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Research Article

THE *IN-VITRO* ANTIOXIDANT PROPERTIES OF TANNINS ISOLATED FROM THE FIBERS OF *CHAMAEROPS HUMILIS* L.

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Abstract

The purpose of this study was to evaluate the antioxidant activity of tannins extract isolated from the fibers of *Chamaerops humilis* L. by three complementary test systems (bioautography HPTLC, DPPH free radical scavenging and ferric reducing antioxidant power FRAP). High performance thin layer chromatography (HPTLC) screening provided qualitatively the antioxidant effect of extract under study. Furthermore, it was found that the tannins had a potent DPPH scavenging potency with IC_{50} value of 0.41mg/mL. Besides, the kinetic behaviour of DPPH radical scavenging activity of extract under investigation allowed us to determine the half life $t^{1/2}$, time reaction (t) and the remaining $DPPH^{\bullet}$ percent. It was also noted that our extract exhibited higher reducing potential which depends upon extract concentration.

Keywords: *Chamaerops humilis*; fibers, tannins; antioxidant activity; kinetic.

Introduction

In the past years, attention has been given to phenolic compounds because they can scavenge or prevent the generation of free radicals. Tannins are the most abundant antioxidants in the human diet and they exhibit many biologically important functions which include protection against oxidative stress, degenerative diseases and typical tanning effect which is important for the treatment of inflamed or ulcerated tissues (Parekh and Chanda, 2007). Plants that have tannins are used for treating intestinal disorders such as diarrhea and dysentery (Dharmananda, 2003). These properties therefore support the use of *C. humilis* in herbal cure remedies. Li and Wang (2003) reviewed the bio-logical activities of tannins and observed that they have antioxidant activity and can be used in cancer prevention, thus suggesting that *C. humilis* has potential as a source of important bioactive anticancer drugs.

Chamaerops humilis has been used in Algerian folk medicine for over many years and has a wide number of traditional uses. It belongs to the Arecaceae family and it is frequently found in the north of Africa especially Mediterranean area (Deysson G., 1979; Dransfield J., 1995; Tuley P., 1995). It occupies also a prime position among the Tlemcen flora. This plant has a long history of folk use in population medical practices for the treatment of diabetes, digestive disorders, spasm, toning and gastrointestinal disorders diseases (Gaamoussi F., 2010; Bnouham M., 2002). Several studies have been shown the beneficial effects of *C. humilis* against chronically diseases such as cancer, ulcer and kidney

stones (Passalacqua N.G., 2006; Dellile L., 2007; Rammal H., 2009).

In view of the considerable importance of the tannins, the present work is aimed for testing these phenolic compounds isolated from the *C. humilis* fibers for their free radical scavenging activity by using different radical scavenge methods.

Materials and Methods

Plant material

The fibers of *Chamaerops humilis* L. were collected from the mountains of Tlemcen Department (Western Algeria) in august 2012. A voucher specimen of the plant was identified and authenticated at the laboratory of botany at the biology Institute. The fibers were ground by an electrical mill mesh and powdered part was stored in a nylon bag in a deep freeze until the time of use (Figure 1).

a

b

c

Figure 1: *Chamaerops humilis*: **a**- whole plant, **b**- heart-fibers-coated, **c**- fibers

Preparation of the tannin extract

A total of 105 g of defatted powder of fibers was contacted with 250 mL of water and 160 mL of acetone in a 500 mL capped flask with timely shaking and stirring for 4 days at room temperature (maceration). After filtrating, and evaporating of the acetone, the aqueous layer was extracted respectively with dichloromethane (2x50 mL) and 4x50 mL with diethyl acetate. The organic layer (AcOEt) was dried over Na_2SO_4, filtered and concentrated to dryness to give crude extract of tannins as a brownish solid (Lin et al., 2006).

Preparation of the methanolic crude extract

About 20 g of powdered fibers material was macerated with 80 mL of methanol for 24 h. The methanol extract was filtered using whatman filter paper and then the methanol was completely removed by vacuum evaporator at 50°C to give viscous mass. The crude extract was weighed and stored at 4°C prior to antioxidant tests.

DPPH free radical scavenging assay

Basically, the DPPH method consists in measuring the ability of a molecule to reduce the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) in methanol and its subsequent bleaching at 517 nm. Briefly, 100 µL of various concentrations of the tannins extract in methanol was added to 1.9 mL of a methanol solution of DPPH (0.004%). The mixture was vigorously shaken and then allowed to stand at room temperature for 30 min in the dark.

The radical scavenging activity of the tannins extract and vitamin C against DPPH was performed spectrometrically using UV-1700 Pharma Spec SHIMADZU spectrophotometer at 517nm and 20°C, as described by Brand-Williams (1995).

A mixture of 100 µL of methanol and 1.9 mL of DPPH solution was used as the control. The scavenging activity was estimated on the percentage of DPPH radical scavenged as the following equation: (Blois, 1958).

$$\%DPPH\ quenched = 100.[(A_{Blank} - A_{Sample}) / A_{Blank}]$$

The commercial known antioxidant, ascorbic acid was used for comparison or as a positive control.

The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted

against extract concentration (0.5; 0.25; 0.125; 0.0625; 0.0312; 0.0156; 0.0078 mg/mL).

High performance thin layer chromatography (HPTLC) assay

Tannins extract isolated from the fibers of *C. humilis* was subjected to high performance thin-layer chromatography (HPTLC) on a silica gel plate (20x20 cm, Silica gel F254, Merck). The solvent system optimized for crude extracts of *C. humilis* was methanol and Chloroform (10: 90 v/v). The tannins crude extract was loaded on a TLC silica gel plate and the plate was developed in a sandwich TLC chamber to a distance of 70 mm. After 15 min air-drying, the plate was sprayed with 0.004% (w/v) DPPH reagent prepared in methanol and 10% (v/v) sulphuric acid, respectively.

The spots on the plates were observed after the plate had been heated at 60°C for 30 min exactly after spraying (Subramanion et al., 2011).

Ferric reducing antioxidant power (FRAP) assay

The reducing power of tannins extract and ascorbic acid was evaluated according to the method of Oyaizu et al. (1986). Different concentrations of tannins crude extract and ascorbic acid (1; 2; 3; 4; 5 mg/mL) in 1mL of distilled water were mixed with 2.5ml of phosphate buffer (200mM, pH 6.6) and 2.5mL of 1% potassium ferricyanide separately and undergoes vortex. The mixture was made homogeneous and incubated at 50º C for 20 min; aliquots of trichloroacetic acid (2.5 mL, 10%) were added to the mixture, which was then centrifuged at 1500 rpm for 10 min (2.5 mL) and finally freshly

prepared $FeCl_3$ solution 3 (0.5mL, 1%) was added to this and mixed uniformly. The absorbance of supernatant was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power (Oyaizu et al., 1986; Kekuda et al., 2010). The ascorbic acid was taken as positive control.

Kinetic analysis

Fitting of the experimental data was carried out by using the Levenberg–Marquardt method (Marquardt, 1963) implemented in Origin v. 6.0 programme for Windows. Recording of spectrophotometric data was taken until the disappearance of $DPPH^{\bullet}$ in the presence of the tannins crude extract occurred.

DPPH radical: DPPH was obtained from Fluka (Buchs, Switzerland). 1 mg DPPH was solved in 25 mL methanol (mother solution (1) 0.04%). This stock solution was used for the measurements, and kept in the dark at ambient temperature when not used.

On the other hand, 5 mg of the studied extract was solved in 4 mL methanol (mother solution (2) 1.25 mg/mL). From this solution, different concentrations were prepared (0.6250; 0.3125; 0. 1562; 0.0780 mg/mL) and used for kinetic behaviour.

To fellow the kinetic behaviour of $DPPH^{\bullet}$ radical scavenging activity of extract under study, 1.5 mL of solution (2) was mixed with 1.5 mL of solution (1), the absorbance was measured after each 30s until it becomes constant.

Results and Discussion

Recently, interest in drugs of herbal origin has significantly increased due to less harmful side effects of natural products and the fact that plants are easily accessible. Scientific research is being conducted all over the world to determine whether plants that are traditionally used to treat various diseases are actually appropriate for their intended use.

Screening and extraction of tannins

The preliminary phytochemical screening tests may be useful in the detection of the bioactive phytochemicals and subsequently may lead to the drug discovery and development (Gami B et al., 2010).

The Phytochemical screening in the present study has revealed the presence of tannins in the fibers of *C. humilis*. The detection test gave a positive reaction with $FeCl_3$ (1%) expressed by blue-greenish coloration.

In this study, 70% acetone (v/v) was used to derive crude tannins extract from *C. humilis* fibers. Djipa et al. (2000) reported that the mixture of acetone-water was believed to be a better solvent for tannin extraction in comparison with other extractants. The isolation experience of tannins gave 0.35% yield.

Determination of free radical scavenging activity by HPTLC and DPPH methods

Antioxidants are known to eliminate oxidative stress by scavenging free radicals and protect biological macromolecules from their toxic effect (Chander R et al., 2005). Therefore, in recent years evaluating plant antioxidant activity and their free radical quenching ability is considered to be an important task in pharmacological studies.

Using the HPTLC bioautography technique, we observe on the TLC plate, the appearance of zones of antiradicalaire activity of pale yellow colour on purple bottom (Molyneux, 2004) for the tannins extract as for the ascorbic acid.

Yellow spots were observed after spraying the plates with DPPH solution indicating, the presence of antioxidant compounds in the extract of tannins.

In conclusion, the bioautography HPTLC method revealed that tannins possessed antioxidant effect.

In the next step of our work, the antioxidant property of the tannins extract was determined and compared to the reference ascorbic acid (Vitamin C), and are illustrated in figure 2.

The scavenging effects of extract increased with their concentrations. Tannins extract (73%) showed potent DPPH radical scavenging activity (Figure 2b) at the concentration of 1mg/mL but lower to standard ascorbic acid (Figure 2a, 93% at concentration of 0.25 mg/mL) . Statistical analysis shows a strong positive correlation, (R^2=0.993) between concentration of studied

samples and antioxidant potential. Besides, both tannins extract and ascorbic acid exhibited high scavenging activity toward DPPH with the IC_{50} values (the amount of antioxidant material required to scavenge 50% of free radical in the assay system) of 0.408 and 0.107 mg/mL, respectively.

Antioxidant Activity of Tannins Extract by Fe Reducing Power Assay (FRAP)

The reducing potential of the tannins crude extract was determined by the ferric reducing antioxidant power (FRAP) method and the results are depicted in figure 3.

The reducing ability of the extracts was evaluated on the basis of absorbance increasing where the curve of FRAP essay of tannins was plotted as absorbance versus different concentrations (Figure 3). As seen in figure 3, the trend for ferric ion reducing activity of tannins extract isolated from C*. humilis* fibers showed the increased absorbance, due to the reducing of Fe^{3+} to Fe^{2+} ions with increasing concentration. The absorbance of the reaction system was highly correlated with the concentration of extract (R^2 = 0.97), and the higher slope of the line indicated higher reducing power of the tested phytochemical. The reducing capacity of the studied extract may serve as significant indicator of its potential antioxidant activity (Bhalodi M et al., 2008; Meir et al., 1995). Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent. Generally, antioxidants are compounds capable of donating a single electron or hydrogen atom for reduction (Schafer F et al., 2001).

Kinetic analysis

Kinetic studies of DPPH-extract reaction were carried out to estimate the scavenging activity as a function of time. Our work aimed also to study the concentration effect of tannin extract on scavenging reaction of the free radical $DPPH^{\bullet}$. In fact, different concentrations were tested (0.625; 0.312; 0.156 and 0.078 mg/mL). Starting our experience, the calibration curve has been established in order to determine the % remnant $DPPH^{\bullet}$ radical after the scavenging reaction. The results of absorbance versus concentration are given in figure 4. The calibration equation for DPPH was y = 0.0095x + 0.0508. Strong correlation was observed between absorbance and DPPH concentrations (R^2= 0.9926).

The free radical scavenging activity of each solution was calculated as percent remnant $DPPH^{\bullet}$ according to the following equation:

$$\% \text{ Remnant } DPPH^{\bullet} = ([DPPH^{\bullet}]_{t=T} / [DPPH^{\bullet}]_{t=0}) \times 100$$

where $[DPPH^{\bullet}]_{t=T}$ was the concentration of DPPH at steady state and $[DPPH^{\bullet}]_{t=0}$ was the initial concentration of $DPPH^{\bullet}$. Table 1 summarized the time reaction at steady state, half life ($t_{1/2}$) and the percentage of remnant $DPPH^{\bullet}$.

Based on the time required for the reaction to reach steady state, one reaction kinetic type (rapid and very fast) were found. At concentrations of 0.078; 0.156 and 0.312mg/mL of the tannins extract, a steady state was attained even after 12min; 7min.30s and 6min.30s of reaction, respectively. Thus, the investigated extract is classified as showing rapid behaviour.

At each time, it was possible to compare the antioxidant activity of extract. For example, in the presence of 0.625mg of studied sample per mg DPPH radicals, after 4min 30s, when the steady state was almost reached, about 10% of the initial DPPH radicals remained in the medium. At this concentration, the tannins extract is classified as showing very fast behaviour.

The findings given in Table 1 showed clearly that the tannin extract exhibited a significant antioxidant effect where the times of reaction at steady state were 12; 7.30; 6.30 and 4.30 for the studied sample at concentration of 0.078; 0.156; 0.312; 0.625mg/mL, respectively. The effectiveness of tannins was expressed *via* the values of half life $t^{1/2}$ which were

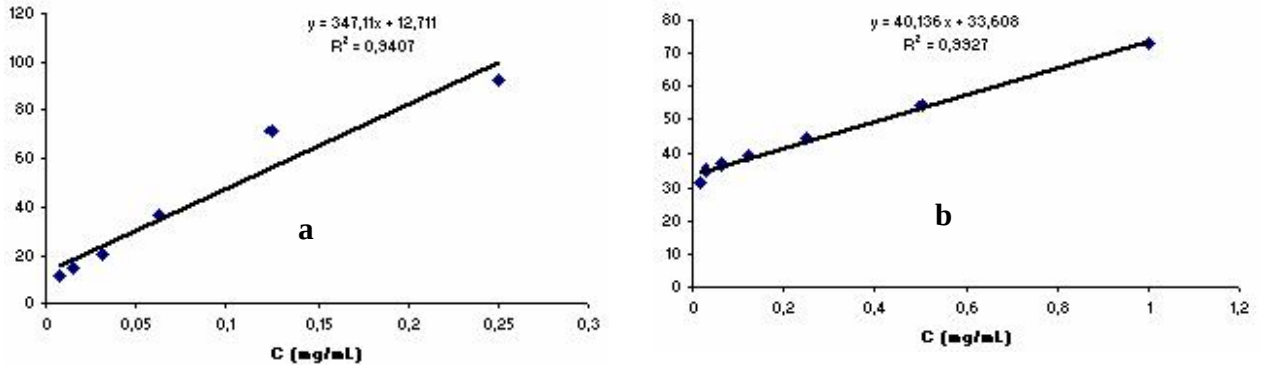

Figure 2: Percentage inhibition of DPPH versus concentration of (**a**: ascorbic acid, **b**: tannins extract)

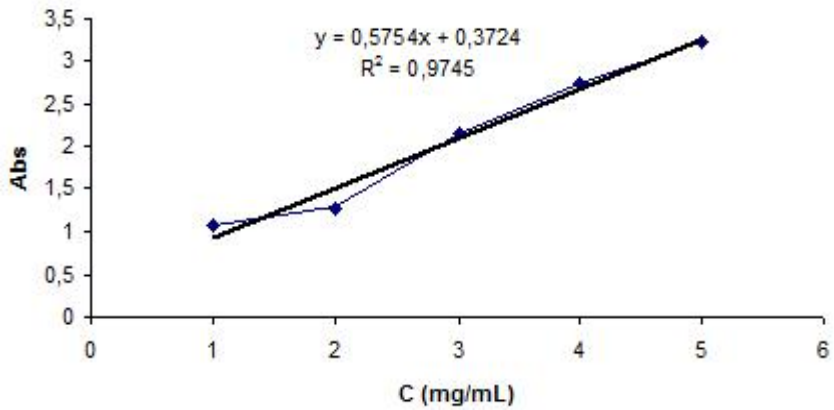

Figure 3: FRAP assay of tannins extract

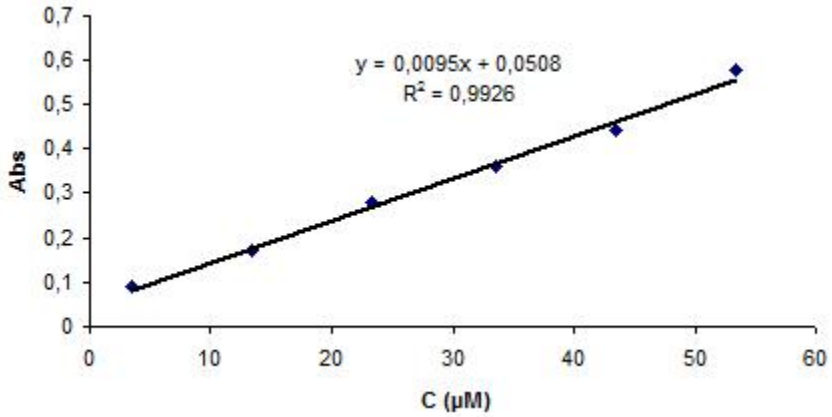

Figure 4: Calibration curve of DPPH

Table 1: Kinetic data of RSA of tannins extract at different concentrations.

Extract	C (mg/mL)	t= T(min)	$t^{1/2}$ (min)	$(DPPH)_R$ (%)
Tannins	0.078	12	3	16.13
	0.156	7.5	2.5	13.05
	0.312	6.5	2	11.02
	0.625	4.5	1	10.00

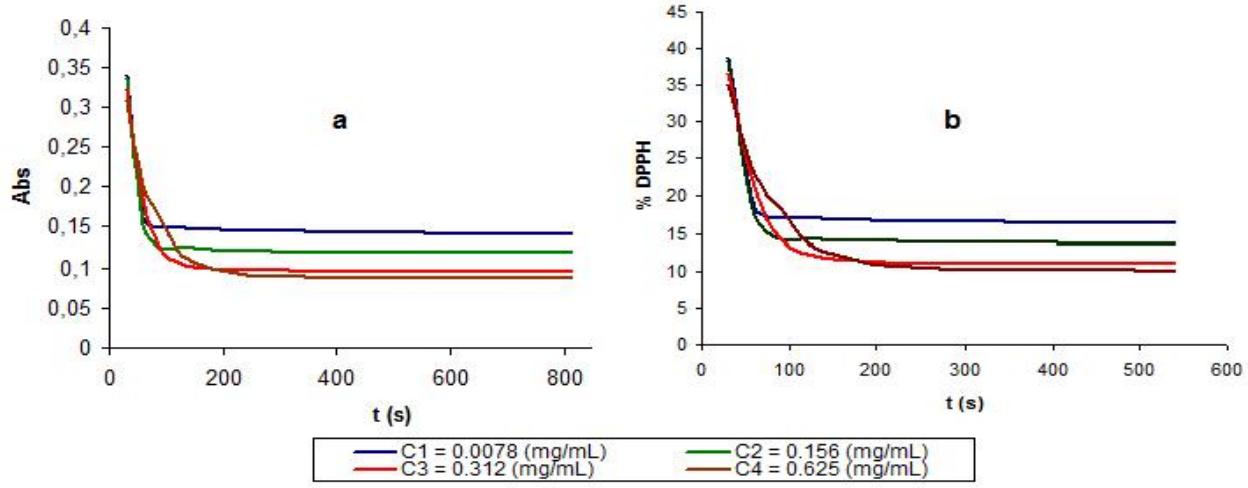

Figure 5: Kinetic behaviour of tannins extract with DPPH• radical
(**a**: Abs= f (t), **b**: % DPPH remained = f (t)).

shorter (1; 2; 2.5 and 3 min) and also by the lower content of remnant (%) DPPH• at the steady state (16.13; 13.05; 11.02 and 10 %).

The observed decrease in percentage of DPPH radical is caused by antioxidant through the reaction between antioxidant molecule and radical results in the scavenging of the radical by hydrogen donation (Brand-Williams W et al., 1995).

For more details, figure 5 illustrated the kinetic profile of DPPH• annihilation by the studied extract. These figures showed especially the absorbance and %DPPH remained as a function of time.

As we can see in table 1 and fig.5, the % of remaining DPPH decreased with increased tannins concentration to a certain point then leveled off. The value of Absorbance (t=x) refers to the content of DPPH scavenged at t = x. In the presence of tannins crude extract a rapid initial decrease of DPPH content is followed by slow subsequent disappearance of DPPH. Tannins can quench free radicals by two major mechanisms: by reduction via electron transfer or by hydrogen atom transfer that may also occur in parallel (Huang et al., 2005). Furthermore, in case of rapid kinetic behaviour, tannins at high concentration reacted within a very short time, and a steady state was reached almost immediately. In contrast, at lowest concentration, a slow kinetic behaviour was observed (Table 1). These results are in harmony with those obtained by Sladjana et al. (2012).

Conclusion

The results of the present study show that the tannins extract of *C. humilis* L. exhibited a significant antioxidant effect. Therefore, this study confirms that the fibers of

the plant under investigation play an important role as a potential source of natural antioxidants. Additional works such as chromatographic separations and spectral analysis of these phytochemicals have to be performed if they are to be used for medicinal purposes.

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