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(RESEARCH ARTICLE)

In vitro antioxidant studies of some terrestrial indigenous plants

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Abstract

The alcoholic extract of the plants *Azadiracta indica, Argimone maxicana, Malvastrum tricuspidatum and Allium sativum* were investigated for their free radical scavenging activity by DPPH and Nitric oxide scavenging method. Ethanolic extract of *A. indica,* and *M. tricuspidatum, A.sativum* and A*. maxicana* showed remarkable free radical scavenging activity. It interacted significantly with free radical DPPH at concentration of 8, 16, 32, 64, 125, 250, 500 and 1000µg/ml. The IC_{50} value was 63.71, 82.16, 64.62 and 73.6 µg/ml respectively as compared to 93.53 µg/ml of standard ascorbic acid. In nitric oxide scavenging method is at all concentration of 8, 16, 32, 64, 125, 250, 500 and 1000µg/ml. The IC_{50} value was 67.87, 74.35, 61.24 and 74.26 µg/ml respectively. All the extracts showed significant free radical scavenging activity with mild variation.

Key words: Antioxidant property; Free radical scavenging activity; Plant antioxidant; *Malvastrum tricuspidatum*

1. Introduction

It is proven that antioxidant products protect the animals against oxidative stress [1] and improved immunity followed stress in animals [2]. The increased production of toxic oxygen derivatives (free radicals) is considered to be a universal feature of stress condition. Free radicals are produced continuously in cells either as an accidental by-product of metabolism or deliberately during phagocytosis. Free radicals attacks the membrane lipids, thereby generating the lipid radicals and these lipid radicals can combine with oxygen producing peroxy radicals. These peroxy radicals can further cause peroxidation of cellular membrane lipids leading to cell necrosis. This chain reaction process is implicated in a number of pathophysiological conditions as well as toxicity in animals, poultry and human beings [3]. Free radicals have been implicated for a variety of conditions including inflammation, atherosclerosis, diabetes, ageing and hepatotoxicities [4]. Antioxidant may offer resistance against oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and many other mechanisms and thus prevent disease [5].

Various synthetic antioxidants are available in the market, which were proved to be good radical as well as lipid-peroxide scavengers. Examples are vitamin C (Ascorbic acid), α – tocopherol and Silymarine etc. But their excess consumption causes various ill effects and the most prominent is *hypervitaminosis* and thus may not be a part of prescription for long-term treatment. Although there are various ayurvedic products in market for specific ailments, which are related to free radical damage mechanism, yet there is need of a *novel* product, which will cover all of them with minimum side effects. Therefore, it's a rising insist to find out new safe and effective antioxidant, which will brawl with the variety of diseases. Literature showed that the antioxidant activity is high in plants [6, 7]. Present study is an effort in that direction which comprised of a study on four plants for antioxidant activity comparing with standard chemical antioxidant.

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2. Materials and Methods

2.1. Plant materials

For antioxidant property following plants was selected. *Argemone maxicana* (Leaves) is a glabrous weed with yellow flowers, native to tropical America. It is widely found in India as a crop weed and is toxic to animals. Literature reveals that it's been widely used in leprosy, skin diseases and inflammation. It is also used as anthelmintic in few cases. Phytochemical review states that it contains vast number of alkaloids, saturated fatty acids and flavonoids. There is no reference regarding *in vitro* antioxidant activity of this plant. Hence the said plant was included in our study. The ethanolic extract of the aerial parts of *M. tricuspidatum* (Leaves) has been reported to have antinociceptive and antibacterial activity. There is no reference in the literature regarding the antioxidant activity but, this plant was found to be hepatoprotective in preliminary clinical studies [8].

Beside these, two plants *Allium sativum* (Bulb) and *Azadiracta indica* (leaves) *were* also incorporated with reported antioxidant activity either *in vivo and in vitro* activity. *Allium sativum* has been reported to have antiallergenic, anti-aging, hepatoprotective and anti-inflammatory, anti-ischemic, antitoxic, antitumor, insecticidal and *in vivo* antioxidant activity. As all these diseases are related to mechanism of free radical damage, it was thought to include this plant in present study to investigate the *in vitro* antioxidant potential of *A. sativum. A. Indica* possesses large number of alkaloids, flavonoids and other phenolic compounds. Literature reveals that phenolic compounds are natural products, which have been shown to possess various biological properties related to antioxidant mechanisms [9].

Ascorbic acid was selected for synthetic antioxidant for comparison with herbal extract.

2.2. Preparation of plant extracts

The plants were collected and shade dried for 2 weeks at 35-45 ^{0}C and crushed to powder in electric grinder. The powder obtained was exhaustively extracted with 95% ethanol in soxhlet extractor. The ethanolic extracts thus obtained were evaporated to dryness by using rotary evaporator (Heidolph, Germany) under reduced pressure, to get syrupy mass. These extracts were used throughout the study.

A preliminary qualitative chemical analysis was carried out to find out the chemical constituents present in each crude extract. For chemical investigation, the crude extract was sub fractioned in separating flask by treatment with different solvent in their chronological order of increasing polarity. Initially, the ethanolic extract was treated with Petroleum ether followed by Solvent ether, ethyl acetate, ethyl methyl ketone, n- butanol and water.

In extract, test for steroids and triterpenoids (Liberamann-buchard test), carbohydrate (Fehling test), Alkaloids (Mayer's test), Glycosides (Lugal's test), Protein (Biuret test), Tannins and phenols (Ferric chloride test), Fixed oil and fat (Spot test), Flavanoids (Shinoda test), Gums and mucilage (Pot. Permanganate test) and saponin (Froth test) were conducted as per standard [10].

3. Methodology

Two methods were conducted to determine radical scavenging activity of plant extracts for assessment of their antioxidant activity.

3.1. DPPH Method (1, 1-diphenyl, 2-picrylhydrazyl radical)

DPPH scavenging activity was measured by the spectrophotometric method [11]. DPPH is stable nitrogen cantered free radical and has been extensively used to characterize an antioxidant compound. The reduction of DPPH radical serves as a quick and simple method to detect the antioxidant potential of compounds, especially those with phenol group. It is known that DPPH react rapidly with compound containing weak N - H or O - H bonds. Electron transport is also an important mechanism for its reduction. It is reversible, reduced and due to its unpaired electron, densely colour. A stock solution of DPPH (200μM) was prepared in ethanol. To this solution, 0.05 ml of test compound dissolved in ethanol was added in different concentration (8-1000 µg/ml). An equal amount of ethanol was added to the control. The reaction was allowed to complete in dark for 20 minutes. The absorbance was noted at 517 nm. Experiment was done in triplicate. Control experiment was done without drug solution but only with the solvent (ethanol).

3.2. Nitric oxide scavenging method

Nitric oxide is a very unstable species under the aerobic condition. It reacts with O_2 to produce the stable nitrates and nitrites through intermediates such as NO_2, N_2O_4 and N_3O_4. The formation of these intermediates was estimated by using Griess reagent as described [12, 13]. In presence of the test compound, which is a radical scavenger, the amount of nitrous acid formed will decrease. The extent of decrease will reflect the extent of scavenging by the drug. Sodium nitroprusside [final concentration 5 mM] in standard phosphate buffer solution PBS was incubated with different concentrations (8-1000 µg/ml) of extract, dissolved in phosphate buffer (0.025M, pH 7.4). The tubes were incubated at 25°C for 5 hours. After 5 hrs of incubation 0.5 ml of Griess reagent (1% sulphanilamide, 25 O-phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride) was added to the incubated solution and the absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyl ethylene diamine were read at 546 nm. The absorbance was taken immediately after the addition of Griess reagent. Control experiment was done without test compounds, but with equivalent amounts of buffer was added in it and the experiment was conducted in an identical manner. Experiment was conducted in triplicate. If the drug solution was colored, different drug concentrations were taken as blank respectively for their specific concentrations.

3.3. Statistical analysis

IC_{50} was calculated by Finney (1971) and F-test by standard methods [14].

4. Results

The results of qualitative detection of active ingredients are displayed in table 1, which revealed that *A.indica* contained steroids, triterpenoids, flavonoids, alkaloids, carbohydrate, fixed oil & fats and; gums and mucilage; *A. sativum*, steroids, triterpenoids, alkaloids, carbohydrate, fixed oil & fats and; protein and amino acid; *A.maxicana*, steroids, flavonoids, and alkaloids; and *M.tricuspidatum* contained steroids, triterpenoids, flavonoids, alkaloids, saponin carbohydrate, fixed oil and fats and; gums and mucilage (Table 1).

Table 1 Preliminary qualitative chemical analysis of ethanolic extracts of various plants

S. No.	Extracts	Steroids	Triterpenoids	Flavonoids	Alkaloids	Saponins	Glycosides	Carbohydrates	Fixed oils & Fats	Tannins & Phenols	Protein & amino acid	Gums & Mucilage
1.	*A. indica*	+	+	+	+	-	-	+	+	-	-	+
2.	*A. sativum*	+	+	-	+	-	-	+	+	-	+	-
3.	*A. maxicana*	+	-	+	+							
4.	*M. Tricuspidatum*	+	+	+	+	+	-	+	+	-	-	+

+: Positive, - : Negative,

In DPPH method, ethanolic extract of *A. indica,* and *M. tricuspidatum, A.sativum* and A*. maxicana* showed remarkable free radical scavenging activity. It interacted significantly with free radical DPPH at concentration of 8, 16, 32, 64, 125, 250, 500 and 1000µg/ml. The IC_{50} value was 63.71, 82.16, 64.62 and 73.6 µg/ml respectively as compared to 93.53 µg/ml of standard ascorbic acid. The free radical scavenging activity of *A. maxicana* extract was more effective than standard ascorbic acid. On the basis of percent scavenging and IC_{50} value, the highest activity was observed in *A. maxicana* followed by *M. tricuspidatum* and *A. indica,* while least in *A. sativum*. The statistical analysis reflected that free radical scavenging property of all plants extract and standard ascorbic acid increased as increase in concentration (Table 2).

Table 2 Percentage scavenging Activity of various ethanolic plant extracts by DPPH method

Concentration (µg/ml)	**Percentage scavenging**				
	Standard	*A. indica*	*M. tricusp.*	*A. sativum*	*A.maxicana*
8	21.84	14.47	15.19	4.23	13.99
16	45.06	28.51	19.35	9.92	35.83
32	45.81	40.08	23.66	13.04	45.16
64	54.47	45.97	31.6	21.3	63.62
125	64.2	51.37	65.39	31.27	64.98
250	70.62	58.72	68.74	40.47	67.34
500	76.57	62.07	78.41	48.15	70.41
1000	93.53	63.71	82.16	64.62	73.6
Mean ± SE	59.01±7.32	45.61±5.72	48.06±9.38	29.13±6.92	54.36±6.90
IC_{50} (µg/ml)	43.16	141.95	102.19	454.43	60.25
F value between treatment – 19.607**			F value between concentration – 42.32**		

** P < 0.01.

Table 3 Percentage scavenging activity of various ethanolic plant extracts by nitric oxide scavenging method

Concentration (µg/ml)	**Percentage scavenging**				
	Standard	*A. indica*	*M. tricusp.*	*A. sativum*	*A.maxicana*
8	15.7	30.91	18.91	10.43	9.5
16	19.49	36.03	26	22.21	13.86
32	21.15	38.39	35.09	23.33	18.13
64	29.44	45.52	42.19	35.09	33.1
125	39.99	50.87	45.5	38.3	44.15
250	58.76	56.58	51.65	48.64	57.58
500	69.94	61.95	60.64	53.9	64.52
1000	83.91	67.87	74.35	61.24	74.26
Mean ± SE	42.29±8.48	48.52±4.33	44.29±5.68	36.64±5.72	39.38±8.13
IC_{50} (µg/ml)	154.68	108.66	155.39	327.09	136.87
F value between treatment – 4.660**			F value between concentration – 54.00**		

** P < 0.01

From the nitric oxide scavenging studies it was found that, extracts were scavenging free nitric oxide radical from the reaction, at all concentration of 8, 16, 32, 64, 125, 250, 500 and 1000µg/ml. The IC_{50} value was 67.87, 74.35, 61.24 and 74.26 µg/ml in *A. indica* and *M. tricuspidatum, A.sativum* and A*. maxicana* respectively.

*A. indica*showed remarkable free scavenging property (48.52 %) than standard ascorbic acid (42.29%) and followed by *M. tricuspidatum,* and *A. maxicana* and least in *A. sativum.* The statistical analysis showed similar trend with increasing concentration. The variation in activity was observed in between two methods, nevertheless, potential antioxidant activity was observed in these tested plants extract (Table 3).

5. Discussion

Similarly many other indigenous plants were also screened for antioxidant property with these methods such as *Annona squamosa* [15], *Luffa echinata* [16], *Aristolochia bracteolate* Lam [17], *Aegle marmelos* fruit [18] and *Panax ginseng* and Indian ginseng [19] in India.

6. Conclusion

The most widely acknowledged behaviour of antioxidants is their interaction with oxidative free radicals. DPPH is relatively stable free radical. Its ethanolic solution showed decrease in absorbance at 517 nm, which is stoichiometric with respect to decolourization of DPPH solution. Dose dependent interaction of these extracts with DPPH, establishes the capability of constituents to scavenge the free radicals. Nitric oxide scavenging activity of *A. indica* and *M. tricuspidatum* were analyzed using sodium nitroprusside and Griess reagent. The ethanol extracts of *A. indica* and *M. tricuspidatum* is a good scavenger of nitric oxide radical produced by sodium nitroprusside. The observations were interesting, especially in view of the fact that the *A. maxicana* showed free radical scavenging properties. These facts can be further exploited for instance, in obtaining a fraction, which will have improved antioxidant activity as compared to the crude extract.

Compliance with ethical standards

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Disclosure of conflict of interest

The author conflict of interest is herbal medicine and toxicology.

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