Research Journal of Chemical and Environmental Sciences
Res J. Chem. Environ. Sci. Vol 6 [3] June 2018: 86-91
Online ISSN 2321-1040
CODEN: RJCEA2 [USA]

Website: www.aelsindia.com/rjces.htm

RJCES

ORIGINAL ARTICLE

Phytochemical analysis, DPPH antioxidant activity and Acute toxicity of bark aqueous extracts of *Pinus halepensis*

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ABSTRACT
The objectives of the present study were to evaluate the phytochemical analysis and DPPH antioxidant activity of Pinus halepensis Qualitative analysis of phytochemicals (flavonoid, terpenoids, alkaloid, saponins, phenol and carbohydrate) and quantitative analysis of total phenolics and flavonoids were prepared by using standard protocols. Antioxidant activity was studied done DPPH assay. The acute toxicity test was applied in Wistar albino rats. Qualitative phytotochemical analysis revealed that the aqueous extract show richness in flavonoids, terpenoids, saponins, phenols and carbohydrates and poor in alkaloids. Total phenol and flavonoid content shows highest concentration in aqueous extract of P. halepensis (34.10mg GA EQ/gm, 3.27mg QEQ/gm). IC50 values of the plant was 34.92µg/ml in P. pinaster. In this study, the toxicity test showed no mortality or behavioral change up to 5000 mg / kg of albino Wistar rats. The results conclude that Pinus halepensis contains antioxidant compounds which protects cells against degenerative effects of Reactive Oxygen Species (ROS).
Keywords: *Pinus halepensis, Antioxidant, acute toxicity, rats*

Received 21.03.2018 Accepted 13.05.2018

INTRODUCTION

Plants possess extraordinary therapeutic virtues. Their uses for the treatment of several diseases in living beings and in particular man is very old [1]. Oye Gureje [2] defines traditional medicine as the sum of knowledge, skills and practices based on theories, beliefs and experiences of different cultures, which are used to prevent, diagnose, alleviate or treat At present, more than 80% of the world's population, especially in underdeveloped countries, use traditional treatments to meet their health and primary care needs [3]. Medicinal plants are used throughout the world in the treatment of cardiovascular pathologies, diabetes and high blood pressure. Some studies have shown that many plants are used in traditional medicine for their so-called hypoglycemic, lipid-lowering activities. and antioxidant [4]. Many herbal plants contains antioxidant compounds which protects cells against degenerative effects of Reactive Oxygen Species (ROS) which is a free radical such as singlet oxygen, superoxide, peroxyl, radicals, hydroxyl radicals [5]. Oxidative stress is defined as an imbalance in the balance between antioxidants and pro-oxidants in favor of antioxidants Antioxidants play a major role in protecting against molecular oxidative damage [6]. Plants are potential source of natural antioxidants. Natural antioxidants or phytochemicals such as flavonoids, phenolic acids, alkaloids, lignins, stilbenes, and tannins are well known free radical scavengers and possess multiple biological activities including anti-oxidant activity [7]. The synthetic antioxidants were create the genotoxic effect [8] and other chronic diseases [9]. So the aim of the present study is to evaluate the DPPH antioxidant activity and phytochemical analysis of plants .

MATERIALS AND METHODS

Chemicals and reagents

All chemicals used were of analytical grade and purchased from Sigma-Aldrich, Mo, USA.

Collection, identification and extraction of plant material

bark of Pinus halepensis was collected in herbalists shops from a local market of El-Oued and were identified by a botanist at the herbarium in the Department of biology, the University of El Oued, Algeria. The plant material was washed using distilled water and then drying in room temperature for 48 to 96 h and then grounded into powder and stored at room temperature until use. The extraction methods

described by Mamta and Parminder [10]. After extraction, the solvents were removed using rotary evaporator, to get gel-like extracts. . The powder was weighed and dissolved in water and stored in a refrigerator at 4 °C for further analysis.

Phytochemical Screening: The methods of Mamta and Parminder (2013) [10] were used to identify the phytochemicals provides in the extracts: alkaloids, saponins, tannins, steroids, flavonoids, terpenoids and glycosides.

Estimation of Total Phenol

The polyphenols are determined by the Folin-Ciocalteu method. This method, initially described by Slinkard and Singleton [11], makes it possible to know the total polyphenolic content of a given sample. The sample of the aqueous extract of the P. halepensis (0.5 ml) and 2 ml of sodium carbonate (75 g / l) were added to 2.5 ml of 10% (v / v) Folin- Ciocalteau with gallic acid as standard. After 30 min of reaction at room temperature, the absorbance was measured at 765 nm. The tests were carried out three times in order to ensure the reproducibility of the results. The total phenolic content was expressed in mg Equivalent of Gallic Acid per gram of sample.

Estimation of Total Flavonoids

Determination of the total flavonoid content of the aqueous extract of the P. halepensis is carried out by the method described by Ahn et al. [12]. 0.5 ml of a 2% AlCl3-ethanol solution was added to 0.5 ml of sample or standard. After 1 h at room temperature, the absorbance was measured at 420 nm. Quercetin was used as a standard for plotting the calibration curve. The tests were carried out three times in order to ensure the reproducibility of the results. The results were expressed in milligram equivalent Quercetin per gram of sample.

***In vitro* Antioxidant activity Assay**

The in vitro antioxidant activity was evaluated by measuring the scavenging power of the DPPH (1,1-diphenyl-2-picryhydrazyl) radical according to the method described by Burits and Bucar [13], where 3ml of various concentrations (5, 10, 15, 25,50, et 60µg/ml) of Pinus halepensis samples were added to 75µL of methanolic solution of DPPH (1.3mg/ml) . Absorbance measurements were read at 517 nm after 30 min of incubation time at room temperature (A1). Absorbance of a blank sample containing the same amount of methanol and DPPH solution acted as the negative control (A0). The percentage inhibition [(A0-A1/A0) × 100] was plotted against the phenol content and IC50 was determined

Acute toxicity test

The test was performed using healthy albino rats of Wistar strain weighing between 209 and 237 g. The animals were divided into three groups of three rats each and administered 0, 2000 and 5000 mg/kg of aqueous extract of P. Halpensis orally. Animals were observed after dosing at least once during the first 30 min, periodically during the first 24 h with special attention given during the first 4 h, and daily thereafter for a total of 14 consecutive days [14].

RESULTS AND DISCUSSION

Phytochemical Screening

The initial phytochemical screening results (shown in Table 1) revealed the presence of a wide range of bioactive secondary metabolites including, phenol, saponins, flavonoids, tannins and carbohydrates and the absence of other bioactive substance such as alkaloids. The secondary metabolites produced by Pinus halepensis possess several interesting biological activities, and are sources of pharmacological principles active against several pathologies [14]. Phenolic compounds such as phenols, flavonoids and tannins are considered major contributors to the antioxidant capacity of plants [15]. These antioxidant compounds could have played a major role in scavenging the reactive oxygen species [16] which interest for the prevention and treatment of various diseases including cancers, inflammatory diseases, diabetes, osteoporosis, cardiovascular and neuro-degenerative diseases [17]. Terpenoid has been widely known for its effects against tumor cells that have the ability to inhibit the growth of cancer cells [18]. Glycosides and flavonoids can inhibit tumor growth and protection against gastrointestinal infections. Saponin is a substance characterized by its surfactant properties and cholesterol binding properties [19]. The presence of each secondary metabolite in *Pinus halepensis* provides a rationale for the traditional use of these plants in the treatment of various health problems.

Table 01: Phytochemical composition and polysaccharide analysis of Aqueous extract of *Pinus halepensis* (+++ presence, --- absence)

Phytochemical	Bark Aqueous extract of *P. halepensis*
Flavonoïdes	+++
Terpenoides	+++
Phénolique	+++
Tannin	+++
Saponoside	+++
Carbohydrate	+++
Alcaloïde	- - -

Phenolic Compounds

Phenolic compounds contain hydroxyl groups (-OH) that facilitate their free radical scavenging activity and act as antioxidants, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity [20]. The Total Phenolic Compounds was expressed in terms of gallic acid equivalents (mg of GAE/gm sample) using the following equation based on the calibration curve: Y = 0.0113x +0.0686 R2 = 0.998 where x was the absorbance and Y was the mg GAE/gm sample. Total phenolic contents of *Pinus halepensis* obtained from water solvent is 33.5 mg GAE/gm (Table 02). Phenolic compounds are well known as antioxidants and directed against free radicals associated with oxidative damage. Tannin and flavonoids act on the complications of diabetes by their antioxidant and anti-enzymatic properties, neutralizing the effect of free radicals and limiting the inflammatory reaction in different tissues [21] .

Table 02: Total Phenol content

Compounds	**Total phenol content mg of GA eq/gm sample**
Aqueous extract of *Pinus halepensis*	34.10± 1.77

Total Flavonoid Content

Flavonoid shows antioxidant activity due to the presence of free -OH groups, especially 3-OH. Plant flavonoids have antioxidant activity in vitro and also act as antioxidants in vivo [22]. The Total Flavonoids Content was expressed in terms of Quercetin equivalents (mg of QE/gm sample) using the following equation based on the calibration curve: Y = 0.035x +0.288 R2 = 0.995 where x was the absorbance and Y was the mg QE/gm sample. Total flavonoid contents of *P. halepensis* obtained from water solvent is systems vary from 3.27 mg of QE/gm (Table 03). Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants [23] . The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure. The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability [24].

Table 03: Total Flavonoid contents

Compounds	**Flavonoid content mg of Quercetin eq/gm dry wt**
Aqueous extract *of Pinus halepensis*	3.27±0.49

DPPH Antioxidant Activity

DPPH, a purple-colored, stable free radical is reduced to the yellow-colored diphenylpicrylhydrazine when antioxidants are added. The antioxidant capacity of the extracts were estimated and compared with ascorbic acid (positive control) using the stable DPPH radical. The results of the experiment for antioxidant activity are shown in Fig. 01. The examination of antioxidant activity of extracts from P. halepensis showed values varied from 5% To 90% of various concentrations. Reactive Oxygen species (ROS)/ Oxidants formed in our body due to exogenous and endogenous factors are found to be responsible for many diseases [25]. Now the research is going on to reveal the potential of phytochemical antioxidants as health benefactors. This is due to their ability to neutralize the free radicals or ROS or oxidants responsible for the onset of cell damage. Flavanoid and other phenolic compounds of plant origin have been reported in scavengers and inhibitors of lipid peroxidation [26].

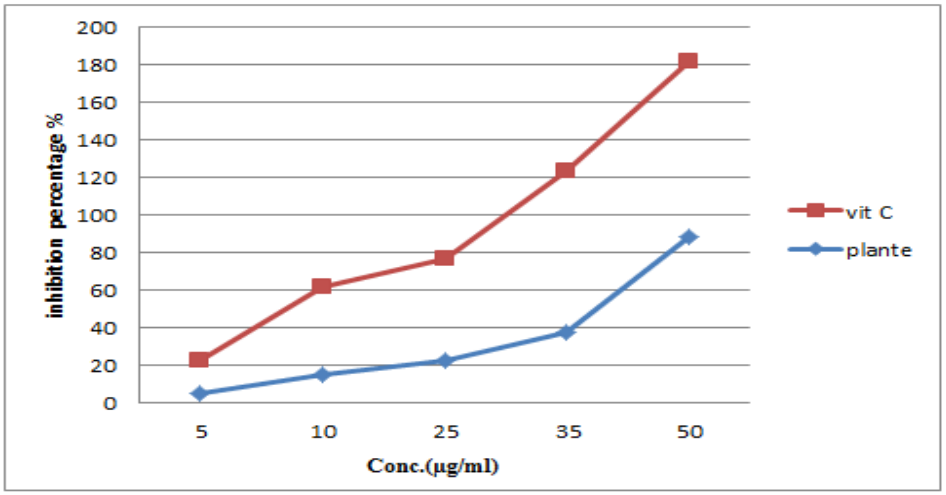

Figure 01: DPPH antioxidant activity of *Pinus halepensis*

IC50 Value

The IC50 of a compound is inversely related to its antioxidant capacity, as it expresses the quantity of antioxidant necessary to decrease the DPPH concentration by 50%, which is obtained by interpolation from a linear regression analysis [27]. Figure 02 shows the IC50 values in the DPPH radical scavenging activity assay of the extracts. It was found that the antioxidant activity in *P. halepensis* (IC50 = 34.92µg/ml). A lower IC50 indicates a higher antioxidant activity of a compound and Huns [28] .

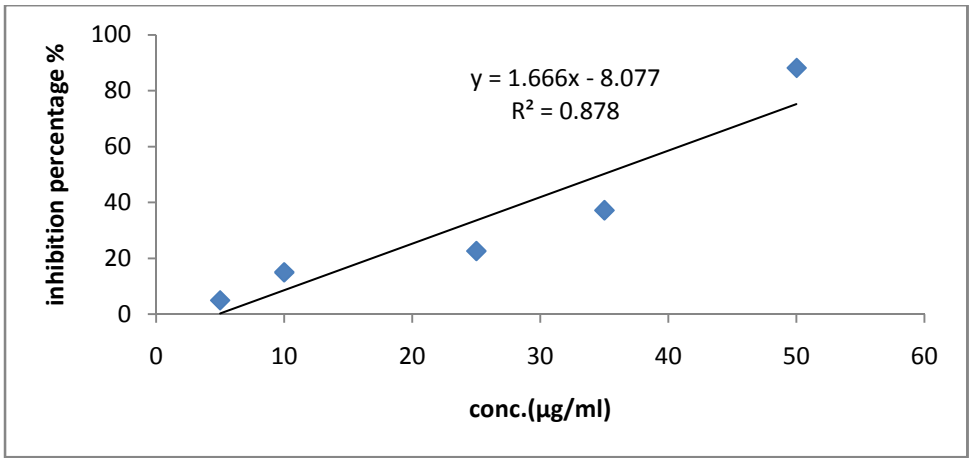

Figure 02 : IC_{50} VALUE

Acute toxicity test

In this experiment the acute toxicity test was performed on albino Wister rats for 14 days. Our plant is used with dose of 0 mg to 5000 mg per kg of weight of rats. The results obtained during this test showed that no mortality was observed before 14 days, which suggests the non-toxic nature of the aqueous extract of *P. halepensis*. The other physiological parameters of the rats were also determined during the experimental period and showed that treatment with the aqueous extract of *P. halepensis* caused no symptoms or complications and also no adverse effects in the rats during the treatment period (Table 4).

Table 4. Effect of aqueous extract of *P. halepensis* on physiological parameters of Wister albino rats.

Parameters	0 h		3 h		24 h		Day- 7		Day-14	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Dead rats	0	0	0	0	0	0	0	0	0	0
Eyes	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N	N	N	N

Test, aqueous extract of P. halepensis (2000 and 5000 mg/kg b.w rats) administered rats,
N, Normal.

CONCLUSION

Phytochemical screening of bark aquous extracts of Pinus halepensis revealed the presence phenols, flavonoids, tannins, saponins and carbohydrates by positive reaction with the respective test reagent and absence of the alkaloids substance. Results obtained in this investigation indicate that the plant extracts of P. halepensis rich in phenolics and exhibited highest antioxidant activities. The finding of this study suggest that this plant could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative diseases.

ACKNOWLEDGEMENT

The first author would like to thank the Department of Cellular and Molecular Biology, Faculty of Sciences of Nature and Life, University of El Oued, Algeria for the permission to utilize the facilities to make this work.

CONFLICT OF INTEREST

We declare that we have no conflict of interest..

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CITE THIS ARTICLE

Kaouachi Aicha and Derouiche Samir. Phytochemical analysis, DPPH antioxidant activity and Acute toxicity of bark aqueous extracts of *Pinus halepensis*. Res. J. Chem. Env. Sci. Vol 6[3] June 2018. 86-91