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PHYTOCHEMICAL CHARACTERIZATION AND FREE RADICAL SCAVENGING CAPACITY OF AQUEOUS AND ETHANOLIC EXTRACTS OF *PARINARI MACROPHYLLA* SABINE (CHRYSOBALANACEAE)

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ABSTRACT

The aim of this work is to perform phytochemical screening and evaluation of the free radical scavenging capacity of aqueous and ethanolic extracts of *Parinari macrophylla* Sabine traditionally used in the management of diabetes in Senegal. Phytochemical characterization tests based on coloured and precipitation reactions revealed mainly phenolic compounds, including flavonoids, tannins and anthraquinones. Specific reagents also revealed on TLC other types of compounds such as reducing sugars, sterols and saponins. Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) were used for evaluation of free radical scavenging capacity and all results were expressed in µmol of trolox equivalent per gram of dry extract. The results of the evaluation of the free radical scavenging capacity showed a higher activity in TEAC for the aqueous barks extract (7832 ± 7µmol TE/g dry extract) and a higher activity in ORAC for the aqueous leaves extract (5961 ± 14µmol TE/g dry extract).

KEYWORDS

Parinari macrophylla Sabine, Phytochemical characterization, TLC and Free radical scavenging capacity.

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INTRODUCTON

Biochemical reactions that take place in the cells and organelles of our body are the driving force that sustained life. In the body and under normal conditions of metabolism, these reactions could lead to the production of free radicals in the mitochondria, through xanthine oxidase, peroxisomes, phagocytosis, inflammatory processes, ischemia and physical exercise. Among these radicals, reactive oxygen species (ROS) and

reactive nitrogen species (RNS) are well known to play a dual role in living beings as both deleterious and beneficial species[1]. The beneficial effects of reactive oxygen species (ROS) occur at low/moderate concentrations, e.g. in defence against infectious agents, induction of a mitogenic response and in the function of a number of cell signalling systems. The harmful effect of free radicals causing potential biological damage is known as oxidative or nitrosative stress[2,3]. It is ironic that these elements, which are essential to life (especially oxygen), have deleterious effects on the human body through these reactive species[4]. This occurs in biological systems when there is an overproduction of ROS/RNS on the one hand and a deficiency of enzymatic and non-enzymatic antioxidants on the other. Excess ROS could damage cellular lipids, proteins, or deoxyribonucleic acid (DNA) inhibiting their normal function. For this reason, oxidative stress is implicated in a number of human diseases as well as in the aging process. In addition, several studies have shown a link between oxidative stress and diabetes and its complications. Under conditions of chronic hyperglycaemia, several mechanisms could be responsible for the production of free radicals: the polyol pathway, the non-enzymatic glycosylation of proteins, the mitochondrial respiratory chain and other mechanisms such as the auto-oxidation of glucose[5], contribute significantly to free radicals production and induce oxidative stress[6-8]. Thus, alongside synthetic antidiabetic drugs, herbs play a critical role in the treatment of type 2 diabetes, particularly in developing countries where most people do not have access to modern drug therapy. The use of herbal compounds or enzyme inhibitors has also encouraged in developed countries, as some side effects associated with the use of synthetic pharmaceuticals were difficult for patients to tolerate[9]. This is the case with acarbose, which although approved by the Food and Drug Administration (FDA) and used to control postprandial glycaemia, reported to cause critical side effects, such as liver disorders[10,11]. To avoid or reduce the adverse effects of these currently used synthetic products, an alternative would be to use compounds of natural origin[12,13]. In this perspective, our study is part of a phytochemical screening of certain plants such as *Parinari macrophylla* Sabine, traditionally used in the management of diabetes in Senegal. *P. macrophylla* (syn. *Neocarya macrophylla*), also referred to as *Gingerbread plum*, is a tree native to western and central Africa belonging to the Chrysobalanaceae family. Some studies have been conducted on the fruits[14, 15], containing an edible pulp and a kernel rich in oil, but little data is available on the leaves and stem barks[16]. In the hereby study, several methods have been used to highlight the antioxidant activity of *Parinari macrophylla* Sabine extracts that would play an essential role in the prevention of complications in diabetics; phytochemical tests have been also carried out to get information about composition of this tree.

MATERIAL AND METHODS

Materials

Reagents and chemicals

All solvents used in this study were analytical grade. Hydrochloric acid and acetic anhydride were purchased from Sigma Aldrich (Steinheim, Germany), sulphuric acid and Neu's reagent from Roth (France). Methanol, formic acid, ammonium acetate and ferric chloride ($FeCl_3$) were purchased from VWR (France), toluene from Fluka (France). Ethanol and glacial acetic acid were purchased from Sigma Aldrich (St-Louis, USA). PEG-400 was purchased from Prolabo (France), chloroform from Fisher (UK). Ethyl acetate and butanol were purchased from Carlo Erba (France). 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±)-6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox), potassium iodide, potassium persulfate, 2, 2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), bismuth subnitrate, formol, α-naphtol and fluoresce in were purchased from Sigma Aldrich (Germany, USA). The phosphate buffer solution (PBS) was prepared as follows: 137mmol/L sodium chloride (NaCl), 2.7mmol/L potassium chloride (KCl), 10mmol/L di-sodium hydrogen phosphate dihydrate (Na_2HPO_4, $2H_2O$), 1.76mmol/L

potassium phosphate (KH_2PO_4) were dissolved in 1 liter of Milli-Q water.

Plant material

The leaves and barks of *Parinari macrophylla* Sabine were harvested manually in Pakour, in the Kolda region of southern Senegal in June 2014. Botanical identification was carried out by the members of the laboratory of pharmacognosy and botany of the faculty of medicine and pharmacy of Dakar. Samples were then dried at room temperature in the laboratory and reduced to powder by an AGREX hammer mill. The powder was packed in tinted plastic bags to limit the oxidation phenomena due to the light radiation prior to analyses.

METHODOLOGY

Extraction method

A quantity of 50g of sample powder (leaves or barks) was macerated in 500mL of solvent (water or ethanol) under automatic stirring for 48 hours. The operation was repeated three times. The extracts were concentrated using a rotary evaporator at 40°C to give 15.56g of aqueous leaves extract (yield 31.1%), 17.14g of ethanolic leaves extract (yield 34.3%) and 13.47g of aqueous barks extract (yield 26.9%). The extraction of barks was carried out only with water, which is the solvent used by the traditional healers.

Phytochemical characterization

Coloured and precipitation reactions

Coloured and precipitation tests were carried out in glass test tubes, on the aqueous and ethanolic extracts from the leaves and the aqueous extract from the bark of *Parinari macrophylla* Sabine. These assays were realized according to Kumar, Houta and Surmaghi works[17-19] with some readjustments to highlight the chemical families of compounds.

Alkaloids

To 0.2g of extract, 2mL of hydrochloric acid (2 M) was added followed by 2mL of Dragendorff reagent. The appearance of an orange precipitate indicated the presence of alkaloids[17].

Flavonoids

Reaction to ferric chloride ($FeCl_3$): To 2mL of extract solution, a few drops of $FeCl_3$ (2%) solution were added. The appearance of a greenish coloration indicated the presence of flavonoids.

Cyanidin reaction: to 2mL of extract solution were added 2mL of hydrochloric alcohol (ethanol 96°, water, concentrated hydrochloric acid (HCl) 2:2:1; v/v/v). A red-orange coloration turning to violet developed, confirming the flavonoids reaction[19].

Tannins

Stiasny's reaction: in the presence of Stiasny's reagent, condensed tannins precipitate. 0.2g dry substance was added to 3mL ammonium acetate (5 M) and 3 to 4 drops of $FeCl_3$ (2%) solution. A blue-black coloration developed, confirming the presence of gallic tannins[19].

Bate-Smith reaction: 0.2g of dry extract was placed in the presence of concentred hydrochloric acid (1mL) and boiled for 5 minutes. The appearance of a brick-red colour indicated the presence of catechic tannins[19].

Anthraquinones

0.2g extract was mixed with 3mL chloroform in a dry test tube and shaken for 5 minutes. Three mL of ammonia solution (10%) were added and stirred. A pink-purple or red colour in the ammonia layer indicated the presence of anthraquinones[20].

Saponins

Approximately 0.2g of the extract was vigorously agitated in the presence of 3mL of distilled water in a test tube and heated to boiling. Foam development could be taken as preliminary evidence of the presence of saponins[20].

Cardiotonicheterosides

They were highlighted by the Keller-Kiliani reaction. For this, 0.2g of extract was dissolved in 1mL of glacial acetic acid containing 1 drop of ferric chloride solution. This mixture was then covered with 1mL of concentrated sulfuric acid. A brown ring obtained at the interface could indicate the presence of a deoxyribose encountered in cardiotonic glycosides[17,20].

Steroids

Two mL of acetic anhydride was added to 2mL of plant extract with 2mL of concentrated sulphuric

acid. The colour changed from purple to blue or green in some samples indicating the presence of steroids[20].

Triterpenoids

Three mL of each extract was added to 2mL of chloroform and 3mL of concentrated sulphuric acid to form a monolayer of reddish-brown coloration of the interface (water-chloroform) showing the presence of triterpenoids[17].

Reducing sugars

To 2mL extract, 2 drops of Molisch reagent were added and stirred. An addition of 2mL of concentrated sulphuric acid to the sides of the test tube resulted in the development of a reddish-violet ring at the junction of the two layers (water-Molisch reagent) indicating the presence of carbohydrates[20].

Planar chromatography

Thin Layer chromatography (TLC) is a complementary method also used for the qualitative analysis of complex mixtures such as plant extracts. Samples were prepared at a concentration of 10mg/mL in a water-methanol mixture (50:50; v/v) and then filtered at 0.45µm. The deposit volume was 10µL. The TLC plates used were based on silica gel (TLC silica gel 60 F254, Merck), fixed on an aluminium support. Ethyl acetate/ methanol/ water/ formic acid (50:4:4:2.5 v/v/v/v) system was used for compounds separation. Specific reagents were used for the research of characteristic structural function. Flavonoids and polyphenols were revealed by NEU, PEG-400 and NEU/PEG reagents at 366nm, sterols and triterpenoids by Liebermann reaction at 366nm, reducing sugars by Molisch reagent.

Evaluation of free radical scavenging capacity

Trolox equivalent antioxidant capacity (TEAC)

For this purpose, 10µL of each extract were deposited in each well of a 96-well microplate and 200µL of $ABTS^{\bullet+}$ at 7mmol/L in PBS buffer solution were added. After 10 minutes of incubation at 37°C, the absorbance was measured at 734nm in a Vario Skan spectrophotometer (Thermo Fisher Scientific). Experiments were carried out in triplicate.

Oxygen radical absorbance capacity (ORAC)

A volume of 10µL of each diluted extract was deposited in a black microplate well followed by 150µL of fluorescein at $8.5.10^{-8}$mol/L in Milli-Q water. After a 10 minutes incubation at 37°C, AAPH at 153.10^{-3}mol/L in PBS buffer solution was automatically distributed to the microplate wells[21-23]. Fluorescence kinetics were then monitored every 5 minutes during 120 minutes using a Vario Skan spectrophotometer with excitation and emission wavelengths at 485nm and 530nm, respectively. Experiments were carried out in triplicate. All results were expressed in µmol of trolox equivalent per gram of dry extract (µmol TE/g of dry extract).

RESULTS AND DISCUSSION

Phytochemical characterization

The results of the phytochemical tests using coloured or precipitation reactions, either in test tubes or using TLC, are summarized in Table No.1.

[a]: The + and - signs indicate the intensity of coloration/precipitation.

These were qualitative tests allowing the identification of basic skeletons characteristic of the main classes of phytochemical compounds. Depending on the test and the family of compound to be highlighted, different colours or stains were observed with varying intensities depending on its content in the analysed sample, providing therefore semi-quantitative data. The use of water or ethanol does not seem to significantly affect the overall qualitative composition of the leaves extracts. According to these results, the extracts of leaves and bark of *Parinari macrophylla* Sabine are essentially rich in phenolic compounds including flavonoids, tannins and anthraquinones. Other types of compounds were also revealed averagely such as reducing sugars, saponins, cardiotonicheterosides, alkaloids, sterols and triterpenes. A study conducted on *Parinari macrophylla* seeds oil showed predominantly alkaloids, saponins, terpenoids and sterols[24].

Evaluation of free radical scavenging capacity

The TEAC and ORAC methods were used to evaluate the free radical scavenging capacity and quantification was performed by running a

calibration range with Trolox. These methods allow the evaluation of the free radical scavenging capacity based essentially on proton or electron donors/acceptors mechanisms. For TEAC method, the radical $ABTS^{\bullet+}$ cation has absorption maxima at wave lengths of 412, 645, 734 and 815nm[25]. In the presence of antioxidant compounds, the free radical $ABTS^{\bullet+}$ is captured, resulting in colour loss and thus a decrease in absorbance measured quantitatively and related to antioxidant concentration[26,27]. As for the ORAC test, it is based on the oxidation of a fluorescent probe (fluoresce in) by free radicals, which are often peroxyl radicals, but can also be hydroxyl radicals. These free radicals are provided by a radical generator (AAPH)[28,29]. During the experiment, the free radicals damage the probe and thus reduce the intensity of the fluorescence. The degree of change in intensity reflected the amount of damage caused by free radicals. The addition of an antioxidant allows the free radicals to be absorbed, reducing the amount of damage received by the probe and prolonging its fluorescence. To quantify the protection conferred by an antioxidant, a measure of the area under the curve of the sample is performed and compared to the area under the curve of trolox used as the reference antioxidant[30]. Results of free radical scavenging capacity using TEAC and ORAC methods of the aqueous and ethanolic extracts are presented in Figure No.1.

EtOHL: ethanolic extract from leaves; AqL: aqueous extract from leaves; AqB: aqueous extract from bark.

Differences in response were noted between the TEAC and ORAC methods. This could be explained by the difference in the mechanism of action between the two methods. Indeed, the TEAC was direct method and involved an electronic transfer whereas the ORAC was indirect method and involved a proton transfer. Similar tendency were obtained from berry study[31]. However, the ORAC index, although used until now for comparative studies, was invalidated in 2012 by the United States Department of Agriculture on the grounds that data obtained *in vitro* by this method can hardly be extrapolated *in vivo*, especially for polyphenols. According to the results, the aqueous and ethanolic extracts of the leaves showed very similar free radical scavenging capacity values (7459.50 and 6599.34µmol TE/g dry extract in TEAC; 5961.40; 5592.77µmol TE/g dry extract in ORAC). This trend in the results obtained for the two solvents would support the use of water as a solvent in the image of traditional therapeutics and for a green method. These values are much higher than those obtained with the fruits of this plant because a study carried out in Niger had shown a free radical scavenging capacity equal to 3.9µmol TE/g dry weight[32]. This could be due to the high content of phenolic compounds in aerial parts compared to fruits. Indeed, phenolic compounds revealed in the leaves and barks could constitute a potential pathway for the use of the plant in the management of diabetes mellitus. Generally, polyphenols, because of their chemical structure with conjugated double bonds and hydroxyl functions, could trap radical species and chelate the transition metals (Fe^{2+} and Cu^{2+}), which were oxidation catalysts. It had therefore been suggested that they reduced oxidative stress, which could create molecular and cellular damage and induce various pathologies (cancers, type 2 diabetes, cardiovascular and neurodegenerative diseases). They were also able to reduce other cardiovascular risk factors involved in the metabolic syndrome (hyperglycemia, hyperlipidemia, insulin resistance and hypertension)[33-36]. In this study, given the small difference in qualitative composition and total antioxidant capacity between leaves and barks extracts, it would make sense to encourage the use of leaves to preserve the plant. Indeed, the abusive use of the plant's bark could lead to its destruction in the Senegalese ecosystem. In this case, reforestation campaigns of *Parinari macrophylla* could be encouraged.

Table No.1: Intensity of coloured and precipitation reactions highlighting chemical families[a]

S.No	Contents	Aqueous extract of leaves (AqL)	Ethanolic extract of leaves (EtOHL)	Aqueous extract of bark (AqB)
1	Alkaloids	+	+	+
2	Flavonoids	+++	+++	++
		+	+	++
3	Tannins	+++	+++	+++
		-	+	+
4	Anthraquinones	+++	+++	+++
5	Cardiotonic heterosides	+	+	+
6	Saponins	++	++	++
7	Steroids	++	++	++
8	Triterpenoids	++	++	+

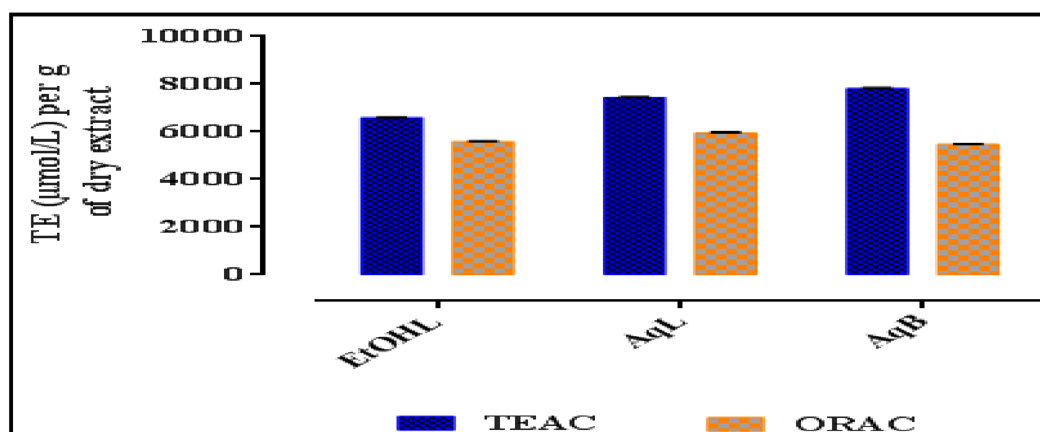

Figure No.1: Free radical scavenging capacity of extracts (µmol TE/g of dry extracts)

CONCLUSION

This study highlighted the phytochemical composition and free radical scavenging capacity of the leaves and barks of *Parinari macrophylla* Sabine. Leaves and barks showed similar qualitative composition and free radical scavenging capacities that could be beneficial to human health. Now that phytochemical characterization tests have revealed a majority of phenolic compounds, identification methods should be implemented to determine the nature of molecules responsible for free radical scavenging capacity.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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