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Characterization of Defatted Cake Prepared from Egyptian Olive's Fruit (Wateeken Cultivar) and Its Biological Activity

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ABSTRACT

In the current study, we characterized the components of a defatted cake prepared from Egyptian olive fruit (Wateeken cultivar) (DCEOF), its phenolic and squalene content, and evaluated its biological activities as radical scavenger capacity, anti-inflammatory, antitumor, and mechanism of action via apoptotic effect. Data obtained showed that methanolic extract showed a high content of phenolic, short-chain fatty acids, and retinol; a derivative of vitamin A. The mean level of squalene was (490 ± 45) while the mean total phenolic level was (5.76 ± 1.5). The extract showed anti-inflammatory activity as measured by inhibition of Albumin denaturation (44.4%) and antiproteinase Activity (92.6%). In addition, the defatted olive cake extract exhibited antiproliferation against HepG2 cells following treatment with different concentrations of extracts (0-200 µg/ml) for 24, 48, and 72 hours. It demonstrated that, after 24 hours, the inhibition percent was increased with dose-dependent (IC50 was 100 mg/ml). The inhibition rate reached maximum after 48 hours and decreased after 72 hours. The apoptotic markers caspase 3 and 9 were increased 2-4 times in response to defatted olive cake extract. It was concluded that the defatted olie cake of the Wateeken cultivar is promising as anti-inflammatory, antitumor activity, and apoptotic activity with high nutritional value with low side effects.

Keywords: *Olive fruit, Defatted olive cake, Phenolic, Antioxidants, Antitumor, Apoptosis*

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INTRODUCTION

Functional foods, medicinal plants, fruits, or flowers are good sources of biologically active compounds that have different biological activities and are used as alternative and complementary medicine (Novais *et al*., 2021; Yu *et al*., 2021). Oxygen or nitrogen peroxy radical species produced inside cells from metabolism, drugs, and pollution can have dangerous effects on cells (Knekt *et al*., 1996; Moreno-González *et al*., 2020). These radicals can induce oxidative damage to subcellular components and subsequently lead to degenerative diseases (Hertog *et al*., 1995; Guinda *et al*., 2010). Squalene (2, 6, 10, 15, 19, 23-hexamethyl tetracosaheaxaene) is a precursor of cholesterol synthesis in animal tissue. It is one of the major components of the epidermal lipids. It protects skin from lipid peroxidation, acting as a peroxide scavenger and antiaging. It was found that a diet supplemented with squalene decreased cholesterol and triglyceride levels and protected against CVD in animals (Duffy *et al*., 2001). The tree *Olea europaea* L. belongs to the family *Oleaceae*. Fruits are the main source of olive oil which is considered to have high nutritional value worldwide. In the Mediterranean region, olive oil is the main dietary component that contributes to the improvement of health status by reducing the incidence of many disorders as metabolic syndrome, cardiovascular disease (CVD), and some tumors (Nijveldt *et al*., 2001). The quality of olive fruit and its composition are affected by species, genetics, acidity, and industrial production conditions (Nijveldt *et al*., 2001). Traditionally, the olive fruit and its leaves have been used in the management of different conditions such as diabetes, hypertension, respiratory infection, antioxidants, skin moister, hair growth, and hypercholesterolemia (Schreyer *et al*., 1998). The monounsaturated fatty acids are the main content of olive oil related to biological and nutritional properties. A previous study reported that olive fruit and oil showed antimicrobial activity against *Salmonella enteritidis* and *Listeria monocytogenes*. It is used for edible purposes in dietary components (Servili *et al*., 2013). The fruit has a powerful anti-oxidant activity and anticancer properties as breast and colon cancer has reported that the amounts of the phenolic and squalene compounds are relatively high in olive fruit and oil. This study aimed to characterize the quality of the Egyptian olive's fruit *Olea europaea* (Wateeken cultivar) by identifying phytochemical components using GC/MS (Gas chromatography/mass spectrum), quantifying the phenolic and squalene content, and assessing its biological activity as antioxidant, anti-inflammatory, antitumor activity against human hepatocellular carcinoma cell line and mechanism of action via apoptosis.

MATERIALS AND METHODS

Preparation of defatted cake prepared from Egyptian olive fruit Olea europaea (DCEOF)

The olive fruit *Olea europaea* (Wateeken cultivar) used in this study was collected from the local Egyptian markets. The fruits were washed and dried to remove moisture. One kilogram of

olive fruit was soaked, chopped, and extracted in one liter of pure methanol at room temperature for 24 h. The extract was filtered using Whatman #1 filter paper. After that, the solvent was removed using a rotatory vacuum till dry. The extract was defatted with pure petroleum ether olive fruit extract. The defatted extract was stored at 20 °C for analysis.

Identification of the composition of DCEOF by GC/MS

A hundred milligrams of extract were dissolved in one ml of high-grade acetonitrile and subjected to analysis. The components of DCEOF were identified by GC/MS (Agilent, USA), Inj = 170 °C, Volume = 1 µL, Split = 1, Carrier Gas is helium, Solvent Delay = 6.00 min, Transfer Temp = 150 °C, Source Temp = 200 °C, Scan: 50 to 620 Da, Column (Elite-5MS, 30m 0.25mmID 0.25um).

Analysis of squalene and phenolic in olive fruit by GC/MS

Briefly, one gm of the fruit was extracted with two ml of pure methanol for 4 hours at room temperature. The solvent was evaporated at 35 °C. The residue was dissolved in 5 ml hexane 5 ml for defatting. The residue was dissolved in 1 ml acetonitrile for subjection to GC/MS. The separation capillary column was 30 mm in length, 0.25 mm Id., and 0.25 mm in thickness. Helium was used as carrier gas. The oven's initial temperature was 100 °C to 270 °C for 20 min (Brahmi *et al*., 2013).

Assay of scavenger capacity of DCEOF using 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH)

The total antioxidant activity of DCEOF was determined according to (Ahmad *et al*., 2021). Serial dilution of extract (10-100 mg/ml) was prepared. Each sample was supplemented with 150 µL of a DPPH solution with a concentration of 400 µM. The sample was placed in an incubator at a temperature of 37 °C for 30 minutes. After incubation, the absorbance of the sample was measured at a wavelength of 517 nm. The obtained value was compared to the value provided by the DPPH solution, and each extract was used as a blank without DPPH. The data was determined by calculating the concentration at which a 50% decrease in the DPPH occurs.

Determination of the anti-inflammatory activity of DCEOF

One mg of extract was dissolved in 1ml DMSO and serial dilutions were prepared (20 to 300 µg/ml). Aspirin (100 µg/ml) was used as a positive control (Allouche *et al*., 2011). The *in vitro* anti-inflammatory activity by the assessment of inhibition of albumin denaturation and membrane stabilization.

Inhibition of albumin denaturation of DCEOF

To one milliliter of extract (1 mg/ml), one milliliter of 1% bovine albumin was added. Adjusting the pH to 6.3, the mixture was heated to 50 °C for 30 minutes and then incubated for 20 minutes at 37 °C. Cooling results in an absorbance of 660 nm. The equation below was used to calculate the percentage inhibition of albumin denaturation: $(A_{control} - A_{sample} / A_{control})$ X100.

Erythrocyte membrane stabilization by DCEOF

A volunteer participant who had not taken any analgesics for two weeks provided the fasting blood sample, which was centrifuged for ten minutes at 4000 rpm. One milliliter of extract was combined with one milliliter of RBC suspension and left to incubate for 30 minutes at 56 °C. After cooling, the mixture was centrifuged at 3000 rpm for 10 minutes to separate the supernatant. At 560 nm, the supernatant's absorbance was measured and contrasted with that of the control sample.

Effect of different concentrations of DCEOF on the viability of HepG2 cell line

The MTT assay is used to measure cellular viability and proliferation. Different concentrations of extract (0-200 µg/ml) were used and incubated for 24, 48, and 72 hours. Then, to each well, 10 µl of MTT reagent was added, and incubated for 3 hours at 37 °C. The absorbance was measured at 500 nm. The 0.3% H_2O_2 (v/v) was used as positive control.

$$Growth\ inhibition\ rate\ (\%) = [(Acontrol - Asample) / Acontrol] \times 100 \quad (1)$$

Determination of caspase 3 and 9 levels in response to different concentrations of DCEOF

HepG2 was treated with 200 ug/ml extract and incubated for 24 hours with a CO2 incubator. The cells were collected and subjected to assay of caspase 3 and 9 by ELISA (enzyme-linked immunosorbent assay) technique kit obtained from BIORAD.

Statistical analysis

The data obtained was analyzed using SPSS, software V 20.0 using principal component analysis. Data was expressed as Mean ± SD. *P* value < 0.05 was considered as significant.

RESULTS AND DISCUSSION

The composition of DCEOF by GC/MS showed different active molecules as shown in **Table 1**. Including Branched alkene squalene [Dodecane, 2,6,11-trimethyl-, Tridecane, 2-methyl-], polyunsaturated fatty acids; Eicosane, Metabolites analogue [Decane, 2,3,5,8-tetramethyl-], Dodecanoic acid, Hexadecane, Sex prehormone [9(11)-Dehydrotestosterone], Sesquterpenoides [Dodecane, 2,6,11-trimethyl-, Undecane, 3,7-dimethyl-, 2,6-Dimethyldecane, Undecane, 3,8-dimethyl]. The squalene exerts an antibacterial effect. Vitamin A derivatives; Retinol, 4,14-Retro-retinol.

Table 1. The chemical composition of DCEOF with relative retention times.

Pk #	RT	Hit	Compound Name	Match	R.Match	Prob.	CAS	Library
1	13.762	1	Dodecane, 2,7,10-trimethyl-	853	859	8.5	544-76-3	RepLib
2	16.658	1	Isocaryophillene	916	920	14.0		Mainlib
3	17.273	1	à-Caryophyllene	917	932	62.6	6753-98-6	RepLib
4	17.713	1	Eicosane	855	867	7.2	31295-56-4	Mainlib

5	18.679	1	Cyclohexanemethanol, 4-ethenyl-à,à,4-trimethyl-3-(1-methylethenyl)-, [1R-(1à,3à,4á)]-	885	907	9.6	639-99-6	RepLib
6	19.224	1	Ledene oxide-(II)	841	866	27.6	1139-30-6	RepLib
7	19.604	1	cis-Z-à-Bisabolene epoxide	796	813	21.7	1139-30-6	Mainlib
8	19.704	1	Nonadecane, 2-methyl-	825	848	9.3	1560-84-5	Mainlib
9	19.899	1	Lanceol, cis	804	811	15.5		Mainlib
10	19.954	1	Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4- dimethyl-	773	782	16.6		Mainlib
11	20.189	1	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,7-octahydro-à,à,4a,8-tetramethyl-, (2R-cis)-	828	872	6.5	1209-71-8	RepLib
12	20.554	1	Cadala-1(10),3,8-triene	676	679	16.2		Mainlib
13	21.335	1	Myristic acid	808	898	65.9	544-63-8	Pfleger
14	21.685	1	(-)-Spathulenol	800	815	28.5	10067-29-5	Mainlib
15	22.415	1	Phytol	743	874	32.9	102608-53-7	Mainlib
16	28.503	1	4,14-Retro-retinol	757	766	20.8	7715-48-2	Mainlib
17	28.963	1	Andrographolide	724	816	6.5	5508-58-7	Mainlib
18	29.108	1	Podocarp-7-en-3-one, 13á-	702	737	36.4	16729-22-9	Mainlib
19	29.168	1	Prasterone	673	693	24.8	57988-82-6	Mainlib
20	29.283	1	4,14-Retro-retinol	718	744	29.0	16729-22-9	Mainlib
21	30.013	1	Androst -5-en-7-one, 3-(acetyloxy)-, (3á)-	724	765	6.7	25845-92-5	Mainlib
22	30.418	1	Retinol	755	760	41.8	68-26-8	RepLib
23	30.854	1	Ferruginol	863	879	83.2	511-15-9	Mainlib
24	31.404	1	9(11)-Dihydrotestosterone	715	809	50.0	24035-43-6	Mainlib

In **Table 2**, the squalene content showed a mean of 490 ± 45, while the phenolic content was 5.76 ± 1.5, and total antioxidant activity was 94.5%. The DCEOF showed anti-inflammatory activity as represented by inhibition of albumin denaturation and protection against erythrocyte cell membrane hemolysis compared with vitamin C as a positive control **(Table 3)**. The GC/MS analysis of DCEOF revealed its higher content of flavonoids, squalene, phenolic, and steroids. Due to the presence of hydroxyl group in Flavonoids and phenolic, it possesses antioxidant ability against free radicals (Meirinhos *et al*., 2005). The phenolic acids and squalene that are synthesized from phenylalanine are potent antioxidants because of their ability of the structure to scavenge free radicals. They are powerful free radical scavengers related to anti-inflammatory activity as indicated by the inhibition of albumin denaturation and prevent erythrocyte hemolysis. Previous studies reported that protein denaturation is a marker of inflammatory indices and tissue injury (Ghomari *et al*., 2019). For that reason, the anti-inflammatory activity of DCEOF is attributed to the content of arachidonic acid that is metabolized to produce anti-inflammatory prostaglandins.

Table 2. The Squalene, total phenolic level, and total antioxidant activity of DCEOF (Mean ± SD).

Parameters	Concentration
Squalene (mg/100 g)	490 ± 45
Total phenolic(mg/100 g)	5.76 ± 1.5
Total antioxidant activity (mg AAE/g extract)	94.5

Table 3. The anti-inflammatory activity of methanolic extract of defatted cake of olive fruit *Olea europaea* (Wateeken cultivar) (Mean of %).

Inhibition of Albumin Denaturation	44.4%
Antiproteinase Activity	92.6%
Aspirin	66.7%

The DCEOF in this study showed a potent anti-inflammatory property against albumin denaturation and erythrocyte hemolysis compared with ascorbic acid. This is attributed to the phenolic content of olive fruit that scavenger peroxy-radicals and protects the cell membrane of RBCs against heat exposure caused by stress and hemolysis. In addition, it inhibits albumin denaturation and coagulation by heat, this may attributed to the protection of non-covalent interaction and disulfide link in albumin that stabilizes. The data obtained showed that olive fruit extract exerts cytotoxicity and anti-proliferative activity against the HepG2 cell line. The effect was dose-dependent. This is following other previous studies (Savarese *et al*., 2007; Sánchez-Quesada *et al*., 2013; Rodríguez-García *et al*., 2019). The anti-proliferative activity may be the presence of active components of phenolic and squalene. This study showed that Wateeken olive fruit contains significantly higher amounts of squalene for that, the anti-proliferative activity may be due to inhibition of HMG-CoA reductase, thus reducing prenylation of the ras oncogene.

Data obtained showed that a cell proliferation assay was conducted on HepG2 cells following treatment with different concentrations of extracts (0-200 µg/ml) for 24, 48, and 72 hours. It was demonstrated that, after 24 hours, there was an increased inhibition percentage with dose dependency **(Figure 1)**. The inhibition rate reached its maximum after 48 hours

(Figure 2) and decreased after 72 hours **(Figure 3)**. The IC50 was 100 mg/ml.

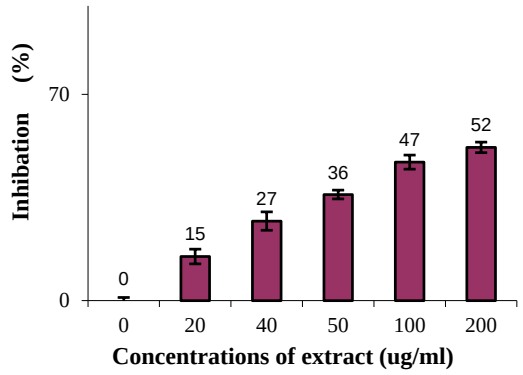

Figure 1. Anti-proliferation activity of- DCEOF after 24 hours of incubation.

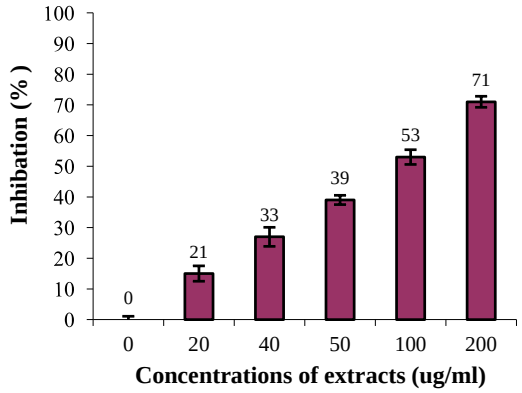

Figure 2. Anti-proliferation activity of DCEOF after 48 hours of incubation.

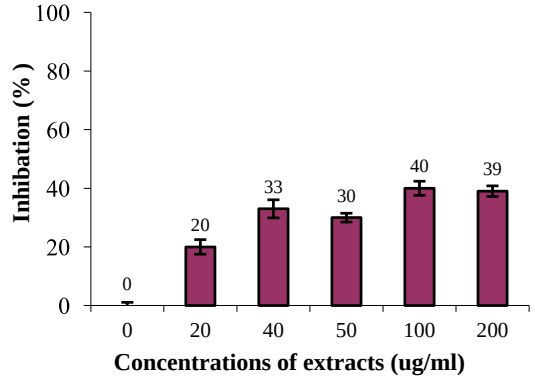

Figure 3. Anti-proliferation activity of DCEOF after 72 hours of incubation.

Programmed cell death (apoptosis) was mediated by signal pathways or mitochondria. HepG2 cell lines exposed to different concentrations of DCEOF caused increased levels of caspase 3 and -9 which mediate the DNA damage and cell death.

The decrease in cell proliferation was statistically significant compared to the control (H_2O_2). The caspase 3 and caspase 9 were found to be more expressed in HepG2 cell lines treated with different concentrations of DCEOF compared with untreated cells ($p < 0.05$) **(Table 4)**.

Table 4. Levels of caspases 9 and 3 as markers of apoptosis were treated with different concentrations of extracts.

Concentration of extract	Caspase-3(µg/ml)	Caspase-9
20 µg/ml	3.23	14.4
40 µg/ml	5.11	15.1
50 µg/ml	6.03	17.8
100 µg/ml	9.90	20.2
200 µg/ml	12.4	23.1

Olive fruit is considered a health-promoting food due to its high nutritional and medicinal impact. Olive fruit is worldwide around a hundred cultivars. In the current study, we collected olive fruit samples from a big store in Egypt. Methanolic extract and GC/MS analysis showed the presence of Dodecane, 2,7,10-trimethyl-, à-Caryophyllene, Eicosane, Nonadecane, 2-methyl-, cis-Z-à-Bisabolene epoxide, Lanceol, Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, Myristic acid, 4,14-Retro-retinol, Phytol, Podocarp-7-en-3-one, 13á- Dietary consumption of olive fruit was associated by prevention of some tumor (Meirinhos *et al*., 2005; Nenadis & Tsimidou, 2009; Ghomari *et al*., 2019). For that, the Egyptian-type Wateeken cultivar was tested as an antioxidant, anti-inflammatory, and cytotoxicity inhibitor against HepG2 cell lines. The antioxidant potential of medicinal plants may be attributed to the presence of active compounds such as Phenolic, terpenoids, and flavonoids. However, olive fruit is rich in phenolics and squalene. The antioxidant property was attributed to these active ingredients. This is in agreement with the study of (Savarese *et al*., 2007; Sabry, 2014; Rosales-García *et al*., 2017; Şahin & Bilgin, 2018) which reported the antioxidant potential of different species of olive fruit depending on geographical variations, water, temperature, and altitude, processing, packaging, and storage. This is approved its property as a preservative from rancidity. Squalene has been shown to reduce cholesterol levels as the main component of some drugs as cosmetic and pharmaceutical formulas (Sánchez-Quesada *et al*., 2013; Rodríguez-García *et al*., 2019; Yu *et al*., 2021).

CONCLUSION

It was concluded that the defatted cake of olive fruit *Olea europaea* (Wateeken cultivar) is rich with active biomolecules that exert significant biological activity as an antioxidant, anti-inflammatory, and antitumor against hepatocellular carcinoma cell line. The mechanism was mediated by an apoptotic pathway with its biological value and no side effects.

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