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IN VITRO CYTOTOXICITY AND FREE RADICAL SCAVENGING ACTIVITY OF ETHANOLIC AND ALKALOIDIC EXTRACTS OF *DELPHINIUM STAPHISAGRIA*

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ABSTRACT

In this study, the *in vitro* cytotoxicity and antioxidant properties of the ethanolic and alkaloidic extract of *Delphinium Staphisagria* seeds were assessed. The antioxidant activity of the plant extracts and the standard was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity. The *in vitro* cytotoxicity was carried out by using the 3-(4,5- dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, against two animals cancer cell lines; Vero cell line, initiated from the kidney of a normal, adult African green Monkey and Neuro-2a (N2a) from a spontaneous tumor of a strain A albino mouse. The DPPH scavenging activity of both extracts was concentration-dependent (increasing from 15.62 µg/ml to 500 µg/ml), exhibited considerably ($P<0.05$) DPPH radical-scavenging activity and was able to inhibit the formation of DPPH radicals with a percentage inhibition of 62.19 and 94 % respectively at the highest concentration. The results showed that the alkaloidic extract (1000-31.25 µg/ml) of *Delphinium Staphisagria* possesses significant IC_{50} compared to the drug positive control on all cancer cell lines used. The lower IC_{50} represent the highest potency of a compound to inhibit the growth of cells and cause toxicity and death of cells. The result obtained in this work demonstrated a high activity at low alkaloids extracts doses (500µg/ml). In conclusion, the results of the present study suggest that *Delphinium Staphisagria* seeds plant have various secondary metabolites and has good quantity of alkaloidic compounds (diterpenoid alkaloids). Plant has potent free radical scavenging activity. Detailed studies on chemical composition, isolation of active constituents and pharmacological evaluation are essential to characterize them as biological antioxidants. The present findings of this study support the view that *Delphinium Staphisagria* seeds are a promising source of potential antioxidant which can be used in treatment of various ailments.

Keywords: *Delphinium Staphisagria*; DPPH; Radical-scavenging; Cytotoxic activity; Diterpenoid alkaloids.

INTRODUCTION

Phytochemicals found in plants have beneficial effect on health or play active role in curing diseases. Plants have the ability to synthesize many secondary metabolites of which only 10% are identified. These metabolites serve remedy for various alemeties like antimicrobial, anti-inflammatory, antihypertensive anti diabetic ect.. [1, 2]. Since these constituents influence human health in beneficial way, analyses of the constituents become important in view of pharmaceutical and health care products.

Certain chronic and degenerative diseases are induced by oxidative stress [3]. Free radical, one of the factors to cause stress, are generated due to

environmental pollutants, radiations, chemicals, toxins, metabolic process especially oxidation, make body cell to deteriorate and cause depletion of innate immunity, change in gene expression, if not used up. Antioxidants take up these free radicals and protect body against pathogenic conditions like anemia, arthritis, inflammation, neruo-degeneration, Parkinson's disease [4, 5]. Hence antioxidant property is important for study.

Antioxidant properties in medicinal plants are attributed to the wide range of amphipathic molecules broadly termed "Polyphenolic compounds". Phenolic products act mainly due to its redox properties, which make them to act as hydrogen donor, reducing agents and singlet oxygen quencher. They also act as metal chelators [6]. In addition to pharmaceutical industry, in the food industry also, these phenolic compounds are of interest because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. Natural phenolic compounds are of interest to scientists, food manufacturers, and consumers due to its beneficial health effects [7].

Due to depletion of INNATE antioxidants, it may be necessary to consume antioxidants as free radical scavenging. Synthetic antioxidants are being replaced by naturally occurring antioxidants, mainly due to low solubility and moderate antioxidant activity [7]. Hence, there is a demand to explore the antioxidant potential of plant species [15].

Medicinal plants presents a potential source of drugs or molecular models for new drugs, in fact, in developing countries, including Morocco, the use of herbal medicine is for a majority of the population an alternative therapy due to the high cost of western pharmaceuticals, health care and the cultural, spiritual point of view of the people of the country [16]. All these makes the knowledge of chemical, biological and therapeutic activities of medicinal plants used in traditional medicine become necessary and led researchers to enhance scientifically the possible beneficial effects of medicinal plants. Natural compounds derived from plants may contribute on the mechanisms behind pathogenesis involved in some diseases like cancer. The varied climate and heterogeneous ecologic condition in Morocco have favoured the proliferation of more than 42,000 species of plants; divided into 150 families and 940 geniuses [8-14]. *Delphinium staphisagria* is an endemic annual or biennial herb from the Mediterranean Basin belongs to the family Renonculaceae, some studies have reported that the extract of *Delphinium staphisagria* have a number of pharmacological activities such as antispasmodic, cathartic, emetic and vermifuge. The seed is used to make a potent insecticide, parasiticide and to destroy vermin [17-19, 20]. Thus, the aim of this study is to evaluate the in vitro cytotoxicity and antioxidant properties of the ethanolic and alkaloidic extract of *Delphinium Staphisagria* seeds.

MATERIALS AND METHODS

Plant material: Plant materials were collected based on ethnopharmacological information, from villages around the region Chefchaoun, northern Morocco with the agreement from the authorities and respecting the United Nations Convention of Biodiversity and with assistance of traditional medical practitioner. A voucher specimen n (N° RAB 65077) was deposited in the Herbarium of Scientific Institute of Rabat in Morocco.

Extraction procedure: Seeds of *Delphinium Staphisagria* were extracted with 80% Ethanol by maceration at room temperature (25°C) over the period of 24 hours. 250 g of seeds material and one liter of 80% Ethanol were used in the extraction. Ethanol, containing the extract, was then filtered through Whatman paper and the solvent was vacuum distilled at 50°C in rotary evaporator. The ethanolic extract was then treated with 0.5 M H_2SO_4 and filtered. The acid solution was extracted with CH_2Cl_2 to give a crude material (6.44g). Acid aqueous phase was neutralized to pH 7 and extracted with CH_2Cl_2 to give a crude materiel (5.36g). Neutral aqueous phase was basified with 20% NaOH to pH 12 and extracted with CH_2Cl_2 to give a crude alkaloidic material (1.45g). Ethanolic and Alkaloid extracts were kept in deep freezer at –20°C until use [20, 21].

Median Lethal Dose Determination: LD_{50} values were determined as described by OECD 423 [18, 20, 23]. Median lethal dose (**LD_{50}**) of the alkaloid extract was determined using female Swiss mice (Animal Center of Mohammed V-Souissi University, Medicine and Pharmacy Faculty-Rabat), weighing 25 ± 2 g. All animals had free access to food and water; they were housed under standard environmental conditions on a 12/12h light/dark cycle. All experiments were conducted in accordance with the Official Journal of the European Committee in 1991. The experiment protocol was approved by the Institutional Research Committee regarding the care and use of animals for experimental procedure in 2010; CEE509. Ethanolic extracts was dissolved in distilled water and given by orally way in a single dose (300mg/kg, p.o.) of body weight. Mice were observed for clinical effects and mortality. The LD_{50} of extract was determined using a

modified up and down method known as Globally Harmonized System (GHS) [20]. Using this method for acute toxicity testing, animals are dosed one at a time. The test consists on a stepwise procedure with the use of three female mice per step. If the first animal survives, then the next animal receives a larger dose, while if the first animal dies the next animal receives a smaller dose. The dose for each successive animal is adjusted up or down depending upon the outcome for the previous animal. This causes the doses to be rapidly adjusted toward the LD_{50} and then be maintained in the region of the LD_{50}. This method is preferred because fewer animals are required; however, it can result in unbalanced numbers in each group [20, 22, 40].

Cytotoxic activity

Cell line and culture conditions: The cell lines tested in this study were Vero cell line, initiated from the kidney of a normal, adult African green Monkey and Neuro-2a (N2a) from a spontaneous tumor of a strain A albino mouse, this tumor line was obtained from the Jakckson Laboratory, Bar Harbor, Maine. Neuro-2a cells produce large quantities of microtubular protein which is believed to play a role in a contractile system which is responsible for axoplasmic flow in nerve cells. Cells were cultured in MEM media (Sigma, USA) supplemented with 10% heat-inactivated FBS 2% antibiotics and L-glutamine. The cells were grown at 37°C in a humidified incubator set at 5% CO2. Cells were subcultured after they formed a monolayer on the flask. The cells were detached by treating them with trypsin-EDTA for 10 minutes and then by adding complete medium to inhibit the reaction.

In Vitro Cytotoxicity Assay: Cytotoxicity of sample on tumor cells was measured by microculture tetrazolium (MTT) assay. For the assays, 96-well microplates were seeded with 100 µl medium containing 2.10^5 cells in suspension. After 24 h incubation and attachment, the cells were treated with dilutions of crude Ethanolic and alkaloidic extracts. Exactly from the stock solution (10 mg/ml), the extract sample was applied in a series of 6 dilutions (final concentrations ranging from 31.25 to 1000 µg/ml) with a final DMSO concentration of 0.1% and was tested in quadruplicate. After 48 h incubation, cell viability was determined by adding (Sigma) tetrazolium salt as cytotoxicity indicator and by reading absorbance at 590 nm with a scanning multiwell spectrophotometer (Spectra Count, Packard, Ont., Canada). Tetrazolium salts are cleaved to formazan dye by cellular enzymes (only in the viable cells). The level of absorbance directly correlates to the metabolically active cells [23, 24]. Mytomicin C (~ 95 % HPLC, sigma-Aldrich) was used as a positive control. All determinations were conducted in triplicate. The inhibition of cell growth was calculated by the following formula:

Percentage inhibition = (**OD** of treated cells **/ OD** of untreated cells) **x** 100

IC_{50} values were calculated as the sample concentration that caused 50% cell death.

Free Radical Scavenging Activity: The free radical scavenging activity of the ethanolic and alkaloidic extracts of *Delphinium Staphisagria* were measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) [Sanchez-Moreno C, 2002]. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of plant extract and was allowed to stand at room temperature for 30 min, and then absorbance was read at 517nm against blank samples. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration, using the equation: % RSA = [(ADPPH−AExtr) / ADPPH] × 100. Where ADPPH is the absorbance value of the DPPH blank sample, and AExtr is the absorbance value of the test solution. AExtr was evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank [25, 26].

Statistical analysis: The results are expressed as mean ± standard error of mean (S.E.M). Significant differences between control and experimental groups were assessed by Student's t test. . The IC50 values were obtained by nonlinear regression using the (Delta graph ….Canada article). Dose-response curves between cell viability percentages and extract concentrations were constructed. The cell viability (%) was calculated as follows:

Cell viability (%) = (Mean OD of treated cells/Mean OD of control cells) x100

RESULTS AND DISCUSSION

The results of *in vivo* acute toxicity during the 14 days show that the treated groups at the administered dose of 300 mg/kg body weight appear normal, presented a significant weight gain in mice group. Weight loss was significant ($P< 0.05$) in the 2000 mg/kg group; the reduction in body weight gain is a simple and sensitive index of toxicity after exposure to toxic substances [27, 28]. There was 100 % mortality recorded in the group treated by the extract at dose 2000 mg/kg body weight. Results showed that treatment with the extract at dose 2000 mg/kg induced some significant changes like skin effects, breathing, and impairment in food intake and water consumption, abdominal contraction, salivation and

hair loss. These results showed that, the ethanolic extract was evaluated as less toxic extract for seeds of *Delphinium staphisagria* (LD_{50}=300 mg/kg, p.o.). However, the alkaloid extract to be the most toxic extract with LD_{50} equate to 200 mg/kg. Under the system of global harmonization of chemicals (GHS), these products are classified category 3 and 4, respectively, due to the LD_{50} was lower than 2000mg/kg. The results from this acute toxicity study indicate that Delphinium staphisagria containing large amounts of MDL-type alkaloids, in addition to high MSAL-type alkaloid content, should be considered potentially **less** dangerous to human than plants with only high MSAL-type alkaloids [40, 41]. Therefore, even though the concentration of the MSAL-type alkaloids is the most important factor, these results suggest that the MDL-type alkaloids play an important role in the toxicity of larkspur plants by exacerbating the toxicity of the MSAL-type alkaloids [41].

The cytotoxicity studies were carried out on the crude extract against a panel of animal cancer cell lines with the MTT bioassay. Cells were treated for 48h by the alkaloidic extract at different concentrations ranging from 1000 to 31.25µg/ml. The results showed that the extract of *Delphinium Staphisagria* possesses significant an IC_{50} value of 31.25µg/ml compared to the positive drug control with an IC_{50} value of 30.40µg/ml on all cancer cell lines used. The lower IC_{50} represent the highest potency of a compound to inhibit the growth of cells and cause toxicity and death of cells [29]. The ethanolic extract doesn't have a cytotoxic effect against Vero and N2a cell lines; the IC_{50} of the extract is more or equal to 1 mg/ml. *Delphinium Staphisagria* is known for its anti-inflammatory, cathartic, emetic, narcotic, nerving, pediculicide and antispasmodic activities [30-32] and these have been attributed to components such as flavonoids, alkaloids (Isoazitine, 19-oxodihydroatisine, and 22-O-acetyl-19-oxodihydroatisine, Azitine, dihydroatisine, delphinine, Neoline, bullatine C (14-acetylneoline), Chasmanine, 14-acetylchasmanine, and the quaternary base atisinium chloride) [32, 33, 41]. Diterpenoid alkaloids, found in plant of the genera Delphinium, are of the C_{18}, C_{19} and C_{20} diterpenoides types have been the targets of considerable interest of medicinal chemists for a broad range of demonstrated pharmacological properties: arrhythmogenic (neurocardiotoxic), local anesthetic, antiarrhythmic, curariform, analgesic, hypotensive, anti-inflammatory, spasmolytic, neurotropic and psychotropic[33-36]. It is possible that this result is related to the tumor origin of animal cancer cell line used. Indeed, the NCI (National Cancer Institute) recommends testing new anticancer agent of 60 strains belonging to seven categories tissue: leukemia, melanoma, lung, colon, kidney, brain and ovary [37, 38]. Thus, we are tempted in perspective, to test the ethanolic and alkaloidic extract on other cell lines, to examine the specific effect on cancer of the different tissue. It is useful and necessary to carry out other investigations to better assess the cytotoxic effect of the extracts of *Delphinium Staphisagria*.

The radical scavenging activity of the extract of *Delphinium Staphisagria* was estimated by comparing the percentage inhibition of formation of DPPH radicals by the alkaloidic and ethanolic extract and those of Trolox. The DPPH scavenging activity of both extracts was concentration-dependent (increasing from 15.62 µg/ml to 500 µg/ml), exhibited considerably ($P<0.05$) DPPH radical-scavenging activity and was able to inhibit the formation of DPPH radicals with a percentage inhibition of 62.19 and 94%, respectively, at the highest concentration (Fig. 1 and 2). In general, the ethanolic extracts showed greater antioxidant activity than alkaloidic extract (Fig. 1 and 2). It can be noted that extract from *Delphinium Staphisagria* show average inhibitory values that give an idea of the interesting antioxidant activity of the extracts of this species and that it may be useful therapeutic agents for treating radical-related pathological damage. Some Further more researchers reported that there is a relationship between the chemical structures of the most abundant compounds in the plants and their above mentioned functional properties [38]. From these results it can be concluded that the major components of *Delphinium Staphisagria* extract (Alkaloides, Flavonoides, Glycosides Anthraniliques in ethanolic extract and Delphinine, 14-acetylneoline, Chasmanine, 14-acetylchasmanine Neoline in alkaloidic extract) could have key roles for their functional properties [39-41]. The result obtained in this work demonstrated a high activity at low alkaloids extracts doses (500µg/ml).

Alkaloids are commonly found to have antioxidant and cytotoxic activities they are detected in the ethanolic and alkaloidic seeds extracts. It is also possible that the minor compounds might be involved in some type of synergism with the other active compounds [40, 41]. Several studies show a very strong correlation between antioxidant activity and alkaloidic compounds. Since alkaloidic compounds are effective hydrogen donors, they are good antioxidants. Alkaloidic compounds have repeatedly been implicated as natural antioxidants in fruits, vegetables, and other plants. Thus, therapeutic

properties of the *Delphinium Staphisagria* can be attributed to the alkaloidic compounds present [40].

CONCLUSION

In conclusion, the results of the present study suggest that *Delphinium Staphisagria* seeds plant have various secondary metabolites and has good quantity of alkaloidic compounds (diterpenoid alkaloids). Plant has potent free radical scavenging activity. Detailed studies on chemical composition, isolation of active constituents and pharmacological evaluation are essential to characterize them as biological antioxidants. The present findings of this study support the view that *Delphinium Staphisagria* seeds are a promising source of potential antioxidant which can be used in treatment of various ailments.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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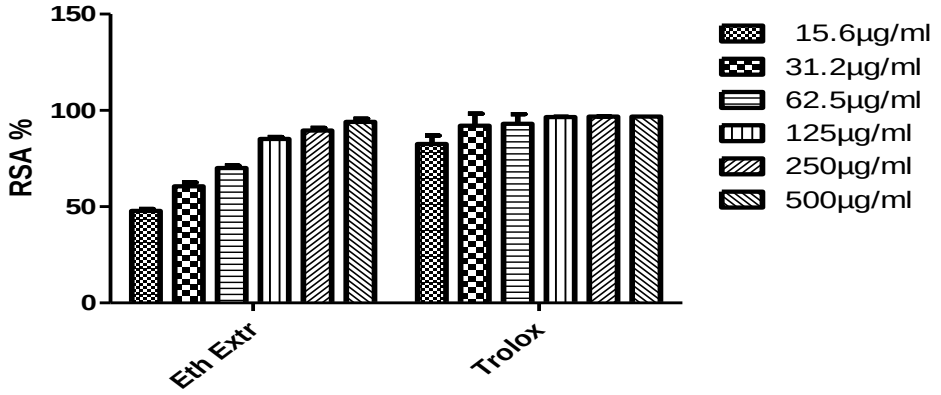

Fig. 1: Free-radical scavenging activity of *Delphinium Staphisagria* ethanolic extract measured using the DPPH assay.

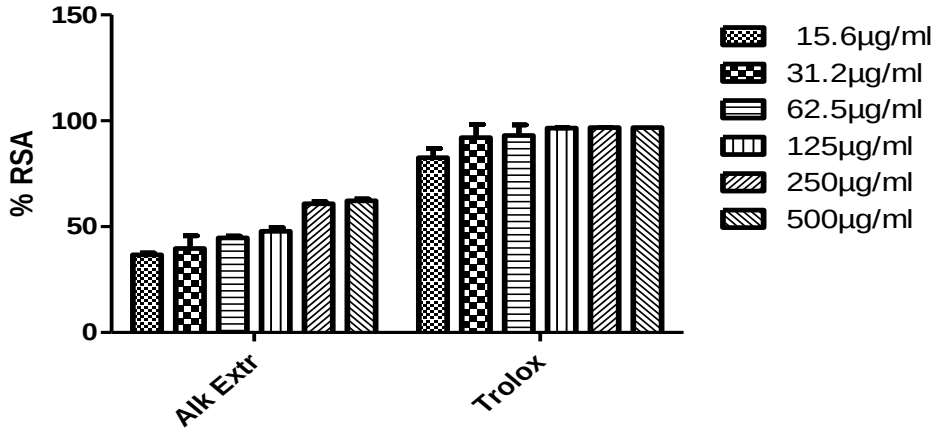

Fig. 2: Free-radical scavenging activity of *Delphinium Staphisagria* alkaloidic extract measured using the DPPH assay.

REFERENCES

1. Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, and Atangbayila TO. Trop J Pharma Res, 2008; 7(3): 1019-1021.
2. Malikarjunn PB, Rajanna LN, Seetharam YN and Sharanabasappa GK. E-J Chem, 2007; 4(4):510-518.
3. Effat S, Aminb G, Farsama H, Jalalizadeha H, Barezia S. Iranian J Pharm Res, 2008, 7 (2): 149-154.
4. Oke JM and Hamburger MO. African J Biom Res, 2002; 5:77-79.
5. Odakoya OA, Sofidiya MO, Familoni OB and Inya-Agha SI. Pakistan J Biol Sci, 2006; 9(8):1438-1441.
6. Rice-Evan CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. Free Radical Res, 1995; 22: 375-383.
7. Huda-Faujan N, Noriham A, Norrakiah A, Babji AS. African J Biotechnol, 2009; 8 (3): 484-489.
8. Alnamer R, Alaoui K, Bouidida El H, Benjouad A and Cherrah Y. J Biol Act Prod Nat, 2012; 1(4): 262 – 272.
9. Alnamer R, Alaoui K, Bouidida El H, Benjouad A and Cherrah Y. J Nat Prod, 2012; 5: 83-92.
10. Alnamer R, Alaoui K, Bouidida El H, Benjouad A and Cherrah Y. J Med Plants Res, 2012; 6(10): 1860-1865.
11. Alnamer R, Alaoui K, Bouidida El H, Benjouad A and Cherrah Y. Adv Pharmacol Sci, 2012; 2012: 1-5.
12. Alnamer R, Alaoui K, Doudach L, Al-Sobarry M, Bouidida El H, Fatiha Chibani, Benjouad A and Cherrah Y. Int J Pharm, 2013; 3(1): 1-6.
13. Alnamer R, Alaoui K, Doudach L, Al-Sobarry M, Bouidida El H, Fatiha Chibani, Benjouad A and Cherrah Y. World J Pharm Res, 2012; (1)5: 1223-1233.
14. Alnamer R, Alaoui K, Bouidida El H, Alwashli A, Benjouad A and Cherrah Y. Int J Universal Pharm Bio Sci, 2013; 2(5): 206-214.
15. Tapsell LC., Hemphill I, Cobiac L, Patch CS, Sullivan DR., Fenech M, Enrys S, Keogh JB, Clifton PM, Williams PG, Fazio VA, Inge KE. Med J Aust, 2006; 185: 4–24.
16. Anowi CF, Utoh-Nedosa UA, Onyegbule AF and Oche G. Int J Pharm, 2012; 2(2): 287-293.
17. El-Hilaly J, Hmammouchi M, and Lyoussi B. J Ethnopharmacol, 2003; 86(2-3): 149–158.
18. Doudach L, Meddah B, Alnamer R, Chibani F, Cherrah Y. Int J Pharm Pharm Sci, 2012; 3(4): 402-405.
19. Huxley A. 1992. Mac Millan Press.
20. Faridi B, Alaoui K, Alnamer R, Cherrah Y and Zellou A. Analgesic activity of ethanolic and alkaloidic extracts of delphinium staphisagria seeds. Int J Uni Pharm Bio Sci, 2013; 2(5): 102-112.
21. Zellou A, Keita A, Cherrah Y, Essassi EM, Hassar M. Thér, 1999; 54: 645-649.
22. Merghoub N, Benbacer L, Amzazi S, Morjaniand H El mzibri M. J Med Plants Res, 2009; 3(12): 1045-1050.
23. Doudach L, Benbacer L, Meddah B, Mzibri M, Faouzi Mya, Hammani K, Elomri A, and Yahia Cherrah. Int J Pharm, 2013; 3(2): 301-307.
24. Sanchez-Moreno C. Food Sci Technol Int, 2002; 8:121–137.
25. Amensour M, Sendra E, Pérez-Alvarez JA, Senhaji NS, Abrini J, López JF. Plant Foods Hum Nutr, 2010; 65:170-178.
26. Raza M, Al-Shabanath OA, El-Hadiyah TM, Al-Majed AA. Scientia Pharm, 2002; 70: 135-145.
27. Teo S, Strlig D, Thomas S, Hoberman A, Kiorpes A, Khetani V. Toxicol, 2002; 79: 183-196.
28. Shokrzadeh M, Saeedi Saravi SS, Mirzayi M. Pharmaco Mag, 2009; 5(20): 316-319.
29. James A Duke and Maryl Fulton. 2nd Edition, P: 697, CRC Press July 2002.
30. Wang JL, Shen XL, Chen QH, Gong QI, Wang W and Wang FP. Chem Pharm Bull, 2009; 57: 801-807.
31. Pelletier SW, Takkar JK, Mody NV, Djarmat IZ and Bhattacharya YA. Phytochem, 1977; 16: 404-405.
32. Rabanal RM, Bonkanka CX, Hernandez-Pérez M and Sanchez-Mateo CC. J Ethnopharmacol, 2005; 96: 591-596.
33. Koster R, Anderson M and Debeer EJ. Fed Proc, 1959; 18: 418-420.
34. Diaz J, Armando J, Carmona PP and Werner H. Phytochem, 2008; 2: 125-129.
35. Diaz J, Marapara JL, Garvin SJ and Werner H. Phytochem, 2005; 66: 733-739.
36. Cragg GM, Boyd MR, Cardellina JH, Newman D, Snader KM and McCloud TG. Vol. 185. Wiley and Sons, Chichester, UK, pp. 178-196, 1994.
37. Farag RS, Daw ZY, Hewedi FM, El-Baroty GSA. J Food Prot, 1989; 52: 665-667.
38. Pelletier SW, Mody NV, Joshi B and Schramm LC. Ed: Willey-Interscience: New York, 1984; 2: 205.
39. Gould GW. J Food Prot, 1996; 59:82-86.
40. Marinova D, Ribarova F and Atanassova M. J Chem Tech Metallurgy, 2005; 40 (3): 255-260.
41. Welch KD, Green BT, Gardner DR, Cook D, Pfister JA and Panter KE. J ANIM SCI, 2012; 90: 2394-2401.