Journal of Medicinal Plants and By-products (2022) Special Issue: 37-41

Original Article

Effects of *Atriplex hortensis* Hydroalcoholic Extract on Phenyl-hydrazine Induced Hemolytic Anemia in Rat

Running title: Anti-hemolytic Activity of *Atriplex hortensis*

Parham Mardi[1], Hasan Fallah Huseini[2], Maryam Ahvazi[2] and Bahareh Tavakoli-Far[3*]

[1]*Faculty of Medicine, Alborz University of Medical Sciences, Karaj, Iran*
[2]*Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran*
[3]*Dietary Supplements and Probiotic Research Center, Alborz University of Medical Sciences, Karaj, Iran*

Article History: Received: 14 October 2020/Accepted in revised form: 01 December 2020

Abstract

Hemolytic anemia is a hematological disorder occurs owing to the excessive hemolysis of red blood cells. *Atriplex hortensis* L. leaves are traditionally used for prevention and treatment of anemia. The present study was performed to assess the effects of *A. hortensis* extract on phenyl-hydrazine induced hemolytic anemia in rat. Thirty-six, six months old, male Wistar rats were randomly divided into six groups of 6 rats each. One group received normal saline and other 5 groups received 40 mg/kg phenyl-hydrazine (PHZ) intraperitoneally at baseline and on day 33 for induction of hemolytic anemia. Four PHZ treated groups received daily oral doses of 50, 100, 200, and 300 mg/kg/day *A. hortensis* extract for 31 days and one PHZ treated group kept as control received saline accordingly. Blood superoxide dismutase (SOD) activity and blood hematological parameters such as hematocrit (HCT), red blood cell (RBC), reticulocyte (Ret) and hemoglobin (HGB) were determined in all groups after 34 days of the study. The blood RBC, HGB and HCT parameters were significantly lowers and Ret increased significantly in PHZ control group compared with the normal saline treated group. In *A. hortensis* extract (100, 200, and 300 mg/kg/day) treated groups the blood HCT, RBC and HGB parameters were significantly increased and Ret decrease, compared with PHZ control group, the blood SOD enzyme activity was significantly improved in all *A. hortensis* treated groups compared with PHZ control group. *A. hortensis* extract improved blood SOD activity and blood HCT, RBC, Ret and HGB parameters indicating inhibition of hemolytic anemia induced by phenyl-hydrazine in rat.

Keywords: Antioxidant, *Atriplex hortensis*, Hemolytic anemia, Traditional medicine

Introduction

Anemia is a reduction in hematocrit (HCT), red blood cell (RBC) and hemoglobin (HGB), which causes the inadequate blood supply to body organs. Severe anemia can cause tissue infarction that leads to organ dysfunction [1-3]. In 2008 global prevalence of anemia was 25 percent according to World Health Organization (WHO) report [4]. Although anemia is not a major risk factor for mortality, it imposed a high avoidable damage in people quality of life. In 2003, according to the Disability-Adjusted Life Year (DALY) index study in Iranian population, the Iron deficiency & other anemias was one of the 10 top disease and injury causes highest mortality with estimated 405 persons in 100000 [5].

Hemolytic anemia is a complex hematological disorder occurs when the hemolysis overtakes the production of RBCs. Hemolysis is a cause of anemia in 5% of cases. Hemolytic anemia is a sign of disorders for diseases such as thalassemia, sickle cell anemia and fauvism [6]. Reticulocytes are immature red blood cells and hemolysis occurs when blood levels of reticulocyte increased [7].
Although in hemolysis the number of RBC and blood supply to organs decreased, more clinically significant symptoms are overproduction of oxidant agents that destruct tissues and organs [8]. Antioxidant agents such as superoxide dismutase (SOD) reduce oxidative stress and can play an imperative role in the prevention of hemolytic anemia [9-11].

*Corresponding Author: Dietary Supplements and Probiotic Research Center, Alborz University of Medical Sciences, Karaj, Iran
Email Address: B.tavakolifar@abzums.ac.ir

Phenyl-hydrazine was used for its antipyretic effects in past, but its adverse hemolytic effects prohibited prescription. This drug is still useful in induction hemolytic anemia in animal models [12].

A. hortensis or red orache, belongs to the Amaranthaceae family. Its leaves decoction is a common traditional therapy for anemia in the northern region of Iran [13]. *A. hortensis* contains many antioxidant agents such as flavonoids [14, 15]. The plant is a source of vitamin C, K, A, magnesium, calcium, fiber, and two major flavonoids, including quercetin 3-O-sulphate-7-O-α-arabinopyranoside and kaempferol 3-O-sulphate-7-O-α-arabinopyranoside [14, 16-18] that may influence anemia. The present study was performed to assess the anti-hemolytic effects of *A. hortensis* in phenyl-hydrazine induced hemolytic anemia in rats.

Material and Methods

Plant Material

Atriplex hortensis L. was collected from the Institute of Medicinal Plants researched Farm (cultivated) in April. Plant's voucher specimen with code number 4582 (MPIH) was deposited in herbarium of the Institute of Medicinal Plants, ACECR, Karaj, Iran. The leaves were washed and dried in the shade at room temperature and crushed for extraction processes.

Extraction Preparation

The crushed leaves were extracted with 80% v/v hydroalcoholic solvent in a percolator for about 72 h. The extraction was repeated two times more and mixed. The rotary evaporator was used for solvent removal for production of dry extract.

DPPH Radical Scavenging Assay

Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of *A. hortensis* extract was assessed using the Han *et al*. method [19-21]. Methanol solution of the DPPH reagent (1.5 mL) was prepared and mixed with diferent concentrations of plant extractand kept in dark place. After 30 minutes the solution absorbance was observed at 517 nm using a spectrophotometer (Human, USA). A standard Ascorbic acid (Merck, Germany) was used as reference.

Radical scavenging activity (RSA) percent was measured as follow:

RSA (%) = (Ac-As/Ac) ×100.

Ac: absorbance of the negative control, As: absorbance of the plant sample or Ascorbic acid.

Findings were stated as IC50, which represented the concentration of antioxidant, which decreases the DPPH radicals to 50%.

Determination of Total Flavonoids

A. hortensis extract in distilled water (1 mg/mL) mixed with sodium nitrite solution (5%, 300 µL). After 5 minutes of standing, aluminum chloride solution (10%, 300 µL), a sodium hydroxide (1 M, 2 mL) and distilled water (3 mL) were added to the mixture. The absorbance was observed at 510 nm using a spectrophotometer (Human, USA). The standard curve was generated using the Rutin (100 up to 1200 µg/mL) as a standared. Findings were stated as milligrams Rutin per gram dry *A. hortensis* extract [18].

Determination of Total Phenolics

Folin-Ciocalteu colorimetric technique was applied to assess the total phenolic contents of *A. hortensis* extract. Briefly, 5 mg of *A. hortensis* extract was disolved in distilled water (5 mL) and Folin-Ciocalteu reagent (500 µL) and sodium carbonate solution (5%, 1 mL) were added to the mixture and kept in dark place for about 30 min. The absorbance was observed at 725 nm using a spectrophotometer (Human, USA). The standard curve was ploted using the Gallic acid (Merck, Germany) as a standared. The data was stated as mg of gallic acid per gram dry *A. hortensis* extract [19].

Animal Treatments and Experimental Design

Thirty six, six months old male Wistar rats were randomly divided into six groups of 6 rats each. One group received intraprotenial (IP) injuction of normal saline and kept as normal control group. Five groups received two time IP injection of phenyl-hydrazine 40 mg/kg dissolved in saline, once at baseline and seconed on day 33 for induction of hemolytic anemia [10, 22]. Four groups PHZ treated received daily oral doses of 50, 100, 200, and 300 mg/kg/day *A. hortensis* (AH) extract by gastric gavage for 31 days and one group PHZ treated kept as control received saline accordingly. The blood hematological parameters such as RBC count, HGB, HCT, Ret and SOD activity [10] was determined at baseline and after 34 days at end of the the study.

Hematological Indices and SOD Evaluation

On day 34, the blood samples were taken under anesthesia from the heart into a CBC tube that contains anticoagulant. Flowcytometry (SYSMEX XT-2000i Fluorescence Flowcytometry) was used for assessment of HGB, HCT, Ret and RBC count[10]. To perform SOD assay, a ZellBio GmbH SOD kit (Biocore Diagnostik, Ulm, Germany) was used. The SOD activity was determined by calorimetr at 420 nm [23].

The present investigation was confirmed by Alborz University of medical science's ethical committee. (IR.ABZUMS.REC.1397.080) (June 13, 2018).

Statistical Evaluation

Findings were stated as the mean ± standard deviation (SD). Statistical assessment was done using SPSS ver.

22. The difference of PHZ and control group was evaluated using independent T-test. One way, ANOVA followed by a Dunnet test was used to evaluate the variance between PHZ and PHZ+AH groups. A *P*-value less than 0.01 was considered a statistically significant level.

Results

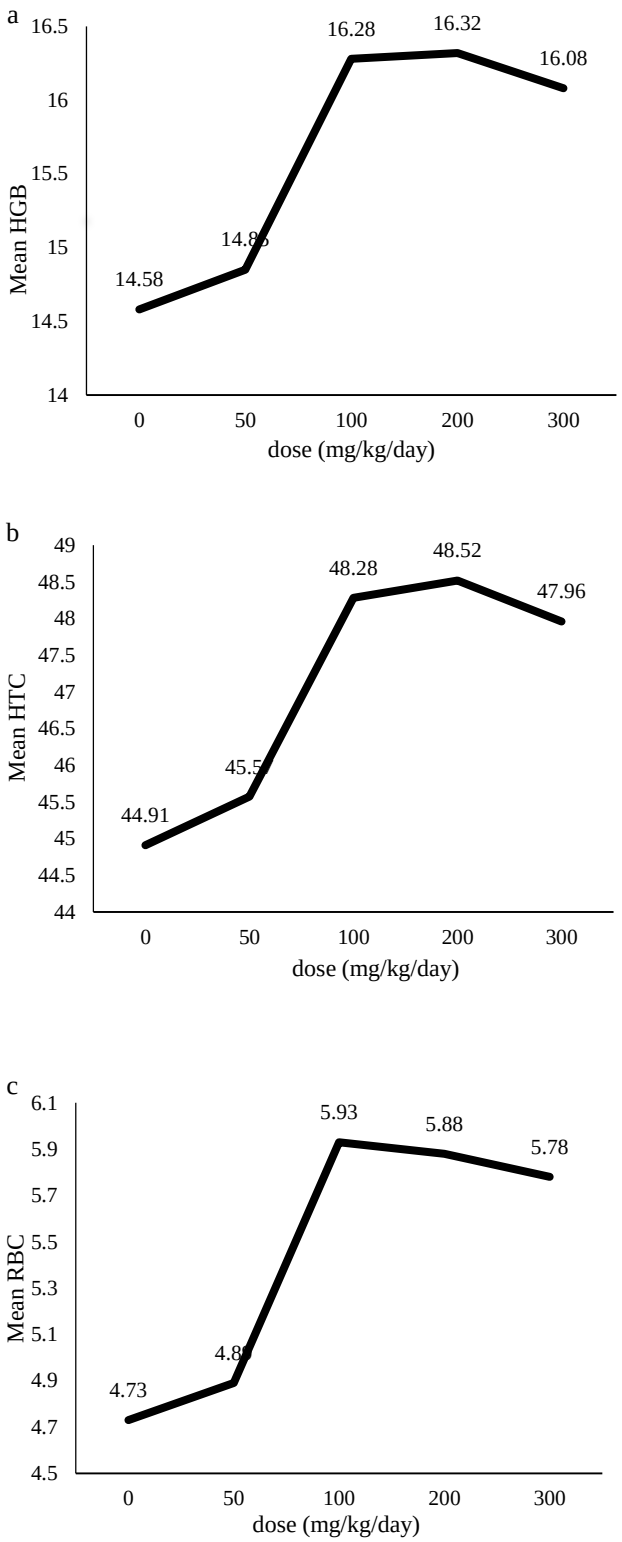

Fig. 1 Hemoglobin (A), Hematocrit (B), and Red Blood Cell (C) dose-response curve.

Phytochemical Analysis

The IC50 of the *A. hortensis* extract and Ascorbic acid were 87.26 ± 0.003 µg/mL (mean ± SD) and 5.626 ± 0.001 µg/mL (mean ± SD), respectively.

The total flavonoid content of *A. hortensis* extract was 54.75 ± 0.143 mg Rutin/g extract, and the total phenolic content of *A. hortensis* extract was 48.23 ± 1.23 mg Gallic acid/g extract.

Hematological Study

Figure 1 discloses the Hemoglobin (A), Hematocrit (B), and Red Blood Cell (C) dose-response curve.

Figure 2 discloses the hemoglobin level at day 34 in different experimental groups (N=6).

The results indicated that RBC, HGB and HTC decreased and Ret increased significantly in the PHZ group compared with the normal control group ($P < 0.01$) (Table 1).

HGB, RBC, HTC and Ret were significantly higher and Ret lower in PHZ+AH (100mg/kg, 200mg/kg, 300 mg/kg) groups, compared to the PHZ group. No significant difference was seen between the PHZ+AH (50 mg/kg) group and the PHZ group in terms of HGB, RBC and HTC. However, RET was significantly higher in PHZ+AH (50 mg/kg) in comparison to PHZ group.

Blood antioxidant study

The SOD enzyme activity was significantly decreased in PHZ treated group compared with normal control group. In all PHZ+AH groups the SOD enzyme activity was significantly higher compared with the PHZ group (Table 2).

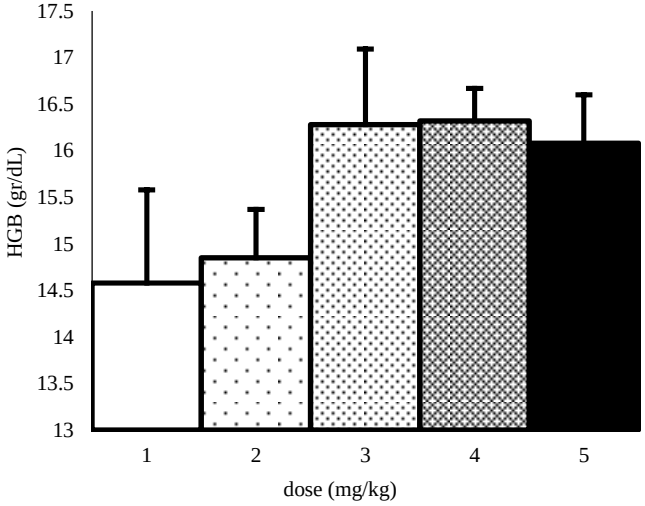

Fig. 2 Hemoglobin level at day 34 in different experimental groups (N=6). Phenyl-hydrazine (PHZ), *A. hortensis* L. extract (AH) 50, 100, 200 and 300 mg/kg/day PHZ + AH 50, 100, 200, and 300 mg/kg groups, compared with the PHZ group.

Journal of Medicinal Plants and By-products (2022) Special Issue: 37-41

Table 1 Hematological finding in rats at day 34. PHZ group compared with control normal group.

Hematological findings	PHZ control group	Normal control group	*P* value*
RBC (cells/mL)	4.73±0.71	5.85±0.51	0.001
HGB (g/dl)	14.58±1.00	16.48±0.94	0.001
HCT (%)	44.91± 2.54	49.20±2.66	0.001
Ret (%)	0.7±1.0	3.55±0.38	0.001

HGB: Hemoglobin, RBC: Red blood cell, HCT: Hematocrit, Ret: reticulocyte, Phenyl-hydrazine (PHZ), *P values were analyzed stated according to independent T-test.

Table 2 Hematological findings in rats on day 34 in PHZ induced hymolytic anemia control and *A. hortensis* L. extract treated groups.

Hematological findings	PHZ group	PHZ+*A. hortensis* (50 mg/kg/day)	PHZ+*A. hortensis* (100 mg/kg/day)	PHZ+*A. hortensis* (200 mg/kg/day)	PHZ+*A. hortensis* (300 mg/kg/day)
HGB (g/dl)	14.58±1.00	14.85±0.52	16.28±0.81	16.32±0.35	16.08±0.52
P-value Compared to PHZ group	-	0.72	0.001	0.001	0.001
RBC (cells/mL)	4.73±0.71	4.89±0.28	5.93±0.45	5.88±0.17	5.78±0.44
P-value Compared to PHZ group	-	0.94	0.001	0.001	0.001
HCT (%)	44.91±2.54	45.57±1.40	48.28±2.09	48.52±1.44	47.96±1.45
P value Compared to PHZ group	-	0.91	0.002	0.003	0.002
SOD (U/mL)	1.73±0.34	2.58±0.68	4.27±0.28	4.26±0.42	4.55±0.23
P-value Compared to PHZ group	-	0.004	0.001	0.001	0.001
Ret (%)	2.7±1.0	2.55±0.58	3.79±0.68	3.90±0.43	3.59±0.92
P-value Compared to PHZ group	-	0.001	0.001	0.001	0.001

HGB: Hemoglobin, RBC: Red blood cell, HCT: Hematocrit, SOD: Superoxide dismutase, PHZ: Phenyl-hydrazine
*P values were analyzed stated according to one way, ANOVA test followed by Dunnett test.

Discussion

Findings of the current investigation revealed that the *A. hortensis* hydroalcoholic extract inhibits hemolytic anemia owing to phenyl-hydrazine injection in the rat model. Phenyl-hydrazine is a strong oxidant, and its metabolites include reactive oxygen species damages RBCs by oxidizing hemoglobin inside erythrocytes cause hemolytic anemia [10]. Theoretically, antioxidant compounds prevent anemia due to oxidative stress by inhibiting free oxygen radicals [24,25].

In the present study, *A. hortensis* showed a strong radical inhibitory activity and SOD activity that may be due to its flavonoids and phenolics content.

Flavonoids are an important plant's metabolite with antioxidant effects [26]. In this study, phytochemistry analysis showed that the plant contains relatively boost phenolic and flavonoid compounds. Flavonoid's antioxidant activity plays an essential role in the treatment of hemolytic anemia symptoms [14].

Two separate studies revealed the high biological effects of *Carica papaya* and *Hymenocardia acida* extract as antioxidant therapeutics which reduced the formation of dense cells, inhibition from oxidative cell injury and finally caused prolongation of RBC life [27,28]. Additionally, the mixture of *Piper nigrum*, *Piper longum*, and *Zingiber officinale* extracts can diminish oxidative stress and inhibit hemolytic anemia [29]. These studies confirmed the results of our study that anti-oxidants are useful in order to cure hemolytic anemia.

Furthermore, Niprisan, a chemical compound extracted from four diverse types of plants including *Pterocapus osun* stems, *Piper guineense* seeds, *Sorgum bicolor* leaves, and *Eugenia caryophyllum* fruit, were traditionally used in Nigeria for sickle cell anemia [30]. Although this mixture reduces sickle cell anemia signs and symptoms, unlike *A. hortensis*, there is no scientific proof that this herbal product enhances hematological indices [31, 32]. Besides, in favor of our study, the anti-hemolytic effects of *Paeonia lactiflora* and *Rehmannia glutinosa* extract were reported in animal studies [33].

The present study was limited to absent other types of *A. hortensis* extract and even its essential oil. Additionally, the lack of histopathological assessment of inner organs, particularly the liver, is another limitation of this survey. However, comparing the findings of 5 different groups and comprehensive assessment of hematological factors are strong points of the present research.

In conclusion, 100, 200, and 300 mg/kg concentrations of *A. hortensis* hydroalcoholic extract inhibits hemolytic anemia caused by injection of phenyl-hydrazine in rat model. Findings discovered that 50 mg/kg concentration

of *A. hortensis* hydroalcoholic extract did not show remarkable effects. However, further researches are critical to assess anti-hemolytic effects of 100, 200, and 300 mg/kg concentrations of *A. hortensis* hydroalcoholic extract on other animal models and even humans.

Acknowledgments

The authors acknowledge the Vice Chancellor for Research, Alborz University research for financial supports and also thank Dr. Mohammad-Javad Qaravi and Mr. Sharif from Fardis Central laboratory for their professional supports.

References

1. Beutler E, Waalen J. The definition of anemia: what is the lower limit of normal of the blood hemoglobin concentration? Blood. 2006;107:1747-1750.
2. Vlagopoulos PT, Tighiouart H, Weiner DE, Griffith J, Pettitt D, Salem DN, Levey AS, Sarnak MJ. Anemia as a risk factor for cardiovascular disease and all-cause mortality in diabetes: the impact of chronic kidney disease. J American Soci Nephrology. 2005;16:3403-3410.
3. Al-Ahmad A, Rand WM, Manjunath G, Konstam MA, Salem DN, Levey AS, Sarnak MJ. Reduced kidney function and anemia as risk factors for mortality in patients with left ventricular dysfunction. J American College Cardiol. 2001;38:955-962.
4. Benoist Bd, McLean E, Egll I, Cogswell M. Worldwide prevalence of anaemia 1993-2005: WHO global database on anaemia. Worldwide prevalence of anaemia 1993-2005: WHO global database on anaemia. 2008.
5. Naghavi M, Abolhassani F, Pourmalek F, Lakeh MM, Jafari N, Vaseghi S, Hezaveh NM, Kazemeini H. The burden of disease and injury in Iran 2003. Populat Health Metr. 2009;7:9.
6. Fauci AS. Harrison's principles of internal medicine: Mcgraw-hill New York. 1998.
7. Piva E, Brugnara C, Spolaore F, Plebani M. Clinical utility of reticulocyte parameters. Clinic Laborator Med. 2015;35:133-163.
8. Fibach E, Rachmilewitz E. The role of oxidative stress in hemolytic anemia. Current Molecul Med. 2008;8:609-619.
9. Friedman JS, Rebel VI, Derby R, Bell K, Huang T.T, Kuypers FA, Epstein CJ, Burakoff SJ. Absence of mitochondrial superoxide dismutase results in a murine hemolytic anemia responsive to therapy with a catalytic antioxidant. J Experiment Med. 2001;193:925-934.
10. Ashour TH. Phenylhydrazine‐induced hemolytic anemia in rats. Res J Med Sci. 2014;8:67-72.
11. Thakur A, Kumar P, Chand D. Superoxide Dismutase and Oxidative Stress: Elucidation of Antiaging and Antioxidative Effect on Mammalian Cell Lines. J Adv Microbiol. 2017;3:54-59.
12. Beutler E. Drug-induced hemolytic anemia. Pharmacol Rev. 1969;21:73-103.
13. Heidari A ZS, Heidari G. Ethnobotanical survey of Namarestagh summer rangelands, Amol (Mazandaran). J Islamic Iranian Traditional Med. 2015;5:330-339.
14. Bylka W, Stobiecki M, Frański R. Sulphated flavonoid glycosides from leaves of *Atriplex hortensis*. Acta Physiol Plant. 2001;23:285-290.
15. Awaad AS, Maitland D, Donia AERM, Alqasoumi SI, Soliman GA. Novel flavonoids with antioxidant activity from a Chenopodiaceous plant. Pharma Biol. 2012;50:99-104.
16. Wright K, Pike O, Fairbanks D, Huber C. Composition of *Atriplex hortensis*, sweet and bitter Chenopodium quinoa seeds. J Food Sci. 2002;67:1383-1385.
17. Carlsson R. Quantity and Quality of Leaf Protein Concentrates from *Atriplex hortensis* L., Chenopodium quinoa Willd. and Amaranthus caudatus L, Grown in Southern Sweden. Acta Agri Scandinavica. 1980;30:418-426.
18. Atanassova M, Aslam M, Sharma S. Studies on nutritional facts of spring herbs collected from Bulgarian market. J Pub Health Catalog. 2018;1:15-20.
19. Han J, Weng X, Bi K. Antioxidants from a Chinese medicinal herb–Lithospermum erythrorhizon. Food Chem. 2008;106:2-10.
20. Gutfinger T. Polyphenols in olive oils. J American Oil Chem Soci. 1981;58:966-968.
21. Yoo KM, Lee CH, Lee H, Moon B, Lee CY. Relative antioxidant and cytoprotective activities of common herbs. Food Chem. 2008;106:929-936.
22. Chikhi I, Allali H, Dib MEA, Medjdoub H, Tabti B. Antidiabetic activity of aqueous leaf extract of *Atriplex halimus* L.(Chenopodiaceae) in streptozotocin-induced diabetic rats. Asian Pacific J Tropic Dis. 2014;4:181.
23. Goudarzvand M, Afraei S, Yaslianifard S, Ghiasy S, Sadri G, Kalvandi M, Alinia T, Mohebbi A, Yazdani R, Azarian SK. Hydroxycitric acid ameliorates inflammation and oxidative stress in mouse models of multiple sclerosis. Neural Regenerat Res. 2016;11:1610.
24. Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Archiv Int Med. 1995;155:381-386.
25. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J Nutr Biochem. 2002;13:572-584.
26. Cook N, Samman S. Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources. J Nutr Biochem. 1996;7:66-76.
27. Mojisola OC, Adebolu EA, Alani DM. Antisickling properties of Carica papaya Linn. J Natural Product. 2008;1:56-58.
28. Mpiana P, Mudogo V, Kabangu Y, Tshibangu D, Ngbolua K, Atibu E, Mangwala K, Mbala M, Makelele L, Bokota M. Antisickling activity and thermostability of anthocyanins extract from a congolese plant, Hymenocardia acida Tul.(Hymenocardiaceae). Int J Pharmacol. 2009;5:65-70.
29. Natarajan KS, Narasimhan M, Shanmugasundaram KR, Shanmugasundaram E. Antioxidant activity of a salt–spice–herbal mixture against free radical induction. J Ethnopharma. 2006;105:76-83.
30. Imaga N. The use of phytomedicines as effective therapeutic agents in sickle cell anemia. Sci Res Essay. 2010;5:3803-3807.
31. Wambebe CO, Bamgboye EA, Badru BO, Khamofu H, Momoh JA, Ekpeyong M, Audu BS, Njoku SO, Nasipuri NR, Kunle OO. Efficacy of niprisan in the prophylactic management of patients with sickle cell disease. Current Therap Res. 2001;62:26-34.
32. Wambebe C, Khamofu H, Momoh J, Ekpeyong M, Audu B, Njoku O, Bamgboye E, Nasipuri R, Kunle O, Okogun J. Double-blind, placebo-controlled, randomised cross-over clinical trial of NIPRISAN® in patients with Sickle Cell Disorder. Phytomedicine. 2001;8:252-261.
33. Lee HW, Kim H, Ryuk JA, Kil K.J, Ko BS. Hemopoietic effect of extracts from constituent herbal medicines of Samul-tang on phenylhydrazine-induced hemolytic anemia in rats. Int J Clin Experiment Pathol. 2014;7:6179.