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Original Article

Comparative Evaluation of Antioxidant Activity of Two Cuscuta Species

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ABSTRACT

The present study was an attempt to investigate two cuscuta species namely C.reflexa and C. australis which were used as traditional medicines in alternativesystems of medicine. World is endowed with a rich wealth of medicinalplants, all these plants are our common heritage, utilizing all these plants for human welfare has moved the concept of the herbal medicine or phytotherapy. Cuscuta species has been well exploited pharmacologically and phytochemically, still there is chance to get an in depth study based on its traditional use and knowledge. These plantsfind good traditional use in diseases of liver and spleen and a cure for dermatitis and boils.And hence were further compared for their Antioxidant potential. The in-vitro assays conducted on both the plant extract fractions indicate them to be significant source of natural anti-oxidant.

Keywords: Anthelminthic, Cuscuta, Herbal medicine

Introduction:

Traditional medicine is the synthesis of therapeutic experience of generations of practicing physicians of indigenous systems of medicine. Herbal drugs constitute only those traditional medicines which primarily use medicinal plant preparations for therapy. (1)Herbal medicines are being used by about 80% of the world population primarily in the developing countries for primary health care in low and medium income countries. (2) In recent decades,the use of herbal medicine has increased in developing countries as theyhave stood the test of time for their safety, efficacy, cultural acceptability and lesser side effects. The chemical constituents present in them are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body. Although modern medicine may exist side by side with such traditional practice,herbal medicines have often maintained their popularity for historical and cultural reasons.

Herbal medicine is now expanding at an astonishing pace due to the great inputs from ethno medicinal practices being pooled from all over the world many of the main food plants contain a fairly good amount of polyphenolic antioxidants. All these compounds play a role in protecting and maintaining our system(3. Therefore evaluation of Indian tradition medicine is possible through the proper exploitation and exploration of wide biodiversity and great ancient treatises of traditional medicine with the light of modern. (4)

Mass screening of plants in the search for new drugs is vastly expensive and inefficient but it would be cheaper and more productive,if we re- examine plant remedies described in ancient texts, a medicinal herb can be viewed as a synthetic lab as it produces and contains a number of chemical compounds, which may be responsible for biological activity (5)

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Free radicals are atoms or groups of atoms with an odd number of electrons and can be formed when oxygen interacts with certain molecules .free radicals are produced continuously in cells either as by products of metabolism or alternatively as in phagocytosis. The most important free radicals in the body are the radical derivatives of oxygen better known as reactive oxygen species These include hydrogen peroxide,hydroxyl radical,nitric oxide and others.(6)It is well known that oxidative stress included by oxygen-free radicals and resultant tissue damage are the hallmarks of several chronic disorders and cell death. The therapeutic potentials of medicinal plants as natural antioxidants on reducing such free radical induced tissue damage and in maintenance of health and protection from some age-related degenerative disorders such as cancer and coronary heart diseases is established.(7,8)

In recent years, there is an increasing interest in finding antioxidant phytochemicals, because they inhibit the propagation of free radical reactions and protect the human body from disease.[9]Recent investigations showed that the antioxidant properties of plants could be co-related with oxidative stress defense and different human diseases including cancer, atherosclerosis and the ageing process.[10] Plant flavonoids and phenolics in general are highly effective free radical scavenging and antioxidants.Hence, antioxidant scavenging activity of plant drugs containing phenolics and flavonoids are assessed to corelate its therapeutic potential.

Cuscuta is a genus of about 170 species of parasitic plants in family cuscutaceae, recent genetic

Research by angiosperm phylogeny group has shown that it is correctly placed in the morning glory family,convovulaceae.(11)

In tropical area it grows more or less continuosly and may reach high into tha canopy of shrubs and trees; while in temperate regions, it is an annual plant restricted to the relative low vegetation that can be reached with new seedlings in each spring. C. reflexa is a parasite on a very wide variety of plants including a number of agricultural and horticultural crop species such as alfaafa, lespedeza, flax, clover, potatoes, chrysanthemum, dahlia, helenium, trumpet vine, ivy, and petunias. In india, the plant is traditionally used for various medicinal purposes. The rural people of india use the juice of this plant as inhalant for treating jaundice and its warm paste is applied in rheumatism, gout, and other affected parts of the body and the paste of whole plant is applied for relieving headache. C. reflexa is a promising drug of plant origin(12)

In traditional Chinese medicine the seeda of C. australis are called Dou Tu Si Zi have been used for thousands of years. According to traditional Chinese medicine practitioners, the seeds of Dou Tu Si Zi have a neutral nature, pungent and sweet taste. Their ethnomedical uses are associated with the treatment of liver and kidney diseases and yin yang deficiencies. It was considered as both the aphrodisiac and longevity herb for slowing down the loss of fluids from the body. Contemporary Chinese herbalists use C. australis in formulas, to treat a range of conditions including impotence, premature ejaculation, frequent urination, ringing in the ears, lower back pain and white discharge from the vagina (leucorrhoea0, dry eyes, blurred vision and tired eyes.

(13)Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Although there are several enzyme systems within the body that scavenge free radicals, the principle micronutrient (vitamin) antioxidants are vitamin E, beta –carotene and vitamin C. The body cannot manufacture thase micronutrients so they must be supplied in the diet.

Scavenging activity of Nitric Oxide;

The absorbance of the chromophore formed during diazotization of the nitrile with sulphanilamide and subsequent coupling with napthylethylene diamine is used as the marker for NO scavenging activity the chromophore formation was not complete in the marker for

NO scavenging activity the chromophore formation was not complete in the presence of different test extracts, which scavenges the NO thus formed from the sodium nitroprusside and hence the absorbance decreases as the concentration of the extracts increases.

Scavenging of Hydrogen Peroxide;

H202 is a weak oxidizing agent and can inactivate a few enzymes directly ,usually by oxidation of essential thiol (–SH) groups. Hydrogen Peroxide can cross cell membrane rapidly. Once inside the cell, H202 can probablyreact with Fe+2and possibly Cu+2 to form hydroxyl radical and this may be the origin of many of its toxic effects It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.

Reducing power assay:

Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of Phenolic antioxidant action in the reducing power assay, the presence of antioxidants in the sample would result in the reducing of Fe3+ to Fe+2 by donating an electron Amount of Fe2+ complex can be then be monitored by measuring the formation of Perl s Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability.

Materials and methods:

Plant Collection

Fresh Whole plant of the C. reflexa and C. australis were collectedfrom barkas Hyderabad (Telangana,) and dried in the

shade at room temperature .Dried material was coarsely powered in grinder .The powered material was passed through 120 mesh to remove fine powder and the coarse powder used for extraction

Authentication

The plants C. reflexa and C. australis weer authenticatedby prasanna p.v officer in charge BOTANICAL SUEVEY OF INDIA ATTAPUR, HYDERABAD by comparing mophological features and a sample vocher specimen of plant was deposited for future references

Extraction:

Extraction was carried out by the using 70% alcohol by macerating plant material for 7days in a covered round bottom flask with vigorous intermittently the extracted solvent was then stained an\d concentrated in rota vapour where the excessive pure solvent was collected separately and the concentrated extracted was obtained. After evaporating, the extract was collected in a porcelain dish. The fractions were separated based on the density of solvents. Following fraction using petroleum ether and ethylacetate the remaining fraction obtained as the aqueous methanolic fractions were air dried and stored in airtight containers. Preliminary phytochemical screening was finally done for these extracts.

Preliminary Phyto-chemical investigation:

The preliminary phyto-chemical investigations were carried out on all three extracts of C. reflexa and C. australis for qualitative identification of phyto-chemical constituents present in each extract and tests were carried out as per the standard methods.(13,14)

Antioxidant Activity

The main characteristic of an antioxidant is its ability to trap free radicals. Ascorbic acid shows high antioxidant properties than other standard antioxidants such as Trolox, Vitamin C, is a mild reducing agent, it is also an electron donor and this property accounts for all its known functions. Reducing Power Assay

In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe3+ to Fe2+ by donating an electron. The amount of Fe2+ complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance indicates an increase in reductive ability.

The reducing power of sample was determined by the method of Yen and Duh. Different concentrations (20 -100 µg/ml) were mixed with 2.5 ml phosphate buffer (200 m M; Ph 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated at 50̊ C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid were added to the mixtures, followed by centrifugation for 10 min. The layer (5 ml) was mixed with 5 ml of distilled water and 1 ml of 0.1 % ferric chloride and the absorbance of the resultant solution were measured at 700 nm. Hydrogen Peroxide Assay

The principle of this method is that there is a decrease in absorbance of H2O2 upon oxidation of H2O2. The ability of the extract to scavenge hydrogen peroxide H2O2 was determined according to the method of Nabavi. A solution of hydrogen peroxide (40 ml) was prepared in phosphate buffer, Ph 7.4. The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Concentrations of the extracts 20-100 µg/ml in distilled water were added to a hydrogen peroxide solution at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as the standard.

Where,

A0= Absorbance of control reaction

A1= Absorbance with the sample

Nitric Oxide scavenging Assay

This assay determines nitric based on the enzymatic conversion of nitrate to nitrite by nitrate reductase in mammalian body. Since most of the NO is oxidized to nitrite (NO2) and nitrate (NO3), the concentrationof anions have been used as quantitative measure of NO production. Sodium nitro preside is aqueous solution. At physiological PH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrate ions that can be estimated by use of greiss reagent spectrophotometrically at 546 nm. This reaction is calorimetric detection. Of nitrite as aza dye product of the greiss reaction in which acidified NO2- produces a nitrosating agent which reacts with sulfinic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative.

The chemical source of NO was sodium nitroprusside (10 mm) in 0.5 M phosphate buffer, pH 7.4, which spontaneously produced NO in an aqueos solution. NO interacted with oxygen to produce stable products, leading to the production of nitrites. After incubation for 60 min at 37̊ C, Griess reagent (mixture of reagent A and B) was added. The same reaction mixture without the extract of sample but with equivalent amount of distilled water served as control. Ascorbic acid was used as positive control.

Where

A0= Absorbance of control reaction

A1= Absorbance with the sample

Statistical analysis

Tests were carried out in two counts and the mean value was represented using Ms Windows based Graph Pad Prism (version 6) software. Results were expressed graphically as mean ± standard deviation.

Results and discussion:

Extraction of powdered plant material was carried by cold maceration in a round bottom flask. The extracts were dried in vacuum dryer and stored in refrigerator.

Table 1: Percentage yield of extracts for C. reflexa and C. Australis

S. No.	Extracts	*C. reflexa* (% w/w)	*C. australis* (% w/w)
1	Petroleum ether	4.8	3.6
2	Ethyl acetate	28.4	31.2
3	Methanol	45.3	48.6

Methanolic and ethyl acetate extracts of both the plants found to show better yield compared to pet ether extract . As the yield obtained specifies the accumulation of constituents in that extract. The methanolic and ethyl acetate extracts of C.reflexa and C.australis were foud to show more accumulated constituents.

Preliminary Phytochemical Screening of Extracts

Table 2: Phytochemical study of C. reflexa

Chemical tests	Fractions		
	P. ether	E. acetate	Methanol
1. Carbohydrates			
a) Molisch's test	+	+	+
b) Fehling's test	+	+	+
c) Benedict's test	+	+	+
2. Non-reducing sugars			
a) Iodine test	-	-	-
b) Tannic acid test	-	-	-
3. Proteins			
a) Biuret test	-	-	-
b) Millon's	-	+	+
4. Amino acid			
a) Ninhydrin test	-	-	+
5. Steroids			
a) Salkowski test	+	+	-
b) Liberman's Burchardt's test	-	-	-
6. Alkaloids			
a) Dragendroff's test	+	+	+
b) Mayer's test	+	+	+
7. Tannins and phenols			
a) $FeCl_3$ test	+	+	+
b) Br_2 water test	+	+	+
c) $KMnO_4$ test	-	-	-
8. Flavonoids			
a) Shinoda test	+	+	+
b) Sulphuric acid test	-	+	+
c) Lead acetate test	-	+	-
9. Glycosides			
a) General test	+	+	+
10. Saponins			
a) Foam formation test	-	-	-

'+' Present '-' Absent

Table 3: Phytochemical study of C. australis.

Chemical tests	Fractions		
	P.ether	E. acetate	Methanol
11. Carbohydrates			
d) Molisch's test	+	+	+
e) Fehling's test	-	+	-
f) Benedict's test	+	+	+
12. Non-reducing sugars			
c) Iodine test	-	-	-
d) Tannic acid test	+	+	+
13. Proteins			
c) Biuret test	+	+	+
d) Millon's	+	+	+
14. Amino acid			
b) Ninhydrin test	+	+	+
15. Steroids			
c) Salkowski test	+	+	-
d) Liberman's Burchardt's test	-	-	-
16. Alkaloids			
c) Dragendroff's test	+	+	+
d) Mayer's test	+	+	+
17. Tannins and phenols			
d) $FeCl_3$ test	+	+	+
e) Br_2 water test	+	+	+
f) $KMnO_4$ test	-	-	-
18. Flavonoids			
d) Shinoda test	+	+	+
e) Sulphuric acid test	+	+	+
f) Zn-HCl test	+	+	+
19. Glycosides			
b) General test	+	-	+
10.saponins			
b) Foam formation test	-	-	-

Ethyl acetate fraction of C.reflexa showed the presence of carbohydrates, alkaloids, tannins, phenols, flavonoids,glycosides and steroids, methanolic extract of C.reflexa revealed the presence of carbohydrates, alkaloids, tannins, flavonoids and phenolics, whereas ethyl acetate extract of C.australis revealed the presence of carbohydrates, alkaloids, tannins, phenols, flavonoids, and steroids and methanolic extract of C.australis revealed the presence of carbohydrates, alkaloids, tannins, phenols, flavonoids,glycosides and steroids.So Ethyl acetate and methanolic extracts were rich in flavonoids and phenolics which possess antioxidant properties.

Table 4: Effect of C.reflexa extracts on invitro Reducing power assay

S. No.	Concentration (µg/ml)	Absorbance (nm)			
		Ascorbic acid	P.ether	E.acetate	Methanol
1	20	-0.012	0.034	o.094	0.020
2	40	0.078	0.046	0.145	0.038
3	60	0.197	0.051	0.160	0.053
4	80	0.325	0.052	0.183	0.053
5	100	0.470	0.058	0.190	0.060

Table 5: Effect of C.australis extracts on invitro Reducing power assay

S. No.	Concentration (µg/ml)	Absorbance (nm)			
		Ascorbic acid	P.ether	E.acetate	Methanol
1	20	-0.012	0.030	o.018	0.020
2	40	0.078	0.040	0.022	0.023
3	60	0.197	0.063	0.072	0.036
4	80	0.325	0.071	0.130	0.047
5	100	0.470	0.078	0.157	0.071

The ethyl acetate fraction of both the species showed higher antioxidant potential than the other two fractions tested. Effect of petroleum ether and Methanol fractions in reduction ability was not significant. However, the three fractions of both species showed antioxidant potential in a dose dependent manner.

Hydrogen Peroxide Assay

Hydrogen peroxide is a reactive oxygen species that accumulates during oxidative stress and is toxic to cell. As a by-product of oxidative stress response and as an integral part of normal inflammatory response to infection, understanding the impact of H2O2 on various model systems has become an important fact of studying many pathophysiological processes.

Table 6: Effect of C. reflexa on Hydrogen peroxide scavenging assay

Concentration (µg/ml)	Hydrogen peroxide assay (% Inhibition)			
	Ascorbic acid	Methanol	P.ether	E.acetate
20	67.84±0.050	66.69±0.000	68.19±0.003	36.39±0.005
40	68.03±0.025	81.95±0.040	79.56±0.000	55.33±0.000
60	68.37±0.122	78.46±0.040	74.67±0.000	63.21±0.006
80	68.81±0.001	76.78±0.122	80.02±0.041	72.18±0.000
100	71.32±0.203	81.95±0.009	86.83±0.039	76.47±0.000

Table 7: Effect of C. australison Hydrogen peroxide scavenging assay

Concentration (µg/ml)	Hydrogen peroxide assay (% Inhibition)			
	Ascorbic acid	Methanol	P.ether	E.acetate
20	67.84±0.050	57.32±0.008	75.37±0.000	62.81±0.205
40	68.03±0.025	65.60±0.040	75.47±0.000	70.38±0.041
60	68.37±0.009	77.36±0.040	81.05±0.000	83.25±0.006
80	68.81±0.001	82.75±0.122	82.35±0.000	84.24±0.042
100	71.32±0.203	89.23±0.009	82.75±0.000	83.84±0.040

Percent inhibition of H2O2 of C. relexa and C. australis were greater than ascorbic acid used as standard. Petroleum Ether (per), Ethyl acetate (EAR), Methanol (MR) fractions of C.reflexa showed 86.83, 76.47 and 81.95 % inhibition respectively at a concentration of 100 µg/ml. C. australis showed 89.23, 82.75 and 83.84 5 inhibitions at 100 µm/ml concentration for Methanol (MA), petroleum ether (PEA) and Ethyl acetate (EAA) respectively.

Nitric oxide assay

Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25̊ C for 150 min resulted in the generation of NO. The fractions effectively reduced the generation of NO, however the % inhibition Was less compared to Ascorbic acid exception of the ethyl acetate fraction of C.reflexa.

Table 8: Effect of C. reflexaon Nitric oxide scavenging assay

Concentration (µg/ml)	Nitric oxide scavenging assay (% Inhibition)		
	Ascorbic acid	Methanol	E.acetate
20	68.68±0.048	50.68±0.146	59.24±0.048
40	64.41±0.007	53.93±0.146	59.03±0.009
60	61.37±0.003	52.27±0.048	65.44±0.006
80	60.75±0.002	52.75±0.048	65.86±0.048
100	59.44±0.005	56.34±0.048	70.00±0.097

Table 9: Effect of C. australison Nitric oxide scavenging assay

Concentration (µg/ml)	Nitric oxide scavenging assay (% Inhibition)			
	Ascorbic acid	Methanol	P.ether	E.acetate
20	68.68±0.048	48.20±0.006	29.17±0.007	48.20±0.048
40	64.41±0.007	49.24±0.007	24.27±0.002	53.86±0.005
60	61.37±0.003	50.48±0.005	21.03±0.008	50.96±0.001
80	60.75±0.002	53.65±0.005	29.37±0.005	51.03±0.048
100	59.44±0.005	54.62±0.048	37.51±0.008	57.93±0.001

The percentage inhibition of nitric oxide by C. reflexa was found remarkable in the ethyl acetate fraction, however C. australis also showed fairly good inhibition but lesser than that of the standard. The percentage inhibition of C. reflexa at 100 µg/ml concentration in MR, PER, EAR as found to be 56.34, 45.79 and 70 respectively and that shown by C.australis was 54.62, 37.51, and 57.93 respectively for MA, PEA and EAA respectively.

Summary and conclusion:

The present investigation on two cuscuta species namely C.reflexa and C. australis after extraction using petether,Ethyl acetate and methanolsolventsfound to show % yield for C.reflexa in petroleum ether, ethyl acetate and methanol was 4.8, 28.4 and 45.3% w/w respectively and the yield for C.australis was found to be 3.6, 31.2 and 48.6% w/w for petroleum ether, ethyl acetate and methanol respectively.

Preliminary phytochemical screening of C.reflexa fractions showed positive results for carbohydrates, alkaloids, tannins, phenols, flavonoids and glycosides. While fractions of C. australis showed positive results for carbohydrates, proteins, amino acids, steroids, alkaloids, tannins, phenols, flavonoids and glycosides. As both the plants showed positive results for the prescence of phenolics and flavonoids and glycosides. As both the plants showed positive results for the presence of phenolics and flavonoids they were presumed to have antioxidant potential.

Ascorbic acid was used as a standard antioxidant as it has highest antioxidant potential. All the fractions from both the species were compared to ascorbic acid.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so they can act as primary and secondary antioxidants. The plant extract fractions could reduce most Fe3+ ions, which had a lesser reductive activity than the standard ascorbic acid. The effect of petroleum ether and aq.methanol fractions of both the species in reductiuve activity were not significant. However, the ethyl acetate fraction showed better antioxidant potential in a dose dependent manner.

Hydrogen peroxide is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals that can initiate lipid peroxidation and cause DNA damage. Hence the ability of plant extracts to scavenge hydrogen peroxide is significant. C.reflexa had good H2O2 scavenging activity as comparable to

the doses of ascorbic acid. Percent inhibition of H2O2 of C. reflexa and C. australis were greater than ascorbic acid. Petroleum ether (PER), ethyl acetate (EAR), methanol (MR) fractions of C. reflexa showed 86.83, 76.47, 81.95 % inhibition respectively at a concentration of 100 µg/ml. C. australis showed 89.23, 82.75 and 83.84 percent inhibition at 100 µg/ml concentration for Methalnol (MA), petroleum ether (PEA) and ethyl acetate (EAA) respectively.

Nitric oxide assay was performed, where the fractions reduced the generation of NO. Dose dependent increase in nitric-oxide radical scavenging activity was observed

Based on the study performed, it can be concluded that cuscuta is a promising genus compromising of numerous species awaiting study. C.reflexa the Indian variety of cuscuta showed antioxidant potential superior than the Chinese variety C.australis. yet both the species have shown promising antioxidant potential. The in-vitro assays conducted on both the plant extract fractions indicate them to be significant source of natural anti-oxidant, which might be helpful in preventing the progress of various oxidative stresses. However, comparative in-vivo anti-oxidant activity of the plants needs to be assessed prior clinical use.

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