

Free Radical Quenching and Immunomodulatory Effect of a Modified Siddha Preparation, Kalpaamruthaa

Shanmugam Arulkumaran,^a Vanu Ramkumar Ramprasath,^a Palanivelu Shanthi,^b and Panchanatham Sachdanandam^{*,a}

^aDepartment of Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai-600 113, Tamilnadu India and ^bDepartment of Pathology, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai-600 113, Tamilnadu India

(Received October 11, 2006; Accepted December 28, 2006)

Kalpaamruthaa (KA), a modified indigenous Siddha formulation constitutes *Semecarpus anacardium* nut milk extract (SA), *Phyllanthus emblica* and honey. The present study attempt has been to investigate the antioxidant and immunomodulatory activities of KA. Antioxidant activity of KA was determined by using ferric thiocyanate and thiobarbituric acid methods. KA showed higher antioxidant activity when compared with vitamin E (α -tocopherol), a well-known antioxidant. Immunomodulatory activities on humoral and cellular immunity were studied by hemagglutination (HA) titre, delayed type hypersensitivity (DTH) and phagocytic index. KA was administered at the dosage level of 100, 150, 200, 250 and 300 mg/kg body weight. Also, KA enhanced the HA titre, phagocytic index and delayed type hypersensitivity in a dose dependent manner, which indicates that KA triggers both humoral and cell mediated immune responses to a greater extent. This improved antioxidant and immunomodulatory activities of KA might be due to the synergistic effect of flavonoids, vitamin C and hydrolysable tannins present in the drug. The current study shows that KA is effective at the dosage level of 200 mg/kg body weight were significantly induced immunomodulatory activity in a dose dependant manner.

Key words — Kalpaamruthaa, *Semecarpus anacardium* nut milk extract, *Phyllanthus emblica*, antioxidant, immunomodulation

INTRODUCTION

In recent times, focus on plant research has been intensified all over the world and a large amount of evidence has been collected to show immense potential of medicinal plants used in various traditional systems.¹⁾ In Indian system of medicine, a large number of herbal drugs have been advocated for various types of diseases/stress related disorders.²⁾ One of their main strategies is to increase body's natural resistance to diseases/stress causing agent rather than directly neutralising the agent itself in practice. These immunomodulatory agents are of plant origin which are claimed to induce paraimmunity, the non-specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions.³⁾ It

is now being recognized that immunomodulatory therapy could provide an alternative to conventional chemotherapy to a variety of diseased conditions.²⁾ Hence, Kalpaamruthaa (KA), a modified indigenous Siddha preparation which constitutes *Semecarpus anacardium* nut milk extract (SA) and *Phyllanthus emblica* was evaluated for its immunomodulatory activity. In order to promote intellect and prevent senility and for longevity, honey was also added to this preparation.⁴⁾ Further, the *in vitro* antioxidant property of KA was assessed because the immunomodulating agents enhance the immune responsiveness against an antigen by controlling the amount of the free radicals generated in the cell.⁵⁾

Semecarpus anacardium (*S. anacardium*) have been highly preferred for its wide range of pharmacological activities such as antioxidant, membrane stabilizing and immunomodulatory properties.⁶⁾ It also has been proved as an alternative therapeutic agent for various types of cancers such as breast and liver cancer.^{7,8)} Satyavati *et al.* (1969)⁹⁾ have reported the anti-inflammatory effect of *S. anacardium* nuts on acute inflammation of both

*To whom correspondence should be addressed: Department of Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai-600 113, Tamilnadu India. Tel.: +91-44-24541767; Fax: +91-44-24540709; E-mail: psachdanandam2000@yahoo.co.in

immunological and non-immunological origin. It also possesses a promising hypolipidemic¹⁰⁾ and anti-atherosclerotic activities.¹¹⁾

Phyllanthus emblica (*P. emblica*) commonly known as Amla, a rich source of vitamin C have been used in Ayurveda as a potent rasayana.¹²⁾ The rasayanas are used to promote health and longevity by increasing defence against diseases, arresting the aging process and revitalizing the body's debilitated conditions.¹³⁾ A wealth of literature is available on the occurrence and health benefits of vitamin C.^{14,15)} *P. emblica* was reported to have an anticancer, immunopotentiating and antioxidant properties^{16,17)} as well to possess anticlastogenic and antimutagenic activities.¹⁸⁾ It also exhibits significant adaptogenic and hepatoprotective effects.¹⁶⁾

Since synergism between flavonoids and ascorbate has been known for a long time and interactions between flavonoids and ascorbic acid have been documented.¹⁹⁾ Also, stabilization of vitamin C at sub-optimal concentrations by flavonoids is well established,²⁰⁾ the combination of SA which has been reported for the presence of carbohydrate, sterols, glycosides, phenols and flavonoids (biflavanones such as, semecarpuflavanone, semecarpetin, anacardiflavanone and tetrahydroamentoflavavone)⁶⁾ with vitamin C rich *P. emblica* was tried for the first time with a view to assess if the combination promotes the immunomodulatory and antioxidant property of SA. In addition to vitamin C, *P. emblica* have been reported for the presence of low molecular weight hydrolysable tannins (emblicanin-A, emblicanin-B, puniglucanin and pedunculagin),²¹⁾ flavonoid rutin, trigalloyl glucose and phyllembic acid.¹⁶⁾ The preliminary phytochemical analysis of the drug, KA gave positive results for the presence of flavonoids, tannins, steroids, triterpenes, proteins and carbohydrate. Further, phytochemical analysis of the drug showed the presence of other compounds such as minerals, vitamins, aminoacids and fiber. Hence, in the light of above facts, the present study aims at investigating improved antioxidant and immunomodulatory properties of *S. anacardium* nut milk extract by combining with *P. emblica* and honey.

MATERIALS AND METHODS

Drug and Chemicals—*S. anacardium* Linn. nut milk extract has been prepared according to Formulary of Siddha medicine (1972).²²⁾ To

this, fresh dried powder of *P. emblica* fruits and honey were added. Linoleic acid and 1,1,3,3-tetraethoxypropane were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). All other chemicals and solvents used were of analytical grade.

Determination of Antioxidant Activity *In Vitro* Study

Ferric Thiocyanate (FTC) Method—The methods of Mitsuda *et al.* (1967)²³⁾ and Osawa and Namiki (1981)²⁴⁾ were slightly modified by Kikuzaki and Nakatani (1993).²⁵⁾ The method was used to determine the amount of peroxide at the initial stage of lipid peroxidation. Briefly, a mixture of 4 mg of the drugs KA and SA in 4 ml absolute ethanol, 4.1 ml of 2.52% linoleic acid in absolute ethanol, 8 ml of 0.05 M phosphate buffer (pH 7) and 3.9 ml distilled water in a screw capped bottle and placed in an oven at 40°C in the dark. To 0.1 ml of this solution, 9.7 ml of 75% ethanol and 0.1 ml 30% ammonium thiocyanate were added. After 3 minutes, 0.1 ml of 0.02 M ferrous chloride in 3.5% HCl was added to the reaction mixture and the absorbance was measured at 500 nm for every 24 hr until the absorbance of the control reached maximum. The control and standard were subjected to the same procedure as the sample but for the control, only solvent was added. In standard, 4 mg of the drugs KA and SA were replaced by 4 mg of vitamin E.

Thiobarbituric Acid (TBA) Method—The method of Ottolenghi (1959)²⁶⁾ was used to determine the TBA values of the samples. The formation of malonaldehyde is the basis for the well-known TBA method for evaluating the level of lipid peroxidation. Briefly, 2 ml of 20% trichloroacetic acid and 2 ml of TBA aqueous solution were added to 1 ml of sample solution prepared as in the FTC procedure and incubated in a similar manner. The mixture was placed in a boiling water bath for 10 minutes. After cooling, it was centrifuged at 800 × *g* for 20 minutes and the absorbance of the supernatant was measured at 530 nm. Antioxidant activity was based on the absorbance on the final day.

Animals—Adult female albino rats of Wistar strain weighing 170 ± 20 g were purchased from King Institute of Preventive Medicine, Chennai, India. The animals were maintained under standard conditions of humidity, temperature (25 ± 2°C) and light (12 hr light/dark). They were fed with standard rat pelleted diet (M/s Pranav Agro Industries Ltd., Pune India) under the trade name Amrut rat/mice

feed and had free access to tap water. Experimental animals were handled according to the University and Institutional Legalisation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Delayed Type Hypersensitivity — Rats were divided into four groups containing 6 rats in each. Delayed type hypersensitivity (DTH) was induced in rats using Sheep red blood cells (SRBC) as an antigen according to Doherty (1981).²⁷⁾ KA and SA at different dose levels (100, 150, 200, 250 and 300 mg/kg b.wt.) were administered on day 0 and continued till the day of challenge. The rats were primed with 0.1 ml of SRBC suspension containing 5×10^9 cells i.p., on day 8 and challenged on day 13 with 0.05 ml of 5×10^9 SRBC on the right hind foot pad. The contralateral paw received equal volumes of saline. The thickness of the footpad was measured at 24 hr after the challenge using vernier caliper. The difference in the thickness of the right hind paw and the left hind paw was used as a measure of DTH.

Humoral Antibody Response — The rats were divided into four groups of six in each. KA and SA at different dose levels (100, 150, 200, 200 and 300 mg/kg b.wt.) were administered on day 0 and continued till the day of the experiment. On day eight, the rats were immunized with 10% suspension of SRBC, i.p. Blood samples were collected from the orbital plexus of individual animals on day 13 and their antibody titre from serum was determined according to Puri *et al.* (1994).²⁸⁾ Briefly, an aliquot (25 μ l) of 2 fold-diluted sera in saline was challenged with 25 μ l of 0.1% v/v SRBC suspension in micro titre plates. The plates were incubated at 37°C for 1 hr and then examined for heamagglutination (HA) and expressed as HA titre.

Phagocytic Index — Phagocytic index was determined by the candida phagocytosis technique according to Wilkinson (1977).²⁹⁾ The buffy coat was isolated from the blood samples and incubated at 37°C for 30 minutes in an incubating medium 0.1 ml HBSS, Hanks solution (0.1 ml inactivated fetal calf serum and 0.1 ml of 2×10^8 heat killed candida species). After incubation, the preparation was centrifuged at $800 \times g$ for 5 minutes and subsequently stained with Leischman stain. The number of positive cells/100 neutrophils gives the phagocytic index.

Statistical Analysis — The values are expressed as mean \pm standard deviation (S.D.). Results were analyzed statistically using student's *t*-test. The significant difference between the groups are considered at $p < 0.05$ level.

RESULTS

Antioxidant Activity

FTC and TBA methods assessed antioxidant activity of KA and compared with vitamin E, a well-known antioxidant. In FTC method, low absorbance values of the drugs indicated high level of antioxidant activity. In general, KA and vitamin E markedly inhibited the oxidation of linoleic acid for a period of 5 days, when compared to the control of KA showed a least absorbance than vitamin E. On the contrary, the control showed no significant changes from the sample until the day 2, but increased significantly on day 3, reached maximum level on day 4 and finally dropped on day 5 due to malonaldehyde content from linoleic acid oxidation (Fig. 1).

Absorbance values from TBA method showed total peroxide values produced by the oxidation of linoleic acid. The higher the absorbance values the lower the antioxidant activity. The control had the highest absorbance value followed by vitamin E, SA and KA. Based on the present results, KA was found to exhibit a profound antioxidant activity (86%) than vitamin E (77%) as shown in Fig. 2.

Delayed Type Hypersensitivity Using SRBC as Antigen

KA at difference dose levels (100 to 300 mg/kg

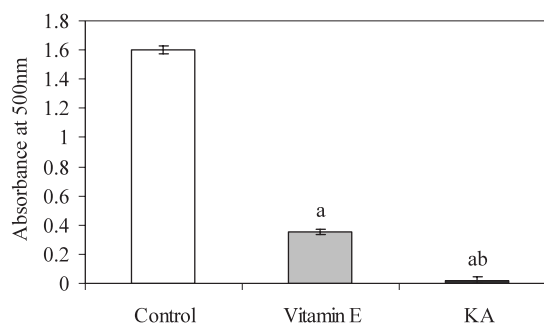


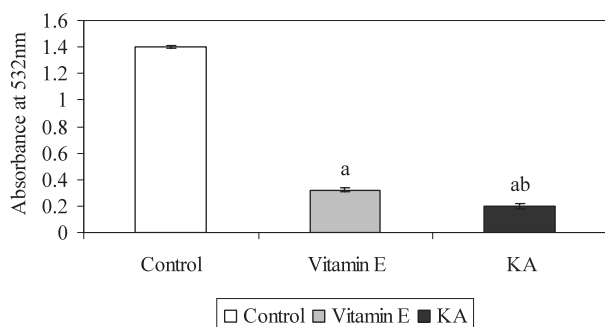
Fig. 1. Antioxidant Activity of Kalpaamruthaa and Vitamin E by FTC Method

Values are expressed as Mean \pm S.D. for six experiments. Comparisons are made between; a: Control Vs Vitamin E and KA, b: Vitamin E and KA. The symbols a and b represent statistical significance at $p < 0.05$.

Table 1. Effect of Kalpaamruthaa on Humoral Antibody titre, DTH and Phagocytic Index

Parameters	Group I (Control)	Group II (100 mg/kg b.wt KA treated)	Group III (150 mg/kg b.wt KA treated)	Group IV (200 mg/kg b.wt KA treated)	Group V (250 mg/kg b.wt KA treated)	Group VI (300 mg/kg b.wt KA treated)
Antibody titre	5.31 ± 0.71	5.62 ± 0.62	7.25 ± 0.64 ^{a,b}	8.12 ± 0.75 ^{a,b,c}	8.14 ± 0.76 ^{a,b,c}	8.15 ± 0.78 ^{a,b,c}
DTH (Paw thickness in percentage)	9.72 ± 0.89	9.83 ± 0.73	10.2 ± 1.09 ^a	15.11 ± 1.10 ^{a,b,c}	14.91 ± 1.92 ^{a,b,c}	14.90 ± 1.81 ^{a,b,c}
Phagocytic index (Number of positive cells/100 cells)	62.03 ± 5.94	62.58 ± 5.91	65.10 ± 6.07	74.11 ± 7.79 ^{a,b,c}	74.13 ± 7.01 ^{a,b,c}	74.14 ± 7.24 ^{a,b,c}

Values are expressed as mean ± standard deviation (S.D.) of six animals. Comparisons are made between; a: Group I Vs Group II, III, IV, V and VI; b: Group II Vs Group III, IV, V and VI; c: Group III Vs Group IV, V and VI; d: Group IV Vs Group V and VI; e: Group V Vs Group VI. The symbols a, b, c, d and e represent statistical significance at $p < 0.05$.

**Fig. 2.** Antioxidant Activity of Kalpaamruthaa and Vitamin E by TBA Method

Values are expressed as Mean ± S.D. for six experiments. Comparisons are made between; a: Control Vs Vitamin E and KA, b: Vitamin E and KA. The symbols a and b represent statistical significance at $p < 0.05$.

b.wt.) produced a significant dose related increase in DTH reaction in rats. The edema reached a peak at 24 hr, the percent edema being 9% for the control group, after which it subsided (Table 1).

Humoral Antibody Response

A significant dose related increase in humoral antibody titres were observed in rats treated with KA (100 to 300 mg/kg b.wt.) as shown in Table 1. The dosage level of 200 mg/kg body weight was found to be the effective dose with a significant ($p < 0.05$) effect.

Phagocytic Index

Significant dose related increase in the phagocytic index was also observed in rats treated with KA (100 to 300 mg/kg b.wt.). On drug treatment, the levels were significantly increased in a dose dependent manner showing a favorable change in groups treated with 200 mg/kg (Table 1).

DISCUSSION

Free radical induced oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders and great importance has been attributed to antioxidants in the prevention and the treatment of diseases.³⁰⁾ In FTC method, though KA and SA showed low absorbance value, KA afflicts the least increase in absorbance values followed by vitamin E without any difference from day 1 to day 2 but on day 3 and day 4, the absorbance was increased and reached a maximum level and that could be due to the maximal oxidation of linoleic acid. On day 5, the reduced absorbance was noted due to the accumulation of malonaldehyde compounds from linoleic acid oxidation and the least absorbance due to KA indicates higher antioxidant activity. The absorbance values from TBA method showed total peroxide values produced by the oxidation of linoleic acid. Since, higher the absorbance values, the lower the level of antioxidant activity, KA showed decreased absorbance when compared to vitamin E.

Since, it had been reported that the additive and synergistic effects of phytochemicals were responsible for the potent antioxidant activities,³¹⁾ the increased activity of KA might be due to the presence of complex mixtures of phytochemicals such as flavanoids, vitamin C, hydrolysable tannins and other phenolic compounds. Reports have demonstrated that flavanoids found in plants might act as antioxidant and studies have shown that flavanoids can replace vitamin E as chain breaking antioxidant in liver³²⁾ because, like α -tocopherol (vitamin E), flavonoids possess chemical structural elements that might be responsible for its antioxidant activity. Flavonoids and vitamin C are both very

strong antioxidant agents and their biological activity are in part found to be synergistic.³³⁾ Further, recently it has been proved that the combination of flavonoids and vitamin C has been found to impart a synergistic antioxidant effect.^{34–36)} For the reason that, ascorbate is reported to have flavonoid protective^{37,38)} and flavonoid-enhancing activities.^{39,40)} But, Ghosal *et al.* (1996),⁴¹⁾ have reported that the potent vitamin C like activity of *P. emblica* is due to the presence of low molecular weight hydrolysable tannins (emblicanin-A and emblicanin-B). Hence, it can be postulated that the enhanced antioxidant effect of KA than SA alone might be attributed to the synergistic effect of flavonoids, vitamin C and tannins present in KA.

DTH is a part of the process of graft rejection, tumour immunity and most importantly, immunity to many intracellular infections of microorganisms, especially those causing chronic diseases.⁴²⁾ In DTH, circulating T cells sensitized to the antigen from prior contact react with the antigen and induce specific immune response, which includes mitosis (blastogenesis) and the release of soluble mediators. This reaction process involved antigen presentation by macrophages, releases interleukin-1 and tumour necrosis factor- α (TNF- α) from activated macrophages, releases Interleukin-2 and IFN- γ from activated T cells⁴³⁾ and promotes phagocytic activity and increases the concentration of lytic enzymes for more effective killing.⁴⁴⁾

To evaluate the effect of KA on humoral response, its influence was tested on SRBC hemagglutination antibody titre in rats. KA was found to significantly enhance the circulating antibody titre at a dose level of 200 mg/kg b.wt when compared to untreated immunized rats. This indicates the enhanced responsiveness of T and B lymphocyte subsets involved in antibody synthesis.⁴⁵⁾

The event of phagocytosis is primarily the removal of microorganisms and foreign bodies, besides imparting the property of the elimination of dead or injured cells. Phagocytic defects are associated with varied pathological conditions in humans.⁴⁶⁾ In view of the pivotal role played by the macrophages in coordinating the processing and presentation of antigen to B cells, KA was evaluated for its effect on macrophage phagocytic activity. Table 1 shows a significant increase of phagocytic index in 200 mg/kg b.wt treated rats at a value of $p < 0.05$ compared to the controls, which indicates that KA has enhanced the capacity of macrophage system.

The enhancement of DTH response, phagocytic index and HA titre provides the first line evidence about the immunomodulatory property of KA by stimulating both cell mediated and humoral immune responses. This significant increase in the immunomodulatory activity of KA could be attributed to the presence of flavanoids, vitamin C, hydrolysable tannins and other phenolic compounds present in the drug. These promising results lend support to the fact that KA exhibits a significant antioxidant activity. In addition to that it also shows immunomodulatory activity in a dose dependent manner and is pronounced at the levels of 200 mg/kg body weight.

Acknowledgement We gratefully acknowledge the financial assistance from UWPFE program (HDP-18), University Grants Commission (UGC), New Delhi, India.

REFERENCES

- 1) Dahanukar, S. A., Kulkarni, R. A. and Rege, N. N. (2000) Pharmacology of medicinal plants and natural products. *Indian J. Pharmacol.*, **32**, S81–S118.
- 2) Pallabi, D. E., Dasgupta, S. C. and Gomes, A. (1998) Immunopotentiating and Immunoprophylactic activities of Immune 21 a polyherbal product. *Indian J. Pharmacol.*, **30**, 163–168.
- 3) Sainis, K. B., Sumariwalla, P. F., Goel, A., Chintalwar, G. J., Sipahimalani, A. T. and Banerji, A. (1997) Immunomodulatory properties of stem extracts of *Tinospora cordifolia*: cell targets and active principles. In *Immunomodulation* (Upadhyay, S. N., Ed.), Narosa Publication House, New Delhi, India, p. 95.
- 4) Khare, C. P. (2004) *Encyclopedia of Indian medicinal plants*, 1st Ed., Springer-verlag Berlin Heidelberg, Germany, pp. 200–203.
- 5) Novoselova, E. G., Safonova, M. V., Gordon, R. Y. and Semiletova, N. V. (1995) Immune function of spleen lymphocytes of rats subjected to chronic irradiation and antioxidant (ubiquinone Q-9) diet. *Int. J. Radiat. Biol.*, **67**, 469–476.
- 6) Premalatha, B. (2000) *Semecarpus anacardium* Linn. nuts-A boon in alternative medicine. *Indian J. Exp. Biol.*, **38**, 1177–1182.
- 7) Sujatha, V. and Sachdanandam, P. (2000) Effect of *Semecarpus anacardium* Linn. nut extract on experimental mammary carcinoma in Sprague-Dawley Rats with reference to tumour markers enzymes. *Phar. Pharmacol. Commun.*, **6**, 375–379.

- 8) Premalatha, B., Muthulakshmi, V., Vijayalakshmi, T. and Sachidanandam, P. (1997) *Semecarpus anacardium* nut extract induced changes in enzymic antioxidants studied in aflatoxin B1-caused hepatocellular carcinoma-bearing Wistar rats. *Int. J. Pharmacol.*, **35**, 1–6.
- 9) Satyavati, G. V., Prasad, D. N., Das, P. K. and Singh, H. D. (1969) Antitumour activity of *Semecarpus anacardium* Linn - A preliminary study. *Indian J. Physiol. Pharmacol.*, **13**, 37–45.
- 10) Tripathi, Y. B. and Pandey, R. S. (2004) *Semecarpus anacardium* L, nuts inhibit lipopolysaccharide induced NO production in rat macrophages along with its hypolipidemic property. *Indian J. Exp. Biol.*, **42**, 432–436.
- 11) Sharma, A., Mather, R. and Dixit, V. P. (1995) Hypocholesterolemic activity of nut shell extract of *Semecarpus anacardium* (Bhilawa) in cholesterol fed rabbits. *Indian J. Exp. Biol.*, **33**, 444.
- 12) Puri, H. S. (2003) 'Rasayana'—Ayurvedic herbs for longevity and rejuvenation, Taylor and Francis, London.
- 13) Govindarajan, R., Vijayakumar, M. and Pushpangadan, P. (2005) Antioxidant approach to disease management and the role of 'rasayana' herbs of Ayurveda. *J. Ethnopharmacol.*, **99**, 165–178.
- 14) Weisburger, J. H. (1995) Vitamin C and disease prevention. *J. Am. Coll. Nutr.*, **14**, 109–111.
- 15) Vanderslice, J. T. and Higgs, D. J. (1991) Vitamin C content of foods: sample variability. *Am. J. Clin. Nutr.*, **54**, 1323S–1327S.
- 16) Jeena, K. J., Joy, K. L. and Kuttan, R. (1999) Effect of *Embllica officinalis*, *Phyllanthus amarus* and *Picrorrhiza kurroa* on N-nitrosodiethylamine induced hepatocarcinogenesis. *Cancer Lett.*, **136**, 11–16.
- 17) Sai Ram, M., Neetu, D., Yogesh, B., Anju, B., Dipti, P., Pauline, T., Sharma, S. K., Sarada, S. K., Ilavazhagan, G., Kumar, D. and Selvamurthy, W. (2002) Cyto-protective and immunomodulating properties of Amla (*Embllica officinalis*) on lymphocytes: an in-vitro study. *J. Ethnopharmacol.*, **81**, 5–10.
- 18) Roy, A. K., Dhir, H. and Sharma, A. (1992) Modification of metal-induced micronuclei formation in mouse bone marrow erythrocytes by *Phyllanthus* fruit extract and ascorbic acid. *Toxicol. Lett.*, **62**, 9–17.
- 19) Bors, W., Michel, C. and Schikora, S. (1995) Interaction of flavonoids with ascorbate and determination of their univalent redox potentials: a pulse radiolysis study. *Free Radical Biol. Med.*, **19**, 45–52.
- 20) Senthilmohan, S. T., Zhang, J. and Stanley, R. A. (2003) Effects of flavonoid extract Enzogenor® with vitamin C on protein oxidation and DNA damage in older human subjects. *Nutr. Res.*, **23**, 1199–1210.
- 21) Bhattacharya, A., Chatterjee, A., Ghoshal, S. and Bhattacharya, S. K. (1999) Antioxidant activity of tannoid principles of *Embllica officinalis* (amla). *Indian J. Exp. Biol.*, **37**, 676–680.
- 22) Narayanaswami, V. and Uthamaroyan, C. S. (1972) *Formulary of Siddha Medicine* 2nd Ed., Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd., Madras, India, p. 197.
- 23) Mitsudha, H., Yasumato, K. and Iwani, K. (1967) Antioxidant action of indole compounds during the autooxidation of linoleic acid. *Eiyo to shoduryou*, **19**, 210.
- 24) Osawa, T. and Namiki, M. (1981) A novel type of antioxidant isolated from leaf wax of *Eucalyptus* leaves. *Agric. Biol. Chem.*, **45**, 735–740.
- 25) Kikuzaki, H. and Nakatani, N. (1993) Antioxidant effects of some ginger constituents. *J. Food Sci.*, **58**, 1407–1410.
- 26) Ottolenghi, A. (1959) Interaction of ascorbic acid and mitochondria lipids. *Arch. Biochem. Biophys.*, **79**, 355–359.
- 27) Doherty, N. S. (1981) Selective effects of immunosuppressive agents against the delayed hypersensitivity response and humoral response to sheep red blood cells in mice. *Agents Actions*, **11**, 237–242.
- 28) Puri, A., Sexena, R. P., Sexena, K. C., Srinivasan, M. and Tandon, J. S. (1994) Immunostimulant activity of *Nyctanthes arbortristis* L. *J. Ethnopharmacol.*, **42**, 31–37.
- 29) Wilkinson, P. C. (1977) Phagocytosis of killed *Candida albicans*. In *Techniques in clinical immunology* (Thompson, R. A., Ed.), Blackwell publication, Oxford, p. 212.
- 30) Maxwell, S. R. (1995) Prospects for the use of antioxidant therapies. *Drugs*, **49**, 345–361.
- 31) Liu, R. H. (2003) Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr.*, **78**, 517S–520S.
- 32) van Acker, F. A., Schouten, O., Haenen, G. R., van der Vijgh, W. J. and Bast, A. (2000) Flavonoids can replace alpha-tocopherol as an antioxidant. *FEBS Lett.*, **473**, 145–148.
- 33) Isler, O., Brubacher, G. and Kiss, J. (1988) Vitamin C. In *Vitamin C and bioflavonoide* (Isler, O., Brubacher, G., Ghisla, S. and Krautler, B., Eds.), Thime verlag, Stuttgart, pp. 396–444.
- 34) Vinson, J. A. and Jang, J. (2001) *In Vitro* and *In Vivo* lipoprotein antioxidant effect of a citrus extract and

- ascorbic acid on normal and hypercholesterolemic human subjects. *J. Med. Food*, **4**, 187–192.
- 35) Milde, J., Elstner, E. F. and Grassmann, J. (2004) Synergistic inhibition of low-density lipoprotein oxidation by rutin, gamma-terpinene, and ascorbic acid. *Phytomedicine*, **11**, 105–113.
- 36) Chen, J., Kang, J., Da, W. and Ou, Y. (2004) Combination with water soluble antioxidants increases the anticancer activity of quercetin in human leukemia cell. *Pharmazie*, **59**, 859–863.
- 37) Sorata, Y., Takahama, U. and Kimura, M. (1988) Cooperation of quercetin with ascorbate in the protection of photosensitized lysis of human erythrocytes in the presence of hematoporphyrin. *Photochem. Photobiol.*, **48**, 195–199.
- 38) Jan, C. Y., Takahama, U. and Kimura, M. (1991) Inhibition of photooxidation of alpha-tocopherol by quercetin in human blood cell membranes in the presence of hematoporphyrin as a photosensitizer. *Biochim Biophys Acta*, **1086**, 7–14.
- 39) Vrijssen, R., Everaert, L. and Boeye, A. (1988) Antiviral activity of flavones and potentiation by ascorbate. *J. Gen. Virol.*, **69**, 1749–1751.
- 40) Kandaswami, C., Perkins, E., Soloniuk, D. S., Drzewiecki, E. and Middleton, E., Jr. (1993) Ascorbic acid enhanced antiproliferative effect of flavonoids on squamous cell carcinoma in vitro. *Anticancer drugs*, **4**, 91–96.
- 41) Ghosal, S., Tripathi, V. K. and Chauhan, S. (1996) Active constituents of *Embllica officinalis*: Part I. The chemistry and antioxidative effects of two new hydrolysable tannins, Emblicanin A and B. *Indian J. Chem.*, **35**, 941–948.
- 42) Elgert, K. D. (1996) *Immunology: understanding the Immune system*. Wiley, New York. p. 306.
- 43) Papas, A. M. (1999) *Antioxidant status, diet, nutrition and health*, 1st Ed., CRC Press, New York. p. 376.
- 44) Kubay, J. (1997) *Immunology*. WH Freeman and Company, New York, p. 436
- 45) Benacerraf, B. (1978) A hypothesis to relate the specificity of T lymphocytes and the activity of I region specific Ir genes in macrophages and B lymphocytes. *J. Immunol.*, **120**, 1809–1812.
- 46) White, C. J. and Gallin, J. I. (1986) Phagocyte defects. *Clin. Immunol. Immunopathol.*, **40**, 50–61.