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Research Article

PHYTOCHEMICAL AND BIOLOGICAL EVALUATION OF *LIATRIS SPICATA* (L.) CORMS

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ABSTRACT

Objective: The following studies investigate the possibility of the use of the corms of *Liatris spicata* (L.) Willd (Asteraceae) cultivated in Egypt as a new herbal drug; especially that corms are, on economical scale, considered as waste products.

Methods: The proximate analysis as well as analysis of amino acids and lipid content was performed. The total phenolic content was carried out using the Folin-Ciocalteu colourimetric method. LD_{50} was determined. The *in-vivo* anti-inflammatory activity was investigated adopting the carrageenan-induced rat paw oedema. The *in-vivo* antioxidant activity was assessed by measuring the ability of the extract to restore glutathione levels in the blood of alloxan induced diabetic rats. The diuretic activity was also assessed.

Results: The proximate analysis revealed the high nutritive value of the corms: carbohydrates (15.2 %) and lipids (0.84 %). The percentage of total amino acids was (16.54 %) with leucine and tyrosine (1.27 and 1.19 %, respectively) being the major essential amino acids. The corms also contain high percentage of phosphorus (405 mg/100 g), stigmasterol (34.56 %) and unsaturated fatty acids represented by docosadienoic acid (40.02 %). The ethanolic extract of the corms was found to be safe up to 5 g/kg body weight the ethanolic extract exhibited diuretic, anti-inflammatory and antioxidant activities and this was in accordance with its high phenolic content (174.2 ± 0.029 GAE/g extract).

Conclusion: The results obtained in this study indicate the high nutritional value of the corms of *Liatris spicata* (L.) Willd. The ethanolic extract of the corms exhibited diuretic, acute anti-inflammatory and antioxidant activity. This adds to the importance of the corms as it can't be considered a waste product anymore.

Keywords: *Liatris*; Proximate analysis; Total carbohydrates; Minerals; Amino acids; Lipid and Phenolic content; Diuretic; Anti-inflammatory; Antioxidant.

INTRODUCTION

Liatris spicata (L.) Willd is a plant belonging to family Asteraceae and widely cultivated in Egypt. The corms were imported from Holland and cultivated to use the inflorescence for ornamental purposes leaving the corms as waste material. This prompted the performance of the following study on the corms of the plant cultivated in Egypt aiming to investigate the possibility of their use as a source of new herbal remedy. Although, it was reported that the plant was used as anodyne, antibacterial, astringent, carminative, diaphoretic, diuretic, emmenagogue, expectorant, stimulant and tonic [1-5].

For herbal drugs standardization is concerned, WHO also emphasize on the need and importance of determining proximate and micronutrients composition of the herbal plants. Such herbal formulations must pass through standardization processes [6]. It was, thus, necessary to study the nutritional value and certain proximate, as well as, the lipid and phenolic content. Also, to study its biological activity such as: diuretic, anti-inflammatory and antioxidant.

MATERIAL AND METHODS

Plant materials

The corms of *Liatris spicata* (L.) Willd were collected in January 2010. The plant was authenticated by Dr. M. Gibali (Senior Botanist). Voucher specimens were deposited at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt (30-5-2013-2).

Chemicals and instruments

All of the chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (USA) and/or Merck Company (Germany). HCl Suprapure® was obtained from Merck. Gallic acid and Folin-Ciocalteau were purchased from Sigma-Aldrich. Indomethacin (Epico, Egyptian Int. Pharmaceutical Industries Co. ARE under license of Merck & Co INC-RAHAWAY, N J.USA), glutathione kit (Wak-Chemie Medical, Germany), vitamin E (Pharco Pharmaceutical Co., Egypt), carrageenan and alloxan (Sigma Co., USA). Shimadzu UV-1650 PC Spectrophotometer (Faculty of Pharmacy, University of Cairo, Cairo, Egypt) was used for absorbance measurements. Perkin-Elmr spectrophotometer for determination of total carbohydrates. Total lipids were estimated using: Soxtherm; Gerhardt laboratory instrument. Eppendrof – Germany. LC3000 Amino acid analyzer.

Proximate analysis

The moisture, ash, acid insoluble ash, water soluble ash and crude fiber were determined in the corms according to the Egyptian Pharmacopoeia [7].

Determination of the total carbohydrates

It was carried out according to phenol-sulfuric acid method described by Dubois [8]. This method is based on complete hydrolysis of the plant material using 1M H_2SO_4 in sealed tube, boiling in water bath for 10 hours, neutralization by barium carbonate, then filtration. The clear solution was made up to known volume; followed by the addition of phenol (5%) and H_2SO_4 (96%) to the known volume of the hydrolysate. The optical density of the obtained yellow orange colour was measured at λ_{max} 490 nm using Perkin-Elmer spectrophotometer against blank.

Determination of the minerals

The concentrations of potassium (K), sodium (Na), calcium (Ca) and iron (Fe) in the corms were determined by using Inductively Coupled Plasma (ICP-AES), Thermo Sci, model: iCAP6000 series after digestion by Advanced Microwave Digestion System Atomic Emission Spectrometry ETHOS 1. Argon plasma was used for excitation of the element atom. The blank values for each element were deduced from the sample values. Phosphorus was determined by spectrophotometric method, using commercial kit according to El- Merzabani [9]. Results are presented in (Table 1).

Analysis of amino acids by HPLC

The total protein was extracted from the corms using 50mM Tris - HCl PH (7.5) and then acid hydrolysis of the protein was carried out

[10]. One ml 6N HCl was mixed with one mg protein in a hydrolysis tube. The solution was frozen in a mixture of dry ice/ethanol in a test tube. The tube was evacuated with a vacuum pump and sealed using gas-burner. The sealed tube was placed in an oven at 110 °C for 72 hours for hydrolysis, and then cooled down in an ice-bath. The solution was centrifuged to precipitate insoluble components. The supernatant was evaporated at 40°C in a rotary evaporator. The residue was then dissolved in a diluting buffer. Flow rate: 0.2 ml/min, pressure of buffer from 0 to 50 bar, pressure of reagent from 0 to 150 bar, reaction temperature 123 °C. results are shown in (Table 2)

Investigation of lipid contents

Powdered air-dried corms of *Liatris spicata* (L.) willd (100g) was extracted with petroleum ether (40-60°C) till exhaustion using Soxhlet apparatus. Petroleum ether extract was evaporated to dryness under vacuum to give a residue of (0.84g). The saponifiable (91.83%) and unsaponifiable fractions (7.75%) were obtained from the petroleum ether fraction and the separated fatty acids were methylated by adopting the method described by Finar (1973) [11] and Vogel (1967) [12]. GLC analysis of un-saponifiable matter (2 µl) and standard material were carried out using Varian 3700 GC with column 200cm glass, 4% OV_{101}+OV_{210} on 80/100 mesh and chromosorb W.H.P; the injector and FID detector temperatures were 150°C and 250°C respectively; carrier gas was nitrogen; flow rate 20ml/min. The program beginning with 100°C for 2 min then increased to 200°C with rate of 10°C/min and isothermally for 25 min. Identification of constituents was carried out by comparison of their relative retention times with the available reference compounds. Quantification was based on peak area integration and internal normalization method. Results are shown in (Table 3). GLC analysis of fatty acid methyl esters (FAME) (2 µl) and standard material were carried out using Varian 3700 with the following condition: Column packed with 3% OV_{17} coated on 80/100 mesh and chromosorb W.H.P; nitrogen was used as the carrier gas at flow rate of 20 ml/min; injector temperature 250°C; detector temperature 320°C with FID. Program beginning with 160°C for 2 min then increased to 300°C with rate of I5°C /min and isothermally for 15 min. Identification of constituents was carried out by comparison of their relative retention times with the available reference compounds. Quantification was based on peak area integration and internal normalization method. Results are shown in (Table 4).

Determination of the total phenolic content

The dried corms (1 kg) were extracted with ethanol 70% by cold maceration till exhaustion. The solvent was then removed by vacuum distillation and the residue was kept in dark at 4 °C. Spectrophotometric determination of the total phenolic content was carried out according to the procedure reported in the European Pharmacopoeia [13], using the Folin-Ciocalteu colourimetric method. Total phenols were expressed as mg of gallic acid equivalents (mg GAE)/g of the dry extract. For the preparation of the calibration curve, 0.8 ml of gallic acid in distilled water (at the concentrations 5, 10, 15, 20, 25, and 50 µg/ml) was thoroughly mixed with 0.4 ml Folin-Ciocalteu reagent and 4 ml distilled water, then diluted to 10 ml with 290 g/L sodium carbonate solution. The absorbance of the resulting solution (blue) was measured after 30 min. at 760 nm.

For each concentration, three replicates were carried out and the average of the obtained absorbance was plotted versus the concentration. The results were recorded in (Table 5) and represented in (Figure 1). The same procedure was carried out with 0.8 ml of the sample (at concentration 1 mg/ml) instead of gallic acid. The phenolic content calculated as gallic acid was deduced from the pre-established standard calibration curve (Figure 1) and calculated according to the following equation:

$$\text{Phenolic content } \left(\frac{\text{mg GAE}}{\text{g extract}}\right) = \frac{\text{Conc.}}{1000} \times \frac{10}{0.8} \times \frac{1000}{x}$$

Conc. = Concentration from equation of the standard calibration curve.

x = Concentration of the extract (mg/ml).

GAE= Gallic acid

Biological activity

Animals

Animals were obtained from the animal house colony, supplied by central services of the Laboratory National Research Center, Giza, Egypt and kept on standard laboratory diet under hygienic conditions. This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy, Cairo University, Cairo, Egypt, following the World Medical Association Declaration of Helsinki (WMA General Assembly, 1964).

Determination of LD_{50}:

LD_{50} of the ethanol extract of the corms of *Liatris spicata* (L.) Willd was performed by oral treatment of male albino mice (25-30 g) adopting Karber's procedure [14]. Preliminary experiments were carried out to determine the minimal dose that kills all animals (LD_{100}) and the maximal dose that fails to kill any animal. Several doses at equal logarithmic intervals were selected in between these two doses; each dose was injected in a group of six animals by subcutaneous injection. The mice were observed for 24 hours and symptoms of toxicity and mortality rates in each group were recorded and calculated.

Preparation of the extract

The extract was freshly suspended in sterile distilled water with few drops of Tween 80 to a final concentration of 200 mg/ml.

***In vivo* diuretic activity**

A total of 18 albino rats (males and non-pregnant females) of body weight 130 to 150 g were used. Rats were allocated randomly into 3 equal groups. Each rat of each group was placed into a separate metabolic cage, with wire mesh floor provided with a conical shaped bottom underneath designed to collect urine in a receptacle without fecal contamination. The test animal was fasted overnight (12-14 hr) but they had free access to fresh water. The first group was kept as normal control while rats of the second group were given an oral dose of 5 mg/kg body weight of Moduretic® (Amiloride and Hydrochlorothiazide), the standard drug serving as positive control, and the other group received orally 100 mg/kg body weight of the extract [15]. The urine output during 2, 4 and 24 hours were collected and measured in graduated cylinder. Results are shown in (Table 6).

***In vivo* anti-inflammatory activity**

The anti-inflammatory activity of the extract was determined, *in-vivo*, by adopting the carrageenan-induced oedema in the hind paws of rats [16]. 18 male albino rats, weighing 130-150 g, were divided into 3 groups (each of 6), and orally treated one hour before induction of oedema. Group 1 receiving saline and served as negative control. Groups 2, were administered the total ethanol extract, at a dose of 100 mg/kg b.wt., Group 3 received Indomethacin, as standard anti-inflammatory drug (20 mg/kg b.wt). Induction of oedema was performed by sub-planter injection of 0.1 ml of 1% Carrageenan [17], in saline into the pad of experimental animal right paw and 0.1 ml saline in its left hind paw. Four hours after drugs administration, the rats were sacrificed. Both hind paws were excised and weighed separately; the difference in weight between both represents the weight of the oedema. Results are shown in (Table 7).

***In vivo* antioxidant activity**

Antioxidant activity was assessed by measuring the ability of the extract to restore glutathione levels in the blood of alloxan induced diabetic rats after the oral administration of 100 mg/kg body weight using Beutler method [18]. Induction of diabetes mellitus was carried out according to the method described by Eliasson and Samet [19]. Vitamin E was used as a standard (7.5 mg/kg b.wt., positive control). Results are shown in (Table 8).

RESULTS AND DISCUSSION

The moisture, crud fibers, ash, acid insoluble ash and water soluble ash were determined in the corms of *Liatris spicata* (L.) Willd. Their percentages were 9.34, 8.2, 10.5, 4.02 and 3.2% respectively as shown in (Table 1). The percentages of lipid and carbohydrate were 0.84 and 15.2%. Analysis of the minerals of the corms of *Liatris spicata* (L.) Willd revealed the presence of phosphorus in high percentages (405 mg/100g). The percentages of potassium, sodium, calcium and iron were 1.94, 0.59, 0.44 and 0.48 mg/100g, respectively. Detection of these elements in the corms adds to its medicinal and dietary importance.

Phosphorus is an essential mineral that is required by every cell in the body for normal function. The majority of the phosphorus in the body is found as phosphate (PO_4). Approximately 85% of the body's phosphorus is found in bone in the form of calcium phosphate salt called hydroxyapatit. Phospholipids (e.g., phosphatidylcholine) are major structural components of cell membranes. All energy production and storage are dependent on phosphorylated compounds. Phosphorus also helps to maintain normal acid-base balance (pH). The effects of hypophosphatemia may include loss of appetite, anemia, muscle weakness, bone pain, rickets (in children), osteomalacia (in adults), increased susceptibility to infection, numbness and tingling of the extremities, and difficulty walking [20]. The total percentage of the amino acids is 16.54 g/100 g corms (Table 2). The main essential amino acid was leucine (1.27 g/100 g corms) followed by tyrosine (1.19 g/100 g corms) and phenylalanine (1.04 g/100 g corms). The lowest percentage of the essential amino acids was isoleucine (0.39 g/100 g corms) followed by threonine, lysine and valine (0.47 g, 0.5 g and o.51 g/100 g corms, respectively).

Table 1: Determination of some nutrients and minerals in *Liatris spicata* (L.) Willd corms

Minerals	**(mg/ 100g)** *	**RDA for adult (mg/ day)[20]**
Potassium	1.94	2000
Sodium	0.59	No more than 2300
Calcium	0.44	800-1200
Iron	0.48	10-15
Phosphorus	405	700
Nutrient	**g%***	
Total Lipid	0.84	
Total Carbohydrate	15.2	
Total ash	10.5	
Acid insoluble ash	4.02	
Water soluble ash	3.2	
Crude fibers	8.2	
Moisture content	9.34	

* Average of three determinations

Proline (4.07 g/100 g corms) was the main non-essential amino acid followed by arginine and glutamic acid (1.95 g/100 g corms and 1.45 g/100 g corms, respectively). The lowest percentage of the nonessential amino acids was both histidine and serine (0.28 g/100 g corms and 0.45 g/100 g corms); followed by glycine, aspartic acid and alanine (0.47 g, 1.17 g and 1.33 g/100 g corms, respectively). According to Al-Zohairy (1992) [21] 100g of of *Liatris spicata* (L.) Willd corms contained the recommended daily dietary allowance and afford the daily dietary requirements of the essential amino acids for infant, child and adult like wise.

Table 2: Percentage of amino acids in *Liatris spicata* (L.) Willd corms

Amino acids		**g/100gm**	**Daily dietary allowance of the amino acids (g /Kg of body weight) [21]**		
			Infant	**Child (10 to 12 years)**	**Adult**
Essential amino acids	Isoleucine	0.39	0.083	0.028	0.012
	Leucine	1.27	0.135	0.042	0.016
	Lysine	0.50	0.099	0.044	0.012
	phenyl alanine	1.04	0.141*	0.022*	0.016*
	Threonine	0.47	0.068	0.028	0.008
	Tyrosine	1.19	0.021	0.004	0.003
	Valine	0.51	0.092	0.025	0.014
Non-essential amino acids	Arginine	1.95			
	Proline	4.07			
	Serine	0.45			
	glutamic acid	1.45			
	Glycine	0.47			
	Alanine	1.33			
	Aspartic	1.17			
	Histidine	0.28			
Total determined amino acids		16.54			
Ammonium ion		2.32			

***Phenyl alanine+tyrosine

Results of GLC analysis of the unsaponifiable matter is presented in (Table 3). Total sterols represent (47.81 %), the major of which is stigmasterol (34.56 %). The saponifiable constituents identified by GLC technique (Table 4), showed the presence of 10 components representing 89.21%. Unsaturated fatty acids represented 49.42% of the total fatty acid. The major component is docosadienoic acid (40.02%).

Table 3: GLC analysis of the un-saponifiable matter of *Liatris spicata* (L.) Willd corms

Identified components	**RR_t***	**Relative area Percentage**
n-Pentadecane	0.38	7.29
n-Hexadecane	0.43	10.87
n-Heptadecane	0.47	9.55
n-Octadecane	0.49	18.54
Stigmasterol	1	34.56
β- Sitosterol	1.05	13.25
% of identified hydrocarbons		46.25
% of identified sterols		47.81

***RR_t; Relative retention time to stigmasterol with R_t =34.64 min.

Table 4: GLC Analysis of Fatty Acid Methyl Esters of *Liatris spicata* (L.) Willd corms

Identified components	RR_t*	Relative area Percentage
Methyl Caprate	0.29	1.32
Methyl Laurate	0.35	7.32
Methyl Myristate	0.44	3.43
Methyl Palmitate	0.55	0.88
Methyl Palmitoleate	0.57	3.05
Methyl Oleate	0.67	5.72
Methyl Behenate	0.86	1.67
Methyl Erucate	0.88	0.63
Methyl Docosadienoate	1	40.02
Methyl Lignocerate	1.02	25.17
% of saturated fatty acids		39.79
% of unsaturated fatty acids		49.42

* RR_t; Relative retention time to docosadienoic acid with R_t = 29.54 min.

The absorbance of the ethanolic extracts of the corms of *Liatris spicata* (L.) Willd was 0.272 ± 0.001 corresponding to the phenolic content which was 174.2 ± 0.029 mg GAE/g extract.

Table 5: Absorbances of the different concentrations of standard gallic acid

Concentration (μg/ml)	Absorbance*
5	0.102
10	0.196
15	0.285
20	0.379
25	0.504
50	0.982

*Average of three determinations.

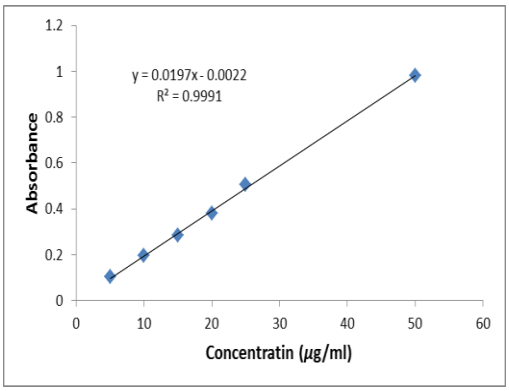

Fig. 1: Calibration curve for standard gallic acid.

The alcoholic extract of the corms of *Liatris spicata* (L.) Willd was safe up to 5g/kg b. wt. The data illustrated in (Table 6) showed that; the tested extract of the corms of *Liatris spicata* (L.) Willd have significant diuretic effect compared to reference drug (Moduretic®). The alcoholic extract (100mg/ kg b. wt.) increased the volume of urine in male rats after two, four and twenty four hours the results were 2.3, 5.4 and 12.9 ml respectively. These results agree with what were reported on the plant [4]. The alcoholic extract exhibited acute anti-inflammatory activity at the tested doses represented by a significant decrease in the weight of the oedema comparing its activity to that Indomethacin (Table 7). This could be attributed to the presence of high percentage of sterol compounds (*β*-sitosterol and stigmasterol). These data go in harmony with that reported on the *β*-sitosterol and stigmasterol [22, 23].

It is well known that there is a strong relationship between total phenol content and the antioxidant activity, as phenols possess strong scavenging ability for free radicals due to their hydroxyl groups. Therefore, the phenolic content of plant may directly contribute to their antioxidant action [24-26]. The alcoholic extracts of the corms of *Liatris spicata* (L.) Willd exhibited pronounced antioxidant activity at a concentration of 100 mg/kg.b.wt. Polyphenolic compounds are also believed to have chemopreventive and suppressive activities against cancer cells by the inhibition of the metabolic enzymes involved in the activation of potential carcinogens or arresting the cell cycle [27]. Nevertheless, a compound with strong antioxidant potential can also contribute to DNA protection and prevent apoptosis [28]. Further studies are therefore required to detect potential anticancer activities of the extract reported her.

Table 6: Effect of the ethanol extracts of corms of *Liatris spicata* (L.) Willd on urine volume

Group (n=6)	Dose mg/kg b.wt.	Volume of urine (in ml)		
		2hrs	4hrs	24hrs
Control	1ml saline	0.8±0.01	2.1±0.1	6.8±0.2
Moduretic®	5	3.9±0.2*	7.1±0.3*	16.7±0.6*
The ethanolic extract of *Liatris spicata*	100	2.3±0.1	5.4±0.2	12.9±0.4*

Values are means of 6 observations

* Statistically significant from the control at p<0.1

Table 7: Acute anti-inflammatory activity of the ethanol extract of the corms of *Liatris spicata* (L.) Willd

Group (n=6)	Dose mg/kg b.wt.	% Oedema		Potency[1]
		Mean±S.E.	% of Change	
Control	1ml saline	59.4±1.6	-	-
The ethanolic extract of *Liatris spicata*	100	27.3±0.6*	54	0,87
Indomethacin	20	22.6±0.4*	62	1

[1]Potency calculated as compared to the standard anti-inflammatory drug Indomethacin

* P < 0.01 Vs. control group

% of change calculated as regard the control group.

Table 8: Antioxidant activity of the ethanol extract of the corms of *Liatris spicata* (L.) Willd and vitamin E drug in male albino rats (n=6)

Group	Blood glutathione (mg %)	% change from control
Control (1ml saline)	36.3±1.4	-
Daibetic	21.4±0.5*	41
Diabetic + Vitamin E (7.5mg/kg.b.wt.)	35.9±1.2	1.1
Diabetic + The ethanolic extract of *Liatris spicata* (100mg/kg.b.wt.)	35.1±0.7	3.3

* Statistically significant different from control group at $p < 0.01$

RERERENCES

1. Grive. A Modern Herbal. Penguin; 1984.
2. John L. The Herb Book. Bantam; 1983.
3. Coon N. The Dictionary of Useful Plants. Rodale Press; 1975.
4. Bown D. Encyclopaedia of Herbs and their Uses. Dorling Kindersley, London; 1995.
5. Moerman D. Native American Ethnobotany. Timber Press. Oregon; 1998.
6. Niranjan RM, Kanaki S. Bioactive Molecules and Medicinal Plants. 2008; 349-369.
7. Egyptian Pharmacopoeia. 4th ed, central Administration of Pharmaceutical Affairs (CAPA), Ministry of Health and Population, Cairo, Egypt, 2005
8. Duboies M, Smith F, Gilles KA, Hamilton JK, Rebers PA. Colortimetric method for determination of sugar and related substances. Anal. Chem. 1956; 28(3): 350.
9. El- Merzabani MM, El- Aaser AA, Zakhary NI. Determination of inorganic Phosphorus in serum. J. Clin. Chem. Clin. Biochem. 1977; 15: 715-718
10. Spackman DH, Stein WH, Moore S. Automatic recording apparatus for use in chromatography of amino acid. Annal.Chem. 1958; 30:1190 – 1206.
11. Finar IL. Organic Chemistry. 6th Ed., Longman group Ltd., England. 1973; 445.
12. Vogel AI. A Text Book of Practical Organic Chemistry. Longmans and Green Co. Ltd., London. 1967.
13. Druckerei CH. European Pharmacopoeia. 4th edition, Beck, Nordlingen, Germany. 2002; 187.
14. Karber G. Determination of LD-50. Arch. Exp. Pathol. Pharmacol. 1931; 162-480.
15. Goldstein S, Brown MS. Methods for urine analysis. 5th ed. M. C. Grawihill Book, Co., New York. 1964.
16. Winter CA, Risley EA, Nuss GW. Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs. Proc Soc Exp Biol Med. 1962; 111: 544-547.
17. Penn GB, Ashford A. The inflammatory response to implantation of cotton pellets in the rat. J Pharm Pharmacol. 1963; 15: 798-803.
18. Beutler E, Duron O, Kelly B. Improved method for the determination of blood glutathione. J. Lab. Clin. Med. 1963; 61: 882-888.
19. Elliason SG, Samet TM: Alloxan induced neuropathies, lipid changes in nerve and root fragments. Life Sciences. 1969; 8(1): 493-498.
20. Food and Nutrition Board. Institute of Medicine: Dietary Reference Intakes - for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. National Academy Press, Washington, DC., 1997; 146-189.
21. Al - Zohairy AD:Human: nutrition. Mosul University, Iraq, 2 nd Ed., 1992; 116 - 118.
22. Gabayya O, Sanchezyzb C, Salvaty C, Chevyx F, Bretonx M, Nourissatyk G, *et al*. Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. Osteoarthritis and Cartilage. 2010; 18: 106-116.
23. Loizou S, Lekakis I, Chrousos GP, Moutsatsou P. Beta-sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells. Mol Nutr Food Res. 2010; 54 (4): 551-8.
24. Wojdylo A, Oszmianski J. Czemerys R. Antioxidant Activity and Phenolic Compounds in 32 Selected Herbs. Food Chemistry. 2007; 105 (3): 940- 949.
25. Bendini A, Cerretani L, Pizzolante L, Gallina-Toschi T, Guzzo F, Ceoldo S, *et al.* Phenol Content Related to Antioxidant and Antimicrobial Activity of *Passiflora* Spp. Extracts. European Food Research and Technology. 2006; 223(1): 102-109.
26. Dlugosz A, Lembas-Bogaczyk L, Lamer-Zarawska E. Antoxid Increases Ferric Reducing Antioxidant Power (FRAP) even Stronger than Vitamin C. Acta Poloniae Pharmaceutica. 2006; 63: 446-448.
27. Newman DJ, Gragg GM, Holbeck S, Saus-ville EA. Natural Products as Leads to Cell Cycle Pathway Targets in Cancer Chemotherapy. Current Cancer Drug Targets. 2002; 2 (4): 279-308.
28. Rajkumar V, Guha H, Kumar RA. Antioxidant and Anticancer Potentials of *Rheum emodi* Rhizome Extracts. Food and Chemical Toxicology. 2011; 49(2): 363-369.