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NOVEL BITTER MELON (*MOMORDICA CHARANTIA L.*) AND OLIVE LEAVES (*OLEA EUROPAEA L.*) PHYTOSOMES: PREPARATION AND ITS EVALUATION FOR ANTI-HYPERGLYCEMIC ACTIVITIES BY ORAL GLUCOSE TOLERANCE TEST (OGTT)

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ABSTRACT

Objectives

The objectives of this study was to prepare and investigate the phytosomes of crude bitter melon and olive leaves extracts for anti-hyperglycemic activities in glucose-induced hyperglycemic rats.

Methods

Phytosomes were prepared by reacting one mole of the phenolic compounds of each extract with two moles of Phosphatydil-choline. Bitter melon and olive leaves phytosomes (150 and 200 mg/kg) were evaluated for oral toxicity.

Results

In the oral toxicity study, no mortality was observed among the rats received the phytosomes at the single dose of 150, 200mg/kg. Hence one tenth of the phytosomes dose tested (20mg/kg). Moreover, it is evaluated for hypoglycemic effects by oral glucose tolerance test in induced hyperglycemic rats. In the orally glucose induced hyperglycemic rats, phytosomes were significantly reduced serum glucose levels at 60, 90 and120 minute ($P<0.001$) than it is extracts. Most significant reduction was observed at 90 minute (26%). Glibenclamide was used as standard drug.

Conclusions

Our results indicated that the bitter melon and olive leaves phytosomes is more potent in anti-hyperglycemic activities than extracts. Clearly, further studies are warranted to improve our understanding of the underlying mechanisms.

KEYWORDS: *Bitter Melon Extract, Olive Leaves Extract, OGTT, Phytosome, Phosphatydil-Choline*

Article History

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder reaching pandemic proportions worldwide [1, 2]. In 2014, WHO reported that the prevalence of DM in adults aged 18 or more was 9%, 90% of them with T 2 DM [3]. More than 382 million individuals from all age groups is diagnosed with DM, which is estimated to reach about 592 million in 2035 [4]. Although, there are many available anti-diabetic drugs such as biguanides and sulphonylureas, yet they have several side effects, including hypoglycemia, liver toxicity, dyslipidemia, and hypertension [5]. Whereas, the natural plants are not only recommended to use from WHO, but also more cost-effective with lower side effects as compared to chemical drugs [6, 7].

Alternative medicine has been used widely in order to treat T2DM by using natural compounds. Nonetheless, limited evidence exists regarding the efficacy, safety, and mechanisms of action for the used natural compounds for the treatment of diabetes [8]. Furthermore, abundant plants products are available in the market that is useful for the treatment of metabolic disorder, including diabetes, nonetheless, these products are water-soluble, which can be easily destroyed by gastric acids and gut enzymes leading to a limited bioavailability [9].

The olive tree (*Olea europaea L.*) is cultivated in several parts of the world, but the Mediterranean region is the superior crop production area with approximately 98% of the world's olive cultivation [10]. Olive leaves are well known in folk medicine to treat hyperglycemia, hypertension, and infectious diseases, especially in Europe that attributed to its high phenolic compounds [11]. Whereas, bitter melon (*Momordica charantia* L.) is a tropical medicinal plant cultivated for its edible fruit. It's a rich in phenolic and saponin compounds, which are linked with higher antioxidant activity [12]. Bitter melon is a well-known agent to decrease blood glucose levels in Asia, Africa, and Latin America, and also its extracts had been called insulin vegetable [5].

Phytosome technology is patented technology based on a combination of various plant's extracts or water-soluble molecules to produce complex lipid molecules called phytosome [13]. Phytosome is highly absorbable and highly bioavailable with greater clinical benefits and without affecting the nutrient safety of the phytosome [14]. Phytosome protects the component of the herbal extracts away from destruction through digestive secretions and gut bacteria [15].Thus, phytosome technology is an effective method of delivery used for the improvement of various dietary supplements activity against diseases [16]. However, to best of our knowledge, studies on providing olive leaves and bitter melon extracts in the phytosome form to treat T2DM are scarce. Thus, the objective of the current study is to prepare crude olive leaves and bitter melon phytosomes, and to evaluate its anti-hyperglycemic activities in glucose-induced hyperglycemic rats by OGTT.

MATERIALS AND METHODS

Reagents

Glibenclamide (Hikma Company, Jordan) D-glucose, ethanol (England), acetone (Lonover chemicals, England), gallic acids (Sigma, USA. MW,170.12 g /mole). Phosphatidylcholine (Soyabean Lecithin Cargill, Belgium E 322. MW, 677.93 g /mole).

Animals

This research was approved by the Department of Nutrition and Food Technology Committee for Animal Experimentation/University of Jordan. 55 male Sprague-Dawley rats, weighing about 150-180 grams and 8 weeks of age were kept at Animal Unit, Scholl of Agriculture, the University of Jordan, Jordan. Each rat was housed in a single metabolically- ventilated plastic cage with a stainless steel wire mesh floor and front. The rats were kept under controlled temperature (22±2ºC) and maintained at a 12:12 hours light: dark cycles (from 19:00 pm to 7:00 am) with free access to water and standard laboratory chow for one week of acclimatization.

Preparation of Plants Material

On Apr 2016, olive (*Olea europaea*) leaves were randomly and directly collected from an olive tree (Baladi variety) from a local farm in Irbid, Northen-Jordan. The Baladi olive trees were of about 20 years old, not irrigated and no phytosanitary treatments had been utilized for the last10 years. Collected leaves were kept in plastic bags. The leaves were washed with running water to remove impurities such as dust, then dried under shade at room temperature and then crush using a mechanical blender (Moulinex Miller, France) and passed through mesh cloth to get leaves powder and stored at 4◦C until use [17].

Fresh bitter melon fruits were purchased from the local market in Irbid, Northern-Jordan. The fruits were washed with running water to remove impurities and then dried in an oven (WTC Binder, USA) at a temperature of 37 ◦C for 5 days and then crush using a mechanical blender (Moulinex Miller, France) and passed through mesh cloth to get plants powder and stored at 4◦C until use [18].

Preparation of Olive Leaves and Bitter Melon Fruits Extracts

Fifty grams of each plant powder was extracted with 500 mL of 95% ethanol. The mixture was mixed on a rotary shaker (New Brunswick Scientific, USA) for two hours at 180 rpm at room temperature 20 ◦C and then for 15 minutes in an ultrasonic bath at 37 °C (Bandelin Electronic-RK-103 H, Germany). The mixture was filtered through Whatman no: 4 and then membrane filter (cellulose type filter, 0.45 µm). After filtration, the solvent was evaporated under vacuum using a rotary evaporator (Büchi, RE 121, Switzerland) to remove solvent under reduced pressure at 38 °C and 120 rpm. All the obtained crude extracts were then transferred to vials covered with aluminum foil and then stored in the dark at room temperature to protect them from photo-oxidation [18, 19].

Total Phenolic, Flavonoids, and Tannins Content

The total phenols content of olive leaves and bitter melon fruits extract were determined by Folin-Ciocalteau assay and were expressed as gallic acid equivalent/gram of extracts (mg GAE/G) [20]. Total Flavonoids was determined by the aluminum chloride colorimetric method [20]. Total tannins were determined by Folin and Ciocalteu method [21].

Phytosome Preparation

Olive leaves and bitter melon phytosomes were prepared by adding 2 moles of phosphatidyl-choline for each one mole of the total phenolic compounds of plant extracts independently. In brief, 2 moles of phosphatidyl-choline was dissolved in a solution containing 200 ml of ethanol, and then one mole of polyphenolic compounds of plant extract was added. The mixture was mixed on a rotary shaker (New Brunswick Scientific, USA) for two hours at 180 rpm at room temperature. After that, ultrasonic bath (Bandelin Electronic-RK-103 H, Germany) was used to enhance the formation of the phytosome structure at 37C for 15 minutes. The solvent was evaporated under vacuum using a rotary evaporator (Büchi, RE 121, Switzerland) at 38 °C and 120 rpm. Finally, 50 ml of acetone was added to each mixture, and then left in the dark at room temperature to precipitate phytosome structure [13, 22].

Microroscopic Scanning

Microscope digital camera (Nicon YS2-H, Japan) was used to confirm the formation of phytosome complex between plant extracts and the phospholipids.

Acute Oral Toxicity Study

Oral toxicity test was performed according to the Organization of Economic Cooperation and Development (OECD) guideline 425. A total of 25 male Sprague-Dawley rats were used. All rats fasted overnight, then divided into five groups with five rats in each group. Group 1 stands as control group received distilled water. Groups (2 and 3) received a single oral dose (150, 200mg/kg) of olive leaves phytosome respectively. Groups (4 and 5) received a single oral dose (150 and 200mg/kg) of bitter melon fruits phytosome respectively. After the phytosomes administration, food withheld for 3-4 hours. Rats were observed post the dose at 0 min, 30 min, 1 h, 2 h, 4 h, 6 h, and every day for 14 days for general toxic signs, morphological behavior and mortality. One tenth of the dose was taken from the OGTT [23].

Oral Glucose Tolerance Test

A test was performed in overnight fasted normal rats. The experimental rats were divided into 6 groups of five rats each. Fasted rats have then received D-glucose solution (2mg/kg/) orally. After thirty minutes, rats were treated as follows: Group 1- stands for normal control group received distal water. Group 2 and 3 were received bitter melon phytosome and extract at the dose of 20mg/kg. Group 4 standard control group received the glibenclamide drug at the dose of 0.25mg/kg. Group 5 and 6 were received olive leaves phytosome and extract at a dose of 20mg/kg. The blood glucose level was estimated before glucose administration (-30 min), 30 min later (0 min) and at 30, 60, 90 and 120 minutes after administration of extracts, phytosome, and standard drug using a glucometer (ACCU-CHEK 68305 Mannheim, Germany) [24].

STATISTICAL ANAYLSIS

Experimental values are expressed as mean ± SEM. One–Way Analysis of Variance (ANOVA) was used. All Pair-wise comparisons were made using Duncan's Multiple Range Test (DMRT) post-hoc test. Statistical significance was considered to be indicated by a p-value ($p<0.001$).

RESULTS

Total Phenol Content

Table 1 shows the total phenolics, flavonoids, and catechin of olive leaves extract and bitter melon extract. The highest value was recorded in olive leaves extract.

Microscopic Scanning

A Microscopic scan showed that the phytosome is a complex cell- like structure in which phyto-constituents of the plants extract present on the inner surface of the phytosome and enveloped by PC (Figure 1).

Acute Toxicity Study

No mortality was observed among the rats received olive leaves and bitter melon phytosomes at the single dose of 150 and 200mg/kg. Hence, one-tenth of the tested phytosome dose was selected (i.e., 20mg/kg).

OGTT

Hyperglycemia induction by intra-gastric gavage of glucose resulted in a one -fold increase in glucose levels of the plasma by comparing groups at 0 minutes with that at -30 minutes (Table. 2). Olive leaves phytosome showed a reduction of blood glucose levels of 10, 20, 26, and 9% at 30, 60, 90, 120 minutes respectively. Whereas, bitter melon phytosome showed a reduction of 12, 14, 26, 10% at 30, 60, 90, 120 minutes respectively. Most significant reduction of glucose levels was observed in the phytosomes of olive leaves and bitter melon and then for glibenclamide drug at 90 minutes.

DISCUSSIONS

The total phenolic compounds of olive leaves and bitter melon extracts were consistency with reported values from different previous reports [25, 26]. Moreover, it is well known that the activities of the olive leaves and bitter melon extracts in management chronic diseases, especially diabetes are due to the antioxidant activities of the phenolic compounds.In this study, the quantitative phytochemicals of each extract responsible for the observed anti-hyperglycemic activities have not been isolated or determined, and further work is now ongoing in our laboratory to determine the phytochemicals content and bioavailability rate.

Microscopic scanning of the produced phytosomes showed a cell-like structure that was strongly in agreement with previous studies [27, 28]. The cell-like the structure of the phytosome resulted from the action of phosphatidyl-choline in which they bind to the phyto-constituents of herbal extract, and engulfing it to produce microsphere which is called phytosome [27]. Therefore, using phosphatidy l-choline in the phytosome structure is to make the structure highly similar to the cell membrane composition that facilitates and accelerate the absorption of the plants material into blood circulation [29].

The OGTT is an important and very necessary parameter to determine the rate of the body ability to consume glucose, and also gives information about the efficacy of a drug and plant extracts in acute hyperglycemia [24]. In this study, olive leaves and bitter melon extracts provided in the phytosome form at the dose of 20mg/kg reported higher glucose reduction of hyperglycemic rats in comparison to non- phytosome form. With regard to the hypoglycemic activities of the tested plants, the olive leaves constituent responsible for these effects is oleuropein and hydroxytyrosol, which are the most phenolic component [30], whereas bitter melon extract contents appear to have similarities with the

structure to the animal insulin-like charantin [31, 32]. Glucose levels were reduced by phytosome form could be attributed to the enhancement of the phenolic compounds of the extracts. In previous studies, the phytosome structure improves the levels of polyphenolic constituents in the blood serum with at least 2-6 times more than non phytosome form. This is actually attributed to the chemical bondages between polyphenol and phosphatidyl-choline molecules [33].

To our knowledge, the finding of this study constitutes the first experimental evidence of the reducing effect of olive leaves and bitter melon phytosomes preparation on glycaemia in laboratory rodents. Moreover, potency and efficacy of it may require further investigation.

CONCLUTIONS

Phytosome structure is a novel delivery system that overcomes the problems that are associated with the using of plants extracts such as poor stability in gastric environment, herbal toxicity, poor pharmacological activity, physical and chemical degradation, and high doses of ingredients that affect the safety and efficacy of the extracts. Olive leaves and bitter melon extracts provided in a phytosome form resulted significant anti-hyperglycemic activities in the glucose- induced hyperglycemic rats in compared to non-phytosome form. Further studies are required to monitor the bioavailability of the active compounds in blood, and other metabolic factors related to the metabolic process of the diabetes .

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APPENDIES

Table 1: Total Polyphenols, Total Flavonoids, and Total Tannins Extracted from Olive Leaves/Bitter Melon

Plant*	Total Polyphenols (mg GAE/g Extract)	Total Flavonoids (mg Rutin /g Extract)	Total Tannins (mg GAE /Extract)
Olive leaves extract	294 ± 6.1	18.18± 5.15	27.35± 10.3
Bitter melon extract	172± 7.45	13.46± 3.55	8.24± 4.6

*Values are represented as the mean of three replicates ± SEM

Table 2: Effect of Provide Olive Leaves and Bitter Melon Extracts and Phytosomes on the Glucose-Induced Hyperglycemia in Rats

Treatment	-30Minute	0 Minute	30 Minute	60 Minute	90 Minute	120 Minute
	Blood Glucose (mg/dl) **	Blood Glucose (mg/dl) %	Blood Glucose (mg/dl) %	Blood Glucose (mg/dl) %	Blood Glucose (mg/dl) %	Blood Glucose (mg/dl) %
Control	76.2 ± 0.86	152.2 ± 0.86 [a] 76	142.8 ±1.20 [a] -9	136.8 ± 0.80 [a] -6	125.8 ± 0.58 [a] -11	112.6 ±1.75 [a] -13
Bitter melon extract	74.2 ±1.16	144.8 ± 0.86 [b] 71	136.4 ±1.40 [b] -8	129.2±1.28 [b] -7	125.2±1.36 [a] -4	112.8 ±1.43 [a] -12
Bitter melon phytosome	74.0 ± 0.71	145.4 ±1.25 [b] 71	133.8 ±1.20 [b] -12	119.8 ±1.02 [c] -14	93.4±1.36 [b] -26	83.2 ±1.28 [b] -10
Glibenclamide drug	74.6 ±1.33	145.2 ±1.11 [b] 71	133.6 ±1.36 [b] -12	111.2 ±1.66 [d] -22	91.2 ±1.16 [b] -20	75.6 ±1.50 [c] -16
Olive leaves extract	76.8± 0.66	145.8 ±1.39 [b] 69	141.6 ± 0.93 [a] -4	132.4 ±1.54 [b] -9	125.0 ±1.52 [a] -7	115.0 ±1.52 [a] -10
Olive leaves phytosome	74.6±0.51	144.6 ±1.36 [b] 70	135.0 ±1.22 [b] -10	115.0 ±1.70 [d] -20	89.0±3.18 [b] -26	79.8 ±1.02 [b] -9
p-value	*0.205*	*0.001**	*<0.001**	*<0.001**	*<0.001**	*<0.001**

*One-Way ANOVA, all Pair-wise comparisons were made using Duncan's Multiple Range Test (DMRT) post-hoc test, and is significant at $p<0.001$.

** Data are presented as mean ±SEM

% Degree of blood glucose levels reduction

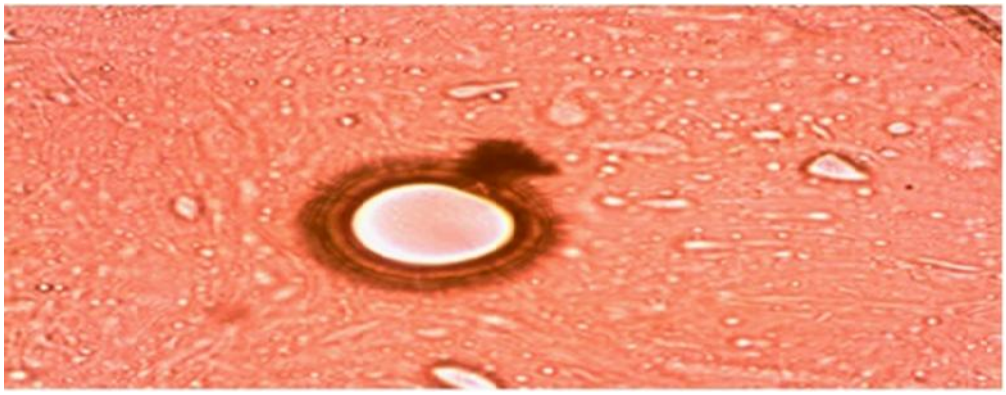

Figure 1: Microscopic Feature of the Phytosome