PHYTOCHEMICAL PROFILE, ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITIES OF OLIVE LEAF EXTRACTS FROM AUTOCHTHONOUS TUNISIAN CULTIVARS

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The aim of the present study was to investigate the biological activities of Tunisian olive leaf extracts and to correlate these activities to their phytochemical composition. The phenolic profile of four Tunisian autochthonous cultivars Chemlali, Sayali, Neb jmel, and Meski was determined using LC/MS-MS. The antioxidant activity of olive leaf extracts was evaluated using DPPH test. The antiproliferative effect was also investigated using MTT assay. The phytochemical screening showed that phenolic content and phenolic class repartition were significantly affected by olive leaf cultivars. Twenty-one components were identified and oleurpein, luteolin 4-glucoside, luteolin 7-glucoside, and apigenin 7-glucoside were the major phenolic components. Among all extracts, Sayali exhibited the strongest antioxidant and antiproliferative activities (IC$_{50}$ 41.36 µg ml^{-1} / EC$_{50}$ 147.11 µg ml^{-1}). The MTT result showed that olive leaf extract reduced MCF-7 cell viability in a dose-dependent manner. Western blot analysis demonstrated that olive leaf extracts exhibited antiproliferative activity through apoptosis induction.

Keywords: olive leaves, biological activities, free radical scavenging, antiproliferative activity, apoptosis

Olive leaves represent an abundant and unexploited source of bioactive compounds (Talhaoui et al., 2018). Indeed, finding new applications for olive leaves is of great value to the economy, to the environment, and to the human health (Rodrigues et al., 2015). Several studies have shown that olive leaf exhibit a large number of biological activities including antimicrobial, anti-inflammatory, antioxidant, and anti-carcinogenic activities (Sabry, 2016). All these beneficial effects have been related mainly to the presence of bioactive phenolic compounds. The phenolic composition of olive leaf is quite complex and strongly influenced by several factors, such as geographical origin, extraction procedure, and cultivar (Abaza et al., 2015).

Recently, there has been great interest in olive leaf as a source of antioxidants, which can significantly delay or prevent oxidation of targeted substrate by scavenging free radicals (Khaliq et al., 2015). Moreover, olive leaves have shown anticancer effects on various tumor cell lines including breast cancer cells (Samet et al., 2014). These mentioned health properties have promoted active research on methods to identify and quantify olive leaves extract. In this regard, the aim of this study was to investigate the chemical composition and the biological potentials of Tunisian olive leaf cultivars.

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1. Materials and methods

1.1. Sample preparation

Olive leaves of autochthonous cultivars (Chemlali, Sayali, Neb jmel, and Meski) were collected in mid-March from an olive orchard located in Mahdia (center of Tunisia, 35 30′ N, 11° 04′ E). The climate of this area is moderate. Olive trees were grown under natural rainfall, without application of pesticides. Fresh leaves were dried at room temperature (25 °C), ground to a fine powder, and conserved at –80 °C until analysis.

1.2. Extraction and phenolic analysis

Phenolic extraction was evaluated according to the procedure described by ABAZA and co-workers (2011). The powdered tissue was added to a mixture of methanol and water (80:20, v/v). The mixture was extracted in the ultrasonic bath for 15 min at room temperature and centrifuged at 5000 r.p.m. for 25 min. The filtrates were evaporated in rotary evaporator (Buchi, Switzerland) below 40 °C.

The samples were analysed by an API 4000 Q-Trap mass spectrometer equipped with a HPLC 1200 series system (Agilent Technologies). The LC-MS was operated in the negative ion mode using multiple reaction monitoring. The chromatographic separation of the analytes was achieved by reverse phase chromatography using an Eclipse XDB-C8-A HPLC column (5 μm particle size, 50 mm length and 4.6 mm i.d.). A binary mobile phase was made of 0.1% aqueous formic acid (A) and methanol (B). The total elution time was 20 min per injection (BEN MOHAMED et al., 2018)

1.3. Determination of the DPPH radical scavenging activity

The capacity to scavenge the free radical DPPH was monitored according to the method employed by ABAZA and co-workers (2011) with some modifications. Briefly, 0.1 ml of olive leaf extract at different concentrations was added to 3.9 ml of methanolic DPPH solution (6×10^{-5} M). The mixture was shaken and left to stand in the dark for 30 min. The radical scavenging activity (% RSA) was calculated according to the formula:

$$\% \text{ RSA} = [(\text{Abs C} - \text{Abs S})/\text{Abs C}] \times 100$$

where Abs C is the absorbance of the control and Abs S is the absorbance of the sample

1.4. Cells culture and treatments

MCF-7 breast cancer cells obtained from American Type Culture Collection (ATCC) were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The phenolic extract was dissolved in dimethylsulfoxide (DMSO) at a concentration ranging from 31.25 μg ml^{-1} to 500 μg ml^{-1} and diluted in DMEM/F12 medium supplemented with 1% DCC-FBS.

1.5. Assessment of cell viability

MCF-7 cells were seeded on 48 well plates (0.2×10^5 cells/well) and grown for 24 h in complete medium. The antiproliferative effect was measured using MTT assay. Seventy-two hours after treatments, fresh MTT was added. After 2 h incubation, cells were lysed with 200 μl of DMSO. The optical density was measured at 570 nm in a spectrophotometer.

1.6. Western blot analysis

Blots were incubated overnight at 4 °C with anti-poly (ADP-ribose) polymerase1 (PARP-1) antibody, anti-cyclin D1. Membranes were stripped and incubated overnight with an anti-glyceraldehyde 3-phosphate dehydrogenase antibody, and immune-reactive bands were visualized with the Western blotting detection system (SIRIANNI et al., 2012).

1.7. Statistical analyses

ANOVA analysis was used to evaluate the phenolic compounds as function of cultivar. The Tukey's multiple comparisons test was used to compare groups. P<0.05 was considered statistically significant. Pearson's correlation and linear regression were used to evaluate the relationship between phenolic composition/antioxidant activity/growth inhibition of MCF-7 cells.

2. Results and discussion

The phenolic extracts of Chemlali, Sayali, Neb jmel, and Meski cultivars were screened for the range of phenolic compounds. All extracts were found to contain high and varying amounts of phenolic compounds. In our study, significant differences were observed for total phenolic contents in extracts. As shown in Table 1, the highest amount was found in Sayali (30.89 mg g^{-1}), while the lowest was registered in Neb jmel extract (23.91 mg g^{-1}). Our results reported that olive leaves represent a potential source of phenolic compounds.

In the present study, a total of 21 compounds were identified including oleuropeosides, flavonoids, alcohol phenols, and simple phenols. Quantitatively a difference in the distribution of phenolic compounds and their corresponding groups was observed. The results from ANOVA showed significant quantitative differences in phenolic compounds according to the cultivar (P<0.05). The obtained result revealed that the phenolic profile of olive leaves is strongly affected by genotype factor. Our results are in accordance with those reported in the literature (KONTOMINAS, 2010; ABAZA et al., 2015).

In all analysed olive leaf cultivars, secoiridoids were by far the main abundant group of phenolic compounds. This dominance of secoiridoids in the phenolic fraction of olive leaves is also reported in the literature (KHALIQ et al., 2015). The main compound in this group was oleuropein. The registered values were 17.68 mg g^{-1} (Sayali); 15.61 mg g^{-1} (Chemlali); 14.93 mg g^{-1} (Meski), and 11.19 mg g^{-1} (Neb jmel). Several authors, by the characterization of the phenolic composition of Greek olive leaves (GOULAS et al., 2009) and Tunisian olive leaves (TAAMALLI et al., 2012) have observed that oleuropein is the main abundant compound in olive leaves of all cultivars.

Furthermore, our results showed that apigenin-7–O-glucoside was present at concentrations ranging from 0.62 mg g^{-1} in Sayali extract to 1.40 mg g^{-1} in Meski extract. Apigenin and luteolin were detected in all samples analysed. Luteolin was found in mean concentration ranging from 0.22 mg g^{-1} (Sayali) to 1.11 mg g^{-1} (Meski). These results are similar to those reported by several authors. MEIRINHOS and co-workers (2005) have reported luteolin-diglucoside and luteolin 4-O-glucoside the most frequent flavonoids in Portuguese olive cultivars. Diosmetin was detected and quantified in all cultivars. The concentration of tyrosol ranged from 0.041 mg g^{-1} in Chemlali to 0.33 mg g^{-1} in Sayali cultivars. The amounts

of hydroxytyrosol varied from 0.92 to 2.10 mg g^{-1} for Chemlali and Sayali cultivars, respectively. The concentration of hydroxytyrosol glucoside in Meski was the highest among all cultivars.

Table 1. Quantification of individual phenolic compounds in the leaves of different cultivars

Phenolic compound (mg of analyte/g dry matter)	Chemlali	Sayali	Neb jmel	Meski
Hydroxytyrosol glucoside	0.077[a]±0.001	0.107[a]±0.002	0.444[b]±0.010	0.599[c]±0.040
Tyrosol	0.041[a]±0.001	0.330[d]±0.001	0.252[c]±0.005	0.211[b]±0.002
Vanillin	0.659[b]±0.003	1.228[c]±0.002	0.600[a]±0.001	0.613[a]±0.007
Hydroxytyrosol	0.924[a]±0.001	2.101[c]±0.012	0.941[a]±0.003	1.152[b]±0.032
Rutin	0.820[d]±0.002	0. 605[c]±0.001	0. 431[b]±0.003	0.243[a]±0.004
Apigenin	1.218[b]±0.008	0.914[a]±0.005	1.308[b]±0.003	1.503[c]±0.047
Luteolin	0.561[c]±0.006	0.226[b]±0.002	0.113[a]±0.001	1.106[d]±0.024
Diosmetin	1.137[d]±0.009	1.463[b]±0.012	0.661[c]±0.002	1.162[a]±0.004
Apigenin-7 glucoside	0.924[b]±0.001	0.619[c]±0.010	1.153[a]±0.001	1.402[b]±0.003
Luteolin -7 glucoside	2.647[b]±0.002	0.978[c]±0.003	1.374[a]±0.002	1.692[b]±0.034
Luteolin-4 glucoside	1.709[c]±0.004	0.777[d]±0.004	1.262[a]±0.004	1.482[b]±0.010
Oleuropein	15.609[b]±0.111	17.684[c]±0.133	11.194[a]±0.078	14.913[b]±0.331
Verbascoside	0.714[c]±0.008	1.594[b]±0.003	1.224[d]±0.012	1.295[a]±0.004
2"-methoxyoleuropein	1.005[d]±0.004	0.457[c]±0.002	0.234[b]±0.002	0.115[a]±0.004
Ligostride	0.290[a]±0.000	0.848[c]±0.026	0.500[b]±0.001	0.329[a]±0.008
Oleuropein aglycone	0.310[a]±0.005	0.394[b]±0.002	1.743[c]±0.010	0.405[b]±0.004
Aglycon oleuropein dialdehydic	0.031[b]±0.01	0.020[a]±0.02	0.066[c]±0.001	0.016[a]±0.002
Olpeuropein derives	nd	nd	0.102[b]±0.003	0.017[a]±0.001
Ligastroside aglycone	0.096[a]±0.003	0.306[d]±0.003	0.178[c]±0.004	0.127[b]±0.002
10-hydroxy oleuropein aglycon	0.009[a]±0.000	0.030[c]±0.001	0.013[b]±0.001	0.033[c]±0.002
Hydroxy D-oleuropein aglycon	0.048[d]±0.000	0.025[b]±0.001	0.016[a]±0.000	0.030[c]±0.001
Total phenols	29.026[c]±0.120	30.898[d]±0.164	23.917[a]±0.082	28.992[b]±0.360

Results are expressed as mean ± standard deviation; significant differences in the same row are showed by different letters (P<0.05); nd: not detected

The obtained results revealed that Sayali extract showed the highest antioxidant potential (IC$_{50}$ value 41.56 µg ml^{-1}). Moreover, all olive leaf extracts were able to scavenge free radicals. These results were in agreement with the report by SALAH and co-workers (2012), who showed that olive leaf extracts present a potent antioxidant activity.

Moreover, all extracts induced the decrease of MCF-7 growth in a dose-dependent manner (Fig. 1). As shown in Table 2, olive leaf extracts from Sayali have shown the highest inhibitory effect against MCF-7 cells with a CE$_{50}$ of 196 µg ml^{-1}, followed by Neb jmel,

Chemlali, and Meski. These results are in agreement with findings of Fu and co-workers (2010). The authors have estimated the CE_{50} values of olive leaves ranging from 200 to 300 µg ml^{-1}.

Table 2. Antioxidant and antiproliferative activities of various extracts of the Tunisian olive leaf cultivars by DPPH and MTT assays

Olive leaf extract	DPPH (IC_{50}) µg ml^{-1}	MTT Test (CE_{50}) µg ml^{-1}
Chemlali	50.17±1.28	213.05±2.13
Sayali	41.56±0.41	147.77±2.78
Neb jmel	58.16±1.21	183.61±1.07
Meski	63.52±0.47	280.07±2.55

The results were given as half maximum effective concentration (IC_{50} and CE_{50}); IC_{50} and CE_{50} values represent the means ± standard deviation of three replications ($P<0.05$).
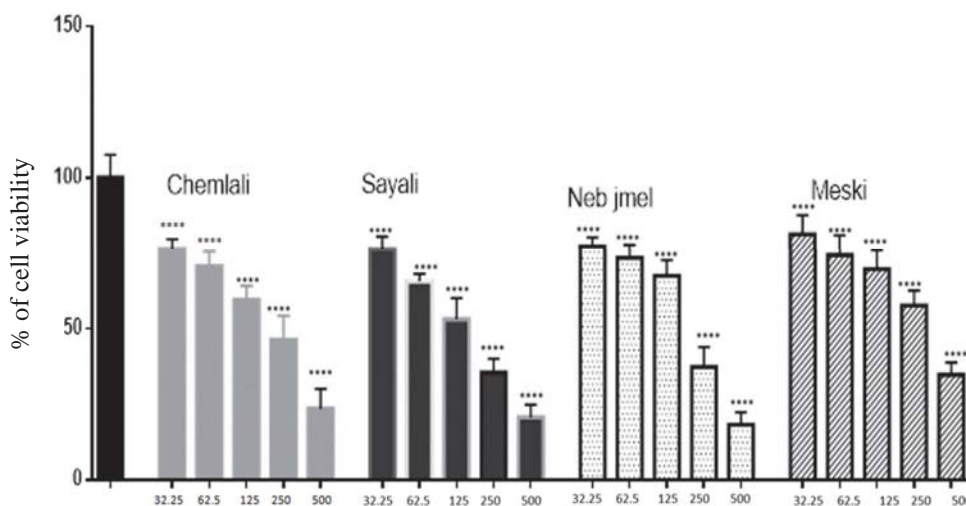

Fig. 1. Effects of different concentration of olive leaf extracts (Chemlali, Sayali, Neb jmel, and Meski) on MCF-7 cell proliferation
Columns are means of three independent experiments each performed with triplicate samples expressed as percent of basal, bars: SD; [xxxx]: significant difference at $P<0.0001$ compared with basal

Western blotting analysis (Fig. 2) revealed the presence of cleaved form of PARP-1 in MCF-7 treated cells. Our result demonstrated that olive leaf extracts induce a slightly upregulated expression of cyclin D1 in treated MCF-7 cells when compared to control cells. The obtained result showed that treatment of MCF-7 cells with olive leaf extracts stimulated the apoptosis efficiently.

As shown in Table 3, a positive linear correlation between the antioxidant capacity values (IC_{50}) and total phenol content was found. The antioxidant activity of olive leaf extracts could be attributed to their high amounts of total phenol ($R^2=0.911$) and total secoiridoids ($R^2= -0.975$). Moreover, antioxidant activity could be attributed to the higher oleuropein amount ($R^2= -0.964$), as well.
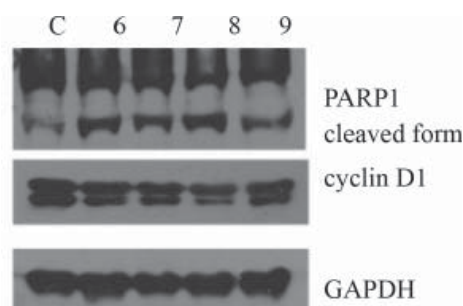
Fig. 2. Effects of olive leaf phenolic extracts on PARP-1 and cyclin D1 activation. Blots are representative of three independent experiments with similar results. (C: Basel; 6: Chemlali; 7: Sayali; 8: Neb jmel; 9: Meski; GAPDH: glyceraldehyde 3-phosphate dehydrogenase antibody)

Table 3. Pearson correlations

Pearson correlations	IC_{50}	CE_{50}
Vanillin	**–0.862**[**]	–0.685[*]
Hydroxytyrosl	**–0.863**[**]	–0.549
Apigenin	0.668[*]	**0.876**[**]
Luteolin	–0.073	**0.935**[**]
Diosmetin	**–0.810**[**]	0.235
Apg-7-GLUC	0.673[*]	**0.850**[**]
Lut-4-GLUC	.522	**0.709**[**]
Oleuropein	**–0.964**[**]	–0.151
Ligostride	–0.578[*]	**–0.798**[**]
Oleuropein aglycone	**0.780**[**]	–0.270
Aglyconolpdialde	**0.805**[**]	–0.335
Olpeuropein derives	**0.834**[**]	–0.145
Ligastroside aglycone	–0.589[*]	**–0.756**[**]
Total phenols	**–0.911**[**]	0.036
Simple phenols	**–0.877**[**]	–0.554
Secoiridoids	**–0.975**[**]	–0.428
Flavonoids	0.200	**0.820**[**]

*: $P<0.05$; **: $P<0.01$

From this study, it was shown that phenolic compounds largely contribute to the antioxidant activities of olive leaf. Our results are in agreement with those reported in the literature. Benavente-García and co-workers (2000) reported that the radical scavenging potential of olive leaf extract depends mainly on its amount of phenolic compounds. On the other hand, the obtained results of correlation of phenolic content and CE_{50} have shown that the anti-proliferative effect in MCF-7 breast cancer cells does not correlate with phenolic content or with antioxidant capacity.

3. Conclusions

The results reveal the richness of Tunisian olive leaves in phenolic compounds. Furthermore, phenolic profile could be used as a fingerprint to discriminate olive leaves by cultivar.

On the other hand, the obtained results showed that Sayali extract exhibited the strongest antioxidant and antiproliferative potentials. In the light of these findings, Sayali extract could be recommended as a source of antioxidant components for therapeutic industries.

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