ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Influence of Extract Derived *In-Vitro* Cell Suspension Cultures of *Echinacea purpurea* Against Some Immunosuppressive Effects.

Heba Mohammed Ibrahim Abdallah[1]*, Gihan Farag Mohammed Asaad[1,2], Somaia Ahmed Nada[1], Hussein Sayed Taha[3] and Medhat M Seif El-Nasr[4].

[1]Pharmacology Department, National Research Centre, Cairo, Egypt.
[2]Pharmacology Department, Pharmacy College, Gizan University, Saudia Arabia
[3]Plant Biotechnology Department, National Research Centre, Cairo, Egypt.
[4]Chemistry of Medicinal Plants Department, National Research Centre, Cairo, Egypt.

ABSTRACT

The present study investigates the therapeutic properties of in *vitro E .purpurea* cell suspension cultures against immunosuppressive effects of cyclophosphamide. Rats were assigned to four equal groups: normal control group, immune-suppressed groups: 2, 3 and 4 injected with cyclophosamide (50 mg/kg /day) i.m. for 3 consecutive days; group 2 kept as control positive, and the other two groups (3 & 4) were administered *E.purpurea* extract at doses 100 mg and 200 mg/kg, p.o., respectively & daily for 21 successive days. Blood samples were withdrawn twice; at the day 11and the 21 for blood cells count and the second for determination of cytokines level, γ-globulin, and MTT-value. Cyclophosphamide treated group showed significant decrease in total leucocytic count, anemia, and thrombocytopenia as well as humoral and cellular immunity markers; γ-globulin and MTT. *E.purpurea* treatment normalized the reduced blood cell elements in dose dependent manner. The extract inhibited production of TNF-α, elevated IL-1 and enhanced both cellular and humoral immune response. It can conclude that extracts derived *in vitro* cell suspension cultures of *E. purpurea* has a therapeutic potential for use in patients with inadequate functioning and regulation of the immune system.

Keywords: *Echinacea, suspension,* immunosuppressive, *cyclophosphamide, rats*.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Plants have been an important source of medicine for thousands of years. Nowadays, the World Health Organization estimates that up to 80 % of people still use traditional remedies for their medicines [1]. Echinacea is widely used in Europe, North America and Australia for the treatment of cold, flu and upper respiratory tract infections [2,3]. Echinacea species such as *E. Purpurea*, *E. angustifolia and E. palleda* are members of the Asteraceae family [4].

E. Purpurea known as purple coneflower and Rudbeckia, is a flowering plant member of the composite family of Asteraceae. It is one of the most popular herbal medicines that have a long history of traditional use for a wide range of diseases [5]. *E. Purpurea* has been reported to act as an immunoregulator, antioxidant, and promoting wound healing promoter [6]. It is widely used as a self-medicating agent for the treatment of common cold, coughs, bronchitis, inflammation of mouth and pharynx [7]. This property was found to be due to enhanced non-specific immune response & free radical scavenging properties elicited by the herb treatment [8].

The principal advantage of plant cell cultures is that it ultimately provides a continuous and reliable source of active agent year-round. In addition, compounds from tissue cultures are more easily purified because of simple extraction processes and the absence of significant amounts of contaminants such as pigments, thus possibly reducing the production and processing costs. Advantages of an *in vitro* system for medicinal plants include: (1) year-round availability of plant materials for extraction of pharmaceuticals produced under controlled conditions; (2) potential regulation of metabolic pathways from which active ingredients or marker compounds are derived; (3) potential genetic modification of cells/tissues to produce specific intermediates or metabolites; and (4) mass micropropagation of desired plants [9].

The chemical composition of *E. Purpurea* extract is very complex. It contains seven groups of medically vital components, including polysaccharides, flavonoids, caffeic acid derivatives, essential oils, alkylamides polyacetylenes. These components are believed to be responsible for the observed immune-stimulatory mechanism of *E. Purpurea* pharmacological activity [10]. The polysaccharides specifically protect surrounding tissue cells from bacterial & pathogenic invasion as an immune-regulator. That involves stimulation of T-cell production, phagocytosis, lymphocytic activity, cellular respiration, and inhibition of hyaluronidase enzyme secretion [11].

E. Purpurea is also thought to have anti-inflammatory effects [12]. It was shown that the inhibitory effect of Echinacea extracts on pro-inflammatory cytokines may be one of the targets by which the herb is capabable of alleviating symptoms of common colds, flu and related infections [13].

Cyclophosphamide (CP) is an alkylating agent which has anticancer property. Its mode of action involves addition of alkyl groups to DNA thus slowing or stopping tumour growth [14]. Besides the cytotoxic effects of CP towards tumor cells, it also affects other cell types in the body most notably the immune cells which protect the body from harmful agents [15]. There is growing interest among biomedical scientists in the ability of some natural products to stimulate the production of immune cells in immunosuppressed animal models. Hence, the present study investigates the therapeutic properties of *E.purpurea* cell culture extract against immunosuppressive effects of CP by evaluating changes in blood cell count, cellular and humoral immunity and anti-inflammatory activity.

MATERIAL AND METHODS

Chemicals

Lipopolysaccharide (Escherichia coli, serotype 055:B5) was purchased from Sigma-Aldrich, Germany. All other chemicals, used throughout the experiment, were of the highest analytical grade available. Kits used to measure serum γ-globulin, TNF-α and IL-1 kits were obtained from RayBio® (USA).

Animals

Adult Sprague–Dawely rats weighing 120-150 gm were obtained from the animal house at the National Research Center (Giza, Egypt), fed a standard laboratory diet and tap water ad libitum. Experimental animals were housed in an air-conditioned room at 22–25 °C with a 12-h light/dark cycle. This study was approved by an ethics committee of the National Research Centre which gave its consent in accordance with the National Regulations on Animal Welfare and Institutional Animal Ethical Committee (IAEC).

In vitro production of *E. Purpurea* cell suspension cultures

Root explants of sterilized and *in vitro* growing seeds of *E. Purpurea* were existed and cultured on solidified nutrient medium (MS) supplemented with 7 g/l agar and 30 g/l sucrose [16]. The MS-nutrient medium was fortified by 3 mg/l NAA + 1 mg/l BA and augmented with 100 mg/l tryptophan. The pH of the culture medium was adjusted to 5.7 with 0.1 M NaOH or 0.1 M HCl before adding agar. The culture medium was dispensed into small jars (150 ml), each one contained 40 ml. Cultures medium was autoclaved at 121°C ± 1 for 20 min. Cultures were incubated in darkness in a growth chamber at a constant temperature of 28 °C ± 1 then incubated under light condition (2000 Lux) from cool white fluorescent lamps, and sub cultured every 4 weeks on new fresh medium. After three subcultures, white calli were initiated and observed. The obtained calli from the previous experiment was saved and re-suspended in an agitated liquid MS medium containing the previous supplementations.

Chemical extraction

The pulverized air –dried plant material was exhaustively extracted by percolation with 80% methanol for three successive times, after filtration of the marc, the solvent was evaporated and the total methanolic extract, was concentrated under reduced pressure using rotavapour. The residue was dissolved in distilled water to be used in the pharmacological study.

Experimental design

After an acclimatization period of one week, 24 healthy rats were randomly assigned to four groups(each of six rats): vehicle control group received normal saline only (10ml/kg, orally), immunosuppressed groups 2,3 and 4 injected with cyclophosphamide (50 mg/kg /day) intramuscularly for three consecutive days; group 2 kept as control positive, and the other two groups (3 & 4) were administered two dose levels of *E. Purpurea* suspension cultures:100 mg and 200 mg/kg, orally , respectively, daily for 21successive days.

Sampling and blood cell counting

Blood samples were withdrawn from the retro-orbital *venues plexus* twice: at the day 11and the 21(the end of experimental period) in to heparinized tubes and used for blood cells counting according to Jain [17]. While in the second blood sample collected into plane test tube, allowed to coagulate and then centrifuged at 3000 rpm for 15 min. The obtained serum was used to determine cytokines level, γ-globulin, and MTT-value.

Evaluation of humoral immunity parameters

γ-globulin levels in blood serum were determined by the precipitation method modified by Siwicki and Anderson [18].

Evaluation of specific cellular immunity parameters (MTT-LPS)

Proliferative response of blood lymphocytes after stimulation with mitogens, lipopolysaccharide (LPS) was determined by proliferative response of blood lymphocytes spectrophotometry (OD 570 nm) using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium (MTT) assay, as described by Mosmann [19].

ISSN: 0975-8585

Determination of cytokines

Serum levels of tumor necrosis factor- alpha (TNF-α), and Interleukin-1 (IL-1) was determined using corresponding commercial kits (Rat TNF-α and Rat IL-1 ELISA kits) according to manufacturer's instructions.

Statistical analysis

Results were analyzed by one way ANOVA using EXCELL Microsoft office 2010.

RESULTS

Production of *in vitro E. purpurea* cell suspension cultures

Results of this part of study are not shown and will be published soon.

The results of the effect of *E. purpurea* extracts against some immunosuppressive effects were recorded on the following parameters as follow:-

Blood picture

Table (1) shows that CP treated group had significant lymphocyopenia, leukopenia, monocytopenia, neutropenia, eosinopenia, basocytopenia, thrombocytopenia and anemia (reduction in all types of blood cell counts) after 11 days. Groups treated with *E.purpurea* cultures significantly elevated and normalized the reduced blood cell elements especially neutrophils and thrombocytic counts in a dose dependent manner. Blood count after 21 days of treatment with CP severely reduced TLC and became worthier than before (after 11 days) when compared with control groups. However, *E.purpurea* improved TLC, DLC, RBC and thrombocyte counts in CP- immunosuppressive groups in a dose dependent manner (Table 2).

Table 1: Effect of *E.purpurea* susp cultures (100 and 200 mg/kg, Os) on RBC , TLC, differential LC, and platelets count in rats treated with cyclophosphamide after 11 days of treatment in rats.

Groups	TLC ($NoX10^3$/ ml)	Differential Leucocytic count ($NoX10^3$/ ml)					RBC ($NoX10^6$/ ml)	Platelets ($NoX10^3$/ ml)
		Lymphocytes	Neutrophils	Monocytes	Eosinophiles	Basophiles		
Control	A 12.14 ± 0.31	A 7.03 ± 0.15	A 2.57 ± 0.021	A 0.90 ± 0.018	A 1.203 ± 0.001	A 0.44 ± 0.011	A 5.86 ± 0.28	A 372.1 ± 2.49
Cyclo	B 7.14 ± 0.11	B 4.88 ± 0.21	B 1.75 ± 0.023	B 0.25 ± 0.004	B 0.22 ± 0.001	B 0.04 ± 0.03	B 3.51 ± 0.23	B 295.7 ± 2.55
E 100	C 9.32 ± 0.27	C 5.25 ± 0.17	A 2.15 ± 0.02	C 0.76 ± 0.01	C 0.56 ± 0.002	C 0.6 ± 0.004	C 4.76 ± 0.18	C 321.6 ± 3.92
E 200	A 12.39 ± 0.34	D 6.95 ± 0.41	A 2.78 ± 0.024	D 1.5 ± 0.01	D 0.85 ± 0.007	D 0.31 ± 0.001	AC 5.12 ± 0.27	A 365.4 ± 3.77

The observation are mean ± S.E.M. (n=6). The different capital letters are significantly different at p< 0.05. (ANOVA one way)

ISSN: 0975-8585

Table 2: Effect of *E.purpurea* susp cultures (100 and 200 mg/kg, Os) on RBC , TLC, differential LC, and platelets count in rats treated with cyclophosphamide after 21 days of treatment in rats.

Groups	TLC (NoX103/ ml)	Differential Leucocytic count (NoX103/ ml)					RBC (NoX106/ ml)	Platelets (NoX103/ ml)
		Lymphocytes	Neutrophils	Monocytes	Eosinophiles	Basophiles		
Control	A 11.81 ± 0.28	A 6.93 ± 0.17	A 2.43 ± 0.011	A 0.95 ± 0.015	A 1.0 ± 0.002	A 0.5 ± 0.001	A 5.4 ± 0.02	A 358.4 ± 2.86
Cyclo	B 5.83 ± 0.21	B 4.1 ± 0.11	B 1.45 ± 0.03	B 0.15 ± 0.008	B 0.12 ± 0.002	B 0.01 ± 0.021	B 3.51 ± 0.08	B 265.9 ± 2.10
E 100	C 8.87 ± 0.30	B 4.65 ± 0.39	A 2.65 ± 0.05	C 0.76 ± 0.04	C 0.41 ± 0.001	C 0.4 ± 0.002	C 4.76 ± 0.026	C 301.6 ± 2.84
E 200	A 11.41 ± 0.34	A 6.32 ± 0.41	A 2.63 ± 0.024	A 1.0 ± 0.01	A 0.95 ± 0.007	A 0.51 ± 0.001	A 5.12 ± 0.06	A 345.6 ± 3.19

The observation are mean ± S.E.M. (n=6). The different capital letters are significantly different at p< 0.05. (ANOVA one way)

Humoral immunity

γ-globulin level showed significant reduction in CP- treated group. Ech.ext (200mg/kg) treatments caused significant increase in γ-globulin level comparing with CP-treatment (Fig 1a).

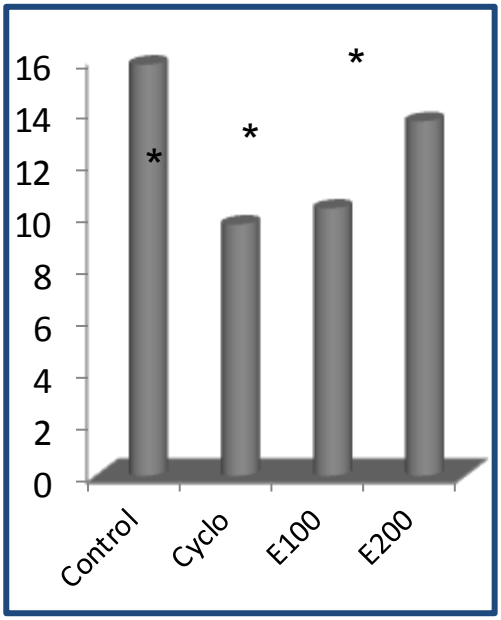


Figure 1.a: Effect of *E.purpurea* susp cultures (100 and 200 mg/kg, p.o.) on serum γ-globulin in rats. The observation are mean ± S.E.M. (n=6). * p> 0.05 vs. control (ANOVA one way)

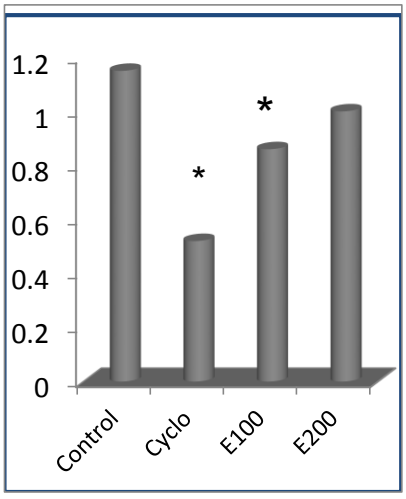


Figure 1.b: Effect of *E.purpurea* susp cultures (100 and 200 mg/kg, p.o.) on serum MTT-LPS reactivity index in rats. The observation are mean ± S.E.M. (n=6). * p> 0.05 vs. control (ANOVA one way)

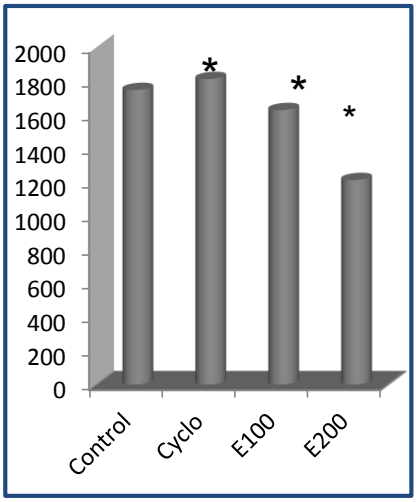
ISSN: 0975-8585

Cellular immunity

Fig (1b) shows that CP-treated group decreased MTT-LPS value similar to that of γ-globulin and this value increased significantly by *E.purpurea* according to the dose level, *E.purpurea* cultures at dose of 200mg/kg normalized MTT-LPS value.

Cytokine production

TNF-α showed significant increase in the group treated with CP compared with the control group. Meanwhile, TNF-α significantly decreased by *E.purpurea* cultures (100 and 200 mg/kg) in CP – immunosuppressed rats in dose dependent manner as illustrated in Fig (2a).



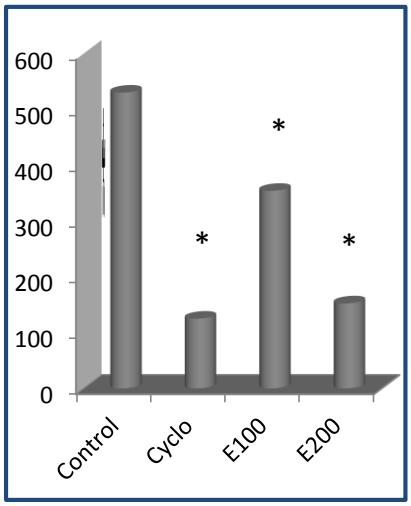


Figure 2.a: Effect of Echnaecia susp cultures (100 and 200 mg/kg, p.o.) on serum TNF-α level. The observation are mean ± S.E.M. (n=6). * p> 0.05 vs. control (ANOVA one way)

Figure 2.b: Effect of Echnaecia susp cultures (100 and 200 mg/kg, p.o.) on serum IL-1 level in rats. The observation are mean ± S.E.M. (n=6) * p> 0.05 vs. control (ANOVA one way)

IL-1 values resulted significant decrease by the treatment with CP when compared with the other treatment groups. However, *E.purpurea* cultures treated group significantly decreased IL-1 in rats treated with 200mg/kg compared with the control group and there was no significant changes were obtained when compared with CP-group. Ech. 100 mg/kg significantly elevated IL-1 than 200 mg/kg and CP alone while did not reached the normal values as shown in Fig (2b).

DISCUSSION

Cyclophosphamide (CP) is known as immunosuppressive drug (15), where the CP group showed highly significant decrease of TLC and DLC on days 11 and 21 comparing with control group. This could be attributed to severe depression of bone marrow that manifested by significant decrease of all types of blood cells, lymphocyopenia, leukopenia, monocytopenia, neutropenia, eosinopenia, basocytopenia, and thrombocytopenia. This result agree with Latha et al. [20] and Smith *et al.* [21] who reported leucopenia, lymphopenia and neutropenia in mice and rats treated with CP.

The stimulation of production of white blood cells in an immunosuppressed animal model has been classified as an immunomodulatory effect [22,23]. The increased neutrophils in the immunosuppressed organisms is crucial for their survival as they make the innate immune system, and mount an immediate non-

specific response to foreign microbial agents [24]. Treatment with *E. purpurea* extract significantly increased TLC as well as lymphocytes, monocytes, neutrophils, basophils and eosinophils compared with CP group showing an obvious degree of protection due to the enhancement of the immune system. These results are in accordance with the findings of Barrett [7] and Widel et al. [25], who reported that Echinacea preparations influenced the leukocyte count. Also Di Carlo et al. [26] reported that the splenic lymphocytes from mice orally treated with Echinacea were shown to be significantly more resistant to apoptosis.

In the current study, administration of cyclophosphamide successfully caused significant immunosuppression. It decreased serum level of γ-globulin and MTT-LPS value indicating its effect on both humoral and cellular immunity. Treatment with *E. Purpurea* extract enhanced both cellular and humoral immunity. It increased serum level of γ-globulin as well as the MTT-LPS value which was normalized at the dose 200mg/kg of the extract. It appears that this herb stimulate the immune function through activation of phagocytosis and fibroblasts, increasing cellular respiration and mobility of leukocytes. The polysaccharides from *E. Purpurea* were found to protect surrounding tissue cells from bacterial and pathogenic invasion as an immunoregulator [5]. Chaves et al. [27] reported that the aqueous extract of Echinacea roots stimulates proliferation of human lymphocytes in vitro and is able to increase specific antibody response in vivo. It has been also suggested that the lipophilic alkylamides isolated from Echinacea species are likely the active components responsible for the immunosuppressive effects of the plant [28].

TNF-α is amplify, propagate, and coordinate proinflammatory signals, resulting in the synchronized expression of effectors molecules that mediate diverse aspects of innate immunity. TNF is capable of eliciting expression of chemokines and adhesion molecules and thus may be critical to the recruitment of neutrophils from the blood [29]. IL-1 is another proinflammatory cytokine which is involved in regulation of immune system. It was first described in 1972 as a lymphocyte-activating factor [30] and later was shown to stimulate of proliferation of T and B cells. In the present study, Echinacea extract decreased the serum levels of the proinflammatory cytokines, TNF-α and IL-1. These results are in hand with Sullivan et al. [31] who examined similar parameters when testing the effects of *E. Purpurea* polysaccharides on macrophages *in vitro*. They found that polysaccharides isolated from *E. Purpurea* stimulated the production of many cytokines including IL-6, TNF-α, IL-12, and NO. There is some other evidence that alkamides present in Echinacea are agonists of cannabinoid receptor type 2, and by stimulation of this receptor alkamides upregulate TNF-α production [32-33, 28]. Bany *et al.* [34] observed a significant inhibition of bacterial and parasite infection in mice treated with *Echinacea purpurea* and *Echinacea angustifolia* extracts. It has been showed that both Echinacea species activate macrophages to produce cytokines, which play the role of cell-type immunity.

CONCLUSIONS

In conclusion, suspension culture *of E. purpurea* is shown to have Immunostimulatory effect in rats treated given cyclophosphamide by stimulation of blood cells production and enhancing both cellular and humoral immunity. The study also provides evidence for possible involvement of cytokines; TNF- α and IL-1 in the *E.purpurea*-mediated immunomodulatory effect.

REFERENCES

[1] Tripathi LF, Tripathi JN. Trop J Pharm Res 2003; 2 (2): 243-253.
[2] Blumenthal M, Goldberg A, Brinkmann J. Herbal Medicine: Expanded Commission E Monographs, Boston (MA Integrative Medicine Communications, 2000.
[3] Baum BR, Binns SE, Arnason JT. HerbalGram 2006; 72:32–46.
[4] Perry NB, Burgess EJ, Glennie V. J Agric Food Chem 2001; 49: 1702–1706.
[5] Cozzolino R, Malvagna P, Spina E, Giori A, Fuzzati N, Anelli A, Garozzo D , Impallomeni G. Carbohyd Polym 2006; 65:263-272.
[6] Kumar KM, Sudha Ramaiah. International Journal of Pharma and Bio Sciences 2011; 2(4): 304-314.
[7] Barrett B. Phytomed 2003; 10: 66-86.
[8] Goel V, Lovlin R, Chang C, Slama JV, Barton R, Gahler R, Bauer R, Goonewardene L, Basu TK. Phytother Res 2005; 19(8):689-694.
[9] Kurz WG, Constabel F. Adv Appl Microbiol 1979; 25: 209–240.
[10] Barnes J, Anderson LA, Gibbons S, Phillipson JD. J Pharm Pharmacol 2005; 57(8); 929–954.
[11] Lee TT, Huang CC, Shieh XH, Chen CL, Chen LJ, Yu B. IJESD 2010; 1(1): 5-9.

[12] Cech NB, Eleazer MS, Shoffner LT, Crosswhite MR, Davis AC, Mortenson AM. J Chromatogr 2006; 1103(2): 219-228.
[13] Sharma M, Anderson SA, Schoop R, Hudson JB. Antiv Res 2009; 83: 165–170.
[14] Baumann F, Preiss R. J Chromatogr B Biomed Sci Appl 2001; 764:173-192.
[15] Hou FX, Hui FY, Tao Y, Wei C. Enviro Toxicol Pharmacol 2007; 24:30-6.
[16] Murashige T, Skoog. Physiol Plant 1962; 15: 473-479.
[17] Jain, NC. Schalm's Veterinary Haematology 4th ed. Lee and Febiger, Pheladelphia, USA, 1986.
[18] Siwicki AK, Anderson DP. Immunostimulation in fish:measuring the effects of stimulants by serological and immunological methods. US Fish Wildlife Service, IFI, Olsztyn, 1993 1: 17.
[19] Mosmann T. J Immunol Methods 1983; 65: 55-63.
[20] Latha PG, Panikkar KR. Phytother Res 1999; 13:517-20.
[21] Smith HW, Winstead CJ, Stank KK, Halstead BW, Wierda D. Toxicol 2003, 194(1-2):129-45.
[22] Vigila GA, Baskaran X. Ethnobot Leaflets 2008; 12:1206-1212.
[23] Shukla SH, Saluja AK, Pandya SS. Pharmacog Res 2010; 2:359–363.
[24] Obameso JO, Akinyele BJ, Oladunmoye MK, Osho IB. African J Microbiol Res 2011; 5(26):4622-4626.
[25] Widel M, Jedrus S, Lukaszczyk B, Raczek-Zwierzycka K, Swierniak A. Radiat Res 2003, 159: 713-721.
[26] Di Carlo G, et al. Pharmacol Res 2003; 48: 273-277.
[27] Chaves F, Chacón M, Badilla B, Arévalo C. Rev Biol Trop 2007; 55(1):113-119.
[28] Woelkart K, Bauer R. Planta Med 2007; 73: 615-623.
[29] Mizgerd JP, Spieker MR, Doerschuk CM. J Immunol 2001; 166:4042-4048.
[30] Gery I, Waksman BH. J Exp Med 1972; 136: 143-155.
[31] Sullivan AM, Laba JG, Moore JA, Lee TD. Immunopharmacol Immunotoxicol 2008; 30(3):553–574.
[32] Clifford LJ, Nair MG, Rana J, Dewitt DL. Phytomed 2002; 9: 249-253.
[33] Raduner S, Majewska A, Chen JZ. J Biol Chem 2006; 281: 14192-14206.
[34] Bany J, Siwicki AK, Zdanowska D. Pol J Vet Sci 2003; 6 (3 Suppl): 3-5.