Phenolic compounds and biological activities of phenolic extract of olive oil mill wastewater issue from the cold extraction of olive oil from Khenchela (Algeria)

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Abstract

This study aimed to the determination of antioxidant, anti-inflammatory, and anticoagulant activities of phenolic compounds present in olive oil mill wastewater (OMW) issue from the cold extraction of olive oil from Khenchela eastern of Algeria.After polyphenols extraction, a quantity and quality analysis by LC-MS was made. The LC-MS (liquid chromatography–mass spectrometry)results were revealed the presence of 20 phenolic compounds in theextract of OMW which were: (quinic acid, gallic acid, protocatechuic acid, caffeic acid, p-coumaric acid, rutin, transfrolic acid, hyperoside (quercetin-3-o-galactoside), luteolin-7-o-glucoside, naringin, 4.5-di-caffeoyquinic acid, quercetrin(quercetin-3-o-rhamonosid), apegenin-7-o-glucoside, salviolinic acid, kampherol, quercetin, naringenin, apegenin,cirsiliol, and cirsilineol). The results of antioxidant activity with DPPH(2,2-diphenyl-1-picrylhydrazyl), ABTS+ (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), and FRAP (ferric reducing ability of plasma)showed that the IC_{50}(half-maximal inhibitory concentration) were(9.62 ± 0.28µg/mL, 7.10 ± 0.11µg/mL and 3.59 ± 0.24µg/mL)respectively. The extract of OMW was found to exhibit the highestinhibitory effectanti-inflammatory activity usinginhibition of protein denaturation(IPD) and membrane stabilizing potential (MSP) tests (80.46 ± 3.81; 87.43 ± 0.66 µg/mL) more than the standard used. In addition, the extract had the best anticoagulation activity in the endogenous and exogenous pathways (44.77 ± 0.25s; 15.84 ± 0.12s). Based on these results, it is right to conclude that OMW is an important source of natural phenolic compounds that have important antioxidant, anti-inflammatory, and anticoagulant activities.

1. Introduction

By-products of olive oil production represent a major disposal problem for the food industry, but they are also promising sources of bioactive compounds. Olive oil mill wastewater (OMW), one of the mains by-products of olive oil production (Cedola et al., 2020). In fact, after oil extraction, OMW contained 98% of the total phenolic content of the olive fruits with concentrations varying from 0.5 to 24 g/L (Alaoui et al., 2016), such as hydroxytyrosol, tyrosol, and flavonoids (Tzathas et al., 2019). Pollution mainly due to the high concentrations of phenolic compounds (Babićet al., 2019).OMW is an aqueous, dark, foul-smelling, and turbid liquid, which includes emulsified grease; it is easily fermentable (Goula and Gerasopoulos, 2017). It characterized by an acidic pH (between 3.0 and 5.9), high conductivity, a solid content ranging from 4.1 to 16.4%. It may contain up to 150 g/L to total solids (TS), water (between 83% and 92%), high chemical oxygen demand COD (between 40 and 220 g/L), also biochemical oxygen demand BOD_5 (between 35 and 170 g/L) (Dermache et al., 2013). All these results in a toxic effect onsoil, microorganisms, plants, and marine organisms (Morón et al., 2018; Basic et al., 2019). Moreover, the main components of OMW are phenolic compounds, sugars, and organic acids, mineral nutrients especially potassium (Dermache et al., 2013). The difference in its compositions is due to different factors such as olive variety, the method of extraction, the technological process separation, the climatic conditions, olive storage time, and the fruit maturity of the olive tree (Dermache et al., 2013; Aggoun et al., 2016). Phenolic compounds have manybiological properties, in particular the antioxidant, anti-

inflammatory, and anticoagulant activities, which used in the pharmaceutical industry (Khana et al., 2020).

Oxidative stress is unsteady in the balance between the defense system of antioxidants and the production of reactive oxygen species (ERO) (Power et al., 2010). This leads to biochemical damage in the cells of the organism due to their molecular consequences, such as alterations in proteins, the appearance of breaks in DNA, or damage to the integrity of the cell membrane by the induction of lipid peroxidation (Shilpa et al., 2017).Oxidative stress has actually been described as a crucial etiological factor involved in various chronic human diseases such as cancer, cardiovascular and neurodegenerative diseases, inflammation, diabetes mellitus, and aging (Uttara et al., 2009).Inflammation and coagulation are two main host-defense systems that interact with each other (Petäjä, 2011). They are implicated in many cardiovascular diseases such as thrombosis and atherosclerosis (Strukova, 2006).Inflammation is a physicochemical process in response to tissue damage resulting from microbial pathogen infection, chemical irritation, and/or wounding (Serhan et al., 2008).Thromboembolic diseases continue to be the leading cause of death throughout the world (Wu et al., 2015). As is well-known, thrombosis is closely related to activating platelet adhesion, aggregation, secretion functions, and activation of intrinsic and extrinsic coagulation systems, which cause blood coagulation and fibrin formation (Xin et al., 2011). Therefore, anticoagulants play a pivotal role in the prevention and treatment of thrombotic disorders (Xu et al., 2016).Diseases arising from blood clotting, including pulmonary emboli, deep vein thrombosis, and cardiovascular diseases are the major causes of death and disability worldwide (Who, 2017).

The main objective of this study is to evaluate the antioxidant, anti-inflammatory, and anticoagulant activities of phenolic compounds present in OMW issue from the cold extraction of olive oil from Khenchela eastern Algeria.

2. Material And Methods

2.1. Physicochemical properties

The olive oil mill wastewater (OMW) was obtained from a modern olive mill situated in Khenchela, eastern Algeria in November 2019.It was collected directly from the decanter, frozen immediately and kept at $^{-}20^{\circ}C$ until use.

Different physicochemical parameters including pH, electrical conductivity (EC), dry matter (DM), organic matter (OM), mineral matter (MM), biological oxygen demand (BOD), and chemical oxygen demand (COD) were performed according to the Standard Methods (APHA, 2005). The pH value was determined with a pH meter (AdwaAD1000). Electrical Conductivity (EC) was determined by a conductivity meter type (inoLab WTW). Dry matter content (DM) was measured by drying at $105^{\circ}C$ for 24 h. Organic matter (OM) was calculated by the differencebetween the dry weight of the OMW and its weight after the calcination. Mineral matter (MM) was determined by weighing after ignition in a muffle furnace type (Nabertherm) at $550^{\circ}C$, for 24 hours. The chemical oxygen demand (COD) was determined using potassium dichromate,

as described by BOD_5 (biological oxygen demand) is determined by the respirometric method. Analyzes were carried out in triplicate.

2.2. Polyphenol extraction methods

The extraction of phenolic compounds was done according to the maceration method. 1 g of OMW powder was mixed with 10 mL of pure methanol, the mixture was vortexed for 15 min, and then let macerate in the dark overnight at 4°C. After maceration, filtration using filter paper is carried out. The macerate was collected and was added to 10 mL of methanol (90%) for a second time, the mixture is vortexed for 15 min and leaves to macerate for 1 hour. The two filtrates are combined and filtered through cellulose paper containing sodium sulfate. Then this solution was concentrated at 40°C in a rotary evaporator type (HAHNVAPOR) and the dry residue is stored.

2.3. Total phenolic content (TPC)

The total phenolic content was determined following the Folin–Ciocalteu method (Makkar et al., 1993) and the results were expressed as milligrams of gallic acid equivalents per milliliter of extract (mg GAE/mL).The total phenolic content (TPC) of extracts was estimated according to the calibration curve prepared from gallic acid ($y = 0.0046 x + 0.0108$, $R^2 = 0.9967$).

2.4. Total flavonoids content (TFC)

The quantification of total flavonoids content was performed by (Ayoola et al., 2008) method and the results were expressedas milligrams rutin equivalents per milliliter of extract (mg RE/mL).The total flavonoids content (TFC) was calculated following the calibration curve prepared from rutin ($y = 0.0103 x + 0.0061$, $R^2 = 0.9963$).

2.5. Tannin condensed content (TCC)

The quantification of condensed contentwas performed according to the method of (Dewanto et al., 2002) by reaction with vanillin in the presence of sulfuric acid. It is expressedin milligrams of catechuic acid equivalent permilliliter of extract (mg CAE/mL). The tannins condensed content (TCC) was estimated according to the calibration curve prepared from catechuic acid ($y = 0.0066 x + 0.0113$, $R^2 = 0.9969$).

2.6. LC-MS separation and identification of phenolic compounds

The analysis for phenolic compounds was performed on a Shimadzu UFLC XR system (Kyoto, Japan), equipped with a SIL-20AXR auto-sampler, a CTO-20 AC column oven, a LC-20ADXR binary pump and a quadripole 2020 detector system. This instrument was equipped with an Inertsil ODS-4 C18 3 µm column (L150×3.0 mm i. d). The column temperature was set at 40°C and the injection volume was 20 µl with a flow rate of 0.5 ml/min. eau95%+MeOh5%+0.2%Acetic acid and 50%ACN + 50%H2O + 0.2%Acetic acid were used as mobile phases A and B, respectively. The analysis was performed using a linear gradient programmed as follows: 0,01–14 min, from 10–20%B; 14–27 min, 0 from 20–55% B ;27–37 min, from 55–100% B ; 37–45 min, 100% B ; 45–50 min 10% B. Dissolving line temperature was 275°C, nebulizing gas flow 1,50L/min, the drying gas was set at 15,00 L/min and Temperature of Heat block was 450°C. LC-

ESI (-) MS mass spectra [M-H] - were acquired using Lab Solutions software. Phenolic compoundswere identified by comparison with retention time of the standards of phenolic compounds. The lab standards were LGC and Sigma Aldrich.

2.7. Antioxidant assays

2.7.1. DPPH free radical-scavenging activity

The antioxidant activity of different extractions was evaluated following Ozgen et al. (2006)method using the free radical DPPH(2,2-diphenyl-1-picrylhydrazyl). The results were given as 50% inhibition concentration (IC_{50}) and compared with the antioxidant standards (BHT, Ascorbic acid and Trolox).

2.7.2. $ABTS^{+}$ free radical scavenging activity

The ABTS (2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) scavenging activity was determined according to the method of (Ozgen et al., 2006). The results were given as 50% inhibition concentration (IC_{50}) and compared with the antioxidant standards (BHT, Ascorbic acid, and Trolox).

2.7.3. FRAP ferric reducing antioxidant power

The FRAP activity was evaluated following (Ozgen et al., 2006). The results were given as 50% inhibition concentration (IC_{50}) and compared with the antioxidant standards (BHT, Ascorbic acid, and Trolox).

2.8. Anti-inflammatory activity

2.8.1. Inhibition of protein denaturation(IPD)

It is determined by the method described by Kandikattu, (2013)with slight modifications.

The principle consists of the inhibition of denaturation of BSA caused by heat (72°C) by the phenolic extract of OMW.1 mL of each concentration of extract added to 1 mL of 0.2% BSA solution prepared in Tris-HCl pH 6.6, then incubated at 37°C for 15 min then in a water bath at 72°C for 5 min.After cooling, the turbidity is measured at 600 nm in a cell spectrophotometer (SPECORD210plus).The same protocol for diclofenac sodium standard (injectable form) which was prepared at different concentrations in ultra-pure distilled water from a 500ppm mother solution and distilled water was used as a negative control.

2.8.2. Membrane stabilizing potential (MSP)

It was measured according to Murugan and Parimelazhagan (2014). An equal volume of blood drawn from a healthy human volunteer who had not taken NSAIDs for two weeks before blood collection, and was mixed with an equal volume of sterilized Alsever solution. This blood solution was centrifuged at 3000 rpm for 10 min, the packed cells were separated and then washed with iso-saline solution, and a 10% (v/v) suspension was prepared with iso-saline. The dosage mixture contains 1 mL of phosphate-buffered saline, 0.5 mL of 10% blood suspension, 0.5 mL of phenolic extract of OMW with different concentrations and 2 ml of hypotonic saline. All test mixtures were incubated at 37°C for 30 min and then centrifuged at 3000 rpm for 20 min. The supernatant was separated and the hemoglobin content was estimated by spectrophotometric reading at 560 nm. The negative control was distilled water and the

positive control was diclofenac sodium at the final concentration.Again, the $IC_{50}$was calculated from a graph defining inhibition against the different concentrations.

2.9. Anticoagulant activity in vitro

2.9.1. Endogenous coagulation pathway (APTT)

Activating partial thromboplastin time (APTT) was determined according to Wang et al. (2010).A pool of platelet plasma consisting of a plasma mixture of 10 healthy untreated volunteers whose APTT and PT were normal. 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°C 100 µLcephalin kaolin was added to the mixture, which was re-incubated for 3 min with agitation at 37°C.The coagulation time was determined using a coagulometer by adding 100 µL of preheated calcium chloride (0.025 M).In parallel, positive control of calciparine (unfractionated heparin) and a negative control test (substitution of the samples with a 0.9% NaCl solution was carried out under the same conditions.An elongation of APTT in the presence of the polyphenols relative to the negative control indicates an anticoagulant effect at the level of this pathway. Clotting time was determined by an automatic coagulation analysis system (CoaDATA 4004).

2.9.2. Exogenous coagulation pathway (PT)

Prothrombin time (PT)was determined according to the protocol described by Wang et al.(2010).This activity consists of measuring the coagulation time of citrated plasma in the presence of an excess of calcium thromboplastin. Using platelet-poor plasma in the presence of calcium thromboplastin.100µL of platelet-poor plasma preheated for 2 min at 37°C was mixed with the phenol extract (90 and 10 µL by order). After 15 min of incubation at 37°C, 200 µL of calcium thromboplastin preheated at least 15 min at 37°C was added to the mixture. Coagulation time was determined by an automatic coagulation analysis system(CoaDATA 4004).

2.10. Statistical study

Data obtained were presented as (mean ± standard) deviation of three dependent determinations. Significant differences between means of total phenolic, total flavonoids, tannins and LC-MS analysis results were determined by Student t-test, and p values (< 0.05) were regarded as significant. Results of antioxidant, anti-inflammatory and anticoagulant activities 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. Physicochemical properties

Table 1
Physicochemical properties of OMW studied

pH	EC (mS/cm)	TSS%	DM (g/L)	OM (g/L)	BOD_5 (g/L)	COD (g/L)
4.9 ± 0.01	12.89 ± 0.09	0.9 ± 0.03	110.8 ± 3.17	53.7 ± 1.16	68 ± 2.28	170 ± 8.5

The physicochemical properties of OMW studied were presented in Table 1.Based on the obtained results, OMW was an acidic liquid effluents (pH = 4.9 ± 0.01), too loaded with mineral and organic matter expressed in terms of a high value of electrical conductivity (EC = 12.89 ± 0.09mS/cm), (BOD_5 = 68 ± 2.28 g/L) and in (COD = 170 ± 8.5 g/L), (DM = 110.8 ± 3.17 g/L), (OM = 53.7 ± 1.16 g/L), and (TSS%=0.9 ± 0.03).

OMW's composition is widely discussed in the literature, and our results are similar to several founded results. OMW is an acidic liquid, with pH values from 3 to 5, and with an electrical conductivity value of 16.79 mS/cm. Generally, it composed of dry matter (6–17%), organic matter (4 to 16%), chemical oxygen demand (COD) (40–220 g/L) BOD_5 (35–110 g/L) and the presence of several phenol-type molecules (0.5– 24 g/L) (El Moudden et al., 2020; Kadi et al., 2020). The quality and quantity of OMW are both very different and are influenced by different factors, such as type of production process, olives, use of pesticides and fertilizers, the area cultivated, weather conditions and stage of ripening olives(El-Abbassi et al., 2017).

3.2. Total phenolic, flavonoids and tannins content

The results of total phenolic, flavonoids and tannin condensed contentswere summarized in Table 2.The results of the test t of Student showed that there is a significant difference between means of total polyphenols, total flavonoids and condensed tannins.

Table 2
Total polyphenols, flavonoids and condensed tannins of phenolic extract of OMW

Secondary metabolites	Total polyphenols (mg GAE/mL)	Total flavonoids (mg RE/mL)	Condensed tannins (mg CAE/mL)
Concentration	10.82 ± 0.11	3.11 ± 0.16	2.43 ± 0.15

Our results showed that OMW is characterized by the richness of phenolic compounds. The phenolic composition obtained from the extract of OMW was total polyphenols (10.82 ± 0.11mg GAE/mL), total flavonoids (3.11 ± 0, 16 mg RE/mL) and condensed tannins (2.43 ± 0.15 mg TAE/mL). The results that we obtained were higher than of those obtained by Kadi et al. (2020).

3.3. Identification and quantification of phenolic compounds by LC-MS analysis

The quantitative analysis results of major phenolic compounds identified in the extract of OMW were summarized in Table 3.

Table 3
LC-MS analysis of phenolic extract of OMW

Nº	Phenolic acids	Retention time	M/h	Concentration ppm
1	Quinic acid	2.030	191	194.53 ± 29.092
2	Gallic acid	4.109	169	65.925 ± 91.78
3	Protocatechuic acid	7.409	153	195.356 ± 58.578
4	Catechin (+)	-	289	N.D.(Peak)
5	Caffeic acid	15.983	179	29.262 ± 9.519
6	Syringic acid	-	197	N.D.(Peak)
7	1,3-di-O-caffeoyquinic acid	-	515	N.D.(Peak)
8	Epicatechin	-	289	N.D.(Peak)
9	p-coumaric acid	22.117	163	21.935 ± 5.604
10	Rutin	24.931	609	2.106 ± 0.445
11	Transfrolic acid	24.280	193	2.708 ± 4.69
12	Hyperoside (quercetin-3-o-galactoside	25.072	463	4.801 ± 0.655
13	Luteolin-7-o-glucoside	25.676	447	15.445 ± 1.365
14	3,4-di-O-caffeoyquinic acid	-	515	N.D.(Peak)
15	Naringin	27.040	579	16.721 ± 0.532
16	Rosmarinic acid	-	359	N.D.(Peak)
17	4,5-di-O-caffeoyquinic acid	27.648	515	676.57 ± 83.712
18	Quercetrin (quercetin-3-o-rhamonosid	27.924	447	12.310 ± 1.248
19	Apegenin-7-o-glucoside	27.906	431	0.732 ± 1.269
20	o-coumaric acid	-	163	N.D.(Peak)
21	Salviolinic acid	28.918	717	33.82 ± 2.943
22	Kampherol	32.879	285	906.831 ± 306.164
23	Quercetin	32.876	301	2.457 ± 0.291
24	Trans cinnamic	-	147	N.D.(Peak)
25	Naringenin	34.783	271	1.367 ± 2.368
26	Apegenin	35.421	269	96.2 ± 10.05

Nº	Phenolic acids	Retention time	M/h	Concentration ppm
27	Luteolin	-	285	N.D.(Peak)
28	Cirsiliol	36.468	329	51.258 ± 2.664
29	Cirsilineol	39.017	343	2.924 ± 5.065
30	Acacetin	-	283	N.D.(Peak)
31	Chlorogenic acid	-	353	N.D.(Peak)
	Total phenols	-	-	2333.258 ± 220.565

Thirty-one (31) compounds were screened by liquid chromatography-mass spectrometry LC-MS. Only twenty (20) compounds were identified and quantified in the extract. They were quinic acid, gallic acid, protocatechuic acid, caffeic acid, p-coumaric acid, rutin, transfrolic acid, hyperoside (quercetin-3-o-galactoside), luteolin-7-o-glucoside, naringin, 4.5-di-caffeoyquinic acid, quercetrin (quercetin-3-o-rhamonosid), apegenin-7-o-glucoside, salviolinic acid, kampherol, quercetin, naringenin, apegenin, cirsiliol, and cirsilineol. The major identified phenolic compounds were kampherol, 4.5-di-o-caffeoyquinic acid, protocatechuic acid, and quinic acid with the concentrations (906.831 ± 306.164, 676.57 ± 83.712, 195.356 ± 58.578 and 194.53 ± 29.092 ppm). Their structures were presented in **Fig. 1**

Several researchers were identified phenolic compounds by HPLC as; Ben Saad et al. (2020) that were identified sixteen peaksby HPLC–DAD–ESI/MS.There wereHydroxytyrosol, Para-hydroxyphenyl acetic acid derivative, Pinoresinol, Apigenin 7-O-glucoside, Luteolin-7-rutinoside, Naringenin, Luteolin-7-glucoside, Syringic acid, 7-β-1-d-Glucopyranosyl-11-methyl oleoside, Elenolic acid, Hydroxy-O-decarboxymethyl oleuropeinaglycon, Luteolin, Apigenin, Diosmetin, and Hydroxytyrosol glycoside.

Antioxidant activities by DPPH.$^{+}$, ABTS.$^{+}$ and FRAP assays

The antioxidant activity of phenolic extract of OMW was shown in Table 4.The free radical scavenging activity determined by DPPH., ABTS.$^{+}$ and FRAP is widely used to estimate antiradical/ antioxidant capacity of phenolic compounds of OMW and compared the results with several reference standards with the aim to achieve more informative and arguably necessary results.

Table 4
Antioxidants activity of phenolic extract of OMW by DPPH, ABTS, and FRAP

	DPPH IC50µg/mL	ABTS IC50µg/mL	FRAP IC50µg/mL
Extract	9.62 ± 0.28	7.10 ± 0.11	3.59 ± 0.24
BHT	20.03 ± 0.25	4.27 ± 0.38	18.81 ± 0.09
Ascorbic Acid	20.84 ± 0.65	2.03 ± 0.14	11.08 ± 0.18
Rutin	10.5 ± 0.36	8.48 ± 0.33	4.72 ± 0.21

The results of DPPH scavenging showed that the phenolic extract of OMWhas exhibited the highest antioxidant activity (IC_{50}: 9.62 ± 0.28µg/mL) closer activity to that of rutin (IC_{50}: 10.5 ± 0.36µg/mL) and more than BHT and ascorbic acid which are in the same group which IC_{50} are (20.03 ± 0.25 and 20.84 ± 0.65 µg/mL), respectively. Similarly, the analysis data of the ABTS assay showed that the phenolic extract of OMW had an IC_{50} higher than rutin (IC_{50}: 7.10 ± 0.11, 8.48 ± 0.33µg/mL) and lower than ascorbic acid and BHT (IC_{50}: 2.03 ± 0.14, 4.27 ± 0.38 µg/mL). From the results of FRAP, phenolic extract of OMW was exhibiting the best antioxidant activity (IC_{50}:3.59 ± 0.24 µg/mL) than rutin (IC_{50}: 4.72 ± 0.21 µg/mL), ascorbic acid (IC_{50}:11.08 ± 0.18 µg/mL) and BHT(IC_{50}: 18.81 ± 0.09 µg/mL).

El Moudden et al. (2020) showed that the phenolic compounds of OMWare characterized by a strong antioxidant activity. In literature, the in vitro antioxidant activity of natural extracts has been widely discussed. These methods involve the presence of oxidizing species such as free radicals or metal complexes oxidized in the presence of an extract, which contains antioxidants capable of inhibiting the generation of radicals. Several studies have shown that the antioxidant activity depends on the concentration of total polyphenols, on the nature and structure of the antioxidants in the extract. In addition, the concentration of total polyphenols was significantly correlated with the antioxidant capacity evaluated by the DPPH and ABTS tests.

According to Stoclet and Schini-Kerth (2011), at the cellular level, certain flavonoids can act on the transmission of signals by protein kinases, including the expression of antioxidant and anti-inflammatory genes and vice-versa, the inhibition of oxidative and inflammatory genes.

The load of the total phenolic content and the type of phenolic compounds can explain the strong antioxidant activity of our extracts. In addition, perhaps this strong antioxidant activity due to the flavonoid contents, especially kampherol(Calderón-Montaño et al., 2011), which is the most abundant flavonoid in our extract (906.831 ± 306.164ppm).

3.4. Anti-inflammatory activity

3.4.1. Inhibition of protein denaturation(IPD)

The results of the denaturing effect of proteins were illustrated in Table 5. The studied phenolic extract of OMW has an inhibitory efficiency of thermal denaturation (IC_{50} = 80.46 ± 3.81 µg/mL) superior to that of the reference anti-inflammatory drug diclofenac sodium (IC_{50} = 83.83 ± 0.21 µg/mL).

Table 5
Anti-inflammatory activity of phenolic extract of OMW

	IPD IC50 µg/mL	MSP IC50 µg/mL
Extract	80.46 ± 3.81	87.43 ± 0.66
Diclofenac sodium	83.83 ± 0.21	95.31 ± 0.69

By their stabilizing effect on proteins, the extract, therefore, has an anti-inflammatory potential, which remains to be confirmed by other *in vivo* tests.

Indeed, the conformation of a protein is linked to the secondary and tertiary structure; it is carried out by means of lower energy bonds (hydrogen bonds, electrostatic, hydrophobic and disulfide bridges), therefore fragile. Denaturation results from a modification of the quaternary, tertiary and secondary structures without fragmentation of the peptide chain under the effect of various chemical or physical agents (Karthik et al., 2013; Marliyahand Ananthi, 2015). The denaturation of a protein causes the induction of the inflammatory reaction by the production of auto-antigens, important factors for developing chronic inflammation (Karthik et al., 2013).

Recent studies have shown that many flavonoids and related polyphenols have significantly contributed to antioxidant and anti-inflammatory activities (Govindappa et al., 2011).

The presence of these bioactive compounds in the OMW extract can contribute to this anti-inflammatory activity.

Therefore, the use of agents that can prevent protein denaturation would be useful for the development of anti-inflammatory drugs (Chatterjee et al., 2012).

3.4.2. Membrane stabilizing potential (MSP)

The results of the membrane-stabilizing effect of the OMW phenolic extract were illustrated in Table 5.According to the results obtained, the extract had a high inhibitory concentration (IC_{50} = 87.43 ± 0.66 µg/mL) compared to diclofenac sodium (IC_{50} = 95.31 ± 0.69 µg/mL).

Stabilization of the red blood cell membrane has been used as a method to study anti-inflammatory activity in vitro because the erythrocyte membrane is analogous to the lysosomal membrane (Marliyah and Ananthi, 2015).

According to the results obtained, the extract showed a significant stabilization of the red blood cell membrane compared to diclofenac sodium.

This stabilization implies that the phenolic extract of OMW can well stabilize the lysosomal membrane. Stabilization of the lysosome is important to limit the inflammatory response by preventing the release of lysosomal constituents from activated neutrophils, such as bacterial enzymes and protease.

Nonsteroidal drugs such as diclofenac sodium work either by inhibiting lysosomal enzymes or by stabilizing lysosomal membranes (SreeKumari et al., 2015).

According to Ghedira (2005), the strong anti-inflammatory activity is probably due to flavonoids, which corresponds to the production of superoxide anions ($O_2.^-$) by the membrane NADPH-oxidase of activated leukocytes and by dismutation to that of the very reactive hydroxyl radical (OH.).

The strong anti-inflammatory of our extract may be due to the kampherol, 4.5-di-o-caffeoyquinic acid, and protocatechuic acid, which are abundant in the extract.

3.5. Anticoagulant activity

The anticoagulant effect of phenolic extract of OMW was measured using two in vitro assay methods: activated partial thromboplastin time (APTT) and prothrombin time (PT) (Kamal et al., 2007).

3.5.1. Endogenous coagulation pathway (APTT)

APTT assay was used to assess the inhibition of intrinsic factors of blood coagulation pathways such as F XII, XI, V, III IX, and prekallikrein (Rizzo et al., 2008). APTT assay use brain lipids and activators instead of platelets to detect VIII, IX, XI and excitatory releasing enzymes in the endogenous coagulation pathway to reflect the effects of endogenous factors on coagulation time.

The results of the anticoagulant activity obtained (Table 6) reveal that the extracts have dose-dependent anticoagulant activity. The clotting time (APTT) in the presence of polyphenolic extracts of OMW and their compounds and heparin had been determined.

The results show that the polyphenolic extract is capable of significantly increasing the APTT ($p \leq 0.01$). The coagulation time of the phenolic extract of OMW obtained are (44.77 ± 0.25 s) was higher than that of the negative control (28.17 ± 0.06s) and positive control (33.1 ± 0.1s). Our results were in the range of those obtained from (Kadi et al., 2020).

Therefore, the extract tested has a good anticoagulant activity with respect to the endogenous pathway.

3.5.2. Exogenous coagulation pathway (PT)

PT assay is assessed to examine the inhibition of the extrinsic coagulation pathway, especially factors V, VII, and X (Rizzo et al., 2008). PT assays are conducted by adding thromboplastin to plasma to reflect the effect of exogenous factors on coagulation time.

In order to research an elongation at the level of the coagulation time, which is defined by an anticoagulant activity of the polyphenolic extracts of OMW with respect to the cascade of this pathway. A normal PT is between 12 and 14 seconds depending on the reagents used (Caquet, 2004).

The results obtained revealed that the incubation time of polyphenolic extracts with plasma significantly influences ($p \leq 0.05$) their anticoagulant power.

Table 6
Anticoagulant activity of phenolic extract of OMW

	APTT (second)	PT (second)
Negative control	28.17 ± 0.06	13.4 ± 0.1
Extract	44.77 ± 0.25	15.84 ± 0.12
Positive control	33.1 ± 0.1	14.1 ± 0.13

From the results obtained (prothrombin time) (Table 6), it appears that the extract is capable of significantly increasing PT. The coagulation times of the phenolic extract of OMW obtained are(15.84 ± 0.12s) was higher than that of the negative control (13.4 ± 0.1s) and positive control (14.1 ± 0.13s). Our results were in the range of those obtained from (Kadi et al., 2020).

Therefore, the extract tested has a good anticoagulant activity with respect to the exogenous pathway.

Tomaru et al. (2005) demonstrated that the anticoagulant activity of heparin results from the inactivation of the coagulation enzymes of the endogenous path via forms a complex with anti-thrombin III.Since thromboplastin time is a coagulation test that explores all of the coagulation factors in the exogenous pathway,it is very likely that the anticoagulant activity of the OMW extract is due to the inhibition of one of these factors, which are activated in cascade.

In total and from the results, it can be deduced that the phenolic extract of OMW exerts an anticoagulant effect on the two coagulation pathways in a dependent manner.

Because thrombotic diseases have developed into the main causes of death, so effective anticoagulant drugs are urgently needed. Based on the above data analysis, it can be concluded that the phenolic extractof OMW has a certain anticoagulant effect in vitro and could be developed as an anticoagulant drug and applied to the treatment of coagulation-related diseases.

According to Ghedira (2005), flavonoids act on the blood vessels in the form of vitamin P activity, which is involved in maintaining the normal vascular permeability.

The strong anticoagulant activity of our extractmay be due to its richness in kampherol, caffeic acid, and its derivatives.

4. Conclusion

The present study has made it possible to highlight the presence of bioactive molecules in olive mill wastewater. They are polyphenols, flavonoids and tannins, endowed with biological properties. OMW seems to present a real and potential interest by their antioxidant, anti-inflammatory and anticoagulant activities. These pharmacological properties of the polyphenols present in OMW allow the possibility of their therapeutic asantioxidant, anti-inflammatory and anticoagulant drugs. The extraction and reuse of polyphenols such as kampherol, 4.5-di-o-caffeoyquinic acid, protocatechuic acid, and quinic acid from OMW. This last is a dangerous source of environmental pollution, give us benefits from its biological properties, and in return, this will reduce its polluting impact, which has become a difficult problem for olive oil-producing countries.

Declarations

Authors' Contributions: All authors contributed extensively to the work presented in this paper, collect data, designed and performed research, wrote, revised and approved the final manuscript.

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

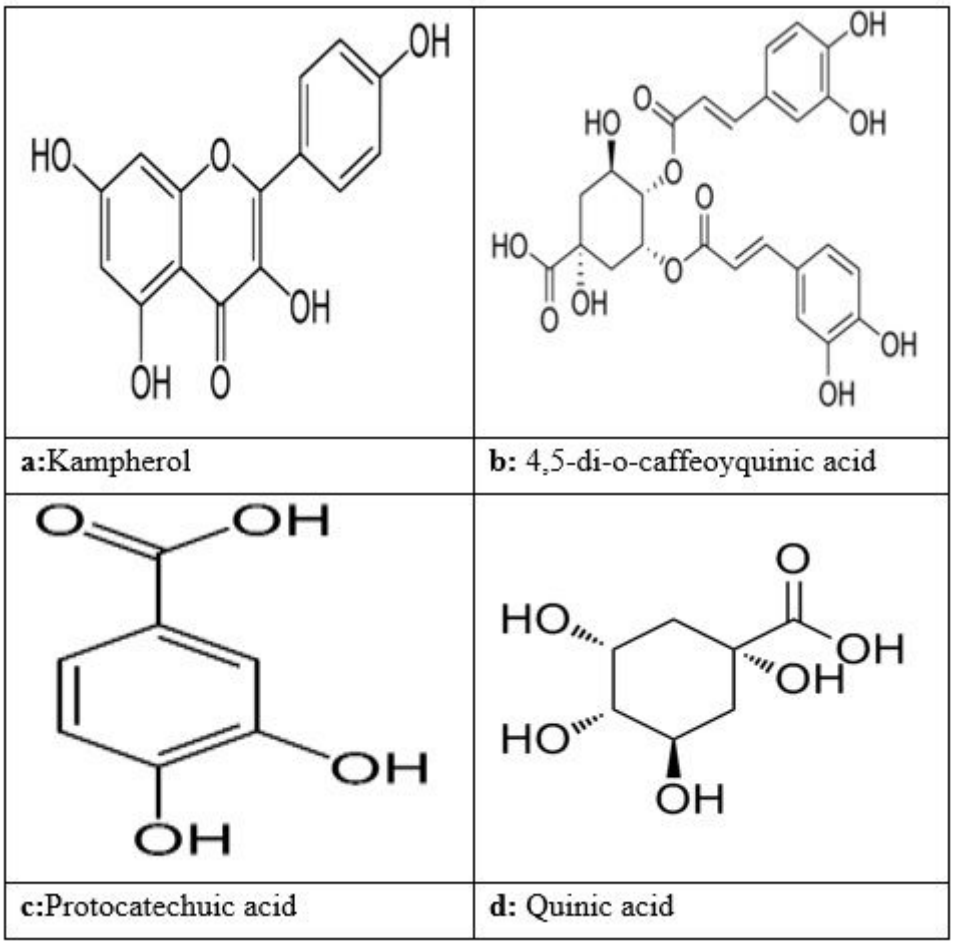

Figure 1

Chemical structure of major phenolic compounds (a: kampherol, b: 4,5-di-o-caffeoyquinic acid, c: protocatechuic acid, d: quinic acid) identified in OMW.