CHEMICAL PROPERTIES OF POLYPHENOLS EXTRACTED FROM OLIVE MILL WASTEWATER OF CHAMLAL VARİETY

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Abstract—The paper is to study the reductive and antioxidant activity of polyphenols extracted from Olive margins wastewater (OMWW) of the Chamlal variety through various tests. The phenolic compounds (PC) are extracted with different organic solvents (ethyl acetate, diethyl ether and n-butanol). The glycosylated forms have undergone acid hydrolysis treatments at different concentrations of HCl to break single bonds and alkaline hyrolysis to hydrolyze the esters. The antioxidant properties of the heterosidic and aglycone forms were evaluated before and after deglycosylation by the DPPH test and the reduction of potassium ferrocyanide. The level of quatity of PC is remarkably higher after deglycosylation thus proving their release from any previous combination, since the amount of ether, butanolic and ethyl acetate extract before treatment is respectively (3.64 ± 0.58). , (12.60 ± 0.26) and (5.22 ± 0.69) mg / g of total solids and amounts after acid chemical hydrolysis to (35.70 ± 0.23), (45.60 ± 0.79) and (39.50 ± 0.50). The results of the evaluation of the activity of extracts obtained from vegetable waters (OMWW) before and after chemical deglycosylation treatments are subjected to a statistical analysis. The fractions, extracted after treatment in 2N HCl medium, containing a higher level of sugars are those which have statistically better anti-radical activity and a better reducing power compared to extracts containing less glycosylated PC. On the other hand, the ether extract, containing the aglycone forms, obtained after hydrolysis with 6N HCl, is statistically more active than the other extracts (butanol and of ethyl acetate). In addition, the exposure time of phenolic molecules to HCl is different, thus showing differences in the activity of extracts that result. This could be due to the nature of the compounds released from the reactions that statistically exhibit very highly significant differences and to the very mechanisms of the reactions that would present differences.

Key words—Olive margin wastewater, Polyphenols, Antioxidant activity, DPPH, Reducing power, Deglycosylation.

1. Introduction

The olive industry is an important economic activity, concentrated mainly in the Mediterranean countries, which account for about 95% of world production, In Algeria 1% in 2001. It mainly produces olive oil, which requires a large quantities of water generating liquid effluents (Olive mill wastewater), and solid (pomace). Olive mill wastewaters (OMWW) are liquid discharges that are very rich in organic matter (phenolic compounds (PC), lipids), often spread as they are in nature, uncontrolled on agricultural soils or sometimes stored temporarily in agricultural soils, exposing the water-soil-plant systems to an unavoidable pollution (Tsioulpas and al, 2002).
Different treatments are applied to them: biological, physical and chemical. Costly and still insufficient, these treatments all reduce their impact on the environment. In order to reduce the costs of the various treatments applied to vegetable waters and to rationalize the management of their discharges or to valorize them, research is oriented on the valorization of their effluents in various fields: composting, agriculture and even in the pharmaceutical industry . Phenolic compounds are inhibitors of oxidation by free radical scavenging, identified as natural antioxidants (Bondia -Pons and *al*, 2009).

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2. Materials and methods

The used vegetable waters (OMWW) come from a modern industrial unit of olives trituration by three-phases centrifugation, located in the region of Sidi Naamane (Tizi-Ouzou), Algeria during the 2012/2013 olive oil campaign. Samples were taken from the garden pond and stored in plastic bottles at -20 ° C until use. They are recovered in the liquid state as produced by the centrifuge of the oil mill.

2.1. Polyphenols extraction

The polyphenols was extracted from the vegetable waters according to two methods either by adding the ethyl acetate or using three solvents by increasing polarity. According to the first method, the ethyl acetate is added to the dilapidated vegetable waters (V / V), the whole is homogenized. After centrifugation at 4000 g for 20 min, the mixture is completely separated into two phases: ethyl acetate rich in (supernatant) and vegetable waters (precipitate). The extraction operation is repeated three times in order to recover the maximum of phenolic compounds (CPMB and CPMD). The organic phase rich in phenolic compounds undergoes evaporation under vacuum in a rotavapor at 40° C (Zahari and*t al*, 2014). According to the second method , the extraction is carried out using the method of Markham (1982) and the method of Bruneton (1993). It is based on the degree of polyphenols solubility in organic solvents (diethyl ether, ethyl acetate and *n*-butanol). In order to separate the compounds into aglycone, monoglycoside and di and triglycoside fractions, previously delipidated vegetable waters are mixed with diethyl ether (v / v) to obtain an organic phase containing the aglycone forms and optionally the aglycones (Eth). The remaining aqueous phase in turn undergoes three extractions with ethyl acetate in order to recover certain aglycone forms in the organic phase but especially the monoglycosides (Acet.). The remaining aqueous phase is mixed with *n*-butanol to recover in particular the di and triglycosides, as well as the C-glycosylated forms. The three fractions harvested are concentrated by evaporation at low pressure at 35°C.

2.2. Hydrolytic treatment

The hydrolytic treatment was carried out using different concentration of hydrochloric acid (HCl) in order to break the bonds of C-O-C linking the sugars to the polyphenols. The extraction method described by ROBLES and *al.* (1998) is based on the hot acid hydrolysis of the glycosides present in the plant material. According the first method of the Heteroside Bonds of Phenolic compounds using 2N HCl, for a glycone research, acid hydrolysis to break the bonds (C-O-C) is carried out on 2g of plant material (lyophilisate) which are placed in the presence of 160 ml of cold 2N HCl. The solutions are then placed in a water bath at 100°C, for 40 minutes. Two acid treatment procedures were performed. After cooling, the CP are extracted from the acidic aqueous phase with different organic solvents to compare them qualitatively and quantitatively later. The dry residues are taken up in methanol (Acet 2N, But 2N ; Eth 2N, But'2N).

According the second method using 6N HCl, the treatment is carried out on the sample in its liquid form, the olive mill wastewaters dilapidated undergo an acid treatment by operating in a solvent consisting of equal volumes of 6N HCl and dilapidated vegetable waters. The mixture is brought to the bubbling water bath for exactly 5 minutes. Two tests were carried out for subsequent comparative purposes, after cooling, the total phenols are extracted as for the previous treatment (Acet 6N, But 6N ; Eth 6N, But'6N).

According the third method based on alkaline hydrolysis adapted by Ribereau-Gayon (1968) method followed by acid hydrolysis. It is conducted in a 2N NaOH medium at room temperature, under a vacuum atmosphere, for 4 hours, this step is followed by an acid treatment, the latter consists of acidification with concentrated HCl until a pH identical to that of 2N HCl, then bring to a boiling water bath for 3 minutes. This type of hydrolysis is intended to break the ester bonds and the C-O-C bonds that can bind the sugar to the polyphenols, possibly. The extracts obtained are symbolized as follows: Acet ALCA, But ALCA; Eth ALCA, But' ALCA.

2.3 Characterization of phenolic extracts

2.3.1 Colorimetry

The quantitative and qualitative content of phenolic compounds of the various extracts obtained from treated and untreated vegetable waters was estimated by the method of Folin-ciocalteu according to Singleton and Rossi (1965) which is based on the reduction in alkaline medium of the mixture phosphotungstic and phosphomolybdic reaction of Folin reagent by reducing groups phenolic compounds, leading to the formation of blue color reduction products. The latter have an absorption maximum at 760 nm whose intensity is proportional to the amount of polyphenols present in the sample. The solutions of the different samples to be assayed and the standard range (gallic acid) are prepared in the same manner and under the same conditions.

2.3.2 Thin layer chromatography (TLC)

The samples are analyzed using commercial ready-to-use plates of size (20x20cm) silica gel.Several solvent systems have been tried:

I: Ethyl acetate / Methanol / Water (v / v / v) (100 / 13.5 / 10) (Radomir and *al*, 2010).

II: Chloroform / Ethyl acetate / Acetic acid (v / v / v) (50/50/10) (CHA *et al*, 2011).

III: Chloroform / Methanol / Acetic Acid / Water (100/15 / 0.3 / 0.5) (Amiot and *al*, 1995).

IV: Chloroform / Ethyl acetate / formic acid (50/40/10) (RIOV and GOTTLIEB, 2006).

V: Acetic acid 2% and 15% (Ribereau-Gayon, 1968).

Only the two systems (II) and (IV) showed good separation.

2.3.3 Evaluation of the antioxidant activity

The reducing capacity of a compound can serve as a significant indicator of its potential for antioxidant activity (Meir and *al*, 1995, Apak and *al*, 2007). The reduction of Fe^{3+} is often used to study the ability of a substance to donate electrons. This property is an important mechanism of the antioxidant action (Ebrahimzadeh *et al*, 2008).

o *Reducing power test on potassium ferrocyanide*

The iron-reducing activity of the phenolic extracts obtained before and after deglycosylation is determined according to the method described by OYAIZU (1986), modified by Chew and *al*. (2009) which is based on the Fe^{3+} reduction present in the $K_3Fe(CN)_6$ complex in Fe^{2+} involving the electron transfer mechanism.Thus 2.5 ml of the CP dilution of concentration of approximately 10 µg/ml were mixed with 2.5 ml of a phosphate buffer solution (pH 6.6) and 2.5 ml of potassium ferrocyanide ($K_3Fe(CN)_6$) (1%). The resulting mixture was incubated at 50°C for 20 min. Then, 2.5 ml of trichloroacetic acid (10%) is added to the mixture to stop the reaction. The mixture is centrifuged at 3000xg for 10 min. The supernatant is recovered, 2.5 ml of the latter are mixed with the same volume of distilled water and 0.5 ml of $FeCl_3$ 0.1% solution. Absorbance is measured spectrophotometrically at 700 nm. The same procedure is performed for the other concentrations of PC before and after hydrolysis treatments, namely 0, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg / ml in distilled water. The same experiment was carried out for gallic acid, caffeic acid, ascorbic acid, and oleuropein at different concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg / ml in distilled water). The tests were repeated 3 times for all the compounds tested.

o *Ability to trap radical species: Reaction between the free radical DPPH • and the antioxidant*

The experimental protocol used is that of Brand-WIlliams *et al.*(1995). The DPPH(α,α-diphenyl-β-picrylhydrazyle) solution is prepared by solubilization of DPPH in methanol. 50µl solutions of phenolic or standard extracts are added to 2ml of DPPH solution. The mixture is left in the dark for 1 hour and the discoloration compared to the negative control which contains only the DPPH solution which is measured at 517 nm. In our test the positive control is represented by a solution of synthetic gallic acid as a standard antioxidant. The concentrations of phenolic extracts of vegetable waters (before and after deglycosylation) in the reaction medium, as well as that of gallic acid used as a control in several tests, are 20; 40; 60; 80; 100; 150; 200 and 250 µg / ml. The decrease in absorbance is evaluated by the percent inhibition (% I) and the IC50 (equivalent quantity extract that neutralizes 50% of DPPH •). A low IC50 corresponds to a high antioxidant or anti-radical activity of the extract.

2.3 Statistical analysis

The statistical processing is done using the STATBOX software version 6.4. The measured variable taken into account is the antioxidant power measured. The two factors studied are: solvent type and concentration, since the nature of the polyphenols found in an extract depends on the type of solvent used for their extraction, and the second factor (the concentration of the phenolic compounds extracts) must imperatively be taken in account since the antioxidant power is variable depending on this setting.

3. Results and discussions

3.1.Characterization of phenolic extracts

Results of qualitative and quantitative polyphenols extracted obtained from vegetable waters are represented in histograms (Fig.1).

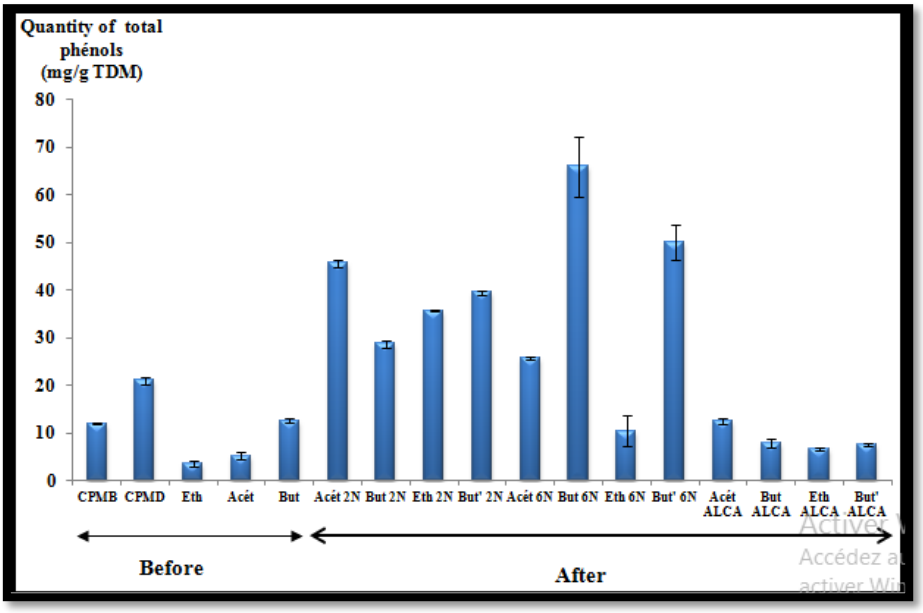

Figure 1 Results of the total polyphenols extracts obtained before and after chemical deglycosylation treatments (acids and alkaline).

According the results in figure 1, the total phenol content which is promising since it reflects the release of certain entities that have glycosidic links with glycan structures. For example, the polyphenols content is estimated at 3.64 ± 0.58 mg gallic acid equivalent GAE/g total dry matter (TDM) of the ether extract obtained without chemical treatment of vegetable waters and raises to 35.70 ± 0.231 mg GAE/g TDM after hydrolysis in 2N HCl medium. Diethyl ether extracts aglycone forms while ethyl acetate is specific for monoglycosylated forms and *n*-butanol for tri-O -glycosides and C-glycosides, for its polar character (Liu and *al*, 2014).

The qualitative analysis of the polyphenolic extracts from raw and delipid vegetable water (OMWW) by thin layer chromatography (TLC) revealed that both extracts have the same qualitative composition and contain a considerable number of constituents visible on the chromatographic profiles (Figure.2 and figure.3).

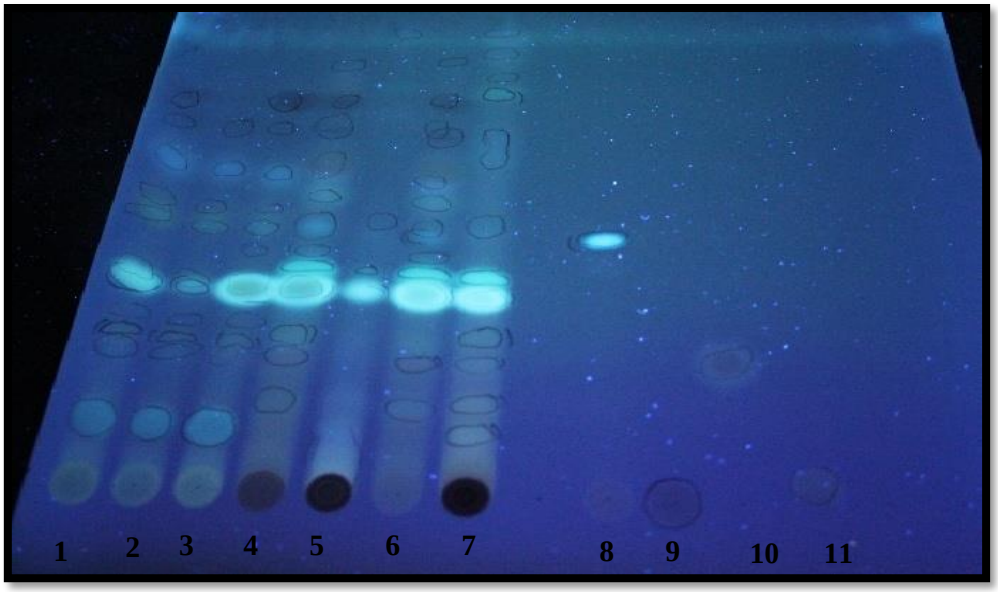

Figure 2. Chromatographic profile of the phenolic extracts obtained before and after treatment with 2N HCl. Visualization under UV lamp at 366 nm. 1. CPMB. 2. CPMB. 3. CPMD.4. Acet2N.5.But 2N.6.Eth 2N.7.But'2N.8.cafeic acid.9.rutin.10.gallic acid.11.oleuropein.

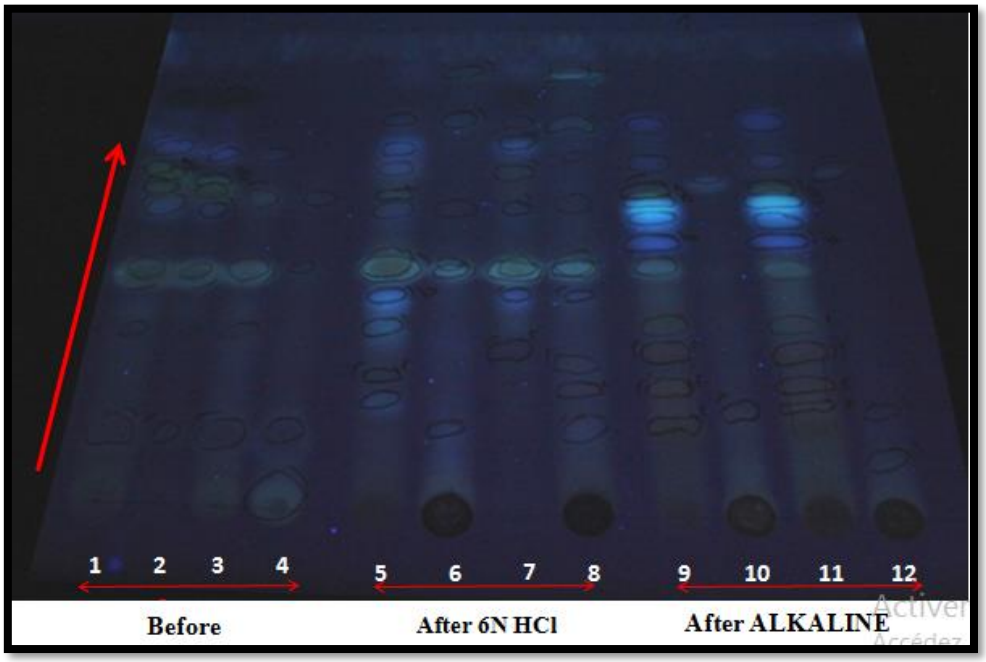

Figure 3. Chromatographic profile of the extracts obtained before and after acid hydrolysis (6N HCl) and alkaline. Reading at 366 nm.1.CPMB 2. Ather Extract (OMWW) 3. Ethyl acetate extract (OMWW) .4. butanolic extract (OMWW)5. Acet 6N. 6.But 6N. 7.Eth 6N. 8.But '6N. 9. Eth ALCA 10.But'ALCA.11.Acét ALCA.12.But ALCA.

The chromatographic profiles prove that polyphenols have indeed been liberated from anterior glycosidic bonds since the extracts obtained before and after deglycosylation show differences in the number of spots that appeared in the butanol extracts, thus confirming their capacity in glycosylated forms, in visible form already and under UV. The polyphenols of the ether extracts seem to migrate further than those of the other extracts thus testifying to their non-polar nature by the removal of sugar by the acid and / or alkaline chemical treatments previously provided (figs. 02 and 03).In addition, the color of the spots (spots of red, blue, yellow color ...) confirms the difference between the aglycone or less glycosylated forms released by the different treatments, this could only be explained by the fact of

the mechanisms of action of each method used specific to it (an example is given of alkaline hydrolysis releasing phenolic acids which appear under UV under clean aspects and specific colors (fluorescence) to the molecule).

3.2. Antioxidant activity

To compare the activity of the various substances, the reducing power PR0.5AU defined as the quantity of a substance in µg per ml of the reaction volume, which gives an absorbance unit of 0.5 to 700 nm is determined (Ardestani and Yazdanparast, 2007).The results of the potassium ferrocyanide reducing power evaluation of the extracts obtained before and after the acidic and alkaline chemical treatments are summarized in Table I.

Table I: Concentrations of extracts obtained before and after deglycosylation giving after reduction of potassium ferrocyanide an optical density of 0.5.

Extract	Concentrations (µg/ml) giving an optical density of 0.5 to 700 nm
CPMB	33,27
Eth	26,88
Acet	23,47
But	24,2
Acet 2N	34,33
But 2N	35,73
Eth 2N	45,25
But' 2N	33
Acet 6N	28,38
But 6N	34,30
Eth 6N	17,72
But' 6N	22,19
Acet ALCA	18,43
But ALCA	16,52
Eth ALCA	21,10
But'ALCA	17,09

These concentrations provide information on the effectiveness of phenolic compounds in reducing potassium ferrocyanide. They are inversely proportional to the antioxidant activity, the lowest concentration corresponds to the most important activity. It is clear that the ether extracts obtained before and after the hydrolysis treatments containing the less glycosylated compounds or even aglycones have less important activities than those of the compounds extracted with n-butanol and ethyl acetate. However, the ether extract (Eth 6N) obtained after hydrolysis with 6N HCl has a lower PR0.5 (17.72 µg / ml) than that of the butanolic extract (22.19 µg / ml) and the extract Acetate (Acet 6N) appears to be more effective (28.38 µg / ml) than butanol extract (34.30 µg / ml). These differences could be explained by the difference in the composition of the phenolic compound extracts (DORMAN and HILTUNEN, 2010). A statistical analysis was carried out in order to concretize the differences existing between the extracts and to interpret both biologically and statistically the existence of these differences. The most plausible reason is the mechanism of action of HCl used at different concentrations.

3.3. Anti-radical activity-DPPH test

The figure 4 shows the concentrations of phenolic extracts obtained before and after deglycosylation treatments as well as those of standards reducing the amount of DPPH to 50% (IC50).

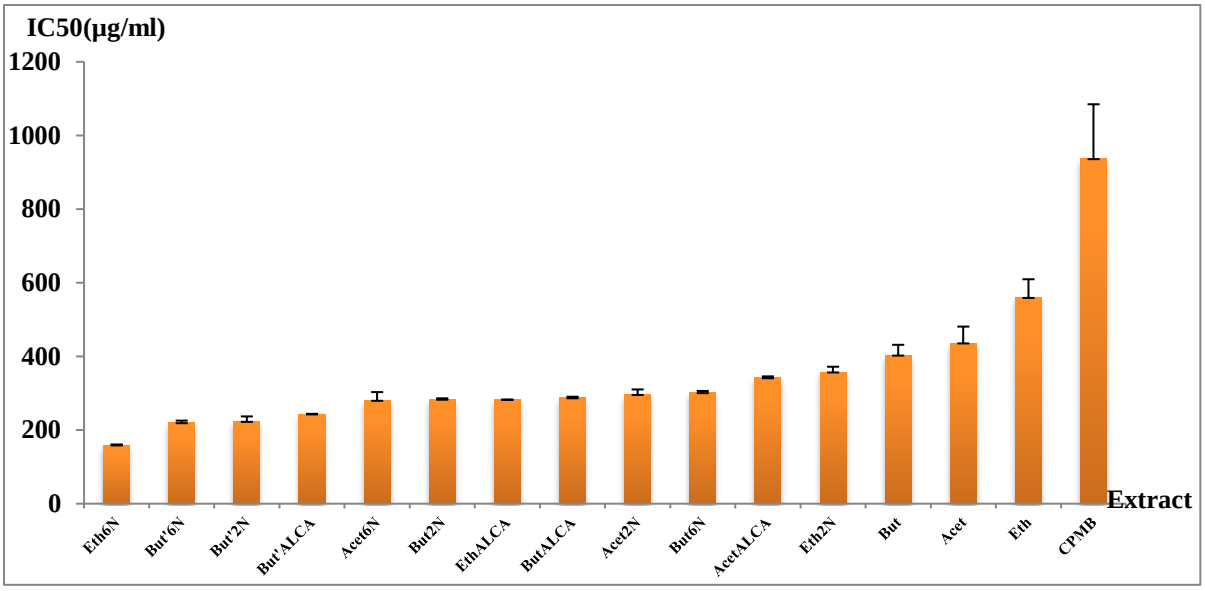

Figure 4. Inhibitory concentrations (µg/ml) of polyphenols extracted before and after chemical deglycosylation treatments reducing the amount of DPPH to 50% (IC50).

The comparison between the activity of the phenolic molecules extracted with *n*-butanol (polar solvent) and those extracted by the less polar solvents (ethyl acetate and ether) shows that the polar forms have a relatively better activity, which could leave assume that the forms found in the butanolic extract possess either more carbohydrate-related structures; or hydroxyl groups (OH) or other .. having a polar character that can contribute to the potentiating of the power to reduce free radicals. This was also noticed and concluded by LIU *et al.* (2014) following their study on butanol, ether and ethyl acetate extracts of phenolic compounds of another plant material as well as that of Dorman and Hiltunen (2010).) thus bringing the same conclusion.

From our study, we note that the methods developed for the extraction of polyphenols and deglycosylation are reliable to a certain degree and release each of the specific products according to the mechanism of action of the hydrolytic reagent. Some hydrolysis methods appear to release aglycones that show better activity than glycosylated forms; others on the contrary release in addition to aglycones, glycosylated forms having escaped hydrolysis and have a better activity. It would appear that sugars in some cases would contribute to the potentiating of antioxidant capacity when their reducing functions are free.

4. Conclusions

The olive oil industry requires a large quantities of water generating liquid effluents (Olive mill wastewater), and solid (pomace). Olive mill wastewaters (OMWW) are liquid discharges that are very rich in organic matter (phenolic compounds (PC), lipids). The paper study the reductive and antioxidant activity of polyphenols extracted from Olive margins wastewater of the Chamlal variety located in the region of Sidi Naamane (Tizi-Ouzou), Algeria. The phenolic compounds (PC) are extracted with different organic solvents (ethyl acetate, diethyl ether and n-butanol). The glycosylated forms have undergone acid hydrolysis treatments at different concentrations of HCl to break single bonds and alkaline hyrolysis to hydrolyze the esters. The antioxidant properties of the heterosidic and aglycone forms were evaluated.

The total phenol content which is promising since it reflects the release of certain entities that have glycosidic links with glycan structures.

The qualitative analysis of the polyphenolic extracts from raw and delipid vegetable water by thin layer chromatography (TLC) revealed that both extracts have the same qualitative composition and contain a considerable number of constituents visible on the chromatographic profiles.

The important anti-oxidant activity of the phenolic compounds (aglycones and glycosylated) extracted from the effluent, it would be interesting to consider incorporating them into products as well for the purpose of food, pharmaceutical and cosmetology, thus increasing their conservation. A biotechnological valorization study of olive by-products can be considered in future work

5. References

[1] Amiot, m.j., m. Tacchini, S.Y. Aubert (1995). Influence of cultivar, maturity stage, and storage conditions on phenolic composition and enzymatic browning of pear fruits. *J. Agric. Food Chem.,* 43: 1132-1137.

[2] Apak R., Güçlü K., Demirata B., Özyürek M., Çelik S.E., Bektasoğlub., Berker K.I. and Özyurt D. (2007). Comparative evaluation of various totalantioxidant capacity assays applied to phenolic compounds with the cuprac assay. *Mol.,* 12: 1496-1547.

[3] Ardestani A. and Yazdanparast R. (2007). Antioxidant and free radical scavenging potential of Achillea santolina extracts. Food Chem., 104 : 21–29.

[4] Bondia-Pons I., Aura A-M., Vuorela S., Kolehmainen M., Mykkanen H., and Poutanen K. (2009). Rye phenolics in nutrition and health. *J. of Cereal Science*., 3: 1–14.

[5] Brand-Williams W., Cuvelier M. E. and Berset C. (1995). Use of free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.*, 28: 25–30.

[6] Bruneton, J. (1993). Pharmacognosie : Phytochimie, Plantes médicinales. 2ème édition, Lavoisier, Paris.

[7] Cha Y.Y., Kim S.K., Ahn C.B. and JE J.Y. (2011). Preparation, characterization and antioxydan properties of gallic acid-grafted chitosans. *Carbohydrate polymers.*, 83: 1617-1622.

[8] Chew Y-L., Goh J-K. and Lim Y-Y. (2009). Assessment of in vitro antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia. *Food Chem.*,116 : 13–18.

[9] Dorman HJ and Hiltunen R. (2010). Ocimum basilicum L.: phenolic profile and antioxidant-related activity. *Nat. Prod. Commun.,* 5(1): 65-72.

[10] Ebrahimzadeh M.A., Pourmorad F. and Hafezi S. (2008). Antioxidant Activities of Iranian Corn Silk. *Turk. J. Biol.*, 32, 1-7.

[11] Liu F, Liaos, Zou Y and Xiao G (2014). Antioxidant activity of *Phellinus igniarius* extracts. *The FASEB Journal*. 28(1) : 830-32.

[12] Markham KR. (1982). Technics of flavonoids identification. *Academic Press* (London); Chap.1 et 2: 1-113.

[13] Meir S., Kanner J., Akiri B. and Hadas S.P. (1995). Determination and involvement of aqueous reducing compounds in oxidative defence systems of various senescing leaves. *J. Agric. Food Chem.,* 43 : 1813–1815.

[14] Oyaizu, M. (1986). Studies on product of browning reaction prepared from glucose amine. *Jpn. J. Nutr*. 44, 307–315.

[15] Profile and antioxidant-related activity. *Nat. Prod. Commun.,* 5(1): 65-72.

[16] Radomir V. Malbaša, Eva S. Lončar and Kolarov L. (2004). TLC analysis of some phenolic compounds in kombucha beverageapteff, 35, 1-280.

[17] Ribereau-Gayon P. (1968). Les composes phénoliques des végétaux.Edition Dunod, Paris, pp 254.

[18] Riov J. and Gottlieb H.E. (2006). Metabolism of auxin in pine tissues:Indole-3- acetic acidconjugation.*Physiologia. Plantarum.,* 50 : 347-352.

[19] Robles C., Greff S., Pasqualani V., Garzino S., Bousquetmelou A., Fernandez C., Korboulewsky N. and Bonin G. (2003). Phenols and Flavonoids in Aleppo Pine Needles as Bioindicators of Air Pollution. *J. Environ.* Qual.32:2265–2271.

[20] Zahari A., Tazi A. and Azzi M. (2014). Optimisation des conditions de traitement des margines par un superoxydant K3FexMnyO8. *J. Mater. Environ. Sci.*, 5 (2): 484-489.