Pharmacognosy Communications
Volume 2 | Issue 3 | Jul-Sep 2012
www.phcogcommn.org

Research Article

Immunomodulatory and anti-oxidant potential of *Alpinia galanga* Linn. rhizomes

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ABSTRACT: **Introduction:** The plant *Alpinia galanga* Linn.(Zingiberaceae) is widely distributed in India. It is a perennial, aromatic and rhizomatous herb which is traditionally used as a carminative, stomachic, disinfectant, aphrodisiac and for the treatment of inflammation. The objective of present study is to evaluate the immunomodulatory and antioxidant effects of different extracts in a dose-dependent manner. **Methods:** Immunomodulatory activity was studied by examining T cell proliferation, splenocyte proliferation and by delayed type hypersensitivity reaction measurement. The antioxidant effect was observed by measuring lipid peroxidation (LPO), reduced glutathione (GSH) content and by determination of superoxide dismutase (SOD) and catalase (CAT) activities. **Results:** The flavonoid fraction of *Alpinia galanga* Linn. extract significantly stimulated ($P < 0.001$) T cell proliferation and splenocyte proliferation in mice spleen at a dose of 100 mg/kg body weight of mice. The aqueous fraction had a lower stimulatory effect than the flavonoid fraction. The presence of quercetin in the flavonoid fraction was confirmed by chromatographic studies. The antioxidant level of the spleen cells also increased following treatment with the flavonoid fraction. **Conclusion:** In conclusion the flavonoid fraction of *Alpinia galanga* Linn. rhizomes have greater immunostimulating effects as well as antioxidant effects in mice.

KEY WORDS: *Alpinia galanga;* galangal; immunomodulatory; antioxidant; flavonoid fraction; quercetin

INTRODUCTION

Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' is based on related principles.[1] Rasayana, listed as a class in the texts of traditional Indian medicine literature, consists of a number of plants reputed to promote physical and mental health, improve defense mechanisms of the body and enhance longevity. These attributes are similar to the modern concept of adaptogenic agents, which are known to afford protection of the human physiological system against diverse stressors.[2] The host defense response of humans is complex and multileveled, involving many cell types with distinct but overlapping roles. One of the earliest cell types to respond to invasion by potentially pathogenic organisms are the phagocytes, which are a key participants in the innate immune response.[3] Thus, the development of novel therapeutics to nonspecifically augment innate immune responses represents an ideal strategy for addressing current concerns of how to combat infectious agents.[4] The immune modulation is a procedure which may alter the immune system of an organism by interfering with its function or even by the modification of the function that may increase or reduce the ability to produce antibodies.

Alpinia galanga Linn. (Zinziberaceae) is a perennial herb which was found earlier in Indonesia, but is now available in many parts of India especially in the shady areas. Root stalk is tuberous and aromatic; the leaves are oblong-lanceolate, with acute margins and white sheath long ligule rounded. Plant contains 0.4% of essential oil (α-pinene, β-pinene, limonene, terpinen-4-ol, α-terpineol, linalool, methyl eugenol, eugenol and 1, 8-cineol).[5] It contains different constituents e.g. quercetin, kaempferol, isorhamnetin, kaempferide, quercetin 3-methyl ether, galangin, 1-acetoxychavicol acetate, 1-acetoxyeugenol acetate, galangal A, B and galanolactone. Through bioassay-guided separation, three new linked neolignans, galanganal and galanganols A-B were isolated form rhizomes of *Alpinia*

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DOI: 10.5530/pc.2012.3.7

galanga Linn. and were screened for nitric oxide production inhibitory action.[6]

A. galanga is used to treat dyspepsia, fevers, urinary incontinence, halitosis, and to reduce hoarseness in throat infections.[7] It is also used as a carminative and is useful in respiratory troubles. One study demonstrated that an alcoholic extract of the rhizomes induced hypothermia, rheumatism and catarrhal infections in mice.[8] Antiulcer activity has been demonstrated for the seeds.[9] It is useful in whooping cough in children. The volatile oil is also used in cough and digestive mixtures.[10] Various compounds have been isolated and screened for biological activities. These include terpenyl ester (2-endo-70 hydroxy-1, 8-cineole) for antimicrobial and antibacterial activity[11], essential oils for antifungal activity[12], hypoglycemic activity[13] and *in vitro* cholinesterase enzyme inhibition[14].

MATERIAL AND METHODS

Plant material and preparation of flavonoid fraction

The rhizomes of *Alpinia galanga* Linn. were procured from the local market, Sagar (M.P.) and authenticated (Bot/her/315) at Department of Botany, Dr. H. S. Gour Vishwavidyalaya, Sagar (M.P.). The rhizomes (200 g) were air dried followed by grinding to a moderately coarse powder. The powdered rhizomes were successively extracted with petroleum ether (40-60 °C), methanol and chloroform water I.P. Finally the extracts were vacuum dried, weighed and the percentage yield was calculated. The dried methanol extract was suspended in distilled water (50 ml) and partitioned in ethyl acetate (50 ml) in a separating funnel. The ethyl acetate layer was separated and tested for the presence of flavonoids. The ethyl acetate fraction gave crimson red colour with a few drops of concentrated hydrochloric acid in the presence of magnesium metal ribbon piece (Shinoda test).[15] All extract fractions were dried under vacuum and stored in closed containers for further studies.

HPTLC fingerprinting

A chromatographic study was carried out to develop solvent system and detect the presence of quercetin in the fractions. The thin layer chromatography (TLC) of different extract fractions (chloroform water, petroleum ether and flavonoid fraction) of *A. galanga* Linn. were carried out on a pre-coated silica gel plate (0.2mm, Merck 60 F-254, Germany) as the stationary phase and toluene: chloroform: acetone (40:25:35) as a mobile phase. The samples (10 µL) of extract fractions and standard compound quercetin (LGC Promochem India Pvt. Ltd., Bangalore) were spotted in the form of bands of width 6 mm with a 100 µL Hamilton syringe on precoated silica gel aluminum plate (10 cm × 10 cm; 0.2 mm, Merck 60 F-254, Germany) with the help of Linomat 5 applicator. The applicator was attached to CAMAG HPTLC system, which was programmed through WIN CATS software.

The linear ascending development was carried out in a 20 cm x 10 cm twin through glass chamber saturated with the mobile phase. The developed plate was dried by hot air to evaporate solvents from the plate. The developed plate was placed in UV chamber and observed at 254 nm. The plate was kept in densitometer (CAMAG Scanner 3) under UV light at 254 nm. The R_f values and finger print data were recorded by WIN CATS software.

Animals

Healthy male Swiss albino mice (5 weeks old) of uniform body weight (25 ± 5 g) with no prior drug treatment were used for *in vivo* studies. The animals were fed on a commercial pellet diet (Hindustan Lever, Bangalore, India), and provided with water ad libitum. The animals were acclimatized to laboratory hygienic conditions for 10 days before commencing the experiment. Permission of the Institutional Animals Ethics Committee was obtained for all animal experimentation (Registration number 379/01/ab/CPCSEA, India). The animals were divided into seven groups each containing six animals. The control group received only vehicle and other treatment groups received different extract fractions.

Chemicals and Reagents

RPMI-1640 medium was purchased from Himedia Laboratories Pvt. Ltd., India. Ethyl acetate, petroleum ether (40-60°C), methanol and chloroform were puchased from Rankem, Ranbaxy Fine Chemicals Ltd., Thane, Mumbai, India. All these chemicals were of analytical grade. TMB/H_2O_2 (3, 3, 5, 5-tetramethylbenzidine and hydrogen peroxide) was obtained from Genei, Banglore, India. 5, 5'-dithiobis (2-nitrobenzoic acid), lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phythaemagglutinin (PHA), superoxide dismutase (SOD), catalse (CAT) and reduced glutathione (GSH) and pyrogallol were purchased from Sigma Chemical Co. (USA). Sodium dodecyl sulfate (SDS), hydrogen peroxide solution, tocopherol and dimethyl sulfoxide (DMSO) were purchesed from Qualigen, Fine Chemicals, Mumbai, India. Dithionitrobenzoic acid (DTNB), trichloracetic acid (TCA), malonyl dialdehyde (MDA), 2-thiobarbituric acid (TBA), butylated hydroxy toluene (BHT), nitroblue tetrazolium (NBT) were purchased from Himedia Laboratories Ltd., Mumbai, India.

Immunomodulatory activity

In-vitro *T-cell proliferation assay*

Antigen-specific T-cell proliferation is a major technique for assessing the functional capacity of $CD4^+$ lymphocytes

to respond to various stimuli (e.g. antigen or mitogen). This method involves isolating peripheral blood mononuclear cells (PBMCs), placing 100,000 cells in each well of a 96-well plate with or without any stimuli, and allowing the cells to proliferate for four days at 37 °C in a 5% CO_2 incubator. Sheep blood was collected from the Military Hospital, Jabalpur, India. Peripheral blood mononuclear cells (PBMCs) were separated from Buffy coat residues by centrifugation. The cells were diluted to $2x10^6$ cells per ml, RPMI 1640 complete medium under sterile conditions, and dispensed in 96-wells micro titer plates (50 ml per well). Proliferation of cells was induced by 50 µl phythaemagglutinin (PHA, 5 µg/ml), and 50 µl test samples were added in appropriate dilution (100 and 200 µg/ml). After 4-day incubation at 37 °C, T-cell proliferation was determined using a modified colorimetric MTT assay.[16] To each well, 25-ml MTT (1 mg/ml) was added and the plate was incubated for 4 h at 37 °C. The formazan product formed was dissolved by adding 50 ml SDS solution and by shaking for 1 h on a microtiter plate shaker. Absorbance values were measured by ELISA reader (Bio-Rad, USA) operating at 550 nm. Controls consisted of peripheral blood lymphocytes with medium (negative).

Splenocyte proliferation assay

Spleens were collected from sacrificed mice under aseptic conditions and store in Hank's balanced salt solution (HBSS, Sigma). They were minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension. The erythrocytes were lysed with ammonium chloride (0.8%, W/V). After centrifugation (4 °C for 10 min), the pelleted cells were washed three times in phosphate buffer saline and re-suspended in RPMI 1640 complete medium. Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique.[17] The cell viability exceeded 95% splenocyte proliferation was assayed[18] and splenocytes were seeded into 4 wells of a 96-well flat-bottom micro titer plate at 5×10^6 cell/ml in 100µl complete medium with LPS (final concentration 10 µg/ml) were added giving a final volume of 200 µl in RPMI 1640 medium. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2. After 44 hrs, 50 µl of MTT solution (2 mg/ml) were added to each well and incubated for a further 4 hrs. The plates were centrifuged (1400, 5 min) and the untransformed MTT was carefully removed by pipetting. To each well 150 µl of a DMSO working solution was added, and the absorbance was evaluated in an ELISA plate reader (Bio-Rad, USA) at 570 nm after 15 min. The stimulation index (SI) was calculated based on the following formula:

$$SI = \frac{\text{Absorbance value for mitogen culture}}{\text{Absorbance value for no stimulated culture (control)}}$$

Effect on sheep red blood cell (SRBC) induced delayed-type hypersensitivity

Delayed type hypersensitivity response to SRBC was induced in mice by the method of Puri[19] and measured as described by Doherty.[20] Six week old mice were divided into seven groups, each consisting of six mice. Mice were immunized i.p. with 10^8 SRBC in 0.5 ml PBS (phosphate buffer saline). Beginning on the day of immunization, the immunized mice were administered p.o. with different extracts at the doses of 100 and 200 mg/kg for 5 days. The control groups received the same volume of saline. Five days post immunization the mice of all the groups were injected with 50 µl of SRBS (1×10^8) in the right front foot. The left front feet of each mouse were injected with the same volume of physiological solution (control). After 24 h the weight of experimental and control feet were determined. The reaction index (foot weight of test animal – foot weight of control) was expressed as an increment of test foot weight in relation to control foot weight.

Antioxidant activity

Reactive oxygen species (ROS) are known to be engaged in several of the critical steps (eg, dendritic cell activation/maturation, release of cytokines, T-cell activation) involved in the skin immune response. Numerous studies have shown that ROS directly involved in the elicitation of immune responses during delayed type of hypersensitivity reactions. At the sites of inflammation, tissue damage has been linked to the release of various pro-oxidants that directly affect dendritic cell maturation or activation or act as a signal to induce expression of different immune regulatory molecules (eg, cytokines).[21]

The antioxidant effect of flavonoid fraction was assessed by measuring the lipid peroxidation, enzymatic antioxidant status catalse (CAT) and reduced glutathione (GSH) in spleen of mice. The spleen was removed and a 10% homogenate was prepared using ice cold Tris-HCL (100 Mm, pH 7.4) buffer using a homogenizer. The homogenate was centrifuged at 2000 rpm for 15 minutes and the supernatant was used for biochemical studies. The detail methods are given following:

Lipid peroxidation

The spleen homogenates (0.5 ml) and 1 ml of 0.15 M potassium chloride were added to test tubes. Peroxidation was initiated by adding 100 ml ferric chloride after incubation at 37°C. The reaction was stopped by adding 2 ml of ice cold mixture of 0.25 N hydrochloric acid containing 15% trichloroacetic acid, 0.30% TBA and 0.05% BHT and reaction mixture heated for 1 h at 80 °C. The sample was cooled and centrifuged. The absorbance of supernatant was measured at 532 nm. Results were expressed in nmol of MDA/mg protein.[22]

Reduced glutathione (GSH)

Reduced glutathione (GSH) level was determined by the method of Moron.[23] Spleen homogenates of mice were immediately precipitated with 0.1 ml of 25% TCA and the precipitate was removed after centrifugation. Free-SH groups were assayed in a total 3 ml volume by the addition of 2 ml of 0.6 mM DTNB and 0.9 ml 0.2 mM sodium phosphate buffer (pH 8.0) to 0.1 ml of the supernatant and the absorbance was read at 412 nm using UV spectrophotometer. The GSH level was determine by standard curve of reduced glutathione.

Superoxide radical scavenging activity

The assay was based on the capacity of the sample to inhibit blue formazon formation by scavenging the superoxide radicals generated in riboflavin-light-nitro blue tetrazolium (NBT) system. The reaction medium contains 2.5 mL of phosphate buffer (pH 7.6), 100 µL riboflavin (20 µg), 200 µL EDTA (12mM), 100µL NBT (0.1 mg) and spleen homogenates sample contained in 100 µL of methanol. The reaction mixture was left to stand for 5-10 minutes. The absorbance was measured at 590 nm. Blank was performed in the same way with 100 µL of methanol instead of test substance (spleen sample).[24]

Catalase (CAT)

Beers and Seizer method was used to determine the activity of the enzyme Catalase (CAT). Three milliliters of reaction mixture containing 1.9 ml of phosphate buffer (0.05 M) pH 7.0, 1.0 ml of hydrogen peroxide (5.0 mM) and 0.1 ml of diluted enzyme (spleen homogenate) was used in this assay. The activity was measured by reading absorbance at 240 nm at 30 second interval for 3 min using UV spectrophotometer. The enzyme concentration was determine by standard Catalase.[25]

Statistical analysis

Data was expressed as the mean standard deviation (S. D.) and statistical analysis was carried out employing Tukey-Kramer multiple comparison test.

RESULTS

HPTLC fingerprinting

The HPTLC fingerprinting of different extract fractions of *Alpinia galanga* revealed several peaks were showed under UV 254 nm and recorded in Figures 1 and 2 (Table 1.). The standard compound quercetin gave single spot at R_f value 0.57 (Figure 2). The pet ether extract revealed 3 spots with R_f values in the range of 0.14 to 0.34 (Table 1). The chloroform water extract showed 6 peaks with R_f values in the range of 0.17 to 0.87 and the flavonoid fraction revealed 7 peaks with R_f values in the range of 0.06 to 0.86 and purity of the sample was confirmed by comparing the absorption spectra at start, middle and end position of the band.

Immunomodulatory activity

In-vitro *T-cell proliferation assay*

The lymphocyte formation was found greater in the group containing chloroform water and petroleum ether extracts at concentration 100 µg/ml in comparison to control group. The flavonoid fraction containing group at 200 µg/ml concentration showed greater lymphocyte formation as compared to 100 µg/ml concentration and control group (Table 2). The flavonoid fraction showed greatest lymphocyte formation (200 µg/ml) than other extract fraction and also comparable to the PHA group.

Splenocyte proliferation assay

The effect of the extracts on the number of antibody producing cells represented as plaque forming cells (PFC) per million spleen cells is shown in Table 3. The group treated with chloroform water extract and petroleum ether extract showed lower PFC and not significant than the control group of animals. The stimulation index was found significantly increased in flavonoid fraction treated group of animals in both doses (100 and 200mg/kg). The flavonoid fraction treatment enhanced the number of PFC in the spleen at both doses tested. The number of PFCs gradually increased, which was approximately two times greater the number of PFCs formed than in controls.

Effect on sheep red blood cell (SRBC) induced delayed-type hypersensitivity

The hypersensitivity reaction due to the influx of neutrophils and macrophages was observed in control mice (Table 4). It was observed that the flavonoid fraction applied at two doses practically does affect the mouse reaction of delayed-type hypersensitivity in both doses and significant increase in the reaction was observed. The CWE

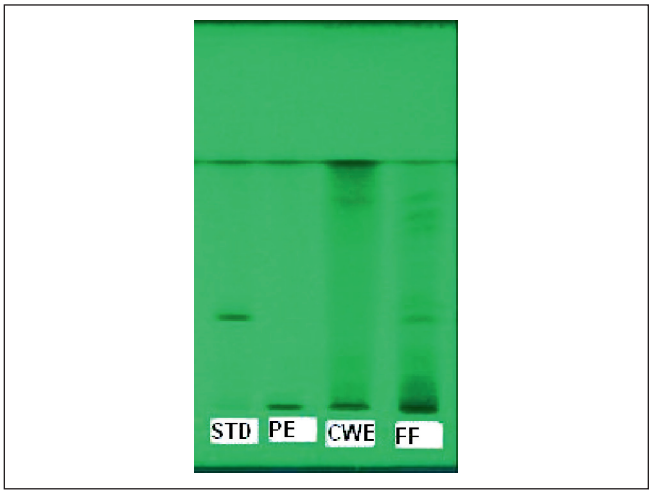

Figure 1: TLC of different fractions with standard quercetin; solvent system: Toluene: chloroform: acetone (40:25:35)

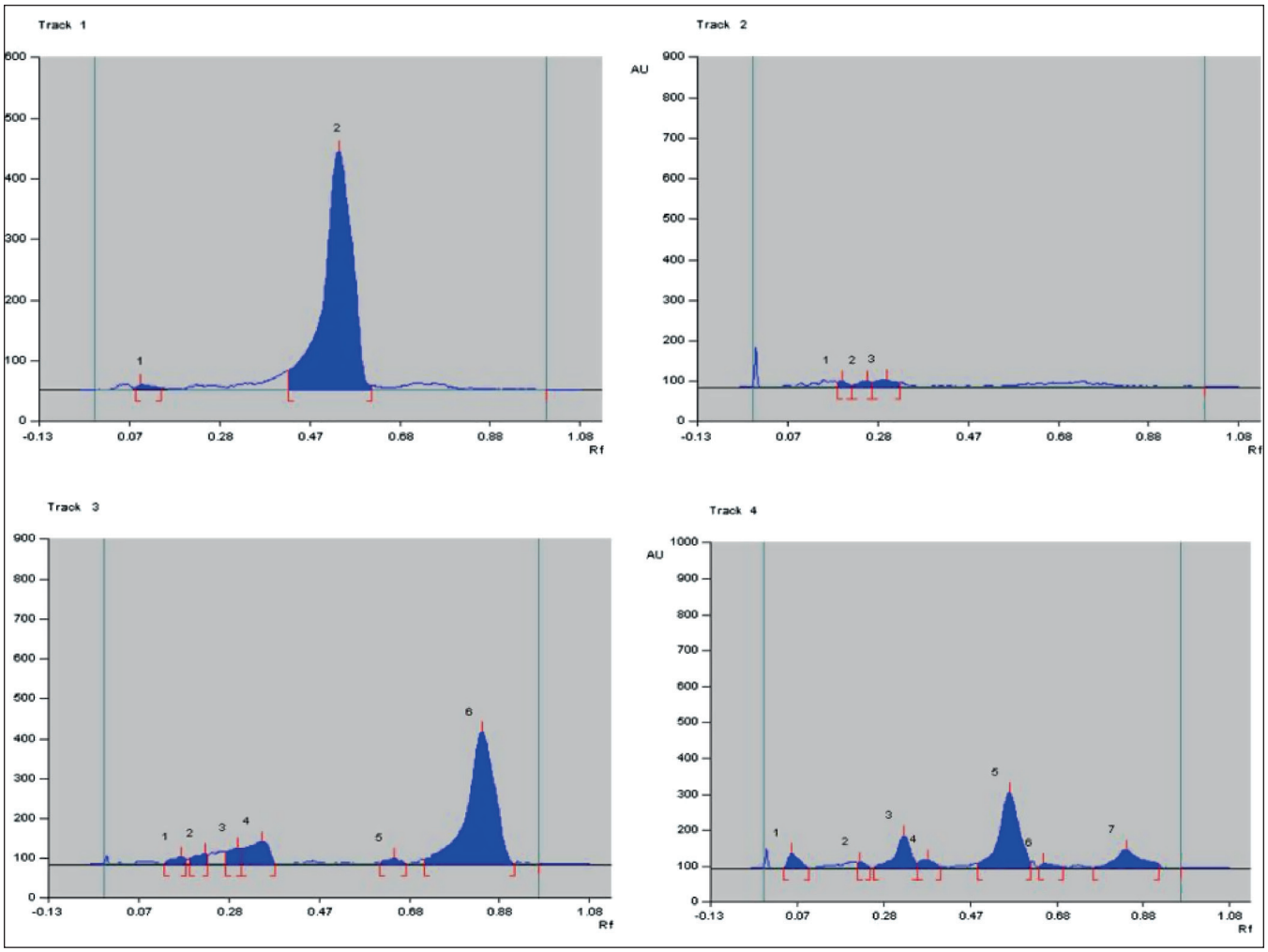

Figure 2: HPTLC profile of different extract fraction of *Alpinia galanga* Linn.
Track 1- Standard (STD); Track 2- petroleum ether extract (PE); Track 3- chloroform water extract (CWE); Track 4- flavonoid fraction (FF)

Table 1: HPTLC profile of the different extract fractions of *Alpinia galanga* Linn

Sample	Peak	Max. R_f	Area
Standard	1	0.10	102.7
	2	0.57	21181.4
PE	1	0.14	187.4
	2	0.22	207.6
	3	0.34	384.1
CWE	1	0.12	138.5
	2	0.26	224.2
	3	0.31	335.8
	4	0.38	734.2
	5	0.64	187.6
	6	0.87	17157.6
FF	1	0.06	204.2
	2	0.24	133.1
	3	0.30	486.5
	4	0.38	253.4
	5	0.57	15714.2
	6	0.63	137.6
	7	0.86	764.8

CWE: chloroform water extract; PE: petroleum ether extract; FF: flavonoid fraction

Table 2: Effect of different extracts on *in-vitro* lymphocyte proliferative activity

Groups	Lymphocyte formation
Control	3814 ± 438.26
CWE (100 µg/ml)	5965 ± 540.21*
CWE (200 µg/ml)	5448 ± 445.37*
PE (100 µg/ml)	4989 ± 430.12
PE (200 µg/ml)	4247 ± 536.33
FF (100 µg/ml)	6359 ± 542.84**
FF (200 µg/ml)	6931 ± 554.31**
PHA (5 µg/ml)	10245 ± 846

All values expressed as mean ± SD (n = 6); CWE: chloroform water extract; PE: petroleum ether extract; FF: flavonoid fraction; PHA: phythaemagglutinin; *P < 0.05, **P < 0.001, when compared to control.

and PE treated group also increases the mouse foot weight but does not increase significantly than the control group of animals. The reaction index was found 63.6 and 71.1 in flavonoid fraction treated group.

Antioxidant activity

The oxidative stress marker studies revealed that in delayed-type hypersensitivity reactions, the levels of LPO was increased, decreased the activities of SOD and CAT and reduced the content of GSH (Table 5). The SOD content of spleen was elevated when the animals were administered flavonoid fraction by intraperitoneal injection (Table 4). The CAT produced by the spleen reached a peak at the dose of 100 mg/kg bodyweight ($P < 0.001$) and 200 mg/kg bodyweight ($P < 0.001$). Flavonoid fraction markedly raised the activity of SOD ($P < 0.001$) in a dose dependent manner in spleen of mice. The chloroform water and petroleum ether extract does not increase antioxidants level significantly in mouse spleen.

Table 3: Effect of different extracts on splenocyte proliferation activity

Groups	PFC/million spleens	Stimulation index
Control	361.1 ± 24.04	1.00
CWE (100 mg/kg)	495.3 ± 32.54*	1.37
CWE (200 mg/kg)	483.8 ± 39.27*	1.34
PE (100 mg/kg)	358.1 ± 28.14	0.99
PE (200 mg/kg)	378.4 ± 29.68	1.04
FF (100 mg/kg)	687.2 ± 57.29**	1.90
FF (200 mg/kg)	765.1 ± 58.21**	2.12

All values expressed as mean ± SD (n = 6); CWE: chloroform water extract; PE: petroleum ether extract; FF: flavonoid fraction. *$P < 0.05$, **$P < 0.001$, when compared to control.

Table 4: Influence of different extracts on mouse reaction of delayed-type hypersensitivity

Groups	Mouse foot weight	Reaction index
Control	98.5 ± 5.07	0.0
CWE (100 mg/kg)	118.7 ± 8.56*	20.2
CWE (200 mg/kg)	143.2 ± 10.51*	44.7
PE (100 mg/kg)	108.7 ± 6.27	10.2
PE (200 mg/kg)	101.5 ± 8.35	03.0
FF (100 mg/kg)	161.2 ± 11.72**	63.6
FF (200 mg/kg)	168.6 ± 13.24**	71.1

All values expressed as mean ± SD (n = 6); CWE: chloroform water extract; PE: petroleum ether extract; FF: flavonoid fraction. *$P < 0.05$, **$P < 0.001$, when compared to control.

DISCUSSION AND CONCLUSION

Immune modulation is an attempt to improve or restore immune responses artificially through the therapeutic use of cytokines, cell receptors, agents or other proteins that have been produced using recombinant DNA technology. Specific immunomodulation may be actively achieved by administration of antigen in a form or by a route that induces a certain type of immune response.[26] Immunomodulatory effect of different extracts on antigen specific cellular immunity can be measured by calculating the delayed type hypersensitivity (DTH) response by measure footpad weight. Upon injection of the antigen, Langerhan's cells process the antigen and present it to local memory T cells, whether they are CD4+ or CD8+. These T cells in concert with activated Langerhan's cells secrete numerous cytokines that cause the early hallmarks of inflammation. Approximately 4 h after injection of antigen, neutrophils rapidly accumulate around the post-capillary venules at the infection site. The neutrophil infiltration rapidly subsides and by 12 h the injection site becomes infiltrated with T-cells and blood monocytes. The endothelial cells lining these venules swell, show increased biosynthetic organelles and become leaky to plasma macromolecules. Fibrinogen escapes to the surrounding tissue from the blood vessels and gets converted to fibrin. Deposition of fibrin, accumulation of T-cells and monocytes within the extravascular tissue space around the injection site cause the tissue to swell and indurate. The endothelial cells secrete vasodilators such as prostacyclin. The vasodilatation caused by the prostacyclin optimises delivery of immune cells to the site of challenge. The endothelial cells remodel the basement membrane and allow the extravasations of plasma macromolecules, especially fibrinogen.[26]

Immunization of a mouse with an antigen such as SRBC results in a lag phase of 3-4 days. The peak serum antibody levels are attained around 7-10 days. The secondary response has a shorter lag period, more rapid onset, reaches a greater magnitude and lasts for longer. Secondary response is characterized by secretion of antibody with a higher affinity for the antigen, isotypes other than IgM predominant.[27]

Table 5: Effect of different extracts on lipid peroxidation and antioxidant level of mice splee

Groups	LPO (nmol of MDA/mg protein)	GSH (µmol/50 mg tissue)	SOD (µg/50mg tissue)	CAT (µmol/50 mg tissue)
Control	7.61 ± 0.72	14.28 ± 0.7	16.24 ± 0.31	13.13 ± 0.8
CWE (100 mg/kg)	7.72 ± 0.57	18.46 ± 1.2	17.62 ± 0.8	14.95 ± 0.7
CWE (200 mg/kg)	8.47 ± 0.84	19.48 ± 1.4*	17.60 ± 1.08	16.94 ± 0.85*
PE (100 mg/kg)	7.59 ± 0.52	14.28 ± 0.82	15.38 ± 1.04	14.18 ± 0.76
PE (200 mg/kg)	8.64 ± 0.92	14.08 ± 1.07	16.08 ± 0.29	13.21 ± 0.89
FF (100 mg/kg)	10.43 ± 1.37*	21.35 ± 1.08*	20.27 ± 1.32*	19.26 ± 1.62*
FF (200 mg/kg)	10.52 ± 1.08*	22.64 ± 1.78*	19.38 ± 1.35*	21.61 ± 1.58*

All values expressed as mean ± SD (n = 6); CWE: chloroform water extract; PE: petroleum ether extract; FF: flavonoid fraction. *$P < 0.05$, when compared to control.

Many diseases, including heart disease, cancer, and even aging, have been linked to excess production of free radicals, such as superoxide, singlet oxygen, and hydroxyl radicals. These free radicals may be generated as a result of oxygen metabolism during the course of normal cellular activity, and may even be helpful in certain settings such as inside a phagocytic vesicle where free radicals may assist in the destruction of invading microorganisms. However, elevated levels of free radicals generated during periods of stress due to infection or exposure to toxic components may result in damage to host tissues.[28] Reduced glutathione, a free radical scavenger, plays a key role in the activation of T cells and macrophages.[29]

The effect of different extract fraction was tested on cell-mediated immunity by measuring T cell proliferation (two concentrations of extract fraction i.e. 100 and 200 µg/ml), splenocyte proliferation and DTH response. The effect was tested at two different dose levels 100 and 200 mg/kg body weight on animals. Results obtained during present investigation showed significant (P<0.001) increase in reaction index (RI) in response to SRBCs at doses 100 and 200 mg/kg with flavonoid fraction. The chloroform water extract group showed significantly increase in RI but less in comparison to flavonoid fraction group. The group of petroleum ether extract had not observed significant reaction index and values obtained were more or less equal to control group of animals (Table 3). The results of lymphocyte and splenocyte proliferation study indicated that lymphocyte formation was found greater in flavonoid fraction as well as in chloroform water extract group at both doses level. It also increases in group of petroleum ether but less in comparison to other extract fraction groups. The splenocyte proliferation study showed greater stimulation index in group of flavonoid fraction as well as in chloroform water extract group than petroleum extract group.

The present results indicated that *Alpinia galanga* was an effective modulator of T cell mediated immune response and DTH response. The antibody production to T-dependent antigen SRBCs requires the co-operation of T-lymphocytes and macrophages.[30] DTH is antigen specific and causes erythema and induction at the site of antigen infection in immunized animals. T-cells are required to initiate the reaction.[31]

Antioxidant enzymes are considered to be a primary defence that prevents biological macromolecules from damage in antioxidant systems.[32] SOD protects cells against O^{2-} by dismutation of the highly reactively superoxide anion and to a less reactive species, H_2O_2 plus O_2.[33] H_2O_2 can be eliminated by glutathione reductase or catalase.[34] Among the oxygen radicals, the hydroxyl radical is the most reactive and severely damages adjacent biomolecules.[35] These radicals react with biological molecules such as DNA, proteins and phospholipids and eventually damage membranes and other tissues. The flavonoid fraction may significantly enhance the antioxidant enzyme activities (SOD, CAT and GSH) to maintain the balance of the prooxidant/antioxidant inside the body.[36] *Alpinia galanga* Linn. contains quercetin, kaempferol, isorhamnetin, kaempferide and quercetin 3-methyl ether. It has been reported that glycoside of quercetin[37] significantly increases the antibody titre, carbon clearance and delayed type hypersensitivity response in mice. Superoxide dismutase (SOD) catalyzes the breakdown of O_2^- to O_2 and H_2O_2, prevents formation of OH^- and has been implicated as an essential defence against the potential toxicity of oxygen. Thus, SOD is one of the essential cofactors for antioxidants that protects against endogenous oxygen radicals generated during pathogenic processes and other changes in the physiological state. In this study, SOD activity significantly increased in mice treated with flavonoid fraction when compared with the normal control *in vivo*.

In conclusion, the results obtained in the present study indicate that the immunomodulatory activity of *A. galanga* is greater in the flavonoid fraction and the aqueous extract. Moreover numerous natural products such as flavonoids, phenols, glycosides, terpenoids, etc. are also prime free radical scavengers. The chromatographic studies support the presence of quercetin in the flavonoid fraction. It was concluded that *A. galanga* may have immunomodulatory potential due to presence of quercetin in the flavonoid fraction although further studies are required to confirm this. The investigations have revealed that GSH and SOD level were reduced during delayed type hypersensitivity reaction. Indeed, free radicals are the signaling entities in T cell activation. The antioxidant effect of flavonoid fraction of *A. galanga* could increase the antioxidant enzymes level and this may be the mechanism for the observed immunomodulation in the mice.

ACKNOWLEDGEMENTS

The authors are grateful to Genei, Banglore (India); The Himalayan Drug Co., Bombay (India); Institute of Microbial Technology, Chandigarh (India) for providing chemicals required for the study.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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