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Free radical scavenging and antioxidant properties of a truffle, *Kalaharituber pfeilii found* in Kalahari deserts of Botswana

Chaturvedi P., Khare K.B.*, Kwape T and Makholwa K
Department of Biological Sciences, University of Botswana, Private Bag 0022, Gaborone, Botswana

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Abstract: Natural anti-oxidants are the good source for scavenging excess of free radicals generated in the body after oxidative stress. The present study is aimed at *in vitro* evaluation of free radical scavenging potentials of two extracts prepared from samples of a truffle, *Kalaharituber pfeilii* collected from Kalahari desert in Botswana. These are water extract (WTE) and methanol extract (MTE).First of all, DPPH-TLC-Semi quantitative assay was used to assess the anti-oxidant properties. This experiment showed that at lower concentration, WTE showed better results than MTE and gallic acid. But at higher concentration, 90% inhibition was noted. Five *in vitro* system; DPPH, ABTS, Hydrogen peroxide, reducing power and Nitric Oxide, were employed to measure the radical scavenging potentials of WTE and MTE. Phenol content of WTE and MTE were determined and it was found that WTE had more phenol content (549mg GAE/g) as compared to MTE. Both the extracts of the truffle showed concentration dependent significant free radical scavenging in all the five assay systems employed. WTE was found to be more effective than MTE. Free radical scavenging activities and anti-oxidant potentials are due to possession of high phenol content.

Key words: DPPH; ABTS; Reducing power; Nitric Oxide Kalahari Desert Truffle.

INTRODUCTION

Production of free radicals during the process of oxidation is natural phenomena in the biological system. Anti–oxidant system of the body is efficient enough to neutralize these free radicals under natural condition and thus renders protection from free radical generated oxidative stress. Some time, oxidative stress surpasses this and body undergoes the state of cellular damage because of free radical induced chain reactions of oxidation (Yu, 1994). Peroxidation of lipid, protein and ultimately the DNA molecule in a chain reaction culminates to the development of physiological disorders like arthritis, diabetes and cardiovascular complications (Wang *et al.,* 1996; Dubost *et al*., 2007). Modern population is at the risk of these diseases because of over stressed working condition and the prevailing mode of fast life style that lead to the consumption of fast food, alcohol, analgesics and antibiotics. Metabolism of these stuffs produces huge amount of free radicals that cannot be controlled by normal antioxidant system of the body. To overcome these situation people should consume fresh food along with green vegetables, fresh fruits and mushrooms because of their richness in nutrients and antioxidants that can scavenge generated surplus free radicals (Enrique, 2012; Yildiz, 2014). Chaturvedi *et al.* (2011) found two most common mushrooms in Botswana, *Agaricus bisporus* and *Plurotus ostreatus* to have good anti-oxidant properties. Unfortunately food of nowadays is nutritionally-deficient, lacks anti-oxidants and is full of pesticides as they are grown with chemical fertilizers and protected from pests by pesticides. Conversely, organically-grown fruits and vegetables, wild fruits have significantly more anti-oxidants, polyphenols, and enzymes.

Desert truffles are nutritious hypogenous fungi exhibiting unusual biological features. They are mycorrhizal and may form either or both of the two main types of associations, ecto- or endomycorrhizae. These fungi inhabit sandy soils and fruit only in years with adequate and properly distributed rains. *Kalaharituber pfeilii* (Hennings) Trappe-Kagan-Zur is one of the most common Kalahari desert truffle species found fruiting from May to July (Trappe *et al*, 2008). The truffles were collected and consumed from the wild by desert dwellers from early stages of civilization (Trappe, 1990). Kalahari Truffles are highly sought after food sources in Kalahari, particularly among marginal groups such as the *Basarwa* (Bushmen) for whom this represent an important source of nutrients as well as food security since they may be dried for later consumption. The *Khoisan* people of the Kalahari Desert locally called these truffles as *mahupu/n'xaba* and used them for centuries. Truffles are hunted in the Kalahari by men and women; they look for cracks in the soil, often humped, caused by expansion of the truffles, which are then extracted with hands or digging sticks (Trappe *et al*, 2008).

The truffles are eaten raw or cooked (boiled, roasted over fire, or buried in hot ashes). Commercial harvest of Kalahari truffles has increased in the last decade and the quantities harvested have been observed to be declining where livestock have been concentrated (Trappe, 1990; Taylor *et al*, 1995). This natural scarce truffle is delicious, nutritious and has a variety of medicinal uses. The desert truffles of Middle Eastern regions have been reported to have the anti-oxidant properties (Abdul Ameer, 2010). It is for this reason that there is a need for scientific validation of the traditional usage of this truffle, and this paper evaluates the anti-oxidant properties of the Kalahari desert truffle.

MATERIALS AND METHODS

Collection of Truffles and preparation of methanol extract (MTE) and water extract (WTE)

The truffles were collected from Kalahari Desert, washed, sun dried and ground to powder. Methanol extract was prepared by soaking the powder with three times volume of the powder with 100% methanol. After 3 days soaking, the extract was filtered and methanol free extract was obtained by evaporating the extract into Butchi type

***Corresponding Author:**
Dr. Khare KB,
Department of Biological Sciences,
University of Botswana,
Private Bag 0022, Gaborone, Botswana.

rotary evaporarizer under reduced pressure. Similar method was used to get the water extract also.

DPPH-TLC-Semi quantitative assay to assess the anti-oxidant properties

To measure the antioxidant activities of truffle extracts, the method described by Yeboah and Majinda (2009) was followed. One mg/ml stock solutions of TME and TWE and reference standard (gallic acid) were used. The spraying reagent was then prepared by dissolving 2 mg of DPPH in 50 ml conical flask. The mixture was shaken well and kept in a refrigerator for about 20 minutes before use. Different amounts (0.1 µL, 0.5 µL, 1.0µL, 5.0 µL, 10.0 µL) of test sample and standard was taken from the stock solution using microliter syringes and finely spotted on the Thin Layer Chromatography (TLC) plates. The solvent on the TLC plates was left to evaporate in the fume hood before spraying. The spotted TLC plates were sprayed with the DPPH forming a purple background on the plates. The radical scavenging activities of the extracts were then observed after 30 minutes.

Spectrophotometric measurement of free radical scavenging activities of extracts by using 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH)

DPPH radical scavenging activity was determined by the method of Yeboah and Majinda (2009). One ml from 0.3 mM alcohol solution of DPPH was added to 2.5 ml of TME and TWE. The samples were kept at room temperature in the dark and after 30 minutes the optical density was measured at 517 nm. The percentage antiradical activity (AA) was determined according to the following formula:

AA%=100-{[(Absorbance of sample-Absorbance of empty sample) x100]/Abs of .control}

Where;

Empty samples = 1ml methanol + 2.5 ml from various concentrations of extract;

Control sample=1ml 0.3 mM DPPH + 2.5 ml methanol.

2-Azobis-3-ethyl benzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity

ABTS radical scavenging activity was determined by the method described by Pellegrini *et al.*, (1999). The ABTS radicals, cations were produced when ABTS (7 mM) reacted with potassium persulfate (2.45 mM) when incubated at room temperature in the dark for 16 hrs. The solution thus obtained was further diluted with phosphate buffer saline (PBS) to give an absorbance of 1.000. Different concentrations of the test sample in 50 µl were added to 950 µl of ABTS working solution to give a final volume of 1 ml. The absorbance was recorded immediately at 734 nm. Gallic acid was used as reference standard. Inhibiting concentrations of extract were tested at 100, 200, 300 and 500mg/ml. Reference standard (gallic acid) was tested at the same concentrations. The percent inhibition was calculated from the following equation:

% inhibition = [(1-Absorbance of sample/ Absorbance of control)] x100

Determination of total phenol content

The total phenol content (TPC) was determined by the method described by Stoilova *et al.* (2007), using Folin-Ciocalteu reagent. One ml of extract or standard solution (Gallic acid) (100-500 mg/ml) were added to a mixture of 10 ml deionising water and 1.0 ml of Folin - Ciocalteu phenol reagent. After 5 minutes, 2.0 ml of 20% sodium carbonate was added to the mixture. After 1 hour of incubation at room temperature in darkness, the absorbances were measured at 750 nm. The TPC was then calculated from the linear regression equation of the standard curve and from this equation, the concentration of gallic was determined for each extract and converted to mg of gallic acid equivalents/g of dry extract (mg GAE/g).

Hydrogen peroxide scavenging

Hydrogen peroxide scavenging of the extract was determined by the method of Iihami *et al.*, (2005). Different concentrations of MTE (10-50mg/ml) in methanol were added to 0.6 ml of H_2O_2 (40 Mm in phosphate buffer). After 10 minutes, absorbance of H2O2 will be measured against a blank containing only phosphate buffer at 230 nm after 15 minutes. H_2O_2 will be used as a control. Percentage scavenging of H_2O_2will be calculated using the following formulae:

Percent scavenging of H_2O_2= [1- abs of standard/abs. of control] x 100

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of extracts was measured by the method of Sun *et al.*,(2003). Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured by the Griess reagent. The absorbance of the chromophore formed was measured at 546 nm. The reaction mixtures (3ml) containing 10Mm sodium nitroprusside in phosphate buffer saline and extract of various concentrations (100-500mg/ml) were incubated at 250C for 150 minutes. After that 0.5 ml of Griess reagent was added to the reaction mixture. The absorbance was then measured at 546 nm. Ascorbic acid was used as a positive control.

Inhibition % = (1-absorbance of sample)/absorbance of control x 100

Determination of reducing power

The reducing power of extracts was determined by the method of Yen and Chen (1995). Different concentration of MTE and WTE (100-500mg/ml) in water were mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and 2.5 ml of 1% potassium ferric cyanide. The mixture was incubated for 20 minutes at 50^0C. 2.5ml of 10% trichloroacetic acid was added to the mixture to stop the reaction, and then it was centrifuged at 3000 rpm for ten minutes. The upper layer of the centrifuged mixture (2.5ml) was mixed with 2.5 ml of distilled water 0.5 ml of 0.1%ferric The absorbances were measured at 700nm. Increase in absorbance is proportional to increased reducing power. Percent increase in the reducing power was determined as follows;

%increase in reducing power= Absorbance of test-Absorbance of control/Absorbance of control x 100

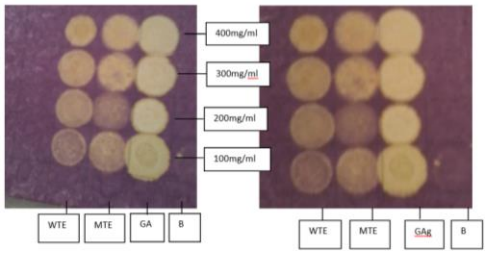
Statistical Analysis

All data were expressed as Mean ± SEM. Analysis of variance was carried out by ANOVA and Tukey's test was used to find out the significant differences. P<0.05 was considered to be statistically significant.

RESULTS

Different concentrations of MTE and WTE spotted on the TLC sheet showed gradually increasing high activities with increasing concentrations as indicated by the increase in intensity of the yellow color over the purple DPPH background after an hour.

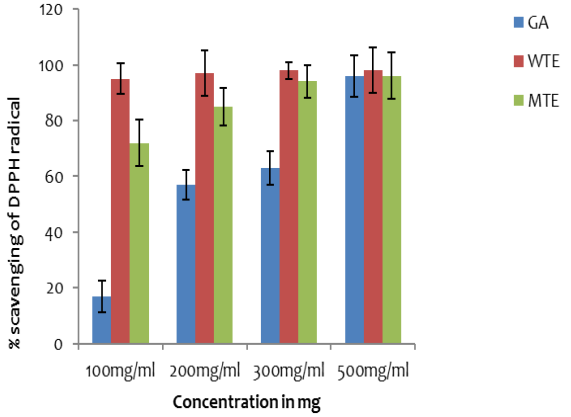
Fig. 1a: Spotted TLC plates observed after 30 minutes while Fig. 1b: shows the spotted TLC after 1 hour.



Key: B-Blank (100% methanol only), WTE-Aqueous extract, MTE-100% methanol extract, GA-Gallic acid

Inhibition of DPPH radicals by MTE and WTE at different concentration of 100 mg/ml, 200mg/ml, 300mg/ml and 500mg/ml presented in Fig. 2 showed that DPPH radical scavenging activities of MTE were concentration dependent. Lowest scavenging activity (75%) was noted at 100mg and highest inhibition (90%) at 300 and 400mg/ml. WTE showed very significant effects even at 100mg/ml (85% inhibition) and 90% inhibition at all other higher concentration. Gallic acid showed less significant effects at lower concentration but at 500mg/ml, the effects were similar to MTE and WTE. Two extracts and gallic acid showed 90% inhibition at higher concentration.

Fig. 2: DPPH free radical scavenging activity of WTE, MTE and Gallic acid



ABTS free radical scavenging activity of MTE and WTE are presented in Table 1. Significant ABTS scavenging activity was seen in MTE at 300mg/ml (IC50) followed by WTE at 400mg/ml while gallic acid showed the similar results at 100mg/ml. Both the extracts of *K. pfeilli* extracts demonstrated high phenol content but the highest estimated phenol content was observed as 549 mgGAE/g inWTE, followed by 393 mgGAE/g in MTE.

Table 1: ABTS radical scavenging activity of various extracts of MTE and WTE

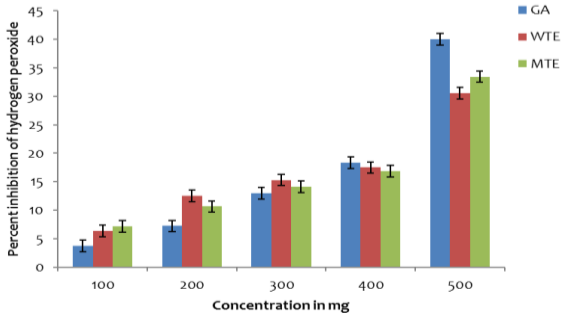
Truffle extracts	Concentration of inhibition [IC50] mg/ml
100% methanol extract (MTE)	300
Aqueous extractextract (WTE)	400
Gallic acid -Standard	100

Table 2: Total Phenol content of MTE and WTE

Truffle extracts	Total phenol content (mg GAE/g)
100% methanol extract (MTE)	393
Aqueous extract (WTE)	549

Both the extracts, WTE and MTE had considerably high phenol content but the highest estimated phenol content was 549 mgGAE/g in WTE, followed by 393 mgGAE/g in MTE (Table 2). The percent hydrogen peroxide scavenging activities of both extracts, WTE and MTE along with the gallic acid at different concentrations (100 – 500m/ml) presented in Fig. 3 demonstrated that the percent scavenging activities in all the cases are dose dependent. At lowest dose 100mg/ml, gallic acid showed the least inhibition (4%) while MTE and WTE showed 7% inhibition. At 500mg/ml, gallic acid had 45% inhibition. MTE at these dose showed 30% scavenging while 23% scavenging was shown by WTE.

Fig. 3: Hydrogen peroxide scavenging activity of WTE, MTE and Gallic acid (standard).



The percent Nitric oxide radical scavenging activities of various concentrations of MTE and WTE presented in Fig. 4 showed that WTE had higher nitric oxide scavenging activity than MTE and both extracts showed concentration gradient response. Gallic acid showed significant response at all the tried concentrations (100-500mg/ml). MTE and WTE showed 80% scavenging at 300mg/ml. A slight increment in the scavenging (90%) was noticed at 500mg/ml but it was not significant.

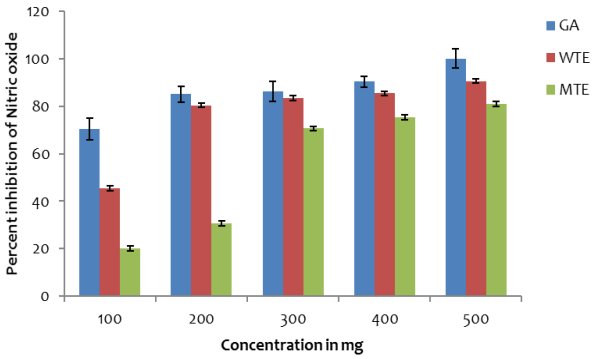


Fig. 4: Percent Nitric oxide radical scavenging activities of various concentrations of MTE and WTE

Results of reducing power (Table 3) shows that both extracts WTE and MTE had reducing power. WTE showed 64.95% reduction power and MTE showed (54.12%) against ascorbic acid that demonstrated 90% reducing power.

Table 3: Determination of reducing power of methanol and water extracts at 500mg/ml

Compound	Absorbance (±SD) at 700 nm	% increase in reducing power
Ascorbic acid (standard)	1.698±0.018	90.15
100% methanol extract (MTE)	1.412±0.0005	58.12
Aqueous extract (WTE)	1.473±0.012	64.95

DISCUSSION

Natural anti-oxidants protect lipids from oxidation and thus provide health benefits in terms of diseases caused by oxidative stress. Medicinal plants, fruits and vegetables including mushrooms are the richest source of anti-oxidants. Taking into consideration of all the results of *in vitro* experiments, it can be concluded that both WTE and MTE of the truffle possess strong anti-oxidant activities. Any substance can be considered as anti-oxidant if it scavenges free radicals. DPPH is considered as proton free radical and scavenging of proton free radical is one of the mechanisms to reduce oxidants in the biological system (Sharma and Bhatt 2009). The results of both DPPH scavenging experiments by TLC method (Semi quantitative assay) and spectrophotometric measurement had indicated the possession of strong scavenging of DPPH radicals by WTE and MTE in concentration dependent manner. Phenolics present in WTE and MTE possess hydroxyl groups that cause the quenching of DPPH radicals. Apart from DPPH scavenging, both extracts were also able to quench the long lived ABTS cation radical (ABTS+). Significant ABTS scavenging activity (IC50) was seen in MTE at concentration of 300mg/ml followed by WTE at 400mg/ml. ABTS radical scavenging activity is due to reduction by donation of an electron from both extracts leading to decrease in absorption indicating high percentage scavenging activities (Nishaa *et al.*, 2012). This can be further supported by the possession of significant reducing power by both extracts, MTE and WTE, which could be due to possession of antioxidant that reduce the Fe_3 + ferrycyanide complex to ferrous by donating an electron (Nandhakumar and Indumathi, 2013). Hydrogen peroxide causes cytotoxicity by giving rise to variety of free radicals like hydroxyl radicals and thus it is worth to remove H_2O_2 through anti-oxidants from biological system ((Gelard and Richard, 1973; Coyle and Kader, 2007; Chaturvedi *et al.*, 2011).

The results of present hydrogen peroxide scavenging experiments showed that MTE and WTE had scavenged H_2O_2 successfully from the reaction mixture in a dose dependent manner. Maximum scavenging at higher doses was almost comparable to that of Gallic acid.

Nitric oxide is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Nishaa *et al.*, 2012), but excess of it might have reverse effects on the antioxidant system. Excess of nitric oxide should be scavenged. In the present study, MTE and WTE had successfully scavenged nitric oxide in the reaction mixture, and it was shown to be concentration dependent. Rich phenolics content of WTE and MTE could be the cause of scavenging by H-atom abstraction or by the reduction of NO by single electron transfer (Klotz and Sies, 2003).

Both extracts of the Kalahari truffles, WTE and MTE had reducing power which could be due to possession of antioxidants that reduce the Fe3+ ferricynide complex to ferrous form by donating an electron (Nandhakumar and Indumathi, 2013).

CONCLUSION

Thus in the present study, WTE and MTE demonstrated significant scavenging of DPPH, ABTS, Hydroxyl and NO radicals, inhibited hydrogen peroxide and showed pronounced reducing power. All these activities are because of possession of phenolic compounds by WTE and MTE that must have scavenged free radicals *in vitro* experimental condition.

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