A Comparative Study on Phytochemical and in Vitro Antioxidant Activity of Herbal Formulation of *Achyranthes Aspera* and *Boerhavia Diffusa* Ethanolic Extracts

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ABSTRACT

The present research was conducted to evaluate the yield, phytochemical and antioxidant activity of ethanolic extracts of two widely used medicinal herbs, *Boerhavia diffusa*, *Achyranthes aspera* and their herbal mixture. Solvent extract was prepared by subjecting plant material to soxhlet extraction. Qualitative and quantitative phytochemical analysis was performed to test the presence of major phytochemical constituents of each plant and their herbal mixture. Evaluation of in vitro enzyme and non enzymic antioxidant activity of plant extracts was studied. The results of the present study reveal that the ethanolic extract showed higher percentage of yield in individual and herbal mixture extracts of *Boerhavia diffusa* and *Achyranthes aspera* among other solvents used in the extraction process.A herbal mixture extract possesses more phytochemical and antioxidant activity, when compared with individual plants. The results of the study also suggest that the herbal mixture of both herbs at an equivalent ratio at the same doses as in individual plants showed synergistic phytochemical and antioxidant activity. Thus, lower doses of a combination of herbs may be used in place of individual higher dose to reduce side effects.

Keywords: Phytochemical, Antioxidant, Synergy, *Boerhavia diffusa*, *Ahcyranthes aspera*.

INTRODUCTION

Reactive oxygen species (ROS) or cellular oxidants, are formed in animal and human biological system, are accountable for oxidative injury in cellular constituents resulting in a variety of degenerative diseases and aging. This oxidative variant promotes age-related disorders, as well as cardiovascular diseases. Those damages in human biological system are prohibited by cellular antioxidants. Antioxidant-based drugs are recognized to avert a range of diseases such as

Alzheimer's disease, diabetes, stroke, cancer and atherosclerosis[1, 2,3]. Different macromolecules like proteins and DNA are injured by free radicals and events of these radicals are retarded by antioxidants via scavenging or inhibiting their development.

The *in vitro* antioxidant property, and radical scavenging activity of polyherbal formulated plants, which contain two medicinal plants *Achyranthes aspera* and *Boerhavia diffusa* have been used widely nowadays [4]. 80% population of developing countries has been predicted to rely on traditional medicines for their major health care[5]. Plants are natural factories that constantly produce compounds in themselves. These compounds acquire abundant biological and pharmacological properties which form a source for the synthesis of various drugs based on natural sources. All over the ages humans have relied on nature to endow for their basic needs, such as medicines for the cure of a wide range of diseases. In recent years the wide utilize of medicinal herbs is reported in the field of toxicities such as hepatotoxicity, cardiotoxicity, renal toxicity, etc. In order to study the synergistic effect of formulated plants, we evaluated, yield, phytochemicals, antioxidant and radical scavenging activity of two widely used herbs, *Achyranthes aspera* and *Boerhavia diffusa* individually and in combinations.

Boerhaavia diffusa L. (Nyctaginaceae) known as 'Punarnava' in the Indian system of medicine is a perennial creeping belongs to a species of flowering plant in four o'clock family, found throughout the wasteland of India. The roots are apparent to be laxative and diuretic are given for the treatment of jaundice, ascites and anasarca. The genus *Boerhaavia*, consisting of 40 species is dispersed in the tropical and subtropical region and also in humid temperature. Out of 40 species of *Boerhaavia*, six species are distributed in India, namely *B. diffusa, B.erecta, B. rependa, B. chinensis, B. hirsute* and *B. rubicunda. Boerhaavia diffusa* is seen in warmer regions of India. Grows as a common weed. Useful parts of the plants are seeds, root and leaves.

Scientific Name: *Boerhaavia diffusa* Linn.

Family: Nyctaginaceae,

Family Name: Horse Purslane, Hog weed,

Common Names; Hindi: Snathikari, Gujarati: Dholia-saturdo, Moto- satoda, Sanskrit: Punarnava, Raktakanda, Shothaghni Bengali: Punurnava, Telugu: Punernava, Marathi: Tambadivasu, Varshabhu, Kannada: Kommegida, Tamil: Mukaratee-Kirei[6].

Achyranthes aspera Linn. (Amaranthaceae) is an annual, pubertal stiff, erect herb usually well-known as Chirchita in Hindi, identified as a weed all over India, tropical Asia and other regions

of the world. *Achyranthes aspera* is reported to have a variety of actions such as immunomodulatory, anti-inflammatory, anti- peroxidative, antibacterial cancer, chemopreventive, hepatoprotective, anti- leprotic, anti-arthritic, reproductive functions, contraceptive and thyroid-stimulating[7].

Saponins of *Achyranthes aspera* are studied to contain phosphorus activity of the heart. Root extract is used in malarial fever, diabetes, hypertension and asthma. The whole plant decoction is used in the treatment of pneumonia and has diuretic properties. *Achyranthes aspera* are also used in abdominal tumor, stomach pain and as astringents for wounds[8]. In this study, the yield, phytochemicals, antioxidant and radical scavenging activity of individual plant and their combined mixtures were examined.

EXPERIMENTAL

Plant Material

The plant materials of *Boerhaavia diffusa* and *Achyranthes aspera we*re collected from Coimbatore district of Tamilnadu, India. The plant was recognized and authenticated by the herbarium center of botanical survey of India; Coimbatore, Tamilnadu.

Preparation of Individual Plant Extract

The roots of the *Boerhaavia diffusa* and *Achyranthes aspera* were dried within the shade for 6-8 weeks and kept at 25°C. It has been ground to a fine powder; 100 g of each plant root powder was passed through sieve no. 40 and stored in airtight bottles separately. The soxhlation extraction process was carried out separately for each plant root extract using different solvents and dried. The extract obtained was subjected to preliminary phytochemical analysis.

Preparation of Polyherbal Formulation:

The roots of Achyranthes aspera and Boerhavia diffusa were shade dried for 6-8 weeks and powdered finely in a mixture and sieved twice to obtain a fine powder. 100 g of dried powder (1:1) of the each plant roots were mixed and extracted with Soxhlet extractor using 70 % ethanol till solvent was colourless. The extract was dried to constant weight was acquired.

10 mg residue of the herbal root mixture and individual plant root extract was dissolved in 10ml of ethanol, boiled in water bath for 5-10 minutes, cooled and centrifuged at 3000 rpm for 10 minutes.The clear supernatant was used for evaluating phytochemical and antioxidant properties.

Qualitative Phytochemical Analysis:

Phytochemical screening tests of the herbal preparation were performed according to standardize procedures. The polyherbal extract was identified to contain the important phytochemicals like flavanoids, alkaloids, phenols, etc., which prove to develop the anti-oxidant potential[9].

Quantitative Phytochemical Analysis:

Determination of Total Flavonoid Content:

The flavonoid content was determined by the use of a slightly modified calorimetry method described previously[10]. A 0.5ml aliquot of appropriately (10 mg/2ml) diluted sample solution was mixed with 2ml of distilled water and subsequently with 0.15ml of 5% $NaNO_2$ solution. After 6 minutes, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 minutes, and then 2ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5ml, and then the mixture was thoroughly mixed and allowed to stand for another 15minutes. Absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent.

Determination of Total Alkaloid Content:

Total alkaloid content was determined according to the method described[11]. One ml of the sample solution (5 mg/ml) was transferred to a separating funnel, and then 5 ml of BCG solution (69.8 mg bromocresol green, 3 ml of 2N NaOH and 5 ml distilled water were mixed and raised to 1 L with distilled water) along with 5 ml of phosphate buffer (2 M sodium phosphate adjusted to pH 4.7 with 0.2 M citric acid) was added. The mixture was shaken, and the complex formed was extracted thrice with chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. The analysis was performed in triplicate and the results were expressed as atropine equivalent.

Determination of Saponins:

0.5 g of the sample was added to 20 ml of 1NHCl and was boiled for 4 h. After cooling, it was filtered and 50 ml of petroleum ether was added to the filtrate to ether layer and evaporated to dryness. 5 ml of acetone ethanol was added to the residue. 0.4 ml of each was taken into 3 different test tubes. 6 ml of ferrous sulphate reagent was added into them followed by 2 ml of conc. H_2SO4. It was thoroughly mixed after 10 min and the absorbance was taken at 490 nm[12].

Determination of Total Phenolics:

The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15min. 5 ml of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark in the 50 ml flask and left to react for 30 min for colour development. This was measured at 505 nm[13]. The analysis was performed in triplicate and the results were expressed as tannic acid equivalents.

Determination of Tannins:

5 g of the ground sample was shaken constantly for 1 min with 3 ml of methanol in a test tube and then poured into a Buchner funnel with the suction already turned on. The tube was quickly rinsed with an additional 3 ml of methanol and the content poured at once into the funnel. The filtrate was mixed with 50 ml of water and analyzed within an hour. For aqueous extractions, 5 ml of water was used for the extraction and for the rinse and the filtrate was added to 50 ml of water. 3 ml of 0.1 ml FeCl3 in 0.1 NH_4Cl was added to 5 ml of the extract and followed immediately by timed addition of 3 ml of 0.008 ml K_2, Fe (CN) $_6$. The absorbance was taken at 720 nm spectrophotometrically[14].

Estimation of Steroid:

To 0.1 ml of plant extract, added 4.9ml of ferric chloride precipitating reagent. Centrifuged and to supernatant, 2.5ml of ferric chloride diluting agent and 4.0 ml of concentrated sulfuric acid was added. A blank was prepared simultaneously by taking 5.0 ml of diluting reagent and 4.0ml of concentrated sulphuric acid. A set of standards (0.5-2.5 ml) were taken and made up to 5.0 ml with ferric chloride diluting reagent. Then added, 4.0 ml of concentrated sulphuric acid. After 30 minutes, the intensity of color developed was read at 540nm against a reagent blank. The amount of steroids in the sample is expressed as mg/dl[15].

Evaluation of *In Vitro* Antioxidant Activity

Assay of Superoxide Dismutase (SOD):

The assay of superoxide dismutase was done according to the method described [16]. In this method, 1.4ml aliquots of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer of pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride and 0.1ml of 50 mM EDTA) was added to 100µl of the sample extract and incubated at 30ºC for 5 minutes. 80 µl of 50 µM riboflavin was added and the tubes were exposed for 10 min to 200 W- philips fluorescent lamps. After the exposure time, 1ml

of Greiss reagent (a mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and the absorbance of the color formed was measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under the assay conditions

Assay of Catalase (CAT):

The enzyme extract (0.5 ml) was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O and incubated for different time period. The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 minutes and the absorbance was read at 610 nm. Catalase activity was expressed in terms of µmoles of H_2O_2 consumed/min/mg protein[17].

Assay of Glutathione Peroxidase (GPx):

Glutathione peroxidase was assayed according to the method described[18] with slight modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of water and 0.5 ml of plant extract was incubated at 0, 30, 60, 90 seconds respectively. The reaction was terminated with 0.5 ml of 10% TCA and after centrifugation; 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1ml of DTNB reagent (0.04% DTNB in 1% sodium citrate). The color developed was read at 412 nm and the enzyme activity is expressed in terms of µg of glutathione utilized/min/mg protein

Estimation of Reduced Glutathione (TRG):

The amount of reduced glutathione in the samples was estimated by the method described[19]. 1ml of the sample extract was treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl dissolved in 100ml water). After centrifugation, 2.0 ml of the protein-free supernatant was mixed with 0.2 ml of 0.4 M Na_2HPO_4 and 1.0 ml of DTNB reagent (40 mg DTNB in 100 ml of aqueous 1% tri sodium citrate). Absorbance was read at 412 nm within 2 minutes. GSH concentration was expressed as nmol/mg protein.

Estimation of Vitamin C:

The assay mixture of vitamin C consisted of 0.1 ml of brominated sample extract, 2.9 ml of distilled water, 1 ml of 2% DNPH reagent and 1-2 drops of thiourea. After incubation at 37ºC for 3 h, the orange-red osazone crystals formed were dissolved by the addition of 7 ml of 80% sulphuric acid and absorbance was read at 540 nm after 30 minutes. Vitamin C concentration was expressed in terms of µg/mg plant tissue[20].

RESULTS AND DISCUSSION

Yield of Plant Extract:

When compared with the different solvents employed for the individual and polyherbal mixture of *Achyranthes aspera* and *Boerhaavia diffusa*, the ethanolic extracts of the plants showed higher percentage of yield followed by the plant extracts of aqueous, methanol, petroleum ether and chloroform as shown in the table 1. Hence the investigation of secondary metabolites and the antioxidant efficacy were carried out on the individual and the polyherbal extract of *Achyranthes aspera* and Boerhaavia *diffusa*.

Qualitative Phytochemical Analysis:

The present study carried out on the plant extracts revealed the presence of medicinally active constituents. The investigated phytochemical characters of the individual and polyherbal extracts of *Achyranthes aspera* and *Boerhaavia diffusa* are summarized in table 2. Alkaloids, tannins, flavonoids, saponin, carbohydrates, phenols, glycosides and steroids were present in all the three ethanolic extracts of plants, *Achyranthes aspera*, *Boerhaavia diffusa* and their polyherbal mixture. Proteins were absent in both ethanolic extracts of *Achyranthes aspera* and *Boerhaavia diffusa*, respectively. Only the polyherbal extracts, showed the presence of proteins (Table 2).

These results revealed that the plant has relatively a number of chemical constituents, which possess several pharmacological actions. Reports have shown that a large amount of active compound constituents of plants is phenolics, alkaloids, tannins and saponins. Alkaloids are very essential in medicine and comprise most of the valuable drugs[21]. Phenolic is considered as potentially noxious for the growth and development of pathogens, whereas tannins are fairly effective bioactive compounds used for therapeutic measures. Flavonoids are the most widely studied phenolic and antioxidant compounds, which play more rigorous roles in ethno-pharmacology.The steroids and saponins were responsible for central nervous system activities[22].

Quantitative Phytochemical Analysis:

Quantitative estimation of the phytoconstituents studied in the ethanolic extracts of *Achyranthes aspera*, *Boerhaavia diffusa* and the polyherbal extracts of both plants was summarized in table 3. From the estimated values of phytochemicals shown in the table 3 the herbal mixture of ethanolic extract showed the highest content of flavonoids, phenolics, alkaloids, tannins, steroids and saponins followed by the presence of moderate amounts of above phytoconstituents in the ethanolic extract of Boerhaavia *diffusa* and lowest amount of the phytoconstituents was found to be presented in the ethanolic extract of *Achyranthes aspera*.

Estimation of Enzymatic Antioxidants:

The level of enzymatic antioxidants such as SOD, CAT, GPx, showed in table 4. SOD, CAT and glutathione peroxidase in the of polyherbal extract of *A. aspera* and *B. diffusa* were found to be high as 39.68 ± 0.47/mg protein, 40.88 ± 0.11µmole of H_2O_2 consumed/min/mg proteins and 272.63 ± 2.34µg of glutathione oxidized/min/mg protein respectively. The ethanolic extract of *A. aspera* showed the lowest antioxidant activity values of SOD, CAT and glutathione peroxidase as 18.97±0.42/mg protein, 22.58±1.02 µmole of H_2O_2 consumed/min/mg proteins and 87.96±2.334µg of glutathione oxidized/min/mg protein among the ethanolic extract of *B. diffusa* and polyherbal extract of plants, *A. aspera* and *B.diffusa*. The SOD, CAT and glutathione peroxidase in the ethanolic extract of *B. diffusa* showed moderate antioxidant activity values, 22.03±0.12/mg protein, 31.26±2.8µmole of H_2O_2 consumed/min/mg proteins and 174.33±1.18µg of glutathione oxidized/min/mg protein among the ethanolic extract of *A. aspera* and polyherbal extract of plants, *A. aspera* and *B.diffusa*.

Natural compounds extracted from plants have shown to possess phytochemical antioxidant properties which are valuable in curing diseases caused by oxidative damage. Oxidative injury in cells has been suggested to take place by reactive oxygen species (ROS) formed as a consequence of ETC in mitochondria. A number of analyses have been suggested that ROS can influence critical measures linked to many disorders [23]. The development of ROS is prohibited by antioxidant coordination: low molecular weight antioxidants and ROS-interacting enzymes such as SOD, regenerating the reduced forms of antioxidant enzymes, catalases and peroxidases[24]. The SOD enzyme eliminates the superoxide radical[25]. It has been noticed as one of the most vital antioxidant defense enzyme that hunt superoxide anion by converting to hydrogen peroxide thus diminish the toxic effect caused by this radical[26].

Catalase is a tetrahedral protein, formed by four heme groups that catalyze the dismutation of hydrogen peroxide into oxygen and water[27]. Glutathione peroxidases are significantly more competent on a molar base than other enzymes[28, 29]. Glutathione peroxidase acts as a, membrane stabilizer, radical scavenger[30] and precursor of heavy metal strap peptides[31]

Estimation of Non-Enzymatic Antioxidants:

The level of non-enzymatic antioxidants such as total reduced glutathione and vitamin C of the individual and herbal mixture extracts showed in table 5. The antioxidant activity of total reduced glutathione and vitamin C present in the polyherbal ethanolic extract of *A. aspera* and *B.diffusa* was found to be 71.31 ± 1.69 nmol / mg protein and 263.66 ± 2.35µg/mg plant tissue, whereas the values of above antioxidant activity in the ethanolic extract of *B. diffusa* were found to be 49.13 ± 2.17 nmol / mg protein and 197.17 ± 0.26 µg/mg plant tissue. Ethanolic extract of *A. aspera* showed the antioxidant activity of total reduced glutathione and vitamin C as 33.12 ± 0.58 nmol / mg protein and 116.99 ± 0.91µg/mg plant tissues. Hence, the activity of total reduced glutathione and vitamin C of the polyherbal ethanolic extract showed higher antioxidant activity when compared with individual ethanolic extract of *A. aspera* and *B. diffusa*.

Glutathione, a chief non-protein thiol in living organisms, plays a crucial role in coordinating the body's antioxidant defense mechanism. Extreme peroxidation leads to huge glutathione consumption. Reduced thiols have long been reported to be important for recycling of antioxidants, vitamin C and vitamin E[32]. Vitamin C is a water soluble antioxidant and serves as a free radical scavenger. It scavenges peroxyradicals. Vitamin C is observed as the initial line natural antioxidant guard in plasma and a potent inhibitor of LPO [33, 34]. It has been found in the cytosol, vacuole, chloroplast and extracellular compartments of the plant cells and it act as a reluctant for various free radicals [35].

CONCLUSION

The individual and polyherbal formulation of *Achyranthes aspera* and *Boerhavia diffusa* showed higher yields in ethanolic extract. Polyherbal formulation of *Achyranthes aspera* and *Boerhavia diffusa* showed synergistic phytochemical and antioxidant activity when compared with the individual ethanolic extracts of *Achyranthes aspera* and *Boerhavia diffusa*. The present study suggests lower doses of mixture of herb may be used in place of individual higher dose to minimise side effects. Thus, polyherbal ethanolic extract of *Achyranthes aspera* and *Boerhavia diffusa* is a potential source of phytochemical and natural antioxidant that might have great

significance as therapeutic agents in preventing or slowing the oxidative stress associated degenerative diseases such as cancer and other numerous human ailments. Further research of these medicinal plants in combination is required to bring out in vivo studies in order to develop natural pharmaceutical products of high value.

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Table-1: Yield of plant extracts in different solvents

EXTRACT	*Achyranthes aspera* (%)	*Boerhavia diffusa* (%)	Herbal formulation (%)
Aqueous	21	23	42
Ethanol	24	26	56
Methanol	19	16	39
Petroleum Ether	17	13	28
Chloroform	11	14	26

Table-2: Preliminary phytochemical screening of ethanolic extracts

Tests	*Achyranthes aspera*	*Boerhavia diffusa*	*Herbal formulation*
Alkaloids			
Dragendroff's test	+	+	+
Wagner's test	-	+	+
Meyer's test	+	-	+
Flavanoid	+	+	+
Saponin	+	+	+
Carbohydrate			
Fehling's test	+	+	+
Benedicts test	+	+	+
Molisch's test	+	+	+
Protein			
Millon's test	-	-	+

("+" indicates presence of phytochemical and "–" indicates absence of phytochemical)

Table-3: Quantitative phytochemical estimation of ethanolic extracts

Ethanolic Extract	Flavonoids (mg RE / g sample)	Total Phenolics (mg TAE / g sample)	Total Alkaloids (mg / g)	Steroids (mg / dl)	Tannins (mg/ 100 g)	Saponins (mg / 100 g)
Achyranthes aspera	28.18 ± 0.44	23.02 ± 0.70	18.32 ± 0.6	9.1 ± 0.24	3.7 ± 0.16	2.77 ± 0.81
Boerhavia diffusa	51.02 ± 0.11	31.14 ± 0.81	24.03 ± 1.02	12.37 ± 0.36	4.9 ± 0.63	5.02 ± 0.01
Herbal formulation	69.25 ± 0.41	42.23 ± 1.51	33.06 ± 0.16	17.09 ± 0.26	9.03 ± 0.04	6.47 ±0.40

Table-4: Enzymatic antioxidant activity of ethanolic extracts

Ethanolic Extract	Superoxide Dismutase (Units / mg protein)	Catalase (µmol of H_2O_2 consumed / min / mg protein)	Glutathione Peroxidase (µg of glutathione oxidized / min / mg protein)
Achyranthes aspera	18.97 ±0.42	22.58 ± 1.02	87.96 ± 2.3
Boerhavia diffusa	22.03 ± 0.12	31.26 ± 2.8	174.33 ± 1.18
Herbal formulation	39.68 ± 0.47	40.88 ± 0.11	272.63 ± 2.34

Table-5: Non enzymatic antioxidant activity of ethanolic extracts

Ethanolic Extract	Total reduced glutathione (nmol / mg protein)	Vitamin C (µg / mg plant tissue)
Achyranthes aspera	33.12 ± 0.58	116.99 ± 0.91
Boerhavia diffusa	49.13 ± 2.17	197.17 ± 0.26
Herbal formulation	71.31 ± 1.69	263.66 ± 2.35