Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(4); 645-650

ISSN: 0975-4873

Research Article

# Human Xanthine Oxidase Inhibitory Effect, Antioxidant *In Vivo* of Algerian Extracts (*Globularia alypum* L.)

Boussoualim N*, Krache I, Baghiani A, Trabsa H, Aouachria S, Arrar L

*Laboratory of Applied Biochemistry, Department of Biology, Faculty of Sciences, University Ferhat Abbas of Setif 1, 19000, Algeria.*

*Available Online: 31st March, 2016*

**ABSTRACT**

Objective The aim of this study consisted in evaluating the antioxidant *in vivo* properties, anti-hemolytic and XOR inhibitory effect of *Globularia alypum* L. (GA) extracts. Methods: GA was submitted to extraction and fractionation to give crude (CrE), chloroformique (ChE), ethyle acetate (EAE) and aqueos (AqE) extracts. Total polyphenols contents of GA extracts were determined. The inhibitory activity of the extracts on the human oxidoreductase was evaluated. antioxidant activities of the CrE, EAE and AqE were tested by an *in vivo* assay in mice, the plasma ability to inhibit DPPH radical was measured. Results: EAE is the most rich in polyphenols (157.74 ± 5.27 mg GAE/mg of extract). GA Extracts inhibited XO in a concentration-dependent manner, the EAE showed the highest inhibitory properties on the XOR activity ($IC_{50}$ = 0.083 ± 0.001 mg/ml), followed by CrE and ChE. The The CrE was found to exhibit the greatest scavenger activity with 48.41 ± 2.763%, followed by AqE and EAE (40.54± 7.51% and 41.79± 1.654%, respectively). Total antioxidant capacity of red blood cells was measured, from the kinetics of hemolysis obtained. The calculated $HT_{50}$ reveal an extension of time for half hemolysis in all treated groups compared with the control group. CrE increase significantly $HT_{50}$ (112.8± 2.427 min). The hemolysis is lagged, indicating that endogenous antioxidants in the erythrocytes can trap radicals to protect them against free-radical-induced hemolysis. Conclusions: *Globularia alypum* L. fractions have a strong inhibitory effect on xanthine oxidoreductase and also have a significant antihemolytic and antioxydant effect.

**Keywords**: *Globularia Alypum*, Xanthine oxidoreductase, *in vivo*-antioxidant activity, hemolysis, polyphenol

## INTRODUCTION

Oxidative stress is a process where the physiological balance between pro-oxidants and antioxidant is disrupted in favor of the former, ensuing in potential damage for the organism[1]. Oxidative stress denotes an imbalance between oxidants (reactive species) and antioxidants in favor of the oxidants at the cellular or individual level, leading to a disruption of redox signaling and control and /or molecular damage[2]. ROS production can induce DNA damage, protein carbonylation, and lipid peroxidation, leading to a variety of chronic health problems[3]. The development of alternative antioxidants from natural origin has attracted considerable attention and many researchers have focused on the discovery of new natural antioxidants aimed at quenching biologically harmful radicals[4,5]. In the Algerian traditional pharmacopoeia, *Globularia alypum* (*Globulariaceae*) locally named "Tasalgha" is one of the most traditional plant remedies, it is a perennial shrub found throughout the Mediterranean area. The plant is known for its uses in the the indigenous system of medicine for a variety of purposes such as hypoglycaemic agent, laxative, cholagogue, stomachic, purgative, sudorific and also in the treatment of cardio-vascular and renal diseases[6,7], rhumatism, gout, typhoid, intermittent fever and diabetes[8-10]. Gout is metabolic disorders in human, associated with an elevated uric acid level in the blood, leading to the deposition of urate crystals in the joints and kidneys, leading to gouty arthritis and uric acid nephrolithiasis. Xanthine oxidase (XOR) catalyses the oxidation of hypoxanthine to xanthine and then to uric acid, the final reactions in the metabolism of purine bases[11]. The treatment for gout is either increasing the excretion of uric acid or reducing the uric acid production. Xanthine oxidase inhibitors (XOIs) are much useful. A potential source of such compounds can be obtained from medicinal plants. Flavonoids and polyphenolic crude extracts have been reported to possess xanthine oxidase inhibitory activity[12]. The aim of this study was to evaluate the therapeutic efficacy of subfractions of GA to inhibit XOR, to investigate it's *in vivo* antioxidant activity and anti-hemolytic effect.

## MATERIALS AND METHODS

*Biological material*

*Globulaia alypum* was collected from SETIF, Algeria in Mai 2010. All other reagents were purchased from Sigma Chemicals (Germany), Fluka and Prolab.

*Extraction of phenolics compounds*

The extractions were carried out using various polar and non-polar solvents. According to the method of Markham (1982)[13] the powdered plant material was extracted with

*Author for Correspondence

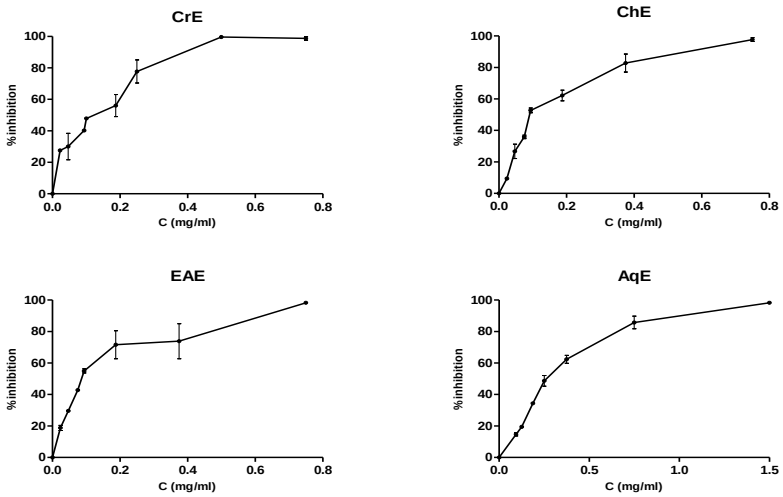


Figure 1: Kinetic of human milk XO inhibition by GA extracts. Values were expressed as mean ± SD of triplicate.

Table 1: Total polyphenols and flavonoids content of GA extracts

| Extract | Total phenol Content(µg EAG / mg E) |
|---|---|
| CrE | 140.24 ± 4.18 |
| ChE | 81.01 ± 1.91 |
| EAE | 157.74± 5.27 |
| AqE | 100.86 ± 0.76 |

Values are expressed as mean ± SD (n=3-4).

methanol (CrE), at room temperature overnight. The CrE extract was combined and concentrated under reduced pressure on a rotary evaporator. CrE successively extracted with hexane, chloroform and ethyl acetate. Each fraction was evaporated to dryness under reduced pressure to give hexane (HE), chloroform (ChE), ethyl acetate (EAE), and the remaining aqueous (AqE) extracts.

*Determination of total phenolics content*

Total phenolic content was determined by the Folin-Ciocalteu method[14]. Two hundred microliters of diluted sample were added to 1mL of 1:10 diluted Folin-Ciocalteu reagent. After 4 min, 800 mL of saturated sodium carbonate solution (75 g/L) was added. After 1h of incubation at room temperature, the absorbance at 765nm was measured in triplicate. Gallic acid (0-160 µg/ml) was used for calibration of standard curve. The results were expressed as microgram gallic acid equivalent (mg GAE)/g of extract.

*Effect of Globularia alypum extracts on XOR*

Xanthine oxidoreductase was purified from fresh human milk by ammonium sulphate fractionation, followed by affinity chromatography on heparin and ion exchange fast protein liquid chromatography, essentially as described for bovine by Sanders et *al.*, 1997[16]. The purity of enzyme was assessed on protein/flavin ratio (PFR = A280 /A450)[15] and on a 12% SDS-PAGE[17]. Total and specific activity of HXOR was assayed spectrophotometrically at 295 nm following the production of uric acid with an extinction coefficient of $9600 M^{-1}cm$[18].

The effect of GA on xanthine oxidation was examined spectrophotometrically at 295 nm following the production of uric acid using an absorption coefficient of 9600 $M^{-1}$ $cm^{-1}$. Different concentrations of tested compounds were added to samples before the enzyme had been added and their effect on the generation of uric acid was used to calculate regression lines and $IC_{50}$ values. The reaction was started by the addition of XO. The enzyme activity of the control sample was set to 100% activity[19].

*In vivo antioxidant activity*

Animal experiments in mice were conducted after the experiment procedures were revised and approved by the ethics committee of the university Ferhat Abbas SETIF1, Algeria. Experiments were performed on adult male mice from the Pasteur Institute of Algeria weighing 25–30 g. They were kept under standardized conditions (temperature 21-24 °C and a light/dark cycle of 12 hours/12 hours) and fed a normal laboratory diet. After 1 week of acclimatization, mice's were divided into one control and three experimental groups with 7-8 animals in each group. The extract was dissolved in normal saline. Animals from group 1,2 and 3 doses expressed on the basis of mg dry extract per kg body mass, namely CrE (160 mg/kg), EAE (160 mg/kg) and AqE (160 mg/kg) for 21 days. The control group of animals was treated as a control and received only saline solution.

*Blood collection*

The blood was collected through direct heart puncture from anesthetized mice. Blood was collected from direct heart puncture from anesthetized mice into tubes containing heparin, and plasma was obtained by low-speed centrifugation (1500g for 10 min). A volume of whole blood is immediately transferred to another tube containing phosphate buffer (300 mOs, pH 7.4) to obtain a dilution of 1:25. The amount of remaining blood is centrifuged at 1500 g for 5 min at 4 ° C. The plasma obtained was aliquoted and stored at -20° C until use for DPPH tests[19].

*Total antioxidant capacity of plasma, and red blood cells*

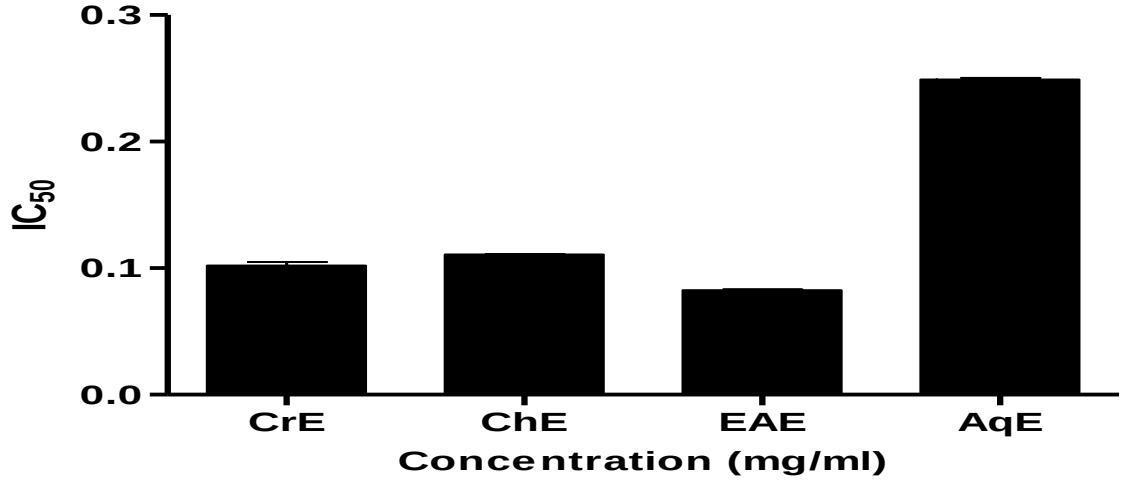


Figure 2: Inhibitory concentration of GA extracts for 50% of XO activity ($IC_{50}$).

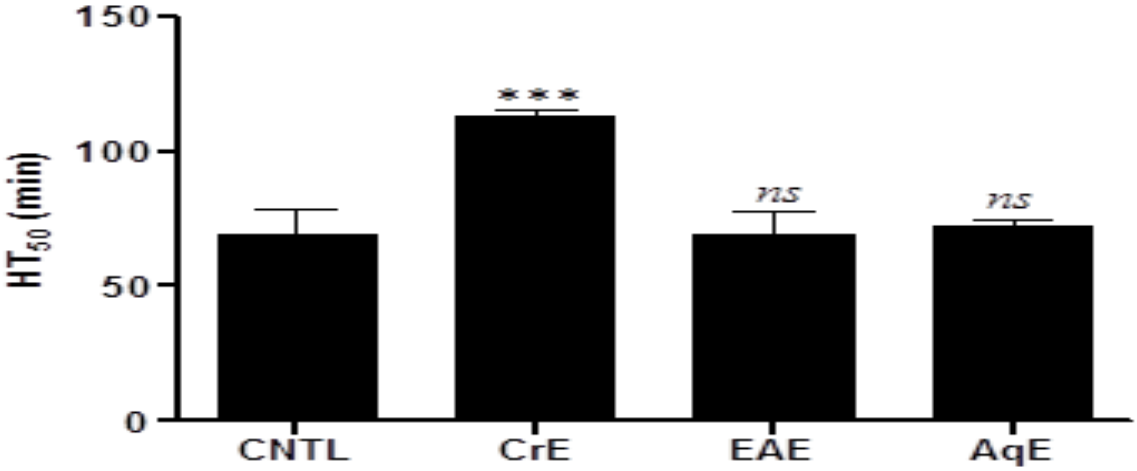


Figure 3: The half-life ($HT_{50}$) for the different group. Comparison was realized against CNTL, *: p≤0.05, p≤0.01, *** : p≤0.001,

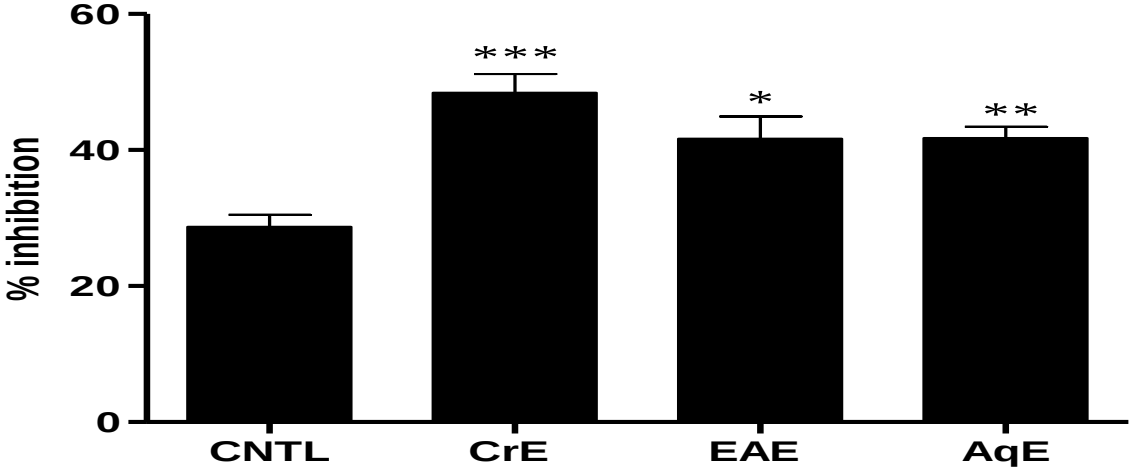


Figure 4: DPPH scavenging activity of GA extracts. Values were expressed as the mean (N=7-8) ± SEM.

Resistance to free radical damage was tested as the capacity of red blood cells (RBCs) to withstand free radical induced hemolysis and was measured, as previously described by Girard et al. (2006)[20] with some modifications, by monitoring the rate of free radical-induced hemolysis, using a microplate titraton. Briefly, whole blood was diluted 1:25, v:v, with phosphate buffer (125 MmNaCl, 10 mM sodium phosphate, pH 7.4 and 50 µL of RBC suspension or whole blood and were assayed using a 96-well microplate coated with a t-BHP as a free radical generator. The kinetics of RBCs resistance to hemolysis were determined at 37°C by continuous monitoring of changes in 620nm absorbance. The time to

reach 50% of total hemolysis was retained for group comparisons[21].

*DPPH radical scavenging activity*

In this test, plasma ability to inhibit DPPH radical was measured[22] DPPH is one of the few stable organic nitrogen radicals and has a maximum of absorption at 517 nm. A volume of plasma was added to the volume of DPPH solution (4mg/100ml methanol) and the reaction mixture was shaken vigorously. After incubation at room temperature for 30 min, the absorbance of this solution was determined at 517 nm, DPPH solutions without serum is used as the control.

*Statistical analysis*

The results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to one-way analysis of variance (ANOVA) and the differences between samples were determined by Tukey's multiple comparison test, using GraphPad program. p value ≤ 0.05 was regarded as significant.

## RESULTS AND DISCUSSION

*Total phenolics content and flavonoids content*

Determination of total phenolic contents of different extracts of GA was done by Folin-Ciocalteu assay, EAE is the richest in polyphenols followed by CrE then AqE. ChE showed the lowest values. (Table 1)

*Purification of human milk xanthine oxidase*

The yield of purification is up to 5.7 ± 1.8 mg/l of milk. Our HXOR has a PFR of 5.10 ± 0.22. SDS-PAGE of XOR showed a major band, and the molecular mass of purified HXOR was estimated to be 150 kDa. The total specific activity was 78.64 ± 17.81 nmole/min/ mg of enzyme.

*Effect of GA extracts on XO*

GA Extracts inhibited XO in a concentration-dependent manner (Figure 1). The EAE showed the highest inhibitory properties on the XO activity ($IC_{50}$ = 0.083 ± 0.001 mg / ml), followed by CrE and ChE with $IC_{50}$ of 0,104 ± 0,002 and 0,111 ± 0,010 mg / ml respectively. Finally, AqE had the lowest inhibitory effect on XOR activity with ($IC_{50}$ = 0.248 ± 0.016 mg /ml) (Figure 2).

Flavonoids have been reported to possess XOR inhibitory activity[23], the presence of phenolics and flavonoids content in the extracts would have contributed towards XOR inhibition. The higher polyphenol and flavonoid content is the EAE and it is the most active extract, but the highest $IC_{50}$ value was of AqE and it has not the lower content of polyphenols. where, glycosidic (EAE) and non-glucosidicpolyphenols (ChE) showed the highest inhibitory effect. In the view of this correlation, our results were confirmed by those obtained with Lychnophora species[24] and *Geranium sibiricum* L.[12]. In addition, these results showed that the XO inhibition could be linked not only on the polyphenols and flavonoids contents of the extract, but also to the nature (structure) of these compounds[11].

*Hemolysis*

Oxidative hemolysis of whole red blood cells is used as a model to study free radical-induced damage of biological membranes and the protective effect of phenolic compounds as known antioxidants.

The antioxidant activity of the GA was confirmed in mice's erythrocytes (RBC) exposed to *t*-BHP, by measuring the erythrocyte membrane resistance to free radical-induced hemolysis. From the kinetics of hemolysis obtained, the calculated HT50 reveal an extension of time for half hemolysis in all treated groups compared with the control group (CNTL) (Figure 3). However, for the CrE increase in HT50 is statistically very significant ($p \leq 0.001$) compared with the control group. The hemolysis is lagged, indicating that endogenous antioxidants in the erythrocytes can trap radicals to protect them against free-radical-induced hemolysis[25]. The results of present study have shown that GA can effectively protect erythrocytes against haemolytic injury induced t-BHP, GA is a powerful scavenger; it could have provided a defence against haemolytic injury by suppressing t-BHP related fall in reduce glutathione. The protective effects of GA may be due to: (1) The nature of phenolic content.in our study, there is no significatif correlation between antihymolitic effect of extracts and their phenolic compound content[11]. (2) The difference in the degree of the flavonoid molecules penetrations in erythrocytes and their ability to penetrate lipid bilayers[26,27]. It was demonstrate that binding of the flavonoids to the RBC membranes significantly inhibits lipid peroxidation, and at the same time enhances their integrity against lysis[28]. Bilto and his collaborator (2012) showed that flavonoids; quercetin, 3,5,7-trihydroxy-4'-meth- oxy flavone-7-rutinoside and 3-hydroxy flavone significantly protected erythrocytes against protein degradation. This inhibition could also be explained by the presence of a hydroxyl group at C-3 in ring C of the flavonoid structure[29]. Dai and his collaborator (2006) have previously reported that flavonols and their glycosides are effective antioxidants protecting human red blood cells from free radical-induced oxidative haemolysis[30].

*DPPH in vivo scavenging assay*

Plasma antioxidant capacity is one of the most commonly used biomarkers to assess the effectiveness of dietary supplementation or antioxidant treatment. Due to the large number of antioxidants present in plasma, several methods have been developed: the ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), total radical absorption potential (TRAP) and the DPPH radical scavenging activity. Sanchez-Moreno (2002)[31]. suggested that the 2,2-di(4-tertoctylphenyl)-1-picrylhydrazyl (DPPH) assay was an easy and accurate method with regard to measuring the antioxidant capacity of fruit and vegetable juices or extracts. In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the anti- oxidants[32]. It appears that the increase in plasma antioxidant capacity is probably attributed to elevated levels of exogenous antioxidants such as ascorbic acid, carotenoids, vitamin E and flavonoids also or phenolic compounds, acquired

following treatment with the extracts of GA, whose antioxidant properties have already been demonstrated in other *in vitro* study[33]. From the obtained results (Figure 4) show that oral administration of CrE leads to increased plasma antioxidant capacity 28.74 ± 1.73% *versus* 28.74 ± 1.730% for the control group. This increase is statistically very significant compared with the control group, however the administration of EAE and AqE has significantly increased ($p \leq 0.01$) the antioxidant capacity (40.54± 7.51% and 41.79± 1.65%). Studies involving plasma have indicated that flavonoids have the ability to delay the consumption of some endogenously present antioxidants in the human body[34]. The increase in plasma antioxidant capacity is probably attributed to the elevated levels of exogenous antioxidants acquired following treatment with GA extracts.

## CONCLUSION

The medicinal plant *Globularia alypum L* is among the plants widely used today in traditional medicine in Algeria, In tests conducted in this study, a significant correlation between polyphenols content and inhibition activity of XO was observed. In our study, extracts of *Globularia alypum L* seem to be of interest and potential for their antioxidant activities that have been established in vivo. These results remain preliminary, it would be interesting to test the activity of fractions and molecules that underlie the various activities detected in the different extracts. In addition, extensive further studies concerning the identification of phenolic compounds by more efficient methods are needed and a toxicological study is very requested.

## ACKNOWLEDGMENTS

This work was supported by the Algerian Ministry of Higher Education and Scientific Research (MERS) and by the Algerian Agency for the Development of Research in Health (ANDRS).

## REFERENCES

1. Ames GBN, Shigenaga MK, Hagen TM. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences of the United States of America. 90: 7915-7930.
2. Lykkesfeldt J, Svendsen O. (2007). Oxidants and antioxidants in disease: Oxidative stress in farm animals. The Veterinary Journal. 173:502–511
3. Bland JS. (1995). Oxidants and antioxidants in clinical medicine: Past, present and future potential. *J Nutr Environ Med*. 5: 255-280.
4. Aligiannis N, Mitaku S, Tsitsa-Tsardis E, Harvala C, Tsaknis I, Lalas S, et al. (2003). Methanolic extract of *Verbascum macrurum* as a source of natural preservatives against oxidative rancidity. *J Agr Food Chem*. 51: 7308-7312.
5. Vági E, Rapavi E, Hadolin M, Vásárhelyiné Perédi K, Balázs A, Blázovics A, Simándi B. (2005). Phenolic and triterpenoid antioxidants from *Origanum majorana* L. herb and extracts obtained with different solvents. *J Agric Food Chem.* 53: 17-21.
6. Bellakhdar J, Claisse R, Fleurentin J, Younos C. (1991). Repertory of standard herbal drugs in the Moroccan pharmacopoeia. *J Ethnopharmacol*. 35: 123-143.
7. Fehri B, Aiache JM. (2010). Effects of *Globularia alypum L*. on the gastrointestinal tract. *J Nat Prod*. 3:141-146.
8. Balansard J, Delphant J. (1948). La globulaire: un purgatif oublié. *Rev Phytothérap*. 12:213-234.
9. Skim F, Kaaya A, Jaouhari J.T, Lazrek H.B, Jana M, El Amri H. (1999). Hypoglycaemic activity of Globularia alypum leaves in rats. *Fitoterapia*. 70: 382-389.
10. Jouad H, Maghrani M, Eddouks M. (2002). Hypoglycaemic effect of Rubus fructicosis L. and *Globularia alypum L*. in normal and streptozotocin-induced diabetic rats. *J Ethnopharmacol*. 81:351-356.
11. Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Van Poel B, Pieters L, Vlietinck AJ, Vanden Berghe D. (1998). Structure activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J Nat Prod*. 61: 71-76.
12. Wu N, ZuY, Fu Y, Kong Y, Zhao J, li X, li J, Wink M, FerthT. Antioxidant Activities and Xanthine Oxidase Inhibitory Effects of Extracts and Main Polyphenolic Compounds Obtained from *Geranium sibiricum L*. *J Agric Food Chem*. 58:4737-4743.
13. Markham KR. (1982). Techniques of flavonoid identification. London Academic Press. Chap1 et 2 (pp. 1-113).
14. Li HB, Cheng KW, Wong CC, Fan KW, Chen F. Jiang Y. (2007). Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. *Food Chem*. 102:771-776.
15. Bray RC. (1975) Molybdenum iron-sulferflavin hydroxylases and related enzymes. In " Boyer P.D (Eds.), the enzymes" 3rd Ed. Academic Press, New York. pp: 299 - 419.
16. Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
17. Sanders S, Eisenthal R, Harrison R. (1997) NADH oxidase activity of human xanthine oxidoreductase. Generation of superoxyde anion. *Biochem Eurp J*. 245: 541-548.
18. Avis PG, Bergel F, Bray RC, James DWF, Shooter KV. (1956). Cellular constituent, the chemistry of xanthine oxidase.Part II. The homogeneity of crystalline metalloflavoprotein fraction. *J Chem Soc* 1212-1219.
19. Trabsa H, Baghiani A, Boussoualim N, Krache I, Khennouf S, Charef N and Arrar L. 2015. Kinetics of Inhibition of Xanthine Oxidase by Lycium arabicum and its Protective Effect against Oxonate- Induced Hyperuricemia and Renal Dysfunction in Mice. Tropical Journal of Pharmaceutical Research. 14 (2): 249-256.

20. Manna C, D'angelo S, Migliardi V, Loffredi E, Mazzoni O, Morrica P, Galletti P, Zappia V. (2002) Protective effect of the phenolic fraction from virgin olive oils against oxidative stress in human cells. *J Agr Food Chem*. 50: 6521-6526.
21. Girard, A., Madani, S., Boukortt, F., Cherkaoui-Malki, M., Belleville, J., Prost, J. 2006. Fructose-enriched diet modifies antioxidant status and lipid metabolism in spontaneously hypertensive rats. *Nutrition*. 22, 758-766.
22. Mansouri, A., Embarek, G., Kokkalou, E., Kefalas, P. 2005. Phenolic profile and antioxidant activity of the Algerian ripe date palm fruit (Phoenix dactylifera). *Food Chemistry*. 89, 411-420.
23. Van Hoorn DEC, Nijveldt RJ, Van Leeuwen PAM, Hofman Z, M'Rabet L, De Bont DBA,Van Norren K. 2002. Accurate prediction of xanthine oxidase inhibition based on the structure of flavonoids. *European Journal of Pharmacology* 451: 111-118.
24. Ferraz Filha ZS, Vitolo IF, Fietto LG, Lombardi JA, Saúde-Guimarães DA. Xanthine oxidase inhibitory activity of Lychnophora species from Brazil ("Arnica"). Journal of Ethnopharmacology Volume 107, Issue 1, 11 August 2006, Pages 79–82
25. Takebayashi J, Chen J, Tai A. 2010. A Method for Evaluation of Antioxidant Activity Based on Inhibition of Free Radical-Induced Erythrocyte Hemolysis. D. Armstrong (ed.), Advanced Protocols in Oxidative Stress II, Methods in Molecular Biology, vol. 594. pp. 287-296.
26. López-Revuelta A, Sánchez-Gallego JI, Hernández-Hernández A, Sánchez-Yagüe J, Llanillo M. (2006). Membrane cholesterol contents influence the protective effects of quercetin and rutin in erythrocytes damaged by oxidative stress. *Chem Biol Interact.* 15;161(1):79-91.
27. Blasa M, Candiracci M, Accorsi A, PieraPiacentini M, Piatti E. (2007). Honey flavonoids as protection agents against oxidative damage to human red blood cells. Chemistry. 104(4): 1635-1640.
28. Kitagawa H, Sakamoto H, Tano Y. (2004). Inhibitory Effects of Flavonoids on Free Radical-Induced Hemolysis and Their Oxidative Effects on Hemoglobin. *Chem Pharm Bul*. 52: 999-1001.
29. Yousif Y. Bilto1*, Sanaa Suboh1, Talal Aburjai2, Shtywy Abdalla1. Structure-activity relationships regarding the antioxidant effects of the flavonoids on human erythrocytes Vol.4, No.9, 740-747 (2012) Natural Science
30. Dai F, Miao Q, Zhou B, Yang L, Liu ZL. (2006). Protective effects of flavonols and their glycosides against free radical-induced oxidative hemolysis of red blood cells. *Life Sci*. 18;78(21):2488-2493.
31. Sa´nchez-Moreno C. (2002). Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci Technol Int. 8* (3): 121-137.
32. Bondent V, Williams B, Bereset C. (1997). Kinetic and Mechanism of Antioxidant Activity Using the DPPH Free Radical Methods. *Lebensmittel Wissenschaft and Technologie*. 3(6): 609-615.
33. Harzallah HJ, Jrah H, Neffati A, Skandrani I, Maaloul E, Chekir- Ghedira L, Mahjoub T. (2010). Antioxidant and antigenotoxic activities of *Globularia alypum* leaves extracts. *J Med Plants Res*. 4(19): 2048-2053.
34. Lotitio SB, Fraga CG. (1998). Catechin Prevents Human Plasma Oxidation. *Free Radical Bio Med*. 24: 435-441.