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Appraisal of organic extracts and isolated compounds of *Azadirachta indica* for *in-vitro* antioxidant, acetylcholinesterase and butyrylcholinesterase inhibitory activity

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Abstract: The current study emphasized on assessment of Antioxidant, Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) inhibitory activity of the crude methanolic and chloroform leaves extracts along with their isolated compounds derived from *Azadirachta indica*. Phytochemical analysis revealed the manifestation of ancillary metabolites like alkaloid, anthraqinones, catechins, flavonoids, phenolic compounds, saponins, tannins and steroids. Methanolic crude extract of *Azadirachta indica* leaves revealed comparable antioxidant activity as that of quercetin and propyl gallate. As far as enzyme inhibitory activity was concerned, a significant AChE enzymes inhibition was observed. These findings confirm the traditional use of *Azadirachticha indica* as medicinal plant in the treatment of mental ailments and anti-inflammatory illnesses. Chloroform crude extract and isolated compounds showed weak antioxidant and enzyme inhibitory activities.

Keywords: Antioxidant, acetylcholinesterase (AChE), butyrylcholinesterase (BChE) inhibition.

INTRODUCTION

In developing countries, the usage of medicinal plants offers a major source of antioxidant and enzymes inhibitory agents with potential medicinal applications. It is estimated that more than 25% of the modern medicines used for various diseases are derived either directly or indirectly from plants (Fridlender *et al*., 2015). Pakistan is blessed with medicinal plants which are considered as the source of various pharmacologically active compounds used for multiple ailments. Medicinal plants are being used since the beginning of civilization to cure ailments. Pharmacological investigation have accredited the value of medicinal plants as a source of bioactive compounds (Matu *et al*., 2012). Many lead compounds have been discovered from plants which cure the suffering humanity from many ailments due to presence of phytochemical compounds & secondary metabolites (Saxena *et al*., 2013). A deficiency of neurotransmitter acetylcholine (ACh) and activation of acetylcholinesterase (AChE) has been detected in the patient’s brain suffering from Alzheimer’s disease (Şenol *et al*., 2010). AChE is found in all nerve or muscle tissue, in placental tissue and in most erythrocytes and while BChE is present in the central and plasma, peripheral nervous system and liver (Santarpia *et al*., 2013). Reactive oxygen species are a class of reactive molecule and their imbalance with antioxidants results in the occurrence of free radicals. The rapid production/accumulation of free radicals may lead to degenerative disorders, cancer, neural disorders etc (Gueraud *et al*., 2010). As the available synthetic antioxidants have many side effects, there is a need for more potent but less toxic antioxidants. Most of the medicinal plants possess large amount of antioxidants such as polyphenols, ascorbic acid, Vitamin E etc (Kasote *et al*., 2015).

The current study was carried out to explore a presence of possible AChE or BuChE inhibitors and antioxidant potential in leaves methanolic and chloroform extract of *Azadirachta indica* which is possibly the most commonly used medicinal plant of Pakistan. All parts of it are enriched with medicinal content. It is well-known and multipurpose medicinal plants having a wide range of biological activity (Feitelson *et al*., 2015, Council, 2002). It is a perennial tree, cultivated in various parts world.

MATERIALS AND METHODS

Collection of plant material and identification

Azadirachta indica leaves were collected from Moghalpura nursery, district Lahore, west part of the Punjab, Pakistan and authenticated by Prof. Dr. Asif Saeed, Faculty of Pharmacy, PU, Pakistan. The herbarium is kept in the department bearing voucher specimen number GC-Herb-Bot-3238 for the future reference.

Preparation of extracts

Plants material was washed in tap water, afterwards rinsed with distilled water and dried in open air fortnightly and grinded to powder form with mechanical mill from Paper Mandi, Lahore, Pakistan. The powder was extracted by maceration in methanol and chloroform by means of the cold maceration method. Both extracts were concentrated

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using rotary evaporator (Heidolph, Germany) and stored at 4°C.

Extraction and isolation

5.0grams of crude methanolic extract was subjected to column chromatography and eluted with hexane, chloroform and methanol in order of polarity to give 40 fractions each of 100ml and were combined after checking TLC into 3 main fractions: A, B & C. Fraction C did not exhibit any biological activity in comparison of fractions A and B and was not further investigated.

Fraction B was sub-fractionated by silica gel chromatography and was eluted with hexane, acetone and methyl alcohol in order polarity to yield 20 fractions each of 50 ml which were pooled in sub-fractions after checking with TLC. Sub-fractions F12-18 eluted with n-hexane: acetone (7:3) was declared as compound B (8 mg) it was further purified by preparative TLC and Sephadex LH20.

Chlorophyll was removed by Sephadex LH20 from Fraction A by Sephadex LH20 and eluate was further concentrated and fractionated by similar method as described above for fraction B to produce compound A (4 mg).

Phytochemical potential

The phytochemical studies of the plant were carried out for identification of phytochemical constituents/ components belonging to various classes such as; alkaloids, catechins, anthraqinones, phenolic compounds, alkaloids, flavonoids, saponins, tannins and steroids (El-Olemy *et al*., 1994).

Antioxidant potential

Antioxidant potential was estimated by 2, 2 - diphenyl - 1 - picryl hydrazyl (DPPH) technique for both crude extracts and isolated compounds (Huang *et al*., 2011). Crude extract and isolated compound were diluted by the 3ml of 0.1mM methyl alcohol solution of DPPH. Quercetin and Propyl gallate were used as standard whose strengths were 1mg/ml. All of the testing tubes were well-maintained for a duration of 30 min. Methyl alcohol was used for not only making dilutions but also for the adjustment of baseline value. Degree of unsaturation was measured using UV spectrophotometer at wavelength of 588nm. Following formula is used for the measurement of free radical scavenging activity (RSA).

$$\% \text{ RSA} = \frac{A_c - A_s}{A_c} \times 100$$

Where

A_s = Sample absorbance ; A_c = Control absorbance

Acetylcholinesterase (AChE) and butyrylcholinesterase (bche) inhibitory assay

Inhibitiry assay of AChE and BChE was carried out by the procedure described by Ellman (Ellman *et al*., 1961). 0.1mM sodium phosphate buffer (100µL) at 8.00 pH, 20µL of enzyme (AChE 0.005 units or BChE 0.5 units) and 20µL test solution thoroughly mixed in methyl alcohol and incubated for a period of about ½ hr. 10µL of DTNB (5, 5' - dithio - bis - [2-nitro benzoic acid]) was also added and reaction was initiated by the addition of 10µL of 0.5mM acetylthiocholine iodide for AChE or butyrylthiocholine chloride for BChE. Reaction mixture was further incubated prior to next process for a period of 10 minutes at 37ºC and absorbance was monitored at 405 nm. 96 well plate reader (Synergy HT, Biotek, USA) was used for the purpose. Reading were taken in triplicate manner. 0.5mM Eserine was used as standard. % inhibition was estimated by following formula.

$$\text{Inhibition (\%)} = \frac{Control - Test}{Control} \times 100$$

IC_{50} values were calculated by EZ - Fit software for Kinetics of Enzyme (Perrella Scientific Inc. Amherst, USA).

STATISTICAL ANALYSIS

Graph pad Prism version 5.00 for windows was used to analyse the data statistically. Results were shown as mean ± standard deviation and compared with ANOVA (one way analysis of variance) among groups. For post hoc analysis, Tukey's test was used. P value ≤ 0.001 was considered significant.

RESULTS

Phytochemical Investigation

The qualitative testing of the crude powder of *Azadirachta indica* leaves was carried out in Chemistry laboratory, FOP, UCP, Lahore for the determination of different phytochemicals and results of this testing are mentioned in table 1.

Antioxidant assay of crude extracts & isolated compounds

Crude extracts of leaves were evaluated with DPPH reagent for free radical scavenging activity. Findings of the test are outlined in the table 2. It showed that crude methanolic extract of *Azadirchta indica* leaves exhibited more antioxidant potential at low dose in comparison to chloroform extract. Substantial variations were observed for reference drugs (quercetin & propyl gallate).

Based on antioxidant assay of above crude extracts, only methanolic extract was fractionated and compounds were isolated. All of the isolated compounds showed significantly weak antioxidant activity as compared to standard drugs (quercetin & propyl gallate) as given in table 3.

AChE and BChE inhibitory activity of crude extracts & isolated compounds

Methanolic and chloroform extracts were subjected to inhibitory assay and it was observed that only methanolic

Table 1: Phytochemical analysis of *Azadirachta indica*

Phytochemical constituent	Phytochemical tests	Results
Alkaloids	Mayer's reagent	+++
	Wagner's reagent	+++
	Hager's reagent	++
	Dragendorff's reagent	++
Anthraquinone Glycoside	Borntragers reagent	+
Cardiac Glycosides	Keller Kiliani reagent	+
Flavonoids	Ferric chloride	+
	Alkaline reagent	+
	Lead acetate	++
Saponins	Foam test	+
	Bromine water test	++
Tannins	Ferric chloride test	–
	Match stick Test	+

– = not found, + = weakly found, ++ = moderately found, +++ = strongly found

Table 2: Antioxidant activity of methanol and chloroform crude extracts

Extracts/ Standard	DPPH activity	
	% RSA (Mean ± S.E.M)	IC_{50} µg/ml (Mean ± S.E.M)
Methanolic Extract	63.56±0.21 ^{ab}	79.22±0.50 ^{ab}
Chloroform Extract	53.33±0.27 ^{ab}	102.3±1.64 ^{ab}
Quercetin	85.65±0.15mM	14.18±1.12
Propyl gallate	80.71±0.14mM	46.31±1.18

Data are shown as mean± SEM. where 'a' shows significant variation from standard quercetin, 'b' shows significant variation from standard propyl gallate at p<0.0001, IC_{50}= concentration of the extract exhibiting 50% of DPPH radical scavenging ability.

Table 3: Antioxidant activity of isolated compounds

S No.	Compounds	Antioxidant activity DPPH radical scavenging ability	
		% RSA (Mean ± S.E.M)	IC_{50} µg/ml (Mean ± S.E.M)
1	Compound A	21.17 ± 0.39^{ab}	-
2	Compound B	18.91 ± 0.51^{ab}	-
3	Quercetin	78.55 ± 0.15mM	12.10±0.12
4	Propyl gallate	80.21± 0.47mM	45.11±0.11

Data are shown as mean±SEM. Where 'a' shows significant variation from standard quercetin, 'b' shows significant variation from standard propyl gallate at p<0.0001, IC_{50}= concentration of the extract exhibiting 50% of DPPH radical scavenging ability.

Table 4: AChE & BChE inhibition of crude extracts

S No.	Compound/ Sample Code	AChE		BChE	
		Inhibition (%) at 0.5 mM (Mean ± S.E.M)	IC_{50} µg/ml (Mean ± S.E.M)	Inhibition (%) at 0.5 mM (Mean ± S.E.M)	IC_{50} µg/ml (Mean ± S.E.M)
1	Methanolic Extract	58.44±0.76^{a}	293.71±0.24	38.74±0.45^{a}	-
2	Chloroform Extract	49.34±0.52^{a}	-	28.34±0.15^{a}	-
3	Eserine Standard	91.27±1.17mM	0.04±0.01	82.82±1.09 mM	0.85±0.01

Data represents mean ±SEM. Where 'a' indicates significant difference from standard eserine at p<0.0001, IC_{50}= concentration of the extract causing 50% inhibition of enzyme activity.

extract was proficient of impeding the AChE enzyme activity. A substantial decline in the ability of AChE enzyme was seen at the potency of 10mg/mL^{-1} as shown in table 4. It revealed an IC_{50} of 293.1±3.21.

There was 41.56% enzymatic inhibition for AChE enzyme at a potency of 10mg/mL^{-1}. However, no significant BChE inhibitory activity was found for either methanolic or chloroform extracts. The isolated components were also assessed for their inhibitory potential. The outcomes of % age inhibition is outlined in tables 5.

Table 5: AChE and BChE 9nhibition (%) of isolated compounds

S. No.	Compound	AChE		BChE	
		Inhibition (%) at 0.5 mM (Mean ± SEM)	IC_{50} µg/ml (Mean ± SEM)	Inhibition (%) at 0.5 mM (Mean ± SEM)	IC_{50} µg/ml (Mean ± SEM)
1	Compound A	39.93±0.86 [a]	-	31.36±0.58 [a]	-
2	Compound B	27.40±0.85 [a]	-	28.61±0.41 [a]	-
3	Eserine Standard	90.72±0.87	0.04 ± 0.001	82.02±0.89	0.85±0.001

Data represents mean±SEM. Where 'a' indicates significant difference from standard Eserine at $p<0.0001$, IC_{50}= concentration of the extract causing 50% inhibition of enzyme activity.

DISCUSSION

Antioxidants are vivacious materials which have the ability to protect the body from damage caused by free radical induced oxidative stress. Free radicals are involved in many disorders like neuro-degenerative diseases, cancer & AIDS (Yaidikar and Thakur, 2015). Antioxidants due to their scavenging activity are useful for the management of those diseases. DPPH stable free radical method is a sensitive way to determine the antioxidant activity of plant extracts (Siddique *et al*., 2010). The use of DPPH provides an easy and rapid way to evaluate antioxidants. The therapeutic potential of natural medicinal plants as an antioxidant in reducing such free radical induced tissue injury, suggests that many plants have antioxidant activities that can be therapeutically useful in treating neurological diseases caused by oxidative stress (Kumbhare *et al*., 2012).

All extracts of *Azadirachta indica* exhibited remarkable antioxidant activity in comparison with Quercetin and Propyl Gallate (reference antioxidants) while its isolated compound A & compound B exhibited comparatively weaker free radical scavenging activity suggesting that our medical plant possesses moderate antioxidant potential to be utilized for treating degenerating neurological diseases. Our results are in line with the findings of Veeru who investigated crude extracts of different medicinal plants and found moderate free radical scavenging activities (Veeru *et al*., 2009).

Acetylcholine (ACh), a neurotransmitter, is scattered in the nervous system and has been implicated to play an important role in cortical activity, modulation of cognitive performance, and a signal transfer in the synapses. Loss of cholinergic innervations is correlated with the degree of dementia and the severity of the neuropathological hallmarks of Alzheimer's disease (AD). AChE inhibitors decrease the hydrolysis of ACh to elevate the endogenous level of ACh in the brain and to boost cholinergic neurotransmission. Therefore, it was considered that elevating the level of ACh might be helpful in attenuating the symptoms of neuronal deficits and treating cognitive dysfunction in the mild to moderate cases of AD (Jivad and Rabiei, 2014).

In order to search for an effective AChE inhibitor from natural sources, different extracts and the compounds isolated form these extracts were screened at conc. of 0.5mM through respective enzyme assay where methanolic extract showed substantial AChE inhibitory activity using Eserine as standard while no significant activity was observed by other extract. Both extracts displayed weak BChE inhibitory activity. Our findings are in line with the work of Sano, who utilized medicinal plant for treating patient suffering from Alzheimer's disease (Sano *et al*., 1997).

Phytochemical exploration of current plant shows the existence of secondary metabolites for example anthraquinones, alkaloids, cardiac glycosides, flavonoids, tannins & saponins. These are responsible for antioxidant and enzymatic inhibition potential (AO and OO, 2015). The substantial antioxidant and AchE inhibitory activities exhibited by crude methanolic extract in comparison to its isolated compounds suggest that these comparatively better activities of crude extracts than that of its isolates may be due to the cumulative effect of different secondary metabolites in it.

CONCLUSION

The results obtained from the in-vitro studies reveals that the methnolic extract of *Azadirachta indica* has a moderate antioxidant activity which may led to several therapeutically and useful preparation and compounds, which generates enough encouragements in exploring more information about these medicinal plants. Development of such modern drug from the medicinal plants should be emphasized for the treatment of various diseases and it therefore confirms their traditional use in the treatment of mental disorders and anti-inflammatory diseases.

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