We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Chapter 6

Enzymatic Polymerization of Rutin and Esculin and Evaluation of the Antioxidant Capacity of Polyrutin and Polyesculin

Latifa Chebil, Ghada Ben Rhouma,
Leila Chekir-Ghedira and Mohamed Ghoul

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60413

1. Introduction

Polyphenols have gained great attention due to their biological and pharmacological activities. Their anti-inflammatory, antioxidant, antimutagenic, anticarcinogenic and antiviral properties were studied in many *in vivo* and *in vitro* systems [1-7]. It seemed that these properties were potentially beneficial in preventing diseases and protecting genome stability. In fact, many of these properties were related to the antioxidant activities of polyphenols [7-10]. However, depending on their structure, the processability of these compounds was limited by their weak stability and low solubility in organic or aqueous solvents [11, 12]. With a view to improve these properties, derivatization of phenolic compounds by enzymatic polymerization was reported by several authors [13-15]. So, it is a useful alternative to chemical catalysis because it can be realized without less hazardous. The two principal enzymes family used in phenolic compounds polymerization process were the laccases and peroxidases. Horseradish peroxidases (HRP) are H_2O_2 dependent. HRP are used in several works to catalyze the polymerization of catechin [14, 16, 17], catechol [18], quercetin, rutin, daidzein 5, 6, 4′-trihydroxyisoflavone [16], 4-hydroxybiphenol [19, 20], 4-[(4-phenylazo-phenyimino)-methyl]-phenol [21], and phenols in various solvents, solvent-aqueous buffers mixture, buffers [22] and in ionic liquids at room temperature [23].

Laccase are also indicated as an efficient catalyst for polymerization of phenolic compounds [24]. Compared to HRP, laccase-catalyzed polymerization without the use of hydrogen

INTECH
open science | open minds

© 2015 The Author(s). Licensee InTech. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

peroxide, as an oxidizing agent. Laccase from different origin (*Trametes versicolor*, *Myceliophthora, Agarucus bisporus, Ustilago maydis, Trametes pubescens, Pycnoporus coccineus, Pycnoporus sanguineus)* have been described for the polymerization of phenolic compounds as rutin [15, 25-29], esculin [28, 30], methoxyphenols, gallic acid, caffeic acid, vanillin, Kaempferol and quercetin [25].

As it has been mentioned previously one of the problem in the use of phenolic compounds, was their weak solubility. The first results of enzymatic polymerization reported that the obtained polymers of rutin and esculin were 4200-folds and 189-folds more water soluble than rutin and esculin, respectively [26, 28]. The solubility of polyrutin was also increased in dimethylformamide (DMF) and dimethyl sulfoxyde (DMSO) [15].

Enzymatic polymerization of phenolic compounds affected also their biological properties. These properties, including antioxidant activities, might be dependent on the molecular weight of the synthesized polymers and the type and the position of the linkages ($\overline{M_w}$, PDI, C-C or C-O bridges). Moreover, depending on the used method for determining antiradical activity (AAPH, DPPH,...) of polyphenols, results were controversial.

As an example, rutin polymerized by laccase from *Pycnoporus coccineus*, *Pycnoporus sanguineus* or *Myceliophthora* led to polymers with a better inhibition of AAPH radical, compared to its monomer [15, 27]. However, Anthoni *et al*. [26] reported that polyrutin, obtained by laccase from *Trametes versicolor* polymerization, had a weaker DPPH radical scavenging activity compared to rutin. This behavior could be due either to the used method of antioxidant activity determination or the degree of polymerization. Oligorutin fractions showed a higher ability of to reduce the genotoxicity induced by H_2O_2 and antimutagenic effect compared to monomeric rutin [28, 29].

For other phenolic compounds, like catechin, kaempferol, esculin and 8-hydroquinoline, polymerization enhanced inhibition effects against free radicals including-oxidation of low-density lipoprotein (LDL) [14] and DPPH radical [25].

Using xanthine oxidase inhibition test, it was well established that enzymatic polymerization of phenolic compounds (rutin, esculin, catechin and epigallocatechin gallate) increased antioxidant activity [14, 15, 26, 28, 30].

Furthermore, the polymerization of 3-methylcatechol by Kawakita et al. [31] led to the formation of polymers with high copper ions adsorption power.

The aim of this work was in one hand, to compare the effect of polymerization on the antioxidant activity of rutin and esculin (Figure 1) and in other hand, to discuss the structure-antioxidant activity relationship. Polyrutin and polyesculin were synthesized by laccase from rutin and esculin, respectively, and carefully separated in different fractions by diafiltration process. Antioxidant activity was evaluated by radical scavenging activity, iron chelating capacity, xanthine oxidase inhibition activity, cupric reducing capacity.

http://dx.doi.org/10.5772/60413

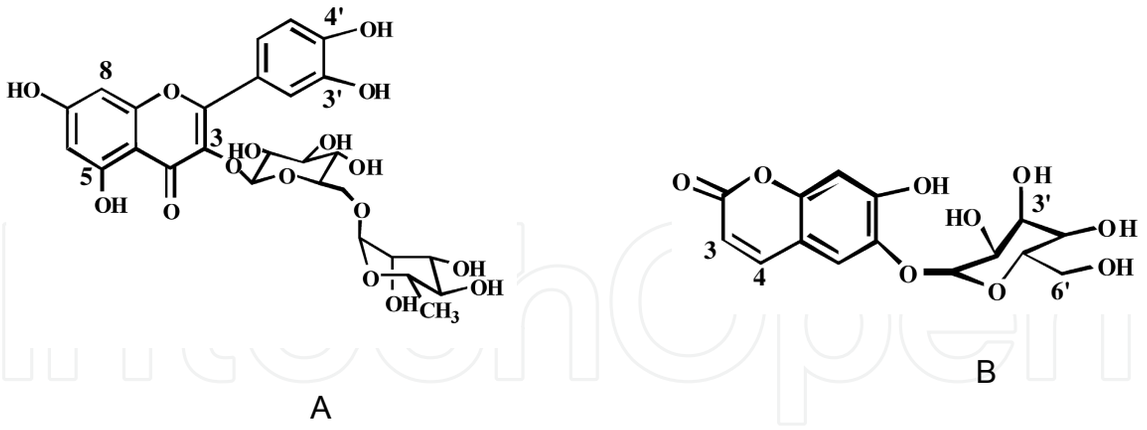


Figure 1. Molecular structure of rutin (A) and esculin (B)

2. Materials and methods

2.1. Chemicals

Laccase from *Trametes versicolor* (E.C. 1.10.3.2., 21.4 U mg-1), rutin hydrate (98%), esculin hydrate (98%), ascorbic acid, 2-deoxyribose, trichloroacetic acid (TCA), thiobarbituric acid (TBA), dimethylsulsulfoxide (DMSO), 2,2′-azino-bis(3methylbenzenothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2-2- diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid (Trolox) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich. All used solvents were HPLC grade from VWR.

2.2. Methods

2.2.1. Polymerization reaction

Polymerization reaction was carried out in the same operating conditions described by Anthoni et al [26, 28]. Rutin or esculin (50 g/L) was suspended in 1 L of a methanol/ water (30:70 v/v) reaction medium. Laccase solution (3 U/ mL) was added to the mixture. The reaction was stirred at 600 rpm, for 24 h for rutin and 72 h for esculin, at 20 °C. We noticed that rutin polymerization reaction didn't evolve beyond 24 h, whereas, esculin polymerization reaction continued till 72h. The Kinetic of polymerization reaction was followed with seize exclusion chromatography (SEC).

2.2.2. Polymers separation and lyophilization

Final reaction media enriched with rutin and esculin polymers was separated, by successive filtration processes on a 15, 5, 3 and 1 KDa membranes in diafiltration process (INSIDE CeRAM™), using a mixture of water/methanol (70:30 v/v) (5 L) as eluent, at 50°C. The transmembranaire pressure (ΔP) was fixed at 2 bars. The state permeate flux (F) was in the

range of 35 l/h/m^2.Then, the fractions were lyophilized (Christ Alpha 1-2 LD freeze dryer). Five fractions were thus obtained and characterized (Table 1).

	Fractions	
	Rutin	**Esculin**
Permeate on Mb 1KDa	R1	E1
Retentate on Mb 1KDa	-	E2
Permeate on Mb 3 KDa	R2	-
Retentate on Mb 3 KDa	-	E3
Permeate on Mb 5 KDa	R3	-
Retentate on Mb 5 KDa	R4	E4
Retentate on Mb 15 KDa	R5	E5

Table 1. Fractions of polyrutin and polyesculin obtained after separation, Mb : membrane; KDa : Kilo Dalton

2.2.3. Seize exclusion chromatography analysis (SEC)

Relative masses of polymers were evaluated by size exclusion chromatography (SEC) (HPLC LaChrom, UV 280 nm LaChrom L-7400, Tosoh TSKgel α 3000 column, 60 °C). Dimethylformamide (DMF) with 1 % LiBr was used as a mobile phase (0.5 mL/min). Molecular mass calibration was obtained using standards of polystyrene and polystyrene sulfonate. The obtained data allowed the determination of number-average molecular mass ($\overline{Mn}$), weight-average molecular mass ($\overline{M_w}$), weight-average molecular mass index (I_M) and polydispersity (PDI) as described by Faix et al. [32].

2.2.4. UV analyzes

The UV spectra of rutin, esculin solutions and their obtained polymers fractions were determined using a UV6000LP spectrometer (Spectra System, Thermofinnigan).

2.2.5. FTIR analysis

The IR analyses were conducted by ATR-FT-IR spectroscopy using a FT-IR spectrometer Tensor 27 (Bruker). The analysis was carried out on monomers and polymers lyophilized powders.

2.2.6. Radical scavenging on ABTS $^{+\cdot}$

The assay was conducted according to protocols presented by Re et al. (1999) and van den Berg et al. (2001) [33, 34]. To generate the ABTS $^{+\cdot}$ radical, the ABTS stock solution (7 mM) and potassium persulphate (2.45 mM) in water were allowed to stand in the dark at room temperature for 12-16 h before use. For the reaction, 10 µl of each sample at various concentrations

http://dx.doi.org/10.5772/60413

(from 800 to 0.25 μM) was added to 990 μl of diluted $ABTS^{+\cdot}$ (absorbance 0.7 at 734 nm) and the absorbance was recorded every min. A standard curve was prepared using a series of concentrations of trolox (from 0 to 15 μM) with 990 μl of diluted $ABTS^{+\cdot}$ solution. The radical scavenging capacity of tested samples was calculated based on the trolox standard curve and expressed as the trolox equivalent antioxidant capacity (TEAC) and as IC_{50}.

2.2.7. Radical scavenging activity on DPPH·

The free radical scavenging capacity of the esculin and rutin and their polymers was determined with 2,2-diphenyl-1-picryl-hydrazyl as described by Bruda et Oleszek [35]. A solution of 1 ml of monomers or polymers (from 10^2 to 4 10^4 μM, concentrations were calculated from $\overline{M_w}$) in methanol, was mixed with 2 ml of DPPH (10 mg/L in methanol/water, 80:20, v/v). A reference sample was prepared by adding 1 ml of methanol in 2 mL of DPPH solution. Monomers and polymers absorbance for each concentration was evaluated at 527 nm, after 15 min, at 23 °C. The antiradical activity was calculated as a percentage of DPPH discoloration using the following equation (1).

$$Antiradical\ activity = \left(1 - \frac{Absorbance\ of\ the\ sample - Absorbance\ of\ polymers}{Absorbance\ of\ the\ reference}\right) * 100 \quad (1)$$

The results were expressed as IC_{50} and TEAC according to the calibration curve (from 0 to 5 μM).

2.2.8. Inhibitory effect on deoxyribose degradation

Inhibitory effects of tested compounds on deoxyribose degradation were determined by measuring the competition between deoxyribose and theses compounds for the hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system (referred to non site-specific assay) or Fe^{3+}/ascorbate/H_2O_2 system (referred to site-specific assay which could indicate the hydroxyl scavenging power of tested molecules by iron chelating power) according to the method described by Halliwell et al. [36] with slight modifications.

The tested sample was added to the reaction mixture containing deoxyribose (10 mM), Fe(III) chloride (10 mM), EDTA (1 mM), and H_2O_2 (10 mM), ascorbic acid (1 mM), 1mM H_2O_2 and 50 mM potassium phosphate buffer (pH 7.4). The mixture was incubated for 1 h at 37°C, TBA (1%) and TCA (2.8%) were added to the above mixture, and then heated for 90 min on water bath at 80 °C. The absorbance at 532 nm was then measured against a blank containing deoxyribose and buffer. For site-specific hydroxyl radical scavenging activity, the procedure was similar to the above method, except that EDTA was replaced by the equivalent volume of buffer. The gallic acid was used as a standard. The percentage of deoxyribose degradation inhibition was calculated using the equation (2).

$$PI(\%) = \left(1 - A_s / A_c\right) * 100 \quad (2)$$

where A_C is the absorbance of negative control and A_S the absorbance of sample solution.

Results of deoxyribose assay in the presence and the absence of EDTA are expressed as IC_{50} and as TEAC.

2.2.9. Xanthine oxidase inhibition assay

The tested samples were solubilized in phosphate buffer (pH 7.5, 50 mM), except rutin which was dissolved in a minimum of DMSO (5 µl) and then in buffer. The assay was conducted as described by Kong et al. [37]. Tests solutions were prepared by adding 1600 µL of buffer, 300 µL of tested solutions (from 4 10^{-6} to 10^{-3} M), 1000 µL of a solution of xanthine (0.15 mM) and 100 µL of a solution of xanthine oxidase (0.2 U/mL). The reaction was monitored for 6 min at 295 nm. Two samples were prepared, the first without tested solutions to determine the total uric acid production, and the second without enzyme to measure the absorbance of tested solutions at 295 nm for the range of concentrations. Results were expressed as the final concentration that results in half-maximal enzyme velocity (IC50) and calculated by standard curve regression analysis and as TEAC according to the calibration curve (from 1 10^{-3} to 5 10^{-1} µM).

2.2.10. Cupric reducing antioxidant capacity (CUPRAC)

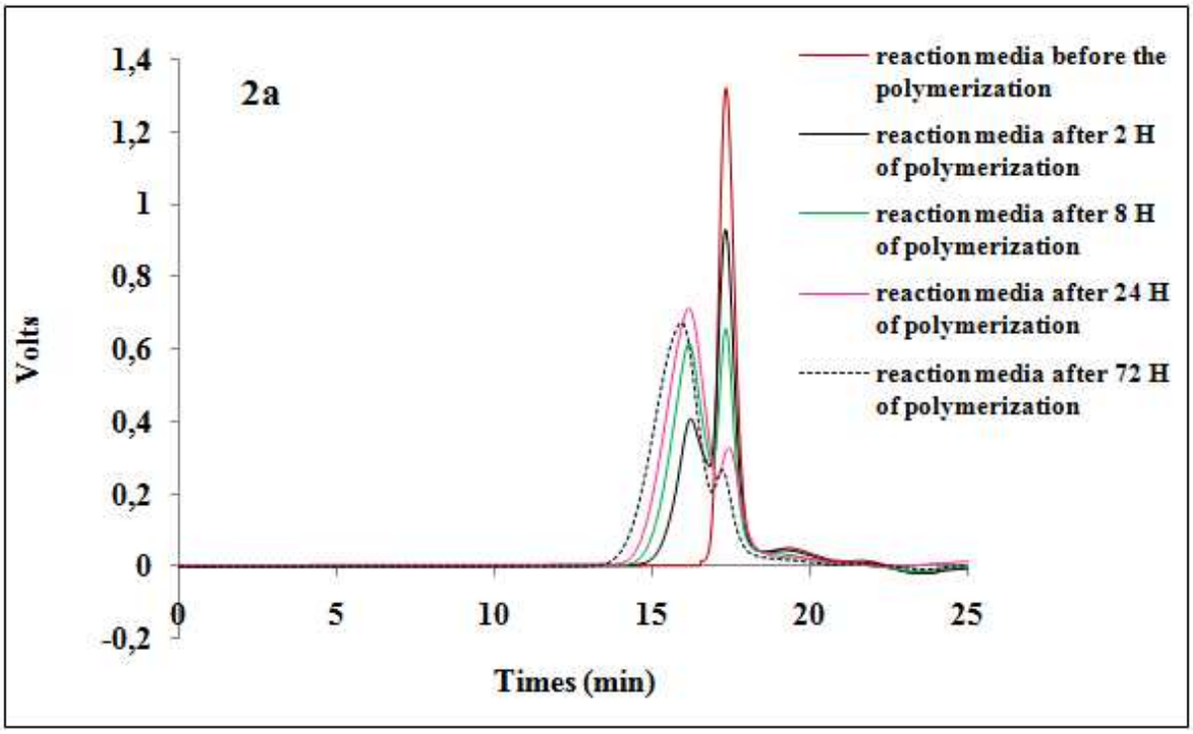
The cupric reducing antioxidant capacity was determined according to the method of Apak et al. [38] To each tube containing 20 µl of tested substrate concentration, we added $CuCl_2$ to a final concentration of 3.12 mM, ethanolic neocuproine solution and NH_4Ac buffer solution (pH=7) to final concentrations of 2.34 × 10^{-3} M and 312 mM, respectively. The total volume was then adjusted with distilled water to 2 ml and mixed well. Absorbance against a reagent blank containing all reagents except $CuCl_2$ and neocuproine was measured at 450 nm after 1h. The results were expressed as equivalent of Trolox according to the calibration curve (from 10 to 10^3 µM).

3. Results

3.1. Polymers synthesis, separation and characterization

Kinetics of esculin and rutin polymerization were monitored by SEC-UV at 280 nm. Once the polymerization was achieved, polymers were separated, by successive diafiltration process. Weight-average molecular mass $\overline{M_w}$, polydispersity (PDI) and weight average molecular mass index (I_M) of obtained fractions (R1-5 and E1-5) were summarized in table 2. These results indicated clearly that the polymerization of the two substrates was occurred and led to polymers of rutin and esculin with high molecular weight (Figure 2).

http://dx.doi.org/10.5772/60413



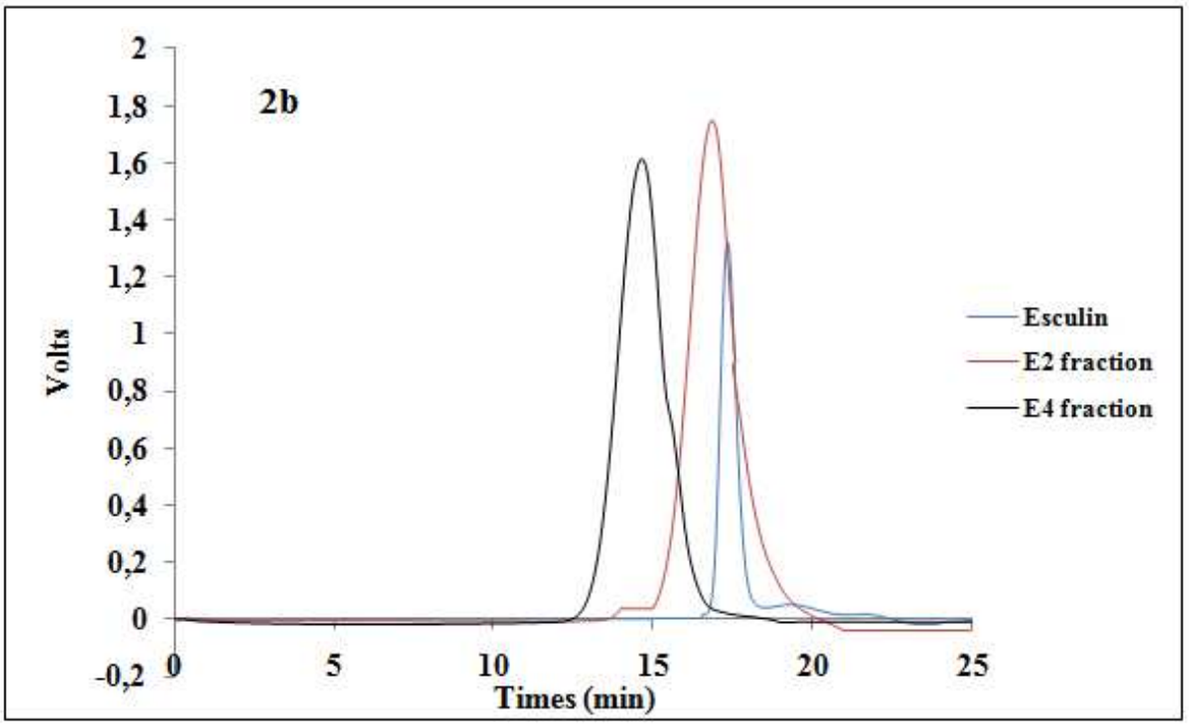


Figure 2. Kinetic of esculin polymerization determined by SEC-UV using dimethylformamide (DMF) with 1 % LiBr as a mobile phase (0.5 ml/min) (2a). SEC-UV analyses of esculin and polyesculin fractions E2 and E4 using dimethylformamide (DMF) with 1 % LiBr as a mobile phase (0.5 ml/min) (2b).

Fractions	$\overline{M_w}$ (g /mol)	PDI	I_M
Rutin (R)	611.21 ± 80.54	1.0024 ± 0.012	1 ± 0.0
R1	2127.42 ± 67.12	1.17 ± 0.03	3.48 ± 0.14
R2	4301.8 ± 102.72	1.37 ± 0.07	7.05 ± 0.16
R3	5069.93 ± 116.2	1.36 ± 0.04	8.30 ± 0.18
R4	7106.54 ± 96.62	1.35 ± 0.08	11.64 ± 0.14
R5	8331.85 ± 146.24	1.42 ± 0.12	13.65 ± 0.22
Esculin (E)	339.36 ± 43.46	1.009 ± 0.09	1 ± 0.0
E1	688.12 ± 40.66	1.31 ± 0.11	2.02 ± 0.12
E2	1021.33 ± 48.51	1.48 ± 0.06	3.009 ± 0.14
E3	3042.1± 86.24	1.39 ± 0.13	8.96 ± 0.25
E4	5080.43 ± 70.96	1.41 ± 0.07	14.97 ± 0.20
E5	6973 ± 68.1	1.54 ± 0.10	20.54 ± 0.20

Table 2. Weight-average molecular mass ($\overline{M_w}$), polydispersity (PDI) and weight-average molecular mass index (I_M) of obtained polyrutin (R1-R5) and polyesculin (E1-E5) fractions.

3.2. UV and FTIR investigations

The UV-visible spectrum of rutin, in methanol/water (30/70 v/v), presented two maxima of absorption at 282 and 359 nm due to the π-π* transition of the aromatic electrons. For polyrutin fractions (R1, R3 and R5) the 359 nm band was larger and presented a hypsochromic shift of 5 nm. Such results could be due to the implication of the B ring of rutin in the formation of polymers. In fact, Anthoni *et al*. [26] and Marckam [39] observed a similar behavior. The latter stated that the presence of a substitution on the 5, 7 and 4' positions of the phenolic rings led to a hypsochromic shift.

The UV spectra of esculin and polyesculin fractions E2, E3, E4 and E5 presented the same peaks with a maximum of absorption at 345 nm while the peaks correspondent to E5 were broader than those of esculin, which could be attributed to conjugated oligomeric structure [15, 40]. The same profile was reported by Anthoni et al. for the esculin polymerization [30].

FTIR spectra of rutin and polyrutin fractions (R1, R3 and R5) (Figure 3), showed a new peaks at 1220 cm^{-1} and at 1465 cm^{-1}.The peak at 1220 cm^{-1} indicated the formation of new ether bonds C-O. The signal at 1465 cm^{-1} could be attributed to a bond C-C while the absence of a peak at 1747 nm on the R1 spectra compared to rutin spectra could be explained by the disappearance of C-H bonds. These results showed that obtained polyrutin fractions were formed through C-C and C-O linkages. In fact, many authors reported that flavonoid polymers were composed of phenylene units and/or oxyphenylene units [24, 26, 41]. Uzan et al. [27] reported that the nucleophilicity of the aromatic A-ring seemed to play a major role as the reactive hydroxylated ring in coupling reactions for the formation of a new bond. They suggested that polymerization of rutin by *Pycnococus* laccases led to formation of polymers through C-C and C-O bonds and

http://dx.doi.org/10.5772/60413

more precisely through C8-C8, C6-O4′ and C8-C5′ linkages. A study of the polymerization of quercetin by Bruno et al. [12], with Horseradish peroxidase (HRP), showed that the highest occupied molecular orbital (HOMO) was concentrated on the catechol group. Therefore, these authors expected the polymerization reaction to take place in the two more negative carbons of that group 2′ and 5′.

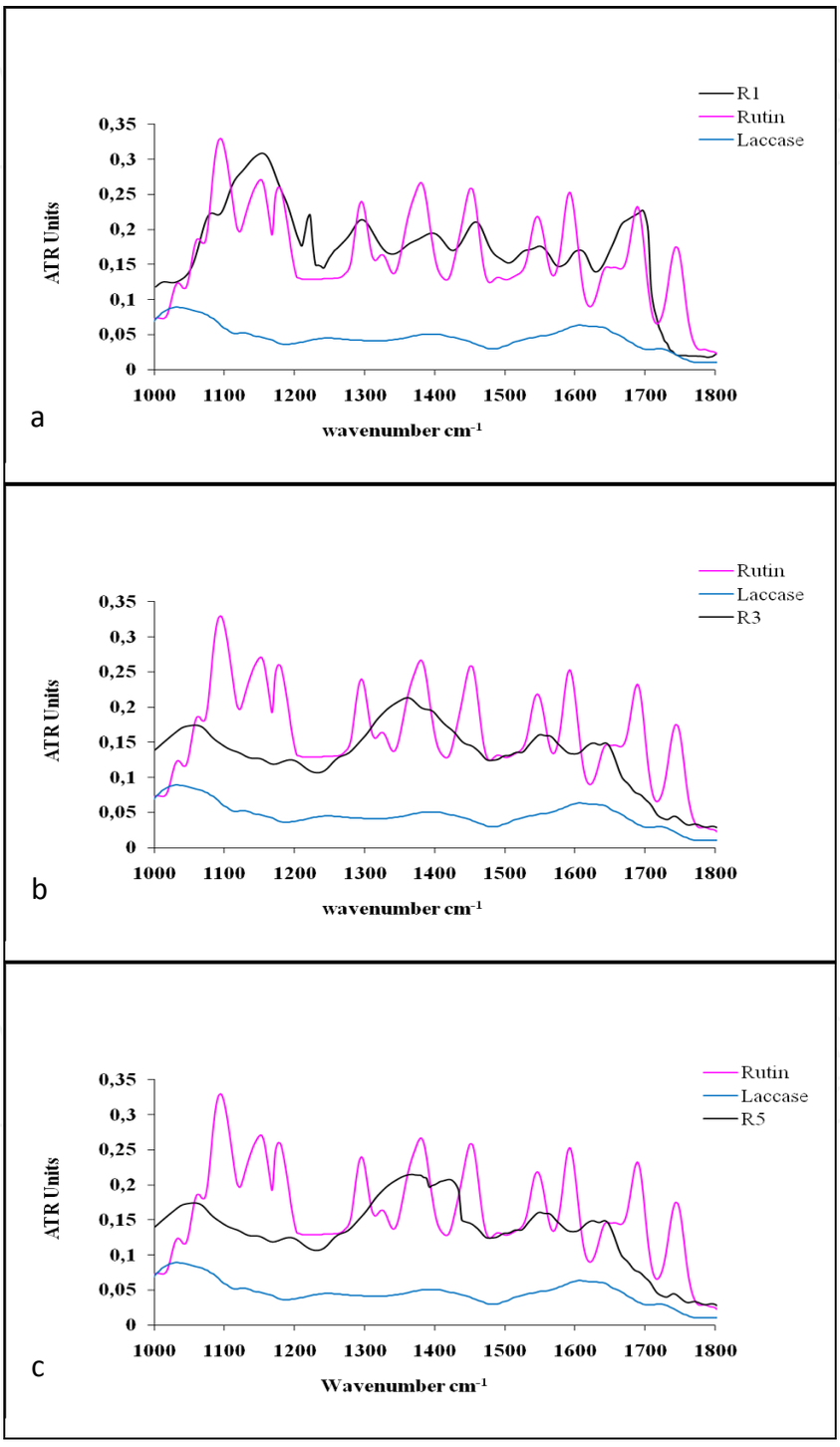


Figure 3. FTIR spectrum of rutin fractions and laccase, R1 (a), R3 (b) and R5 (c).

As for rutin, FTIR spectra of polyesculin fraction E2, E3, E4 and E5 showed a new peak at 1400 cm^{-1} compared to the spectra of esculin. This could be due to a formation of C-C bonds. In fact, Anthoni et al [30] reported the formation of C-C and C-O linkages, involving both the phenolic and the glucosidic part of the coumarin during the esculin polymerization. Moreover, an *in silico* structure investigation of oligoesculin by the same authors suggested the preferential formation of C8-C8 linkage between esculin units during the polymerization reaction.

The obtained and reported data of UV and FTIR suggested that different linkages (C-C, C-O) could be achieved depending to monomer, enzyme and operating conditions (pH, temperature, medium). This might affects the antioxidant activity of the polymer.

3.3. Evaluation of antioxidant activity of rutin and polyrutin fractions

Different methods were used to evaluate the antioxidant activity (free radicals scavenging activity, iron chelating capacity, xanthine oxidase inhibition activity and cupric reducing capacity) of esculin, rutin and their derivatives. Results were summarized in Table 3.

	ABTS		DPPH		Hydroxyl radical		XO inhibition		Iron chelation		CUPRAC
	IC_{50} (µM)	TEAC (µM)	IC_{50} (µM)	TEAC (µM)	IC_{50} (µM)	TEAC (10^{-2} µM)	IC_{50} (µM)	TEAC (10^{-3} µM)	IC_{50} (µM)	TEAC (10^{-3} µM)	TEAC (µM)
R	320±12	3.89±0.2	1.1±0.1	113.4±13.5	18.6±1.6	101±0.08	962±16	5±0.1	58.3±5	1±0.1	315±18
R2	440±14	2.83±0.25	3.9±0.2	62.0±8.2	25.7±1.75	75±0.09	119.02±14	47±4	49.7±6	1.1±0.1	411±27
R3	540±22	2.31±0.42	18±0.9	56.9±5.25	30±1.5	62±0.06	29.74±7	190±2	38.3±1	1.5±0.2	483±13
R5	640±24	1.95±0.48	38±0.2	43.2±6.75	38.32±1.9	49±0.05	14.12±1.5	400±16	36.5±4	1.6±0.1	527±29
E	450±2	0.003±0.1	9200±9	0.021±0.005	5600±64	0.3±0.01	779±33	7±0.5	6800±9	0.8±0.1	29±1.5
E2	110±19	0.1±0.02	500±43	3.9±0.4	1600±13	1.1±0.05	301±21	18±0.3	2100±5	2.7±0.2	89±4
E3	30±1	0.41±0.05	500±32	3.9±0.3	353±21	5.3±0.07	160±9	35±1.5	650±25	9±0.1	328±10
E4	9±0.5	0.83±0.08	480±25	3.7±0.5	150±8	12.5±0.5	154±14	36±1	423±12	10±0.5	538±4
E5	1±0.1	1.23±0.4	480±39	3.7±0.3	70±4	26.9±1.5	141±6	40±0.8	180±5	30±1.2	898±34

Table 3. Antiradicals, xanthine oxidase inhibition, iron chelating and CUPRAC activities of rutin, esculin and their polymer fractions. Results are represented by the means ± SD of three experiments. TEAC: Trolox equivalent antioxidant capacity. IC50: The half maximal inhibitory concentration; ABTS: 2,2′-azino-bis(3methylbenzenothiazoline-6-sulfonic acid) diammonium; DPPH: 2-2- diphenyl-1-picrylhydrazyl; XO: xanthine oxidase. CUPRAC: Cupric reducing antioxidant capacity.

3.3.1. Free radicals scavenging activity of rutin and polyrutin fractions

Results in Table 3 showed that IC_{50}, related to polyrutins, increased progressively versus $\overline{M_w}$. The fraction R5, presenting the highest $\overline{M_w}$, led to highest IC_{50} values (640, 38 and 38.32

http://dx.doi.org/10.5772/60413

μM) compared to IC_{50} values obtained in presence of rutin (320, 1.1 and 18.6 μM) respectively for ABTS, DPPH and hydroxyl radicals. These results suggested that higher is the $\overline{M_w}$ lower is the antiradical activity. The low antiradical activity of polyrutin fractions observed in this study was in accordance with that reported by other authors [15, 26, 27].

3.3.2. Xanthine oxidase inhibition activity of rutin and polyrutin fractions

For XO inhibition activity (Table 3), the IC_{50} values of polymer fractions appeared to be lower than the IC_{50} value of rutin (962 μM). The results indicated that IC_{50} decreased when the $\overline{M_w}$ arised, which traduced the better ability of polyrutins to inhibit XO compared to monomeric rutin. The fraction R5 illustrated the highest XO inhibition power, 68-folds better than monomeric rutin. The strong XO inhibition observed for polyrutin fractions was in accordance with other studies dialled in enzymatic flavonoid polymerisation [14, 15, 25, 26, 42].

3.3.3. Iron chelating properties of rutin and polyrutin fractions

All polyrutin fractions exhibited higher degree of iron chelating ability (Table 3). This activity grow with the increase of $\overline{M_w}$. The polyrutin fraction R5 presented the highest iron chelating power with an IC_{50} value of 36.5 μM compared to 58.3 μM, in presence of the monomer.

3.3.4. CUPRAC of rutin and polyrutin fractions

The cupric ion (Cu^{2+}) reducing abilities of rutin and polyrutin fractions (R2, R3 and R5) were shown in Table 3. It appeared that the cupric ion (Cu^{2+}) reducing powers of different tested compounds were in the following order R5 (TEAC of 527 μM)>R3 (TEAC of 483 μM)>R2 (TEAC of 411 μM)> rutin (TEAC of 315 μM), meaning that cupric ion (Cu^{2+}) reducing ability increased with the increase of $\overline{M_w}$.

3.4. Evaluation of antioxidant activity of esculin and polyesculin fractions

3.4.1. Free radicals scavenging activity of esculin and polyesculin fractions

Polyesculin fractions presented lower IC_{50} values than those of monomeric esculin which indicated their stronger antiradical activity (Table 3). Polyesculin fraction E5 was the most potent one. It was respectively for ABTS, DPPH and hydroxyl radicals 450, 19 and 80 folds more active than esculin (450, 9200, 5600 μM). Unlike rutin, the antiradical activities increased with $\overline{M_w}$ when ABTS and hydroxyl radical methods were used. However, for DPPH the IC_{50} remained constant, about 480 μM, for all tested fractions. So, DPPH scavenging activity seemed to be independent to the degree of polymerisation.

3.4.2. Xanthine oxidase inhibition activity of esculin and polyesculin fractions

Results in Table 3 showed that for all polyesculin fractions, IC_{50} were lower than that of the monomer (779 μM). This activity was linked to $\overline{M_w}$ and decreased as $\overline{M_w}$ increased. The fraction E5 presented the lowest IC_{50} and therefore the highest XO inhibition activity, 5-folds higher than monomer.

3.4.3. Iron chelating properties of esculin and polyesculin fractions

Polyesculin fractions exhibited high degree of iron chelating activity, according to the site-specific hydroxyl radical-scavenging assay (Table 3). Results showed that iron chelating capacity was high as the $\overline{M_w}$ increases. The best iron chelating power was observed in the presence of the E5 fraction (IC_{50}=180 μM), which was 37-folds better than esculin (IC_{50}=6800 μM).

3.4.4. CUPRAC of esculin and polyesculin fractions

Table 3 indicated that polyesculin fractions presented higher TEAC than esculin. This activity rose as the $\overline{M_w}$ increased. Therefore, the best cupric reducing antioxidant capacity was seen with the E5 fraction which was 30-folds more active than esculin.

3.5. Structure-antioxidant activity relationship

The structure-antioxidant activity relationship of monomeric flavonoids and coumarins was well investigated. According to many authors [43] free hydroxyl groups on C4', C3' and C7 played a major role in antiradical activity of rutin and esculin. However, few data are available about the behaviour of these activities with polymerization. In this work we observed a decrease of polyrutin antiradical activities with $\overline{M_w}$ increase. This decrease could be attributed to the loss of these groups during the rutin polymerization reaction.

For high iron chelating power and CUPRAC, hydroxyl groups on C5, C3 and the 4 oxo (for flavonoids) and hydroxyl groups and catechol moiety (for coumarins) were essential. So, high iron chelating and cupric reducing antioxidant capacities observed with polyrutin and polyesculin fractions suggested that these groups were not implicated in the linkage occurred in rutin polymerization reaction [44-47].

For high xanthine oxidase inhibition activity, several works reported the importance of the presence of a double bond between C2 and C3 and free hydroxyl groups on C5 and C7 [26, 48-50]. High inhibition of the xanthine oxidase obtained in the presence of polyrutin and polyesculin fractions implicated that these groups are not affected during the polymerization reaction.

4. Conclusion

Polyphenolic polymers of rutin and esculin were synthesized using a laccase from *Trametes versicolor*. These polymers were fractioned by diafiltration process.

The analyses of rutin polymers by FTIR showed the presence of new C-C and C-O bonds and the desperation of a C-H bond on monomer. These results suggested that polyrutin were synthesized through phenylene and oxyphenylene units. For polyesculin fraction, FTIR analyses indicated the presence of only C-C bond.

Free radical scavenging activity of rutin was decreased by the enzymatic polymerization while polyesculin fractions showed a high antiradical activity compared to monomeric esculin. This

http://dx.doi.org/10.5772/60413

behaviour suggested that the antioxidant activity depend on the position of linkage through the polymerization reaction. For esculin, it seemed that the polymerization didn't affect groups implicated in the antioxidant activity. This could explain the high antioxidant activity values observed for polyesculin.

Both polyrutin and polyesculin fractions exhibited a high XO inhibition activity, iron chelating and cupric reducing antioxidant capacities.

Abbreviations

AAPH: 2,2′-azobis (2-amidinopropane)dihydrochloride ; ABTS: 2,2′-azino-bis(3methylbenzenothiazoline-6-sulfonic acid) diammonium salt; ATR: Attenuated Total Reflectance; $CuCl_2$: Copper (II) chloride; DMF: dimethylformamide; DMSO: and dimethyl sulfoxyde; DPPH: 2-2-diphenyl-1-picrylhydrazyl; EDTA: Ethylenediaminetetraacetic acid; FTIR: Fourier Transformed InfraRed analysis; H_2O_2: Hydrogen peroxide; HOMO: Highest occupied molecular orbital; HRP: Horseradish peroxidises; I_M: weight average molecular mass index; LDL: Low-density lipoprotein; LiBr: Lithium bromide; $\overline{Mn}$: Number-average molecular mass; $\overline{M_w}$: Weight-average molecular mass; NH_4Ac: Ammonium acetate; NH_4Ac: Ammonium acetate; TBA: Thiobarbituric acid; TCA: Trichloroacetic; TEAC: Trolox equivalent antioxidant capacity; Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid; XO: Xanthine oxidase

Acknowledgements

The authors are grateful to the Lorraine region in France, the French Ministry of Foreign Affairs (French Institute of Cooperation (IFC)), and to the Ministry of Higher Education and Scientific Research in Tunisia for the financial assistance of this study.

Author details

Latifa Chebil[1*], Ghada Ben Rhouma[1,2], Leila Chekir-Ghedira[2,3] and Mohamed Ghoul[1]

*Address all correspondence to: latifa.chebil@ensaia.inpl-nancy.fr

1 Laboratory of Biomolecules engineering, ENSAIA, University of Lorraine, Vandoeuvre-lès-Nancy, France

2 Unity of Pharmacognosy/Molecular Biology, Faculty of Pharmacy, University of Monastir, Monastir, Tunisia

3 Laboratory of Cellular and Molecular Biology, Faculty of Dental Medicine, University of Monastir, Monastir, Tunisia

References

[1] Yoshino K, Ogawa K, Miyase, T, Sano, M. Inhibitory Effects of the C-2 Epimeric Isomers of Tea Catechins on Mouse Type IV Allergy. Journal of Agricultural and Food Chemistry 2004;52(15) 4660-4663.

[2] Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. The Journal of Clinical Investigation 2001; 107(2) 135-142.

[3] Chen HJ, Chung CP, Chiang W, Lin YL. Anti-inflammatory effects and chemical study of a flavonoid-enriched fraction from adlay bran. Food Chemistry 2011;126(4) 1741-1748.

[4] Bordoni A, Hrelia S, Angeloni C, Giordano E, Guarnieri C, Caldarera CM, Biagi PL. Green tea protection of hypoxia/reoxygenation injury in cultured cardiac cells.The Journal of Nutritional Biochemistry 2002;13(2) 103-111.

[5] Nakagawa K, Ninomiya M, Okubo T, Aoi N, Juneja LR, Kim M, Yamanaka K, Miyazawa T. Tea Catechin Supplementation Increases Antioxidant Capacity and Prevents Phospholipid Hydroperoxidation in Plasma of Humans. Journal of Agricultural and Food Chemistry 1999;47(10) 3967-3973.

[6] Takano K, Nakaima K, Nitta M, Shibata F, Nakagawa H. Inhibitory Effect of (-)-Epigallocatechin 3-Gallate, a Polyphenol of Green Tea, on Neutrophil Chemotaxis in Vitro and in Vivo. Journal of Agricultural and Food Chemistry 2004;52(14) 4571-4576.

[7] Bhouri W, Sghaier MB, Kilani S, Bouhlel I, Dijoux-Franca MG, Ghedira K, Ghedira LC. Evaluation of antioxidant and antigenotoxic activity of two flavonoids from Rhamnus alaternus L. (Rhamnaceae): Kaempferol 3-O-[beta]-isorhamninoside and rhamnocitrin 3-O-[beta]-isorhamninoside, Food and Chemical Toxicology 2011;49(5) 1167-1173.

[8] Jovanovic SV, Steenken S, Tosic M, Marjanovic B, Simic MG. Flavonoids as Antioxidants. Journal of the American Chemical Society 1994;116(11) 4846-4851.

[9] Yen GC, Chen HY. Antioxidant Activity of Various Tea Extracts in Relation to Their Antimutagenicity. Journal of Agricultural and Food Chemistry 1995;43(1) 27-32.

[10] Ben Sghaier M, Boubaker J, Skandrani I, Bouhlel I, Limem I, Ghedira K, Chekir-Ghedira LC. Antimutagenic, antigenotoxic and antioxidant activities of phenolic-enriched extracts from Teucrium ramosissimum: Combination with their phytochemical composition. Environmental Toxicology and Pharmacology 2011;31(1) 220-232.

[11] Tommasini S, Raneri D, Ficarra R, Calabrò ML, Stancanelli R, Ficarra P. Improvement in solubility and dissolution rate of flavonoids by complexation with β-cyclodextrin. Journal of Pharmaceutical and Biomedical Analysis 2004;35(2) 379-387.

[12] Bruno FF, Trotta A, Fossey S, Nagarajan S, Nagarajan R, Samuelson LA, Kumar J, Enzymatic synthesis and characterization of polyquercetin. Journal of Macromolecular Science, Part A: Pure and Applied Chemistry 2010;47(12) 1191-1196.

[13] Racicot K, Favreau N, Fossey S, Grella AR, Ndou T, Bruno FF. Antioxidant potency of highly purified polyepicatechin fractions. Food Chemistry 2012;130(4) 902-907.

[14] Kurisawa M, Chung JE, Kim YJ, Uyama H, Kobayashi S. Amplification of Antioxidant Activity and Xanthine Oxidase Inhibition of Catechin by Enzymatic Polymerization. Biomacromolecules 2003 a;4(3) 469-471.

[15] Kurisawa M, Chung JE, Uyama H, Kobayashi S. Enzymatic Synthesis and Antioxidant Properties of Poly(rutin). Biomacromolecules 2003 b;4(5) 1394-1399.

[16] Mejias L, Reihmann MH, Sepulveda-Boza S, Ritter H. New Polymers from Natural Phenols Using Horseradish or Soybean Peroxidase. Macromolecular Bioscience 2002;2(1) 24-32.

[17] Bruno FF, Nagarajan S, Nagarajan R, Kumar J, Samuelson LA. Biocatalytic synthesis of water-soluble oligo(catechins). Journal of Macromolecular Science - Pure and Applied Chemistry 2005;42 A(11) 1547-1554.

[18] Nabid MR, Zamiraei Z, Sedghi R, Nazari S. Synthesis and characterization of poly(catechol) catalyzed by porphyrin and enzyme. Polymer Bulletin 2011;64(9) 855-865.

[19] Angerer PS, Studer A, Witholt B, Li Z. Oxidative Polymerization of a Substituted Phenol with Ion-Paired Horseradish Peroxidase in an Organic Solvent. Macromolecules 2005;38(15) 6248-6250.

[20] Tseitlin GM, Beksaev SG, Medzhitov DR. Enzymatic polymerization of phenols. Polymer Science - Series A 2006;48(8) 798-802.

[21] Turac E, Sahmetlioglu E. Oxidative polymerization of 4-[(4-phenylazo-phenyimino)-methyl]-phenol catalyzed by horseradish peroxidase. Synthetic Metals 2010;160(1-2) 169-172.

[22] Akita M, Tsutsumi D, Kobayashi M, Kise H. Structural change and catalytic activity of horseradish peroxidase in oxidative polymerization of phenol. Bioscience Biotechnology and Biochemistry 2001;65(7) 1581-1588.

[23] Eker B, Zagorevski D, Zhu G, Linhardt RJ, Dordick JS. Enzymatic polymerization of phenols in room-temperature ionic liquids. Journal of Molecular Catalysis B: Enzymatic 2009; 59(1-3) 177-184.

[24] Kobayashi S, Uyama H, Tonami H, Oguchi T, Higashimura H, Ikeda R, Kubota M. Regio- and chemo-selective polymerization of phenols catalyzed by oxidoreductase enzyme and its model complexes. Macromolecular Symposia 2001 b;175(1) 1-10.

[25] Desentis-Mendoza RM, Hernandez-Sanchez H, Moreno A, Rojas del C E, Chel-Guerrero L, Tamariz J, Jaramillo-Flores ME. Enzymatic Polymerization of Phenolic Com-

pounds Using Laccase and Tyrosinase from Ustilago maydis. Biomacromolecules 2006;7(6) 1845-1854.

[26] Anthoni J, Lionneton F, Wieruszeski JM, Magdalou J, Engasser JM, Chebil L, Humeau C, Ghoul M. Investigation of enzymatic oligomerization of rutin. Rasayan J. Chem. 2008;1(4) 718-731.

[27] Uzan E, Portet B, Lubrano C, Milesi S, Favel A, Lesage-Meessen L, Lomascolo A. Pycnoporus laccase-mediated bioconversion of rutin to oligomers suitable for biotechnology applications. Applied Microbiology and Biotechnology 2011;90(1) 97-105.

[28] en Rhouma G, Chebil L, Krifa M, Ghoul M, Chekir-Ghedira L. Evaluation of mutagenic and antimutagenic activities of oligorutin and oligoesculin. Food Chemistry 2012;135(3) 1700–1707.

[29] en Rhouma G, Chebil L, Mustapha N, Krifa M, Ghedira K, Ghoul G, Chekir-Ghedira L. Cytotoxic, genotoxic and antigenotoxic potencies of oligorutins. Human and Experimental Toxicology 2013;32 (8) 881-889.

[30] Anthoni J, Humeau C, Maia E, Chebil L, Engasser JM, Ghoul M. Enzymatic synthesis of oligoesculin: structure and biological activities characterizations. European Food Research and Technology 2010;231(4) 571-579.

[31] Kawakita H, Hamamoto K, Ohto K, Inoue K. Polyphenol polymerization by horseradish peroxidase for metal adsorption studies. Industrial and Engineering Chemistry Research 2009;48(9) 4440-4444.

[32] Faix O, Lange W, Salud EC. The use of HPLC for the determination of average molecular weights and molecular weight distributions of milled wood lignins from *Shorea polysperma* (Blco.). Holzforschug 198135(1) 3-9.

[33] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 1999;26(9-10) 1231-1237.

[34] van den Berg R, Haenen GRMM, van den Berg H, van der Vijgh W, Bast A. The predictive value of the antioxidant capacity of structurally related flavonoids using the Trolox equivalent antioxidant capacity (TEAC) assay. Food Chemistry 2000;70(3) 391-395.

[35] Burda S, Oleszek W. Antioxidant and antiradical activities of flavonoids. Journal of Agricultural and Food Chemistry 2001;49(6) 2774-2779.

[36] Halliwell B. Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. American Journal of Medicine 1991;91(3 C) 14S-22S.

[37] Kong LD, Cai Y, Huang WW, Cheng CHK, Tan R.X. Inhibition of xanthine oxidase by some Chinese medicinal plants used to treat gout. Journal of Ethnopharmacology 2000;73(1-2) 199-207.

[38] Apak R, Güçlu K, Özyürek M, Karademir SE. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. Journal of Agricultural and Food Chemistry 2004;52(26) 7970-7981.

[39] Markham K. Techniques of flavonoid identification. Academic Press; 1982.

[40] Bilici A, Kaya I, YildIrIm M, Dogan F. Enzymatic polymerization of hydroxy-functionalized carbazole monomer. Journal of Molecular Catalysis B: Enzymatic 2010;64(1-2) 89-95.

[41] [41]Uyama H, Kobayashi S. Enzyme-catalyzed polymerization to functional polymers, Journal of Molecular Catalysis B: Enzymatic 2002;19-20 117-127.

[42] Uyama H. Artificial Polymeric Flavonoids: Synthesis and Applications, Macromolecular Bioscience 2007;7(4) 410-422.

[43] Seyoum A, Asres K, El-Fiky FK. Structure-radical scavenging activity relationships of flavonoids, Phytochemistry 2006;67(18) 2058-2070.

[44] Fernandez MT, Mira ML, Florêncio MH, Jennings KR. Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. Journal of Inorganic Biochemistry 2002;92(2) 105-111.

[45] Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. The Journal of Nutritional Biochemistry 2002;13(10) 572-584.

[46] Guo M, Perez C, Wei Y, Rapoza E, Su G, Bou-Abdallah F, Chasteen ND. Iron-binding properties of plant phenolics and cranberry's bio-effects. Dalton Transactions 2007; (43) 4951-4961.

[47] Mladenka P, Zatloukalová L, Filipský T, Hrdina R. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. Free Radical Biology and Medicine 2010;49(6) 963-975.

[48] Hayashi T, Sawa K, Kawasaki M, Arisawa M, Shimizu M, Morita N. Inhibition of Cow's Milk Xanthine Oxidase by Flavonoids. Journal of Natural Products 1988;51(2) 345-348.

[49] Nagao A, Seki M, Kobayashi H. Inhibition of xanthine oxidase by flavonoids, Bioscience, Biotechnology and Biochemistry 1999;63(10) 1787-1790.

[50] Lin CM, Chen CS, Chen CT, Liang YC, Lin JK. Molecular modeling of flavonoids that inhibits xanthine oxidase. Biochemical and Biophysical Research Communications 2002;294(1) 167-172.

IntechOpen

IntechOpen