Research Article

Evaluation of Antioxidant Action of Polyherbal Formulation in Oxidative Stress Induced Animals by Assessment of Reactive Oxygen Species Level - an *In vivo* Study

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Accepted on: 08-07-2016; **Finalized on:** 31-08-2016.

ABSTRACT

Sida cordifolia Linn. and *Glycyrrhiza glabra* Linn. are two well-known rejuvenator medicinal plants which have been ascribed many medicinal properties in Ayurvedic literature. Their roots contain many polyphenols, Flavonoids, saponins, alkaloids and glycosidic compounds. Their pharmacological actions include hypoglycaemic, anti-microbial, antioxidant, antiulcer, anti-mutagenic, hepato-protective, antipyretic and anti-inflammatory activities. In this study, *in vivo* antioxidant analysis of aqueous extract of the combination of roots of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. has been evaluated in animals, especially rats, after inducing acute and chronic oxidative stress using potassium dichromate. Total phenol content determined using Folin-Ciocalteu reagent was found to be 28.60 µg Gallic acid/mg. Acute toxicity study using mice showed no significant toxic symptoms or mortality up to 1500 mg/kg dose. Total Antioxidant Capacity by Phosphomolybdate Assay in concentrations of 3, 6 and 9 mg/ml was evaluated to be 16.48 ± 80, 18.97 ± 8.87 and 20.98 ± 8.93 (µg/mg) equivalents of ascorbic acid. Determination of Reactive Oxygen Metabolites (d-ROMs Test) to evaluate the level of hydroperoxides in terms of U. CARR units indicated that effect of agents causing chronic or acute oxidative stress can be mostly neutralized by research drug polyherbal formulation administration. Similarly, Determination of Reactive Oxygen Species by ELISA kit in the form of Optical Density values suggests that effect of acute and chronic oxidative stress is drastically reduced due to simultaneous administration of research drug. Therefore, experimental evaluation of in vivo antioxidant properties of aqueous extract of the polyherbal formulation using the chronic and acute oxidative stress models in rats indicated its significant efficacy as an antioxidant which is non-toxic and able to counter the negative effects of induced oxidative stress in animals, possibly due to presence of polyphenolic compounds and flavonoids in high concentrations in its abstract.

Keywords: antioxidant, oxidative stress, polyherbal, ROS, ROM, *in vivo*, *Sida cordifolia, Glycyrrhiza glabra*.

INTRODUCTION

Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer. Plants and foods are recognized as important sources of natural antioxidants and thus play an important role in the prevention and treatment of diseases related to oxidative stress by decreasing the reactive oxygen species. Plants have an innate ability to biosynthesize a wide range of non-enzymatic antioxidants capable of attenuating ROS-induced oxidative damage. Several *in vitro* methods have been used to screen plants for their antioxidant potential, and in most of these assays they revealed potent antioxidant activity. However, prior to confirming their *in vivo* therapeutic efficacy, plant antioxidants have to pass through several physio-pharmacological processes. Consequently, the findings of *in vitro* and *in vivo* antioxidant potential assessment studies are not always the same. Nevertheless, the results of *in vitro* assays have been often extrapolated to the therapeutic application of plant antioxidants without undertaking sufficient *in vivo* studies.[1] The aim of the present study was to assess the antioxidant potential of a new polyherbal formulation as a safe and significant antioxidant compound.

Therefore, *in vivo* antioxidant analysis of aqueous extract of the combination of roots of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. in equal amount has been done during the present study in animals models, especially in rats, after inducing the oxidative stress by using potassium dichromate as oxidative stress agent using two different methods. In the first method, it is administered as a single dose and in the second one, the dose was administered daily up to 26 days to generate the acute and chronic oxidative stress following two standard published methods. In the present context, Potassium Di-chromate ($K_2Cr_2O_7$), a potential Cr (VI) compound, was able to generate reactive oxygen radicals in laboratory animals as evidenced from significantly higher oxygen metabolites compared to untreated controls. Chromium (Cr) is a naturally occurring heavy metal found commonly in the environment in two valence states: trivalent Cr (III) and hexavalent Cr (VI). Studies by animal model also found many harmful effects of Cr (VI) on mammals. Once inside the cell, Cr (VI) is reduced to its lower oxidation states Cr (V) and Cr (IV) and then Cr (III) by low molecular weight molecules, enzymatic, and non-enzymatic reductants.[2] These reactive chromium intermediates are capable of generating a whole spectrum of reactive oxygen species (ROS), which is an important characteristic of Cr (VI) metabolism.[3] Excessive quantity of ROS generated by these reactions can cause injury to cellular proteins, lipids, and DNA leading to a state known as oxidative stress.[4-5] Therefore, one of the most important damage caused by extraneous Cr (VI) is massive

production of ROS during the reduction of Cr (VI) in the cell. Under oxygen-dependent stresses, endogenous antioxidants (vitamins, anti-oxidative enzymes, tripeptides and reductants) can quench the reactive oxygen species (ROS) or can terminate the process of lipid peroxidation thus neutralizing the ill effects. A number of phenolic compounds containing plant extracts act as potential antioxidants in ameliorating the degenerative effects from oxidative stress. Therefore, supplementation of inherent anti-oxidants from external sources of potent medicinal plants becomes necessary to combat oxidative stress.

Sida cordifolia belonging to the Malvaceae family is one of the most useful medicinal plants in Ayurvedic literature. Also known as *Bala*, it is a small, erect, annual downy shrub. The leaves of the plant are chordate-oblong or ovate-oblong and fruits with a pair of awns on each carpel. The tap root of the plant is odourless with slightly bitter taste and grayish yellow colour which constitute a cluster 5-15 cm long with few lateral roots of smaller size. It has been used as a cooling, astringent, aromatic, stomachic, diuretic and tonic in Ayurvedic system of medicine for curing of diseases like asthma, cough, fever, wound, skin diseases, heart diseases, facial paralysis, muscle and joint pain, swelling, inflammation, urinary infection, skin diseases, lack of sexual desire and unwanted weight loss. Its roots and seeds contain alkaloid ephedrine, vasicinol, vasicinone, β-sitosterol and stigmasterol and N-methyl tryptophan while the leaves of *Sida cordifolia* contain small amounts of both ephedrine and pseudoephedrine. Its pharmacological actions include hypoglycaemic, wound healing, anti-microbial, antioxidant, anti-inflammatory, analgesic, adaptogenic and hepato-protective activities.[6-11]

Glycyrrhiza glabra Linn. also called Liquorice root belongs to the Fabaceae family. It is a perineal herb/sub-shrub found in the subtropical and temperate zones. The plant attains a maximum height up to 2 m. The underground stem grow horizontally up to 2 m length, highly branched consisting of short tap root with large number of rhizomes. The diameter of the root varies from 0.75 to 2.5 cm, grey-brown exterior and yellow interior. Externally, it is longitudinally wrinkled with patches of cork. It has a characteristic pleasant sweet taste. Leaves alternate, pinnate, yellow green leaflets 4-7 pairs are covered with soft hairs on underside. Flowers appear in axil of terminal and axillary leaves in raceme, pea-like, lavender to purple in colour. Seed pod is 2-2.5cm long containing 2-5 seeds. Flowering & fruiting is from August to February. Its underground stems and roots are used medicinally for treatment of cough, hyperacidity, skin diseases, eye diseases and as a tonic, rejuvenator, demulcent, expectorant, etc. The chief constituent of liquorice is glycyrrhizin, which is present in the drug in the form of the potassium and calcium salts of Glycyrrhizic acid. Glycyrrhizin is 50 times sweeter than Sucrose. Glycyrrhizic acid is not a glycoside since it yields on hydrolysis one molecule of Glycyrrhetic acid and two molecules of Glycuronic acid but no sugar. Glycuronic acid is, however, very closely related to the hexose sugars, and Glycyrrhetic acid has a haemolytic action like that of the saponins. Liqourice also contains glucose (up to 3.8 per cent), sucrose (2.4 to 6.5 per cent), bitter principles, resins, mannite, asparagines (2 to 4 per cent) and fat (0.8 per cent). Its pharmacological activities are reported to be muscle depressant, anti-microbial, hypo-lipidaemic, anti-antherosclerotic, antiviral, hypotensive, hepato-protective, anti-exudative, spasmolytic, antidiuretic, antiulcer, anti-mutagenic, antipyretic, antioxidant, anti-inflammatory, anti-nociceptive and expectorant.[6,8,11-13]

The polyherbal formulation has been prepared by adding equal amounts of dried parts of the roots of *Sida cordifolia* (Bala) and *Glycyrrhiza glabra* (Yashthimadhu) to find out its rejuvenator action on the basis of modern standard methods to assess the level of ROS after introducing oxidative stress in animal models. These two plants have been used since ancient times in the Ayurvedic system of medicine (Charak Samhita Chikitsa Sthanam) as a tonic & aphrodisiac, to increase muscle strength & sexual desire, and to cure respiratory and abdominal diseases in the form of single drug and in combined form. This is a new formulation which has not been evaluated till now although it is likely to exhibit sustained and significant antioxidant action due to the synergetic effect of the phenolic and flavonoidic compounds present in this polyherbal formulation and the pharmacological properties of its constituent herbs.

The antioxidant level of aqueous extract of research formulation was determined in the serum of blood of animals collected from the tail of each rat following standard methods for experimental determination of Oxygen Reactive Metabolites and Oxygen Reactive Species. These levels were analyzed in comparison to the readings of normal control group. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. Examples of ROS include peroxides, superoxide, hydroxyl radical and singlet oxygen. In a biological context, ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically. This may result in significant damage to cell structures. Cumulatively, this is known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation. Ionizing radiation can generate damaging intermediates through interaction with water, a process termed radiolysis. Since water comprises 55–60% of the human body, the probability of radiolysis is quite high under the presence of ionizing radiation. In the process, water loses an electron and becomes highly reactive. Then through a three-step chain reaction, water is sequentially converted to hydroxyl radical (*OH), hydrogen peroxide (H_2O_2), superoxide radical ($^{\bullet}O-2$) and ultimately oxygen (O_2). The hydroxyl radical is extremely reactive and immediately removes electrons from any molecule in its path, turning

that molecule into a free radical and thus propagating a chain reaction.

MATERIALS AND METHODS

Collection of Plant

The roots of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. were purchased from the approved crude drug supplier of Katwa Chowrasta, district Burdwan. The plant samples were authenticated by the Botanical Survey of India at Shibpur, Howrah, India and herbarium was kept in the museum of the Dravyaguna department of the Institute.

Chemicals

Glacial acetic acid (ID: CD3C630227) was obtained from Merck Specialties Pvt. Ltd., Mumbai, Ferrous sulphate (ID: 064963) from Sisco Research Laboratories Pvt. Ltd, Mumbai; N, N-diethyl-p-phenylenediamine (ID: 24855816) and Sodium Acetate (ID: 24899538) from Sigma-Aldrich, Mumbai, while ROS Elisa kit was procured from Elabscience (Lot No: AK0016FEB18047).

Animals

Swiss albino mice of either sex, weighing about 20-30 gm, and albino (Wistar) rats of either sex, weighing about 120-130 gm, were used for different *in-vivo* evaluation. All animals were procured from the Government of West Bengal approved breeder, M/s Satyacharan Ghosh, Kolkata and housed under standard environmental conditions with fixed 12 h light/dark cycles and a temperature of approximately 25°C in animal house of IPGAE&R (Reg. No 1180/ac/08/CPCSEA). The animals were kept in standard polypropylene cages and provided with food (standard pellet diet) and water *ad libitum*. These animals were acclimatized for a period of 14 days prior to performing any experiments. All experimental protocols were approved by the Institutional Animal Ethics Committee.

Preparation of Extracts

The roots of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. were washed, sun dried and crushed to particle size of 80 mesh. This coarse powder of each plant sample taken in equal amounts for the preparation of the polyherbal formulation was sequentially extracted with petroleum ether (60°C-80°C), chloroform, acetone, ethanol and water using soxhlet apparatus. These extracts were filtered using a Buckner funnel and Whatman No. 1 filter paper at room temperature and concentrated at reduced temperature and pressure using rotary evaporator. All obtained extracts were stored in refrigerator below 10°C for subsequent experiments.

The aqueous extract of the roots was used in the present study.

Analysis of Phytochemical Constituents

Systematic analysis using standard methods was done for ascertaining the physical parameters such as moisture content, ash value, extractive value and also for ascertaining the presence of different phyto-chemical constituents such as alkaloids, amino-acids, reducing sugars, tannins, saponins, anthroquinones, steroids, terpenoids, flavonoids and salicyclates (Furniss).[14-15]

Total Phenolic Content

Total phenol content was determined using the Folin-Ciocalteu regent. To 0.5 ml aliquot of sample dried aqueous extract of polyherbal formulation, 2.5 ml of Folin- Ciocalteu's reagent (10 %) and 2 ml of 7.5% of sodium carbonate were added. The absorbance was read after 30 min incubation period at room temperature at 760 nm colorimetrically. A standard calibration plot was generated at 760 nm using known different concentration of Gallic acid (100, 200, 300, 400, and 500 µg/ml). The concentration of phenol in the test samples was calculated from the calibration plot. Total phenolic content was expressed as mg Gallic acid Equivalents (GAE). All determinations were performed in triplicates and the results were expressed as mg Gallic acid equivalents per gm sample extract.[16-17]

Total Antioxidant Capacity by Phosphomolybdate Assay

The total antioxidant capacity was determined following the method of Prieto. The assay is based on the reduction of Mo (VI) – Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3ml polyherbal formulation at different concentrations (300, 600 & 900 µg/ml) was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95ºC for 90 min. Then the absorbance of the solution was measured at 695 nm using a UV-VIS spectrophotometer (shimadzu UV 2450) against blank after cooling to room temperature. Alcohol (0.3 ml) in place of the extract was used as the blank. The total antioxidant activity is expressed as the number of gm equivalents of ascorbic acid. The calibration curve was prepared by mixing ascorbic acid (7.5, 15, 30, 60 and 150 µg/ml) with Alcohol.[18]

Acute Toxicity Study

Acute toxicity study was carried out on healthy Swiss *albino* mice following OECD guidelines. The animals of both sexes (n=6) were selected by random sampling technique and divided into 5 groups of 6 animals each.

A single oral dose of the extract of polyherbal formulation was administered orally at the level of 300, 600, 900, 1200 and 1500 mg/kg body weight respectively.

All the animals were observed for appearance of toxic symptoms including muscle spasm, loss of righting reflex, tremors, behavioural changes, locomotion, convulsions and mortality for 1, 2, 4, 8 and 24 h. Long term supervision was continued for a period of 14 days for observing any occurrence of toxic symptoms and mortality.[19]

***In-vivo* Antioxidant Analysis**

The in-vivo antioxidant analysis of the aqueous extract of the Polyherbal formulation has been done in animals especially in rats after inducing the oxidative stress by using potassium dichromate as oxidative stress agent in two different ways. Following the standard published methods, acute oxidative stress is produced by administration of a single dose in the animals while chronic oxidative stress is caused through administration of the dose daily up to 26 days.[20-21] The antioxidant level was determined in the serum of the blood of animals which was collected from the tail of each animal following the standard methods for the experimental determination of Oxygen Reactive Metabolites and Oxygen Reactive Species. The analysis of each method was repeated three times and readings were analyzed in comparison to the readings of the control group of animals.

The animal were grouped for this study in the following manner –

Group A

Control (Distilled water 10 ml/kg bwt, p.o)

Group B

Potassium Dichromate ($K_2Cr_2O_7$) at 0.4mg/kg bw, i.p. for 26 days

Group C

Polyherbal formulation at 600 mg/kg bw, p.o along with Potassium Dichromate ($K_2Cr_2O_7$) at 0.4mg/kg bw, i.p. for 26 days

Group D

Polyherbal formulation at 600 mg/kg bw, p.o. for 26 days

Group E

Polyherbal formulation at 600 mg/kg bw, p.o. for 26 days and Single Dose of Potassium Dichromate ($K_2Cr_2O_7$) at 15mg/kg bw, i.p on 26th Day

Group F

Single Dose of Potassium Dichromate ($K_2Cr_2O_7$) at 15mg/kg bw, i.p on 26th Day

The regular observation of each animal was done and all important parameters such as weight, food intake, behavioral symptoms and mortality were noted throughout the study. The blood was collected from the tail of the rat at room temperature on day 27. Blood plasma was separated and stored at 4°C and used for the determination of Reactive Oxygen metabolite (DROM) and Reactive Oxygen Species (ROS) assessment.

Using these methods, chronic oxidative stress was introduced for animals in Groups B and C, and comparison of the data between these two groups indicated the efficacy of the polyherbal formulation in countering the chronic oxidative stress. Similarly, Groups E and F were subjected to acute oxidative stress and comparison was made between these two groups. All experimental groups were also compared to the control group to assess the normative oxidative stress levels.

Determination of Reactive Oxygen Metabolites (d-ROMs Test)

This test detects the derivatives of reactive oxygen metabolites (d-ROMs) which is used to evaluate the level of hydroperoxides, which are markers and amplifiers of oxidative damage, produced by the attack of free radicals, a large class of substances.

The derivative of Reactive Oxygen Metabolites (d-ROMs) test is a photometric test for measurement of the concentration of hydro - peroxides (ROOH) in biological samples which are substances that belong to a broad class of Reactive Oxygen Metabolites (ROMs). The d-ROMs test uses the principle of Fenton's reaction. Hydroperoxides are converted into radicals that oxidize N,N-diethyl-para-phenylendiamine and that can be detected through spectrophotometric procedures as U.CARR. (Carratelli units). In this test, Chromogen (N, N-diethyl-paraphenylendiamine) is used which indicates the oxidative reaction by changing colour and intensity of color is measured photometrically at 505 nm. 20 µl of Serum with 20µl of FeSO4 and 20µl of D.E.P.P.D were added and thereafter 1.94 ml of 0.1 M of Acetate buffer was added and incubated for 1 min and vortexed. The absorbance of the all samples was taken at 505 nm wavelength in the spectrophotometric methods using the shimadzu no-2450 instrument. Thus, it becomes possible to quantify the level of hydro-peroxides available in the sample. The results of the d-ROMs test are expressed in arbitrary units, called CARRATELLI UNITS (CARR U), where 1 CARR U is the equivalent of 0.08 mg/100ml of H_2O_2.[22]

Determination of Reactive Oxygen Species by ELISA Kit

ROS are constantly generated and eliminated in the biological system and are required to drive regulatory pathways. Under normal physiological conditions, cells control ROS levels by balancing the generation of ROS with their elimination by scavenging system. But under oxidative stress conditions, excessive ROS can damage cellular proteins, lipids and DNA, leading to fatal lesions in cell that contribute to carcinogenesis. ROS are produced as a normal product of cellular metabolism. In particular, one major contributor to oxidative damage is hydrogen peroxide (H_2O_2) which is converted from superoxide that leaks from the mitochondria. Catalase and superoxide dismutase ameliorate the damaging effects of hydrogen peroxide and superoxide by converting these compounds into oxygen and hydrogen peroxide (which is later converted to water), resulting in the production of benign molecules.[23]

Sandwich-ELISA ROS ELISA kit (Elabscience, Lot No: AK0016FEB18047) has been used in this method for determination of the level of ROS in serum of the blood of rats. The micro ELISA plate is pre-coated with an antibody

specific to ROS. Standards or samples are added to the micro ELISA plate and combined with the specific antibody. Then the biotinylated detection antibody specific for ROS and Avid in-Horseradish Peroxidase conjugate is added to each micro plate well successively and incubated. Free components are washed away.

The substrate solution is also added to each well. Only those wells that contain ROS, biotinylated detection antibody and Avid in-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of Sulphuric acid solution and the colour turns yellow. The optical density (OD) is measured spectometrically at a wavelength of 450nm. The OD value is proportional to the concentration of ROS. The concentration of ROS in the sample is calculated from standard curve.

All the reagents were set aside to bring them to room temperature. All the reagents were mixed thoroughly by gently swirling before pipetting. The assay process has been followed as mentioned in the literature of kit which is given below:

Assay Procedure

Samples should be clear and transparent and be centrifuged to remove suspended solids.:

Allow serum samples to clot for 2 hours at room temperature or overnight at 4℃ before centrifugation for 15 minutes at 1000×g.

Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin. Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay.

All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. It's recommended that all samples and standards be assayed in duplicate.

1. Add Sample: Add 100µL of Standard, Blank, or polyherbal formulation per well. The blank well is added with Reference Standard & Sample diluent. Solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix it gently. Cover the plate with sealer we provided. Incubate for 90 minutes at 37℃.
2. Biotinylated Detection Ab: Remove the liquid of each well, don't wash. Immediately add 100µL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.
3. Wash: Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 350µL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, remove remained Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper.
4. HRP Conjugate: Add 100µL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37°C.
5. Wash: Repeat the wash process for five times as conducted in step 3.
6. Substrate: Add 90µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.
7. Stop: Add 50µLof Stop Solution to each well. Then, the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.
8. OD Measurement: Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters.
9. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.

Statistical Analysis

The data generated for each experiment was considered for calculation of the Mean ± S.E.M. for different groups of rats and comparison of each test was done with the control group. Statistical evaluation of data was done following Students't-test and by ANOVA. The level of significance was fixed between $p < 0.05$ and $p < 0.01$.

RESULTS

Analysis of Phytochemical Constituents

Evaluation of the physical parameters of polyherbal formulation indicated that while the moisture content was 7.1 % w/w, the extractive value of aqueous extract was 7.912 % w/w.

The total ash content was 9.72 % w/w, the acid insoluble ash was 0.13 % w/w and water soluble ash was 7.87 % w/w. Among phytochemical constituents, alkaloids, flavonoids, tannins, carbohydrates and saponins were found to be present in the aqueous extract of the polyherbal formulation.

Total Phenol Content (TPC)

The concentration of total phenols in the polyherbal formulation was calculated from the Standard curve of Gallic acid (Fig. 1) and expressed as µg Gallic acid

equivalent of phenol/gm of sample. The aqueous extract of the Polyherbal formulation showed 28.60 µg Gallic acid per mg of dried sample).

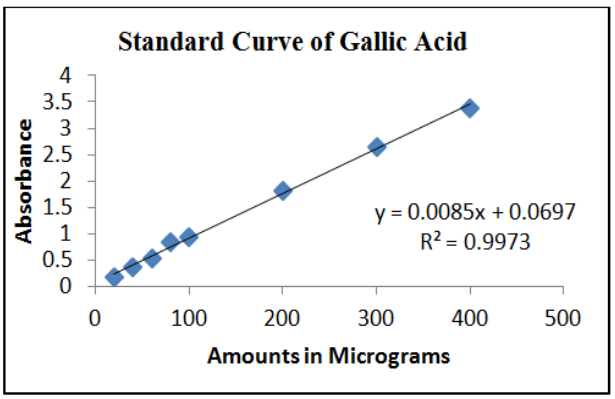

Figure 1: Standard Curve of Gallic Acid

Total Antioxidant Capacity by Phosphomolybdate Assay

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 acidic pH with a maximal absorption at 695 nm. It was expressed as the number of equivalents of ascorbic acid as a reference using the Standard curve for Ascorbic acid (Fig. 2) to determine the antioxidant capacity of the polyherbal formulation. Total antioxidant content of the aqueous extract of polyherbal formulation in different concentrations of 3 mg/ml, 6 mg/ml and 9 mg/ml expressed as gram equivalents of ascorbic acid was evaluated to be 16.48 ± 80, 18.97 ± 8.87 and 20.98 ± 8.93 (µg/mg) equivalent to Ascorbic Acid respectively as shown in Table 1.

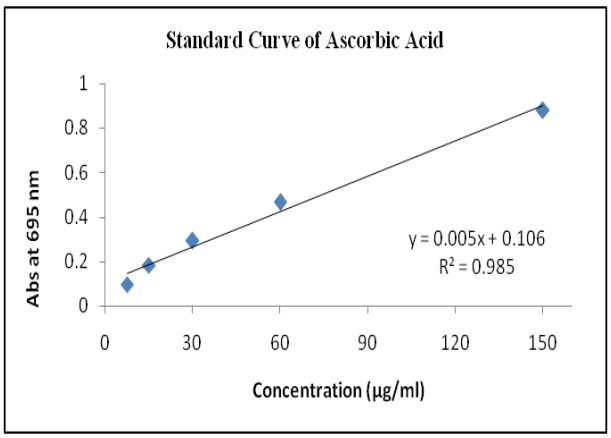

Figure 2: Standard Curve of Ascorbic Acid

Table 1: Total Antioxidant Capacity of Aqueous Extract of *Sida cordifolia*

Concentration (mg/ml)	Anti-oxidant Content (µg/mg) equivalent to Ascorbic Acid
3.0	16.48 ± 8.17
6.0	18.97 ± 8.87
9.0	20.98 ± 8.93

Acute Toxicity

During acute toxicity tests of the aqueous extract of the polyherbal formulation up to the dose of 1500 mg/kg, the test animals showed no significant toxic symptoms like sedation, convulsion, diarrhea, irritation, etc. and no signs of behavioral changes. No mortality was reported up to 24 hrs and even later during the subsequent 14 days at 1500 mg/kg dose. The therapeutic dose for the experiments is selected as 600 mg/kg on the basis of symptoms and mortality because some physical changes are observed in some animals at higher dose up to 1500 mg/kg; however, these symptoms subsided within one to two hour and no mortality occurred.

***In-vivo* d-ROM (Determination of Reactive Oxygen Metabolites) Anti-Oxidant Assay**

The determination of Reactive Oxygen Metabolites [d-ROMs] test is a photometric test for measurement of the concentration of hydro-peroxides (ROOH) in biological samples which has been used to find out the U. CARR values which are directly proportional to the oxidative stress in different groups of animals. The CARR unit values of the different groups obtained during the d-ROMs test have been summarized and shown in Table 2 and Figure 3. Comparison of the CARR values of all other groups with the Control Group A (80 ± 4.81) indicates the level of oxidative stress under various experimental conditions in these different groups.[24]

Chronic oxidative stress is produced in case of Groups B (166 ± 6.96) and C (94 ± 8.97) by daily administration of $K_2Cr_2O_7$ in animals. CARR values of Group B indicate that oxidative stress levels more than double as compared to the control group (Group A) after daily administration of $K_2Cr_2O_7$. However, the results of Group C strongly suggest that very high oxidative stress levels due to chronic administration of $K_2Cr_2O_7$ drastically reduce when there is concurrent administration of the polyherbal formulation in the animals. In fact, the CARR values of Group C are just over half the level noticed in case of Group B. At the same time, the CARR values of Groups C and A are quite close indicating that effect of agents causing chronic oxidative stress in animal subjects can be countered and neutralized to a great extent by the polyherbal formulation administration, bringing them very close to the control group.

The impact of acute oxidative stress is studied in case of Groups E (100 ± 9.69) and F (125 ± 6.98) by one-time introduction of $K_2Cr_2O_7$ in research animals on the 26th day. Data of Group F indicates very high CARR values in comparison to the values in case of the control group (Group A) signifying high acute oxidative stress in animals. CARR values obtained in case of Group E animals suggest that the effect of acute oxidative stress is drastically reduced due to simultaneous administration of polyherbal formulation in test animals. Thus, the CARR values of Group E are low as compared to Group F and higher than those of the control group.

The values of Group D (82 ± 9.03) are quite similar to those of Group A, indicating almost no additional oxidative stress generation in the animal subjects due to continuous daily administration of the research drug.

The results of the in-vivo d-ROMs test in induced acute and chronic oxidative stress showed that the polyherbal formulation reduced the oxidative stress levels in both kind of experiments, but the impact of oxidative stress was counteracted more in case of chronic than in case of acute stress conditions.

Table 2: CARR Values in Different Groups of Animals

Group	Treatment Protocol	CARR 1 U. CARR = 0.08 mg H^2O^2/dl
A	Control (distilled water 10 ml/kg bdwt)	80 ± 4.81
B	$K_2Cr_2O_7$ treated group	166 ± 6.96
C	Polyherbal formulation & $K_2Cr_2O_7$ treated group for 26 days	94 ± 8.97 †
D	Polyherbal formulation treated group for 26 days	82 ± 9.03 ‡
E	Polyherbal formulation for 26 days and single dose of $K_2Cr_2O_7$ treated group on 26th day	100 ± 9.69
F	Single dose of $K_2Cr_2O_7$ treated group on 26th day	125 ± 6.98

Values are presented as Mean ±SEM. n = 6.

† $p < 0.001$; ‡ $p = 0.005$

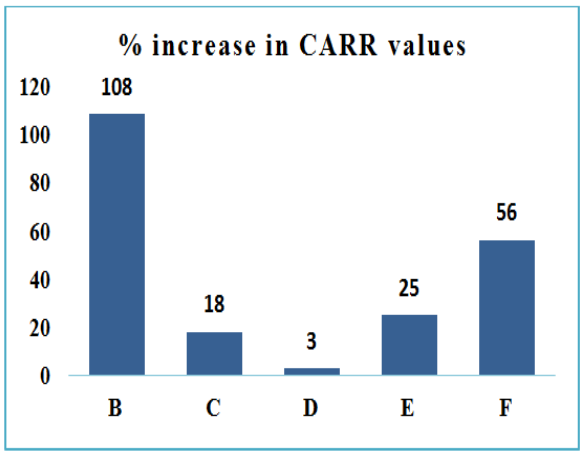

Figure 3: Percentage Increase in CARR Values in Experimental Groups

Table 3: Absorbance of Standard in Different Concentrations

Concentration (ng/ml)	Optical Density
0.16	0.259
0.31	0.379
0.63	0.450
1.25	0.588

ROS Anti-Oxidant Assay (Determination of Reactive Oxygen Species)

The sandwich-ELISA ROS kit was used to measure the concentration of Reactive Oxygen Species in the serum of rats after introducing oxidative stress by $K_2Cr_2O_7$. The results obtained were compared with those shown in the standard curve (fig. 4) in different concentrations. The optical density (OD) was measured spectrometrically at a wavelength of 450 nm as shown in table 3 because the OD value is directly proportional to the concentration of ROS. The ROS value equivalent to standard (in ng per ml) of all groups as shown in table 4 is compared with the control group A (0.15 ± 0.039) to indicate the level of oxidative stress under various experimental conditions in these different groups.

Chronic oxidative stress is produced in case of Groups B (0.76 ± 0.078) and C (0.29 ± 0.052) by daily administration of $K_2Cr_2O_7$ in animals.[25] ROS values of Group B (fig. 5) indicate that oxidative stress levels are more than five times as compared to the control group (Group A) after daily administration of $K_2Cr_2O_7$. However, the results of Group C strongly suggest that very high oxidative stress levels due to chronic administration of $K_2Cr_2O_7$ drastically reduce when there is concurrent administration of the polyherbal formulation in the animals. In fact, the ROS values of Group C are around one third of the level noticed in case of Group B. At the same time, the ROS values of Groups C and A are quite close indicating that effect of agents causing chronic oxidative stress in animal subjects can be countered and neutralized to a great extent by the research drug administration.

The values of Group D (0.17 ± 0.043) are quite similar to those of Group A, indicating almost no additional oxidative stress generation in the animals due to continuous daily administration of the research drug.

The impact of acute oxidative stress is studied in case of Groups E (0.44 ± 0.065) and F (0.69 ± 0.083) by one-time

introduction of $K_2Cr_2O_7$ in research animals on the 26th day. Data of Group F indicates that the ROS values are more than four times in comparison to the values in case of the control group, signifying high acute oxidative stress in animals.

ROS values obtained in case of Group E animals suggest that the effect of acute oxidative stress is drastically reduced due to simultaneous administration of research drug in test animals. Thus, the ROS values of Group E are less than two-third in quantum as compared to Group F and a little higher than those of the control group.

The results showed that the research drug reduced the oxidative stress levels in both acute and chronic oxidative stress experiments, but the impact of oxidative stress was counteracted more in case of chronic than acute oxidative stress conditions.

Table 4: ROS Values of Different Groups

Group	Treatment Protocol	ROS equivalent to standard (in ng per ml)
Group A	Control (distilled water 10 ml/kg bw)	0.15 ± 0.039
Group B	$K_2Cr_2O_7$ treated group	0.76 ± 0.078
Group C	Polyherbal formulation & $K_2Cr_2O_7$ treated group for 26 days	0.29 ± 0.052
Group D	Polyherbal formulation treated group for 26 days	0.17 ± 0.043
Group E	Polyherbal formulation for 26 days and single dose of $K_2Cr_2O_7$ treated group on 26th day	0.44 ± 0.065
Group F	Single dose of $K_2Cr_2O_7$ treated group on 26th day	0.69 ± 0.083

Values are shown as mean ± SEM. n = 6.; Values are statistically significant ($p < 0.01$).

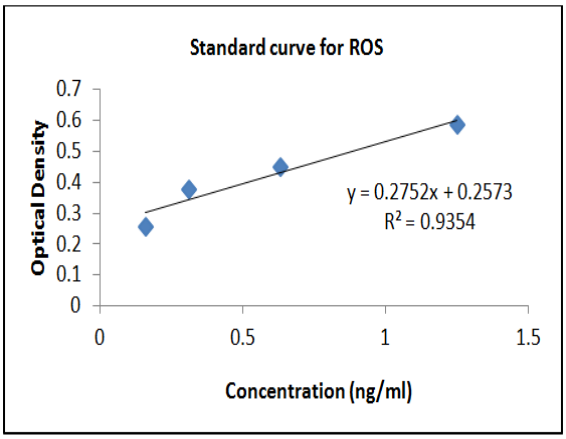

Figure 4: Standard Curve for ROS

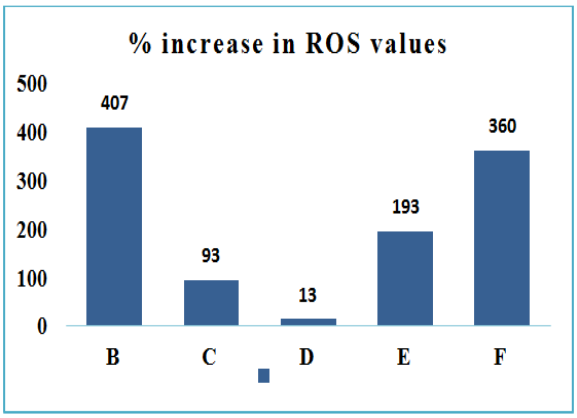

Figure 5: Percentage of Increase in ROS values in Experimental Groups

DISCUSSION

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that may damage cells. Antioxidants such as thiols or ascorbic acid (vitamin C) terminate these chain reactions. Plant antioxidants such as ascorbic acid and flavonoids have been shown to be the best exogenous antioxidants. Indeed, these compounds not only restrain ROS production by scavenging free radicals, but also help boost endogenous antioxidant defences of the body.[1,16]

In all living organisms and particularly in humans, there is a delicate balance between the production and elimination of the so-called free radicals by our antioxidant defence systems. The physiology of all living beings contains endogenous defence systems that protect structural and functional biomolecules from attack by free radicals. These defence systems react with the reactive species before they can attack the biological structures, lessening their potential damaging action.

If this "antioxidant barrier" is absent, the free radicals will quickly react with the biomolecules that are essential for life, such as DNA, lipids and proteins, causing severe cell damage and even death of the cells themselves.

The alteration of this balance, indicated as oxidative stress, causes cellular lesions which, if severe and continued over time, lead to an acceleration in the ageing process and the onset of a large number of diseases.

There are many medicinal plants which have been prescribed for the prevention and cure of common human diseases as per the Ayurvedic system of medicine as a single drug or in combination with other drugs since ancient times as a rejuvenator, energy booster and tonic. Most of these plants have been reported on account of their *in vitro* antioxidant activity. Bala (*Sida cordifolia*) and *Glycyrrhiza glabra* (Yashthimadhu) are two of the prominent and potent Ayurvedic medicines due to their rasayan action (rejuvenator, tonic, emollient, aphrodisiac, etc.). They have been traditionally used in treatment of diseases of the respiratory system, blood, throat, urinary system and reproductive system due to the presence of flavonoidal compounds in their roots.[7]

The aqueous extract of polyherbal formulation was found to contain 28.60 µg Gallic acid equivalent/mg of dried extract of total phenol content indicating high concentration of phenolic compounds. It also contains substantial amount of preliminary phyto-constituents such as alkaloids, tannins and flavonoids. No mortality was found in the experimental animals (mice) up to the maximum dose of 1500 mg/kg body weight of the polyherbal formulation during acute toxicity study. The total antioxidant capacity of its aqueous extract in different concentrations of 3mg/ml, 6mg/ml and 9mg/ml expressed as the number of gram equivalents of ascorbic acid was assessed to be 16.48 ± 80, 18.97 ± 8.87 and 20.98 ± 8.93 (µg/mg) equivalent to Ascorbic Acid respectively, thereby indicating its substantial and significant antioxidant capacity.

The *in vivo* antioxidant action of polyherbal formulation was evaluated scientifically during this study. The results of the two experiments related to induced oxidative stress by $K_2Cr_2O_7$ in the rat model showed the concentration in the serum of the animal of the Reactive oxygen metabolites (d-ROM) and Reactive oxygen species (ROS) and compared the antioxidant action of the polyherbal formulation with the control group. The % increase in CARR values in the d-ROM test showed that maximum oxidative stress is found in Group B (108 %) and Group F (56 %), and it is comparatively much lower in Group E (25%), Group C (18 %) and Group D (3%). Therefore, it is clear that high level of oxidative stress was found in both the groups B & F where the research drug was not administered to the animal in case of both chronic and acute oxidative stress.

On the other hand, groups C & E showed low level of oxidative stress due to oral administration of the polyherbal formulation to the animals for 26 days which gradually counteracted the bio-chemical action of $K_2Cr_2O_7$ and protected cellular destruction by less generation of the reaction oxygen species. The *in vivo* antioxidant property of the polyherbal formulation could be due to high concentration of the phenolic compounds equivalent to antioxidant Gallic acid found in its extracts.

The antioxidant property of the compounds is well correlated with the content of their phenolic compounds. Phenols contain good antioxidant, anti-mutagenic, and anticancer properties.

Flavonoids are the naturally occurring polyphenolic compounds representing one of the most prevalent classes of compounds in vegetables, nuts, fruits, and beverages such as coffee, tea, and red wine.[1,16]

The result of ROS test showed that the maximum percentage increase in oxidative stress was found in Group B (407%) and Group F (360 %) where the polyherbal formulation has not been orally administered to the animals under both acute and chronic oxidative stress conditions.

However, Group E (193%), Group C (93 %) and Group D (13%) showed low levels of the oxidative stress after orally prescribing the polyherbal formulation to the animals continuously for 26 days.

The data obtained in ROS test clearly suggests that administration of the polyherbal formulation during both acute and chronic conditions of oxidative stress counteracted the adverse action of $K_2Cr_2O_7$ and protected the healthy cells by reduced generation of the reaction oxygen species.[26]

Plants have an innate ability to synthesize non-enzymatic antioxidants. However, under biotic and abiotic stress conditions, the production of reactive oxygen species (ROS) increases in the plants, resulting in induction of oxidative stress.

In response to increased oxidative stress, plants augment the production and accumulation of several low molecular weight antioxidants (e.g., vitamin C, vitamin E, phenolic acids, etc.) and high molecular antioxidant secondary metabolites such as tannins, which confer antioxidants to most plants under *in vitro* studies by functioning as free radical scavengers, reducing agents, and metal chelators.[27-28]

Under *in vivo* conditions, the antioxidant potential of polyphenols is predominately dependent on their concentrations in the bloodstream after absorption from the gastrointestinal tract, as well as their modifications during metabolism.

The chemical structures of polyphenols will also influence the *in vivo* antioxidant potential of polyphenols, as they determine the conjugation reactions with methyl, sulfate or glucuronide groups and the nature and amounts of metabolites formed by the gut microflora absorbed at the colon level.[29-31]

CONCLUSION

Experimental evaluation of *in vivo* antioxidant properties of aqueous extract of the polyherbal formulation by Phosphomolybdate Assay and d-ROM and ROS techniques using the chronic and acute oxidative stress models in rats indicated its significant efficacy as an antioxidant specially in case of chronic test which is non-toxic and able to mitigate and counter the negative

effects of oxidative stress produced by administration of $K_2Cr_2O_7$ in experimental animals.

This antioxidant activity of the research drug could be attributed to the presence of the polyphenolic compounds and flavonoids in high concentrations in its aqueous abstract.

Acknowledgement: This research work was supported by grants from the Department of Science and Technology, Government of West Bengal and the authors are grateful for this support.

We are thankful to Shri Saumya Kanti Sasmal, Ph.D. scholar, Department of Dravyaguna, Institute of Post Graduate Ayurvedic Education and Research, Kolkata for providing the necessary help during the experimental work.

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Source of Support: Nil, **Conflict of Interest:** None.