

RESEARCH COMMUNICATION

Immunomodulatory Activities of *Centella asiatica* and *Rhinacanthus nasutus* Extracts

Khanittha Punturee¹, Christopher P Wild², Watchara Kasinrer³, Usanee Vinitketkumnuen^{1,*}

Abstract

Centella asiatica (CA) and *Rhinacanthus nasutus* (RN) have been used for treatment of various illnesses, but the mechanisms of action remain largely unknown. This study focused on the influence of CA and RN extracts on cell-mediated and humoral immune responses. In human peripheral blood mononuclear cells (PBMCs), CA (water extract) and RN (water and ethanol extracts) significantly increased proliferation and the production of IL-2 and TNF- α . In contrast, an ethanol extract of CA inhibited human PBMC mitogenesis and the production of IL-2 and TNF- α . BALB/c mice treated with CA extracts (100 mg/kg bw) showed higher responses to both primary and secondary antibodies against BSA when compared with non-treated group. Only the secondary antibody response was increased in RN extract-treated mice. The present study revealed immunomodulating activity of CA and RN extracts with regard to both non-specific cellular and humoral immune responses. The data available to date suggest that they may have chemopreventive or anticancer potential.

Key Words: *Centella asiatica* - *Rhinacanthus nasutus* - humoral immune response - cell-mediated immune response

Asian Pacific J Cancer Prev, 6, 396-400

Introduction

The immune system plays a vital role in the defense against infections. Its integrity and efficiency is important during chemotherapeutic intervention for the treatment of many diseases (Ishizuka et al., 1995). Modulation of the immune response to alleviate disease then has been of interest for many researchers. In traditional medicine different plant parts are believed to have specific medicinal properties including the ability to stimulate the body's disease-fighting mechanisms (Craig, 1999; Jones, 1996). In recent years, natural products from the plant kingdom have been investigated for their immune modulating potential against infections and neoplastic diseases such as Echinacea, ginseng and astragalus (Block and Mead, 2003).

Centella asiatica (L.) Urban, belongs to the family Umbelliferae, has been used for centuries as a medicinal plant. In Thailand, it is commonly known as "Buabok" and normally is drunk as a tea or juice. This plant has been used in Thai alternative medicine in the treatment of conditions as diverse as mental disorders, inflammation, rheumatism,

circulatory problems, asthma and bronchitis, epilepsy and immune system deficiencies (Farnsworth and Bunyapraphatsara, 1992). *C. asiatica* has been subjected to extensive experimental and clinical investigations (Brinkhaus et al., 2000). The plant showed evidence of wound healing (Suguna et al., 1996; Shukla et al., 1999; Coldren et al., 2003), anti-tumor activity (Babu et al., 1995), anti-anxiety activity (Bradwejn et al., 2000), anti-viral activity (Yoosook et al., 2000), anti-hepatoma activity (Lin et al., 2002) and cognition-enhancement in rats (Veerendra Kumar and Gupta, 2002; Gupta et al., 2003). *Rhinacanthus nasutus* (L.) Kurz belongs to the family Acanthaceae. It is a medicinal plant that is widely distributed in Southeast Asia, however it is known as "Tongpunchang" in Thailand. This plant is also commonly considered as a treatment for a number of common disorders including cancer, fungal infections, eczema, pulmonary tuberculosis and herpes virus infections (Rojanapo et al, 1990; Sendl et al, 1996; Kernan et al, 1997).

The medicinal effects of *C. asiatica* and *R. nasutus* for health promotion may be due to immunomodulating activity

¹ Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand 50200 ² Molecular Epidemiology Unit, Epidemiology and Health Services Research, School of Medicine, University of Leeds, Leeds LS2 9JT, UK ³ Department of Immunology, Faculty of Associated Medical Science, Chiang Mai University, Chiang Mai, Thailand 50200

*Corresponding author: Dr. Usanee Vinitketkumnuen, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand 50200. Tel.: +66-53-945325; fax: +66-53-894031. E-mail address: uvinitke@mail.med.cmu.ac.th

of these plants. Therefore, in the present study, we evaluated immunomodulating properties of *C. asiatica* and *R. nasutus* extracts on both cell-mediated immune response and humoral immune response.

Materials and Methods

Chemicals

Phytohemagglutinin (PHA), pokeweed mitogen (PWM), Trypan Blue, Histopaque and RPMI 1640 medium were obtained from Sigma Chemical Company, USA. Tritiated-thymidine was obtained from Amersham Company, Germany. Peroxidase conjugated rabbit anti-mouse IgG and IgM were purchased from Zymed laboratory, USA. Lipopolysaccharide (LPS) from *E. coli* 0111: B4, fetal bovine serum (FBS), MTT [3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], N-(1-naphthyl)-ethylenediamine dihydrochloride, sulfanilamide and sodium nitrite were purchased from Sigma-Aldrich Chemical Company, Dorset, UK. Ninety six-well flat bottom tissue culture plates were purchased from Nunc Inc, Hereford, UK. IL-2 (human) and TNF- α (human and mouse) ELISA assay kit were purchased from eBioscience Company, Wembley, UK.

Plant Materials

Whole fresh plants of *C. asiatica* and *R. nasutus* were obtained from local market in Chiang Mai, Thailand and identified by Dr. Chusie Trisonthi at the Department of Biology, Faculty of Sciences, Chiang Mai University. All fresh plants were washed with tap water, sliced into small pieces, dried and ground to a fine powder. One hundred grams of powder were extracted with 500 ml of distilled water or of 80% ethanol. The supernatants were filtered through Whatman filter paper. The filtrates were evaporated using a rotating evaporator (60°C) followed by lyophilized to dryness. The residue was dissolved in distilled water, adjusted to 50 mg/ml of final concentration and sterilized by Millipore filter membrane (pore size: 0.22 μ m).

Human Lymphocyte Proliferation Assay

The peripheral blood mononuclear cells (PBMCs) were separated from whole blood of three healthy donors by Ficoll-Hypaque gradient centrifugation. The PBMCs were prepared under sterile conditions in RPMI-1640 medium containing 10% fetal calf serum. The viability of PBMCs that determined by the trypan blue exclusion test was more than 98%. The concentration of PBMCs was final adjusted to 1.0×10^6 cells/ml. One hundred micro liters of cell suspension were cultured with different concentrations of plant extracts and PHA or PWM in a 96-well flat-bottomed microplate at 37°C in a 5% CO₂ incubator. One triplicate series of wells was used as negative control (without extracts and mitogens) and positive control with mitogens. After 72-h incubation, cell proliferation was estimated by adding 0.2 μ Ci of [³H]-thymidine per well during the final 18-h culture. [³H]-thymidine incorporation into cells was measured as

count per minute (CPM) using a liquid scintillation counter (β -counter). Stimulation index (SI) was determined by dividing (the mean CPMs of treated cultures - negative controls) by (the mean CPMs of positive controls - negative controls).

IL-2 and TNF- α determination

Human PBMCs (5×10^5 cells/ml) were cultured in RPMI-1640 for 18 h in the presence or absence of plant extracts. For stimulation of interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α , PHA (5 μ g/ml) and LPS (10ng/ml) were used, respectively. Culture supernatant were collected and stored at -80°C until tested. For determination, the ELISA kits were used to measured IL-2 and TNF- α secretion in culture supernatant following the manufacturer's instructions.

Animals

Male Balb/c mice (8-12 weeks of age, weighing 20-25g) were obtained from the animal resource facilities of the faculty of medicine, Chiang Mai university, Thailand and the animals were kept in air controlled room with a 12-h light/dark cycle and fed with normal mouse chow. Mice were divided into treated and control groups, 6 mice each. In treated groups, mice were fed with different concentrations of *C. asiatica* or *R. nasutus* extract/kg bw and the control mice were received distilled water throughout the whole experiment.

Humoral Antibody Responses to BSA

One week after the administration of the crude water extract, pre-immunized blood was collected, allowed to clot for 30 min, and centrifuged at 1000xg for 15 min at 4°C. Serum was stored at -20°C for use as a non-immunized control. Two weeks after mice immunization with 1 mg of bovine serum albumin (BSA) in PBS by intra-peritoneal (i.p.) injection blood was similarly collected. On the next day, mice were boosted with BSA and blood was again collected two weeks later.

Measurement of Antibody Production in Serum

Anti-BSA IgG and IgM antibodies in serum were measured by the ELISA method. The 96-well plates were coated with 2% BSA in PBS pH 7.4 and incubated overnight at 4°C. Plates were blocked by 5% skimmed milk for 2 hr at 37°C. Diluted serum samples or PBS control were added directly into the wells and incubated for 1 h at 37°C. After plates were washed six times with 0.05% Tween 20 in PBS, horseradish peroxidase (HRP)-conjugated anti-mouse IgG or IgM were added and incubated for 1 hr at 37°C. This reaction were developed by adding TMB substrate for 15 min at room temperature in dark and optical density was measured at 450 nm using ELISA plate reader.

Statistical Analysis

Data presented are means \pm SDs with analysis by the Mann-Whitney U test to determine significance (p value <

0.05).

Results

Effects of C. asiatica and R. nasutus extracts on the mitogen-induced human PBMCs proliferation

In vitro immunomodulating activity of *C. asiatica* and *R. nasutus* extracts were assessed using lymphocyte activation assay. Comparison of the cell proliferation in non-treated and extracts-treated cultures showed no direct mitogenic activity. Water extract of *C. asiatica* significantly increased PWM-induced lymphocyte proliferation with dose response manner and slightly increased PHA-induced lymphocyte proliferation (Fig. 1a). However, ethanol extracts of *C. asiatica* inhibited mitogens-induced lymphocyte mitogenesis (at concentration >50 µg/ml) (Fig. 1a). The viability of lymphocytes was also determined by trypan blue exclusion to confirm that this inhibition effect was not caused by the cytotoxicity of ethanol extract itself. This result indicated that *C. asiatica* has both immunostimulant and immunosuppressive activities. Both water and ethanol extracts of *R. nasutus* significantly increased lymphocyte proliferation induced by either PHA or PWM (Fig. 1b.). These results showed immunomodulating activity of plant extracts on cell-mediated immune response.

Effects of C. asiatica and R. nasutus extracts on IL-2 and TNF-α production

Human PBMCs were used for the production of IL-2 and TNF-α. Water extract of *C. asiatica* at concentration 500 µg/ml increased PHA-stimulated IL-2 as well as LPS-stimulated TNF-α production. In contrast, ethanol extract of *C. asiatica* inhibited IL-2 and TNF-α production (Fig.

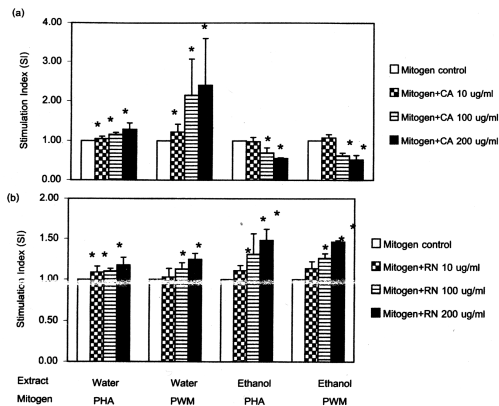


Figure 1. Effects of (a) *C. asiatica* (CA) and (b) *R. nasutus* (RN) Extracts on Human PBMCs Proliferation Induced by Mitogens. Human PBMCs were incubated for 72 h with either PHA or PWM as mitogen in the presence of either water or ethanol extracts (treated) or with medium alone (control). Then, [³H]-thymidine was added in the culture at 18 h before cell harvesting and [³H]-thymidine incorporation was determined. Stimulation index (SI) was calculated and each value represents mean ± SD of three donors. Statistical significance versus mitogen control: * *p*<0.05.

2). Both water and ethanol extract of *R. nasutus* increased PHA-stimulated IL-2 as well as LPS-stimulated TNF-α production in human PBMCs culture when cells were exposed to extract for 18 h (Fig. 3).

Effect of C. asiatica and R. nasutus extracts on humoral immune response in vivo

Water extracts of *C. asiatica* and *R. nasutus* increased lymphocyte proliferation induced by PWM (Fig. 1). PWM is a mitogen that can induce the proliferation of specific group of cells called B-lymphocytes. B-lymphocytes are

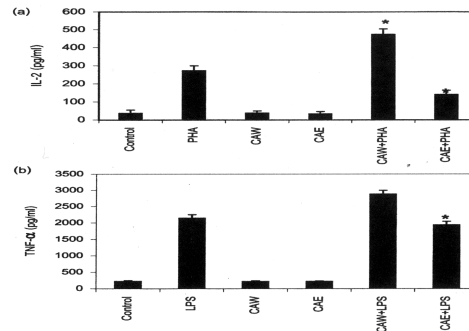


Figure 2. Effects of *C. asiatica*, Water Extract (CAW) and Ethanol Extract (CAE), on (a) IL-2 and (b) TNF-α Production in Human PBMCs. Human PBMCs was cultured in the presence or absence of 500 µg/ml of *C. asiatica* extract. PHA (5µg/ml) and LPS (10ng/ml) were used as positive control to stimulate IL-2 and TNF-α, respectively. Statistical significance versus mitogen control: * *p*<0.05.

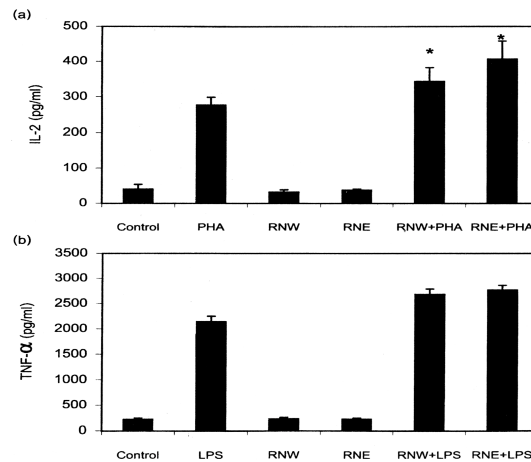


Figure 3. Effects of *R. nasutus*, water extract (RNW) and Ethanol Extract (RNE), on (a) IL-2 and (b) TNF-α Production in Human PBMCs. Human PBMCs was cultured in the presence or absence of 500 µg/ml of *R. nasutus* extract. PHA (5µg/ml) and LPS (10ng/ml) were used as positive control to stimulate IL-2 and TNF-α, respectively. Statistical significance versus mitogen control: * *p*<0.05.

responsible for the specific antibody production in the immune system. We then evaluated the effects of water extract of *C. asiatica* and *R. nasutus* on *in vivo* antibody production. BALB/c mice treated with water extract of *C. asiatica* (100 mg/kg bw) significantly increased both primary (IgM) and secondary (IgG) antibody responses to BSA when

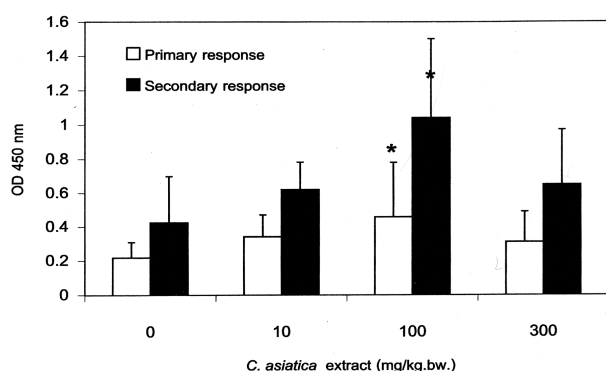


Figure 4. Effects of Water Extract of *C. asiatica* on Antibody Production against BSA. BALB/c mice were fed with extract and immunized twice with 1 mg BSA. Sera were collected 2 weeks after each immunization. Primary antibody (IgM) and secondary (IgG) antibody against BSA were determined by ELISA method. Statistical significance versus mitogen control: * $p < 0.05$.

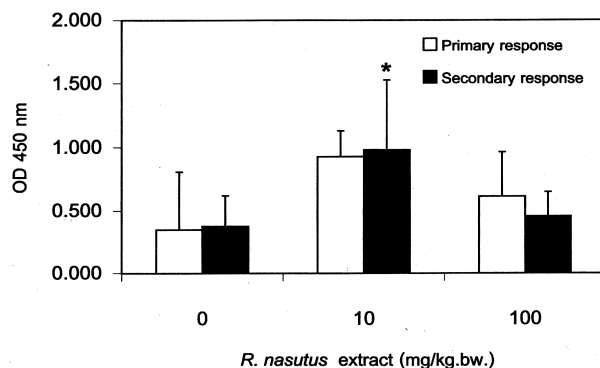


Figure 5. Effects of Water Extract of *R. nasutus* on Antibody Production Against BSA. BALB/c mice were fed with extract and immunized twice with 1 mg BSA. Sera were collected 2 weeks after each immunization. Primary antibody (IgM) and secondary (IgG) antibody against BSA were determined by ELISA method. Statistical significance versus mitogen control: * $p < 0.05$.

compared with non-treated control group (Fig. 4). *R. nasutus* extract-treated mice produced higher amount of secondary antibody production against BSA than non-treated mice (Fig. 5). Interestingly, the observed effect was not dose-response manner. Mice fed with higher concentration of extracts showed no difference in the specific antibody production when compared with non-treated control mice.

Discussion

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years. Modulation of immune responses to alleviate disease has been one mechanism of interest. A number of medicinal plants have been shown to stimulate or inhibit immune responses (Craig, 1999; Block and Mead, 2003; Agarwal and Singh, 1999).

C. asiatica has been used in the treatment of conditions such as inflammation, rheumatism, asthma and immune

system deficiencies (Farnsworth and Bunyapraphatsara, 1992). Some studies have shown that it can affect the immune reactions through their anti-inflammatory actions (Chen et al, 1999). Recent studies showed the potential cytotoxic and anti-tumor properties of *C. asiatica* (Babu et al, 1995; Bunpo, 2004). Beside direct cytotoxic to tumor cells, *C. asiatica* may prevent carcinogenesis by modulating of immune response. Our data showed that water extract of *C. asiatica* exerted immunostimulating activity on mitogen-stimulated proliferation of human PBMCs. PHA was used to stimulate T and B cell proliferation whereas PWM was used for B cell proliferation. The stronger affect was observed in PWM-induced PBMCs proliferation, indicating the stimulation of B lymphocytes. Water extract of *C. asiatica* also increased the production of IL-2 and TNF- α in human PBMCs. IL-2 enhances the proliferation of activated T lymphocytes and activates B lymphocytes stimulating the proliferation and secretion of immunoglobulin (Thorpe, 1998). These results support effects of extracts on human PBMC proliferation. *In vivo* study, BALB/c mice treated with water extract of *C. asiatica* (100 mg/kg.bw.) showed the higher responses to both primary and secondary antibodies against BSA when compared with the non-treated group. Thus *in vivo* results correlated with *in vitro* proliferative responses to B cells. and indicate that active components could be directly absorbed to produce effects.

In contrast to water extract, ethanol extract of *C. asiatica* showed immunosuppressive activity. It decreased the proliferation of mitogen-stimulated human PBMCs and the production of IL-2 and TNF- α . The viability of cells was also determined by trypan blue exclusion test and the cells showed high viability (>95%). This confirmed that the inhibitory effect observed in this condition could not be consider as cytotoxic effect of ethanol extract of *C. asiatica*. TNF- α is a cytokine produced by many types of cells in response to inflammation, infection, and environmental challenges (Rosenblum and Donato, 1989). Overproduction is associated with various diseases including infectious diseases, autoimmune disease and cancer. Thus, the inhibition of TNF- α production by ethanol extract of *C. asiatica* may be important. This result also correlated with our recent study, which showed the inhibition of nitric oxide and TNF- α production in J774.2 mouse macrophages by an ethanol extract of *C. asiatica* (Punturee, 2004).

In conclusion, the present study revealed immunostimulating activity of *C. asiatica* and *R. nasutus* extracts regarding both non-specific cellular immune responses and humoral immune responses. Although, the exact mechanism of this effect is not clear, it may be mediated by interactions between active components of extracts and cell surface molecules or growth factors involved in mitogen activation. Another possible action of extracts may be interference with cell signaling. Although further investigations are warranted, the data available to date suggest that *C. asiatica* and *R. nasutus* may have chemopreventive or anticancer actions through

immunostimulating activity.

Acknowledgements

The investigators wish to extend their appreciation to the Royal Golden Jubilee Scholarship Thailand, 1999 for generous financial support for the project.

References

- Agarwal SS, Singh VK (1999). Immunomodulators: a review of studies on Indian medicinal plants and synthetic peptides: Part I. Medicinal plants. *Proc Indian Natl Sci Acad*, **65**, 179-204.
- Babu TD, Kuttan G, Padikkala J (1995). Cytotoxic and anti-tumour properties of certain taxa of Umbelliferae with special reference to *Centella asiatica* (L.) Urban. *J Ethnopharmacol*, **48**, 53-7.
- Block KI, Mead MN (2003). Immune system effects of Echinacea, ginseng, and astragalus: a review. *Int Cancer Ther*, **2**, 247-67.
- Bradwejn J, Zhou Y, Koszycki D, Shlik J (2000). A double-blind, placebo-controlled study on the effects of Gotu Kola (*Centella asiatica*) on acoustic startle response in healthy subjects. *J Clin Psychopharmacol*, **20**, 680-4.
- Brinkhaus B, Lindner M, Schuppan D, Hahn EG (2000). Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. *Phytomedicine*, **7**, 427-48.
- Bunpo P, Kataoka K, Arimochi H, et al (2004). Inhibitory effects of *Centella asiatica* on azoxymethane-induced aberrant crypt focus formation and carcinogenesis in the intestines of F344 rats. *Food Chem Toxicol*, **42**, 1987-97.
- Coldren CD, Hashim P, Ali JM, et al (2003). Gene expression changes in the human fibroblast induced by *Centella asiatica* triterpenoids. *Planta Med*, **69**, 725-32.
- Craig WJ (1999). Health-promoting properties of common herbs. *Am J Clin Nutr*, **70**, 491S-9S.
- Farnsworth NR, Bunyapraphatsara N (1992). Thai medicinal plants: recommended for primary health care system. Mahidol University, Thailand, pp. 111-114.
- Gupta YK, Veerendra Kumar MH, Srivastava AK (2003). Effect of *Centella asiatica* on pentylentetrazole-induced kindling, cognition and oxidative stress in rats. *Pharmacol Biochem and Behavior*, **74**, 579-85.
- Ishizuka M, Kawatsu M, Yamashita T, Ueno M, Takeuchi T (1995). Low molecular weight immunomodulators produced by microorganisms. *Int J Immunopharmacol*, **17**, 133-9.
- Jones FA (1996). Herbs-useful plants. Their role in history and today. *Eur J Gastroenterol Hepatol*, **8**, 1227-31.
- Kernan MR, Sendl A, Chen JL, et al (1997). Two new lignans with activity against influenza virus from the medicinal plant *Rhinacanthus nasutus*. *J Nat Prod*, **60**, 635-7.
- Lin LT, Liu LT, Chiang LC, Lin CC (2002). In vitro anti-hepatoma activity of fifteen natural medicines from Canada. *Phytother Res*, **16**, 440-4.
- Punturee K, Wild CP, Vinitketkumneun U (2004). Thai medicinal plants modulate nitric oxide and tumor necrosis factor- α in J774.2 mouse macrophages. *J Ethnopharmacol*, **95**, 183-9.
- Rojanapo W, Tepsuwan A, Siripong P (1990). Mutagenicity and antimutagenicity of Thai medicinal plants. *Basic Life Sci*, **52**, 447-52.
- Rosenblum MG, Donato NJ (1989). Tumor necrosis factor a: A multifaceted peptide hormone. *Crit Rev Immunol*, **9**, 21-44.
- Sendl A, Chen JL, Jolad SD, et al (1996). Two new naphthoquinones with antiviral activity from *Rhinacanthus nasutus*. *J Nat Prod*, **59**, 808-11.
- Shukla A, Rasik AM, Jain GK, et al (1999). In vitro and in vivo wound healing activity of asiaticoside isolated from *Centella asiatica*. *J Ethnopharmacol*, **65**, 1-11.
- Suguna L, Sivakumar P, Chandrakasan G (1996). Effects of *Centella asiatica* extract on dermal wound healing in rats. *Indian J Exp Biol*, **34**, 1208-11.
- Thorpe R (1998). Interleukin-2. In: Cytokines, (Mire-Sluis, AR and Thorpe, R Eds), Academic Press Ltd., London, pp. 19-33.
- Veerendra Kumar MH, Gupta YK (2002). Effect of different extracts of *Centella asiatica* on cognition and markers of oxidative stress in rats. *J Ethnopharmacol*, **79**, 253-60.
- Yoosook C, Bunyapraphatsara N, Boonyakiat Y, Kantasuk C (2000). Anti-herpes simplex virus activities of crude water extracts of Thai medicinal Plants. *Phytomedicine*, **6**, 411-9.