Contribution to the Chemical and Biological Study of Eucalyptus Leaves Extracts: Effect on Frying Oil

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***Abstract*—In this research, various extracts (petroleum ether, ethyl acetate, methanol and water) of Eucalyptus stricklandii were screened for their global chemical composition, antioxidant, antilipidic peroxidation and anti-inflammatory activities. Tannins (102.89-248.73 mg catechin equivalent/Kg dry mass), flavonoids (19.3-15.34 g quercetin equivalent/Kg dry mass), phenolics (54.18-305.24 g gallic acid equivalent/Kg dry mass) and anthocyannins (394.86-4781.02 mg cyanidin-3-glucoside equivalent/Kg dry mass) of various extracts were evaluated. Free radical scavenging capacity of all samples was carried out. In all samples, methanol extract showed the best result (IC_{50}=4.39±4.75 mg/L) in DPPH assay which was compared to vitamin C (IC_{50}=4.4±0.2 mg/L). In application, by frying assay, we can conclude that a protection is afforded for soya oil enriched with methanolic extract. This result enhances the antioxidant effect of extracts of our plant. Methanolic extract exhibited good anti-inflammatory activity (IC_{50} =9.66±0.57 mg/L). The aim of this study is to investigate the chemical composition and their biological activities to contribute to the amelioration of frying oil.**

***Index Terms*—Eucalyptus, lipid peroxidation, antioxidant activity, frying oil**

I. Introduction

The genus *Eucalyptus*, native from Australia, belongs to the Myrtaceae family and comprises more than 800 species [1]. *Eucalyptus* trees are used for several fields. It represents the most important hard wood forestry crops worldwide and provides a major source of pulp wood for high quality paper production. The timber is used for construction and fuel [2] and the gum is used for diarrhoea and as an astringent in dentistry [3]. This genus is known to be a rich source of bioactive natural products including tannins, flavonoids [4].

This tree is used for timber and shade, and every year they cut the leaves, which will be discarded, to retrieve the timber. To valorize this by-product, we investigated a study of chemical and biological secondary metabolites contained in *Eucalyptus* leaves.

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To our knowledge no study for biological activities, especially antioxidant and anti-inflammatory was made on leaf extracts of *Eucalyptus stricklandii*. In the present study, we analyzed qualitatively and quantitatively the constituents of leaves *E. stricklandii* various extracts. Antimicrobial, antioxidant, lipid peroxidation and anti-inflammatory activities of samples from *E. stricklandii* were also investigated. Correlations between chemical composition and biological activities were studied. In application, by frying assay, we tested soya oil enriched with antioxidant samples of *Eucalyptus* extract.

II. Material and Methods

A. Collection of Plant Material

E. stricklandii leaves were picked on April 2009 from Hajeb Layoun arboretum, located in Kairouan governorship in Tunisia. Specimens were identified in the Regional Station of the National Institute of Research in Farming genius, Waters and Forests. The voucher specimen was deposited at the Department of Biology. The arboretum was established in April 1960 and plant was imported from Austria. They were stored at a dry place during fifteen days. Dried leaves were subject to preparation of various extracts.

B. Preparation of Extracts

Extraction method was solvents sequential with croissant polarity. Solvents used were: petroleum ether, ethyl acetate, methanol and water. 10 g of harvested leaves, finely crushed, were stocked in 100 mL of petroleum ether during 16 hours under frequent agitation at ambient pressure and temperature. The mixture was filtered using Watman paper (GF/A, 110 mm). Solvent was evaporated using rotary evaporator under vacuum at 35 ℃. Then, the firstly lixiviate powder was lixiviate with ethyl acetate in the same condition with petroleum ether. The same procedures are applied for following solvents. Extracts were kept in amber vials and stored at 4 ℃ for its further analysis.

C. Total Amount of Phenolics

The total phenolics of each extract were determined by the Folin–Ciocalteu method [5]. Preparation of Folin and

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Ciocalteu reagent 2 N (Fluka): dissolve 10 g sodium tungstate and 2.5 g sodium molybdate in 70 mL water. Add 5 mL 85% phosphoric acid and 10 mL concentrated hydrochloric acid. Reflux for 10 h. Add 15 g lithium sulfate, 5 mL water, and 1 drop bromine. Reflux for 15 min. Cool to room temperature and bring to 100 mL with water. Hexavalent phosphomolybdic/ phosphotungstic acid complexes with the following structures are formed in solution. The diluted aqueous solution of each extract (0.1 mL) was mixed with Folin–Ciocalteu reagent (0.5 mL at 0.2 N). This mixture was allowed to stand at room temperature for 5 min and then sodium carbonate solution (0.4 mL at 75 g/L) was added. After 1 h of incubation, the absorbances were measured at 765 nm against water blank using a Helios spectrophotometer (Unicam, Cambridge, U.K.). A standard calibration curve was plotted using gallic acid (0 to 200 mg/L). The results were expressed as mg of gallic acid equivalents (GAE)/1g of dry mass.

D. Condensed Tannin Content

Catechins and proanthocyanidins reactive to vanillin were analyzed by the vanillin method [6]. One milliliter (0.5 mL) of each extract solution was placed in a test tube and 2 mL of vanillin (1% in 7 M H_2SO_4) in an ice bath and then incubated at 25 ℃. After 15 min, the absorbance of the solution was read at 500 nm. Concentrations were calculated as gram catechin equivalents (CE)/kg dry mass from a calibration curve.

E. Total Flavonoids Determination

The total flavonoids were estimated according to the Dowd method as adapted by [7]. A diluted methanolic solution (0.5 mL) of each extract was mixed with a solution (0.5 mL) of aluminum trichloride ($AlCl_3$) in methanol (2%). The absorbance was read at 415 nm after 15min against a blank sample consisting of a methanol (0.5 mL) and extract (0.5 mL) without $AlCl_3$. Quercetin was used as reference compound to produce the standard curve, and the results were expressed as gram of quercetin equivalents (QE)/kg of dry mass.

F. Determination of Total Anthocyanin Content

Total anthocyanin content was measured with the pH differential absorbance method, as described by [8]. Briefly, absorbance of the extract was measured at 510 and 700 nm in buffers at pH 1 (hydrochloric acid-potassium chloride, 0.2 M) and 4.5 (acetic acid-sodium acetate, 1 M). The wavelength reading was performed after 15 min of incubation. Anthocyanin content was calculated using a molar extinction coefficient (ε) of 29600 (cyanidin-3-glucoside) and absorbance of A= $[(A_{510} - A_{700})\ pH1.0 - (A_{510} - A_{700})\ pH4.5]$. Results were expressed as milligram cyanidin-3-glucoside equivalent (C3GE)/kg of dry mass.

G. Free Radical Scavenging Activity: DPPH Test

Antioxidant scavenging activity was studied using 1, 1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by [9] with some modifications. 1.5 mL of various dilutions of samples (extracts or essential oil) was mixed with 1.5 mL of 0.2 mM methanolic DPPH solution. After an incubation period of 30 minutes at 25 ℃, the absorbance at 520 nm was measured. The wavelength of maximum absorbance of DPPH, was recorded as $A_{(sample)}$, using spectrophotometer (Helios, Unicam, Cambridge UK). A blank experiment was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as $A_{(blank)}$. The free radical-scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

$$\%\ inhibition = ((A_{(blank)} - A_{(sample)})/A_{(blank)})\ x100$$

Antioxidant activity of standard or samples was expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH concentration. Ascorbic acid was used as a standard. All measurements were performed in triplicate.

H. Determination of Antioxidant Activity Using AAPH/ Linoleic Acid Assay

The antiperoxyl radical efficiency of extracts was determined by measuring the AAPH-induced oxidation of linoleic acid. This test was applied only for extracts with better results in DPPH assay. The method described by [10] was employed. Aqueous dispersion of linoleic acid (16 mM) was prepared in 0.05 M borate buffer solution (pH 9), with Tween 20 as emulsifier and sodium hydroxide as a clarifying agent. This solution was distributed in 0.5 mL aliquots and stored at -20 ℃ until needed. AAPH solution (40 mM) was prepared in 0.05 M phosphate buffer (pH 7.4); aliquots were stored at -20 ℃ until required. The test was carried out as follows. In a quartz cuve containing 2810 μL of phosphate buffer (0.05 M, pH 7.4), 30 μL of linoleic acid dispersion were mixed with 10 μL of crude extract in methanol, at various concentrations. In the assay carried out with standard antioxidant, 10 μL trolox at various concentrations in methanolic solution, were employed. The oxidation of linoleic acid was started by adding 150 μL of AAPH solution. In the assay without antioxidant (control), 10 μL of methanol were employed instead of sample. A blank of AAPH was prepared with 2840 μL of phosphate buffer, 10 μL of methanol and 150 μL of AAPH solution. All cuvettes were incubated at 37 ° C. The rate of oxidation was monitored at regular intervals, by recording the increase in absorption at 234 nm caused by diene hydroperoxides. The AAPH absorbance at 234 nm changes as the compound decomposes. Therefore, the AAPH-blank measures were subtracted from each experimental point. Absorbance at 234 nm was plotted as a function of time; the inhibition time (T_{inh}) estimated as the point of intersection between the tangents to the inhibition and propagation phase. The antioxidant power was expressed as rate of inhibition (R_{inh}, min $Conc^{-1}$), which represents the slope of the linear regression (R^2 above 0.99) calculated by plotting T_{inh} versus sample concentration. In the case of crude extract, R_{inh} was expressed in terms of both min per mg L^{-1} of dry extract. In the case of standard, R_{inh} was expressed in terms of min per mg L^{-1} of pure antioxidant.

I. Frying Studies

To assess the role of natural extracts from *E. stricklandii* during the frying, soya oil provided from the industry "Slama Frère, Tunisia, were subject to heating at a constant temperature and in the lack of food. Two amber glass bottle (1 L) were used: the first one contains only 1 L of soya oil, the second one contains 1L of soya oil enriched 1 g/L of *E. stricklandii* methanolic extract. These oils were heated, separately, in an electric fryer at 180 ℃ (+/- 3 ℃) for 6 hours a day, during 4 consecutive days. At the end of each heating cycle (6 hours) a quantity of oil was removed for characterization. The samples were stored at 4 ℃ until analysis.

J. Determination of Free Fatty Acid

When glycerol bonds break, a fatty acid separates from the triglyceride molecule. This free fatty acid results in an increase in the acidity of the oil. The free fatty acids appear during hydrolysis and oxidation of triglycerides. Thus, more oil is altered; its degree of acidity is high. This parameter reflects the percentage of free fatty acids found in 100g of oil, generally expressed in oleic acid. The Measurement of the acidity of vegetable oils is carried out according to [11]. It consists of taking a mass of 10g oil to which is added a volume of 50 mL of neutralized ethanol. The titration is done with a solution of sodium hydroxide (0.1 mol/L) in the presence of the colored indicator phenolphthalein until persistent pink color. The acidity is determined by the following formula:

$$Acidity\ (\%) = \frac{(V*C*M)}{(10*m)}$$

V: volume of standard solution of sodium hydroxide (mL); C: concentration of standard solution of sodium hydroxide (mol/L); M: molecular weight of the fatty acid used for the expression of the result (g/mol); m: mass of oil sample (g)

Oleic acid is selected for expression of the acidity to the soybean oil (M = 282 g/mol).

K. Determination of Peroxide Value

The peroxide value of the three different sample of soya oil was determined by using standard procedures according to [12]. The peroxides present in the oil oxidizes is determined quantitatively by titration with sodium thiosulphate (0.01 N) and expressed as milliequivalents of oxygen per kilogram of oil. The oil sample was dissolved in a mixture of acetic acid/chloroform (3:2), followed by the addition of saturated potassium iodine (KI) and kept into dark for a period of 5 min. The titration was done with the solution of sodium thiosulfate $Na_2S_2O_3$ (0.01 N) in the presence of starch (as an indicator). When the solution becomes colorless, the reaction was stopped and the volume of sodium thiosulfate solution is noted. The Peroxides values are expressed as milliequivalents of oxygen/kg of oil. A blank test is performed in the same conditions but in the absence of oil. The index is calculated using the following formula:

$$IP = \frac{((V-V0)*T)}{m} *1000$$

IP: Peroxide (meq O_2/kg); V: Volume of thiosulfate added (mL); V0: volume of the blank (mL); T: The Normality of sodium thiosulfate solution; m: mass of sample (g).

L. Statistical Analysis

All data were expressed as means ± standard deviations of triplicate measurements. The confidence limits were set at $P < 0.05$. Standard deviations (SD) did not exceed 5% for the majority of the values obtained.). Correlation coefficients (R^2) to determine the relationship between chemical composition and antioxidant or biological activity were calculated

III. Results And Discussion

A. Various Extracts

TABLE I. Extraction Yields (%) of E. strickl and ii Various Extracts

Samples	Yield (%)
Petroleum ether	9.32 ±0.02
Ethyl acetate	8.30 ±0.02
Methanol	14.28 ±0.04
Water	5.18 ±0.01

The yields of various *E. stricklandii* extracts were presented in Table I. Methanol extract has the higher yield (14.28%), followed by petroleum ether extract (9.32%), ethyl acetate extract (8.3%), and then water extract (5.18%). No data of *E. stricklandii* leaves extracts has been found in literature. According to [13] the yield of ethyl acetate extract from the commercial *Eucalyptus* leaves from the Japon Food additive association, was about 5.04% (50 g plant material in 50 mL solvent, 4 times), however the extraction yield of ethyl acetate of *E. stricklandii* of our study was 8.3% (10 g, 100 mL ethyl acetate, one time overnight under agitation). The yield of 100 g powder of *E. microtheca* leaves [14] extracted twice with 500 mL methanol was 14%, while, the yield of methanolic extract of our study was about 14.28%. About the water extract [15] have found that the yield of *E. globulus* leaves hot water extract was about 0.14% (1 Kg of powder plant was extracted with 3 L hot water at 90 ℃, during 30 min). This result doesn't match with our findings since water extract of *E. stricklandii* was about 5.18% (10 g, 100 mL water, one time, overnight under agitation). Concerning petroleum ether extract, the yield of *E. camaldulensis* from Bangladesh was about 9.25% according to the study of [16]. This result concords with our study (petroleum ether extract yield of *E. stricklandii* was about 9.32%). These differences in the yield extraction may be related to *Eucalyptus* species, experimental test and also the abundance of polar and non-polar compounds. Our plant has an important set

of global extraction yield. We should mention that examples in other articles are direct extractions; what results is a higher yield than ours, since we have use a fractionation for the plant material by the use of successive extraction.

Global chemical composition of various *E. stricklandii* extracts showed that methanolic extract possesses the most important level of phenolic compounds (305.24±2.71 GAE g/Kg dry mass) followed by water extract (216.43±2.75 GAE g/Kg dry mass), ethyl acetate extract (93.23±1.11 GAE g/Kg dry mass) and petroleum ether (54.18±0.93 GAE g/Kg dry mass). *E. stricklandii* is very rich in phenolics, which gives it an economic interest for a valorisation. The amount of total tannins varied according to the solvent of extraction. In fact water extract has the most important concentration (248.73±0.43 CE mg/Kg dry mass), follow-up by methanol extract (186.27±0.75 CE mg/Kg dry mass), ethyl acetate extract (181.24±0.33 CE mg/Kg dry mass) and petroleum ether extract (102.89±0.12 CE mg/Kg dry mass). Concerning flavonoids they were abundant in methanolic and water extract with an amount successively of 19.3±0.06 and 15.34±0.05 QE g/ Kg dry mass. No flavonoids were detected in petroleum ether and ethyl acetate extracts. Anthocyanins were present in all *E. stricklandii* extracts. Ethyl acetate extract was the richer one (4781.02±34.36 C3GE mg/Kg dry mass), followed by petroleum ether extract (1631.56±35.32 C3GE mg/Kg dry mass), methanolic extract (1559.38±31.96 C3GE mg/Kg dry mass) and finally water extract (394.86±29.74 C3GE mg/Kg dry mass).

A comparison with the literature showed that the total phenolics was 11.9 GAE mg/g from the commercial eucalyptus leaf extract from the Japan food additive association[13]. It was also cited by [17] that the amount of total phenolics extracted with methanolic solutions from three *Eucalyptus* ranged from 116 to 138 mg Tannin Acid Equivalents (TAE)/g dry matter. Distilled water extracts contained 101 to 126 mg TAE/g dry matter. This result wasn't in accordance with *E. stricklandii* extracts of the current study since it contained phenolics more than those of literature. The found results showed that *E. stricklandii* is rich in phenolic compounds which are responsible for many pharmaceutical and medicinal effects. They are capable of trapping free radicals and activate other antioxidants in the body. Phenolic substances are capable of activating the natural anticancer mechanism defense. Hydrolysable tannins as condensed; not only increase the resistance against different cancers, they even have anticancer activity that diminishes, and sometimes even completely eliminates tumors [18].

B. Antioxidant Activity

The antioxidant activities of natural extracts vary according to the chemical composition of the plant, and also depend on several conditions such as the analytical method applied. Besides, it was cited that many mechanisms are implicated in the antioxidant activity. For this reason, it is necessary to carry out more than one kind of antioxidant capacity measurement. In this paper, *E. stricklandii* extracts were evaluated for the antioxidant efficiency using the DPPH assays. Lipidic peroxidation was performed only for some extracts.

C. Antioxidant Activity of Various Extracts

For DPPH assay (Fig. 1), methanol extract possessed the most important activity (IC_{50}=4.39±4.75 mg/L), followed by water extract (IC_{50}=8.23±1.18 mg/L), ethyl acetate extract (IC_{50}=35.8±3.45 mg/L), then petroleum ether extract (IC_{50}=94.18±3.46 mg/L). Ascorbic acid was used as positive control and exhibited IC_{50} equal to 4.4±0.2 mg/L which has almost the same antioxidant activity of our methanolic extract. The phenolics composition of various extracts seems to have a notable effect on the values of IC_{50} by DPPH assay (R^2=0.98).

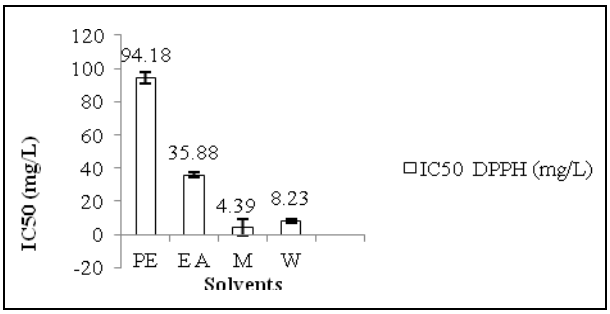

Figure 1. Free radical scavenging capacity *E. stricklandii* extracts

D. Peroxidation LIpidic

Based on the results of antioxidant activity by DPPH assay, we investigated the lipidic peroxidation activity only for methanolic and water extracts, since it showed better antioxidants activities. Samples were screened for antioxidant activity using 2,2'-azobis (2-aminodinopropane) hydrochloride (AAPH), induced oxidation of linoleic acid in vitro model. This method is convenient for determining the efficiency of an antioxidant [10] since peroxyl radical is the predominant free radical in lipidic oxidation in food and biological systems. Antiperoxyl radical efficiency was expressed as rate of inhibition (R_{inh} min mg^{-1} L) of extract. The antioxidant activity is closely linked to the R_{inh} value: more the R_{inh} is increasing, more antioxidant activity is interesting. The result of elaborated data was reported in Table II. It is the first time that the lipidic peroxidation of Eucalyptus extracts was treated since no information found in the literature concerning this plant extract. Our experiments showed that the rate of inhibition of methanolic extract is more important than the aqueous one since the values of R_{inh} (min mg^{-1} L) were respectively 33.42±0.97 and 4.29±0.24, but in both cases they were less important than the standard α –tocopherol which has an R_{inh} (min mg^{-1} L) value about 331±11.

TABLE II. LIPIDIC PEROXYDATION OF E. STRICKLAND II EXTRACTS. R_{INH} (MIN.L.MG^{-1}). R INH: RATE OF INHIBITION.

Samples	R_{inh} (min.L.mg^{-1})
Methanol	33.42 ±0.97
Water	4.29 ±0.24
α-tocopherol	331 ±11

E. Frying Studies

Frying oil plays an important role in the preparation of fried foods since they serve as a medium of heat transfer, and they supply the texture and flavor properties to food. As cooking is normally done at high temperatures (between 160 and 180 ℃) and in the presence of air and humidity, the frying oil is subjected to physical and chemical alterations mainly oxidation reactions. The aim of this part of study is to investigate the benefits of *E. stricklandii* methanolic extract.

F. Free Fatty Acid Content

Free fatty acids content is considered as an indicator of oil quality in food industry. The acidity percentage was found to increase with an increase in frying cycles. The acidity of soya oil during the frying cycle 24 hours varies from (0.15 to 0.44%), however concerning soya oil enriched by *E. stricklandii* methanolic extract the acidity varies from (0.16 to 0.32%). All results were resumed in Fig. 2. The increase in fatty acid was related to the cleavage and oxidation of double bonds. The free fatty acids appear during hydrolysis and oxidation of triglycerides. Thus, more oil is altered; more its degree of acidity is high [19].

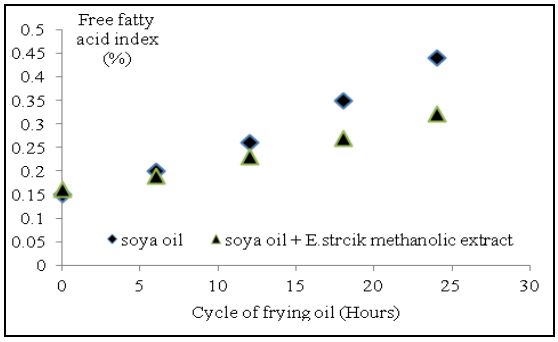

Figure 2. Variation on free fatty acid content (%) during 24 hours of frying of soya oil only, soya oil with *E. stricklandii* methanolic extract

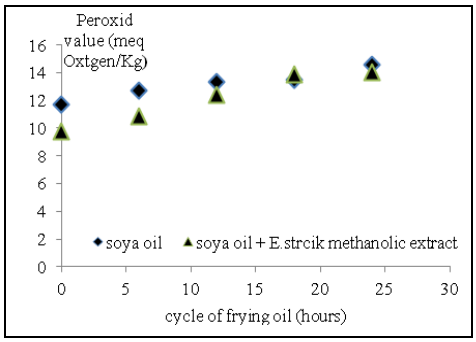

Figure 3. Peroxides values (milliequivalent of O_2 per Kg) of soya oil only, soya oil with *E. stricklandii* methanolic extract

G. Peroxide Value

The determination of the peroxide value is a criterion for the assessment of primary reactions of oxidation of oils. This is an indirect measure of the amount of peroxides (ROOH). products generated during the early stages of oxidation. After frying. the peroxide value has increased. The peroxide value of soya oil only. during the frying cycles. varied from 11.7 to 14.6 meq O_2 /Kg. however it has a value of 9.76 to 14 meq O_2 /Kg for soya oil enriched with *E. stricklandii* methanolic extract. Results are shown in Fig. 3. Peroxides values may increase after the oil is taken from the fryer. for this reason it is generally not considered as a parameter of deterioration of frying oil quality [20].

IV. Conclusion

In conclusion, the results reported in this paper demonstrate that various extracts of *E. stricklandii* exhibited biological activities such as antioxidant, antilipidic peroxidation, they could be used in food industries as conservative agent. Concerning the frying assay, we can conclude that a protection is afforded for soya oil enriched with *E. stricklandii* methanolic extract. This result enhances the antioxidant effect of extracts of our plant. It is envisaged to purify the methanolic extract that gave a good antioxidant activity to identify the molecule(s) responsible for this activity.

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