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Original Article

Exploring the Phytoconstituents, Differential Pharmacology, and Interaction with Empagliflozin of *Olea europaea* Leaves Extracts

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ABSTRACT

Background: The *Olea europaea* L. leaves have long been used in treating various illnesses. Its pharmacological activity based on the phytoactive constituents; therefore, optimizing the extraction process should intensify the benefits. **Aim:** The study aimed to optimize the extraction process for *O. europaea* L. leaves, evaluate their antioxidant and anti-inflammatory activities, and explore their influence on the biochemical parameters of diabetic animals. **Materials and Methods:** The differential pharmacology of the extracts and combinatorial therapy with the antidiabetic agent; empagliflozin, were explored. The aims were accomplished after several *in vitro* and animal studies: quantification of flavonoid and phenol content, measurement of the antioxidant activity, identification of the active constituents, and assessment of hepatic and renal functions, lipid profile, and glycemic status. In addition, molecular biology tools were used to measure the expression of the inflammatory mediators IL-6 and IL1beta. **Results:** Findings reveal that the hydroalcoholic binary system reinforced by the sonication yields the highest polyphenol (44.40±1.414 mg/g dry extract equivalent to gallic acid) and total flavonoids (31.0700±1.202 mg/g dry extract equivalent to quercetin). Extract by the same system showed high substantial antioxidant activity. HPLC-MS/MS reveals oleuropein and its aglycon, o- and p-coumaric acid, hydroxytyrosol acetate, and betaine compounds. A significant reduction in the average weight was recorded in diabetic mice (29.79±2.88 g) compared to the control (32.61±2.57 g). A significant reduction in producing the inflammatory mediators IL-6 and IL1 beta was measured. **Conclusion:** Olive leaves are a potential addition to conventional medicines to enhance the health profile of diabetic mice.

Keywords: Olive, *Olea europaea* L., Diabetes, Phytoanalysis, IL-6, IL1 beta, Phytotherapy, Biodiversity, Sustainability.

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INTRODUCTION

Diabetes Mellitus (DM) is one of the top ten leading causes of death worldwide.[1] The exact etiology of the disorder is still under debate. Both types I and II are characterized by high blood sugar levels and are caused by environmental and genetic factors.[2] Despite extensive research and the high impact on the global economy, DM remains insufficiently understood, therefore, limited efficient treatments available in markets.[3]

There is an increasing reliance on traditional medicine and heredity recipes in treating DM, along with other diseases, which stands as an unending practice. The ethnopharmacology of selected herbal remedies has confirmed biological activities in ameliorating blood sugar levels after several *in vitro, in vivo*, and clinical studies.[4] The hypoglycemic activity was linked to the contained secondary metabolites such as polyphenols, alkaloids, and terpenoids.[5]

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The Olive tree, *Olea europaea* L., a cultivated plant for 6000 years. Olive's cultivation started in the Mediterranean region, particularly in the Middle East, according to published reports (Zohary D, Spiegel-Roy P. Beginnings of Fruit Growing in the Old World: Olive, grape, date, and fig emerge as important Bronze Age additions to grain agriculture in the Near East. Science. 1975 Jan 31;187(4174):319-27). In more recent research, archaeologists from Jordan and France declared a small village in Southern Jordan called Hadeib Al-Reeh, as the oldest world site for olive cultivation. The *O. europaea* L. cultivar was found to be the most common in Jordan.[6]

Olive has been used by folk for its nutrient values and claimed medicinal purposes since the earliest Egyptians era.[6] Antioxidant properties, anti-inflammatory, antimicrobial, hypotensive, laxative, and antiplatelets are among the claimed biological activities in addition to their traditional use in treating DM.[7] The biological activities referred to the contained secondary metabolites such as polyphenols, amino acids, vitamins, and minerals.[8]

Olive oil is rich in bioactive phytoconstituents that were identified via various chromatography and spectroscopy techniques. These include nonpolar compounds such as squalene, tocopherols, sterols, triterpenes and polar polyphenolic compounds. Some of them are flavonoids that were detected in minute amounts in the oil, such as apigenin and luteolin. Other active constituents were detected such as tyrosol and derivatives, secoiridoids, oleacein, oleocanthal, and oleuropein.[9,10]

Olive extracts were previously investigated for their possible activity against diabetes and its complications. In the animal model, the plant was found to significantly enhance the glycemic profile, improve the biochemical parameters of diabetic mice and prevent diabetes complications such as renal impairment and hepatic damage.[11]

The antidiabetic activity of olives was estimated to be through the antioxidant activity of its phytoconstituents, hence the pathology of diabetes and its complications is associated with oxidative stress. The suggested mechanism of action was concluded after several *in vitro* and *in vivo* experiments, in which blood glucose and cholesterol levels improved in diabetic mice after administering oleuropein and hydroxytyrosol extracted from olive leaf.[12]

In a clinical study, the leaf extract was found as a potential remedy to retard and impede starch digestion, therefore, maintain normal blood sugar levels after a carbohydrate-rich meal in diabetic humans. The results suggested using olive extract as an adjunct hypoglycemic therapy.[13]

On the other side, empagliflozin is a synthetic antidiabetic agent that acts by inhibiting the Sodium-Glucose Co-Transporter-2 (SGLT-2) found in the proximal tubules in the kidneys.[14] Thus, it reduces renal glucose reabsorption and increases urinary excretion. Empagliflozin passed the FDA requirements and got approval in 2014 as a therapeutic agent for type II diabetes.[15]

The current research study aims to optimize the extraction process of *O. europaea* L. leaves and to explore the extracts' hypoglycemic activity by itself and as a combinatorial therapy with the known antidiabetic agent, empagliflozin. The overarching objective is to strengthen the link between traditional medicine and evidence-based pharmacology.

MATERIALS AND METHODS

Plant collection

Fresh leaves of *Olea europaea* L. trees were collected from private farms in Al-Karak, 130 Km south of Amman, on December 2021. The botanical information for *Olea europaea* can be verified at The Plant List database WFO Plant List | World Flora Online. The leaves were dried in shade, crushed until reduced size, and kept at 25°C until used. Plant identification and authentication was done by the Royal Society for the Conservation of Nature (RSCN), Amman-Jordan and a voucher sample was kept in the herbarium.

Plant extraction

Dried leaves were macerated in the extractive solvent in a ratio of 5:1 mg/mL for 4 hr. Five extractions were performed separately in different extractive conditions as follows: system I (Distilled water (D. water) at 25°C), system II (D. water at 60°C), system III (D. water at 100°C), system IV (D. water at 60°C and pH =3 (adjusted with aq. HCL of 0.2N)), system V (binary system of ethanol: D. water (4:1) at 25°C), and system VI (binary system of ethanol: D. water (4:1) at 25°C enhanced by sonication).

Quantification of total phenol and total flavonoids

Quantitative analysis of polyphenol content was performed following Folin-Ciocalteau specifications.[10] The absorbance was measured at λ_{max}=760 nm, the total concentration was computed as a gallic acid equivalent. Total flavonoids were quantified using a colorimetric reaction following the specifications adopted from a previously published report.[11] The absorbance was measured at λ_{max}=510 nm, the total concentration was computed as a quercetin equivalent. The absorbance was measured using a microplate reader (Spectra max M2, Molecular Devices, Sunnyvale, CA, USA).

Antioxidant activity

The antioxidant activity was assessed based on the radical scavenging effect of the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) following previously published specifications.[10] The absorbance was measured at t λ_{max} = 517 nm using a Spectrophotometer (Bio-Tek Instrument, USA). Ascorbic acid was used as the reference. The radical scavenging activity was computed using the following formula: % DPPH scavenging=100x[(Abs control)-(Abs Sample)]/(Abs control).

High Performance Liquid Chromatography-Mass Spectroscopy (HPLC/MS-MS)

A crude sample of each extract was prepared by dissolving in Dimethyl sulfoxide-DMSO and diluting with Acetonitrile before being injected into the automated system of LC-MS. The exact retention time and m/z were used to identify the phytoconstituents. A Bruker Daltonik® (Bremen, Germany) Impact II ESI-Q-TOF System equipped with Bruker Dalotonik Elute UPLC system (Bremen, Germany) was used for screening compounds of interest. Authentic standards were used for identifying m/z with high-resolution Bruker TOF MS and the exact retention time of each analyte after chromatographic separation.

The exact experiment specifications were adjusted as follows: The instrument operation involved an ApolloII ion funnel electrospray source. The capillary voltage, nebulizer gas, nitrogen flow rate, and dry temperature were set at 2500V, 2.0 bars, 8 L/min, and 200°C, respectively. Our system's high accuracy (<1 ppm) and resolution (50000 FSR) were pivotal, with a TOF repetition rate of up to 20 kHz. Chromatographic separation occurred on a Bruker solo 2.0_C-18 UHPLC column (100 mm x 2.1 mm x 2.0 µm), maintaining a flow rate of 0.51 mL/min and a column temperature of 40°C. The analysis, conducted in both positive and negative modes, had a total duration of 35 min with an injection volume of 3 µL. The mobile phase consists of acidified water with 0.05% formic acid (A) and acetonitrile (B).

Animal model

Development of a diabetic animal model using streptozotocin

A total of 105 Albino mice, with an average weight of 32.61±2.57, were obtained from the animal house and acclimatized and used for the experiments concerning the ethical guidelines adopted by the Faculty of Pharmacy. Diabetes was induced using streptozotocin and confirmed after measuring blood sugar values from the animals' tails as specified in a previously published report.[5]

A random mix of 90 diabetics and 15 non-diabetic experimental animals were grouped into seven groups of 15 animals each. The groups were treated as follows: Group 1 (negative control: D. water only, NC), Group 2 (diabetic mice), Group 3 (diabetic treated with empagliflozin (10 mg/kg), Group 4 (diabetic treated with the extract (300 mg/kg) and empagliflozin (10 mg/kg), Group 5 (diabetic treated with the extract (20 mg/kg) and empagliflozin (10 mg/kg), Group 6 (diabetic treated with the extract (20 mg/kg), and Group 7 (diabetic treated with the extract (300 mg/kg). All the treatments were given through the intraperitoneal route.

Empagliflozin (98% purity) was purchased from Carbosynth, UK. Streptozocin (75% alpha-anomer basis, 98% HPLC) was purchased from Sigma-Aldrich Chemical Co. Ltd., UK, and the solvents and chemicals were purchased from local suppliers.

Biochemical Analysis

Following the respective treatments. The blood samples were collected at different times during one month of treatments in plain and EDTA tubes. The blood samples were centrifuged at 2500 rpm for 10 min and stored at -20°C until used. Blood glucose level was measured using a glucometer (Glucolab, Infopia Co. Ltd.). Serum levels of cholesterol, triglyceride, Low Density Lipoprotein (LDL), High-Density Lipoprotein (HDL), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), total bilirubin, creatinine, and urea, were determined using chemistry analyzers (Abbott Alinity Series).

At the time of measurement, serum insulin concentrations were estimated using an ELISA kit according to the manufacturer's instructions.

Statistical analysis

A one-way Analysis of Variance (ANOVA) will be used to investigate any significant differences between groups, followed by ' 'Dunnett's *post hoc* test unless stated otherwise. The data will be analyzed by SPSS© 22 (SPSS, Inc., USA). For all statistical analyses, a *p*-value of less than 0.05 is considered statistically significant, and a *p*-value of less than 0.001 is considered highly statistically significant.

RESULTS

Extraction procedure

The binary system, ethanol: D. water (4:1) at 25°C, enhanced by sonication (system VI), was found to extract the highest yield. The least obtained yield was after extracting the leaves with the acidified water at 60°C. The percentage yield obtained by each of the systems I, II, III, IV, V, and VI was approximately 9.2, 9.8, 13.4, 9.0, 16.0, and 44.6%, respectively. The yield percentage was calculated based on the following equation: The weight of the dried extract/weight of the fresh leaves x 100%.

Quantification of total phenols and total flavonoids

According to the colorimetric assays results, the obtained total phenol calculated in equivalence to gallic acid was found to be the highest (44.40±1.414 mg/g dry extract) in system VI extract, followed by system V extract (27.80±1.31) and the least in the extract obtained by system I (12.1500±3.323 mg/g dry extract). Total phenols acquired by the other extracting systems were at comparable levels.

On the other hand, the computed average of the obtained total flavonoids calculated in equivalence to quercetin was found to be the highest in system VI (31.070±1.20 mg/g dry extract). The least concentration of total flavonoids was by extracting via system IV which found to be 7.54±3.01 mg/g dry extract. A multiple comparison analysis for the total polyphenol and total flavonoid

content in each plant extract (200 µg/mL) is demonstrated in Tables 1 and 2, respectively.

Antioxidant activity of *O. europaea* L. leaves extracts

The antioxidant effect, in the mean of radical scavenging activity, evinced that the extract obtained by system VI had the highest substantial dose-related activity over 30 min compared to the other tested extracts. Nevertheless, none of the investigated agents could be identified as equally effective as V.C. The V.C. was included for comparison purposes. Detailed antioxidant activities were demonstrated in Figure 1.

HPLC/MS-MS identification of the phytoconstituents content

According to the available library, several phytoconstituents have been identified by means of signals' retention time comparison and accurate molecular weight. The glycosylated seco-iridoid, oleuropein and its aglycon form are among the characteristic identified phenolic compounds. Ortho- and para-coumaric acid, hydroxytyrosol acetate, and betaine compounds were also detected. The analyzed sample was a representative of the extract by using system VI. The automated generated data by the Bruker® system is shown in Table 3.

Animal studies

A significant reduction in the average weight of the experimental animals was noticed after diabetes induction by streptozotocin injection. The average weight in normal mice was 32.61±2.57, and in diabetic mice 29.79±2.88 g (p value≤0.001). No significant variation in the animal weight was measured after one day of diabetic development (p value=0.75; >0.001).

Glycemic profiles and Animals weight

The analyzed results of plasma insulin measurements, by multiple comparisons test (LSD at α 0.05), for pairwise comparisons of several groups according to the treatment duration, confirmed no significant variation in the plasma insulin concentration between group members in the first week of treatment compared to those in the second week. On the other hand, the changes in insulin levels between the groups in the third and fourth weeks were found to be significant. The recorded insulin levels were the highest in group 4 after 3 and 4 weeks of treatments. Figure 2A shows the effect of groups of treatment on plasma insulin levels.

According to the blood glucose level, both group 1 and group 2 had a significant variation in blood glucose levels at all the measured time intervals compared to the other tested groups. Additionally, the glucose levels between members of Group 2 were significant difference compared to the other groups (groups 3-6). In short, glucose levels in group 2 were significantly different than the reported data in the other tests.

Besides, the measured levels in group 7 members were not significantly different than those measured in group 2 members which indicated that the profile of blood glucose levels was significantly improving during the 25-day study in all the tested animal groups except for those in group 7. Figure 2B illustrates the variation of the measured blood glucose levels in the 7 tested groups. Collectively, the effect of treatment duration on the blood

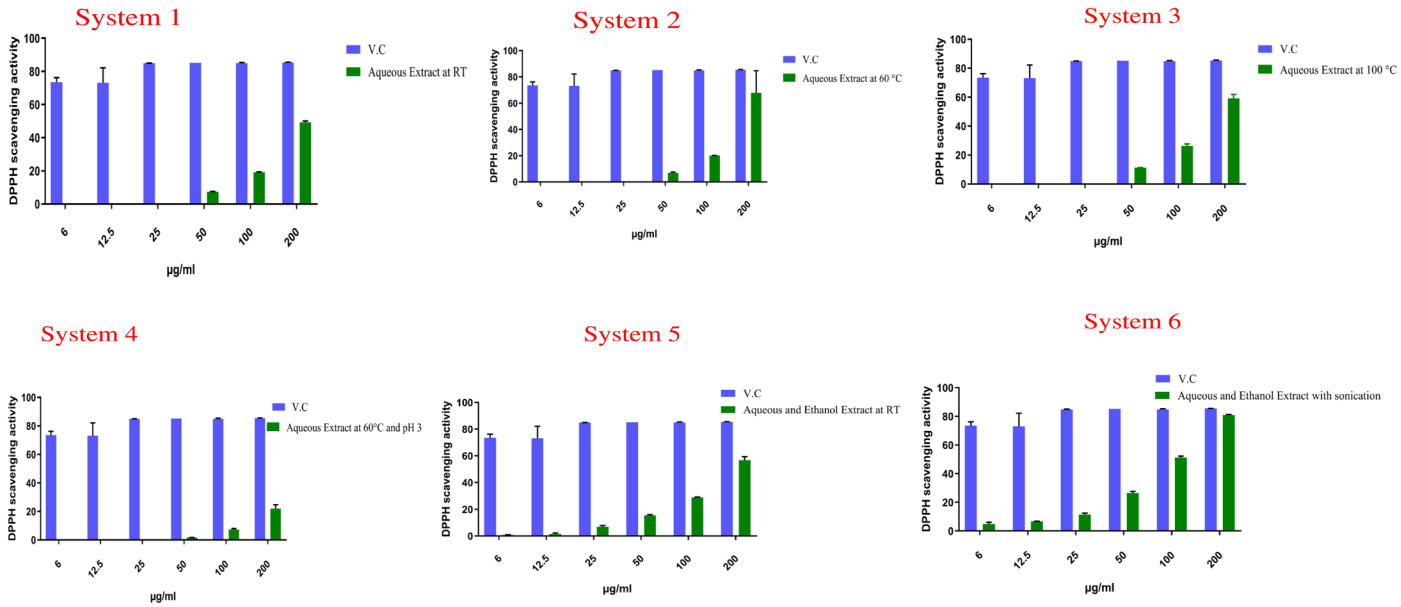

Figure 1: The modulatory radical scavenging effect of *O. eusropaea* L. leaves extracts vs the extract concentration µg/mL. Blue bars represent V.C., and green bars represent the extract type. The results are expressed as means of the three measurements±SD, n=3-4 independent replicates.

Table 1: Comparison analysis of total polyphenols content in *O. europaea* leaf extracts calculated equivalent to gallic acid (mg/g±SD dry extract).

		Descriptive		ANOVA		Multiple Comparisons LSD		
		Mean	Std. Deviation	F	Sig.	Group F	Group A-E	Sig.
200 µg/mL (Plant exrtact)	Water extract at room temp	12.1500	3.32340	28.867	p<0.001	Ethanol and water extract with sonication	Water extract at room temp	p<0.001
	Water extract at 60°C	13.7000	3.39411				Water extract at 60°C	p<0.001
	Water extract at 100°C	14.4000	2.54558				Water extract at 100°C	p<0.001
	Water extract at 60°C and pH 3	19.7500	5.58614				Water extract at 60°C and ph3	p<0.001
	Ethanol and water extract at room temp	27.8000	1.13137				Ethanol and water extract at room temp	0.002
	Ethanol and water extract with sonication	44.4000	1.41421					

Table 2: Comparison analysis of total flavonoids content in *O. europaea* L. leaf extracts calculated equivalent to quercetin (mg/g±SD dry extract).

		Descriptive		ANOVA		Multiple Comparisons LSD		
		Mean	Std. Deviation	F	Sig.	Group F	Group A-E	Sig.
200 µg/mL (Plant extract)	Water extract at room temp	14.2550	3.74059	7.524	.015	Ethanol and water extract with sonication	Water extract at room temp	.045
	Water extract at 60°C	16.4800	6.88722				Water extract at 60 c	.080
	Water extract at 100°C	22.1150	5.21138				Water extract at 100°C	.363
	Water extract at 60°C and pH 3	7.5350	3.00520				Water extract at 60°C and pH 3	.009
	Ethanol and water extract at room temp	20.7850	1.57685				Ethanol and water extract at room temp	.256
	Ethanol and water extract with sonication	31.0700	1.20208					

glucose profiles, of the tested groups, was demonstrated in Figure 2C.

In the manner of animal weight, a repeated measure ANOVA determined the mean value of the weight, which showed no significant differences between groups at different times and different groups within other groups.

Liver and Kidney functions, and lipid profile

Liver enzymes and total bilirubin levels were used as parameters of liver function. No significant variations in the measured levels of both aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) between the tested groups. On the contrary, Alkaline Phosphatase (ALP) and total Bilirubin (BILT) levels exhibited significant variations among the tested animal groups. To compare the results, multiple comparisons were conducted with the LSD at α 0.05, revealing that the ALP levels in normal mice (M=52, SD=0.5) were notably lower compared to those in diabetic mice and diabetic mice receiving various treatments. The BILT results indicate that normal mice and mice in groups G2-5 exhibit no significant differences. However, both G6 and G7 displayed significant increases in BILT values compared to normal mice. The data highlights that groups G4 and 5 displayed only minor enhancements in liver function,

Table 3: HPLCL/MS-MS identification of phytoconstituents from the *O. europaea* L. leaves extracts by extractive system VI.

RT min	m/z meas.	M meas.	Ions	MS/MS	Name	Molecular Formula
4.55	165.05	164.0473	$[M+H]^+$	True	p-Coumaric acid	$C_9H_8O_3$
3.63	165.05	164.0474	$[M+H]^+$	True	p-Coumaric acid	$C_9H_8O_3$
4.55	165.05	164.0473	$[M+H]^+$	True	o-Coumaric acid	$C_9H_8O_3$
0.26	118.08	117.079	$[M+Na]^+$, $[M+H]^+$, $[M+H.H_2O]^+$	True	Betain	$C_5H_{11}NO_2$
2.98	197.08	196.0734	$[M+H]^+$,$[M+H.H_2O]^+$	True	Hydroxytyrosol acetate	$C_{10}H_{12}O_4$
3.63	165.05	164.0474	$[M+H]^+$	True	o-Coumaric acid	$C_9H_8O_3$
7.57	361.13	378.131	$[M+H]^+$,$[M+H.H_2O]^+$	True	Oleuropein-aglycon	$C_{19}H_{22}O_8$
8.16	541.19	540.184	$[M+Na]^+$, $[M+H]^+$	True	Oleuropein	$C_{25}H_{32}O_{13}$

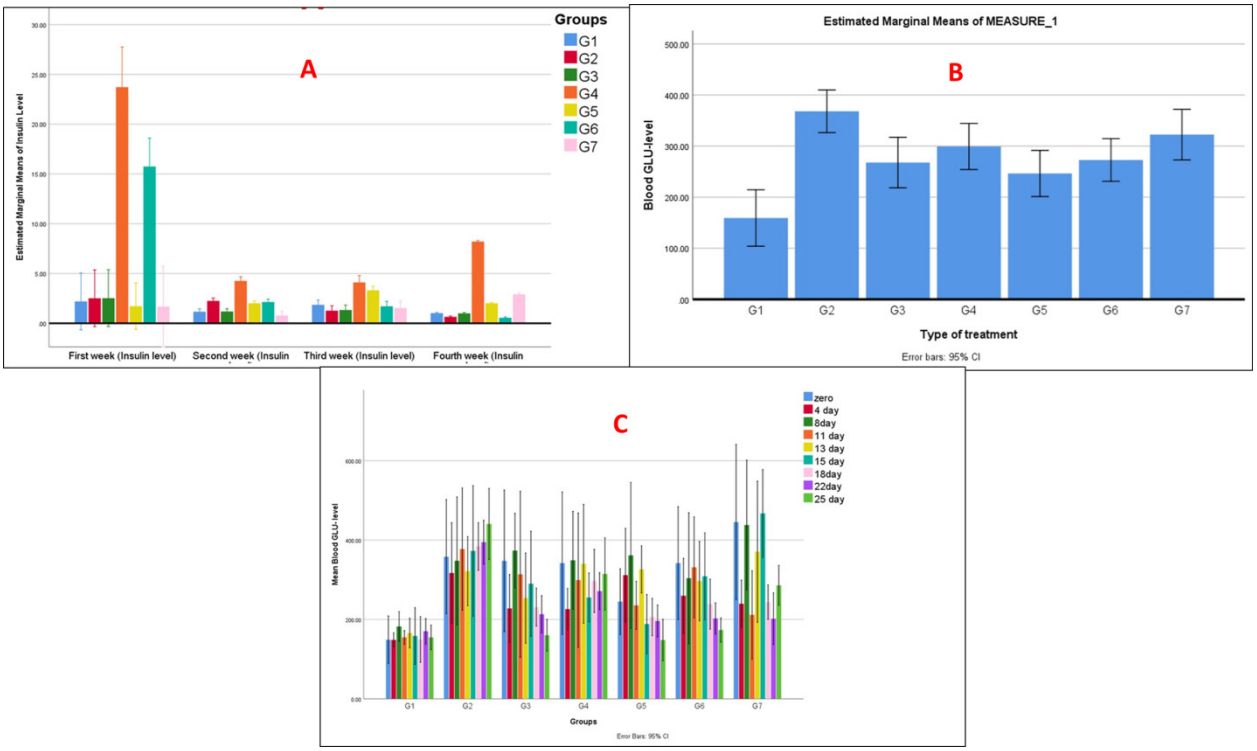

Figure 2: The glycemic profiles. A shows the effect of groups of treatment on plasma insulin levels, 2B illustrates the variation of the measured blood glucose levels in the 7 tested groups, and C the effect of treatment duration on the blood glucose profiles, of the tested groups. G1: normal, G2: diabetic (no treatment), G3: diabetic+empagliflozin, G4: diabetic+extract 300 mg/Kg+empagliflozin, G5: diabetic+extract 20 mg/Kg+empagliflozin, G6: diabetic+extract 20 mg/Kg, G7: diabetic+extract 300 mg/Kg.

which may be attributed to the short treatment duration. Figure 3A displays the effect of groups of treatment on liver function.

Urea and creatinine levels were used as parameters of kidney function. The obtained biochemical data confirmed no significant differences in urea levels among the tested groups, while the measured creatinine levels were in significant disparity. Creatinine levels in the control group (non-diabetic) were markedly lower than the other tested groups even those receiving treatment. Figure 3B displays the effect of groups of treatment on kidney function.

The serum levels of cholesterol and triglycerides were used as a parameter of the lipid profile. The obtained results confirmed a significant variation in triglyceride levels among the tested animal groups. Contrariwise, the measured cholesterol levels reported no significant variations. A remarkably significant increase in both parameters was measured in treated mice (either diabetic or diabetic receiving herbal treatments) compared to the control (non-diabetic) group. Although some variations between the treated groups were observed, the treatment failed to reverse the hyperlipidemic effect on the experimental animals in the given time. For instance, the average triglyceride measured in group 7 was found to be significantly lower than in group 4. Figure 3 C displays the effect of groups of treatment on lipid profile. The results generated and analyzed by multiple comparisons test (LSD at α0.05) reveal the development of a diabetic model in all the tested animal groups.

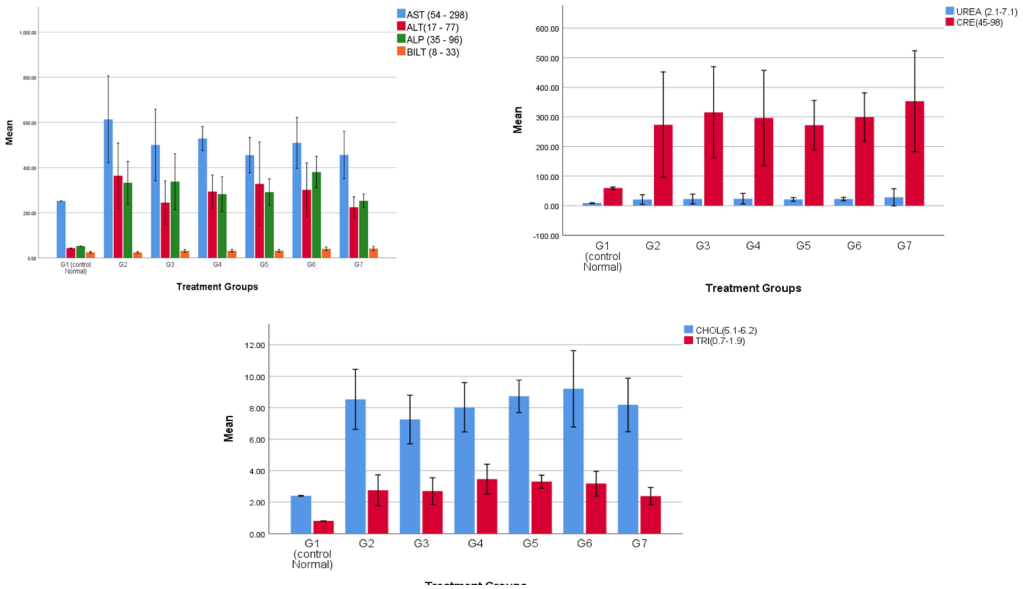

Figure 3: Biochemical parameters of groups of treatments. A represents liver function tests, B represents kidney function tests, and C represents the lipid profile. G1: normal, G2: diabetic (no treatment), G3: diabetic+empagliflozin, G4: diabetic+extract 300 mg/Kg +empagliflozin, G5: diabetic+extract 20 mg/Kg+empagliflozin, G6: diabetic+extract 20 mg/Kg, G7: diabetic+extract 300 mg/Kg.

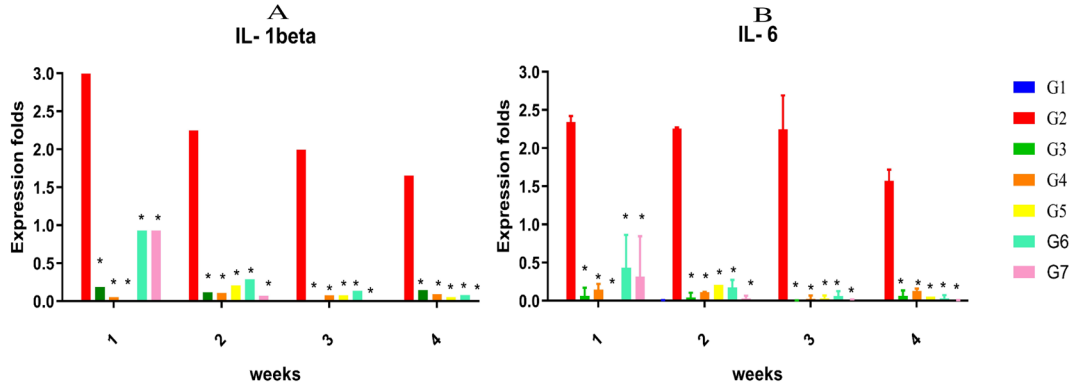

Figure 4: The effect of *Olea europaea* L. leaf extracts on the gene expression of IL-1beta (A) and IL-6 (B) over the treatment time. Values are the mean of three dependent replicates. *p<0.05, compared to diabetic mice, G2). the results were analyzed using one-way ANOVA.

Inflammation assay

At the molecular level, either treating by the conventional hypoglycemic agent, empagliflozin, or leaf extracts reveals a significant reduction in producing the inflammatory mediators IL-6 and IL1beta. These results were confirmed by an RT-PCR analysis, whereas significant downregulations of the induction of both IL-6 and IL1beta mRNA productions ($p<0.05$) compared to diabetic mice (group 2) were reported. Figure 4 demonstrated the effectiveness of various treatments during the trial period on gene expression of IL-6 IL-1beta separately.

DISCUSSION

Recently, a tight association between oxidative stress and metabolic disorders has been confirmed after several preclinical, clinical, and epidemiological scientific studies.[18] The depletion of biosynthesized antioxidants compared to the massive production of oxidizing molecules should lead to the accumulation of oxidized lipids and proteins, disruption at the cellular level, and consequently, to evolving of diseases and disorders.[19]

Polyphenols and flavonoids are secondary metabolites with confirmed antioxidant properties. The most efficient leaves extracting system was system VI (ethanol: D. water (4:1), 25ºC enhanced by sonication). The adopted system has been recommended by several various studies to obtain a better yield of polyphenols and flavonoid metabolites.[20] Enriching the medical plant extract with the highest possible antioxidants could be achieved by increasing the efficiency of flavonoids and polyphenol extractions, which in turn, is achievable by enhancing the hydroalcoholic solvent system with ta sonication step. The other used extractive systems are capable of extracting these secondary metabolites but to a lower extent compared to the sonication-enhanced system, as per discussed in previous studies.[21] Adding a sonication step was found to effectively increase the concentration of antioxidant compounds.[22] The role of sonication treatment is by increasing solvent porosity, which plays an important role in accelerating the solute's dissolution and diffusion and the heat transformation, which will improve the extraction efficiency in comparison with maceration, decoction, and even in Soxhlet's extraction method.[23] Endorsing this technique in extracting polyphenols and flavonoids from star fruit and Thymus serpyllum separately has evidence of efficacy.[22,23]

In aspects of pharmacological activity, treating with the natural remedy along with the conventional hypoglycemic agent, empagliflozin enhances the glycemic profile in diabetic animals after treating solely with the extract over the experiment time. These findings were compatible with a conclusion made by a systematic review of the published reports that the crude olive oil intake could be beneficial in preventing and treating diabetes mellitus type II.[24]

The improvement in the glycemic profile of diabetic animals is linked to the detected phytoconstituents in the prepared extract. The identified compounds have been previously reported to have antidiabetic activity after *in vitro* and *in vivo* experiments.

Starting with oleuropein: it was found that administering oleuropein along with vitamin C intensifies insulin secretion from the pancreatic β-cells and attenuates the effect of streptozotocin-induced diabetes in experimental animals.[25]

Furthermore, plasma betaine is inversely associated with multiple metabolic disorders, including insulin resistance, type II diabetes, and hyperlipidemia. Obese individuals were given betaine 3300 mg twice a day for ten successive days in a randomized placebo-controlled trial. Then, fasting blood sugar, insulin resistance, and other biochemical functions were analyzed. Betaine supplementation significantly reduced the fasting blood glucose level but it had no effect on the insulin sensitivity and intrahepatic glycosides, while the total cholesterol level increased in patients who received the supplements compared to those having placebo.[26]

Additionally, *O. europaea* L. leaves extract contains P-coumaric acid, a common constituent in higher plants. The acid has confirmed biological activities against oxidative stress, inflammation, and cancers.[27]

Moreover, it's hypoglycemic and hypolipidemic activities have been elucidated via an *in vivo* approach in diabetic rats. A daily dose of 100 mg/Kg of P-coumaric acid, and then Glucose Transporter (GLUT2 mRNA) expression of the pancreas. The obtained results revealed a significant decrease in blood glucose levels and glucogenic enzymes and an increased level of insulin. Besides, total cholesterol and triglyceride levels decreased in animals' plasma and tissues.[28] Similar findings were obtained after administering hydroxytyrosol to diabetic animals.[29]

Although the results obtained didn't firmly confirm the positive role of olive extracts on the lipid profile and renal and hepatic health; previous data had linked the intake of olive oil contributed in decreasing thrombosis risk in hypercholesteremic animal models,[30] and in humans.[31]

In Jordan, olive leaves have been used for ages in treating various disorders relying on traditional recipes and folkloric medicine.[32] The reduction of the inflammatory mediator's production matched the expectations. The proposed mechanism of action was through suppression of the IKKβ/NF-κB inflammatory pathway, which in turn is considered a significant factor in inducing insulin resistance. Therefore, both the crude leave extract and oleuropein could enhance insulin secretion and improve lipid profiles.[33]

CONCLUSION

There is a unanimous agreement on the role of natural products in enriching the health sector with therapeutic agents and health-boosting compounds. It has been an inspiration for scientists to simulate its active components' biosynthesis in discovering new medicines. Optimizing the extraction processes of active principles from natural sources is a warranty to maximize biological activities. The significance of the current study is to ascertain the best extraction conditions for the efficient use of the active phytoconstituents in olive leaves. Using a hydro-alcoholic solvent system enhanced by sonication was the most productive. *In vivo* studies confirm the positive effect of the leaf extracts in enhancing the glycemic profile, and liver and kidney functions, and combat inflammation either by itself or in combination with the conventional antidiabetic agent, empagliflozin.

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CONFLICT OF INTEREST

The authors declare that are no conflict of interest.

ETHICAL STATEMENT

All procedures are conducted in accordance with the guidelines for the use and care of laboratory animals, and the protocol was approved by the scientific and ethical committee, Faculty of Pharmacy, Mutah University-Approval number 5/2022/2023.

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