Research Article

Comparative Evaluation of Antioxidant Activity of Four Indigenous Herbal Plants and their Polyherbal Extract

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ABSTRACT

In a normal cell, there is appropriate oxidant: antioxidant balance. However, this balance can be shifted, when oxidant species is increased or when levels of antioxidants are diminished. This stage is called oxidative stress. Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc. Numerous polyherbal formulations, which are combinations of different herbal extracts/fractions, are used for the treatment of these diseases, as herbal drugs are a rich source of natural antioxidants. The aim of this study was to compare the antioxidant activity of the extracts of four different plants viz *Tinospora cordifolia* (Menispermaceae), *Withania somnifera* (Solanaceae), *Azadiracta indica* (Meliaceae), and *Centella asiatica* (Apiaceae) with their polyherbal extract and explore the synergic action for designing new drug combinations that are individually sub therapeutic but efficacious in combinations. The crude alcoholic extract of these individual plants and their polyherbal combination were studied for their antioxidant activity by Nitric oxide scavenging assay, Hydroxyl radical scavenging assay, Reducing power capacity, Superoxide free radical scavenging assay and Total antioxidant activity. The crude extracts showed different level of antioxidant activity and is a potential source of antioxidants and thus could prevent many radical related diseases.

Keywords: Hydroxyl radical scavenging assay, Nitric oxide scavenging assay, Oxidative stress, Polyherbal combination, Reducing power capacity, Superoxide free radical scavenging assay, Total antioxidant activity.

INTRODUCTION

An antioxidant is a chemical that prevents the oxidation of other chemicals. They protect the key cell components by neutralizing the damaging effects of free radicals, which are natural by-products of cell metabolism.[1,2] Free radicals are chemical species that posses an unpaired electron in the outer valence shell of the molecule. This is the reason, why free radicals are highly reactive and can react with proteins, lipids, carbohydrates and DNA. These free radicals attack the nearest stable molecules, stealing its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, resulting in the destruction of living cells.[3] Free radicals may be either oxygen derived (ROS, reactive oxygen species) or nitrogen derived (RNS, reactive nitrogen species). The oxygen derived molecules are O-2 [superoxide], HO [hydroxyl], HO2 [hydroperoxyl], ROO [peroxyl], RO[alkoxyl] as free radical and H2O2 oxygen as non-radical. Nitrogen derived oxidant species are mainly NO [nitric oxide], ONOO [peroxy nitrate], NO2 [nitrogen dioxide] and N_2O_3 [dinitrogen trioxide].[4,5] In a normal cell, there is appropriate oxidant: antioxidant balance. However, this balance can be shifted, when oxidant species is increased or when levels of antioxidants are diminished. This stage is called oxidative stress. Oxidative stress results in the damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates. Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc.[6]

In the search of plants as a source of natural antioxidants, some medicinal plants and fruits have been extensively studied for their antioxidant activity and radical scavenging in the last few decades.[7] There is an increasing interest in the measurement and use of plant antioxidant for scientific research as well as industrial (e.g., dietary, pharmaceutical and cosmetics) purposes. This is mainly due to their strong biological activity, excluding those of many synthetic antioxidants which have possible activity as promoters of carcinogenesis. Therefore, the need exists for safe, economic, powerful and natural antioxidants to replace these synthetic ones. Obviously, there has been an increasing demand to evaluate the antioxidant properties of direct plant extracts.[8]

Thus herbal drugs are rapidly becoming popular in recent years as an alternative therapy. Numerous polyherbal formulations, which are combinations of different herbal extracts/fractions, are used for the treatment of diseases. For developing a satisfactory antioxidant herbal formulation, there is a need to evaluate the formulation for desired properties such as antioxidant activity.[9]

Tinospora cordifolia is a widely used shrub in folk and Ayurvedic systems of medicine. It is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae. It is distributed throughout tropical

Indian subcontinent and China. It is reported to possess anti-spasmodic, anti-inflammatory, anti-allergic, anti-diabetic, anti-oxidant properties.[10]

Withania somnifera is a small and erect evergreen woody under shrub that grows up to a height of 1-m tall and belongs to the family of Solanaceae locally known as Ashwagandha. This plant is capable of growing wildly not only in all the drier parts of the subtropical Bangladesh i.e. in Nator, Savar, and North-western parts of Bangladesh but also in India, Congo, South Africa, Egypt, Morocco, Jordan, Pakistan and Afghanistan. The roots are the main portions of the whole plant as they possess wide number of the therapeutic agents. The crude aqueous extract of the plant contains the phenolics and flavonoids which are said to be the potent antioxidants.[11]

Azadiracta indica belonging to family Meliaceae, is well known in India and its neighboring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. All parts of the plant have been used for medicinal purposes including fruits, seeds, leaves, roots and barks. Leaf & bark extract of *Azadiracta indica* has been studied for its anti-oxidant activity.[12]

Centella asiatica belonging to family Apiaceae, is a perennial herb that has been used for centuries in Ayurvedic medicine to treat several disorders, such as insanity, asthma, leprosy, ulcers and eczema and for wound healing.[14,15]

The aim of this study was to compare the antioxidant activity of the extracts of the above four different plants with their polyherbal extract and explore the synergic action for designing new drug combinations that are individually sub-therapeutic but efficacious in combinations.

MATERIALS AND METHODS

Materials

Collection of crude drug

The authenticated crude drugs were collected from Kerala Ayurveda Limited, Angamali, Ernakulam, Kerala.

Crude drug

Stems of *Tinospora cordifolia,*

Roots of *Withania somnifera* ,

Whole plants of *Centella asiatica,*

Leaves of *Azadiracta indica*

Polyherb

Equal proportion of coarsely powdered crude drug

Plant Extracts

Alcoholic extract of Polyherbs

Alcoholic extract of *Tinospora cordifolia*

Alcoholic extract of *Withania somnifera*

Alcoholic extract of *Centella asiatica*

Alcoholic extract of *Azadiracta indica*

Preparation of Plant extracts

The sun dried crude drugs were subjected to physical evaluation. The standardized coarse powdered crude drugs (sieve size 60) were subjected to alcoholic extract by Soxhlet Extractor.

Preparation of Polyherbal extracts

Crude drugs were powdered to coarse size (sieve size 60) separately and mixed in equal ratio by weight. Alcoholic extract of mixed crude drugs were prepared by Soxhlet Extraction Method.

Methods

Hydroxyl Radical Scavenging Activity

This assay is based on the qualification of the degradation product of 2 deoxy ribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+} - ascorbate-EDTA –H_2O_2 system (The Fenton reaction). The reaction mixture contained in the final volume of 1 mL.2 deoxy 2 ribose (2.8mM) KH_2PO_4—KOH buffer (20 mM pH 7.4), $FeCl_3$ (100µm), EDTA (100µm), H_2O_2 (1.0mM), ascorbic acid (100µm) and various concentrations (0-200µg/ml) of the test sample. After incubation for 1hour at 37°C, 0.5 ml of the reaction mixture was added to 1ml of 2.8% TCA, then 1ml aqueous TDA was added and the mixture was incubated at 90°C for 15 minutes to develop the colour. After cooling the absorbance was measured at 532nm against an appropriate blank solution.[16]

Nitric Oxide Scavenging Activity

Nitric oxide (NO.) has also been involved in a variety of biological functions, including neurotransmission, vascular homeostasis, antimicrobial, and antitumor activities. Despite the possible beneficial effects of NO., its contribution to oxidative damage is also reported. This is due to the fact that NO can react with superoxide to form the peroxynitrite anion, which is a potential oxidant that can decompose to produce OH and NO. The procedure is based on the principle that, sodium nitro prusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Large amounts of NO. may lead to tissue damage.

Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitro prusside (5mmolL^{-1}) in phosphate buffered saline pH 7.4, was mixed with different concentration of the extract (250-2500µg mL^{-1}) prepared in methanol and incubated at 25°C for 30minutes.A control without the test compound, but an equivalent amount of methanol was taken. After 30minutes, 1.5mL of the incubated solution was removed

and diluted with 1.5mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphathyl ethylene diamine dihydrochloride was measured at 546nm and the percentage scavenging activity was measured with reference to the standard.

% Inhibition of the NO radical=$[A_0 – A_1]/A_0$ x 100 where A_0 is the absorbance before the reaction and A_1 is the absorbance after reaction has taken place with Griess reagent.[17]

Reducing Power Activity

The reducing power of extract was determined by the method of YEN and DUH (1993). Different concentration of extract was mixed with 2.5ml of phosphate buffer (pH 6.6). Weighed the different concentrations of extract (50,100,200µl) to it add 2.5ml of phosphate buffer (pH 6.6) and 2.5ml of 1% potassium ferri cyanide was added and boiled it for 20 minutes at 50°C. To it added 2.5 ml of TCA and centrifuged it for 10 minutes at 2000rpm.Collect the supernatant and added 1ml of distilled water, 250µl of 0.1% ferric chloride and the absorbance was read at 700nm.[18]

Super oxide free radical scavenging activity

Super oxide is biologically important as it can form singlet oxygen and hydroxyl radical.

Overproduction of super oxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. Super oxide anion are generated in PMS-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan.0.02ml of extracts, 0.05ml of Riboflavin solution (0.12mM), 0.2 ml of EDTA solution [0.1M], and 0.1 ml NBT (Nitro-blue tetrazolium) solution [1.5mM] were mixed in test tube and reaction mixture was diluted up to 2.64ml with phosphate buffer [0.067M]. The absorbance of solution was measured at 560nm using DMSO as blank after illumination for 5 min and difference in OD was determined after 30 minutes incubation in fluorescent light. Absorbance was measured after illumination for 30 min. at 560 nm on UV visible spectrometer.[19]

% scavenging/Inhibition = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

Total Antioxidant Activity

The total antioxidant capacity was evaluated by the phosphomolybdenum method.[18] 0.3ml extract was obtained with 3ml of reagent solution (0.6ml H_2SO_4, 28mM sodium phosphate and 4mM ammonium molybdate). The tube containing the reaction solutions were incubated at 95c for 90 minutes. The absorbance of the solution was measured at 695nm against blank after cooling to room temperature (Methanol 0.3ml) in the place of extract was used as blank. The antioxidant activity is expressed as number of gram equivalent of ascorbic acid. The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by the following equation: A=(c x V/m) Where, A = total content of antioxidant compounds, mg/gm plant extract, in ascorbic acid equivalent c = the concentration of ascorbic acid established from the calibration curve, mg/ml, V = the volume of extract in ml, m = the weight of crude plant extract, gm.[20]

RESULTS AND DISCUSSION

Hydroxyl radical scavenging activity

Hydroxyl radical is one of the most potent reactive oxygen species in the biological system that reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell. Scavenging of hydroxyl radical of different extracts is presented in figure 1. The percentage of hydroxyl radical scavenging activity of *Tinospora cordifolia* extract was found to be 70% which is highest among five extract at 200 µg/ml compared to antioxidant activity of standard ascorbic acid which was 85.88 % at the same concentration. The combined extract also showed significant activity with a scavenging value of 65.23 % at the same concentration. Among the five extracts *Tinospora cordifolia*, Combined polyherbal extract and *Azadiracta indica* extract showed good activity with an IC_{50} value of 55.53, 58.82 and 61.7µg/ml respectively compared with ascorbic acid value of 14.179 (Table 1).

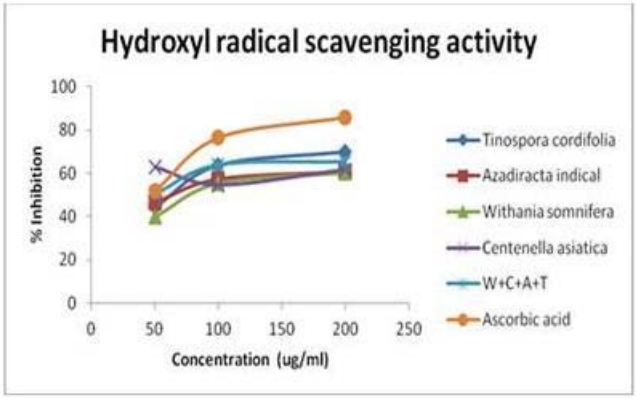

Figure 1: Hydroxyl radical scavenging activity

Table 1: IC_{50} values of different extracts in hydroxyl radical scavenging assay

Extracts/standard	IC_{50}(µg/ml)
Azadiracta indica	61.7
Tinospora cordifolia	55.53
Withania somnifera	102.27
Centenella asiatica	366.9
W+C+A+T(polyherbal extract)	58.82
Ascorbic acid	14.179

Nitric Oxide Scavenging Assay

Nitric oxide is a very unstable species and reacting with oxygen molecule produce stable nitrate and nitrite which can be estimated by using Griess reagent. In the presence of a scavenging test compound, the amount of nitrous acid will decrease which can be measured at 546 nm. Alcoholic extract of *Withania somnifera* and *Tinospora cordifolia* has potent nitric oxide scavenging activity (IC_{50} value 131.25 µg/ml and 137.5 µg/ml) while alcoholic extract of *Centenella asiatica* has showed the least nitric oxide scavenging activity (IC_{50} value 575.69 µg/ml), while the polyherbal combination has a good nitric oxide scavenging activity (IC_{50} value 154 µg/ml) (Table 2). *Withania somnifera, Tinospora cordifolia* and the polyherbal extract showed maximum activity of 73.41%, 64.55% and 59.49% respectively at 200 µg /ml, where as gallic acid at the same concentration exhibited 81.23 % inhibition. (Figure 2)

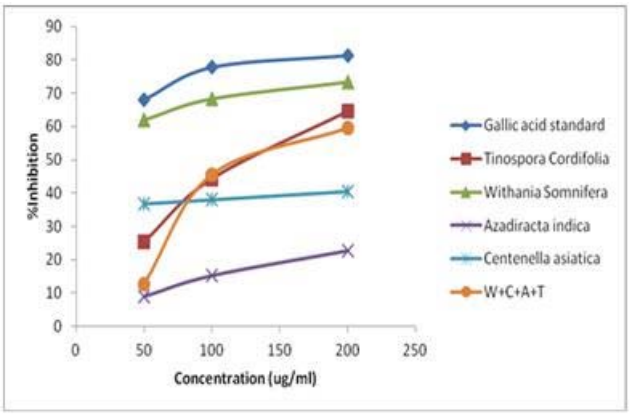

Figure 2: Comparative Nitric oxide scavenging activity of the four individual extracts and their combination with gallic acid standard

Table 2: IC_{50} values of different extracts in nitric oxide scavenging assay

Extracts/standard	IC_{50}(µg/ml)
Azadiracta indica	496.46
Tinospora cordifolia	137.5
Withania somnifera	131.25
Centenella asiatica	575.69
W+C+A+T(polyherbal extract)	154
Gallic acid	20.21

Reducing Power Capacity Assessment

Reducing power of the fractions was assessed using ferric to ferrous reducing activity as determined spectrophotometrically from the formation of Perl's Prussian blue colour complex. Reducing power of different extracts was compared with ascorbic acid. Among the extracts the *Azadiracta indica* extract exhibited the most reducing power, while the polyherbal extract had a better reducing power than *Withania somnifera* but a comparatively lesser value when compared to the ascorbic acid standard. This result indicates that the extracts may consist of polyphenolic compounds that usually show great reducing power and that the polyherbal product has a good reducing power capacity comparable to that of the standard ascorbic acid. (Figure 3)

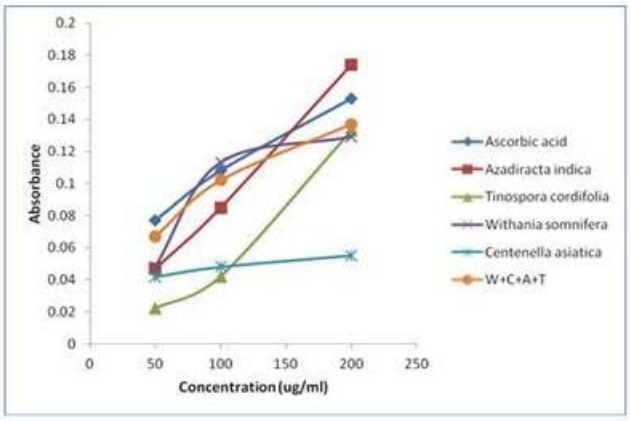

Figure 3: Comparative reducing power capacity of the four individual plants and their polyherbal formulation

Superoxide free radical scavenging activity

Although superoxide anion is a weak oxidant, it ultimately produces powerful and dangerous hydroxy radicals as well as singlet oxygen which contribute to oxidative stress. Alcoholic extract of *Withania somnifera* root has potent nitric oxide scavenging activity (IC_{50} value 99.05 µg/ml) while the polyherbal combination has a good nitric oxide scavenging activity (IC_{50} value 126.83 µg/ml) (Table 3). *Withania somnifera* showed maximum activity of 77.67%, at 200 µg /ml, comparable to ascorbic acid standard which at the same concentration exhibited 77.07 % inhibition. The polyherbal combination showed maximum activity of 68.75% at 200 µg/ml. (Figure 4)

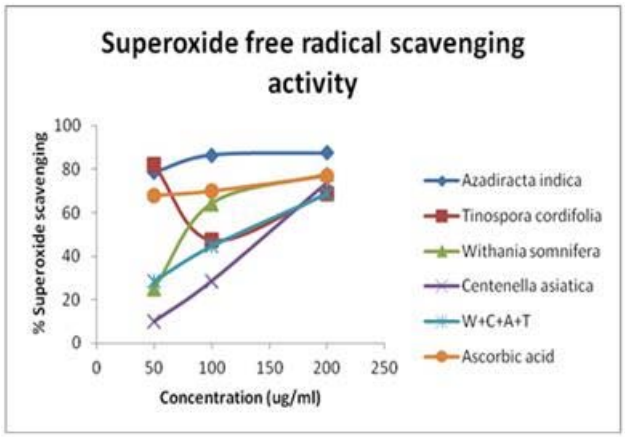

Figure 4: Superoxide free radical scavenging activity

Total antioxidant activity

The total antioxidant capacity (TAC) was based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of green phosphate/ Mo(V) complex at acid pH. It evaluates both water-soluble and fat-soluble antioxidants (total antioxidant capacity). Electron transfer occurs in this assay which depends upon

the structure of the antioxidant. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid. Polyherbal combination of four different extracts was found to possess the highest total antioxidant capacity (Table 4). Total antioxidant capacity of the extracts was found to decrease in the following order: polyherbal combination extract > *Centenella asiatica* extract > *Withania somnifera* extract > *Tinospora cordifolia* extract > *Azadiracta indica* extract.

Table 3: IC_{50} values of different extracts in Superoxide radical scavenging assay

Extracts/standard	IC_{50}(µg/ml)
Azadiracta indica	537.66
Tinospora cordifolia	466.77
W+C+A+T(polyherbal extract)	126.83
Withania somnifera	99.05
Centenella asiatica	145.58
Ascorbic acid	24.2

Table 4: Total antioxidant activity of the four different extracts and their polyherbal combination

Extracts/standard	Total Antioxidant Capacity (mg/gm, Ascorbic Acid Equivalent)
Azadiracta indica	76.57±12.1
Tinospora cordifolia	81.842±7.82
W+C+A+T(polyherbal extract)	310.2±9.11
Withania somnifera	87.15±5.76
Centenella asiatica	129±15.32

Values are the mean of triplicate experiments and represented as mean ± SD

CONCLUSION

All the conducted experiments in the present study are based on crude extract and are considered to be preliminary and more sophisticate research is necessary to reach a concrete conclusion about the findings of the present study. To sum up, these findings together demonstrate that the polyherbal extract is an excellent candidate for further bio-guided investigation so as to develop a polyherbal formulation. Therefore, in depth extensive study should be an urgency to sort out bioactive compounds and fix its therapeutic dose. These extracts can be used against damaging effects of free radicals and can inhibit immunological incompetence and neurodegenerative disorders, carcinogenesis and delay aging.

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