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Phytochemical analysis and in vitro antioxidant stress properties of methanol acetate and ethyl extracts of *Tirmania Nivea* and *Tirmania Pinoyi*

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ABSTRACT

For thousands of years, truffles have been used as essential foods in different cultures around the world because of their rich nutritional value and their pleasant and characteristic smell. We have studied the effect of truffles (*Tirmania Nivea* and *Tirmania Pinoyi*) extracts on the antioxidant stress properties issued from the Moroccan desert. Antioxidant and anti-free radical activities were studied using three analytical methods: trapping capacity of 1,1-diphenyl-2-picrylhydrazyl, phosphomolybdate, and reducing ferric antioxidant capacity; in addition, phenol and flavonoid levels were measured. The results of the FRAP, DPPH and PPM tests of T. Nivea were respectively 4.112±0.217, 0.142±0.006, 2.235±0.110 mg/mL for methanols and 3.424±0.034, 0.137±0.025, 0.858±0.010 mg/mL for ethyl acetate extracts. The results of the tests of *T. pinoyi* were respectively 3.670±0.572, 0.102±0.004, 0.907±0.014 mg/mL for methanols and 3.404±0.096, 0.080±0.003, 0.693±0.057 mg/mL for ethyl acetate extracts. For this work, we propose a valorization of the Moroccan truffle in the prevention of oxidative stress.

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INTRODUCTION

Since the beginning of civilization, desert macrofungi (truffles) have been exploited for food and medicine. The truffle belongs to the genera *Tirmania* and Terfezia of the Terfeziaceae family, order Pezizales, edible fungi, mainly endemic to the semi-arid and arid areas of North Africa and the Mediterranean region (Bouatia *et al.*, 2018).

Macrofungi have been classified as food and medicines only for the royal family. No citizen has been allowed to benefit from it. During Greek and Roman rule, these mushrooms were imported from Libya and sold in southern Europe (Enshasy *et al.*, 2013).

The collection of truffles is carried out by competent and experienced specialists for this type of flora. Sometimes, animals such as dogs and pigs are used to facilitate the discovery of underground fungi. Truffles are very rich in volatile compounds

which explains the choice of this exploration technique. For a long time, truffles are consumed raw or cooked as precious food. They have also been used for the benefit of traditional medicine. This use was due to its high fibre, fatty acids, proteins, vitamins, amino acids, minerals, terpenoids, sterols, carbohydrates, and aromatic compounds (Bokhary and Parvez, 1993; Kalač, 2009). Indeed, the natural properties attributed to truffles and their derivatives have served as a field of exploration for scientists to study their added value (Hamza *et al.*, 2016).

The metabolic mechanism in humans is capable of producing free radicals responsible for the oxidation of biomolecules such as lipids, proteins, DNA which can cause cell-death and tissue-damage. (Dubost *et al.*, 2007). It has been observed that oxidizing enzymes, as well as compounds such as ascorbic acid, tocopherols, and phenols, have a protective effect against damage caused by free radicals formed.Regular consumption of natural antioxidants can prevent and correct the imbalance of the antioxidant system caused by accelerated ageing, responsible for the development of certain diseases such as cardiovascular disease, cancer, diabetes, and other age-related diseases (Kris-Etherton *et al.*, 2002). *Tirmania* is a significant source of food for the North African population; it is found in the deserts of Morocco, where it grows spontaneously. In this study, we have sought to further our knowledge of the antioxidant activities of *T. Nivea* and *T. Pinoyi* truffles in the arid areas of Morocco. Phytochemical screening and antioxidant properties were studied for methanol and ethyl acetate extract.

MATERIALS AND METHODS

Extraction of *Tirmania* phytochemical compounds

Four grams of *Tirmania* powder were weighed and soaked separately in 15 mL of an organic solvent such as methanol and ethyl acetate. The mixture was maintained under magnetic agitation or 24 hours at room temperature for 24 hours. The soaked powder was filtered, and the raw extracts were stored at low heat for analyzing.

Phytochemical characterization

Qualitative tests for the characterization of chemical groups (secondary metabolites) were performed using extracts of methanol and ethyl acetate from *T. Nivea* and *T. pinoyi* using the standard procedures defined above.

Saponins test

1 gram of the raw extract was homogenized in 3 mL of distilled water for 15 seconds and stored at ambient temperature for 15 minutes. The presence and persistence of moss (more than 1 cm high) indicate the presence of saponins (Angone *et al.*, 2013).

Anthraquinones analysis

1 gram of extract was mixed with 2 mL of chloroform. The supernatant was collected, and 10% aqueous KOH (v/v) was added to the test tube. After stirring, the red colouration indicated the existence of anthraquinones (Angone *et al.*, 2013).

Polyphenols analysis

The reaction with ferric chloride (FeCl3) was used to reveal the presence of polyphenols. 2 mL of methanol and ethyl acetate extracts were prepared and mixed with 1 or 2 drops of ferric chloride (solubilized in 2% ethanol). The dark blue or green colour indicates the existence of polyphenols (Angone *et al.*, 2013).

Tannins analysis

5 grams of extract per material was homogenized with 10 mL of 80% methanol, stirred for 15 minutes, and the soluble fraction was transferred into test tubes. The presence of tannins was tested by adding FeCl3 (1% in water). The dark or blue colouration indicated the presence of Gallic tannins, while the brown-green colouration indicated the existence of catechetical tannins (Angone *et al.*, 2013).

Flavonoids analysis

Alkaline reagent test: truffle extracts treated with 2 or 3 drops of sodium hydroxide in solution. Formation of an intense yellow colour, which becomes colourless by adding a few drops of sulphuric acid, indicating the presence of flavonoids (Yadav *et al.*, 2017).

Anthracenosids analysis

The ether extract (4mL) was concentrated to 2mL and stirred with 2 mL of 25% ammonia solution. The appearance of a cherry red solution on the top layer indicates the presence of models (anthracenoside aglycones) in the oxidized form (Nabatanzi *et al.*, 2015).

Sterols analysis

An extract was prepared using 1 gram of powdered sample and 20 mL of macerating ether for 24 hours in a water bath. The extract obtained was used to detect sterols.

10 mL of ether macerate was dry evaporated. 1 mL of chloroform was added to the residue. The solution obtained was divided into two assay pieces. One to 2 mL of concentrated sulfuric acid was placed in the bottom of one of the assay tubes, the other serving as a control. The formation of a reddish-brown

Table 1: Qualitative phytochemical screening of *Tirmania Nivea* and *Tirmania Pinoyi*

Tests/ Species	*Tirmania Pinoyi*	*Tirmania Nivea*
Polyphenols	+++	++
Flavonoids	+++	+
Tannins	+++	++
Anthracenosids	+	+
Alkaloids	+++	-
Anthraquinons	+++	++
Saponosids	+++	+++
Sterols	+	+

Note: (+++), (++) and (+) signs indicate high presence of active ingredient, medium, and low active ingredient respectively. (-) the sign indicates the absence of an active ingredient.

Table 2: Total phenolic content and total flavonoid content of *T. Pinoyi* and *T. Nivea*

Truffles extracts	TPC (mg GAE / 100 g of DM) (Mean±SD)	TFC (mg QE / 100 g of DM) (Mean±SD)
tn methanol extract	9.90±1.50	1.60±0.40
tp methanol extract	30.50±24.8	0.20±0.10
tn Acetate extract	47.00±0.60	0.40±0.10
tp Acetate extract	13.00±0.80	0.30±0.10

Values are considered assignificant at $p < 0.05$
TPC: Total phenolic content; TFC: Total flavonoid content
tn: *Tirmania Nivea*; tp: *Tirmania Pinoyi*

Table 3: Antioxidant and antiradical properties of *Tirmania Pinoyi* and *Tirmania Nivea* extracts

Matrix/Tests	IC_{50} (mg/mL)		
	Test Phosphomolybdate	Test FRAP	Test DPPH
tn methanol extract	2.235±0.110	4.112±0.217	0.142±0.006
tp methanol extract	0.907±0.014	3.670±0.572	0.102±0.004
tn Acetate extract	0.858±0.010	3.424±0.034	0.137±0.025
tp Acetate extract	0.693±0.057	3.404±0.096	0.080±0.003
Ascorbic acid standard	0.100±0.001	0.338±0.043	0.020±0.001

Values are considered assignificant at $p < 0.05$
tn: *Tirmania Nivea*; tp: *Tirmania Pinoyi*

or purple ring where the two phases meet indicates the presence of sterols (Nabatanzi *et al.*, 2015).

Alkaloids analysis

The Truffle extract was homogenized with 1% v/v HCl, heated and filtered. This filtrate was used for the subsequent analysis.

Mayer's analysis

The filtrate was treated with the Mayer reagent. Yellow colour precipitation is considered an indication of the existence of alkaloids (Yadav *et al.*, 2017).

Determination of total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteu method (Ghadage *et al.*, 2017; Wolfe *et al.*, 2003). A spectrophotometer measured the absorbance of the developed blue colour at 760 nm. The results were expressed in milligrams of gallic acid equivalent per gram of extract.

Determination of Total Flavonoid Content (TFC)

The aluminium chloride colourimetric method was used to quantify the total flavonoid Content (Chougui *et al.*, 2013). The optical density of the extracts and standard solutions was read at 430 nm. The concentration of flavonoids was expressed as milligram equivalents of quercetin per gram of extract.

Antioxidant capacity

DPPH radical scavenging assay

Antioxidant activity was evaluated using a solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 100 ml of refrigerated methanol (Ghadage *et al.*, 2017; Brand-Williams *et al.*, 1995). 750 μL of truffle extract was introduced into test tubes with 1.75 mL of DPPH solution. The mixture was vortexed and kept in the dark for 30 min. A spectrophotometer measured the absorbance at 517 nm.

Ferric reducing antioxidant power (FRAP) assay

For the different extracts of *T. Nivea* and *T. Pinoyi*, the test was performed using the method described by Chougui *et al.* (2013). After 30 minutes of incubation in the dark at room temperature, the absorbance was measured with a spectrophotometer at 700 nm (Gordon, 1990). The increase in absorbance of the reaction mixture shows a high reducing power.

Phosphomolydate (PPM) assay

The phosphomolybdate method using ascorbic acid as the standard is used to determine antioxidant capacity (Odunola *et al.*, 2015). In a test tube, the 500 μl volume of the extract solution is added to 2.5 ml of the reagent (sulphuric acid 0.6 M, sodium phosphate 28 mM, and ammonium molybdate four mM). The tubes were closed and incubated in a water bath at 95°C for 90 minutes. The absorbance was measured by UV spectrophotometer at 695 nm relative to the blank, after cooling the samples to room temperature. The results were expressed in milligram equivalent of ascorbic acid per 100 grams of extract.

Statistical Analysis

Data are reported as mean $\pm$ standard deviation. Analyses were performed in triplicate. A unidirectional ANOVA test was performed to analyze the significance of the difference between the different extracts studied. The test is significant if $p < 0.05$. The IC50 was determined graphically by a linear regression method.

RESULTS AND DISCUSSION

Phytochemical characterization

Phytochemical screening of the truffles revealed some differences in the constituents of the two truffles tested Table 1.

T. Pinoyi was positive for all phytochemicals tested; *T. Nivea* showed the absence of alkaloids. All truffles had antioxidant activity. The presence of polyphenols in all truffles is probably responsible for the free radical scavenging effects observed.

Determination of total phenolic and flavonoid contents (TPC) and (TFC)

Phenolic compounds are the main secondary metabolites of truffles. They have several pharmacological activities useful in the prevention of chronic diseases. In addition to their antioxidant activities, they can act on free radicals by reducing their oxidative power. The results obtained for the phenol and flavonoid levels of *T. Nivea* and *T. Pinoyi* are presented in Table 2.

Phenolic compounds are potent antioxidants; they exert their activity by capturing or trapping free radicals such as pyroxylated and hydroxylated radicals or peroxynitrite and superoxide anions (Carocho and Ferreira, 2013). Phenolic compounds derived from truffles are classified into several families, the main one being flavonoids. The latter have more marked antioxidant or anti-free radical activities than the others (Almeida *et al.*, 2012). In addition to other antioxidants, flavonoids have been more effective in their mechanism of action by trapping oxidants or free radicals (Bravo, 1998). These results are similar to the results in the literature for other desert truffle extracts (Al-Laith, 2010) From our findings on the inhibitory concentration, 50 of soluble extracts of *T. Nivea* and *T. Pinoyi*. We can suggest that phenolic compounds may be the main actors of antioxidant or anti-radical activity. The concentration of total phenol was much superior in *T. Nivea* ethyl acetate extract than in *T. Pinoyi* ethyl acetate and methanol extracts. T. Nivea has relatively high contents of flavonoids, phytochemicals compounds that are responsible for antioxidant activity and have been primarily reviewed for their health benefits. Phenolic compounds in general and flavonoids, in particular, can contribute to the inhibition of the pro-oxidant effects of proteins, DNA, and lipids. (Yeddes *et al.*, 2013).

Antioxidant capacity

Oxygen is essential in the physiology of life. Also, it can be a source of increased cell damage due to oxidative damage (Elmouttaleb *et al.*, 2012). Oxidative stress is triggered when there is a disorder between the reactive species of oxygen produced and the antioxidant capacity of the cell concerned (Ahmad *et al.*, 2009).

Several in-vitro tests were performed to determine antioxidant properties to obtain active substance content (Almeida *et al.*, 2012). Many chronic diseases are due to the action of free radicals. Antioxidants or anti-free radicals fight against these free radicals and protect organisms against various dis-

eases. The mechanism of action is the trapping of reactive oxygen species or the strengthening of antioxidant defence mechanisms (Umamaheswari and Chatterjee, 2008; Toure *et al.*, 2015).

The antioxidant activity, estimated by three different analyses ,showed moderate reducing power of methanol and ethyl acetate extracts Table 3 for *T. Pinoyi* and *T. Nivea* (IC50 = 4.112±0.217 and 3.670±0.572 mg/mL ; 3.424±0.034 and 3.404±0.096 mg/mL respectively), elimination of DPPH radicals (IC50 = 0.142±0.006 and 0.102±0.004 mg/mL ; 0.137±0.025 and 0.080±0.003 mg/mL respectively), phosphomolybdenum inhibition (IC50 = 2.235±0.110 and 0.907±0.014 mg/mL ; 0.858±0.010 and 0.693±0.057 mg/ml).

Depending on the effect of extracts of *T. Nivea* and *T. Pinoyi* on the colour intensity of the diphenyl-1,2-diphenyl-1-picrylhydrazyl solution, their anti-free radical capacity is measured (Almeida *et al.*, 2012). The assay method is used by trapping the DPPH radical by adding another free radical or an antioxidant. The action of the latter causes a discolouration of the initial solution. The low intensity of the colour is relative to the strength of the antioxidant substance or its concentration. The strong anti-radical activity of the tested compound is demonstrated by a significant decrease in the absorbance of the reaction solution. DPPH is a stable radical that produces a violet staining solution in methanol, at room temperature. The DPPH solution gives a high absorbance in the visible spectrum at 515 nm (Saeed *et al.*, 2012; Krishnaiah *et al.*, 2011). In this work, the samples (*T. Nivea* and *T. Pinoyi*) tested with methanol extracts, and ethyl acetate showed a low inhibition concentration. According to these results, we notice that truffle extracts may contain phytochemical components capable of trapping potential damage by giving hydrogen to a free radical.

They were using the method based on the formation of phosphomolybdenum compounds by the reduction of Molybdenum (VI) to Molybdenum (V) by extracts. The antioxidant capacity of the different extracts is determined by UV-Visible spectrophotometry with maximum absorption at 765 nm. The results showed that *T. Nivea* and *T. Pinoyi* had a strong antioxidant capacity for phosphomolybdenum reduction. Recently, it has been shown in numerous studies that flavonoids and related polyphenols are significant, responsible for the phosphomolybdate scavenging capacity of medicinal plants. (Sharififar *et al.*, 2009; Khan *et al.*, 2012).

The reducing power of the different extracts and the reduction in intensity are responsible for the test solution changing from a yellow to a green colour. The reduction of the ferric complex to ferrous form is due to the presence of reducers or chelators in the solution. The ferrous form can be measured by spectrophotometer at 700 nm. It has been shown in the literature that anti-radical properties are possible by adding a hydrogen atom that is capable of breaking the chain of free radicals (Gordon, 1990; Ramaswamy *et al.*, 2015). The reducing power of the extracts depended on the concentration. At 700 nm, there is a proportionality between absorbance and reducing concentration. The antioxidants present in *T. Nivea* and *T. Pinoyi* extracts have caused the reduction of the ferric complex into a ferrous complex. This mechanism has demonstrated the presence of antioxidant compounds.

CONCLUSION

The truffle extract *Tirmania Nivea* and *Tirmania Pinoyi* from the desert of Morocco has shown, according to three methods of analysis, antioxidant and anti-radical activities to varying degrees. This work is an update on the nutraceutical potential and antioxidant compounds of *T. Nivea* and *T. Pinoyi*, an edible wild desert truffle. It is interesting to note that Burmese truffles appear to be an essential source of several natural antioxidants. Besides, these truffles appreciated by consumers could be used as high antioxidant foods to alleviate oxidative stress responsible for certain chronic diseases. Bioactive compounds from truffles may be a promising alternative source that could be used as important therapeutic agents in the pharmaceutical industry against many diseases.

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Conflict of Interest

The authors declares that they have no competing interest.

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