Laccase-Catalyzed Oxidation of (+)-Catechin in a Hydro-Organic Biphasic System and Biological Activities of Corresponding End-Products

Dr. Issa Nizar [a,b*]

(Received 14 / 3 / 2019. Accepted 22 / 9 /2019)

□ ABSTRACT □

In the present study, a green procedure for the synthesis of nature identical stable compounds by laccase-catalyzed oxidation of catechin in a hydro-organic biphasic system was investigated. Catechin was chosen because it is one of the effective flavonoids present in the human diet with various biological activities. Additionally, this work aimed to exploit the laccase-catalysed oxydation of catechin in a hydro-organic biphasic system in order to increase the solubility of the resulting products, effectiveness in the prevention of their subsequent browning and to improve their biological activities. Myceliophthora thermophila laccase-catalyzed oxidation of (+)-catechin, was carried out in aqueous and hydro-organic medium to synthesize new active molecules. The ultrafiltration of the commercial laccase allowed for the elimination of phenolic contaminants and increased the specific activity by a factor of 2. The oxidation of (+)-catechin in a biphasic hydro-organic system (perfectly mixed) consisting of ethyl acetate and sodium–phosphate buffer resulted in intermediate stable orange products. Compared to the aqueous medium, the biphasic hydro-organic medium presenented significant advantages such as decreased the activity of the laccase, an increased effectiveness in the prevention of browning and an increased solubility and stability of the resulting intermediate end products. Thereby, the biphasic medium facilitates the separation of these products which were soluble only in the organic phase of this system. These intermediate end products showed higher anti-leishmania activity against L. tropica promastigotes than (+)-catechin and glucantime. Additionally, enzymatic oxidation led to amplifying the radical scavenging activity against the superoxide anion and cytotoxic activity toward HUVEC cells compared with a (+)-catechin monomer. Furthermore, oxidation end products inhibited xanthine oxidase activity. Consequently, this enzymatic procedure in hydro-organic medium could provide new compounds presenting optical, antileishmanial, antioxidant and cytotoxic interest.

Keywords: Laccase, Enzymatic oxidation, (+)-Catechin, Anti- Leishmania promastigotes, Xanthine oxidase, Cytotoxicity

* [a] **Assistant Prpfessor, Biology departmant, faculty of sience, damascus university, damascus syria.**
[b] **Leishmania centre of epidemiological and biological research, Damascus University, Damascus, Syria**

الأكسدة الأنزيمية المحفزة بإنزيم اللاكاز للكاتشين في وسط ثنائي الطور (عضوي/مائي) والفعاليات البيولوجية للمنتجات النهائية الموافقة

د. نزار عيسى* [1,2]

(تاريخ الإيداع 14 / 3 / 2019. قبل للنشر في 22 / 9 / 2019)

□ ملخّص □

تم في هذه الدراسة التحري وبطريقة صديقة للبيئة عن أمكانية تركيب مركبات مشابهة للطبيعية ومستقرة عن طريق الاكسدة الانزيمية للكاتيشين المحفزة بإنزيم اللاكاز في وسط ثنائي الطور (عضوي _مائي). اختير الكاتيشين كونه يشكل احدى الفلافونيدات الاكثر فعالية والموجودة في غذاء الانسان، بالإضافة الى امتلاكه للعديد من الخصائص الحيوية المهمة. كذلك، هدف هذا البحث الى استغلال الاكسدة الانزيمية للكاتيشين المحفزة بإنزيم اللاكاز في وسط ثنائي الطور (عضوي _مائي) بهدف زيادة انحلالية النواتج ومنع اسمرارها اللاحق وتحسين خصائصها الحيوية. تمت أكسدة الكاتيشين المحفزة بإنزيم اللاكاز من فطر Myceliophthora thermophila في وسط مائي ووسط ثنائي الطور (عضوي _ مائي) بهدف إنتاج جزيئات حيوية جديدة. سمحت تقنية الترشيح الفائق لإنزيم اللاكاز التجاري بإزالة الملوثات الفينولية وزيادة الفعالية النوعية للإنزيم بمقدار ضعفين. أفضت أكسدة الكاتيشين في نظام ثنائي الطور(عضوي – مائي) الممزوج بشكل تام والمكون من دارئة فوسفاتية والاتيل اسيتات إلى نواتج برتقالية اللون وذات ثباتية عالية. بالمقارنة مع الوسط المائي، يقدم الوسط ثنائي الطور (عضوي – مائي) العديد من الايجابيات المهمة مثل التقليل من الفعالية الإنزيمية والزيادة في الوقاية من تطور تفاعلات السمرة الأنزيمية اللاحقة، وكذلك زيادة انحلالية وثباتية النواتج الوسيطة النهائية للتفاعل. وبناء عليه، يمكن القول بأن الوسط ثنائي الطور يسهل فصل هذه النواتج والتي تكون عادة منحلة فقط في الطور العضوي لهذا النظام. في الواقع، أبدت هذه النواتج الوسيطة النهائية في الزجاج فعالية مضادة لليشمانيا وخصوصا مشيقات الليشمانيا المدارية L. tropica اكبر من الكاتيشين والدواء المرجعي (الغلوكانتيم). علاوة على ذلك، مكنت الأكسدة الأنزيمية من زيادة الفعالية الممخلبة لشوارد الأكسجين المؤكسدة الحرة والسمية الخلوية تجاه الخلايا المشتقة من البطانة الداخلية للحبل السري (HUVEC) بالمقارنة مع جزيء الكاتيشين الأحادي غير المؤكسد. أيضا، أظهرت النواتج النهائية لأكسدة الكاتيشين قدرة تثبيطية لفعالية أنزيم الأكزنتين اوكسيداز والمسبب لتراكم حمض البول في الجسم. وبالنتيجة، مكنت هذه الطريقة الأنزيمية في وسط ثنائي الطور(عضوي – مائي) من الحصول على مركبات جديدة والتي تجسد العديد من الخصائص المهمة مثل اللون والفعالية المضادة لليشمانيا والمضادة للأكسدة والسمية الخلوية والفعالية المضادة لأنزيم الاكزنتين اوكسيداز، مما يجعلها مرشحة للاستخدام في العديد من الصناعات الصيدلانية والغذائية والطبية.

الكلمات المفتاحية: اللاكاز، الأكسدة الأنزيمية، –(+) الكاتيشين، الفعالية المضادة لمشيقات الليشمانيا، اكزنتين اوكسيداز، السمية الخلوية.

[1]* – مدرس– قسم علم الحياة الحيوانية، كلية العلوم، جامعة دمشق، دمشق، سورية

[2] – مركز الدراسات الوبائية والبيولوجية لطفيليات الليشمانية، جامعة دمشق، دمشق، سورية

Introduction

The laccases (p-diphenol oxidase, E.C. 1.10.3.2) belong to a small group named blue copper enzyme. They contain at least four neighbor copper ions directly involved at the active site (Solomon EI, 1996). These enzymes oxidize mono-, ortho- and para-diphenols, methoxy-phenols, polyphenols, lignins, polyamines, and aryl diamines (Thurston CF, 1994). Laccases and related polyphenol oxidases (PPOs) enzymes oxidize phenols into reactive quinonesor semi-quinone free radicals(Scheme 1). The quinones, as the semi-quinones free radicals produced by PPOs are very reactive intermediate species and powerful electrophiles, they can undergo non-enzymatic reactions including covalent coupling to form dimers, oligomers and polymers through C–C, C–O and C–N bonds (Claus H, 2004). Moreover, the instability of these products in aqueous medium seemed to be clearly diminished in organic one (Mustafa R, 2005). Thus, we investigate a biphasic system consisting of ethyl acetate and sodium–phosphate buffer as a reaction medium.

Hydrogen peroxide, hydroxyl radicals, peroxide anions, and superoxide anions are generally known as reactive oxygen species (ROS) inducing aging and many kinds of diseases such as mutagenesis and carcinogenesis. One very important enzyme that has been reported to increase during oxidative stress is xanthine oxidase (XO). Therapeutic use of inhibitors of xanthine oxidase has been proposed in the prevention of ischemia–reperfusion injury (Rose S, 1998). Thus, antioxidation and XO inhibition are an important pharmacological action, and antioxidant agents possessing both ROS scavenging and XO inhibition abilities may be used as protective agents in a number of related diseases.

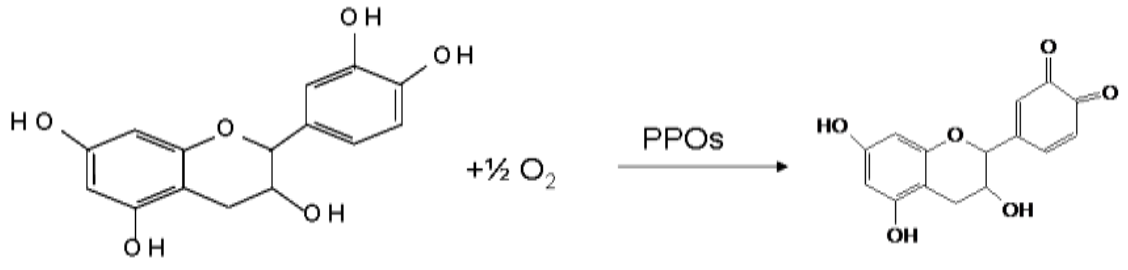
The biological and pharmacological effects of flavonoids, including antioxidant, anti-mutagenic, anti-carcinogenic, probiotic, anti-microbial and anti-inflammatory properties, have been demonstrated in numerous human, animal and in vitro studies, and these properties are potentially beneficial in preventing diseases and protecting the stability of the genome (Jankun J, 1997;Soleas G, 1997). Many of these activities have been attributed to antioxidant actions of catechin, which is a member of the flavonoids and one of the most abundant and best-studied groups of plant polyphenols (Jovanovic SV, 1994;Yen GC, 1995). However, the activities of flavonoids generally persist for limited short periods in vivo. In addition, several low-molecular-weight flavonoids have been shown to act as pro-oxidants and generate reactive oxygen species, such as hydrogen peroxide. Catechin as a polyphenolic compound was also reported to have pro-oxidant effects at lower dosages in the aqueous phase (Rice-Evans CA, 1996). In contrast, a relatively high-molecular-weight fraction of extracted plant polyphenols was reported to exhibit enhanced physiological properties, such as antioxidant, anti-carcinogenic, and antileishmanial activity (Hagerman AE, 1998;Saito M, 1998;Tadesse A, 2015).

Leishmaniasis are group of diseases caused by intracellular protozoan from more than 20 Leishmania species in the family of Trypanosomatidae. This protozoan are transmitted via the bites of infected female sandflies (WHO, 2017). There are about 12-15millions infected people around the world, the mortality rate is 70,000 deaths per year. An estimated 1.5 to 2 million new case occurred each year (Reithinger R, 2007). There are three types of leishmaniasis: cutaneous, mucocutaneous and visceral leishmaniasis, also known as Kala-azar (Tiuman, 2011). The most common form is cutaneous leishmaniasis, the majority of its cases occur in Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia and the Syrian Arab Republic (WHO, 2017). Cutaneous leishmaniasis (CL) caused by many leishmanial species such as L. tropica, L. major, and L. aethiopica in the old world and L. mexicana, L. amazonensis, L. panamensis, L.

baraziliensis in the new world. Cutaneous leishmaniasis (CL) is presented as crusted nodules, papules, plaques, or ulcerative nodular lesions on the face, hands, or feet which are self-healing within a few months but may leave scars, or they may develop into a diffused form with variants of clinical manifestations. The lack of human vaccine for leishmaniasis makes the chemotherapy the only way to control the disease. Current antileishmanial treatment depends on antimonials drugs as the first line of treatment such as stibogluconate of sodium (Pentostam®), N-methylglucantime(Glucantime®). Other drugs that may be used include Pentamidine (Pentacarinato®), and Amphotericin B (Fungizone®), that are the second line of antileishmanial drugs (Monzote, 2009). These drugs have a lot of limitations such as high toxicity, high price, the lack of patient competence due to parenteral administration for a long treatment, and the occurrence of new resistant strains. Because of these limitations, the development of new medications that can replace the traditional ones is necessary. This new treatment is expected to be effective against resistant strains, has a low price, could be administrated orally, and has low toxicity.

From this reason, we have designed laccase-catalyzed oxydation of catechin in a hydro-organic biphasic system in order to increase the solubility of the resulting products, and their effectiveness in the prevention of subsequent browning and to improve their biological activity including antileishmanial. We report, herein, the laccase-catalysed oxydation of (+)-catechin in a hydro-organic biphasic system and evaluate the inhibitory activity against xanthine oxidase, antileishmanial effects against Leishmania tropica and cytotoxicity toward Human umbilical vein endothelial cells (HUVEC) of the resultant end products, compared with a (+)-catechin precursor.

(Scheme 1). PPOs-catalyzed oxidation of catechin (Wu LQ, 2002)**:**



Catechin

1

Quinone

Abbreviations:

PPOs (Polyphenol Oxidases) XO (Xanthine Oxidase) EDTA (EthyleneDiamineTetraacetic Acid)
ROS (Reactive Oxygen Species) BCA (Bicinchoninic Acid) NRU (Neutral Red Uptake)
HUVEC Human umbilical vein endothelial cells DMSO (Dimethy Sulfoxide)
XTT (Sodium-2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide)
HPLC (High Performance Liquid Chromatography) TFA (Trifluoroacetic Acid)

2. Materials and Methods:

2.1. Chemicals and enzyme

The used chemicals are:

-(+)-Catechin (Scheme 1), Sigma- Aldrich (France),purity 99%

-Sodium phosphate (monobasic and dibasic), Prolabo (France), purity 99%.

-Syringaldazine,Ega-chemie, purity 99%.

- Dimethyl sulfoxid (DMSO), ethyl acetate, acetone and methanol (HPLC grade), Merck (Germany)

-Glucantime (GTM), SANOFI AVENTIS (FRANCE)

- RPMI medium containing heat-inactivated fetal bovine serum (HFBS) ,100 µg /mL of streptomycin/ and 100 U /mL of penicillin,Euroclone (France).

-The bicinchoninic acid (BCA) kit, Sigma–Aldrich (France).

-Sodium-2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) kit,Roche (France). Trypan blue, trifluoroacetic acid: TFA (98%) and other chemicals with highest quality available, Sigma- Aldrich (France).

2.2. Laccase characterization

An industrial laccase named Suberase® (Novo Nordisk A/S, Bagsvaerdt, Denmark) was bought from the Society Novozymes in a liquid form. The Suberase® is a fungal laccase from Myceliophthora thermophila , which is considered as a member of the family of polyphenol oxidases, produced by submerged fermentation of a genetically modified Aspergillus oryzae. The enzymatic preparation was supplied as a brown liquid with a density of approximately1.15 gmL^{-1}. It was completely miscible in with water. The Suberase® was partially purified by the ultrafiltration device with a cell Amicon-8200 (20 ml) equipped with a membrane of cellulose acetate (cut off 9 kDa) (SARTORIUS - 14539-63-G) under nitrogen to avoid oxidation. Protein concentration in Suberase® was determined by bicinchoninic acid (BCA) method (Slocum TL, 1991). The laccase activity at different pH and different temperatures was spectrophotometrically determined by measuring the increase of the absorbance at 525 nm caused by the oxidation of syringaldazine (Aljawish A, 2014;Mustafa R, 2005).

2.3. Enzymatic oxidation of catechin

Laccas-catalyzed oxidation of (+)-catechin (5 mM, final concentration) in a magnetic stirred batch reactor was carried out at 30°C. The reaction mixture (250 ml) was sodium–phosphate buffer (50 mM, pH 7.5) only or hydro-organic biphasic medium (perfectly mixed) composing of 175 ml ethyl acetate (70% organic phase), 50 ml sodium–phosphate buffer 50 mM, pH 7.5 (20% aqueous phase) and 25 ml of methanol solution, 50 mM catechin. The reaction was started by adding 0.65 ml of purified laccase (13.5 U/ml). For the control, laccase was not added. The reaction kinetic was monitored at various interval times by withdrawing samples (0.1 ml) from the reaction mixture of the hydro-organic media (in emulsion) and from each phase (aqueous and organic phase). The enzymatic reaction was stopped by the addition of methanol (0.9 ml) containing 0.03% (v/v) of TFA (Lacki K, 1996).

2.4. HPLC and UV-visible analysis

The measures of the enzymatic oxidation of the (+)-catechin and the analyses of reaction mixtures were performed by means of high performance liquid chromatography (HPLC) using a Shimadzu Class-VP HPLC system with a computer controlled system containing upgraded Class-VP 6.1 software. Separations were carried out on a reversed phase column

LiChroCART RP-18 (Merck, 25 x 0.4 cm, particle size 5 µm). The detection was conducted between 200 and 800 nm on a multichannel photodiode- array detector (SPD-M10AVP). The elution was performed using a gradient of solvent A: water/TFA (100:0.03; v/v) and solvent B: acetonitrile/solvent A (80:20; v/v) at a flow rate of 0.7 ml/min. The steps of the gradient were as follow: linear gradient from 15% to 20% B in 5 min, from 20% to 50% in 10 min, from 50% to 60% in 5 min, from 60% to 80% in 15 min and from 80% to 100% in 5 min followed by the washing and the reconditioning of the column. Each analysis was performed triplicate. The UV-visible spectrum of oxidation products was performed between 200 and 600 nm using the spectrophotometer UV-visible (Shimadzu UV-1605).

2.5. Large scale laccase-catalysed oxidation products

In order to produce large quantities of end products and to control dissolved oxygen, temperature, pH, redox potential and agitation, the synthesis was carried out in a 0.5 liter stirred batch bioreactor according to conditions described previously. Each parameter was controlled via a biocontrollerApplikon, ADI 1030 (Schiedam, Hollande). The stirring control was performed using an agitator P100 ADI 1032.

2.6. Recovery of laccase-catalysed oxidation end products

After 4h of the (+)-catechin oxidation reaction, and to ensure a complete oxidation of substrate, the products were recovered by a rotary evaporator under reduced pressure, then freeze-dried for 48 h. After that, the recovered products were stored in a desiccator until use.

2.7. Anti- Leishmania promastigotes activity of laccase-catalysed oxidation products

2.7.1. Parasites and parasites culture

The SYR 113 strain of L. tropica was kindly provided by the leishmania center of epidemiological and biological research, Damascus University, Syria. The parasites were isolated from skin lesions of the patient. The parasites, stored in liquid nitrogen, were thawed and cultured as promastigotes at 26ºC in RPMI 1640 medium (Euroclone, france) supplemented with 10% of heat-inactivated fetal calf serum,1% L-glutamine,100µg/ml of streptomycin and 100 U/ml of penicillin. Subcultures were made at no later than the fourth passage.

2.7.2. Leishmania promastigotes viability evaluation by XTT assay

To study the anti- Leishmania promastigotes activity of (+)-catechin and its oxidation end products, 12 well microplates were filled with 990µl of RPMI 1640 medium supplemented with 10% of heat-inactivated fetal calf serum, 1% L-glutamine, 100µg/ml of streptomycinand and 100 U/ml of penicillin, containing 106 promastigotes/well, after counting in a Neubauer chamber. After 24 h of incubation at 26°C, the cells were exposed to 10 µl of the tested molecules solubilised in DMSO (final concentration of DMSO was 1%) at various concentrations and incubated for 48 h at 26°C. Three controls were used in the microplate, which contain 990µl of diluted promastigotes: first with 10µl of PBS and second with10µl of DMSO solution (final concentration of DMSO was 1%) and third (positive control) with10µl of glucantime solution at different concentrations. The XTT (sodium-2, 3- bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) assay is a parasites viability method, based on the ability of parasites viable cells to reduce tetrazolium salt (XTT) by the mitochondrial dehydrogenase of the parasite to a water-soluble formazan (Williams C, 2003). After 48 h of incubation, 200µl of the culture

medium was transferred into 96 well ELISA plate and 50 µl of tetrazolium salt (XTT, 50µg/ml) were added. The microplate was then incubated with the cells and tetrazolium salt (XTT) solution for 4 h at 37°C. The absorbance of this final solution was measured at 450 nm using a microplate reader (multiscan GO W382TA, Thermo Scientific MIB, France). The relative cell viability was calculated according to the following Eq. (1):

$$\textbf{Relative cell viability}(\%) = \left(1 - \left(Abs_{treated\ cell} / \text{Abs control}\right)\right) \times 100 \qquad 1$$

Abs $_{\text{treated cells}}$ and Abs $_{\text{control}}$ are the absorbance values at 450 nm of sample with treated parasite and control, respectively. Each experiment was repeated three times. The results were expressed as IC50 mean values±standard deviation of three determinations. IC50 was defined as the concentration of a molecule leading to 50% of cell inhibition growth. The cytotoxicity was evaluated as proposed by Viana et al. (2015).

2.8. Xanthine oxidase inibition of laccase-catalysed oxidation end products

The XO activity with xanthine as the substrate was measured spectrophotometrically as mentioned by Noro et al. (1983) with the following modifications: Assay mixtures (1.7 ml) were prepared by adding 1000 µl of xanthine (final concentration 50 µ mole), 4µL of hydroxylamine (final concentration 200 mM), 400 µL of EDTA (final concentration 100 µmole), 196 µl of phosphate buffer solution (pH7.5, 200 mM) and 100 µL of laccase-catalysed oxidation products and (+)-catechin dissolved in a small amount of DMSO (final concentration 0 -250 µg/ml). The reaction was started by adding 300µl of xanthine oxidase (final concentration 7.5 mU/ ml) in a phosphate buffer solution (pH 7.5, 200 mM). The mixture (total 2 ml) was incubated for 30 min at 37°C prior to the measurement of uric acid production by measuring the UV absorbance at 290 nm. The reaction was stopped by adding 300µl of HCl (0.58 M). The uric acid production was calculated from the differential absorbance with a blank solution in which the xanthine oxidase was replaced by buffer solution.A test mixture containing no added molecules was prepared to measure the total uric acid production. The XO inhibition activity was expressed as the percentage of inhibition of the forming uric acid, calculated according to the following Eq. (2):

$$\textbf{PI} = \left(1 - OD_A / OD_B\right) \times 100$$

$$\textbf{PI : Percentage of Inhibition} \qquad 2$$

$$\textbf{OD}_{\textbf{A}}\textbf{ : absorbance of the assay with the testedmoleculesat } \lambda = \textbf{290 nm}$$

$$\textbf{OD}_{\textbf{B}}\textbf{ : absorbance of the assay without the testedmoleculesat } \lambda = \textbf{290 nm}$$

The results were expressed as IC_{50} mean values±standard deviation of three determinations. IC_{50} was defined as the concentration of a molecule leading to 50% of forming uric acid inhibition.

2.9. Superoxide scavenging activity of laccase-catalysed oxidation end products

To detect superoxide, the coloring reagent (final concentration of 300 µg/ml sulfanilic acid, 5 µg/ml of N-(1-naphthyl)- ethylenediaminedihydrochloride, and 16.7% (v/v) acetic acid) was added to the reaction medium used for the determination of the xanthine oxidase inhibition activity. The mixture was allowed to stand for 30 min at room temperature, then the absorbance at 550 nm was measured (Cos et al.,1998). The test was performed for various concentrations of samples used in the xanthine oxidase inhibition test. Positive control was allopurinol, which was used in the treatment of gout. The antioxidant activity

was expressed as the percentage of inhibition of the superoxide radicals, calculated according to the following Eq. (3):

$$\mathbf{PI} = \left(1 - \boldsymbol{OD_A} / \boldsymbol{OD_B}\right) \times 100$$

PI : Percentage of Inhibition **3**

OD_A : absorbance of the assay with the testedmolecules at $\lambda = 550$ nm

OD_B : absorbance of the assay without the testedmolecules at $\lambda = 550$ nm

The results were expressed as IC_{50} mean values±standard deviation of three determinations. IC_{50} was defined as the concentration of a molecule leading to 50% of superoxide radicals inhibition.

2.10. Cytotoxic properties of laccase-catalysed oxidation end products

2.10.1. Cells and cell culture

HUVEC cells (Human umbilical vein endothelial cells) were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK, No. 07073101) and cultivated in Endothelial Basal Medium (Sigma–Aldrich, France). The medium was prepared by the addition of growth supplement (3%) (Sigma–Aldrich, France). HUVEC cells were cultivated between passages 1 and 7. The cells were usually split when 80% confluence was reached (5days). They were first rinsed with Dulbecco's phosphate-buffered saline without calcium (D-PBS) and then trypsinised with a solution containing 0.25% trypsin and 1 mM EDTA (GIBCO, USA) and counted by Thoma cell under an optical microscope. For maintenance of the cell line, the cells were seeded at $1.4x10^4$cells/ml in flasks. All experiments of cytotoxicity evaluation were performed between the passage 3 and 5.

2.10.2. Cell viability evaluation by neutral red uptake (NRU) assay

To study the cytotoxic activity of (+)-catechin and its derivatives, 96-well microplates were filled with190µl of diluted HUVEC cells at 5.10^4cells/well. After 24 h of incubation at 37°C under 5% CO_2 atmosphere, the cells were exposed to 10 µl of the tested molecules solubilised in DMSO (final concentration of DMSO was 0.25%) at various concentrations and incubated for 48 h at 37°C under 5% CO_2 atmosphere. Two controls were used in two columns of the microplate which contain190µl of diluted cells: one with 10µl of D-PBS and another with10µl of DMSO solution (final concentration of DMSO was 0.25%).

The NRU (neutral red uptake) assay is a cell viability method, based on the ability of viable cells to bind a neutral red dye within the membranes of intracellular lysosomes (Mingoia RT, 2006). After 48 h of incubation, the culture medium (200µl) was removed from each well of the microplate. The attached cells were rinsed with equal volume of D-PBS at pH 7.2. Then, 200 µl of neutral red solution (50µg/ml) freshly prepared in the culture medium was added to each well. The microplate was then incubated with the cells and the neutral red solution for 3 h at 37°C. After incubation, the neutral red solution was eliminated and the attached cells were rinsed with D-PBS. The attached cells were solubilised in 200µl of an ethanol/acetic acid/water solution (50%, 1%, 49%, v/v/v) to release the neutral red remained within the lysosomal or cytoplasmic compartments. The plates were then shaken at room temperature for 10 min. The absorbance of this final solution was measured at 540 nm using a microplate reader (multiscan GO W382TA, Thermo Scientific MIB, France). The relative cell viability was calculated according to the following Eq. (4):

$$\mathbf{Relative\ cell\ viability}\,(\%) = \left(1 - \left(\boldsymbol{Abs}_{\textit{treated cell}} / _{\textit{Abs control}}\right)\right) \times \mathbf{100} \qquad \mathbf{4}$$

With Abs $_{treated}$ cells and Abs $_{control}$ being the absorbance values at 540 nm of sample with treated cells and control, respectively. Each test was carried out in four times, and each experiment was repeated three times. The results were expressed as IC_{50} mean values±standard deviation of three determinations. IC_{50} was defined as the concentration of a molecule leading to 50% of cell mortality. The cytotoxicity was evaluated as proposed by Kong et al. (2009).

3. Results and discussion

3.1. Partial purification of laccase

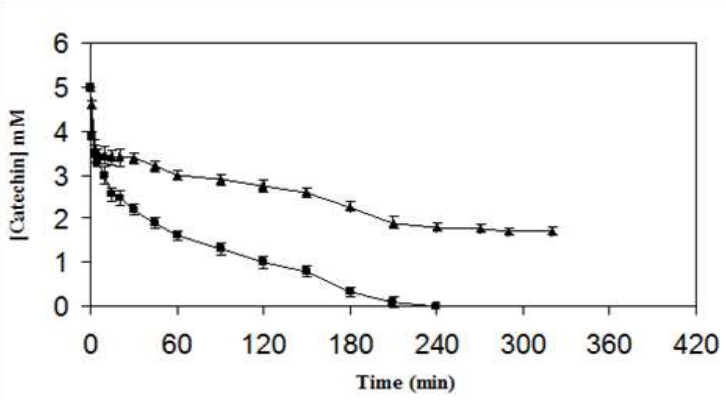
Laccase was partially purified with a yield of 82 % of total activity by ultrafiltration. A 2-fold increase in enzyme activity and almost 2.5 fold of total protein elimination compared to that of the raw laccase have been obtained (Table 1).

(Table 1). Purification of laccase from Myceliophthora thermophile

Step	Protein (µg/ml)	Total activity (µmo/min/ml)	Specific activity (µmo/min/ µg protein)	Purification (fold)	Yield of activity (%)
Raw laccase	20000	5200	0.26	1	100
Ultrafiltration	8000	4240	0.53	2	82

3.2. Kinetics and analysis of oxidation catalysed by purified laccase

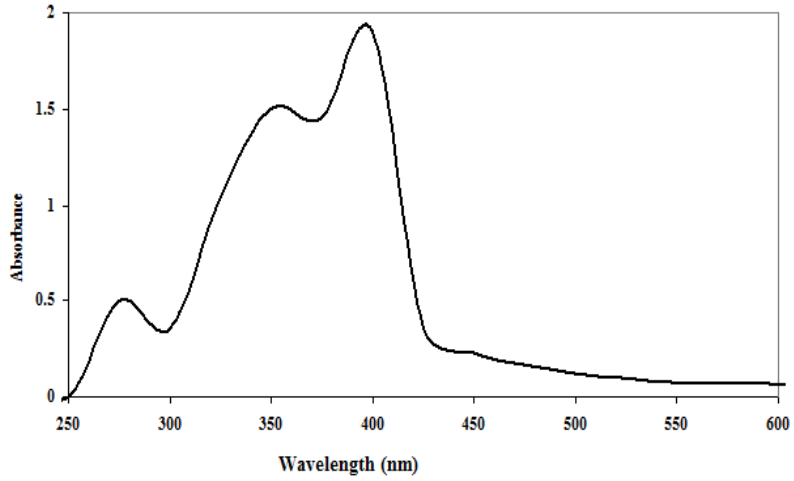
HPLC analyses show considerable differences between the oxidation of (+)-catechin in the two media (Figure 1). The oxidative rate of (+)-catechin in aqueous medium was higher than that of (+)-catechin in biphasic medium. Thus, the complete oxidation of (+)-catechin was achieved after 240 min in aqueous medium, while the complete consumption of (+)-catechin was not achieved in biphasic medium during the reaction time of 320 min. Moreover, it was verified that without laccase, (+)-catechin was not chemically oxidised by auto-oxidation.



(Fig.1). Oxidation of (+)-catecin by Laccase from Myceliophthora thermophila in hydro-organic biphasic

medium in emulsion (▲) and in aqueous medium (■).Values are the mean ± standard deviations of three independent experiments.

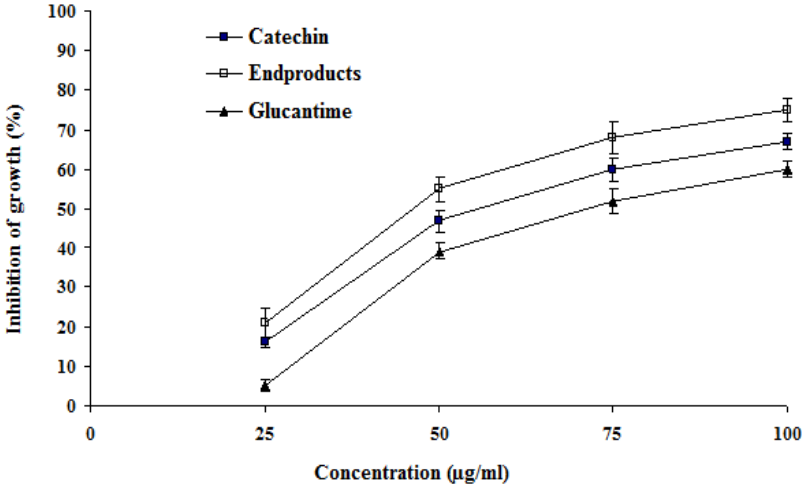
In UV-visible measurements, (+)-catechin showed one peak at 280 nm and in the case of the organic phase end-products an additional broader peak was seen at ~400 nm (Figure 2).



(Fig.2).UV-visible spectrum of end-products from laccase-catalyzed oxidation of (+)-catechin in the organic phase (ethyl acetate) of the hydro-organic medium.

3.3. Anti-leishmanial activity of laccase-catalysed oxidation endproducts

The XTT assay was used to evaluate the viability of the promastigotes treated with molecules tested at several concentrations (25, 50, 75 and 100 µg/ml) over 48 h. The results were expressed as IC_{50} mean values with standard deviations. In this study, (+)-catechin and its oxidation end products showed significant leishmanicidal activity against L.tropica promastigotes with increasing concentration (Figure 3). However, oxidation end products of (+)-catechin displayed marked in vitro anti-promastigotes activity with higher inhibition growth effect toward L.tropica promastigotes than that of the (+)-catechin monomer and glucantime at different used concentrations as indicated in fig. 3. Consequently, the oxidation of (+)-catechin increased the anti-leishmania activity against L.tropica promastigotes of this phenolic compound. Additionally, it was found that (+)-catechin (IC_{50} = 50 ± 4.7 µg/ml) exhibited a higher anti-promastigotes activity towards L.tropica promastigotes, compared to glucantime (IC_{50} = 75 ± 5.8 µg/ml).



(Fig.3). Antileishmanial activity in vitro of (+)-catechin and its end products in the organic phase (ethyl acetate) of the hydro-organic medium against promastigotes of L. tropica. Values are the mean ± standard deviations of three independent experiments.

3.4. Xanthine Oxidase (XO) activity measurement of laccase-catalysed oxidation endproducts

Both inhibition of XO and the scavenging effect on the superoxide anion were measured in one assay in comparison with allopurinol which is a pharmaceutical component commonly used as a XO inhibitor. Inhibition of XO results in a decreased production of uric acid, which can be measured spectrophotometrically, and a decreased production of superoxide, measured by the nitrite method. For each substance tested two IC_{50} values can be calculated: 50% inhibition of XO (50% decrease of uric acid production) and 50% reduction of the superoxide level. The half-maximal inhibitory concentrations (IC_{50}) of the allopurinol, (+)-catechin and its laccase- catalysed oxidation products are listed in table 2. Allopurinol, which is widely used as a XO inhibitor, constituted the reference molecule, with an IC_{50} value of 0.4µg/ml.

(Table 2). IC_{50} Values of allopurinol, (+)-catechin and its laccase-catalysed oxidation end products for inhibition of Xanthine Oxidase and reduction of superoxide level. IC_{50} mean values±standard deviation of three determinations.

Compound	Xanthine oxidase inhibition IC_{50}(µg/ml) ± SD	Superoxide radical scavenging IC_{50}(µg/ml) ± SD
Allopurinol	0.4±0.02	0.36±0.03
(+)-catechinnc	nc	-
Oxidation end products	90±0.08	29±0.06

nc= no calculation possible for this compound
-, pro-oxidant effect

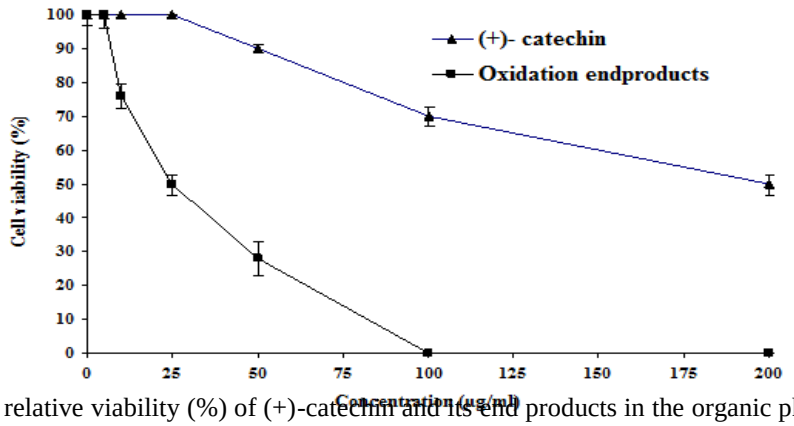
3.5. Radical scavenging activity measurement of laccase-catalysed oxidation endproducts

The antioxidant activity of the laccase-catalysed oxidation end products was evaluated in term of superoxide anion scavenging activity using the nitrite method. (+)-catechin monomer can not be considered as superoxide radical scavenger, while the laccase-catalysed oxidation end products greatly scavenged superoxide anions (IC_{50} = 29 ± 0.06µg/ml), compared to monomeric (+)-catechin (Table 2).

3.6. Cytotoxic activity of laccase-catalysed oxidation products

In the present work, the effect of (+)-catechin and its oxidation end-products on HUVEC cellsgrowth was investigated by the neutral red uptake (NRU) assay. No cytotoxic effect was observed on the growth of HUVEC cells, using 0.25% (v/v) of DMSO (the final concentration in the assay) (data not shown). The neutral red uptake (NRU) assay was used to evaluate the viability of the cells treated with compounds tested at several concentrations (5, 10, 25, 50, 100 and 200 µg/ml) over 48 h. The results were expressed as IC_{50} mean values with standard deviations.

At the concentrations of 5, 10 and 25µg/ml, the (+)-catechin monomer did not present any cytotoxic effect towards HUVEC cells, while it showed considerable cytotoxicity at relatively high concentrations. The oxidation end products exhibited a dose dependent cytotoxic activity on HUVEC cells and were shown to be more toxic (IC_{50} = 25 ± 4 µg/ml) than (+)-catechin monomer (IC_{50} = 200 ± 3 µg/ml) (Figure 4). Consequently, the oxidation and the dimerization of (+)-catechin increased the cytotoxic effect of this flavonoid.



(Fig. 4). Cell relative viability (%) of (+)-catechin and its end products in the organic phase (ethyl acetate) of the hydro-organic medium toward HUVEC cells. Values are the mean ± standard deviations of three independent experiments.

4. Discussion

Enzymatic oxidation of flavonoid is very important in biochemistry because the subsequent coupling reactions are involved in some biosynthetic pathways, such as tannin and melanin formation (Ihara N, 2005). The oxidative coupling of (+)-catechin using peroxidase or polyphenol oxidase as catalyst was reported to form oligomeric compounds with complicated structures (Kurisawa M, 2003). This is the first report that describes the oxidation end products obtained by the action of laccase from Myceliophthora thermophila on catechin in hydro-organic biphasic system. To remove inactive peptides or proteins and

to avoid the interaction between the interesting phenols and contaminated phenols, which were initially present with the industrial laccase, the industrial laccase was partially purified. The purified enzyme gave a single protein band on SDS–PAGE with an apparent molecular mass of ~83 KDa which is consistent with the molecular mass (85 KDa) of laccase Myceliophthora thermophila (Aljawish et al., 2014). The optimal activities were determined to be at pH 7.5 and a temperature of 30°C, using syringaldazine as a specific substrate (data not shown). In fact, after 4 h of the oxidation reaction, the laccase was inactive, probably due to the reaction between the intermediate products (free radicals from quinones or semi-quinones) and histidine groups of the enzyme active site linked to the binuclear copper (Whitaker JR, 1995;Whitaker JR., 1994). In aqueous medium, the yield of the oxidation products was negligible, due to the higher activity of both enzyme and semi-quinone radical in this medium. In contrast, a completely different behavior was observed in the biphasic hydro-organic medium in emulsion. The intermediate product, which polymerize quickly in water, are prevented from doing so in biphasic medium due to their transfer into the organic solvent (Rojo M, 2001). Consequently, their stability is enhanced in this medium. Nevertheless, in the biphasic medium, a maximum yield of orange colored end products can be obtained after 4 h of the reaction (corresponding to a substrate conversion of 64%). HPLC analyses of the reaction mixture during the oxidation of (+)-catechin by purified laccase in the two media revealed the presence of several peaks corresponding to various products. However, at the end of the reaction in aqueous medium, the condensation of intermediate products was very important, therefore, no isolated peaks were detectable under HPLC conditions. In the hydro-organic medium in emulsion, the HPLC analyses showed the formation of three principal products (λmax 280 nm) whose polarity is lower than that of (+)-catechin, while in the organic phase of the hydro-organic medium, the analyses showed the formation of one principal end product (λmax 400 nm) whose polarity is lower than that of (+)-catechin (data not shown). Indeed, the UV-visible measurements indicated that (+)-catechin mostly converted into its oligomers; the aromatic carbon-carbon linkage between (+)-catechin molecules is formed via the oxidative coupling of (+)-catechin Further work using HPLC/LC–MS is in progress to elucidate the structure of these obtained oligomers. These findings are in good agreement with the results reported by other authors (Ma HL, 2009) who found the formation of probable dimer from laccase-catalysed oxidation of (+)-catechin in organic solvent. Additionally, Mustafa et al. (2005) reported that the laccase-catalysed oxidation of ferulic acid in a biphasic hydro-organic system (perfectly mixed) consisting of ethyl acetate and sodium–phosphate buffer resulted in intermediate (dimmers) stable yellow products. Additionally, these orange colored end products only soluble in the organic phase was obtained by evaporation of the organic solvent under vacuum. In addition, these colored products can be solubilised in other organic solvents such as methanol and ethanol or in vegetable oils, but they are not soluble in water.

The anti-leishmania promastigotes activity of (+)-catechin and its oxidation end products in the organic phase of the hydro-organic medium were evaluated toward L.tropica promastigotes parasites by the XTT assay in comparison with glucantime which was used as a reference drug. Prior to the assay, the effect of 1% (v/v) DMSO (the final concentration of DMSO in the assay) was investigated and no growth inhibition was observed on the growth of L.tropica promastigotes parasites (data not shown). However, antileishmania activity has been reported in several compounds extracted from medicinal plants belonging to diverse chemical groups, including flavonoids and quinones

(Hamidizadeh N, 2017; Tadesse A, 2017; Dos Reis MBG, 2013; Viana SM, 2015). In previous studies, the in-vitro investigations have indicated that luteolin and quercetin exert their antileishmaniasis effects through inhibition of the catalyzer activity of DNA topoisomerase II, which stimulates disruption of cell cycle and results in L. donovani promastigotes apoptosis. In addition, quercetin, luteolin and (+)-catechin can have antileishmanial effect on L. amazonensis by inhibiting arginase enzyme and can be used as oral complementary medicines for treatment of Leishmania (Dos Reis MBG, 2013; Das BB, 2006;Manjolin LC, 2013). Thus, the leishmaniostatic activity of (+)-catechin is probably related to its inhibitory effects of the enzymes parasite. Furthermore, (+)-catechin oxidation products (IC_{50} = 44 ± 6.8 µg/ml) presented higher antileishmanial effect toward L. tropica promastigotes, compared to its initial compound (IC_{50} = 50 ± 4.7 µg/ml) and glucantime (IC_{50} = 75 ± 5.8 µg/ml). This higher antileishmanial activity could be attributed to the enzymatic dimerisation of the (+)-catechin precursor. In fact, the oxidation procedure increased the number of radical species sources (quinones or semiquinones) and concentration of phenolic moieties in the molecules being responsible for the prevention of growth and proliferation of L. tropica promastigotes (Viana SM, 2015;Kurisawa M, 2003). Quinones are highly redox active molecules and with their semiquinones radicals can lead to formation of reactive oxygen species (ROS), including O_2, H_2O_2 and ultimately to hydroxyl radicals. The formation of ROS could probably explain the in vitro leishmanicidal activity of the oxidation end products in this study, since promastigotes are readily susceptible to killing by H_2O_2 in vitro (Viana SM, 2015;Zarley JH, 1991;Bolton JL, 2000). However, this source of quinone free radicals seems to be often more readily apparent in vitro than in vivo (Viana SM, 2015).

Increased XO activity is not only assumed to be important in mediating oxidative injury in ischemia–reperfusion but also the enzyme responsible for the formation of uric acid associated with gout, which causes painful inflammation in the joints (Chiang HC, 1994;Matsumura F, 1998;Manach C, 1999). The monomeric (+)-catechin has no effect on xanthine oxidase inhibition (IC_{50} not calculated) due to the presence of a hydroxyl group at C-3, while the laccase-catalysed oxidation end products exhibited marked XO-inhibiting activity (IC_{50} = 90 ± 0.08µg/ml), corresponding to the results reported by other authors (Tapia A, 2004;Cos P, 1998). In fact, hydroxyl groups which make a negative contribution to the inhibition of xanthine oxidase are in the 2', 8 and 3 positions (Van Hoorn DEC, 2002). The markedly amplified XO inhibition activity of the laccase-catalysed oxidation end products is considered to be due to effective multivalent interaction between XO and the oligomeric chains of the oxidation end products. Similarly, enhanced activity of XO inhibition was observed following the oxidative polymerization of catechin derivatives(Kurisawa M, 2003;Ihara N, 2005) and the polycondensation of catechin with aldehydes(Chung JE, 2004;Kim YJ, 2004).

Reduction of molecular oxygen to superoxide anions by XO, generating hydroxyl radicals and uric acid, is an important physiological pathway. However, superoxide anions in excess damage biomacromolecules both directly and indirectly by forming hydrogen peroxide or highly reactive hydroxyl radicals (Floyd R, 1990). Conversely to (+)-catechin, its oxidation end products can be considered as superoxide radical scavengers, exhibiting IC_{50} value of 29 µg/ml. Indeed, (+)-catechin had pro-oxidant activity in lower concentrations and this activity is consistent with results often reported for tea polyphenol, (+)-catechin and rutin at low dosages in the aqueous and organic phases (Li C, 2000 ;Kurisawa M, 2003;Ihara N, 2005). Since compounds capable of inhibiting XO can also

positively affect the ability to scavenge superoxide anions, the XO inhibitory activity of laccase-catalysed oxidation end products might partly contribute to the superoxide-scavenging activity of these endproducts. However, the XO inhibitory activity (IC_{50} = 90 ± 0.08µg/ml) was lower than the superoxide radical scavenging activity (IC_{50} = 29 ± 0.06µg/ml) of the oxidation end products. Therefore, the strong inhibitory effect of the end products on superoxide radical scavenging was predominantly a result of superoxide radical scavenging rather than XO inhibition. These results demonstrated that the laccase-catalysed oxidation end products possessed much higher potential for both superoxide anion scavenging and XO inhibition, compared with (+)-catechin monomer. Consequently, the oxidation end products might be an uncompetitive inhibitor of XO with respect to xanthine as a substrate, consistant with the results reported by other authors (Ihara N, 2005;Kim YJ, 2004). In previous studies, the in vitro cytotoxic activity of green tea catechins (58-127µmole) toward human cancer cells was shown to be correlated with the induction of apoptosis, activation of caspases, inhibition of protein kinases, modulation of cell cycle regulation and inhibition of cell proliferation (Yang CS, 1999;Babich H, 2007). In addition, in vitro cytotoxicity studies of green tea catechins have shown tumor cells to be more sensitive than normal cells (Ahmad N., 2000;Babich H., 2005). Moreover, several flavones and flavonols exert cytotoxicity toward cultured human normal cells, i.e., human lung embryonic fibroblasts (TIG-1) and human umbilical vein endothelial (HUVE) cells through increasing intracellular ROS levels (Babich H, 2005;Matsuo Y, 2004). Thus, it is suggested that (+)-catechin at high concentrations was probably incorporated into cells, increased intracellular ROS levels, and then exerted cytotoxicity. The laccase-catalyzed oxidation of phenolic compounds in organic media was expected to increase the lipophilicity of their endproducts (quinones) and consequently their ability to interact with the cell membrane and their transfer through it (Ma HL, 2009;Mustafa R, 2005). Consequently, as the lipophilicity of oxidation end products was higher than of that (+)-catechin monomer, due to the enzymatic oxidation, their cytotoxic activity was higher than that of (+)-catechin monomer against HUVEC cells. Additionally, the high cytotoxic activity of the end products oxidation could be attributed to the presence of the carbonyl group in their structure. In fact, this toxic effect could be explained by the inhibition of fatty acids synthesis of cells by carbonyl group (Brusselman k, 2005).

5. Conclusion

The oxidative reaction of (+)-catechin by the laccase from M. thermophile laccase in a biphasic hydro-organic medium in mild controlled temperature and pH (30°C and pH7.5) illustrates an interesting potential significantly enhanced for preparation and separation of stable colorful end products, compared to aqueous medium. These end products were only soluble in the organic phase of our system. Further structure determination of the oxidation products is in progress. Additionally, oxidation end products showed a higher anti-promastigotes activity towards L. tropica promastigotes, much greater superoxide scavenging and XO inhibitory activity compared to the (+)-catechin monomer. These end products can be considered as antileishmanial candidate drug and antioxidant agents, but more studies are needed to find out its activity against amastigote and appropriate route of administration. In the present study, evidence was pointed out that although the parental molecule (+)-catechin was rather less toxic towards HUVEC cells, its synthesized end products exhibited higher cytotoxicity activities. The high cytotoxic effects of these end products may indicate that these novel compounds might possess improved anti-tumor properties. Further antiproliferative activity determination towards $CaCO_2$ human colon

cancer cells, of the oxidation products is in progress. These new compounds with colour, anti-leishmanial and antioxidant functionalities, presenting at the same time high cell cytotoxicity may find useful applications in several domains, such as food colorants, or as antioxidant additives for food preservation, anti-leishmanial drugs and anti-tumor agents. Furthermore, previous studies demonstrated that these end products tend to bind to other macromolecules,i.e.,chitosan,pectin,hemicelluloses, amine-terminated polyhedral oligomericsilsesquioxane (POSS),carnosine, etc. (Aljawish A, 2012;Aljawish A, 2016;Karaki N, 2016;Ihara N, 2005) as a method to enhance their biofunctions properties.

References

Ahmad N, Gupta S, Mukhtar H (2000) Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor_B in cancer cells versus normal cells. Arch. Biochem. Biophys. 376 : 338–346.

Aljawish A, Chevalot I, Madada N, Paris C, Muniglia L (2016) Laccase mediated-synthesis of hydroxycinnamoyl-peptide from ferulic acid and carnosine. Journal of Biotechnology 227: 83–93.

Aljawish A, Chevalot I, Jasniewski J, Paris C, Scher J, Lionel Muniglia L (2014) Laccase-catalysed oxidation of ferulic acid and ethyl ferulate in aqueous medium: A green procedure for the synthesis of new compounds. Food Chemistry 145: 1046–1054.

Aljawish A, Chevalot I, Piffaut B, Rondeau-Mouro C, Girardin M., Jasniewski J (2012) Functionalization of chitosan by laccase-catalysed oxidation of ferulic acid and ethyl ferulate under heterogeneous reaction conditions. Carbohydrate Polymers 87: 537–544.

Babich H, Krupka M.E, Nissim HA, Zuckerbraun HL(2005). Differential in vitro cytotoxicity of (−)-epicatechingallate (ECG) to cancer and normal cells from the human oral cavity. Toxicol. In Vitro 19 : 231–242.

Babich H, Zuckerbraun HL, Weinerman SM (2007) In vitro cytotoxicity of (−)-catechingallate, a minor polyphenol in green tea. Toxicology Letters 171:171–180.

Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ (2000). Role of quinones in toxicology. Chem. Res. Toxicol. 13(3): 135-60.

Brusselman k, Vrolix R, Verhoeven G, Swinnen VJ (2005) Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acids synthase activity. J.Biol.Chem 280: 5636-5645.

Chiang HC, Lo YJ, Lu FJ (1994) Xanthine oxidase inhibitors from the leaves of Alsophilaspinulosa (Hook) Tryon. J Enzyme Inhib 8:61–71.

Chung JE, Kurisawa M, Kim YJ, Uyama H, Kobayashi S (2004) Amplification of antioxidant activity of catechin by polycondensation with acetaldehyde. Biomacromolecules 5:113–118.

Claus H (2004) Laccases: Structure, reactions, distribution. Micron 35(1–2): 93–96.

Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Van Poel B (1998) Structure activity relationship and classification of flavonoids as inhibitors of xanthine-oxidase and superoxide scavengers. J Nat Prod 61: 71–76.

Das BB, Sen N, Roy A, Dasgupta SB, Ganguly A, Mohanta BC (2006) Differential topoisomerase I-DNA cleavage complex by selected flavones and induction of Leishmaniadonovani bi-subunit camptothecin: activity of flavones against camptothecin-resistant topoisomerase I. Nucleic Acids Res 34:1121-32.

Dos Reis MBG, Manjolin LC, MaquiaveliCdC, Santos-Filho OA, da Silva ER (2013) Inhibition of Leishmania (Leishmania) amazonensis and Rat Arginases by Green Tea EGCG, (+)-Catechin and (2)-Epicatechin: A Comparative Structural Analysis of Enzyme-Inhibitor Interactions. PLoS ONE 8(11): e78387. doi:10.1371/ journal.pone.0078387.

Floyd RA (1990) Role of oxygen free radicals in carcinogenesis and brain ischemia. FASEB J 4: 2587–2597.

Hagerman AE, Riedl KM, Jones GA, Sovik KN, Ritchard NT, Hartzfeld PW, Riechel TL (1998) High molecular weight plant polyphenolics (tannins) as biological antioxidants. J Agric Food Chem 46:1887–1892.

Hamidizadeh N, Ranjbar S, Asgari Q, Hatam G (2017) The evaluation of quercetin and luteolin efficacy on cutaneous leishmaniasis in mice infected with Leishmania major. J Pharm Negative Results 8:43-8.

Ihara N, Kurisawa M, Chung JE, Uyama H, Kobayashi S (2005) Enzymatic synthesis of a catechin conjugate of polyhedral oligomericsilsesquioxane and evaluation of its antioxidant activity. ApplMicrobiolBiotechnol 66: 430–433.

Jankun J, Selman SH, Swiercz R, Skrzypczak-Jankun E (1997) Why drinking green tea could prevent cancer. Nature 387:561.
Jovanovic SV, Steenken S, Tosic M, Marjanovic B, Simic MG(1994) Flavonoids as antioxidants. J Am ChemSoc 116:4846–4851.
Karaki N, Aljawish A , Muniglia L, Humeau C, Jasniewski J (2016) Physicochemical characterization of pectin grafted with exogenous phenols. Food Hydrocolloids 60 : 486–493.
Kim YJ, Chung JE, Kurisawa M, Uyama H, Kobayashi S (2004) Superoxide anion scavenging and xanthine oxidase inhibition of (+)-catechin-aldehyde polycondensates. Amplification of antioxidant property of (+)-catechin by polycondensation with aldehydes. Biomacromolecules 5:547–552.
Kong NH, Jiang T, Zhou ZC, Fu, JL (2009) Cytotoxicity of polymerized resin cements on human dental pulp cells in vitro. Dental Materials 25(11): 1371–1375.
Kurisawa M, Chung JE, Kim YJ, Uyama H, Kobayashi S (2003) Amplification of Antioxidant Activity and Xanthine Oxidase Inhibition of Catechin by Enzymatic Polymerization. Biomacromolecules 4: 470-471.
Lacki K, Duvnjak, Z (1996) Modeling the enzymatic transformation of 3,5- dimethoxy,4-hydroxy cinnamic acid by polyphenoloxidase from the white-rot fungus Trametesversicolor. Biotechnology and Bioengineering 51(3): 249–259.
Li C, Xie B (2000) Evaluation of the antioxidant and pro-oxidant effects of tea catechinoxypolymers. J Agric Food Chem48: 6362–6366.
Ma HL, Kermashab S, Gaoc JM, Borges RM, Yu XZ (2009) Laccase-catalyzed oxidation of phenolic compounds in organic media. Journal of Molecular Catalysis B: Enzymatic 57: 89–95.
Manach C, Texier O, Morand C, Crespy V, Regerat F, Demigne C, Remesy C (1999) Comparison of the bioavailability of quercetin and catechin in rats. Free Radic. Biol. Med. 27: 1259.
Manjolin LC, dos Reis MB, MaquiaveliCdo C, Santos-Filho OA, da Silva ER (2013) Dietary flavonoids fisetin, luteolin and their derived compounds inhibit arginase, a central enzyme in Leishmania (Leishmania) amazonensis infection. Food Chem 141: 2253-62.
Matsuo Y, Sawai H, Funahashi H (2004) Enhanced angiogenesis due to inflammatory cytokines from pancreatic cancer cell lines and relation to metastatic potential. Pancreas 28 : 344-52.
Matsumura F, Yamaguchi Y, Goto M, Ichiguchi O, Akizuki E, Matsuda T, Okabe K, Liang J, Ohshiro H, Iwamoto T, Yamada S, Mori K, Ogawa M (1998) Xanthine oxidase inhibition attenuates kupffer cell production of neutrophil chemoattractant following ischemia– reperfusion in rat liver. Hepatology 28 :1578.
Mingoia RT, Nabb DL, Yang CH, Han X (2006) Primary of rat hepatocytes in 96-well plates: effects of extracellular matrix configuration on cytochrome P450 enzyme activity and inductibility and its application in in vitro cytotoxicity screening. Toxicology In vitro 21: 165-173.
Monzote L. current leishmaniasis treatment (2009). The Open Antimicrobial Agents Journal.1(3):9-19.
Mustafa R, Muniglia L, Rovel, B, Girardin M (2005) Phenolic colorants obtained by enzymatic synthesis using a fungal laccase in a hydro-organic biphasic system. Food Research International 38(8–9): 995–1000.
Noro TOY, Miyase T, Ueno A, Fukushima S (1983) Inhibition of xanthine oxidase from the flowers and buds of Daphne genkwa. Chem Pharm Bull 31: 3984-3987.
Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free RadicBiol Med 20:933–956.
Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. (2007) Cutaneous leishmaniasis. The Lancet Infectious Diseases. 7(9):106-111.
Rojo M, Go`mez M, Isorna P, Estrada P (2001). Micellar catalysis of polyphenol oxidase in AOT/cyclohexane. Journal of Molecular Catalysis B: Enzymatic 11 : 857–865.
Rose S, Fiebrich M, Weber P, Dike J, Buhren V (1998) Neutrophil activation after skeletal muscle ischemia in humans. Shock 9: 21.
Saito M, Hosoyama H, Ariga T, Kataoka S, Yamaji N (1998) Antiulcer activity of grape seed extract and procyanidins. J Agric Food Chem 46: 1460–1464.
Slocum TL, Deupree, JD (1991) Interference of biogenic-amines with the measurement of proteins using bicinchoninic acid. Analytical Biochemistry195(1): 14–17.
Solomon EI, Sundaram UM, Machonkin, TE (1996)Multicopper oxidases and oxygenases. Chemical Reviews 96: 2563–2605.
Soleas GJ, Diamandis EP, Goldberg DM (1997)Wine as a biological fluid: history, production, and role in disease prevention. J Clin Lab Anal 11:287–313.
Tapia A, Rodriguez J, Theoduloz C, Lopez S, Feresin GE, Schmeda-Hirschmann G (2004) Free radical scavengers and antioxidants from Baccharisgrisebachii. Journal of Ethnopharmacology 95 : 155–161.
Tadesse A, Hymete A, Bekhit AA, Hailu A (2017) In vitro activity of wushwush green tea extracts against

Leishmania major promastigotes. International Journal of Chemical & Pharmaceutical Analysis 4: 1-6.
Tadesse A, Ariaya H, Adnan AB, Salahuddin FM (2015) Quantification of total polyphenols, catechin, caffeine, L-theanine, determination of antioxidant activity and effect on antileishmanial drugs of ethiopian tea leaves extracts. Phcog Res 7: S7-14.
Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140: 19–26.
Tiuman T, Santos A, Nakamura T, Nakamura C. (2011) Recent advances in leishmaniasis treatment.International Journal of infection disease. 15(3):111-118.
Van Hoorn DEC, Nijveldt RJ, Van Leeuwen PAM, Hofman Z,Rabet LM, De Bont DBA, Norren KV (2002) Accurate prediction of xanthine oxidase inhibition based on the structure of flavonoids European. Journal of Pharmacology 451: 111-118.
Viana SM, Ferreira MAD, Guerra PV, Viana GSB, Teixeira MJ (2015) In vitro and in vivo evaluation of quinones from AuxemmaoncocalyxTaub. on Leishmaniabraziliensis. Journal of Medicinal Plants Research 9(5) : 132-139.
Whitaker JR (1995) Polyphenol oxidase. Food Enzymes Structure and Mechanism: 271–307.
Whitaker JR (1994) Principle of Enzymology for the Food Science. New York: Marcel Dekker : 271–556. drug discovery for Leishmania. Journal of Microbiological Methods55 : 813– 816.
Williams C, Espinosab OA, Montenegroc H, Cubillac L,Capsona TL, Barrıa EO, Romerob LI (2003) Hydrosolubleformazan XTT: its application to natural products drug discovery for Leishmania. Journal of Microbiological Methods55 : 813– 816.
WHO EMRO.WHO. [Online].; 2012 [cited 2017 NOV 29. Available from: http://www.emro.who.int/pdf/neglected-tropical-diseases/information-resources-leishmaniasis/cl-factsheet.pdf?ua=1
Wu LQ, Embree HD, Balgley BM, Smith PJ, Payne GF (2002) Utilizing renewable resources to create functional polymers: chitosan-based associative thickener. Environ. Sci. Technol 36: 3446-3454.
Yang CS, Lambert JD, Hou Z, Ju J, Lu G, Hao X (2006) Molecular target for cancer preventive activity of tea polyphenols. Mol. Carcinog. 45 : 431–535.
Yang, CS, Lee M.J, Chen L, (1999) Human salivary tea catechin levels and catechin esterase activities: implications in human cancer prevention studies. Cancer Epidemiol. Biomarkers Prev. 8: 83–89.
Yen GC, Chen HY (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity. J Agric Food Chem 43:27–32.
Zarley JH, Britigan BE, Wilson ME (1991). Hydrogen peroxide mediated toxicity for Leishmaniadonovanichagasipromastigotes: role of hydroxyl radical and protection by heat shock. J. Clin. Investig. 88(5):1511-1521.