Quantitative estimation of phytochemicals and in vitro antidiabetic activity of chloroform fraction of *Marrubium vulgare* L. leaf.

Shabir Ahmad Dar[1]*, Manik Sharma[1], Jagrati Tripathi [2], Rajendra Chauhan [3], Dixhya Rani [4], Anil Bhushan[4]

[1]P.G. Department of Zoology career College Bhopal, Madhya Pradesh, India,

[2]Department of Botany, Govt. College Khimlasa, Sagar, Madhya Pradesh, India,

[3]Govt. Motilal Vigyan Mahavidyalaya, Bhopal, India,

[4]Natural Product Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Jammu, 180001, India.

Abstract

Diabetes mellitus a chronic metabolic disorder characterized by hyperglycaemia together with altered carbohydrate, lipid and protein metabolism due to inadequate amount of insulin secretion by pancreas. Various approaches are used to have a control/curb over this increased life threatened disease. Among these approaches, one of the most widely used one is, use of natural inhibitors which reduces gastrointestinal glucose production and absorption through the inhibition of carbohydrate digesting enzymes such as α- amylase and α-glucosidase. Through these strategic managements post prandial increase of blood glucose after a mixed carbohydrate diet can be reduced significantly. Keeping this therapeutic approach in mind we estimated the phytochemical contents of methanolic extract of *M. vulgare* L. leaf and its chloroform fraction and evaluate the vitro antidiabetic activity of chloroform fraction. The assay suggested that chloroform fraction containing highest content of terpenoids (636.81 ± 22.95 mg Le/g extract) exhibited dose dependent increase in inhibitory effect on α-amylase enzymes (IC_{50} 487.33±2.65 µg/ml) and α-glucosidase (IC_{50} 392.95±7.38 µg/ml). From the current study in which chloroform fraction (Chf fr) showing significant inhibitory effect on carbohydrate digestive enzymes, we depicted that the activity could be due to presence of terpenoids.

Key words: α-amylase, α-glucosidase, diterpenoids, total phenol content, total terpenoid content.

1. Introduction

Diabetes mellitus a silent killer primarily defined as a condition characterized by hyperglycemia give rise to severe long-term complications like atherosclerosis, macular edema, diabetic ketoacidosis diabetic microangiopathy, diabetic neuropathy, diabetic nephropathy and diabetic retinopathy [1]. There has been a surge in the number of diabetes mellitus cases since 2000, which has become leading cause of deaths worldwide. According to World Health Organization, around 1.5 million people died worldwide due to diabetes in 2019. There are about 463 million people with diabetes worldwide in the age group of 20-79 years, of whom 79% reside in developing countries [2]. In addition, the International Diabetes Federation [2], also estimated that the prevalence of this figure would rise to 700 million in 2045. As far as India is concerned studies highlighted that the prevalence of diabetes is high and also there is a rapid increase in the urban population. It was estimated that about 134 million adults will be affected with diabetes in the year 204 [3].

M. vulgare L. (Lamiacea) commonly known as white horehound is a popular herbal that is often used as a domestic remedy for coughs, colds, wheeziness etc [4,5]. *M. vulgare*, Leaves which is mostly being studied showed analgesic, hypoglycemic, antispasmodic anticancer, gastroprotective activities [6-10]. Previously antidiabetic studies were in vivo; to our best knowledge this in vitro antidiabetic study is the first one till date. In this study we not only screen chloroform fraction of methanolic extract of *M. vulgare* leaf but also estimated quantitatively the phytoconstituents present in that fraction.

2. Material and Methods

2.1. Plant material and extraction

The fresh leaves of *M. vulgare* were collected from Pampore (Pulwama) in the month of June 2018 and authenticated by Department of Botany (Centre for Biodiversity & Taxonomy) University of Kashmir and sample of the plant material was deposited in the herbarium of the Department of Taxonomy, University of Kashmir under voucher specimen number, *Marrubium vulgare*- 2678 KASH [Ref No: F (voucher-specimen CBT/KU/18)]. Further the collected leaves were washed, dried for 3 weeks and then made into coarse powdered. The powdered leaf (300 g) was subjected to soxhletion using methanol (5 L) as solvent. After three siphoning the infusion was filtered through Whatman filter paper No. 1 and evaporated to dryness under vacuum at 40 ^{0}C using Rotary evaporator to get 56 g (18.6.%) methanol crude extract. Later from this methanol extract 50 g was dissolved in water and sequentially fractionated into n-hexane, chloroform and aqueous to obtain their respective fractions. All the fractions as well as crude methanol extract was preserved in refrigerator till further use.

2.2. Quantitative estimation of phytochemicals

2.2.1. Determination of total terpenoid content

Total terpenoid assay was performed using the procedure followed by Ghorai et al. [11] with slight modification. 200 µl of MeOH extract and its hexane, chloroform and aqueous fractions (3 mg/mL in methanol) were mixed with 1.5 ml of chloroform, and then allowed to rest for 3 min. Concentrated H_2SO_4 (100 µl) was added to each sample taken in 2.5 mL appendorf, then incubated for 2 h at room temperature in dark. After 2 hours of incubation, terpenoids settled down as dark reddish brown precipitate. The supernatant was carefully decanted and the precipitate was dissolved in 1.5 ml methanol. 100 µl from each sample in appendrofs were transferred in a 96 well plate for spectrophotometric analyses. Linalool (1.56 – 100 mg/200 µl, R^2= 0.999) was used to prepare a standard curve. The Absorbance was recorded at 538 nm in a spectrophotometer against methanol. The assay was performed in triplicate and concentration was expressed as equivalent to mg linalool/ g extract.

2.2.2. Determination of total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu spectrophotometric method following the procedure adopted by kaur et al. [12]. Briefly 100 µl of MeOH, hexane, chloroform and aqueous extract solutions (4 mg/mL in DMSO) were mixed with 100 µL of 1 N Folin–Ciocalteu reagent followed by 5 min incubation in dark. After 5 minutes, 200 µL of 20 % Na_2CO_3 was added to each sample taken in in appendrofs to avoid precipitation. The samples were then transferred to 96 well plates and absorbance was measured at 730 nm using microplate reader Tecan Infinite

M Nano Elisa plate Reader (Austria). Concentration of phenolic compounds was calculated using the standard curve of gallic acid (2–10 µg; R^2 = 0.989). The results were expressed as gallic acid equivalent mg GAE/g extract.

2.2.3. Determination of total flavonoid content

For flavonoid content, 100 µl of crude extract and its fractions (50 mg/mL in DMSO) were mixed with 30 µL of a 5 % $NaNO_2$ solution and then incubated for 5 min, then after 300 µL of 10 % $AlCl_3.H_2O$ solution was added followed by addition of 200 µL of 1 M NaOH and 200 µL of distilled water after 6 min. Absorbance was read at 510 nm in a plate reader and total flavonoids were calculated using quercetin as standard (2–20 µg; R^2 = 0.981). The results were expressed as quercetin equivalent mg QR/g of extract.

2.3. In vitro methods employed in antidiabetic studies

2.3.1. α-Amylase Inhibitory Assay

This assay was carried out according to the procedure followed by Kim et al. [13] with slight modification. Briefly, 100 µl of different concentrations (100-1000 µg/mL) of chloroform fraction were added to 100 µl of 50 mM sodium phosphate buffer (pH 6.8) containing α-amylase solution (1.5 mg/mL). This solution was incubated at 25^0C for 20 min, after which 100 μL of 1% soluble starch solution in 50 mM sodium phosphate buffer (pH 6.8) was added and then again incubated at 25^0C for 10 minutes. The reaction was stopped by adding 500 μL of dinitrosalicylic acid (DNS) reagent followed by incubation in boiling water for 5 min and then cooled at room temperature. The resulting mixture was then diluted with 5 mL distilled water and the absorbance was measured at 540 nm. A control was prepared using the same procedure replacing the extract with distilled water.

2.3.2. α-Glucosidase Inhibitory Assay

The assay was based on the method described by Mir et al. [14] with few modifications. Briefly 30 µL of phosphate buffer solution, 30 mL sample solution with various concentrations (100-1000 µg/mL) and 20 µL *P*-nitrophenyl glucopyranoside (PNPG) substrate at concentration of 5 mM were put in 96 well microplate. This mixture was incubated at 37°C for 5 min. After 5 min, 20 µL of α-glucosidase solution 0.15 U/mL was added in each well to obtain total volume of 100 mL. The mixture was incubated for 15 min to get the complete hydrolysis reaction. After 15 min, the reaction was stopped by adding 100 µL of 200 mM Na_2CO_3. Absorbance was measured at 405 nm using a microplate reader. The assay was performed in triplicate.

Calculation of Percent Inhibition.

The data obtained from the activity test was processed to get percentage inhibition using the following formula:

$I\% = [(Ac - As)/(Ac)] \times 100$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

The concentration of the plant extracts required to scavenge 50% of the radicals (IC_{50}) was calculated by using GraphPAD Prism Software Version 5.0.

Statistical analysis

Variation in concentration of total phenol, flavonoid and terpenoid content in MeOH extract and its fractions were analyzed by one way ANNOV at 95% level. These analyses were performed in Graph pad prism 5.0.

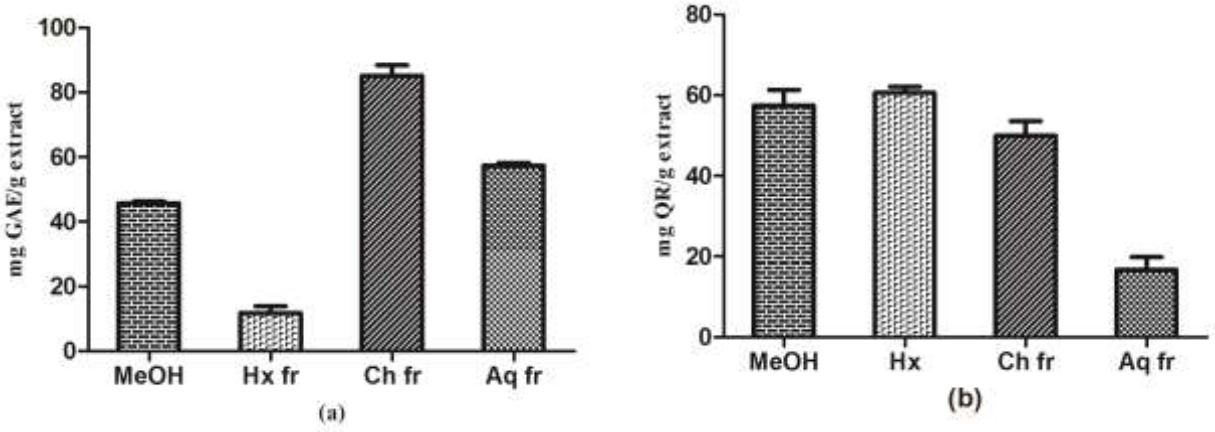
3. Results and Discussion

3.1. Total phenol, flavonoid and terpenoid content

Phenolic compounds which are considered to be important constituents of plant are responsible for various pharmacological activities including antioxidant [15]. The association of redox properties of phenolic compounds allows them to donate hydrogen atoms, electrons and as reducing agent [16]. The total phenolics of 4 samples (MeoH, Hx fr, Ch fr and Aq fr) and expressed in gallic acid equivalents (GAE) per gram dry weight of extract depicted in **Fig. 1a** showing highest content in chloroform fraction (85.15± 5.59 mg GAE/g extract). Earlier studies showed 93.42±1.04 to be the highest content of phenolics in ethanol extract of whole plant obtained by using microwaves [17]; however through simple extraction processes like percolation and maceration total phenolic content of leaf in separate studies were observed to be ranging from 6.02±0.01 to 81.21±0.69 mg GAE/g DW [18,17,19]. Another important class of secondary metabolites possessing various biological properties like antibacterial, antiviral and anti-allergic activities is flavonoid [20]. Studied on its quantitative estimation reported 66.3 mg catechin equivalents/g in DW in crude ethyl estate and an amount of 81.21±0.69 and 26.30±0.31 mg RE/g DW in methanol and acetone extract of leaf of the herb [21,19]. In ethanolic extract of whole plant, total flavonoid content was reported to be 37.7 ± 1.66 and 23.25 ± 0.94 mg of RUE/g through percolation and microwave extraction processes [17]. In our present study the total flavonoid content ranged from 16.76 ± 3.05 to 60.55± 1.55 mg QR/ g of extract (**Fig. 1b**).

Terpenoids which are reported to possess numerous biological activities including inhibition of cholesterol synthesis [22] was found to be highest in chloroform fraction (636.81 ± 22.95 mg Le/g extract) and lowest in hexane fraction (336.81 ± 27.74 mg Le/g extract) [**Fig. 1c**]. Non availability of data related to total terpenoid content in *M. vulgare* made us unable to compare our results.

The above phytochemical parameters (total phenol, flavonoid, terpenoid) estimated in the present study clearly indicate that leaves of *M. vulgare* contain highest concentration of terpenoids than that of phenolcs and flavonoids.



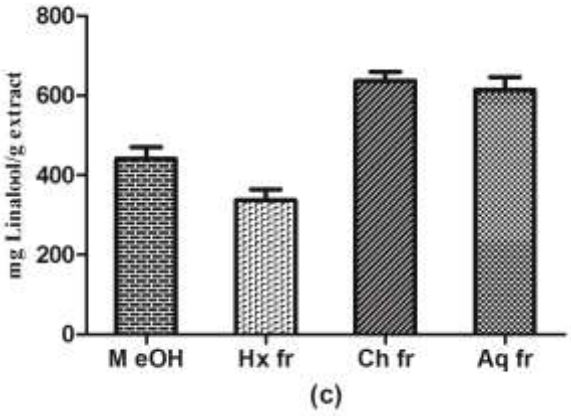


Figure 1: Total phenol (a) total flavonoid (b) and total terpenoid (c) content in the Methanol leaf extract and its fractions. All values are presented as mean ±SD and the means are significantly different (p<0.05) as determined by one way ANOVA.

3.2. Evaluation of in vitro α-amylase inhibitory activity of chloroform fraction

Diabetes mellitus which is increasing at an alarming rate throughout the world though several antidibetic agents are used but these commercially available antidiabetic agents are not free from side effects. Various new therapeutic approaches are being introduced for its management and treatment and among them is natural inhibitors (α-amylase & *α*-Glucosidase) which delay the degradation of carbohydrate and causes a decrease in the absorption of glucose; as a result the elevation of postprandial blood glucose level reduces [23].

In this in vitro study when subjected to different concentration of chloroform fraction (Ch fr) , the inhibition of α-amylase was observed to be concentration dependent (**Table 1**). Comparing the results with IC_{50} value of ascorbic acid (277.43±5.94) it is found that the fraction possess good inhibitory activity with IC_{50} value of 487.33± 2.65µg/mL but low to that of standard.

Table 1: α-amylase inhibition by chloroform fraction *of M. vulgare* leaf and standard Acarbose

Sample	Concentration (µg/mL)	% inhibition	IC_{50} (µg/mL)
Ch fr	200	25.12±0.52	
	400	42.71±0.46	
	600	56.08±0.95	487.33±2.65
	800	65.46±1	
	1000	72.52±0.45	
Acarbose	200	41.97±0.76	
	400	58.31±0.98	
	600	70.47±0.98	277.43±5.94
	800	79.21±0.54	
	1000	85.24±0.35	

3.3. Evaluation of in vitro α-glucosidase inhibitory activity of chloroform fraction

Alpha-glucosidase enzymes which are mainly distributed along the brush border of the intestinal epithelium plays an important role in the catabolism of sugars, so by suppressing the activity of these digestive enzymes, degradation of starch and oligosaccharides can be delayed which in turn can reduce the absorption of glucose as well as elevated postprandial blood glucose level. Such a process can be achieved by plant based inhibitors like α-glucosidase inhibitor, which in this study showed a significant action on α-glucosidase enzyme. The percentage inhibition at 100-1000 µg/mL concentration of chloroform fraction (Ch fr) showed a concentration dependent increase varying from 79.56 ± 0.47% to 30.26 ± 0.8 % (**Table 2**)

Table 2: α-glucosidase inhibition by chloroform fraction of *M. vulgare* and standard Acarbose

Sample	Concentration (µg/mL)	% inhibition	IC_{50} (µg/mL)
Ch fr	200	30.26±0.8	
	400	47.72±0.62	
	600	62.06±0.88	392.95±7.38
	800	76.79±1.1	
	1000	79.56±0.47	
Acarbose	200	43.52±0.52	
	400	55.61±1.41	
	600	76.67±0.31	269.23±4.99
	800	84.88±0.37	
	1000	87.75±0.31	

The concentration required to inhibit 50% (IC_{50}) activity by Ch fr was found to be 392.95±7.38 mg/mL and that of standard drug acarbose was 269.23±4.99 mg/mL. The result of α-glucosidase inhibitory activity depicts that Chloroform fraction of *M. vulgare* leaf exhibited good inhibitory activity of enzyme α-glucosidase by comparing with the standard acarbose, though not as much as that of standard drug but the difference is very less.

There are no previous records related to in vitro study on inhibitory action of α-amylase and α-glucosidase of *M vulgare* due to which we were unable to compare the results, however the in vivo studies [24,25,26,27] so far done showed significant antidiabetic activity exhibited by crude extracts of leaf on wistar albino rats. These studies were restrict to crude extracts of aerial parts only and suggested that presence of flavonoids and verbascoside derivatives could be responsible for antidiabetic activity [24, 25, 27]. In the present study which to our best knowledge is the first study till date in which we not only evaluated in vitro study of saturated fraction but reported that terpenoids could be responsible for inhibitory action on α-amylase and α-glucosidase.

4. Conclusion

In conclusion, the present study revealed that saturated fraction (Ch fr) containing highest amount of terpenoids exhibited considerable α-amylase and α-glucosidase inhibitory activities. Moreover in this study we report that terpenoids could be responsible in the management of diabetes, so need further validation through in vivo antidiabetic studies by diterpenoids from *M. vulgare* leaf which are main components of aerial part of the herb.

Acknowledgment

The authors are thankful to Natural Product Chemistry Division, CSIR-IIIM Jammu for providing necessary facilities for carrying out this study.

Conflict of Interest

All contributors of this manuscript declare no conflict of interest in this study

References

1. Chaudhary N, Tyagi, N. 2018. Medicinal plants used for Diabetes mellitus: An Overview. *International Journal of Research and Development in Pharmacy & Life Sciences* 7(4): 3022-3029.
2. IDF Diabetes Atlas. 9th Edition. Brussels: *International Diabetes Federation*, 2019.
3. Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, Colagiuri S, Guariguata L, Motala AA, Ogurtsova K, Shaw JE. 2019. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045:

Results from the International Diabetes Federation Diabetes Atlas. *Diabetes Research and Clinical Practice*. 157:107843.

4. Grieve A. 1984. Modern herbal. Penguin ISBN 0-14-046-440-9
5. Chevallier A. 1996. The encyclopaedia of medicinal plants. Dorling Kindersley, London. ISBN 9-780751-303148
6. De Souza MM, De Jesus RAP, Cechinel-Filho V, Schlemper V. 1998) Analgesic profile of hydroalcoholic extract obtained from *Marrubium vulgare*. Phytomedicine 5(2): 103-107.
7. Novaes AP, Rossi, C. Poffo E, Pretti Junior AE, Oliveira V, Schlemper R, Niero V, Cechinel-Filho, Burger C. 2021. Preliminary evaluation of the hypoglycemic effect of some Brazilian medicinal plants. *THERAPIE-PARIS* 56(4): 427-430.
8. Schlemper V, Ribas A, Nicolau M, Cechinel Filho V. 1996. Antispasmodic effects of hydroalcoholic extract of *Marrubium vulgare* on isolated tissues. *Phytomedicine* 3(2): 211-216.
9. Yamaguchi K, Liggett JL, Kim NC, Baek SJ. 2006. Anti-proliferative effect of horehound leaf and wild cherry bark extracts on human colorectal cancer cells. *Oncology reports* 15(1): 275-281.
10. Paula de Oliveira A., Santin JR, Lemos M, Klein Junior LC, Couto AG, Meyre da Silva Bittencourt C, Filho VC, Faloni de Andrade S. 2011. Gastroprotective activity of methanol extract and marrubiin obtained from leaves of *Marrubium vulgare* L. (Lamiaceae). *Journal of Pharmacy and Pharmacology* 63(9): 1230-1237.
11. Ghorai N, Chakraborty S, Gucchait S, Saha SK., Biswas S. Concentration in plant tissues using a monoterpene, Linalool as standard reagent.
12. Kaur T, Hussain K., Koul S, Vishwakarma R, Vyas D. 2013. Evaluation of nutritional and antioxidant status of *Lepidium latifolium* Linn.: a novel phytofood from Ladakh. *PLoS One* 8(8): e69112
13. Kim YM, Wang MH, Rhee HI. 2004. A novel α-glucosidase inhibitor from pine bark. *Carbohydrate research* 339(3): 715-717.
14. Mir MA, Sawhney SS, Jassal MM S. 2015. In-vitro antidiabetic studies of various extracts of Taraxacum officinale. The *Pharma Innovation* 4(1, Part B): 61
15. Balasundram N, Sundram K, Samman S. 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food chemistry* 99(1): 191-203.
16. Afanas' ev IB, Dcrozhko AI, Brodskii AV, Kostyuk VA, Potapovitch AI. 1989. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochemical pharmacology* 38(11): 1763-1769.
17. Vineet M, Arun N. 2016. The pharmacognostical evaluation of the *Marrubium vulgare* Linn collected from the Pulwama district of Jammu and Kashmir state in India. *Journal of Chemical and Pharmaceutical Research* 8(10): 7-15
18. Amri B, Martino E, Vitulo F, Corana F, Kaab LBB, Rui M, Rossi D, Mori M, Rossi S, Collina S. 2017. *Marrubium vulgare* L. leave extract: Phytochemical composition, antioxidant and wound healing properties. *Molecules* 22(11): 1851.

19. Amessis-Ouchemoukh N, A bu-Reidah IM, Quirantes-Pine R, Madani K., Segura-Carretero A. 2014. Phytochemical profiling, in vitro evaluation of total phenolic contents and antioxidant properties of *Marrubium vulgare* (horehound) leaves of plants growing in Algeria. *Industrial Crops and Products* 61:120—129.
20. Middleton E, Kandaswami C, Theoharides TC. 2000. The effectsof plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews* 52(4): 673—751.
21. Bouterfas K, Mehdadi Z, Elaoufi MM, Latreche A, Benchiha W. 2016. Antioxidant activity and total phenolic and flavonoids content variations of leaves extracts of white Horehound (*Marrubium vulgare* Linne) from three geographical origins. *In Annales pharmaceutiques francaises* 74(6): 453-462.
22. Indumathi C, Durgadevi G, Nithyavani S, Gayathri PK.. 2014. Estimation of terpenoid content and its antimicrobial property in *Enicostemma litorrale*. *International Journal of ChemTech Research* 6(9): 4264-4267.
23. Rhabaso LR, Chiasson JL. 2004. Glucosidase inhibitors. In: Defronzo RA, Ferrannini E, Keen H, Zimmet P. (Eds.), International Textbook of Diabetes Mellitus, Vol. 1, Edn 3, John Wiley and Sons Ltd., UK, 901-914.
24. Boudjelal A, Henchiri C, Siracusa L, Sari M. 2012. Compositional analysis and in vivo anti-diabetic activity of wild Algerian *Marrubium vulgare* L. infusion. *Fitoterapia* 83(2): 286–292.
25. Elberry AA, Harraz FM, Ghareib SA, Gabr SA, Nagy AA, Abdel-Sattar E. 2015. Methanolic extract of *Marrubium vulgare* ameliorates hyperglycemia and dyslipidemia in streptozotocin-induced diabetic rats. *International Journal of Diabetes Mellitus* 3(1): 37-44.
26. Ghlissi Z, Atheymen R, Sahnoun Z, Zeghal K, Mnif H, Hakim A. 2015. The Effect of *Marrubium vulgare* L. on Hyperglycemia-Mediated Oxidative Damage in the Hepatic and Renal Tissues of Diabetic Rats. *International Journal of Pharma and Chemical Research* 1(2): 97-106.
27. Chakir ARS, Elbadaoui K, Alaoui TI. 2015. Antidiabetic activities of methanolic extracts of *Marrubium vulgare* leaves in rats. *International Journal of Pharmaceutical and Phytopharmacological Research* 4(5): 258-263.