Research Article

Free Radical Scavenging Potential of A Poly Herbal Formulation

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ABSTRACT

The present study was carried out to evaluate the free radical scavenging activity of a poly herbal extract and its cytotoxicity in BRL-3A cell line by SRB assay. Different solvent extracts (petroleum ether, chloroform and hydroethanolic) of the formulation was tested for its DPPH assay, Super oxide radical, Hydroxyl radical and Nitric oxide radical scavenging activity. In cytotoxicity assay, hydro ethanolic extract was found to be toxic (CTC >100) than other two solvent extracts. The potential scavenging activity and its cytotoxicity suggests that the hydro ethanolic extract of the poly herbal formulation has significant antioxidant properties and can be used as a potent drug in several diseases.

Keywords: BRL-3A, DPPH, FRAP assay, Poly herbal extract, SRB assay.

INTRODUCTION

In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants in various traditional systems.[1] Natural antioxidants have been known to play an important role in alleviating the deleterious effects induced by free radicals by blocking the initiation or propagation of oxidizing chain reactions.[2] Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer.[3] Polyherbal formulations are preferred than a single herb by the traditional healers for the management of several diseases and disorders. When herbs are combined together, they become more potent and effective due to their activating or catalyzing influence.

Herbal formulations are enriched with enormous amount of free radical scavenging molecules, such as vitamins, terpenoids, phenolic acids, lignins, tannins, flavonoids, alkaloids, amines and other metabolites, which stimulate the function of immune system under several disease conditions and aging process. The aim of the present study was to determine the free radical scavenging activity of a polyherbal formulation and its cytotoxicity.

MATERIALS AND METHODS

Chemicals

NBT, hemoglobin powder, EDTA, DPPH, were obtained from Precision company Limited, Tamil Nadu.

Plant Material and Extraction

Poly herbal formulation contains *Semecarpus anarcadium* (seeds), *Curculigo orchioides* (rhizomes), *Asparagus racemosus* (root), *Plumbago zeylanica* (root) and *Tinospora cordifolia* (plant). These plants are equally weighed, shade dried and coarsely powdered. 100 g of dried powder was cold macerated with 50 % hydro ethanol, petroleum ether and chloroform with occasional stirring for 3 days. After 3 days, the suspension was filtered through a fine muslin cloth and the filtrate was evaporated to dryness at low temperature (<40^0 C) under reduced pressure in a rotary evaporator. The yield of plant extract was 2.35% for petroleum ether 3.57% for chloroform extract and 9.64% for hydro ethanolic extract. The samples were stored in an air-tight desiccator and used for further analyses.

DPPH Spectrophotometric Assay

A methanolic solution of 0.5 ml of DPPH (0.4 mM) was added to 1 ml of the different concentrations (125, 250, 500, 1000 µg/ml) of plant extracts and allowed to react at room temperature for 30 minutes. Methanol served as the blank. After 30 min, the absorbance was measured at 518 nm and converted into percentage radical scavenging activity as follows.[4]

$$\text{Scavenging activity (\%)} = \frac{\text{A Control} - \text{A sample}}{\text{A Control}} \times 100$$

Super Oxide Radical Scavenging Activity

Super oxide radical ($O_2^{\cdot-}$) was generated from the photo reduction of riboflavin and was detected by nitro blue tetrazolium dye (NBT) reduction method. The assay mixture contained sample with 0.1 ml of Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 ml of EDTA (0.1M EDTA), 0.05 ml riboflavin (0.12 mM) and 2.55 ml of phosphate buffer (0.067 M). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Ascorbate was used as the reference compound.[5]

Hydroxyl Radical Scavenging Activity

The assay is based on quantification of degradation product of 2-deoxy ribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+} -Ascorbate – EDTA –H_2 O_2 system (Fenton reaction). The reaction mixture contained 0.1 ml deoxyribose (2.8mM), 0.1 ml EDTA (0.1 mM), 0.1 ml H_2 O_2 (1mM), 0.1 ml Ascorbate (0.1mM), 0.1 ml KH_2PO_4-KOH buffer, P^H 7.4 (20mM) and various concentrations of plant extracts in a final volume of 1 ml. The reaction mixture was incubated for 1 hour at 37^0 C. Deoxyribose degradation was measured as TBARS and the percentage inhibition was calculated.[6]

Nitric Oxide Radical Scavenging Activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions. The reaction mixture (3ml) containing 2 ml of sodium nitroprusside (10mM), 0.5 ml of phosphate buffer saline (1M) were incubated at 25^0C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was pipetted and mixed with 1 ml of sulphanilic acid reagent (0.33%) and allowed to stand for 5 min for completing diazotization.

Then 1 ml of naphthylethylene diamine dihydrochloride (1% NEDA) was added, mixed and allowed to stand for 30 min. Sodium nitroprusside in aqueous solution at physiological P^H spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions which can be estimated by the use of Griess Illosvery reaction at 540 nm.[7]

Quantitative Estimation of Phytochemicals

Total Phenols

To 0.25 g of sample, added 2.5 ml of ethanol and centrifuged at 2ºC for 10 min. The supernatant was preserved. Then, the sample was re-extracted with 2.5 ml of 80% ethanol and centrifuged. The pooled supernatant was evaporated to dryness. Then, added 3 ml of water to the dried supernatant. To which added 0.5 ml of Folins phenol reagent and 2 ml of sodium carbonate (20%). The reaction mixture was kept in boiling water bath for 1 min. the absorbance was measured at 650 nm in a spectrophotometer.[8]

Flavonoids

0.2 g of the plant material was ground with ethanol-water in 2 different ratios namely 9:1 and 1:1 respectively. The homogenate was filtered and these two ratios were combined. This was evaporated to dryness until most of the ethanol has removed. The resultant aqueous extract was extracted in a separating funnel with hexane or chloroform. The solvent extracted aqueous layer was concentrated 0.5 ml of aliquot of extract was pipetted-out in a test tube. 4 ml of the vanillin reagent (1% vanillin in 70 % conc. H_2SO_4) was added and kept in a boiling water bath for 15 min. The absorbance was read at 360 nm. A standard was run by using catechol.[9]

Cytotoxicity by SRB Assay

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different concentrations of the extract (1000, 500, 250, 125, 62.5 µg) were added to the cells in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, microscopic examination was carried out and observations were recorded for every 24 h. After 72 h, 25 µl of 50% trichloro acetic acid was added to the wells gently in such a way that it forms a thin layer over the extract to form an overall concentration of 10%. The plates were incubated at 4^o C for 1 h. The plates were flicked and washed five times with water to remove traces of medium, extract and serum, and air-dried. They were stained with SRB for 30 min. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. Tris base (10 mM, 100 µl) was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using micro plate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated as mentioned.[10]

% Growth Inhibition =

100- Mean OD of individual test group X 100 / Mean OD of control group

RESULTS

Different concentrations of the plant extract (125, 250, 500, 1000 µg / ml) and rutin decolorized the DPPH solution. The maximum DPPH scavenging activity of 57.36% was observed for hydro ethanolic extract at 1000 µg/ml whereas for rutin it was found to 60.1%. The IC_{50} of the hydro ethanolic extract and rutin were 550 µg /ml and 350 µg /ml respectively (Table 1).

The percentage scavenging of superoxide radical surged with the enhanced concentration of the polyherbal extract. The maximum scavenging activity of different herbal extracts and quercetin at 1000 µg/ml was found to be 68.66 %, 76.7 %, 73.3 % and 57.4% respectively (Table 2).

The nitric oxide scavenging method showed significant activity of poly herbal extract when compared to the standard Mannitol. The IC_{50} value of the solvent extracts and the standard was found to be 620 µg /ml, 580 µg /ml, 520 µg/ml and 610 µg/ml respectively (Table 3). The hydro ethanolic extract at a higher concentration of 1000 µg/ml showed maximum inhibition of nitric oxide radical than other extracts.

Table 1: DPPH radical scavenging activity of the herbal formulation

Concentration of the poly herbal extract(µg /ml)	Percentage scavenging activity of DPPH radical			
	Petroleum ether extract	Chloroform extract	Hydro ethanolic extract	Rutin
125	14.0±1.54	15.75±1.37	15.7±0.95	16.95±1.42
250	29.1±1.13	22.28±0.88	26.5±0.97	27.5±1.28
500	37.06±0.68	28.53±1.17	40.48±0.80	37.28±1.01
1000	48.56±1.11	56.71±1.55	57.36±1.74	61.20±1.09
Ic 50	980 µg /ml	650 µg /ml	550 µg /ml	350 µg /ml

Table 2: Super oxide radical scavenging activity of the herbal formulation

Concentration of the poly herbal extract(µg /ml)	Percentage scavenging activity of superoxide radical			
	Petroleum ether extract	Chloroform extract	Hydro ethanolic extract	Quercetin
125	12.33±0.13	14.46±1.15	16.54±1.89	13.5±1.25
250	26.36±1.04	26.53±1.36	27.53±0.74	27.60±1.15
500	47.75±1.39	35.46±1.33	46.7±1.65	42.37±1.60
1000	68.66±1.47	76.7±1.62	73.3±1.67	57.4±1.25
Ic 50	500 µg /ml	330 µg /ml	350 µg /ml	750 µg /ml

Table 3: Nitric oxide radical scavenging activity of the herbal formulation

Concentration of the polyherbal extract (µg /ml)	Percentage scavenging activity of nitric oxide radical			
	Petroleum ether extract	Chloroform extract	Hydro ethanolic extract	Mannitol
125	25.9±0.75	29.86±1.80	28.6±0.75	13.01 ± 1.16
250	40.56±1.43	42.9±0.56	42.9±0.77	28.02 ± 0.91
500	53.36±1.13	49.24±0.66	68.9±0.52	37.96 ± 0.91
1000	76.95±1.59	80.18±1.36	92.2±1.28	78.14 ± 2.09
Ic 50	620 µg /ml	580 µg /ml	520 µg /ml	610 µg /ml

Table 4: Hydroxyl radical scavenging activity

Concentration of the poly herbal extract (µg /ml)	Percentage scavenging activity of hydroxyl radical			
	Petroleum ether extract	Chloroform extract	Hydro ethanolic extract	Ascorbate
125	23.39±1.35	25.75±1.60	24.59±1.36	28.80 ± 2.77
250	44.30±1.20	39.82±1.36	42.98±1.34	43.30 ± 2.05
500	66.34±0.95	66.0±2.41	62.57±0.76	72.10 ± 2.06
1000	75.35±1.54	70.4±1.85	72.92±2.9	86.60 ± 2.77
I c50	300 µg /ml	500 µg /ml	480 µg /ml	230 µg /ml

Table 5: Quantitative Estimation of Phytochemicals

Phenolic compounds	Petroleum ether extract	Chloroform extract	Hydro ethanolic extract
Phenols mg(**pyrocatechol/g of plant tissue**)	14.25±0.98	14.65±0.54	15.44±0.45
Flavanoids mg **(catechin/g of plant tissue)**	8.9±1.45	9.0±1.21	9.3±1.25

The maximum scavenging of hydroxyl radical was observed with 1000 µg/ml of petroleum ether extract and its inhibitory percentage was 75.35%. Ascorbate is used as a standard and the maximum scavenging effect was observed as 86.60% (Table 4).

Polyphenols are the major plant compounds with potential antioxidant capacity. The total phenolic content of the poly herbal extract was found to be 14.25, 14.65, and 15.44 mg of pyrocatechol for pet ether, chloroform and hydroethanolic extract. Flavonoids are secondary metabolites exhibit antioxidant activity. The flavonoid content for different extract was recorded to be 8.9, 9.0, 9.3 mg of catechin / g plant tissue (Table 5).

In cytotoxicity assay, the Ic 50 values were found to be 61.2 µg/ml, 91.33 µg/ml and 935 µg /ml for pet ether, chloroform and hydro ethanolic extract (Figure 1) respectively.

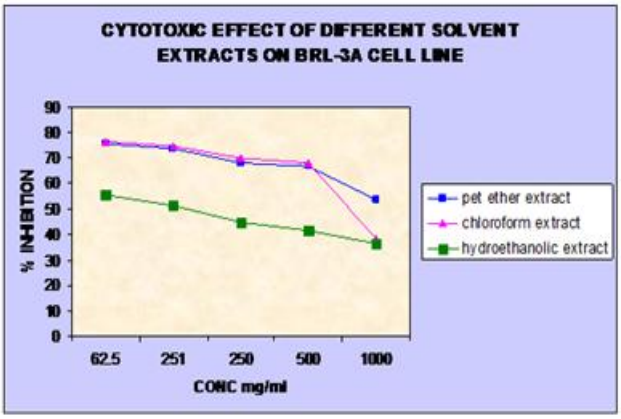

Figure 1: Cytotoxic effect of different solvent extracts on BRL-3A cell line

DISCUSSION

Reactive oxygen species (ROS) such as superoxide anions ($O2^{-}$), hydroxyl radical ($OH^{\cdot}$), and nitric oxide (NO) inactivate enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation. The increased production of toxic oxygen derivatives are considered as a universal feature of stress.[11] Antioxidant principles from natural resources possess versatileness in their magnitude of activities and provide enormous scope in correcting oxidative stress produced by the human body. Combination of herbs can make a premium anti oxidant herbal formulation, which can be used for the treatment of many diseases associated with free radical generation.

DPPH assay is being used widely as a preliminary test which provides information on the reactivity of test compound. This method is based on the reduction of alcoholic DPPH solution in the presence of hydrogen donating antioxidant (AH) due to the formation of non-radical form DPPH-H.[12] Antioxidant react with DPPH which is a stable free radical and is reduced to the DPPH-H and as a consequence the absorbance decreased from the $DPPH^{\cdot}$ radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds present in the poly herbal extract in turns of hydrogen donating ability.[13]

The super oxide radical is ubiquitous in aerobic cells. superoxide ions are produced from molecular oxygen due to oxidative enzymes of body as well as via non-enzymatic reaction such as auto oxidants by catecholamines.[14] In this study, superoxide radical reduces NBT to a blue colored formazan. The decrease in absorbance with antioxidants indicates the consumption of superoxide anion in the reaction mixture.[15]

Hydroxyl radicals are the major active oxygen species causing LPO and enormous biological damage.[16] The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipid and protein.[17] Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological process including neurotransmission, vascular homeostasis, antimicrobial and antitumor activities. However, excess production of NO is associated with several diseases. It would be interesting to develop potent and selective inhibitors of NO for potential therapeutic use.[18]

The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO-). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The poly herbal extract inhibits nitrite formation by directly competing with oxygen.[19] The present study proved that the extracts studied has more potent nitric oxide scavenging activity than the corresponding standard.

Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups and may contribute directly to antioxidative action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans.[20] The mechanism of action of flavonoids is through scavenging or chelation.[21] Flavonoids are important secondary metabolite of plant, modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It has been confirmed that the pharmacological effect of flavonoids correlating with their antioxidant activities.[22]

The BRL 3A immortal rat liver cell line was selected in the present study as a convenient *in vitro* model and it is well characterized for its relevance to toxicity models.[23] The cytotoxicity testing is based on one or more mammalian cell lines being grown under conditions where they are actively growing and undergoing mitotic cell divisions. [24] The SRB assay is a sensitive, simple, reproducible and more rapid than the formazan based assays and gives better linearity, and has a stable end-point.[25]

In this study, among the solvents tested in buffalo rat liver cell line, hydro ethanolic extract was found to be toxic when compared to other solvents like petroleum ether and chloroform. When the cell is lysed, greater amount of dye is also released which increase the absorbance. The higher amount of phytochemicals and secondary metabolites in the hydro ethanolic extract contributes its cytotoxicity in the normal liver cell line.

CONCLUSION

Polyherbal formulation has complex and inconsistent composition particularly for its antioxidants and secondary metabolites. These components play a direct role in fighting against several diseases. The present study confirms that hydro ethanolic extract of the poly herbal formulation was found to be cytotoxic and have high antioxidant effect when compared to other non polar solvent extracts. Further research is needed to evaluate the effect of these extracts in cancer cell lines and clinical studies should be carried out to establish its potency.

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