

Immunomodulatory and Antioxidant Activity of *Curculigo orchioides* Gaertn

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ABSTRACT: *Curculigo orchioides* Gaertn. (Family: Hypoxidaceae), commonly known as Kali-Musli, is found to be growing in is found to be growing in tropical and subtropical Asia from India to Malasia. Rootstocks are highly valued as a tonic, bitter, restorative, aphrodisiac. Methanol extract (Ext M) and its fractions and polysaccharide rich aqueous extract (Ext PS) prepared from the marc left after the preparation of methanol extract, were evaluated for immunomodulatory. Ext M, which was found to be more effective than Ext PS, in carbon clearance assay and *E. coli* induced abdominal peritonitis in mice, was further fractionated into ethyl acetate soluble fraction (Ext E) and saponin fraction (Ext S). Ext E performed marginally better than Ext S in haemagglutination (HA) titre, delayed type hypersensitivity (DTH) and plaque forming cell (PFC) assays. Only Ext M was subjected to *in vitro* antioxidant activity, since Ext M and its both fractions (Ext E and Ext S) showed similar immunomodulatory activity. Ext M exhibited substantial activity in DPPH antiradical, super oxide scavenging, nitric oxide scavenging, lipid peroxidation and protection against superoxide induced damage to erythrocytes. A marker compound, 3,5-diacetoxy 2-methoxy 6-methyl phenyl acetic acid, was isolated from Ext E and estimated using HPTLC method.

Key words: *Curculigo orchioides*, immunostimulatory, antioxidant activity, HPTLC.

INTRODUCTION

Curculigo orchioides Gaertn. is a small stemless herb found in India in the sub tropical Himalayas from Kumaon eastwards and from Western Ghats to Konkan southwards. It is commonly known as Kalimusli in Hindi. The rootstock is considered as a Rasayana drug [1] and used as an alterative, demulcent, restorative, aphrodisiac, depurative, hepatoprotective and in skin diseases [2]. In China it is used for the treatment of decline in the strength [3]. Rootstocks of *C. orchioides* was evaluated for their effect on specific and non specific immune responses. Role of free radicals have been implicated in immune system related disorders, thus the active parent extract was studied employing different *in vitro* test models.

MATERIALS AND METHODS

Animals

Swiss albino mice of either sex weighing 25-30 g were used in the present study. The distribution of animals in groups, the sequence of trials and the treatment allotted to each group were randomized. The animals

had free access to standard pellet diet and clean tap water under standard condition circadian cycle, humidity and temperature. The protocols were permitted by the institutional animal ethical committee.

Plant material and extraction

Dried rootstocks of *C. orchioides* were collected from local market of Ahmedabad, in the month of October, 2005. The authenticity of the rootstocks was established and the voucher specimen (LM 147) was deposited in the Pharmacognosy department, L. M. College of Pharmacy, Ahmedabad. Dried powder (250g, 60#) was defatted with petroleum ether (60° to 80°, 1 h; 3 X 500ml), to yield yellow semisolid residue (Ext P, 0.8% w/w). Defatted powder was exhaustively extracted with methanol (Ext M) and concentrated (5.48% w/w), dried and made aqueous to extract with ethyl acetate (3 X 500 ml, 2.31% w/w) and *n*-butanol (3 X 500 ml) successively. The latter was concentrated and taken in alcohol and excess of solvent ether was added to precipitate saponins. The precipitates were separated, redissolved in alcohol and excess of solvent

ether was added to achieve a purified saponin fraction (Ext S, 1.4% w/w). The marc left after methanol extraction was dried, extracted with water (3 X 500 ml), and polysaccharides were precipitated out by adding excess of acidified ethanol in water extract (Ext PS, 0.68% w/w).

Phagocytic activity

Swiss albino mice were divided into (n = 8) three groups. Group I was control receiving distilled water, group II and III were given Ext M and PS respectively (100, 200, 300 mg/kg body weight, p.o.) for 5 days. The method was executed as reported earlier [4]. The clearance of carbon particles from blood was determined at 650 nm and phagocytic index (PI) was calculated as slope of time by concentration curve of test divided by that of control.

Evaluation of Immunoprophylactic effect

The strength of pathogenic *E. coli* was standardized to induce 100% mortality (10^9 cells/30 gm body weight) and administered to mice as reported earlier [5], which were grouped and dosed (optimum dose for 15 days) as above. Apart from mortality, the extent of bacteraemia was determined by enumeration of colony forming units (cfu) on Mac Conkey's agar medium (Hi Media, India) from samples retrieved at 1, 12 and 24h after challenge.

Haemagglutinating antibody (HA) titre and Delayed type hypersensitivity (DTH) response

The assays were done as described earlier [6] with grouping and dosing (7 days) as in immunoprophylactic effect. Blood from SRBC (8th day) immunized animals were collected (11th day) for two fold dilution in microtitre plates (Polylabs, India) with phosphate buffer saline. The haemagglutination titre was considered as the reciprocal of the highest dilution of test serum giving agglutination. For delayed type hypersensitivity the animals were challenged with subplanter injection of SRBC on 11th day followed by Vernier caliper aided measurement of edema. The difference between the pre and post challenge foot thickness expressed in mm was taken as a measure of delayed type hypersensitivity (DTH).

Plaque forming cell (PFC) assay

The animals grouped as above, were administered optimal dose for 7 days, followed by procurement of spleens from euthanized animals on the 8th day. As reported earlier [7], spleen cells were separated in RPMI-1640 medium and layered with agarose (Hi-Media, India) in presence of SRBC and fresh guinea pig serum. The plaques were expressed as count per 10^5 spleen cells.

Study of antioxidant activity

Antiradical activity (free radical scavenging activity) by DPPH method

Antiradical activity was measured by observing decrease in absorbance at 516 nm of a methanol solution of colored DPPH (1, 1-diphenyl-2-picrylhydrazyl, a stable free radical) brought out by the test at various concentrations (20, 30, 40, 50, 60 and 70 μ g/ml) as reported earlier [8]. Ascorbic acid was used as a reference standard and DPPH alone in methanol as control. The activity was expressed as an effective concentration at 50% (EC₅₀) i.e. the concentration of the test solution required to give a 50% inhibition calculated as reported.

Superoxide radical scavenging activity

Superoxide anion radical scavenging assay was performed by monitoring the reduction of nitroblue tetrazolium (NBT) to a blue colored formazan as reported elsewhere [9], which was measured at 590 nm at regular interval of 30 sec up to 2.5 min and terminally at 4 min. The EC₅₀ from % Inhibition of superoxide radical scavenging activity was calculated.

Erythrocyte membrane stabilizing activity

The assay was carried out according to the procedure described by Navarro et al. [8], wherein the hemolysis of RBC was induced with superoxide radical by a riboflavin-light-NBT system. The % protection provided by test (300 μ g/ml) was calculated as described previously. Hydrocortisone was taken as a reference standard and control was prepared without test solution.

Lipid peroxidation

As reported earlier [10, 11] in presence of hydroxyl radical and deoxyribose, lipids from liver homogenate of wistar rats, fragmentize to malonyl dialdehyde (MDA) that binds to 2-thiobarbituric acid (TBA) to form pink MDA-TBA chromogen was estimated at 532 nm. Test (6, 8, 10, 12, 14 and 16 μ g/ml) and α -tocopherol taken as a reference standard showing antioxidant activity were expected to interfere with the above process by scavenging hydroxyl radical, evident from malondialdehyde content calculated as reported earlier. % Inhibition and EC₅₀ was calculated as above.

Hydroxyl radical (OH $\cdot$) scavenging activity

The formaldehyde formed during the oxidation of dimethylsulphoxide (DMSO) by Fe⁺³ - ascorbic acid was used to detect hydroxyl radicals [10, 12]. The reaction mixture with different concentrations of test sample (10, 100 and 1000 μ g/ml) dissolved in phosphate buffer or 100 μ l ascorbic acid (2 mM) was incubated for 30min at 37°. The reaction was stopped by adding 125 μ l of trichloroacetic acid (17.5% w/v)

and the formaldehyde formed, followed by another reaction for 10 min at 50°, was determined by measuring the absorbance of reaction mixture at 412 nm.

Statistical analysis

Results expressed as the mean $\pm$ SEM were examined for statistical significance by one way analysis of variance (ANOVA factor) followed by posthoc analysis of Tuckey's t test with the level of significance set for $p \leq 0.05$. The r^2 values were calculated from the regression curve of the mean values.

Isolation of the marker 3, 5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid

100mg of Ext E was loaded on a glass column (30 X 1cm), packed with silica gel G (10g, 160-200#, Spectrochem Pvt. Ltd.) as a stationary phase. Gradient elution was performed using toluene: ethyl acetate (100:0 to 0:100). The eluted fractions monitored by TLC (toluene-10 : ethyl formate-2) and rechromatographed. Fractions 50-53 yielded chromatographically pure compound (R_f 0.43), which was subjected to melting point and spectral analysis (FT-IR-8400S, Shimadzu; GC-MS-Q5050, Shimadzu; NMR, Bruker Advance-400)

High performance thin layer chromatography (HPTLC) of the marker compound

The isolated marker compound was used as working standard. For test solution, 10mg of ethyl acetate extract (Ext E) taken in volumetric flask and volume was adjusted to 5ml with methanol to get a concentration of 2mg/ml.

Chromatographic conditions were as follows:

Instrument	:Camag linomat IV (semi automatic spotting device) equipped with Camag TLC scanner 3 and Camag CATS 4 integration software.
Stationary phase	:Precoated TLC plate of silica gel G 60 F ₂₅₄ (Merck).
Mobile phase	:Toluene: Ethyl formate (10:2)
Spotting parameter	:3-18 μ l of standard solution of 3,5-diacetoxy-2-methoxy-6-methyl phenyl acetic acid (2 mg/ml)
Test sample	: 40 μ l
Temperature	: 25 $\pm$ 2°.
Migration distance	: 8 cm.
Detection	:Faint blue colored florescent spot in U.V light at 366 nm.

Estimation of 3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid

Graded concentration of standard 3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid solution (2mg/ml; 3, 6, 9, 12, 15 and 18 μ l) was spotted on methanol washed silica gel G 60 F₂₅₄ TLC plate (Merck) with Camag linomat IV automatic spotter. 40 μ l of test sample solutions were used for spotting. The plate was developed in mobile phase toluene : ethyl formate (10:2) and scanned at 366 nm. Data of peak height and peak area were recorded. Standard curve of peak area by concentration was plotted and the concentration of the compound was determined by comparing the area of standard solution from calibration curve.

RESULTS AND DISCUSSION

Plants and other natural products have been in use since ages for health and maintenance of life. The vedic literature, the most authentic, ancient Indian scripture, gives the reference of many plants for different diseases and their prevention. In Ayurveda, Rasayana plants are particularly recommended for the treatment of epidemic diseases [13]. Apart from immunostimulating activity, they have also been evaluated for their anabolic, antistress, adaptogenic, nootropic, antioxidant and antiaging effects [14].

Carbon clearance assay reveals effect of phagocytosis in *in vivo* systems emphasizing on the innate and non specific immunity [15]. The phagocytic index (PI) of Ext M (PI of 2.1, 1.6 and 0.8 at 300, 200 and 100 mg/kg body weight) suggested higher phagocytic activity than Ext PS (PI of 1.2, 0.74 and 0.4 at 300, 200 and 100 mg/kg body weight), indicating a significant activation of the macrophages and reticuloendothelial system [16]. The dose of 300 mg/kg body weight was found to be optimum without any overt signs toxicity and hence was used for other assays.

In *E. coli* induced peritonitis the mortality within 24 h is generally considered due to septic shock followed by failure of major organs under siege of bacteria. The positive immunoprophylactic effect of Ext M (37.5% mortality), as compared to control (100% mortality) and Ext PS (75% mortality) against *E. coli* induced peritonitis, indicated that Ext M might have enhanced the capacity of the monocyte-macrophage system [5]. This was evident from enhanced bacterial clearance (60.33 $\pm$ 3.05% reduction in cfu) with the use of Ext M in comparison to the control (17.40 $\pm$ 3.38% reduction in cfu; $p < 0.05$) and Ext PS (22 $\pm$ 4.64% reduction in cfu). Ext M being more effective than Ext PS, its fractions Ext E and Ext S were further evaluated for their immunomodulator potential in specific immune responses.

In haemagglutinating antibody titre assay, Ext E (576 ± 98.63 ; $p < 0.05$) and Ext S (384 ± 44.89 ; $p < 0.05$) performed better than the control (128 ± 19.59). A similar pattern was observed in plaque forming assay, where Ext E (212 ± 1.78 ; $p < 0.05$) and Ext S (160.2 ± 2.12 ; $p < 0.05$) outperformed control (104 ± 1.66). Presensitization with suboptimal doses of antigen, sheep red blood cells (SRBC) is known to generate helper T-cells for humoral response and also memory cells for delayed type hypersensitivity (DTH) response to SRBC [17]. The enhancement in number of PFC, from spleen cells, and antibody titre, in the circulation, are the functions of B-cells. These also suggest the positive adjuvant effect, a significant immunopotentiating action on humoral response, which may be mediated by T-lymphocytes, macrophage and complement [5, 18]. Release of cytokines from sensitized T-lymphocytes in response to SRBC is an important marker of cell mediated immunity because of which DTH model was realized [18]. Pretreatment with Ext E, 0.095 ± 0.0036 (143.88%), and Ext S, 0.080 ± 0.0068 (105.12%), increased DTH response to SRBC in mice as compared to control, 0.039 ± 0.0019 ($p < 0.05$) suggesting immunopotentiating effect of both the fractions. In conclusion, the results obtained in the present study indicated that the immunomodulatory activity of *C. orchioides* can be localized to both Ext E (containing phenolics) and Ext S (saponin rich fraction). Due to equipotent activity of Ext E and S, the parent fraction Ext M was examined for its interaction with reactive oxygen species (ROS) in various ROS-generating *in vitro* chemical reactions. Reactive oxygen species (ROS) such as superoxide, hydroxyl radical, iron- O_2 complexes, H_2O_2 and lipid peroxides that are generated by several reactions are down stream inflammatory mediators and result into substantial tissue damage. These radicals react with biological molecules such as DNA, proteins and phospholipids and eventually damage membranes and other tissues [19]. Several proteins and biomolecules in the living organism act as free radical scavengers. Moreover numerous natural products like flavonoids, phenols, glycosides, terpenoids, lignans etc. are also prime free radical scavengers [20-22]. The dose dependent scavenging of DPPH radical indicated by decrease in optical absorbance due to the trapping of

the unpaired electron, by Ext M ($EC_{50} = 29.97$, $r^2 = 0.95$) was comparable to that of ascorbic acid ($EC_{50} = 11.02$, $r^2 = 0.98$). Reduction of NBT in superoxide ($O_2^{\cdot -}$) anion scavenging assay [23] indicated inhibitory effect of Ext M ($EC_{50} = 116.84$, $r^2 = 0.95$) with scavenging potential similar to that of ascorbic acid ($EC_{50} = 111.27$, $r^2 = 0.99$). The activity, for many phenolic antioxidants, is said to depend on hydrogen atom donation leading to the formation of secondary radical species [24]. The lipids in the red cell membrane are highly unsaturated and packed with hemoglobin [25]. Thus illumination in presence of porphyrins causes superoxide peroxidation aggregation and eventually hemolysis, wherein Ext M yielded $30.64 \pm 2.7\%$ protection similar to that of ascorbic acid ($38.45 \pm 1.3\%$). Supportive to this, NADH and NADPH aided [26] liver lipid peroxidation induced by iron-ADP-ascorbate system MDA formation was effectively curbed by Ext M ($EC_{50} = 10.20$, $r^2 = 0.95$) in comparison to that of α -tocopherol ($EC_{50} = 68.75$, $r^2 = 0.98$). Antioxidant compounds effective in preventing NADPH dependent lipid peroxidation act by inhibiting the enzymatic reduction mediated by molecular oxygen bound to the iron-ascorbate complex. $ADP-Fe^{+2}$ promote both oxygen uptake and MDA formation and ascorbic acid induces lipid peroxidation apart from reducing and stabilizing the MDA modifications of protein and DNA [27]. The hydroxyl radical is highly reactive oxygen species and is thought to be a major factor responsible for oxidative injury of enzymes, lipid membranes and DNA strand breakage [28], although the reaction affecting the stereochemistry of lipids is reversible [29]. Ext M ($EC_{50} = 113.76$, $r^2 = 0.99$) out-competed DMSO in scavenging hydroxyl radicals, thus reducing formaldehyde produced. Collectively it can be said that Ext M has potent antioxidant activity as vouched by *in vitro* assays for different kinds of radicals and oxygen species.

Column chromatography of Ext E performed using Toluene : Ethyl formate (100:0.1) and rechromatographed with toluene yielded solid (3.4 mg) compound having m. p. $156-160^\circ$. The identity was developed by subjecting it to spectral analysis (IR, NMR, MS) (Table 1) and was concluded to be 3, 5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid (Fig 1).

Table 1: IR, NMR and MS spectral data of 3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid.

Spectra	Spectral data
IR	3270-3400 (broad), 2854, 2616.17, 1730, 1639 (broad), 713.6 cm^{-1}
NMR	1.22 (s, 3H, $-\underline{CH}_3$), 2.47 (s, 3H, $-\underline{OCOCH}_3$), 2.48(S), 3.99 (3H, $-\underline{OCH}_3$), 5.30 (benzylic proton), 7.15, 7.23 (aromatic proton)
MASS	296 (M^+), 266 ($-\underline{OCH}_3$), 222 ($-\underline{COCH}_2$), 180 ($-\underline{CH}_3$), 136 ($-\underline{COOH}$), 123 (butate)

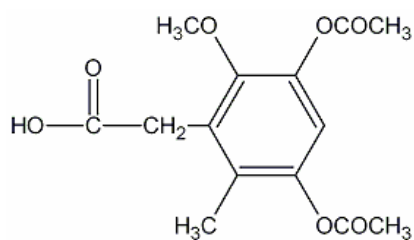


Figure 1. Structure of 3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid

Considering, 3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid as a chemical marker, HPTLC method was developed to estimate its content in drug. Estimation of it was done by HPTLC method, using toluene : ethyl formate (10:2) as a mobile phase, silica gel 60 F₂₅₄ as a stationary phase. The plate was scanned at 366nm (Fig. 2).

The HPTLC patterns of the test sample and working standard (3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid) revealed that the peaks corresponding to R_f

0.42 in both test and standard showed blue fluorescence and were superimposable. By single level HPTLC analysis, 3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid content was found to be 0.14% w/w in the rootstock.

The proposed HPTLC method was validated using different validation parameters such as linearity, precision, specificity, limit of detection and limit of quantification (Table 2).

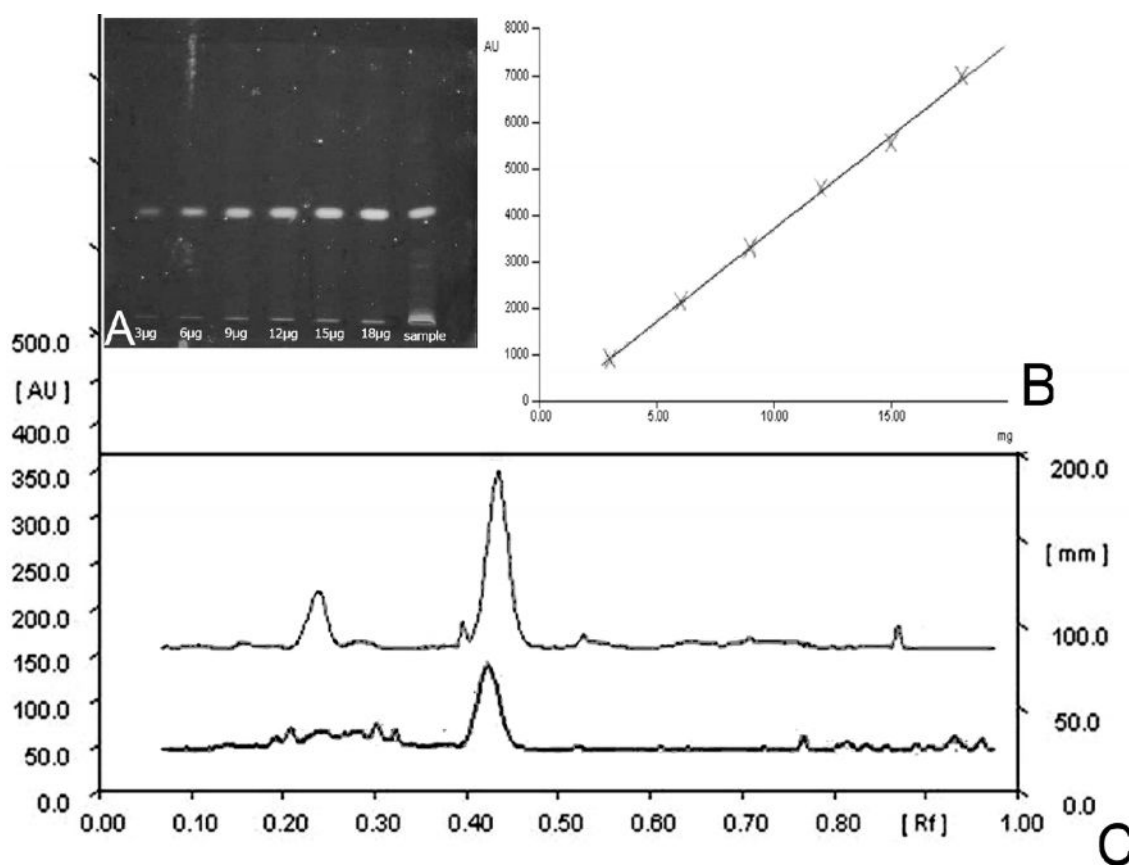


Figure 2. HPTLC of 3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid.

A. chromatogram at 366 nm of tracks 3, 6, 9, 15, 18 µg and sample from left to right.

B. Calibration curve of the compound.

C. Densitometric chromatogram of 3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid (lower) at 366nm and ethyl acetate fraction.

Table 2: Summary of validation parameters of 3,5-diacetoxy-2-methoxy-6-methyl-phenyl acetic acid.

Sr. No.	Parameters	Results
1	Linearity	0.9991
2	Precision (% C.V.)	
	▪ Repeatability of Measurement	0.26
	▪ Repeatability of Application	1.45
	▪ Interday	1.3 – 3.5%
	▪ Intraday	1.2 – 3.1%
3	Range	3 - 18µg/spot
4	Limit of Detection	3 µg/spot
5	Limit of Quantification	6 µg/spot
6	Accuracy	98.33 - 99.66%
7	Specificity	Specific

Overall it can be said that Ext M seemed to work on both specific and non-specific immune pathways, whereas Ext PS appeared to stimulate pro-inflammatory immune responses. Moreover, antioxidant potential of Ext M could be correlated to lower incidence of mortality in *E. coli* induced peritonitis, which may be due to neutralization of release of reactive oxygen species triggered by neutrophil and other immune cells.

REFERENCES

1. Kirtikar K.R., Basu B.D., Indian Medicinal Plants. Vol-2. Calcutta, India: Prabhasi Press; 1975. p. 2469.
2. Anonymous, Wealth of India. Vol-2. New Delhi, India: Council of Scientific and Industrial Research; 1950. p. 400.
3. Kubo M., Namba K., Nagamoto N., Nagao T., Nakanishi J., Uno H., Nishimura H. A., new phenolic glucoside, curculigoside, from rhizomes of *Curculigo orchoides*. *Planta Med* 1983;47:62-63.
4. Gonda R., Tomoda M., Shimozu N., Kanari M., Characterization of an acidic polysaccharide from the seeds of *Malva verticillata* stimulating the phagocytic activity of cells of the RES. *Planta Med* 1990;56:73-76.
5. De P., Dasupta S.C., Gomes A., Immunopotentiating and immunoprophylactic activities of 'Immune 21', a polyherbal product. *Indian J Pharmacol* 1998;30:163-168.
6. Puri A., Saxena R.P., Sumati Guru P.Y., Immunomodulatory activity of Picrolive, the iridoid glycoside fraction of *Picrorhiza kurroa*. *Planta Med* 1992;58:529-532.
7. Jerne N.K., Nordin A.A., Immunological Speculation. *Science*: 401-405.
8. Navarro M.C., Mantilla M.P., Martin A., Jeimenz J., Utrilla M.P., Free radical scavenging activity and antihepatotoxic activity of *Rosamarinus tomentosus*. *Planta Med* 1993;59:312.
9. Beauchamp C., Fridovich I., Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem*, 1971;44:276.
10. Rekka E., Kolstee J., Timmermen H., Bast A., The effect of some H₂-receptor antagonists on rat hepatic microsomal cytochrome P-450 and lipid peroxidation *in vitro*. *Eur J Med Chem*, 1971;24:43-54.
11. Liu C.F., Lin C.C., Lin S.C., Hepatoprotective and therapeutic effects of tetramethyl pyrazine on acute econazole induced liver injury. *Planta Med*, 2002;68:510-514.
12. Klein S.M., Cohen G., Cederbaum A.L., Production of formaldehyde during metabolism of dimethyl sulfoxide by hydroxyl radical generating systems. *Biochem*, 1981; 20:606.
13. Tripathi Y.B., Chuarasia S., Tripathi E., Upadhyay A., Dubey G.P., *Bacopa monniera* Linn. as an antioxidant: Mechanism of action. *Indian J Expt Biol*, 1996;34:523-526.
14. Yamini B., Tripathi P., Pandey E., Role of alcoholic extract of shoot of *Hypericum perforatum* Linn. On lipid peroxidation and various species of free radicals in rats. *J Expt Biol*, 1999;37:567-571.
15. Ponkiske C.A., Indap M.M., *In vivo* and *in vitro* evaluation for immunomodulatory activity of three marine animal extracts with reference to phagocytosis. *Indian J Exp Biol*, 2002;40:1399-1402.
16. Kristin I., Jurcic K., Fischer B., Wagner H., Immunologically active polysaccharide from *Cetraria islandica*. *Planta Med*, 1994; 60:527-531.
17. Sainis K.B., Gudgil M.B., Phondke G.P., Modulation of delayed type hypersensitivity to sheep erythrocytes by niridazole. *Indian J Exp Biol*, 1983; 21:304-309.
18. Barua C.C., Gupta P.P., Patnaik G.K., Goel R.K., Kulshrestha D.K., Dubey M.P., Dhawan B.N., Immunomodulatory effect of *Albizia lebbeck*. *Pharm Biol*, 2000;38:161-166.

19. Vuillaume M., Reduced oxygen species, mutation, induction and cancer initiation. *Mutation Research*, 1987;186:43-72.
20. Fether J., Cosmos G., Vereekei A., Free radical reactions in medicine. Heidelberg, Berlin: Springer Verlag; 1991. p. 1-42.
21. Faure M., Lissi E., Torres R., Videla L.A., Antioxidant activities of lignans and flavonoids. *Phytochem*, 1991;29:3773-3775.
22. Cao G., Sofic E., Prior R.L., Antioxidant activity of tea and common vegetables. *J Agric Food Chem*, 1997;44:3426-3431.
23. Halliwell B., Gutteridge J.M.C., Role of free radicals and catalytic metal ions in human disease: An overview. *Methods in Enzymology*, 1989;1-85.
24. Slater T.F., Eakins M.N., *New Trends in Therapy of Liver Diseases*. Edited by Bertelli A. Basel: Karger; 1975. p. 84-89.
25. Takayanagi R., Takeshige K., Minakami S., NADH and NADPH dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and an antioxidant role of ubiquinol. *Biochem J*, 1980;192:853-860.
26. Chiu D., Lubin B., Shohet S.B., Free Radicals in Biology. Vol 5, New York: Pryor WA ed., Academic Press; 1982. p. 115-160.
27. Chojkeir M., Houglum K., Solis H.J., Brenner D.A., Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts- A role for lipid peroxidation. *J Biol Chem*, 1989;264:957-962.
28. Fridovich I., The biology of oxygen radical threats and defenses. Oxygen Radicals in the Pathophysiology of Heart Disease. Edited by Single PK. Lancaster, Dordrecht, Boston, USA: Kluwer Academic Publishers; 1988. p. 1-11.
29. Dix T.A., Aikens J., Mechanisms and biological relevance of lipid peroxidation initiation. *Chem Res Toxicol*, 1993;6:2-18.
