLS ANTIOXIDANT POTENTIAL AND ACETYLCHOLINE ESTERASE INHIBITION ACTIVITY OF THE EXTRACTS AND COLUMN FRACTIONS OF *L. ephalotes* AND *J. regia*

Sample	Total phenolics [a]	Antioxidant activities			AChE	
		DPPH [b] (%)	IC_{50} (µg)	FRAP [c]	Inhibition[d] (%)	IC_{50} (µg)
JRH	33.8 ± 0.8	47.6 ± 0.3	-	0.510 ± 0.005	78.7 ± 1.1	175 ± 4
JRC	136.3 ± 1.2	79.6 ± 0.5	319 ± 5	0.840 ± 0.009	70.0 ± 0.9	182 ± 3
JRH-1	250.2 ± 0.9	1.5 ± 0.5	-	0.579 ± 0.011	51.3 ± 0.8	247 ± 6
JRH-2	54.2 ± 1.5	2.6 ± 0.7	-	0.943 ± 0.016	42.7 ± 1.0	-
JRH-3	44.2 ± 0.5	6.1 ± 0.3	-	0.976 ± 0.013	48.5 ± 0.8	-
JRH-4	68.6 ± 0.7	9.5 ± 0.7	-	0.535 ± 0.008	44.0 ± 0.5	-
JRH-5	85.1 ± 1.1	28.0 ± 0.4	-	0.787 ± 0.010	41.5 ± 0.9	-
JRH-6	106.6 ± 1.2	17.8 ± 1.1	-	0.355 ± 0.014	42.1 ± 0.7	-
JRH-7	61.4 ± 1.2	5.9 ± 1.1	-	0.318 ± 0.004	31.6 ± 0.3	-
JRH-8	369.2 ± 1.2	80.8 ± 1.1	283 ± 4	0.672 ± 0.012	57.6 ± 0.9	230 ± 4
LCH	115.8 ± 1.2	42.5 ± 1.1	-	0.351 ± 0.004	73.5 ± 1.5	108 ± 3
LCC	475.2 ± 1.2	1.7 ± 1.1	-	0.331 ± 0.009	71.1 ± 1.1	93 ± 2
LCE	864.5 ± 1.2	83.3 ± 1.1	271 ± 6	1.304 ± 0.013	60.3 ± 1.4	214 ± 5
Gallic acid	-	93.2 ± 0.9	5 ± 1	-	-	-

[a] mg GAE/g of extract, [b] 0.5mg/mL, [c] absorbance at 595 nm, [d] 250 µg, S.D (±) was calculated using MS Excel 2007

disturbance. In traditional medicines several plants have been used to treat neuropharmacological disorders. Therefore medicinal plants could provide better lead compounds as acetylcholine esterase inhibitors.

Antioxidants can play an important role in disease prevention and health maintenance. There is an increasing interest in the role of free radical-mediated damage in the etiology of human diseases. Free radicals formed during oxidation process occurring in various products and biological systems are known to be responsible for oxidative deterioration, health damage and accelerated aging[12]. Consequently, antioxidants have become an essential part of preservation technology and contemporary health care. The potential toxicity of some synthetic antioxidants, however, has intensified research efforts to discover and utilize antioxidants from natural sources[13]. A number of synthetic antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ) have been added to food stuffs but, are reported to cause liver disorders[14]. Previously some work has been carried out on the total phenols and reducing properties of *J. regia* bark extracts[15].

In traditional medicines several plants have been used to treat neuropharmacological disorders. Therefore, medicinal plants could provide better lead compounds as acetylcholine esterase inhibitors. The present study has been directed towards the identification of enzyme inhibition DPPH radical scavenging and reducing abilities of *L. cephalotes* and *J. regia* extracts/fractions using *in vitro* models.

EXPERIMENTAL

Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid and quercetin were purchased from Sigma-Aldrich (USA). All other solvents, chemicals and reagents of analytical grade were from Merck (Germany).

Collection of plant material: *L. cephalotes* was collected from Narowal region, Pakistan, while seed of *J. regia* were purchased from local market and identified by Dr. Zaheer-ud-Din Khan (Taxonomist) at Department of Botany, Government College University, Lahore.

Extraction: The aerial parts of *L. cephalotes* (LC) were shade dried, powdered and extracted with methanol. The crude extract was concentrated on rotary evaporator, dissolved in distilled water and fractionated with *n*-hexane, chloroform and ethyl acetate resulting LCH, LCC and LCE respectively. The seeds of *J. regia* (JR) were extracted in n-hexane and ethanol successively. The crude ethanolic extract was partitioned with *n*-hexane and chloroform yielded JRH and JRC respectively. The *n*-hexane extract was further loaded on column and obtained eight different fractions (JRH-1, JRH-2, JRH-3, JRH-4, JRH-5, JRH-6, JRH-7 and JRH-8).

Determination of total phenolics: The total phenolics in extracts and fractions were determined using Folin-Ciocalteu reagent[14]. 40 µL of each sample (5 mg/mL) was mixed with 0.25 mL of Folin-Ciocalteu reagent and 0.8 mL of 10 % sodium carbonate solution. The mixture was allowed to stand for 0.5 h and the absorption was measured at 765 nm against a blank which contained 40 µL of methanol in place of sample. The total phenolics content was expressed as gallic acid equivalents in mg/g of extract (Table-1).

DPPH radical scavenging assay: The radical scavenging ability of different extract/fraction was measured using the method of Shahwar *et al.*[14]. Methanol solution (1.0 mL) of all the extract/fraction at various concentrations (0.01-1.5 mg/mL) was added to 1.0 mL (0.2 mg/mL) methanol solution of DPPH and kept in dark. The decrease in absorbance at 517 nm was noted after 0.5 h.

The % age scavenging of radical was determined by the following formula.

$$\text{\% age inhibition of DPPH} = \frac{A - B}{A} \times 100$$

where, A is the optical density of blank and B is the optical density of sample.

Ferric reducing antioxidant power: Ferric reducing antioxidant power assay was carried out according to the method of Shahwar *et al.*[16]. 150 µL of ferric reducing antioxidant power reagent was mixed with 1.25 mg/mL of sample and read the absorbance at 595 nm after 15 min.

Acetylcholine esterase assay: Acetylcholine esterase inhibitory activity was measured by the spectrophotometric method[16]. Acetylthiocholine iodide was used as substrate in the assay. The reaction mixture contained 1500 µL of (100 mM) tris buffer (pH 7.8), 1000 µL of DTNB, 200 µL (50, 100, 150, 200, 250 µg/mL) of test compound solution and 200 µL of acetyl cholinesterase solution (erythrocytes), which were mixed and incubated for 15 min (25 ºC). The reaction was initiated by the addition of 200 µL acetyl thiocholine iodide. The hydrolysis of acetyl thiocholine iodide was monitored at 412 nm after 30 min. Galanthamine was used as positive control. All the reactions were performed in triplicate. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = (E-S)/E \times 100$$

where; E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound.

RESULTS AND DISCUSSION

Enzymes are the primary targets for the development of new drugs because of the simplicity of enzyme based assays. The inhibitor interacts with the enzyme or enzyme-substrate complex with a decrease in the rate of reaction. Plant extracts and their secondary metabolites have been used as inhibitors of various classes of enzymes. Several thousand plant extracts have been screened against acetylcholine esterase from different parts of the world[17-19]. Shahwar *et al.*[14] and Lee *et al.*[20] have demonstrated the acetylcholine esterase inhibition activities from various plants. All extracts/fractions of *J. regia* and *L. cephalotes* were evaluated against acetylcholine esterase and results indicated that *n*-hexane extracts of both plants exhibited remarkable activity. Maximum activity was shown by *n*-hexane extract of *J. regia* (78.7 ± 1.1 %, IC_{50} = 175 ± 4 µg) followed by *n*-hexane extract of *L. cephalotes* (73.5 ± 1.5 %). The column fractions of *J. regia* also exhibited significant results (31.6 ± 0.3 to 57.6 ± 0.9) as shown in Table-1 (Fig. 1).

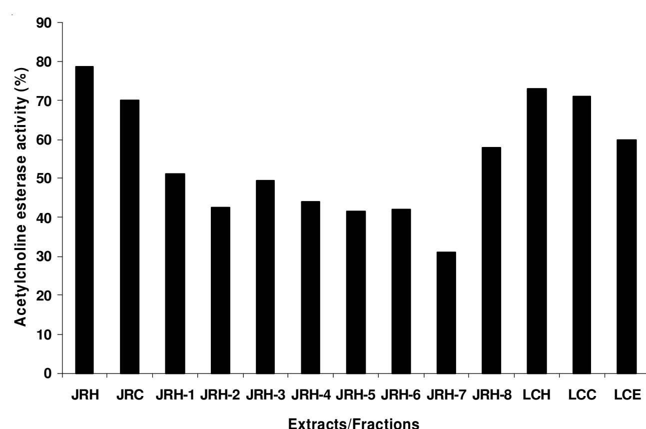

Fig. 1. Acetylcholine esterase inhibition potential of extracts/fractions of *J. regia* and *L. cephalotes*

Total phenols of the extracts/fractions were calculated as gallic acid equivalent using Folin-Ciocalteu reagent. Among the two extracts of *L. cephalotes*, ethyl acetate extract (LCE) showed highest amount of total phenols (864.5 ± 1.2 mg GAE/g crude extract). DPPH assay has been widely used to determine the free radical scavenging activity of various plants extracts and its purified compounds[21-23]. Ethyl acetate extract of *L. cephalotes* exhibited maximum antiradical activity (83.3 ± 1.1 % with IC_{50} value 271 ± 6 µg/mL) which also contained highest amount of total phenols (Table-1). The column fraction of *n*-hexane extract and ethyl acetate extract of *J. regia* demonstrated 80.8 ± 1.1 % and 79.6 ± 0.5 % DPPH inhibition activity respectively (Fig. 2). Many authors suggested that DPPH scavenging activity of oily extracts of walnuts and other plants is due to the presence of tocopherols which are the principal components in them[24]. Reducing power of all extracts/fractions were also evaluated using ferric reducing antioxidant power assay and it was found that all extracts/fraction showed remarkable reducing activity (Table-1).

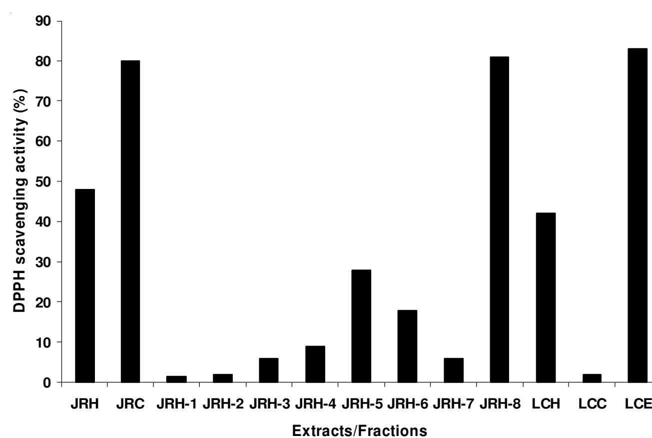

Fig. 2. DPPH scavenging activity of extracts/fractions of *J. regia* and *L. cephalotes*

Recent work on walnut seed have found to contain various classes of polyphenols identified mainly as tannins[25], most of which were demonstrated to contribute the overall antioxidant activity of the walnut extract with different *in vitro* and *in vivo* antioxidant estimation models[26]. In addition, some monomeric phenolics such as gallic acid and ellagic acid have also been reported in the walnut extracts and shown to inhibit plasma and LDL oxidation *in vitro*[27]. This implies that, in addition to tannins, some low molecular weight phenolics are present in walnuts. However, no significant work has been carried out on their isolation structure elucidation, enzyme inhibition and contribution to the total antioxidant activity of walnuts.

Conclusion

It was concluded from the results that ethyl acetate extracts of both plants showed acetylcholine inhibition potential, DPPH scavenging and reducing properties while most of the column fractions of walnut showed poor activity in DPPH assay and moderate behaviour in others models. In addition, this investigation of enzyme inhibition potency and antioxidant activity of the extracts/fractions provide a chemical basis for some of the health benefits claimed of these plants in foods and folk medicine. Several nuts such as walnuts and peanuts are among these dietary plants known to have significant antioxidant contents[28-30]. These results also indicated that both plants might be used as natural antioxidants and alternatives to synthetic antioxidants such as butylated hydroxytoluene.

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