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Antioxidant activity, total phenolic compounds and ld50 bioassay (toxicity) activity of the fermented wood "Nikhra" fractions of combretum hartmannianum, terminalia laxiflora and acacia seyal

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Abstract

This study characterizes antioxidant activity, total phenolic compounds and LD50 bioassay (Toxicity) of the fermented wood "Nikhra" fractions of Combretum hartmannianum, Acacia seyal and Terminalia laxiflora. Antioxidant potential using DPPH assay, the content of total phenolic compounds in the fractions was determined spectrometrically according to the Folin−Ciocalteu procedure and calculated as gallic acid equivalents (GAE) and toxicity using the brine shrimp Artemia salina standard method. These trees exhibited far stronger antioxidant activity and contained significantly higher levels of phenolics. All fractions proved to be non toxic against A. salina expect ethyl acetate and chloroform fractions of A. seyal and chloroform fractions of C. hartmannianum which possessed slight toxicity.

Key words: Antioxidant activity; Phenolic compounds; Phenolic acids; Flavonoids; Tannins; Free radical; Toxicity activity.

Introduction

flowering plant family, Combretaceae. They are native to Eritrea, Ethiopia, Keny, Somalia, Sudan, Tanzania, Uganda. Acacia seyal Genus is Acacia Mill.–acacia Family Fabaceae (John B Hall,1994). Acacia seyal, which is native to Egypt, Eritrea, Ethiopia, Ghana, Iran, Israel, Kenya, Malawi, Mali, Mozambique, Namibia, Niger, Nigeria, Saudi Arabia, Senegal, Sudan, Syrian Arab Republic, Tanzania, Uganda, Yemen, Republic of Zambia, Zimbabwe *(Kamali and Mohammed, 2007).*

Biological definition of antioxidant ''any substance that when present at low concentrations compared to that of an oxidizable substrate would significantly delay or prevent oxidation of that substrate (Halliwell and Gutteridge, 1995). A lot of medicinal plants and their purified constituents have shown beneficial therapeutic potentials (Khalaf et al., 2008). Flavonoids and phenolic compounds are widely distributed in plants that have been reported to exert multiple biological effects including antioxidant, free radical scavenging, anti-inflammatory, and anticarcinogenic (Miller, 1996). Further investigation on the isolation and identification of antioxidant components in the plant may lead to chemical entities with the potential for clinical use(Al-Fartosy, 2011). Recently there has been an upsurge of interest in the therapeutic potentials of plants, as antioxidants in reducing, free radical, induced tissue injury. Combretum and Terminalia are a genus of large trees of the

Polyphenolic compounds have antioxidants effect by quenching the free radicals of biological systems with their phenolic ring and multiple hydroxyl moieties; many phenolic activities, reactive oxygen, nitrogen, and chlorine species such as superoxide, hydroxyl radical, peroxyl radicals, hypochlorous acid, and peroxynitrous acid. Polyphenols can also chelate metal ions leading to a decrease in metal ion prooxidant activity (Boersma et al., 1999).

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The Combretaceae family have been reported to contain antioxidant activities. 24 African Combretum species have antioxidant potential(Masoko et al., 2007). The methanolic extract of C.hartmannianum leaves was markedly effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of ß-carotene, this extract inhibited the formation of peroxides in sunflower oil and showed the highest antioxidative activity compared with 20 mg BHA (Mariod et al., 2004).There are publications regarding Acacia extracts on free radical inhibition and antioxidant (Chang and Tung, 2009). Acacia species (A. nilotica, A. seyal and A. laeta) (leaves, flowers and pods) total antioxidant capacity, DPPH free radical scavenging activity and reducing power of the methanolic extracts of studied parts were evaluated A. nilotica and A. seyal extracts showed less inhibitory concentration 50 (IC50) compared to A. laeta extracts which means that these two species have the strongest radical scavenging activity whereas A. laeta extracts have a lower radical scavenging activity. A positive correlation between saponins and flavonoids with total antioxidant capacity and DPPH radical scavenging activity was observed (Abdel-Farid et al., 2014).

Materials and Methods

Materials

Collection of plant materials

The fermented wood "Nikhra" of three plant species were harvested in March 2010 from Western Kordofan state, Sudan. They were carefully washed, oven-dried for 1 h at 50°C and put in the shade in an aerated place till complete drying, then were ground into a fine powder.

Preparation of plant extracts

Weight 25g powder of the fermented wood "Nikhra" three plant species was added to a Soxhlet apparatus along with a solvent methanol for extraction of chemicals. The liquid extract was evaporated to dryness by vacuum distillation and stored at 4°C for further analysis (Fyhrquist et al., 2002). Methanol extracts were fractionated sequentially by distilled water and organic solvents as ethyl acetate, chloroform and petroleum ether.

Statistical analysis

Means and ± Standard Deviation (SD) were analyzed using one-way ANOVA (Tukey's studentized range) using the program SPSS 19.0 for Windows. Differences were considered significant at $p<0.05$.

Free radical scavenging assay (Antioxidant)

Radical scavenging assay is spectrophotometric test using a methanolic solution of the stable free radical 2, 2-diphenylpicrylhydrazyl (DPPH) as a reagent. DPPH method based on increase in alcoholic DPPH solution by reduction of stable DPPH nitrogen radicals in presence of H binding antioxidants. The hydrogen atoms or electrons donation ability of the corresponding fractions and some pure compounds was measured from the bleaching of purple coloured of DPPH methanolic solution, whereas, the DPPH solution in a dark violet coloured and has a strong absorption range at 517 nm. It loses its color when transformed to DPPH-H and the absorption level decreases. This decrease in absorption shows the cytochiometric decrease in DPPH.

Free radical scavenging Method

In the experiment 10 µl from the fractions (5mg/ml) were added to 90 µl of the 300 µM DPPH solution and placed in a 96-well microtiter plate. The test samples were dissolved in DMSO while DPPH was prepared in ethanol. The mixture was incubated in the dark at room temperature for 30 min. After incubation, the absorbance of the remaining DPPH was read against a blank at 517 nm using multiplate reader spectrophotometer. Propylgallate was used as the positive control, where as DMSO as a negative. All tests and analyses were carried out in triplicate. The inhibition of free- radical DPPH in percent (%) or the capacity to scavenging the DPPH radical (radical scavenging activity) was expressed as EC50 value (mg ml-1) (the concentration demanded to inhibition the 50% of DPPH radical scavenging activity (Luís et al., 2009); calculated using the following equation:

[(A control –A sample)/ A control] x100

A= Absorbance

The test is done as describe by (Chun et al., 2005).

Total phenolic content (TPC) Method

phenolics concentration in plant fractions was determined using spectrophotometric method (Singleton et al., 1999). Samples solutions of the fractions in the concentration of 1 mg/ml were used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of samples solutions of fractions, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% NaHCO3. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu reagent dissolved in water and 2.5 ml of 7.5% of NaHCO3. The samples were thereafter incubated in a thermostat at 45oC for 45 min. The absorbance was determined using spectrophotometer at $\lambda max = 765$ nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in fractions was expressed in terms of gallic acid equivalent (mg of GAE/g of fraction).

Brine shrimp lethality assay (Toxicity)

The Nikhra fractions of A. seyal, T. laxiflora and C. hartmannianum were tested for their cytotoxicity using the brine shrimp Artemia salina standard method as described by(Meyer et al., 1982) with a minor modification. This is a rapid, inexpensive, general bioassay, has been developed for screening and monitoring of physiologically active natural products.

Materials used in toxicity assay

Sample, A. salina (shrimp eggs), sea salt (38 g/L of D/W, pH 7.4), hatching tray with perforated partition, lamp to attract brine- shrimp larvae, micro pipette (5, 50, 500 µl), vial tray, 9 vial samples and organic solvent (methanol). The eggs of brine- shrimp A. saline are readily available as fish food in pet shops. They are stored at a low temperature (4ºC), and remain viable for years. Half hatching tray (a rectangular dish 22x32cm) was filled with filtered brine solution, then (50 mg) eggs of brine were sprinkled and incubated at 37ºC. When the eggs were placed in artificial seawater they hatch within 48 h,

providing large numbers of larvae. They can be used for 48-72 hours after the initiation of hatching, and after 72 hours they should be discarded.

Toxicity assessing procedure

A rectangular dish (22x32cm) was divided into two unequal halves with plastic divider of 2 mm artificial seawater (2with several holes and filled with 28g sea salt/L, Sigma). Approximately 50 mg eggs (Artemia saline Sera Heidelberg Germany) were sprinkled in the larger compartment, which was darkened, while the smaller compartment was illuminated. 0.5 ml of 100, 1000 and 10,000 ppm concentrations of the extract prepared in respective solvent (methanol) 20 mg of extracts were dissolved in 2 ml of 5, 50, 500 µl, the concentrations were (10,100 and 1000 µl/ml respectively) was poured in vials (3vials/concentrations) and kept at room temperature to evaporate methanol. After 24 hours, phototropic nauplii (brine- shrimp larvae) were collected by a Pasteur pipette from the lightened side, and 10 shrimps were transferred to each vial. The vials were placed under the illumination at room temperature, and the volumes were made up to 5ml with sea water, and then incubated at(25 -27ºĊ) for 24 hours under illumination. Other vials were supplemented with solvent and reference cytotoxic drugs (Etoposide) as negative and positive control respectively. The numbers of survivors were counted after 24 hours. The data were analyzed with Finney computer program and the lethal concentrations 50% (LD50) were determined.

Results and Discussion

When the Nikhra" fractions of Combretum hartmannianum, Terminalia laxiflora and Acacia sayal tested for their antioxidant potential using DPPH assay, the ethylacetate fractions exhibited the highest antioxidant power among other fractions (91+0.02> 90+0.01 > 89+0.01%, respectively (Table 1). This could be attributed mainly to presence of polyphenols e.g. flavonoids (Estevinho et al., 2008).

The IC50 values of scavenging DPPH radicals for fractions of A. seyal T. laxiflora and C. hartmannianum on DPPH radical were in the following order: ethyl acetate < chloroform < petroleum ether < aqueous, (0.482±0.073, 0.496±0.102, 0.831±0.208 and0.921±0.073) mg/ml, (ethyl acetate < aqueous < chloroform), (0.347±0.026, 0.463±0.487, 2.771±0.118), (Petroleum ether < Chloroform< Ethyl acetate < Aqueous), (0.366±0.071, 0.413±0.073, 0.460±0.026 and 3.219±0.095) respectively Table (1). This study revealed that the ethyl acetate fractions of A. seyal, T. laxiflora and C. hartmannianum have prominent antioxidant activity Table (1). These phenolics are responsible for this height antiradical activities. The highest antioxidant activity of the three plants studied was found in the ethyl acetate extracts which showed the highest phenolic content of 404.96-594.60 mg GAE/g, a significant relationship between antioxidant capacity and total phenolic content was found, indicating that phenolic compounds are the major contributors to the antioxidant properties of these plants (Dudonne et al., 2009).

Table 1. Antioxidant Power, Total phenol content (TPC) and LD50 bioassay (Toxicity) of different fractions of fermented wood "Nikhra" of C. hartmannianum, T. laxiflora and A. seyal.

Plant fractions	RSA±SD (DPPH)	IC50 ±SD (mg/ml DPPH)	TPC (mg GAE/g fractions)	Toxicity	LC50 (mg/ml)
A. seyal P	78±0.01	0.831±0.208	175.93	No	**386.3872**
A. seyal C	86±0.03	0.496±0.102	461.85	slight	**43.3315**
A. seyal E	91±0.02	0.482±0.073	424.65	slight	**27.2092**
A. seyal A	89±0.03	0.921±0.073	410.79	No	**67.7551**
T. laxiflora P	44±0.24	0.433±0.021	382.35	No	**168.9470**
T. laxiflora C	90±0.01	2.771±0.118	506.56	No	**242.3399**
T. laxiflora E	90±0.01	0.347±0.026	594.60	No	**78.6234**
T. laxiflora A	89±0.07	0.463±0.487	747.05	No	**126.8730**
C. hartmannianum P	84±0.01	0.413±0.073	460.39	No	**115.5829**
C. hartmannianum C	71±0.23	0.366±0.071	473.52	slight	**44.3499**
C. hartmannianum E	89±0.01	0.460±0.026	404.96	No	**55.4412**
C. hartmannianum A	52±0.18	3.219±0.095	363.38	No	**59.5492**
Propyl gallate (control)	91±0.03	0.0312±0.053			

Petroleum ether= P Chloroform= C Ethyl acetate= E Aqueous= A

A. seyal fractions show high DPPH radical scavenging activity of pods 66.67%,, leaves 66.27%, fractions showed the lowest IC50 0.07, 0.076 mg/ml, respectively (Abdel-Farid et al., 2014). fractions of C. hartmannianum bark demonstrated potent antioxidant effect with IC50 range from 0.94–2.24 mg/ml, (Hassan et al., 2014). The antioxidant activity of the extracts measured by DPPH free radical showed high reduction of 50% DPPH in C. hartmannianum leaves extract followed by Guiera senegalensis roots and Guiera senegalensis leaves (Mariod et al., 2006).

Phenolic content of fractions of the Nikhra of C.hartmannianum, T. laxiflora and A. sayal was determined by Folin−Ciocalteu method. The results of this colorimetric method, expressed as mg gallic acid equivalents are shown in Table (1). T. laxiflora fractions presented the highest phenolic content which were reported as 747.05> 594.60> 506.56> 382.35mg GAE/g for aqueous, ethylacetate, chloroform and petroleum ether fractions, respectively Table (1). C. hartmannianum fractions presented the second highest phenolic contents which were reported as 473.52>460.39>404.96>363.38 mg GAE/g, chloroform,

petroleum ether, ethylacetate and aqueous fractions respectively Table (1). The A. seyal fractions presented moderate phenolic contents which were reported as 461.85>424.65>410.79>175.93mg GAE/g, chloroform, ethylacetate, aqueous and petroleum ether, fractions respectively Table (1), chloroform fraction of A. seyal gave the highest phenolic content of 461.85mg GAE/g followed by ethyl acetate 424.65, aqueous 410.79 and petroleum ether175.93 fractions mg GAE/g. Abdel-Farid et al., (2014) reported a high content of phenolics *for the* methanolic extracts *of A. seyal* pods and leaves. Aqueous phase of T. laxiflora gave the highest phenolic content of 747.05 mg GAE/g followed by ethyl acetate 594.60, petroleum ether 382.35 and chloroform 206.56 mg GAE/g respectively, The total phenolic compounds of fractions of the root, stem and leave of T.glaucescens 96.50±0.25 mg GAE/g (Aberoumand and Deokule, 2008). Finally chloroform fractions of C. hartmannianum gave high phenolic content of 473.52 mg GAE/g followed by petroleum ether 460.39, ethyl acetate 404.96 and aqueous, 363.38 mg GAE/g respectively. The total phenolic compounds of methanolic extract of C.hartmannianum leaves was 253.4 mg/g (Mariod et al, 2006), these results agree with our study results and different due to parts used in study.

In vitro toxicity of Nikhra of Combretum hartmannianum, Terminalia laxiflora and Acacia sayal fractions against the brine shrimp (Artemia salina) are presented in Table (1). All fractions proved to be non toxic against A. salina expect ethyl acetate and chloroform fractions of A. seyal and chloroform fractions of C. hartmannianum which possessed slight toxicity. Nguta and Mbaria, (2013) reported that A. *seyal* root methanolic extracts was considered to be non toxic. In vitro toxicity of extracts of the roots and stem bark of T. brownii(Combretaceae) against the brine shrimp (Artemia salina) larvae with LC50 values ranging from 113.75– 4356.76 and 36.12–1458.81 µg/ml, respectively. The genus *Terminalia*, which are known to contain cytotoxic compounds such as hydrolysable tannins (Mbwambo et al., 2007).

Conclusions

In conclusion, the results obtained confirmed the high antioxidant activity of the fermented wood "Nikhra" fractions as the presence of polyphenols compounds, T. laxiflora fractions presented the highest phenolic content, followed by C. hartmannianum then A. seyal. All fractions proved to be non toxic against A. salina expect ethyl acetate and chloroform fractions of A. seyal and chloroform fractions of C. hartmannianum which possessed slight toxicity.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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