

STANDARDIZATION OF A POLYHERBAL PREPARATION (POL-6) FOR TREATMENT OF OXIDATIVE, INFLAMMATORY AND IMMUNE DISORDERS

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Received: 14 Jan 2016 Revised and Accepted: 11 Feb 2016

ABSTRACT

Objective: The objective of this study was to develop and standardize a polyherbal preparation (POL-6) and evaluate its use in treating oxidative, inflammatory and immune disorders.

Methods: The individual hydroalcoholic extractions of *Hypericum perforatum*, *Bacopa monnieri*, *Centella asiatica*, *Withania somnifera*, *Ocimum sanctum* and *Camellia sinensis* dried plant materials were done, and POL-6 was prepared by mixing them in (1:2:2:2:2:1) proportions. POL-6 has been standardized on the basis of organoleptic properties, physical characteristics, and physicochemical properties. The antioxidant activities were evaluated using free radical scavenging methods for 1,1-diphenyl-2-picrylhydrazyl, (DPPH) and nitric oxide assays. Anti-inflammatory activity was done by albumin denaturation and HRBC membrane stabilization assays. Cell viability assay was used for immunomodulatory activity measurement by cultivating peripheral blood mononuclear cells.

Results: The results obtained from physical and chemical parameters evaluation shows that the contents of preparation present are within permissible limits. In *in-vitro* antioxidant studies POL-6 showed significant antioxidant activity when tested in 1-1 Diphenyl-2-picrylhydrazyl and nitric oxide assays. In *in-vitro* anti-inflammatory investigation, POL-6 exhibited significant inhibition against albumin denaturation and significant protection against membrane stabilization assay. POL-6 also exhibit significant immunomodulatory activity at low doses, when tested in cell viability assay.

Conclusion: The results from this study rationalize the medicinal use of POL-6 in oxidative, inflammatory and immune disorders.

Keywords: Polyherbal preparation, Antioxidant, Anti-inflammatory, Immunomodulatory

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INTRODUCTION

Efficacy of medicinal plants in the management of diseases is indubitable, and the World Health Organization has recognized its use in the primary health care delivery system. Based on a combination of traditional usage and ongoing scientific research, the potential of plant-derived antioxidant components for the prevention of some diseases and improvement in health quality has attracted much research attention [1-2]. Due to a reduction in dose, convenience, and ease of administration herbal preparations have attained wide recognition in comparison to crude plant materials and extracts. These preparations are popular worldwide as therapeutic agents, in various ailments that impact the quality of life. It should be noted that herbal formulations have been valued for their added efficacy due to the synergistic effect of many herbs. Since free radicals are implicated in a number of physiological disorders and with the polyherbal drugs of Ayurveda used in the treatment of diverse physiological disorders, there is a strong case to believe that polyherbal drugs exert their therapeutic actions by their ability to scavenge free radicals or by their antioxidant potential [3]. The production of free radicals occurs endogenously during cellular metabolism in all forms of aerobic living systems; e. g. oxidative phosphorylation in mitochondria, liver, mixed function oxidases, bacterial phagocytosis, xanthine oxidase activity, transition metal catalysis, drug, and xenobiotics metabolism. They have a fundamental role in modulating various physiological functions and represent an essential part of aerobic life and metabolism. Excessive generation of these radicals creates homeostatic imbalance that disrupts the antioxidant defense system of the body and generates oxidative stress, which may be responsible for the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, aging, cardiovascular and neurodegenerative diseases [4,5]. Increased production of free radicals and oxidative damage may result in excessive production

and release of pro-inflammatory mediators, cytokines, chemokines *i.e.* TNF- α , interleukines, and recruitment of adhesion molecules. Alteration of the immune response may increase or decrease the immune responsiveness called as immunomodulation. To cure various diseases by immunomodulation has been a very interesting concept in the field of natural products emphasizing on strengthening host defenses. The immunomodulating response of active ingredient is shown either by immunosuppression or immunostimulation. In Ayurveda, modulation of the immune response by plants is classified as *Rasayana* *i.e.* place of ras essential vehicle of life emphasizing the promotion of health [6].

As per literature *Hypericum perforatum* (St John's-wort) family Hypericaceae, *Bacopa monnieri* (Brahmi) family Scrophulariaceae, *Centella asiatica* (Gotu kola) family Apiaceae, *Ocimum sanctum* (Tulsi) family Labiatae, *Withania somnifera* (Ashwagandha) family Solanaceae, *Camellia sinensis* (Tea plant) family Theaceae have good anti-oxidant properties [7-10]. In one of our previous studies, we found that combination of *Hypericum perforatum*, *Bacopa monnieri* and *Camellia sinensis* shows synergistic antioxidant activities [7]. Thus, the present investigation was conducted with the objective of standardization of an Ayurvedic polyherbal preparation for treating oxidative, inflammatory and immune disorders.

MATERIALS AND METHODS

Chemicals

1-diphenyl-1-picrylhydrazyl (DPPH), rutin, naphthyl ethylene-diamine dichloride, and standards Gallic acid, tannic acid and quercetin were purchased from Sigma Chemicals. Ferric chloride, vanillin, trichloroacetic acid (TCA), Folin-Ciocalteu's reagent, aluminum chloride (AlCl₃) were purchased from Himedia Pvt Ltd. All other chemicals used in the present study were of analytical grade.

Plant materials

The dried roots of *Withania somnifera* (Bath number: EBD-18), dried whole plant of *Centella asiatica* (Bath number: ERD-040), *Bacopa monnieri* (Bath number: ERD-92) and dried leaves of *Ocimum sanctum* (Bath number: RHD 283), *Camellia sinensis* (Bath number: ERM-23) has been purchased from Natural Remedies, Bangalore. The aerial parts of *Hypericum perforatum* L. have been collected from the herbal garden of the Jaypee University of Information Technology, Wagnaghat, Dist. Solan, Himachal Pradesh (H. P.). The identification of the plant *Hypericum perforatum* L. was done by Dr. Bhupendra Dutt, Research Officer, Department of Forest Products, College of Forestry, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, H. P. A voucher specimen of the said plant has been deposited to UHF-Herbarium (Field book number: 13420). The individual plants were coarsely powdered with a mechanical grinder and passed through sieve number 60 and stored in an airtight container. The coarsely powdered individual plants were subjected to defatting with petroleum ether (30-40 °C). The defatted air dried individual plant powder has been extracted by Soxhlet apparatus using 70 % v/v of ethanol (hydroalcoholic) at 50 °C for 48 h. The solvent was recovered by evaporation under reduced pressure using rotary evaporator (Heidolph, Germany). The semisolid mass has been further lyophilized (New Brunswick) at -80 °C for 24 h. The obtained dry powder of individual plant was stored at 2-8 °C till further use.

Preparation of polyherbal preparation (POL-6)

For the preparation of POL-6, powdered extract of each plant (*Hypericum perforatum*, *Bacopa monnieri*, *Centella asiatica*, *Withania somnifera*, *Ocimum sanctum* and *Camellia sinensis*) was weighed separately and mixed in (1:2:2:2:1) proportions using a double cone blender. The mixture was passed through sieve number 40 to obtain a homogeneous blend. Further, it was packed in tightly closed container and stored in a cool and dry place [11].

Organoleptic evaluation of POL-6

Sensory characteristics of POL-6 like colour, odour, shape, size, taste, texture and fracture were identified using organoleptic evaluation [12].

Physicochemical investigation of POL-6

Loss on drying

Loss on drying is the loss of mass expressed as percent w/w. 10 g of samples of POL-6 were accurately weighed in a dried and flat weighing bottle and dried at 105 °C for 5 h. The percentage was calculated with reference to initial weight [13].

Determination of total ash

A high ash value is indicative of contamination, adulteration substitution or carelessness in preparing the preparation. Ashing involves an oxidation of the components of the products. For its determination, 2 g powdered material of POL-6 was placed in a suitable tared crucible of silica previously ignited and weighed. The powder was spread into an even layer and weighed accurately. The material was incinerated by gradually increasing the heat, not exceeding 450 °C until free from carbon, cooled in desiccators, weighed and percentage ash was calculated by taking in account the difference of empty weight of crucible & that of the crucible with total ash.

Determination of acid insoluble ash

The ash obtained as above was boiled for 5 min with 25 ml of dilute hydrochloric acid; the insoluble matter was hot water and collected on an ashless filter paper, washed with ignited to constant weight. The percentage of acid-insoluble ash with reference to the air-dried drug was calculated.

Determination of solvent extractive values of POL-6

Alcohol soluble extractive value

Five gram of coarsely powdered air-dried POL-6 was macerated with 100 ml of alcohol in a closed flask for twenty-four hours, frequently shaking during six hours and allowing standing for eighteen hours. It

was then filtered rapidly; taking precautions against loss of solvent. 25 ml of the filtrate was evaporated to dryness in a tared flat-bottomed shallow dish at 105 °C to constant weight and weighed. The percentage of alcohol-soluble extractive was calculated with reference to the air-dried drug and is represented as % value [13].

Water soluble extractive value

Five-gram g of coarsely powdered air-dried POL-6 was macerated with 100 ml of chloroform water in a closed flask for twenty-four hours, frequently shaking during six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent. 25 ml of the filtrate was evaporated to dryness in a tared flat-bottomed shallow dish at 105 °C to constant weight and weighed. The percentage of water-soluble extractive was calculated with reference to the air-dried drug and is represented as % value [13].

Determination of pH

1% solution of POL-6 was prepared in distilled water, and pH was determined using pH meter.

Physical characteristics of POL-6 powder

Bulk density and tape density

The term bulk density refers to a measure used to describe a packing of particles or granules. The equation for determining bulk density (D_b) is: $D_b = M/V_b$, Where M is the mass of the particles and V_b is the total volume of the packing. The volume of the packing can be determined in an apparatus consisting of a graduated cylinder mounted on a mechanical tapping device (Jolting Volumeter) that has a specially cut rotating can. 100 g of weighed POL-6 powder was taken and carefully added to the cylinder with the aid of a funnel. Typically the initial volume was noted, and the sample was then tapped until no further reduction in volume was noted. The initial volume gave the Bulk density value and after tapping the volume reduced, giving the value of tapped density [12, 13].

Angle of repose

In general, powders with the angle of repose greater than 50 degrees have unsatisfactory flow properties, whereas minimal angle close to 25 degrees correspond to very good flow properties. The fixed funnel and the free standing cone method employs a funnel that is secured with its tip at a given height, which was taken 2.5 cm (H), above the graph paper that is placed on the flat horizontal surface. The powder was carefully poured through the funnel until the apex of the conical pile just touched the tip of the funnel. Angle of repose was calculated by the formula

$$\tan \alpha = H/R \text{ or } \alpha = \arctan H/R$$

Where α is the angle of repose, R being the radius of the conical pile.

Hausner's ratio

Hausner's ratio is related to interparticle friction and as such can be used to predict the powder flow properties. Powders with low interparticle friction such as coarse spheres; have a ratio of approximately 1.2, whereas more cohesive, less flowable powders such as flakes have a Hausner's ratio greater than 1.6. The equation for measuring the Hausner's ratio is:

$$\text{Hausner's ratio} = D_f / \text{pour density}$$

Where D_f = Tapped density and D_0 = Bulk density.

Carr's index

Carr's index is a method of measuring the powder flow from bulk density. The equation for measuring Carr's index is (% compressibility) = $\left(D_f - \frac{D_0}{D_f} \right) \times 100$

Where D_f = Tapped density and D_0 = Bulk density.

Phytochemical screening of POL-6

POL-6 was subjected to phytochemical screening tests to evaluate the presence of chemicals constituents like alkaloids, flavonoids, proteins, glycosides, fixed oils, phenolics, sugars and tannins [14].

Determination of total flavonoid content

Total flavonoid content (TFC) in POL-6 was determined by aluminum chloride assay using colorimetric estimation and using rutin as a reference compound. A volume of 0.5 ml of POL-6, 2 ml of distilled water added in 0.15 ml of sodium nitrite (5% w/v). After 5 min, 0.15 ml of aluminum trichloride (10%) was added and incubated for 6 min and after incubation, 2 ml of sodium hydroxide (4% w/v) was added. The final volume was made up to 5 ml with distilled water. The mixture was allowed to incubate for 15 min, and when reaction mixture turns to pink, absorbance was measured against the blank at 510 nm. The TFC was expressed in mg of rutin equivalents per gram of POL-6 [15].

Determination of total phenolic content

The total phenolic content in POL-6 was estimated according to Folin-cioaltea phenol reagent method. The solution of gallic acid was prepared in 80% methanol for the standard curve. Folin-cioaltea reagent was added to 100 µl of sample in ratio 1:10. The solution was mixed and incubated at room temperature for 1 min followed by addition of 1.5 ml of 20% sodium carbonate. The final mixture was shaken and incubated for 90 min in dark at room temperature. The absorbance was taken at 725 nm and the phenolic content was expressed as gallic acid equivalents GAE/g of the sample [16].

Condensed tannin quantification

A volume of 400 µL of POL-6 was added to 3 mL of a solution of vanillin (4% in methanol) and 1.5 mL of concentrated hydrochloric acid. After 15 min of incubation, the absorbance was read at 500 nm. Tannin content was expressed as mgCE/g of sample, using a catechin calibration curve [17].

Evaluation of free radical scavenging activity of POL-6

Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The free radical scavenging activity of POL-6 was determined according to the ability of material to bleach the stable DPPH radicals. 0.5 ml of DPPH was mixed to 0.5 ml aliquot of standard or test solution in different concentrations: 5, 10, 20, 40, 80, 100 µg/ml. Control test tubes (Absorbance control) were loaded with 0.5 mL of DMSO and 0.5 mL DPPH. After incubation at 37°C for 30 min in the dark, the absorbance was taken at 517 nm. Ascorbic acid was used as a standard. When the reading was complete, the percentage of inhibition of samples was calculated from obtained absorbance by the equation [18].

$$\text{Scavenging DPPH (\%)} = \frac{[\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}]}{\text{Abs}_{\text{control}}} \times 100$$

From the obtained percentage inhibition values IC₅₀ was calculated which represents the level where 50% of radicals scavenged by test or standard sample.

Nitric oxide scavenging assay

Under aerobic conditions, nitric oxide reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent. Griess reagent was prepared by mixing 1% sulphanilamide in 5% v/v phosphoric acid and 0.01% naphthylethylenediamine in distilled water in equal volumes.

The solution of sodium nitroprusside (5 mM) in standard phosphate buffer (0.025 M, pH-7.4) was prepared and incubated with different concentrations of standard and test sample 5, 10, 20, 40, 80 and 100 µg/ml at 37 °C for 5 h. An equivalent amount of methanol was taken as control. After 5 h, 0.05 ml of incubated solution was removed and diluted with 0.5 ml of Griess reagent. The absorbance was read at 546 nm. Ascorbic acid was used as a standard. The percentage scavenging by test samples at each concentration was calculated using following formula:

$$\text{Scavenging NO (\%)} = \frac{[\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}]}{\text{Abs}_{\text{control}}} \times 100$$

From the obtained percentage inhibition values IC₅₀ represents the level where 50% of radicals scavenged by test or standard sample [8].

Anti-inflammatory activity of POL-6

Albumin denaturation assay

The reaction mixture was consisting of test samples and 1% aqueous solution of bovine albumin fraction. Both test and standard drugs (Diclofenac sodium) were diluted in concentrations: 25, 50, 100, 250 and 500 µg/ml). 5 ml of BSA was transferred to a tube containing 50 µg/ml of test/standard. The control tube consists of 5 mL 0.2% w/v BSA solution with 50 µl methanol. The samples were heated at 72 °C for 5 min and after cooling, the turbidity was measured spectrophotometrically at 660 nm [8]. Percentage inhibition was calculated using formula

$$\% \text{ Inhibition} = \frac{[\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}]}{\text{Abs}_{\text{control}}} \times 100$$

Membrane stabilization assay

Human blood was obtained from a healthy volunteer, who has not taken any analgesic medication for two weeks and transferred to a heparinized centrifuge tube and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride in water). Blood was centrifuged at 3000 rpm for 15 min. Packed cells were washed with isosaline (0.85% pH 7.2), and a suspension was made with isosaline (10%). Different concentrations of sample: 25, 50, 100, 250 and 500 µg/ml were prepared in isosaline. The assay mixture contained 0.5 ml of HRBC suspension, phosphate buffer (0.15M pH 7.2), 2 ml hyposaline (0.36%) and 1 ml of various concentrations of sample and incubated at 37 °C for 30 min. Then, the mixture was centrifuged at 3000 rpm for 20 min. Diclofenac sodium was used as reference standard. The absorbance of the supernatant solution was estimated using spectrophotometer at 560 nm [19]. Percentage protection was calculated by formula

$$\% \text{ protection} = [100 - (\text{OD of test}/\text{OD of control})] \times 100$$

Immunomodulatory activity of POL-6

Preparation and cultivation of Peripheral blood mononuclear cells (PBMC)

Human blood was collected from healthy volunteers. PBMC were isolated from blood using Histopaque (Himedia) according to manufacturer's instructions. The blood sample was diluted with the same volume of PBS and was carefully layered on Histopaque. The mixture was subjected to centrifugation at 400 g for 30 min at 18–20 °C. Carefully undisturbed lymphocyte layer was transferred out. The lymphocytes were washed and pelleted down with three volumes of PBS twice and resuspended in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotics (100 µg/ml penicillin and streptomycin). Cell counting was performed to determine the PBMC number with an equal volume of trypan blue [20].

Cell viability assay

MTT assay was used to access the effect of plant extracts on cell viability. MTT is a pale yellow substance reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not reduce significant amounts of MTT. PBMC (5×10⁵ cells/ml) were seeded in 10% RPMI-1640 medium in a 96-well plate (in triplicate). After overnight incubation, the medium was replaced with fresh 10% RPMI-1640 medium containing POL-6. PBMC treated with Concanavalin A and DMSO was used as positive and negative controls respectively. Incubation of all plates was done at 37 °C, 5% CO₂ for selected time period. For MTT assay, 10 µl MTT (5 mg/ml) was added to each well to generate formazan, and then cells were incubated in a humidified atmosphere with 5% CO₂ at 37 °C for 4 h. After removing the supernatant, 100 µl DMSO was added to dissolve the purple crystal. The optical density of each well was measured at 570 nm by a microplate reader.

The percentage of proliferation was calculated by the following formula:

$$\% \text{ Proliferation} = \frac{[\text{OD sample} - \text{OD control}]}{\text{OD control}} \times 100$$

RESULTS AND DISCUSSION

Organoleptic evaluation of POL-6

As part of standardization procedure, the prepared POL-6 was tested for relevant physical and chemical parameters. The organoleptic evaluation revealed that POL-6 is a powder with dark brown color, pungent odor, bitter taste and fine texture.

Physicochemical investigation and solvent extractive values of Pol-6

Quality tests on POL-6 were performed for Loss on Drying at 105 °C (4.12±0.121), Water-soluble extractive value (%) (46.138±0.213), Alcoholic soluble extractive value (%) (69.48±0.485), Total ash (%)

(15.30±0.023), Acid insoluble ash (%) (0.62±0.012), pH (1% aq. Soln.) (7.14±0.217) and were found to be within standard ranges. The results are expressed as the mean (n=3)±Standard deviation (SD) and shown in table 1.

Physical characteristics of POL-6

Physical properties of the Pol-6 like Bulk density, Tap density, Carr's compressibility index, Hausner's ratio, Angle of repose were determined, and results were tabulated in table 2. Carr's index is a method of measuring the powder flow from bulk density, and Hausner ratio can be used to predict the powder flow properties as it is related to the inter-particle friction

Table 1: Physicochemical characteristics of POL-6

S. No.	Parameters	Percentage mean±SD
1	Loss on Drying 105 °C	4.12±0.121
2	Water soluble Extractive Value (%)	46.14±0.213
3	Alcoholic Soluble Extractive Value (%)	69.48±0.485
4	Total Ash (%)	15.30±0.023
5	Acid Insoluble Ash (%)	0.62±0.012
6	pH	7.14±0.217

The results are expressed as mean (n=3)±Standard deviation (SD)

Table 2: Physical characteristics of POL-6

S. No.	Parameters	Results
1	Bulk Density	0.389±0.001 (g/ml)
2	Tapped Density	0.612±0.011 (g/ml)
3	Carr's Index	37.24 %
4	Hausner's Ratio	1.38±0.2
5	Angle of Repose	27.2±0.3

The results are expressed as mean (n=3)±Standard deviation (SD)

Table 3: Phytochemical screening of POL-6

Compound	Detection method	Inference
Flavonoids	Shinoda test,	+
	Zinc hydrochloride test	+
	Alkaline reagent test	+
Alkaloids	Dragendroff's reagent	+
	Hager's reagent	+
Tannins	Ferric Chloride Test	+
	Lead acetate test	+
Steroids & triterpenoids	Salkowski Test	+
Coumarin glycosides	Coumarin Glycoside	+
Amino acids	Millon's test	+
	Ninhydrin test	+
Carbohydrates	Molisch's test	+

Presence (+)

Phytochemical screening of POL-6

The qualitative phytochemical analysis of POL-6 revealed the presence of flavonoids, alkaloids, carbohydrate, glycosides, steroids, tannin and phenolics (terpenoids). The observations made during phytochemical analysis are shown in table 3.

Total phenolic, flavonoid and tannin content in POL-6

Plant-based phenolic compounds have an antioxidant activity by scavenging free radicals which give credence for the management of various ailments associated with oxidative stress. Moreover, it also arrests the inflammatory cascade by preventing the decomposition of hydroperoxides into free radicals [21]. Total phenolic content of POL-6 was calculated as mg of gallic acid equivalents/g dry weight (mg GAE/g DW). The phenolic content estimated in POL-6 was 316.66±0.28 mg GAE/g. The total flavonoid content measured in POL-6 was 481.14±0.34 mg QR/g. Tannin content was calculated as mg CE/g DW using a quercetin calibration curve (range 0–400 µg/ml). The tannin content found in POL-6 was 100.43±0.41 (table

4). Excessive free radicals can cause extensive damage to the tissues and biomolecules leading to a pathological condition like inflammation, cell death, and organ failure. The scavenging of free radicals using antioxidants may offer resistance to oxidative stress and cell damage [22]. Although all the plants used for the preparation of POL-6 possess significant antioxidant properties but their synergistic effect has been tested using DPPH and nitric oxide assays.

Free radical scavenging activity of POL-6

DPPH radical scavenging assay

The principle of DPPH test is based upon the fact that if there is a presence of an antioxidant molecule in tested sample then DPPH radicals are scavenged, and yellow-coloured compound diphenyl hydrazine is formed [22]. DPPH radical scavenging activity of POL-6 was compared with ascorbic acid (standard) and presented in table 5. The results showed the degree of discoloration, indicates higher the free radical scavenging activity. The IC₅₀ values were calculated and found to be ascorbic acid (18±0.18 µg/ml) and POL-6 (22.54±0.21 µg/ml).

Table 4: Total phenolic, flavonoid, and tannin content in Pol-6

Polyphenol content (mg of GAE/g DW)	Flavonoid content (mg of QR/g DW)	Tannin content (mg of QR/g DW)
316.66±0.28	481.14±0.34	100.43±0.41

The results are expressed as mean (n=3)±SD

Nitric oxide radical scavenging assay

Nitric oxide reacts with oxygen to form nitrites (oxides of nitrogen) which are inhibited by the tested samples. The scavenging activity of POL-6 against nitric oxide formation was comparable with a standard drug. In nitric oxide assay, the IC₅₀ values of Ascorbic acid (17.99±0.17 µg/ml) and POL-6 (21.38±0.17 µg/ml) was found (table 5). Obtained results showed that POL-6 possess a significant antioxidant activity even at its very low doses. Inflammation is a biological defensive response to remove injurious stimuli as well as initiate the healing process. A simple and viable protein denaturation and HRBC membrane stabilization methods are used to study the anti-inflammatory in-vitro activity of POL-6.

Anti-inflammatory activity of POL-6

Albumin denaturation assay

The denaturation of proteins is a well-documented cause of inflammation; hence, this assay was adopted in assessing the properties of the POL-6 in stabilizing the protein from denaturation process. Results showed that the % inhibition done by POL-6 at the lowest conc. 25 µg/ml was 30.51±1.28 (table 6).

HRBC membrane stabilization assay

POL-6 was studied for its ability to stabilize human RBC membrane lyses in hypotonic saline. The results obtained were also compared with standard anti-inflammatory agent Diclofenac sodium.

Results showed that the % protection done by POL-6 at the lowest conc. 25µg/ml was 38.23±0.92 (table 6).

Immunomodulatory activity of POL-6

Lymphocyte proliferation assay

The POL-6 was tested for its ability to proliferate human lymphocytes. The results revealed that PBMC stimulation was significantly elevated with the POL-6 at 25 µg/ml and showed 61% proliferation. After 24 h of incubation, the lymphocytes that have been treated with Concanavalin A (positive control) showed 52% proliferation and with PBMC showed 3% proliferation. The present study established the immunostimulation effect of POL-6 at low concentrations and immunosuppression at high concentrations on human PBMC (table 7).

The polyherbal preparations enhance the therapeutic efficacy by improving the bioavailability thereby reducing the doses required for individual components in the preparation. Synergism also attenuates undesirable side effects. The prepared polyherbal preparation POL-6 was screened for various standardization parameters as per Ayurvedic pharmacopoeia standards. The results obtained from the study showed that the contents of preparation present are within permissible limits, and their standardization gives a point of view for further research investigation.

Table 5: Free radical scavenging activity of POL-6

Sample name	IC ₅₀ /DPPH (µg/ml)	IC ₅₀ /NO ₂ (µg/ml)
POL-6	22.54±0.21	21.38±0.13
Ascorbic acid	18±0.18	17.99±0.17

The results are expressed as mean (n=3)±Standard deviation (SD).

Table 6: Anti-inflammatory activity of POL-6

Conc. (µg/ml)	Albumin denaturation assay (% Inhibition)		HRBC membrane stabilization assay (% protection)	
	POL-6	Diclofenac sodium	POL-6	Diclofenac sodium
25	30.51±1.28	42.03±1.09	38.23±0.92	49.03±1.69
50	41.13±1.03	59.14±1.03	51.62±1.06	60.05±1.03
100	64.12±1.74	71.04±0.92	67.38±0.74	75.04±1.12
250	76.32±1.13	85.13±1.01	79.14±0.89	87.03±1.21
500	89.38±1.25	94.12±1.13	90.92±1.04	95.84±0.93

The results are expressed as mean (n=3)±Standard deviation (SD)

Table 7: Immunomodulatory activity of POL-6

S. No.	Concentration (µg/ml)	% Lymphocyte proliferation
1.	25	61±4.08
2.	50	21±1.01
3.	100	15±2.09
4.	200	-21±2.12

The results are expressed as mean (n=3)±Standard deviation (SD)

CONCLUSION

The present study also showed that POL-6 have significant antioxidant, anti-inflammatory and immunomodulatory activities. The beneficial effects of POL-6 in oxidative, inflammatory and

immune disorders may be attributed to the presence of multiple ingredients with multiple modes of actions.

The results from this study rationalize the medicinal use of POL-6 in oxidative, inflammatory and immune disorders.

However, further studies are required to identify the active ingredients and their contribution in overall beneficial effects of POL-6.

ACKNOWLEDGEMENT

The authors thank Science and Engineering Research Board (SERB), Department of Science and Technology, Government of India for financial support to this work (SR/FT/IS-07/2012).

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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