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Anti-Inflammatory Activity Of Polyphenols From Olive Oil Mill Wastewaters

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Abstract

Olive oil mill wastewater (OMW) is a by-product of the olive oil industry. It is very polluting and causes serious environmental problems, but is very loaded with polyphenols, which have antioxidant and anti-inflammatory power. This study aimed to evaluate in vitro the anti-inflammatory potential of the phenolic extract of OMW from two varieties, Chemlal and Zlitni, extracted from a modern olive mill located in Khenchela, eastern Algeria. The maceration method did the extraction of the polyphenols. The dosage of total polyphenols and flavonoids content was determined by Folin-Ciocalteu and aluminum trichloride methods successively. Two tests made the anti-inflammatory activity, inhibition of protein denaturation (IPD) and membrane-stabilizing potential (MSP), comparing with sodium diclofenac. The results obtained showed that the phenolic extract of OMW of Chemlal was found to exhibit the highest inhibitory effect anti-inflammatory activity (IPD: IC_{50} = 91.27 ± 2.73 µg/mL, and MSP: IC_{50}=98.41 ± 1.22 µg/mL) more than the phenolic extract of Zlitni and the standard used (sodium diclofenac). The in vitro assays carried out show that the phenolic extract of OMW has an important source of natural anti-inflammatory agents, which can be used in the pharmaceutical industry and on the other hand reducing its dangerous impact on the environment.

Paper type: Research paper

Keywords: Anti-inflammatory, polyphenols, flavonoids, olive mill wastewater, olive oil

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Introduction

The olive oil industry is particularly significant in Mediterranean nations, which is an important part of their economic activity (Al-Hmoud *et al.*, 2020; Esteves *et al.*, 2020). The major waste product from the extraction of olive oil in highly contaminated acidic liquid form is olive oil mill wastewater (OMW) (Rawajfeh *et al.*, 2021). It has a strong purple-brown or brown-red to black hue because of the presence of tannins and phenolic compounds with low molecular weight, as well as an olive oil odor. Its composition is varied and complicated, mostly comprising sugars, lipids, polyalcohol, proteins, polyphenols, and organic acids (Hocaoglu *et al.*, 2018; Genç *et al.*, 2020). Because the extraction process requires a considerable amount of water, this sector creates a large amount of OMW, which is high in polyphenols. It can pollute ecosystems because it is slowly biodegradable (Gueboudji *et al.*, 2021b). OMW has a more varied content. Many factors influence this variability, like the quality of the olives, their level of maturity, the extraction technique, as well as the quality of water used during the oil recovery stage (Battista*et al.*, 2016). 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. Inflammation is a physicochemical process in response to tissue damage resulting from microbial pathogen infection, chemical irritation, and/or wounding (Xiong *et al.*, 2022).

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Nowadays, with its enormous volume and high concentration of organic compounds, the treatment of OMW is exceedingly challenging. OMW is now industrially vaporized in order to recover polyphenols (Rahmanian *et al*., 2014). Olives and their derivatives may find application in the biopharmaceutical industry since they are high in phenol compounds (Gueboudji *et al*., 2021a; De Leonardis *et al*., 2007). Indeed, phenolic compounds exhibit a wide range of biological characteristics, including antioxidant, anticoagulant, and anti-inflammatory effects (Khan*et al*., 2020; Gueboudji *et al*., 2021c). This study aimed to evaluate in vitro the anti-inflammatory potential of the phenolic extract of olive mill wastewater from two varieties, Chemlal and Zlitni, extracted from a modern olive mill in Khenchela, eastern Algeria.

1 Materials and Methods

1.1 Sampling

OMW samples were collected from a modern olive oil mill with a cold extraction system (temperature not exceeding 25°C) located at Khenchela, eastern Algeria. It is an oil mill constructed in Italy that was built in 2016. The OMW samples were collected during the harvest season in February 2020. The samples of the olive oil mill are from two varieties of olive which were Chemlal and Zlitni. All analyzes were carried out in triplicate.

1.2 Polyphenol Extraction Method

The maceration method did it after drying the samples of OMW according to the method described by (Gueboudji *et al*., 2021d). One g of OMW powder was mixed with 10mL of pure methanol. Then, it vortexes for 15 minutes and kept macerating overnight at 4°C in the dark. After maceration, filtering using filter paper is performed. The macerate was collected and added to 10mL of methanol (90%) for a second time; the combination was vortexes for 15 minutes before being left to macerate for 1 hour. The two filtrates are mixed and filtered through sodium sulfate-containing cellulose paper. The solution was condensed in a rotary evaporator (HAHNVAPOR) at 40°C, and the dry material was stored.

1.3 Qualitative phytochemical analysis of OMW

Six tests were carried out. To test for alkaloids, use 2mL of Wagner's reagent to 1mL of OMW (reagent composition). The appearance of a reddish-brown precipitate (Inamdar *et al*., 2014 confirms the presence of alkaloids). To test for phenols, dilute 1mL of an extract with 2mL of distilled water and a few drops of 10% $FeCl_3$. The presence of phenols is showed by the presence of a blue or black colour (Ganatra *et al*., 2013). Test for saponins: In a test tube, 2mL of extract and 2mL of distilled water were combined to determine the presence of saponins. For a few seconds, the mixture was vigorously agitated to see whether any persistent foam formed. Saponins are confirmed by the presence of foam (Inamdar *et al*., 2014). Test for flavonoids: they were tested by adding a few drops of 10% ferric chloride solution to roughly 2mL of extract. The presence of flavonoids is showed by the development of a green or blue colour. To test for steroids, 2mL of chloroform and 0.2 mL of concentrated H_2SO_4 were added to 1mL of extract. The presence of steroids is showed by the development of a red-coloured precipitate. Test for tannin; a few drops of dilute ferric chloride solution were added to 1mL of extract. The development of blue-black or greenish-black precipitate indicates the presence of tannin (Ganatra *et al*., 2013).

1.4 Quantitative Phytochemical Screening of OMW

1.4.1 Total Phenolic Content (TPC)

The total phenolic content was measured using the Folin–Ciocalteu technique described by (Siangu *et al.*, 2019) with slight modification. The results were given in milligrams of gallic acid equivalents per milliliter (mg GAE/mL). Triplicate analyzes were performed. The total phenolic content (TPC) of extracts was estimated according to the calibration curve prepared from gallic acid (y=0.0048x+0.0111, R^2=0.9778), with x, is the concentration of gallic acid mg/mL, y, is the absorbance at 760nm

1.4.2 Total Flavonoid Content (TFC)

The total flavonoid content was determined using the protocol described by (Tona *et al*., 2020) with slight modifications. The findings were measured in milligrams of quercetin equivalents per milliliter (mg QE/mL). Triplicate analyzes were performed. The total flavonoid contents (TFC) was calculated following the calibration curve prepared from quercetin (y=0.0109x+0.0073, R^2=0.9684), with x, is the concentration of quercetin mg/mL, and y, is the absorbance at 510nm.

1.5 In vitro anti-inflammatory activity

1.5.1 Inhibition of protein denaturation (IPD)

IPD was estimated using the method described by (Tona *et al*., 2020), with slight adjustments. The basic idea is that the phenolic extract of OMW prevents BSA (bovine serum albumin) denaturation produced by heat (72°C). One milliliter of each different extract was added to 1mL of 0.2% BSA solution made in Tris-HCl pH 6.6, then incubated at 37°C for 15 minutes, followed by 5 minutes in a 72°C water bath. After cooling, the turbidity is measured in a cell spectrophotometer at 600nm (SPECORD 210 plus). Diclofenac sodium standard (injectable form) was produced using the same technique in ultra-pure distilled water from a 500ppm mother solution, with distilled water serving as a negative control.

1.5.2 Membrane stabilizing potential (MSP)

MSP was calculated using the method of (Murugan and Parimelazhagan, 2014). An equivalent volume of blood was collected from a healthy human volunteer who had taken none NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) for two weeks before blood collection and combined with an equal volume of sterile Alsever solution. This blood solution was centrifuged for 10 minutes at 3000rpm, the packed cells were separated and rinsed with iso-saline solution, and iso-saline was used to make a 10% (v/v) suspension. One milliliter phosphate-buffered saline, 0.5 mL 10% blood suspension, 0.5 mL phenolic extract of OMW with various concentrations, and 2 mL hypotonic saline represent approximately the dose combination. All test combinations were incubated for 30 minutes at 37°C before being centrifuged for 20 minutes at 3000rpm. The hemoglobin concentration was measured using a spectrophotometric measurement at 560nm after the supernatant was separated. Diclofenac sodium at the final concentration was used as a positive control and distilled water as a negative control. The IC_{50} was measured once again using a graph that indicated inhibition at different concentrations.

1.6 Statistical study

Data obtained were given as (mean±standard deviation) of three dependent determinations. Significant differences between means of total phenolic and total flavonoids results were evaluated by Student t-test, and p-values (<0.05) were regarded as significant. Results of anti-inflammatory activity were subjected to statistical analysis of variance (ANOVA) using ECXEL STAT (version 2014) package at $p<0.05$ significant levels.

2 Results and Discussion

2.1 The phytochemical analysis

The results of the phytochemical analysis of OMW are illustrated in **Table 1**. The phytochemical analysis of a phenolic extract of OMW revealed the presence of alkaloids, phenols, flavonoids, steroids, and tannins in the two extracts, while it revealed the absence of saponins in the two extracts.

Table 1 Phytochemical analysis of OMW.

Phytochemicals	Chemlal	Zlitni
Alkaloids	+	+
Phenols	+	+
Saponins	-	-
Flavonoids	+	+
Steroids	+	+
Tannin	+	+

(+):presence of phytochemicals (-): absence of phytochemicals

2.2 Total Phenolic and Flavonoids Contents

The results of total phenolic and flavonoid contents are presented in **Figure 1**. According to the results obtained in Fig. 1, the content of OMW in phenolic contents differs between the extracts of the two varieties. The extracts of Chemlal contain higher TPC concentration (1.02±0.02mg GAE/mL) than the TPC concentration recorded in the Zlitni extracts (0.93±0.05mg GAE/mL). Many researchers have determined the level of polyphenols in OMW: value of (950±14.2µg GAE/ mg of extract) quantified by (Gueboudji *et al*., 2021a), values of (0.8±0.02mg GAE/mL) quantified by (Kadi *et al.*, 2020) A range of (3.02±0.18 to 5.20±0.21g/L gallic acid) is recorded by (Di Mauro *et al.*, 2017). The content of flavonoid from OMW of the two varieties was almost similar (0.46±0.07mg QE/mL for Chemlal and 0.48±0.08mg QE/mL for Zlitni). The level of flavonoids of OMW was largely determined by many researchers such as (Gueboudji *et al*., 2021a) who found a value of (80.6±17.27µg QE/mg of extract), Kadi *et al.*, (2020) found a

value of (0.065±0.001mg QE/mL) and (0.056±0.005 QE/mL), and the range of (0.95±0.17-2.28±0.23g/L catechin) recorded by Di Mauro *et al.*, (2017).

2.3 Anti-inflammatory activity

The anti-inflammatory activity of OMW extract was determined using protein denaturation and membrane-stabilizing potential methods. The results were represented in **Figure 2**.

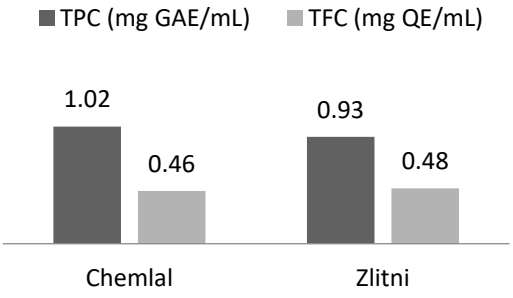

Fig. 1 Total phenolic and flavonoid contents of OMW.

2.3.1 Inhibition of protein denaturation (IPD)

The investigated phenolic extract of OMW from Chemlal has a thermal denaturation inhibitory efficiency (IC_{50}=91.27±2.73µg/mL) that is more than that of the reference anti-inflammatory medication diclofenac sodium (IC_{50}=92.98±1.31µg/mL) and higher than Zlitni (IC_{50}=97.36±0.5µg/mL). The extract has an anti-inflammatory potential as a result of its protein stabilizing action, which has to be validated by additional in vivo testing. Indeed, a protein's conformation is connected to its secondary and tertiary structure; it is performed by lower energy connections (hydrogen bonds, electrostatic, hydrophobic, and disulfide bridges), making it unstable. Denaturation is caused by a change in the quaternary, tertiary, and secondary structures without fragmentation of the peptide chain caused by different chemical or physical causes (Karthik *et al*., 2013; Marliyah and Ananthi, 2015). Denaturation of a protein enables the inflammatory response to be triggered by the formation of auto-antigens, which are essential contributors to the development of chronic inflammation (Marliyah and Ananthi, 2015). Many flavonoids and related polyphenols have been showed in recent research to contribute considerably to antioxidant and anti-inflammatory properties (Gueboudji *et al*., 2021). The presence of these bioactive chemicals in the OMW extract may explain its anti-inflammatory effect. As a result, compounds that inhibit protein denaturation might be beneficial in the development of anti-inflammatory drugs (Chatterjee *et al*., 2012).

2.3.2 Membrane stabilizing potential (MSP)

According to the findings, the extract significantly stabilized the red blood cell membrane when compared to diclofenac sodium. The extract of Chemlal had a higher comparable effect (IC_{50}=98.41±1.22µg/mL) as diclofenac sodium (IC_{50}=99.78±0.92µg/mL), whereas Zlitni had a lower power (IC_{50}=107.2±0.1µg/mL). Because the erythrocyte membrane is like the lysosomal membrane, stabilization of the red blood cell membrane has been used to examine anti-inflammatory effects in vitro (Marliyah and Ananthi, 2015). This means that the phenolic extract of OMW can effectively stabilize the lysosomal membrane. The lysosome must be stabilized in order to minimize the inflammatory response by limiting the release of lysosomal components, such as bacterial enzymes and protease, from active neutrophils. Nonsteroidal anti-inflammatory drugs, such as diclofenac sodium, act by either inhibiting lysosomal enzymes or stabilizing lysosomal membranes (Kumari*et al*., 2015). In a previous study, pre-treatment of LPS-stimulated cells with this fraction decreased the production of nitric oxide (NO), showing its potential as an anti-inflammatory agent (Silvan *et al.*, 2019).

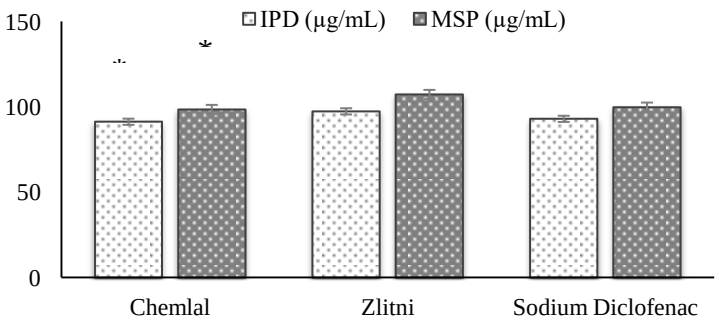

Fig. 2 The anti-inflammatory activity of OMW.

Conclusions

The present study reported for the first time evaluation of in vitro anti-inflammatory activity of phenolic extracts of OMW of two varieties, Chemlal and Zlitni with two tests: inhibition of denaturation of proteins (IDP); and membrane stabilizing potential (MSP). The investigated phenolic extract of OMW from Chemlal had presented the more thermal denaturation inhibitory

efficiency and a comparable effect of MSP as the used standard while the extract of Zlitni had presented the lower power for the two tests. Chemical examination reveals the presence of polyphenols, flavonoids and tannins that may be responsible for the anti-inflammatory properties. The phenolic extract of OMW has an important source of natural anti-inflammatory agents, which can be used in the pharmaceutical industry on the one hand, and on the other hand, reducing its dangerous impact on the environment. OMW bioactivities increase the valorization of olive oil by-products. In conclusion, the obtained results are interesting. However, the mechanisms underlying the observed effects are unknown. It would therefore be wise in the future to deepen the phytochemical study of this effluent by trying to identify and purify the phenolic compounds responsible for this biological activity and to test it in vivo.

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Nomenclature

BSA	=bovine serum albumin	[-]
GAE	=gallic acid equivalent	[-]
IC_{50}	=half maximal inhibitory concentration	[-]
IPD	=inhibition of protein denaturation	[-]
MSP	=membrane stabilizing potential	[-]
NSAIDs	=non-steroidal Anti-inflammatory drugs	[-]
OMW	=olive oil mill wastewater	[-]
QE	=quercetin equivalent	[-]
TFC	=total flavonoid content	[-]
TPC	=total phenolic content	[-]

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