



Evaluation of the Anticoagulant Effect of Phenolic Extracts of Two Olive Mill By-products: Olive Mill Wastewater and Olive Mill Pomace

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Abstract

In the Mediterranean basin, olive oil production represents an important economic sector for some countries. Currently, production is constantly growing and is done at the expense of the environment. In addition to its main product, which is virgin olive oil, it generates two by-products, olive mill pomace (OMP) and olive mill wastewater (OMW). These by-products have heretofore little economic value despite their large constitutions of polyphenols that are of great importance in the pharmaceutical industry. This study aimed to assess the anticoagulant effect of the phenolic extract of OMP and OMW from cold extraction in the region of Khenchela, northeastern Algeria. This activity was chosen for its essential role in the prevention and treatment of cardiovascular diseases and their serious complications, which threaten public health and constitute the leading cause of death worldwide. The extraction of the polyphenols was done by the maceration method. The content of total polyphenols was estimated by the Folin-Ciocalteu method. The amount of flavonoids was determined by the aluminum chloride method. The anticoagulant activity of the polyphenols was evaluated in vitro using the Kaolin-cephalin coagulation time (KCT) and prothrombin time (PT) tests. The coagulation times obtained in normal plasma in the presence of these polyphenols indicated that they exert a great anticoagulant activity. It was very highly significant on both endogenous and exogenous coagulation pathways for OMW extracts and significant for OMP extracts. This activity was observed more for the endogenous pathway of coagulation than the exogenous pathway. In conclusion, the polyphenols of olive oil pomace and olive mill wastewater can be used in the pharmaceutical field as an anticoagulant against the complications of thrombotic diseases.

Keywords: Olive mill by-products, Anticoagulant effect, Phenolic extract, Olive mill wastewater, Olive mill pomace, Polyphenols.

İki Zeytin Değirmeni Yan Ürününün Fenolik Ekstraktlarının Antikoagülan Etkisinin Değerlendirilmesi: Zeytin Değirmeni Atıksu ve Zeytin Değirmeni Pirina

Öz

Akdeniz havzasında zeytinyağı üretimi bazı ülkeler için önemli bir ekonomik sektörü temsil etmektedir. Şu anda üretim sürekli büyüyor ve çevre pahasına yapılıyor. Ana ürünü olan sızma zeytinyağına ek olarak, zeytin değirmeni pirina (OMP) ve zeytin değirmeni atıksu (OMW) olmak üzere iki yan ürün üretmektedir. Bu yan ürünler, farmasötik endüstrisinde büyük önem taşıyan geniş polifenol yapılarına rağmen şimdiye kadar çok az ekonomik değere sahiptir. Bu çalışma, kuzeydoğu Cezayir, Khenchela bölgesinde soğuk ekstraksiyondan OMP ve OMW'nin fenolik ekstraktının antikoagülan etkisini değerlendirmeyi amaçladı. Bu aktivite, halk sağlığını tehdit eden ve dünya çapında önde gelen ölüm nedenini oluşturan kardiyovasküler hastalıkların ve bunların ciddi komplikasyonlarının önlenmesi ve tedavisindeki önemli rolü nedeniyle seçilmiştir. Polifenollerin ekstraksiyonu maserasyon yöntemi ile yapılmıştır. Toplam polifenollerin içeriği Folin-Ciocalteu yöntemiyle tahmin edildi. Flavonoidlerin miktarı alüminyum klorür yöntemiyle belirlendi. Polifenollerin antikoagülan aktivitesi, Kaolin-sefalin pıhtılaşma Süresi (KCT) ve protrombin zamanı (PT) testleri kullanılarak in vitro olarak değerlendirildi. Bu polifenollerin varlığında normal plazmada elde edilen pıhtılaşma süreleri, bunların büyük bir antikoagülan aktivite sergilediklerini gösterdi. OMW özleri için hem endojen hem de eksojen pıhtılaşma yollarında çok yüksek derecede anlamlıydı ve OMP özleri için önemliydi. Bu aktivite, eksojen yoldan daha çok endojen pıhtılaşma yolu için gözlemlendi. Sonuç olarak, zeytinyağı pirina ve

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zeytin değirmeni atıksuyunun polifenolleri, farmasötik alanda trombotik hastalıkların komplikasyonlarına karşı antikoagülan olarak kullanılabilir.

Anahtar Kelimeler: Zeytin değirmeni yan ürünleri, Antikoagülan etki, Fenolik ekstrakt, Zeytin değirmeni atıksu, Zeytin değirmeni pirina, Polifenoller.

1. Introduction

Olive oil production is an important economic sector for various nations in the Mediterranean region, including Algeria (Gueboudji et al., 2021b). Due to its organoleptic and healthful characteristics, the use of olive oil has significantly increased in recent years. Consequently, the amount of wastes and by-products generated by olive production and the olive oil industry has risen, creating substantial environmental and economic difficulties. However, due to the high amount of bioactive compounds in these wastes and by-products, their recovery represents both a significant problem and an attractive potential for the olive oil industry (Gullón et al., 2020).

Now, productivity is continually increasing at the expense of the environment. In addition to its principal product, virgin olive oil, it generates two by-products: olive mill pomace (OMP), a solid waste, and vegetable waters or olive mill wastewater (OMW), a liquid waste (Roussas et al., 2009; Gueboudji et al., 2021b). Despite their high concentrations of polyphenols, which are important in the pharmaceutical sector, these by-products have had little commercial value in the previous (De Leonardi et al., 2007; Gueboudji et al., 2021a). All plants contain phenolic compounds, although their qualitative and quantitative distribution varies among species, organs, tissues, and physiological phases, as it does with other secondary metabolites. They have many chemical structures and are a testament to plants' remarkable biosynthetic potential, which allows humans to employ them in sectors as diverse as food processing and pharmaceuticals (Macheix et al., 2006).

Blood clotting disorders, such as pulmonary emboli, deep vein thrombosis, and cardiovascular disease, are important causes of mortality and disability globally (WHO, 2017). Thrombotic diseases have a significant impact on world health, since they are the main cause of death and morbidity (Wendelboe et al., 2016). Although their shown efficacy, current antithrombotic medicines, or anticoagulants, have a number of disadvantages. Warfarin-based therapy has several drawbacks, including the necessity for regular monitoring of the medication plasma concentration, an indirect action mechanism that influences a number of coagulation factors, and a significant risk of bleeding (Yeh et al., 2015). Anticoagulant treatment is utilized to treat the disorder, and three types of anticoagulants are often employed: vitamin K antagonists (e.g., warfarin), unfractionated heparin, and low-molecular weight heparins. However, because they are multi-targeted, most of these medications have disadvantages such as an increased risk of bleeding and the need for ongoing laboratory testing (Ibrahim et al., 2020).

This study aimed to assess in vitro the anticoagulant effect of the phenolic extract of OMP and OMW from cold extraction in the region of Khenchela, northeastern Algeria. This activity was chosen for their essential role in the prevention and treatment of cardiovascular diseases and its serious complications, which threaten public health, and constitute the leading cause of death worldwide.

2. Material and Method

2.1. Vegetable Material

OMW and OMP samples were obtained from a modern olive oil mill with a cold extraction system (temperature not exceeding 25 °C) situated in Baghai Wilaya of Khenchela, northeastern Algeria. It is an olive mill that was made in Italy in 2016. The OMW and OMP samples were taken in January 2019 during the harvest season. OMW was collected in plastic bottles and stored at -4 °C until use. OMP is first air-dried, then crushed and split into 50 g fractions in plastic bags. These bags are kept frozen at -4 °C until they are needed. In this study, all analyses were performed in triplicate.

2.2. Blood Samples

Blood samples were taken at the central laboratory of Ahmed Ben Bella Hospital in Khenchela, Algeria. The blood is obtained on either citric acid dextrose (ACD) or heparin.

2.3. Extraction and quantification of phenolic compounds

2.3.1. Polyphenol Extraction Method

It was performed using the method described by Uysal et al. (2019), with some modifications. OMW and OMP drying were carried out prior to extracting the phenolic components. The maceration technique was used to extract phenolic compounds. It is obtained by adding 100 mL of a polar organic solvent, methanol, to the delipidated extract. Filtration using filter paper is carried out after 10 minutes of stirring. The resulting phenolic extract is separated from the solvent by rotational evaporation under vacuum at 40 °C and kept in 2 mL of DMSO. This dry extract is collected and kept in closed dark tubes in the refrigerator at -18 °C until it is used. Extraction was done in triplicate.

2.3.2. Total Phenolic Content (TPC)

The total phenolic content was measured using the Folin-Ciocalteu technique described by Siangu et al. (2019) with slight modification. 200 µL of diluted plant extract is mixed with 1 mL of Folin Ciocalteu reagent (FCR) diluted 10 times in distilled water. After 4 minutes, 800 µL of sodium carbonate (Na₂CO₃) at a concentration of 7.5 g/L are added. After incubating the reaction mixture for 2 hours at room temperature and in the dark, the absorbance is measured at 765 nm. The calibration curve is carried out with gallic acid at different concentrations, under the same conditions and the same steps of the assay. The total phenolic content (TPC) of extracts was calculated using a calibration curve ($y = 0.0049x + 0.011$, $R^2 = 0.98$). The results were given in grams of Gallic acid equivalents per 100 grams of dry matter (g GAE /100 g DM).

2.3.3. Total Flavonoid Content (TFC)

It was estimated using the methodology given by Siangu et al. (2019) with minor changes. The determination of the total flavonoid content is carried out by the aluminum chloride (AlCl₃) method. Briefly, one milliliter of extract diluted in methanol, as well as the standard flavonoid quercetin also prepared in methanol is added to 1 mL of AlCl₃ (2% methanolic solution). After 10 minutes of reaction, the absorbance is read at 430 nm. The calibration curve is carried out with quercetin at different

concentrations, under the same conditions and the same steps of the assay. The total flavonoid content (TFC) was determined using the quercetin calibration curve ($y = 0.011x + 0.0073$, $R^2 = 0.97$). The findings were measured in grams of quercetin equivalents per 100 grams of dry matter (g QE/100 g DM).

2.4. Anticoagulant Activity

The anticoagulant activity was evaluated *in vitro* against the two coagulation pathways (the endogenous pathway and the exogenous pathway) on a pool of normal-depleted plasmas and using two global time-series tests; the Kaolin-cephalin coagulation time (KCT) and the prothrombin time (PT).

The platelet-poor plasma pool is a mixture of plasmas depleted from 10 young adults as healthy untreated volunteers, whose KCT and PT are normal and comparable. The blood of each volunteer is taken by venipuncture in a plastic tube on an anticoagulant solution of sodium citrate at 3.2% and at a rate of 1 volume for 9 volumes of blood (1: 9, v / v). The blood is then centrifuged for 10 minutes at 3000 rpm to obtain a plasma poor in platelets. The standard plasma obtained is stored at low temperature ($-10\text{ }^{\circ}\text{C}$) until use (Athukorala, et al., 2007; Liang et al., 2018).

2.4.1. Prothrombin Time Test (PT)

It was determined according to the method described by Liang et al. (2018) with slight modification. The coagulation time of citrated plasma in the presence of an excess of calcium thromboplastin is measured in this activity using platelet-poor plasma in the presence of calcium thromboplastin. The phenol extract (90 μL and 10 μL , respectively) was combined with 100 μL of platelet-poor plasma that had been warmed for 2 minutes at $37\text{ }^{\circ}\text{C}$. After 15 minutes of incubation at $37\text{ }^{\circ}\text{C}$, 200 μL of calcium thromboplastin was added to the mixture, which had been warmed for at least 15 minutes at $37\text{ }^{\circ}\text{C}$. Coagulation time was determined by an automatic coagulation analysis system (CoaData 4004).

2.4.2. Kaolin-Cephalin Coagulation Time Test (KCT)

It was determined according to the method described by Liang et al. (2018) with slight modification. The activity of the phenolic extract was established on a volume of 100 μL whose plasma is 90 μL was mixed with 10 μL of extract. After 15 min of incubation at $37\text{ }^{\circ}\text{C}$, 100 μL cephalin kaolin was added to the mixture, which was re-incubated for 3 min with agitation at $37\text{ }^{\circ}\text{C}$. Using a coagulometer, the coagulation time was measured by adding 100 μL of warmed calcium chloride (0.025 M). In parallel, positive control of unfractionated heparin and a negative control test (substitution of the samples with a 0.9% NaCl solution) was performed under identical circumstances.

An increase in KCT in the presence of polyphenols compared to the negative control implies an anticoagulant impact at this route level. Clotting time was determined by an automatic coagulation analysis system (Coa DATA 4004).

2.5. Statistical Study

Data obtained were presented as (mean \pm standard) deviation of three dependent determinations. Significant differences between means of total phenolic and total flavonoids results were determined by the Student t-test, and p values (< 0.05) were regarded as significant. Results of anticoagulant activity were subjected to statistical analysis of variance (ANOVA) using

ECXEL STAT (version 2014) package at $p < 0.05$ significant levels.

3. Results and Discussion

3.1. Total phenolic and flavonoid contents

Figure 1 showed the total phenolic and flavonoids contents of the extracts of olive oil mill wastewater (OMW) and olive oil mill pomace (OMP).

Based on the total polyphenol and flavonoid contents data shown in Figure 1, it appears that the two extracts of OMW and OMP are high in polyphenol and flavonoid contents.

Indeed, the polyphenol concentrations of OMW and OMP are of the order of (TPC = 8.86 ± 0.1 g GAE/100g DM) and (TPC = 1.04 ± 0.08 g GAE/100g DM), respectively.

In terms of flavonoid content, it appears that OMW has a higher value (TFC = 1.32 ± 0.25 g QE/100g DM) than OMP, which has a value of (TFC = 0.17 ± 0.01 g QE/100g DM). For OMW and OMP, the flavonoids / polyphenols ratio is in the range of 14.90% and 16.35%, respectively. According to these findings, the variance of flavonoids is not proportional to that of polyphenols; this may be explained by the predominance of non-flavonoid polyphenols.

According to the data, OMW and OMP are characterized by the richness of phenolic compounds. The results obtained were superior to those obtained by (Gueboudji et al., 2021a).

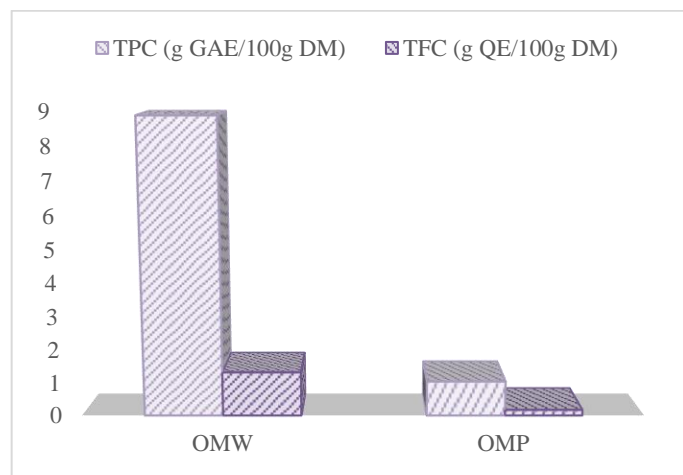


Figure 1. Total phenolic and flavonoid contents of OMW and OMP

3.2. Anticoagulant Activity

3.2.1. Prothrombin Time Test (PT)

The results obtained for the prothrombin test of olive mill wastewater (OMW), olive mill pomace (OMP) and the negative control (C-) were illustrated in Figure 2. A normal PT is between 12 and 14 seconds depending on the reagents used (Caquet, 2011). The incubation time factor of the two phenolic extracts of OMW and OMP examined with plasma is evaluated to identify the best incubation time that allows for strong anticoagulant action. The results showed that the incubation period of phenolic extracts with plasma had a substantial ($P < 0.05$) impact on their anticoagulant power (Figure 2). Indeed, the incubation of the negative control during the different times (5, 10, 15 and 20 minutes) does not influence the coagulation time, whereas in the presence of olive

extracts the prolongation of the PT is remarkable and time-dependent.

The incubation for 20 minutes is the time that made it possible to obtain a significant anticoagulant activity ($p < 0.05$) and higher than that of the incubation at 5 and at 10 minutes and very close to that induced by the different extracts when incubating at 15 minutes, which may explain by far the choice of 15 minutes as a standard incubation time in performing the PT test for the investigation of the anticoagulant properties of different biological and synthetic substances for therapeutic use.

In the incubation time of 15 minutes, there was an elongation of PT of the order of (82.2 ± 0.28 s) (6 times high) and (43.1 ± 0.16 s) (3 times high) by comparing to that of the negative control (13.5 ± 0.02 s) in the presence of phenolic extracts of OMW and OMP, respectively.

The phenolic extract of OMW exhibits the highest anticoagulant activity compared to that of OMP regardless of the incubation time (5, 10, 15, and 20 minutes). The results obtained were comparable to those found by Kadi et al. (2020).

As a result, the extract examined exhibits high anticoagulant action in the exogenous route. Heparin's anticoagulant action is caused by the inhibition of natural coagulation enzymes through the formation of a complex with anti-thrombin III. Because thromboplastin time is a coagulation test that investigates all of the coagulation factors in the exogenous route, the anticoagulant action of the OMW extract is most likely related to the inhibition of one of these factors that are activated in cascade (Tomaru et al., 2005; Liang et al., 2018).

From this study, it is assumed that the anticoagulant activity of the phenolic extracts of OMW and OMP may be due to the synergistic effect of the different classes of polyphenols, and other compounds present in these extracts.

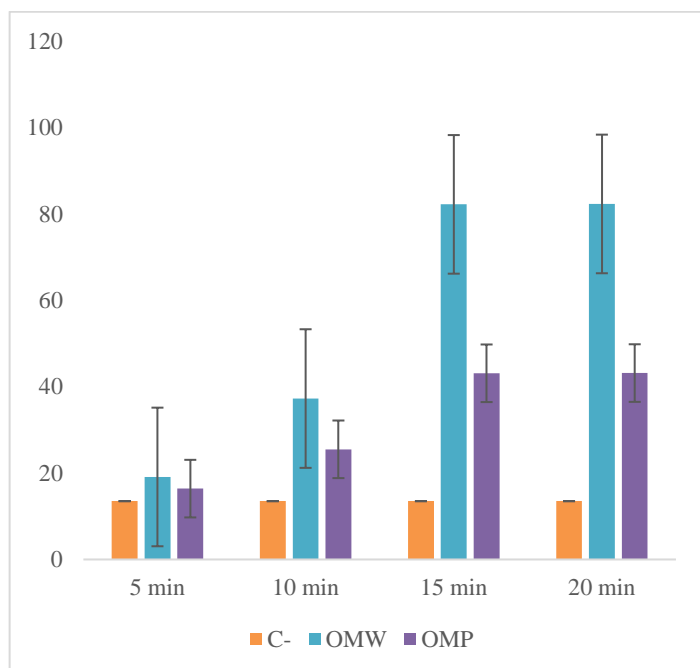


Figure 2. Effect of incubation time of phenolic extracts of OMW and OMP with plasma on PT

3.2.2. Kaolin-Cephalin Coagulation Time Test (KCT)

Figure 3 represented the kaolin partial thromboplastin (KCT) times of the phenolic extracts of OMW and OMP compared to the negative control (C-) and the positive control (C+). A longer clotting time compared to the negative control where the sample is replaced by physiological water reflects the anticoagulant activity of the material tested. The heparin that was used as a reference in this test, to compare its anticoagulant capacity to that exerted by the phenolic extracts OMW and OMP, is low molecular weight heparin (LMWH) in the form of a ready-to-use solution for injection characterized by high anti-Xa activity and low anti-IIa or antithrombin activity (Fraxiparine® Nadroparin calcium 2850 anti-Xa IU / mL).

In the light of the results obtained, it appeared that the two extracts are able to significantly lengthen the coagulation time ($P < 0.05$) with values of the order of (185.4 ± 2.42 s) for the extract of OMW and (70.3 ± 1.77 s) for the extract of OMP, compared to that of the negative control (25.1 ± 0.51 s). The prolongation of the coagulation time induced by the two extracts made OMW extract the first to have the greatest anti-coagulant activity than the second OMP extract by a minimum difference of (115.1 ± 0.65 s).

OMW and OMP extracts had a less anticoagulant effect than that of heparin (positive control) whose coagulation time was (277.6 ± 2.85 s); thus, it caused a lengthening of the KCT of the order of (252.5 ± 2.34 s) compared to the negative control. Therefore, in order to have effective anticoagulant activity, it is probably necessary for the polyphenols of OMW and OMP to act synergistically with each other and/or with other compounds present in its extracts.

Statistical analysis showed the existence of a linear relationship between anticoagulant activity and the different concentrations of the extracts. Therefore, it appears that the two phenolic extracts of OMW and OMP were able to significantly ($P < 0.05$) extend the KCT. The results obtained were comparable to those found in Kadi et al. (2020).

As a result, the phenolic extracts of OMW and OMP examined exhibited high anticoagulant action in terms of the endogenous route.

Consequently, the anticoagulant effect of phenolic extracts from olive oil mill wastewater and olive oil mill pomace might be related to the total content of polyphenols and flavonoids, when flavonoids/polyphenols ratio was low when the anticoagulant effect was high.

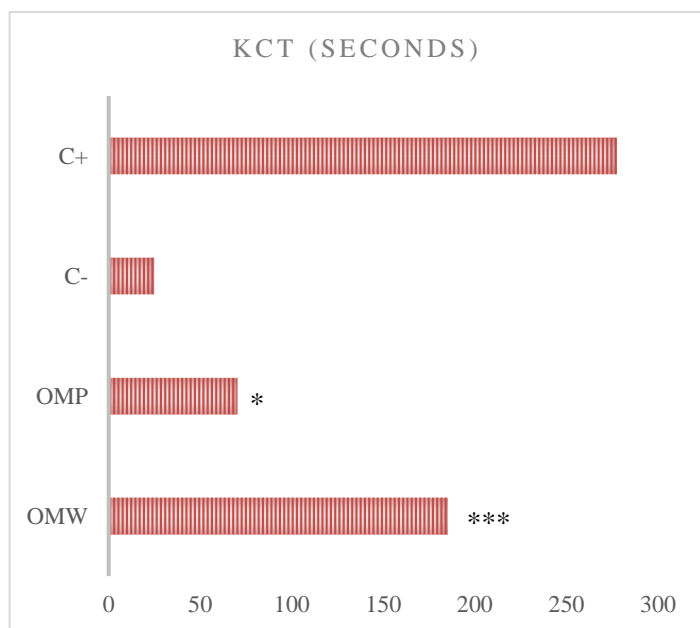


Figure 3. Kaolin partial thromboplastin time (KCT) of phenolic extracts of OMW, OMP, and heparin

4. Conclusions and Recommendations

This study evaluated the anticoagulant effect of the phenolic extracts of OMW and OMP. The study of the effect of the two extracts on the two coagulation pathways (endogenous pathway and exogenous pathway) made it possible to establish that the two extracts exert an anticoagulant effect on the two coagulation pathways with a more marked effect on the endogenous route than exogenous route and more important for OMW than for OMP. In conclusion, this study needing more tests in vivo to might said the polyphenols of olive oil pomace and olive mill wastewater could be used in the pharmaceutical sector as an anticoagulant against the complications of thrombotic diseases.

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