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Antioxidant and cyclooxygenase (COX) inhibitory activities of *Ailanthus altissima* (Mill.) Swingle leaves

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Abstract

The present study aimed to investigate antioxidant and cyclooxygenase (COX) inhibitory activities of *Ailanthus altissima* leaves extracts. N-hexane and methanol-aqueous extracts of *Ailanthus altissima* leaves were tested for their antioxidant activities, using DPPH (1,1-Diphenyl-2-picrylhydrazyl), ferric reducing antioxidant power, cholinesterase, elastase, and tyrosinase assays. The results showed that methanol-aqueous extract has a significant radical scavenging activity, reducing power, and mild cyclooxygenase (COX) inhibitory activity and n-hexane was less active.

Keywords: *Ailanthus altissima*, leaves, antioxidant activity, cyclooxygenase (COX) inhibitory activity.
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Introduction

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide are important mediators for the initiation of lipid peroxidation in biomembranes where the content of unsaturated fatty acids is relatively high, leading to destruction of the cellular membrane, and is related to the development of many chronic disorders such as Alzheimer's disease and other neurodegenerative diseases (Richardson 1993). Epidemiological studies have revealed that intake of antioxidants such as vitamin E and vitamin C reduced the risk of coronary heart disease, stroke and cancer (Ames et al. 1993; Kontos 1989). Antioxidant is an inhibitor of lipid peroxidation. Several synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and butylated hydroxyquinone (BHQ) are commercially available and currently used; however, their safety and toxicity are some problems of concern (Anderson et al. 1996). Hence the development of alternative antioxidants from natural sources has drawn much attention. Many investigators have found different types of antioxidants in various kinds of plants from terrestrial and oceans (Lim et al. 2002). *Ailanthus altissima* (Mill.) Swingle, tree of heaven is used in traditional medicine for treatment of dysentery, gonorrhea, hemorrhoids and a remedy for cough, gastric and intestinal upsets (Perry 1980). The bark of *A. altissima* is prescribed to treat anemia, hemorrhage and spermatorrhea (Adamik and Brauns 1957). It is also used as antispasmodic, antiasthmatic, cardiac depressant, astringent and for treatment of epilepsy (Watt and Breyer 1962). Previous phytochemical studies on *A. altissima* have demonstrated the presence of quassinoids (Chiarlo and Pinca1965; Chiarlo and Tacchino 1965, Furuno et al. 1981; Casinovi et al. 1983; Niimi et al. 1987; Kubota et

al. 1996) as well as indole alkaloids (Ohmoto et al. 1981; Varga et al. 1981; Ohmoto and Koike 1984; Souleles and Waigh 1984; Souleles and Kokkalou 1989), lipids and fatty acids (Chiarlo and Pinca 1965; Bory and Clair 1989; Kapoor et al. 1990; Kucuk et al. 1994), phenolic derivatives (Souleles and Philianos 1983; Barakat 1998; El-Baky et al. 2000) and volatile compounds (Mastelic and Jerkovis 2002). Extracts of *A. altissima* and some isolated compounds have demonstrated medicinal properties such as antituberculosis, antimalarial, antitumor and antiherpes activities (Hwang et al. 2002; Crespi et al. 1988; Kraus et al. 1994; Rahman et al. 1997; Bray et al. 1987; Tamura et al. 2000; Ohmoto and Koike 1989; Ohmoto and Sung 1983; Ohmoto et al. 1985). In our continuing search for bioactive metabolites from natural resources, in this study we encountered antioxidative activity and reducing power of this plant.

Materials and methods

Experimental

All the reagent grade chemicals, purchased from Sigma, Aldrich and Merck chemical companies, were used without further purification. Thin layer chromatography was performed on Merck silica gel GF-254 and an RP-18F-254 precoated plate. Identification was done with UV and typical TLC indicating solution (*p*-anisaldehyde/sulfuric acid/acetic acid mixture). UV spectra and initial rates were recorded on an HP 8453 UV spectrophotometer.

Plant material

Samples of *A. altissima* leaves were collected from Zoo Garden, Giza, Egypt in April 2007. The plant was authenticated by Prof. Dr. Kamal El-Batanony (Professor of Taxonomy, Faculty of Science, Cairo University). All samples were air dried and then powdered and kept in well-closed containers in dark. A voucher specimen was deposited in the NRC herbarium No. (11324).

Extraction methods

The air-dried powder (1 kg) of *A. altissima* leaves was treated with aqueous methanol (70 methanol: 30 water) in a continuous extraction apparatus until exhaustion. The methanolic extract was defatted with n-hexane several times. The n-hexane phases were combined and concentrated to give n-hexane extract fraction.

Free Radical Scavenging Activity

The DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging activity test was carried out according to the method first employed by M.S. Blois (Heim 2002). The 100µl of sample solution was added to 900µl of DPPH solution in ethanol (1.01×10^{-4} M). After incubation at room temperature for 30 min., the absorbance of this solution was determined at 518 nm using spectrophotometer and the remaining DPPH was calculated. All experiments were carried out in triplicate and repeated at least three times. Results were expressed as percentage decrease with respect to control values. The each fraction was evaluated at the final concentration of 100 $\mu g.ml^{-1}$ in the assay mixture.

Ferric Reducing Antioxidant Power (FRAP)

The Ferric Reducing Antioxidant Power (FRAP) assay was performed as previously described by Benzie and Strain 1999 according to Blois (1958). The experiment was conducted at 37°C at pH 3.6 condition with a blank sample in parallel. In the FRAP assay, reductants in the sample reduce Fe^{3+}/TPTZ (tripyridyltriazine) complex, resent in stoichiometric excess, to the blue ferrous form, with an increase in absorbance at 593 nm using spectrophotometer. The difference in absorbance between the blank and each sample is proportional to the total ferric reducing/antioxidant power (FRAP value) of the antioxidants in the sample. Aqueous solution of known Fe^{3+} ($FeSO_4.7H_2O$) was used for calibration. The final results were expressed as micromole ascorbic acid equivalent (AE)

per gram sample ((μmol VCE/g (Vitamin C equivalent/g)).

Cholinesterase assay

ChE-catalyzed hydrolysis of the thiocholine esters was monitored by following production of the anion of thiocholine at 412 nm by the Ellman's coupled assay (Benzie and Strain 1999; Ellman et al. 1961). Assays were conducted on HP8452A or HP8453A diode array UV-visible spectrophotometers and the cell compartments were thermostated by circulating water or Peltier temperature controller, respectively. Acetylthiochline (ATCh) and butyrylthiocholine (BuTCh) were used as substrates for AChE and BuChE, respectively.

Elastase assay

The elastase assay was conducted by the spectroscopic method (Rust et al. 1994). The substrate Suc-$(Ala)_3$-PNA is dissolved in 0.4 M Hepes buffer (pH 6.8) to make 5 mM. One milligram of porcine pancreatic elastase (EC 3.4.21.36) is dissolved in 2.8 mL Hepes buffer to make 0.5 unit enzyme solution. The initial rate, slope of the absorbance change for the initial 30-60 sec, is measured at 410 nm for 60 sec. The reaction is begun by adding 20 μL enzyme solution to the cuvette having 275 μl Hepes buffer, 100 μl sample in MeOH, 100 μl of Suc-$(Ala)_3$-PNA and 5 μl. The enzyme inhibition potency is expressed with % inhibition and calculated by the following equation. Inhibition (%) = 100- 100 (sample/control)

Tyrosinase assay

Tyrosinase inhibition activity is measured by the dopachrome method (Benzie and Strain 1999). Tyrosinase activity was determined spectrophotometrically by measuring the oxygen-dependent conversion of L-3,4-dihydroxyphenylalanine (L-DOPA, Singma-Aldrich). L-DOPA is oxidized by tyrosinase to form quinine, which immediately reacts with 3-methylbenzthiazolinone-2-hydrazone (MBTH) to from a red colored complex. In a 1ml cuvette 100 μL of 50 mM MBTH was added to 800 μL 18mM L-DOPA in 100 mM sodium phosphate pH 6.5 saturated with air (Kawagishi et al. 1993). The reaction was started with 100 μL 5 μg/ml tyrosinase and the increase in absorbance was measured at 484 nm and 30 nm. The inhibition activity is calculated by the following equation.

Tyrosinase Inhibition (%) = {(D-C) - (B-A)} / (D-C) x 100

A and B are the absorbance of the sample before and after incubation, respectively. C and D are the absorbance of the control without the sample before and after incubation, respectively.

COX assay

The murine macrophage cell line RAW 264.7 (from Korean Cell Line Bank) was cultured in DMEM (Dulbecco's modified eagle medium) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Hyclone Laboratories, Inc., USA). Macrophages were grown at 37°C and with 5%CO_2 in fully humidified air. Macrophages were transferred to 96-well plates (1×10^6/well, 200 mL) and were incubated with LPS (*E. coli*, serotype 055:B5, Sigma, 1 $mg.Ml^{-1}$) and various concentrations of samples for 18h. All test samples were first dissolved in dimethylsulfoxide (DMSO) and further diluted with medium and stored at -20°C. PGE_2 concentration in supernatants was measured by EIA (Enzyme Immunoassay) according to manufacturer's guide (from Cayman Chemical Co., USA) (Xie et al. 1991).

Phytochemical screening of *A. altissima* leaves Methanol-aqueous and n-hexane extracts of *A. altissima* leaves were screened for the following tests, Molish's test for the detection of the presence of carbohydrates and/or glycosides (Conalez and Delgano 1962), test

for tannins (Conalez and Delgano 1962; Clauss 1961; Wall et al. 1954), test for alkaloids and/or nitrogenous bases (Fulton 1932), Shinoda test for detection of the presence of flavonoids (Geissman 1962; Harper 1939), froth test for detection of saponins (Conalez and Delgano 1962; Harborne 1973) Bourchard's and Salkowisky's, Liebermann's tests for unsaturated sterols and/or triperpenes, quassinoids and coumarins test (Farnsworth 1966).

Results

The results of DPPH radical scavenging activity test and ferric reducing antioxidant power of Egyptian *A. altissima* leaves extracts in comparison with well-known synthetic and natural antioxidants were given in Tables 1 and 2. Since it is becoming clear that measurement of antioxidant activities of a sample through a single *in vitro* or *in vivo* method may not provide a good prediction of its efficacy in human subjects, the antioxidant activity of each fraction in this study was analyzed using FRAP as well as radical scavenging activity on DPPH.

Table 1. Biological activities of *A. altissima* extracts

	AChE inhibition (%)	BuChE inhibition (%)	DPPH radical scavenging activity (%)	Elastase inhibition (%)	Tyrosinase inhibition (%)	Reducing power activity (%)	COX-1 inhibition (%)	COX-2 inhibition (%)
Concentration	0.1 mg/mL					0.05 mg/mL	4 μg/ml	
A. altissima n-hexane	0.0	0.0	7.3	11.2	0.0	83.0	0.0	0.0
A. altissima MeOH	10.6	18.0	88.5 RC50=36.8 μg/mL	3.9	0.0	88.7	25.3	17.9

RC_{50}: reagent concentration to have 50 % activity

Table 2. Reducing power of *A. altissima* extracts and known compounds

	sample	n=2	duplicate	average	concentration
Control	Quercetin	0.766	0.804	0.785	0.02 mg/ml
	Ascorbic acid	0.639	0.702	0.671	
A.altissima extracts	*A. altissima n*-hexane	0.408	0.426	0.417	0.05 mg/ml
	A. altissima methanol	0.427	0.478	0.452	

According to phytochemical content of *A. altissima* leaves extracts (Table 3), *A. altissima* methanol-aqueous extract have a variety of chemical compounds including carbohydrates, tannins, lots of flavonoids, sterols and terpenes, coumarins, and quassinoids while n-hexane extract has sterols and /or terpenes and coumarins.

Table 3. Phytochemical screening of *A. altissima* leaves

Chemical Constituents	Methanol- aqueous extract	*n*-hexane extract
1. Carbohydrates and/or glycosides	+	-
2. Tannins	+	-
3. Alkaloids and/or nitrogenous bases	-	-
4. Flavonoids	+++	-
5. Sterols and/or triterpenes	+	+
6. Saponins	-	-
7. Coumarins	+	+
8. Quassinoids	+	-

\+ denotes the presence of the constituents

\- denotes the absence of the constituents

Discussion

Methanol-aqueous extract of *A. altissima* showed strong radical scavenging activity and it also showed very strong reducing power comparable to the known natural antioxidants, quercetin and ascorbic acid. The activity of methanol- aqueous extract was in accordance with the presence of flavonoids. While radical scavenging activity and reducing power are good *in vitro* indications of the general antioxidative capacity of the substances, the secondary radical sources resulting from the short-lived primary ROS such as superoxide, hydroxy or peroxy radicals can be a lot more specific and dangerous. Oxidized form of LDL is a very good example which, therefore acts as a major physiological risk factor for cardiovascular diseases. Oxidation of LDL by transition metals takes place *in vivo,* but probably represents only one of the many oxidation mechanisms that occur within the arterial wall. We also measured their enzyme inhibitory activities against enzymes involved in the inflammation, COX-1 and COX-2, neurotransmitor regulating enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), elastin degrading enzyme elastase and the melanin synthesizing enzyme tyrosinase, although *A. altissima* extracts showed weak COXs inhibitory activity. None of n-hexane and methanol extracts showed anti-inflammatory activity (Tables 1 and 2). *A. altissima* leaves methanol extract contained high content of the flavonoid compounds and this can explain why this extract has a potent antioxidative avtivity and mild COX inhibition activity, n-hexane extract has sterols, triterpenes and coumarins, and do not have these active flavonoids and this explain the strong antioxidant activity of methanol extract and than n-hexane extracts. The flavonoid compounds that such as kaempferol, apigenin and some flavonoid glycosides isolated from *A. altissima* leaves methanol-aqueous extract previously showed significant antioxidant activity (Kelly et al. 2002). Thus, the significant antioxidant activity of the methanol -aqueous extract is due to the presence of the flavonoid contents that are present in this extract in considerable amounts.

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