



## **Effect of *Olea europaea* L. and *Juglans regia* L. Extracts on Human Cancer Cell Line Viability with Studying of Hypoglycemic and Antiglycation Properties**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author AD designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript, contributed to the implementation of experiments and managed the literature searches. Author RK contribute in design experiments and literature searches. Authors CAM, MAM, SH and HY carried out the experiments. Authors EA and TI helped the authors CAM, MAM, SH and HY in laboratory to carried out the experiments. All authors read and approved the final manuscript.*

### **Article Information**

DOI: 10.9734/JPRI/2021/v33i1431269

#### Editor(s):

- (1) Dr. Juan Carlos Troiano, University of Buenos Aires, Argentina.
- (2) Dr. Asmaa Fathi Moustafa Hamouda, Jazan University, Saudi Arabia.

#### Reviewers:

- (1) Adedirin Oluwaseye, Sheda Science and Technology Complex, Nigeria.
  - (2) Moan Jéfer Fernandes Costa, Universidade Federal do Rio Grande do Norte, Brazil.
- Complete Peer review History: <http://www.sdiarticle4.com/review-history/65731>

**Original Research Article**

**Received 15 December 2020**

**Accepted 21 February 2021**

**Published 19 March 2021**

### **ABSTRACT**

There is an urgent and continuous need to discover new sources of medicinal plants to obtain useful compounds with health properties. So the purpose of this study was to investigate activity of *Olea europaea* L and *Juglans regia* leaves extract on Hela cell line viability, antidiabetic and Antiglycation. The aqueous extracts were obtained from leaves. Alloxan 180 mg /kg body was used to induce diabetes. Mice with blood glucose level of  $\geq 200$  mg/dl were considered as diabetic and were received 10 mg/kg of body weight of Extracts. For Antiglycation, SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) were prepared for the appearance of high molecular weight products. Hela cell line were cultured in RPMI-1640 medium for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay analysis. The results showed that the

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mean of glucose levels decreasing in mice that treated with 10 mg/Kg extracts of *O. europaea* and *J. regia*. Also SDS-PAGE showed that extracts of *J. regia* were better than of *O. europaea* in inhibition of glycation induced protein cross-linking at all studied concentrations. MTT assay showed that the Cytotoxicity was increased with increasing the doses of extract and the cytotoxic effects of *J.regia* extract were higher than *O.europaea* at all concentrations. This study showed promising results.

**Keywords:** *Olea europaea* L; *Juglans regia* L; hypoglycemic; antiglycation; Hela cell line; MTT.

## 1. INTRODUCTION

Plants and natural products have been shown to present interesting biological and pharmacological activities and are used as therapeutic agents. Medicinal plants play an important role in general health and treatment of diseases for thousands of years and still using in traditional medicine systems around the world [1].

Cancer is one of the main causes of human diseases, is cause by abnormal cells that division uncontrolled. It is evaluated that there will be approximately 20 million new cancer cases in the whole world by the end of the year 2025 [2], so there are needs for finding more effective and safe treatment options for cancers, that is which have promoted researchers all over the world to discover new anticancer agents; plants have been used in treating cancer historically because plants have ability to produce secondary metabolites; a lot of these natural products have pharmacological and biological activities and are used as chemotherapeutic agents [3].

Diabetes is also one of the most common chronic diseases in nearly all countries. Diabetes is ranked seventh among the diseases that are leading to cause death and it is ranked third when fatal complications are taken into the account, and the number of people suffering from the disease worldwide is increasing at an alarming rate with a projected 366 million people likely to be diabetic by the year 2030 compared to an estimated 171 million sufferers in 2000 [4]. Protein glycation plays a key role in the development of chronic complications associated with diabetes such as neuropathy, nephropathy retinopathy and atherosclerosis [5-6], Protein glycation is initiated as a non-enzymatic reaction between reducing sugars and proteins followed by a series of reactions leading to formation of advanced glycation end products (AGE).

Alloxan is one of the chemical compounds used for the induction of diabetes [7-8]. The

diabetogenic dose of alloxan varies considerably amongst species, age and metabolic state of the animal. Alloxan induced diabetes mechanism: Alloxan is reduced to dialuric acid and re-oxidized to alloxan producing alloxan radicals and reactive oxygen species (ROS) which undergo dismutation (by superoxide dismutase, SOD) to form hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Hydroxyl radicals (\*OH) may also be formed by side reactions. These \*OH cause  $\beta$ -cell DNA fragmentation, leading to apoptosis [9].

Several studies confirm the potential therapeutic efficiency of some medicinal plants in treatment of diabetic patients and prevent glycation especially polyphenols and other natural antioxidants, so the daily intake of natural products can play a beneficial role in preventing the diseases. Many traditional treatments have been recommended in the alternative system of medicine for treatment of diabetes mellitus. *Olea europaea* L. with phenolic contents [10] and *Juglans regia* L. with flavonoids [11] are two of the medicinal plants that are used as a treatment for diabetes. Plants have historically been used in treating cancer and are recognized for their ability to produce secondary metabolites. There is an urgent and continuous need to discover new sources of medicinal plants to obtain useful compounds with health properties [12]. So the purpose of this study was to investigate antidiabetic effects of *O. europaea* L and *J. regia* leaves extract in Alloxan-diabetes induced mice, and to study Antiglycation effects against induced protein cross-linking and to investigate activity on Hela cancer cells viability.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Sample

The *J. regia* L. and *O. europaea* L. leaves were collected from Amman, Jordan. Mice and Hela cell line were obtained from Biotechnology and genetic engineering department, Philadelphia University.

## 2.2 Extraction Procedure

The leaves were washed with distilled water and placed in oven at 37°C to dry. Then, the leaves were completely pulverized. 200 g was soaked in 1000 ml distilled water and placed on incubator with shaking for 24 hours at 37°C. The solution was filtered through a filter paper and dried in the oven at 39°C [13].

## 2.3 Experimental Animal

Adult and healthy mice (*Mus musculus*) of 30-35 g were used.

## 2.4 Diabetic Induction

Alloxan was used to induce diabetes. Mice (33 g) were injected with subcutaneous injection 180 mg /kg body (5,94 mg/ 33 g) of freshly prepared of alloxanin [14]. Diabetes mellitus was confirmed by testing blood glucose after 24 hours, and mice with blood glucose level of  $\geq 200$  mg/dl were considered as diabetic.

## 2.5 Experimental Design

The total of 40 mice (n= 10) were grouped randomly into 4 groups as follows: Group I: Normal control (NC): mice of this group did not receive induction and treatment. Group II: Diabetic control (DC): diabetic mice of this group did not receive treatment. Group III: Treated diabetic group (TDO): diabetic mice of this group received 10 mg/kg of body weight of *O. europaea* Extract. Group IV: Treated diabetic group (TDJ): diabetic mice of this group received 10 mg/kg of body weight of *J. regia* Extract.

## 2.6 Detection of Glycation Induced Protein cross-linking

Working solutions were prepared as described by Perera and Ranasing [15] with slight modification: 200 mM phosphate buffer (pH 7.4) containing 0.02% sodium azide, chicken egg lysozyme, Fructose (500 mM), *O. europaea* and *J. regia* L. leaves extracts (5, 10, 15 and 20 mg/ml). Solutions were incubated 20 days at 37°C.

## 2.7 SDS-Page

SDS-polyacrylamide gels (12%) were prepared according to Laemmli [16] for the appearance of high molecular weight products. Aliquots from the incubation mixtures were heated with the SDS sample buffer at 95°C for 3 min, before loading

to the gel. Molecular weight markers (10–225 kDa) were used to assess the approximate size of the high molecular weight products. After separation at pH 8.6, protein bands were visualized by staining with Coomassie blue.

## 2.8 Culture of Hela Cells

Hela cells were cultured in RPMI-1640 medium with 10% FBS and 1% antibiotics, Maintained at 37°C and 5% CO<sub>2</sub> atmosphere, Cells were subcultured every 2-4 days with trypsin/EDTA.

## 2.9 MTT Assay for Cytotoxicity

Cells were seeded in a 96-well flat-bottom microtiter plate at a density of  $1 \times 10^4$  cells/well and allowed to adhere for 24 hours at 37°C in a CO<sub>2</sub> incubator. After 24 hours of incubation, culture medium was replaced with a fresh medium. Cells were then treated with various concentrations (6.25, 12.5, 25, 50 and 100  $\mu$ L) of plant extracts for 24 hours at 37°C in a CO<sub>2</sub> incubator. After 24 hours of incubation, culture medium was replaced with a fresh medium. Subsequently, 10  $\mu$ L of MTT working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 4 hours at 37°C in a CO<sub>2</sub> incubator. The medium was then aspirated, and the formed formazan crystals were solubilized by adding 50  $\mu$ L of DMSO per well for 30 min at 37°C in a CO<sub>2</sub> incubator. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 540 nm. And the viability was calculated from following formula:

$$\text{Viability\%} = (\text{Test OD} - \text{blank OD}) / (\text{Control OD} - \text{blank OD}) \times 100$$

## 3. RESULTS AND DISCUSSION

### 3.1 Antidiabetic Effects

The results showed that the mean of glucose levels of normal control mice were about  $111.5 \pm 0.05$  mg/dL, while the diabetic control mice that received Alloxan were significantly higher than control about  $440 \pm 0.01$  mg/dL. Mice that treated with 10 mg/Kg of *O. europaea* and *J. regia* extracts showed decreasing in the glucose levels to become ( $197 \pm 0.07$  mg/dl and  $181.5 \pm 0.08$  mg/dl) respectively after 7 days of treating diabetic mice, Table 1 and Fig. 1. The antidiabetic effect of an alcohol extract of *Olea europaea* L. leaves was studied, where the

streptozotocin-induced diabetic rats received oral administration of the leaves extract for 14 days and showed significantly decreased the serum glucose [17]. In another study, blood sugar decreased meaningfully when alloxan-induced diabetic rats were treated with leaves extracts of *J. regia* (200 mg/kg) [18]. The antidiabetic effect is due to the presence of phenolic and flavonoids compounds in the extracts, the flavonoids supports the regulation of carbohydrate digestion, insulin secretion and glucose uptake [19]. A study on 200,000 women and men estimated the combination between dietary intake of flavonoids and type 2 diabetes lowers the risk of diabetes [20].

### 3.2 Detection of Glycation Induced Protein Cross-Linking

SDS-PAGE of aliquots were collected and compared after 20 days of incubation in the presence of plant extracts. SDS-PAGE showed that extracts of *J. regia* were better than extracts of *O. europaea* in inhibition of glycation induced protein cross-linking at concentrations (5, 10, 15 and 20 mg/ml) (Fig. 2). So, *J. regia* has high antiglycation properties and agree with Ahmad and his colleagues who supposed that *J. regia* extract could have therapeutic uses in inhibit chronic diabetic complications and slowing aging [21]. Extracts of *J. regia* at all used concentrations (5, 10, 15 and 20 mg/ml) prevented formation trimer (~36 KDa) and tetramer (~48 KDa) products, while high molecular weight products trimer and tetramer of lysozyme appeared when lysozyme was incubated in the presence of *O. europaea* extract at concentrations (5, 10, 15 and 20 mg/ml). Lysozyme was used as a model protein in many studies to display glycation associated protein cross-linking [22]. This current study gave an idea about the approximate inhibition to the cross-link formation using extracts by visual observation. Many antidiabetic plants and spices showed cross-link inhibitory effects, extracts of *Pterocarpus marsupium* and *Coriandrum sativum* have shown inhibitory effects on glycation induced protein cross-linking and *Syzygium*

*aromaticum* showed complete inhibition on the formation of cross-linking with 25 µg/ml extracts [23]. In our study, results with fructose clearly demonstrated that the extent of cross-linking was good because its effects occur at a more manageable rate, and Perera and Ranasinghe proposed that fructose is better than glucose and ribose, in detecting glycation induced cross-link formation [15].

### 3.3 MTT Assay

The results obtained from this study using MTT assay (Viability%) showed the Cytotoxicity of *J.regia* and *O.europaea* extracts on death of Hela cells compared to control group. The inhibitory effect of plant extract was dependent on concentration of extract and the species of plants, in Table 2 and Fig. 3 the Cytotoxicity was increased with increasing the doses of extract and the cytotoxic effects of *J.regia* extract were higher than *O.europaea* at all studied concentrations. The highest Cytotoxicity was observed at 100 µL after 24 hours at 37°C in a CO2 incubator. Also, the percentage of cell survival was lower at 6.25 µL. These anti-cancer properties in *O.europaea* are thought to presence phenolic compounds, especially oleuropein and hydroxytyrosol, oleuropein and hydroxytyrosol were studied for its effects on growth in MCF-7 human breast cancer cells using MTT assay, these compounds decreased cell viability and induced cell apoptosis in MCF-7 cells [24]. Oleuropein present in *O. europaea* leaves gave it very important scientific value due to its several biological properties including anticancer, Ruzzolini and his colleagues found that Oleuropein at a dose of 500 µM was able to stimulate apoptosis, while at dose of 250 µM was non-toxic [25]. Salimi et al. [3] assayed Cytotoxicity effects of various *J. regia* leaf extracts in Human oral squamous carcinoma (BHY), colon adenocarcinoma (HT-29) and breast adenocarcinoma (MCF7) cell lines with MTT assay. Their results indicated that MCF7 and BHY cells were best inhibited by total extracts after 48-h treatment, while HT-29 cells were not inhibited by them [3].

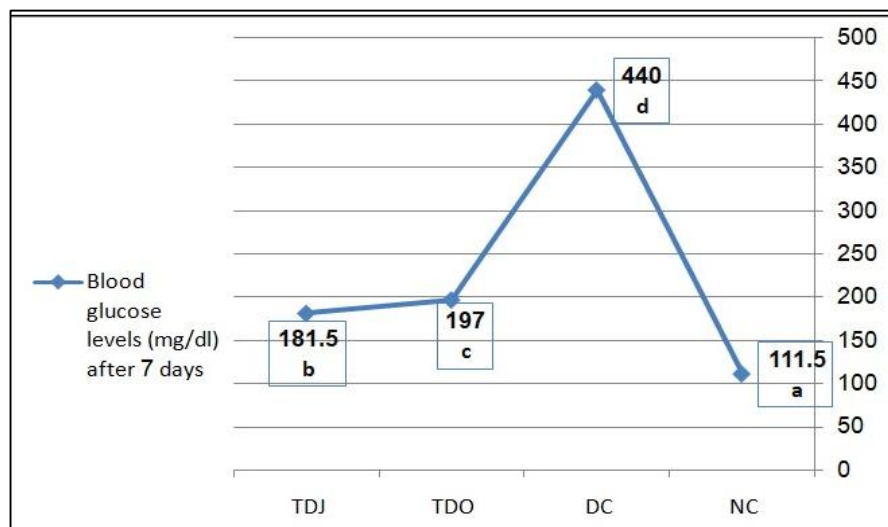
**Table 1. Effect of treatment with an aqueous extract of *O. europaea* and *J. regia* on blood glucose level after 7 days. Mice were received extract 10 mg / kg of body weight**

NC	Blood glucose level mg/dl (Means±SD)		
	DC	TDO	TDJ
111.5 ± 0.05 a	440 ± 0.01 d	197 ± 0.07 c	181.5 ± 0.08 b

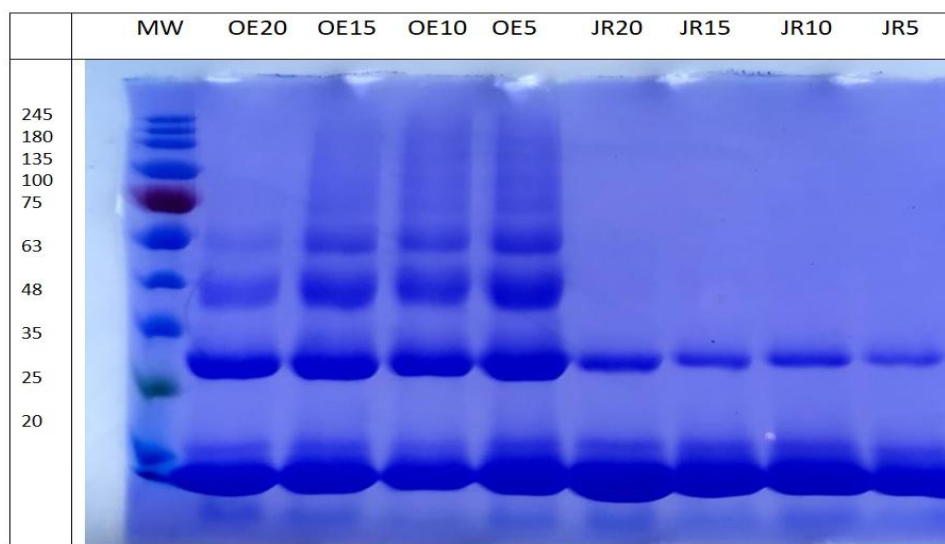
NC: Normal control, DC: Diabetic control, TDO: treated diabetic with *O. europaea* Extract, TDJ: treated diabetic with *J. regia* Extract.

**Table 2. The cytotoxic effect of 6.25, 12.5, 25, 50 and 100  $\mu$ L of *J.regia* and *O.europaea* extracts on death of Hela cells at 24 hours**

Concentration $\mu$ L	Viability%	
	<i>J.regia</i>	<i>O.europaea</i>
6.25	28.68	47.1
12.5	23.52	44.27
25	20.1	42.5
50	18.6	38.27
100	16.52	24.85



**Fig. 1. Blood glucose levels (mg/dl) after 7 days. NC: normal control, DC: diabetic control, TDO: treated diabetic with 10 mg/kg of *O. europaea* Extract, TDJ: treated diabetic with 10 mg/kg of *J. regia* Extract**



**Fig. 2. Effect of plant extracts at 20, 15, 10 and 5 mg/ml on glycation induced protein cross-linking. SDS-PAGE was conducted using aliquots collected on day 20. MW: Molecular weight markers, OE: *O. europaea*, JR: *J. regia***

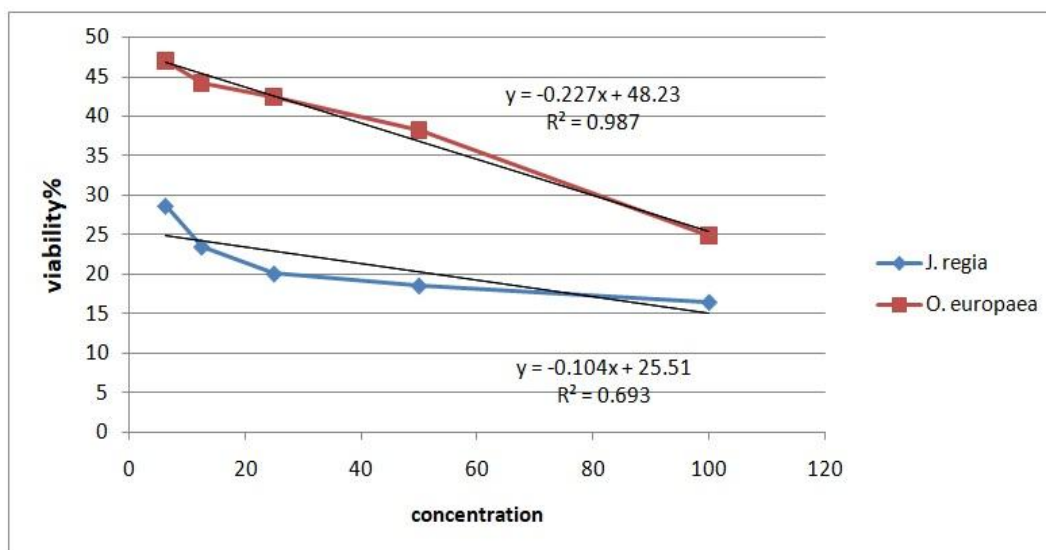


Fig. 3. The cytotoxic effect of 6.25, 12.5, 25, 50 and 100 µL of *J.regia* and *O.europaea* extracts on death of hela cells at 24 hours

#### 4. CONCLUSION

We have demonstrated that both of *J. regia* and *O.europaea* aqueous leaves extracts have properties as antidiabetic, antiglycation and anticancer, but *J. regia* was more effectively.

#### CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

#### ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s).

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:  
The peer review history for this paper can be accessed here:  
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