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Original Article

In vitro antioxidant activity of the methanol extract of *Basella alba* L (Basellaceae) in rat testicular homogenate

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Abstract

The methanol extract of *Basella alba* (MEBa) has been shown to improve male fertility. In order to further understand its action mechanism, its antioxidant effect was investigated *in vitro*. To this respect, the extract was tested on antioxidant enzyme activities and lipid peroxidation using rat testicular homogenate. Furthermore, the effect of the MEBa on oxidation of β -carotene, reduction of ferric ions and scavenging of free radicals was investigated; in addition, phytochemical analyses were performed. The MEBa slightly reduced diphenyl-picrylhydrazyl (DPPH) radical, and inhibited lipid peroxidation and β -carotene oxidation. The MEBa also reduced ferric ions and stimulated the activities of glutathione reductase, catalase and superoxide dismutase. These findings suggest antioxidant properties of MEBa, that could be attributed to phenolic compounds revealed by phytochemical studies of the extract. The MEBa antioxidant properties may thus sustain its various biological activities, such as improvement of male reproductive function.

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INTRODUCTION

Oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, aging, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases, male infertility and others [1-3]. It is defined as “the imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage” [4]. Antioxidants are substances which possess free radical chain reaction breaking properties. Antioxidants include enzymes (superoxide dismutase, catalase, glutathione peroxidase and reductase), glutathione and vitamins (e.g. vitamin E, C), as well as many dietary components and compounds from medicinal plants [5, 6]. In fact, a great number of aromatic, medicinal plants contain chemical compounds exhibiting antioxidant properties [6].

The medicinal plant *Basella alba* is used in the West region of Cameroon to treat sexual asthenia and infertility in males [7]. Its methanol extract (MEBa)

stimulates steroid hormones and cytochrome P450 aromatase mRNA in Leydig cell cultures [8-10]. *In vivo* studies revealed a stimulatory effect of MEBa on testosterone levels in male rats. MEBa treatment also resulted in improvement of the fertility of male rats exposed *in utero* to flutamide, an anti-androgen drug [10, 11]. Although antioxidant compounds such as ascorbic acid, carotenoids and phenolics have been found in *B. alba* leaves [12, 13], only the study of Maisuthisakul *et al* [14] has assessed the free radical-scavenging capacity of this plant. However different methods have shown significant and varying contributions to total antioxidant potential [15], emphasizing the need to apply other assays in addition to the free radical-scavenging test, in order to define the antioxidant activity of MEBa. The present study thus aimed to investigate the *in vitro* antioxidant activity of MEBa, through evaluation of its effect on enzyme activity, lipid peroxidation, β -carotene oxidation, ferric ion reduction as well as anti-radical potential.

MATERIALS AND METHODS

Plant material and preparation of the methanol extract of *Basella alba*

Fresh leaves of *B. alba* (identified at the Cameroonian National Herbarium as specimen No 40720) were collected in Dschang (West region of Cameroon), dried at room temperature and ground in powder. The MEBa was obtained through successive extraction in hexane, methylene chloride and methanol, as described earlier [10, 16].

Animals

Animals used for the preparation of testicular homogenate were 2.5-month-old male Wistar albino rats of approx. 200 g body weight from the animal house of the Department of Biochemistry, University of Yaounde I, Cameroon. They were housed in plastic cages, given food and water *ad libitum*, and were handled according to ethical guidelines of the Cameroon National Veterinary Laboratory.

Chemicals

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, β -carotene and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma Chemicals (St. Louis, MO, USA). 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) was purchased from Sigma Aldrich (Lyon, France). Other reagents were of high quality grade.

Assay of anti-radical activity

Anti-radical activity of the MEBa was evaluated by assessing their ability to reduce DPPH radical according to Brand *et al* [17]. To this end, DPPH solution was introduced in tubes and the MEBa (0.01, 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) or trolox (0.01, 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) diluted in 0.025% dimethyl sulfoxide (DMSO) were added. The change from the radical to the non-radical form leads to the disappearance of the purple coloration of DPPH, which was followed by spectrophotometry at 517 nm. The percentage of discoloration was calculated.

Oxidative degradation of β -carotene (β -CLAMS)

The β -CLAMS (beta-carotene-linoleic acid model system) method is based on the discoloration of β -carotene by the peroxides generated during the oxidation of linoleic acid (a free radical chain reaction) at high temperature [18]. In brief, the solution containing 0.02% of β -carotene and 0.001% of linoleic acid were introduced in tubes. Trolox, MEBa (0.01, 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) or 0.025% DMSO (blank) were then added into the tubes. Furthermore, the tubes were incubated for 5 min at 50°C and absorbance monitored at 470 nm with 10 min intervals for 3 h.

Ferric reducing capacity test (FRAP)

Trolox, MEBa (0.01, 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) or 0.025% DMSO (blank) were added into test tubes containing FRAP reagent (TPTZ 10 mM in acetate buffer pH 3.6, 300 mM). Standards were also prepared using FRAP reagent and ferrous sulphate solution (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM). All tubes were incubated at room temperature for 5 min and absorbance read at 593 nm. Fe^{2+} -TPTZ concentrations characterizing the reducing activity of different compounds were determined from calibration curve of ferrous sulphate [19].

Inhibition of lipid peroxidation

MEBa, trolox (0.01, 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) or vehicle (0.025% DMSO) were added to preincubated testicular homogenate (containing 4 mg/ml of protein). Lipid peroxidation was non-enzymatically initiated using ascorbate (5 mM), ammonium iron(II) sulfate $[(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2]$ (15 μM), and ADP (10 mM). After 15 min of incubation at 37°C, the reaction was stopped by addition of thiobarbituric acid reagent containing trichloroacetic acid, and thiobarbituric acid reactive substances (TBARS) were assayed [20]. The percentage of inhibition was computed.

Glutathione reductase (GR) activity

Trolox, MEBa (0.01, 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) or 0.025% DMSO (blank) were added into test tubes containing ethylene diamine tetraacetate (EDTA) buffer (pH 7.5, 1 mM), 5,5' dithio(2-nitrobenzoic acid) (DNTB, 3 mM) and NADPH (2 mM). The reaction was initiated with the addition of reduced glutathione (GSH) (20 mM) and 50 μl of testicular homogenate. The 5-thio-2-nitrobenzoic acid (TNB) complex formed was read at 412 nm for 30 min with 30 seconds interval, and enzyme activity expressed as mmol of NADPH per mg of protein [21].

Catalase activity

Trolox, MEBa (0.01, 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) or blank (0.025% DMSO) were added into test tubes containing H_2O_2 (30 mM) and phosphate buffer (pH 7.2, 0.1 M). Testicular homogenate (200 μl) was added, and absorbance recorded at 240 nm after 30, 60 and 90 seconds. Catalase activity was expressed as IU/mg protein [22].

Superoxide dismutase (SOD) activity

Carbonate buffer (pH 10.2, 50 mM) was introduced in all test tubes and the testicular homogenate (134 μl) was added to the tubes except the blank. Trolox, MEBa (0.01, 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) or 0.025% DMSO were added in the tubes, and the reaction initiated by adding epinephrine (0.6 mg/l). The absorbance was then read at 480 nm after 20 and 80 seconds. SOD activity was expressed as IU/ mg protein [22].

Phytochemical studies of MEBa

Chemical tests for the screening and identification of the groups of bioactive chemical constituents such as phenols, alkaloids, steroids, flavonoids, saponins, limnoids, coumarins and terpenoids, were conducted in MEBa using the standard procedures as described elsewhere [23, 24].

Statistical analyses

The half efficient concentration (EC₅₀) of MEBa (concentration of MEBa required to scavenge 50% DPPH or to induce a 50% inhibition of any other parameter) was determined, and differences between treatments assessed by one factor ANOVA followed by the Student-Newman-Keuls test. The P values less than 0.05 were considered statistically significant. All analyses were performed using GraphPad Prism 5 software.

RESULTS

Effect of MEBa on non-enzyme antioxidant parameters

The effect of MEBa on the discoloration of DPPH, degradation of β-carotene, reduction of ferric ions and lipid peroxidation is summarized in Table 1. When compared to the reference antioxidant compound trolox, MEBa moderately inhibits β-carotene oxidation (EC₅₀: 37.63 ± 12.02 μg/ml vs. 0.66 ± 0.15 μg/ml, P < 0.001) and slightly increases the discoloration of DPPH (EC₅₀: > 200 μg/ml vs. 15.23 ± 0.57 μg/ml, P < 0.001). However, MEBa showed similar effect as trolox in preventing lipid peroxidation (EC₅₀: 2.78 ± 0.47 μg/ml vs. 2.27 ± 0.52 μg/ml, P > 0.05), although it significantly reduced ferric ions (EC₅₀: 0.63 ± 0.19 μg/ml vs. 3.62 ± 0.4 μg/ml; P < 0.001).

Effect of MEBa on antioxidant enzyme activities

The concentrations of MEBa ≥ 0.1 μg/ml stimulated GR activity (P < 0.01) and the same observation was noted with the reference compound trolox (Fig.1A). Both MEBa and trolox showed biphasic pattern on catalase activity (Fig.1B). The maximum stimulation of the enzyme was observed with 1 μg/ml and 10 μg/ml of trolox and MEBa (P < 0.001 for both) followed by a substantial decrease of the activity of this enzyme. SOD activity (Fig.1C) was stimulated with MEBa and trolox and MEBa also showed biphasic effect with significant stimulatory activity at 100 μg/ml (P < 0.05).

Phytochemical content of MEBa

MEBa contains phenols (Table 2), a group of compounds exhibiting a variety of antioxidant properties. The extract also displayed significant amounts of steroid and terpenoid compounds, saponins, coumarins and limnoids.

Table 2. Phytochemical compounds in the methanol extract of *Basella alba* (MEBa)

Compounds	Intensity of the coloration	Result of the test
Terpenoids	+++	Strongly positive
Steroids	++	Positive
Phenols	+++	Strongly positive
Saponins	++	Positive
Coumarins	+	Positive
Limonoids	+	Positive
Flavonoids	-	Negative
Alkaloids	-	Negative

Table 1. Effect of the methanol extract of *Basella alba* (MEBa) on non-enzyme antioxidant parameters

Concentration (μg/ml)	DPPH discoloration (%)		β – CLAMS test (%)		FRAP test (mM)		Lipid peroxydation (%)	
	Trolox	MEBa	Trolox	MEBa	Trolox	MEBa	Trolox	MEBa
200 μg/ml	100	30.07 ± 0.28	100	87.25 ± 2.54	0.21 ± 0.01	0.16	93.15 ± 2.61	95 ± 7.07
100 μg/ml	100	16.01 ± 0.31	100	47.70 ± 4.20	0.20 ± 0.01	0.15 ± 0.01	85.21 ± 0.30	88.15 ± 4.46
10 μg/ml	31.07 ± 5.37	4.9 ± 0.51	88.59 ± 1.22	24.18 ± 1.63	0.17 ± 0.01	0.09 ± 0.01	63.13 ± 9.72	55.44 ± 7.69
1 μg/ml	5.23 ± 0.05	4.56 ± 0.04	53.48 ± 2.78	15.24 ± 0.49	0.02 ± 0.01	0.08	33.54 ± 9.13	30.54 ± 6.3
0.1 μg/ml	0.65 ± 0.92	3.26 ± 1.82	39.11 ± 1.75	4.34 ± 1.10	0	0.08	0	7 ± 2.83
0.01 μg/ml	0	0.33 ± 0.06	9.03 ± 2.48	0	0	0.02 ± 0.02	0	0
0.00 μg/ml	0	0	0	0	0	0	0	0
EC ₅₀ (μg/ml)	15.23 ± 0.57	>200*	0.66 ± 0.15	37.63 ± 12.02*	3.62 ± 0.40	0.63 ± 0.19*	2.27 ± 0.52	2.78 ± 0.47

Data are mean ± SEM of 3 independent experiments; *P < 0.001 value different from the positive control (trolox) (Student-Newman-Keuls test).

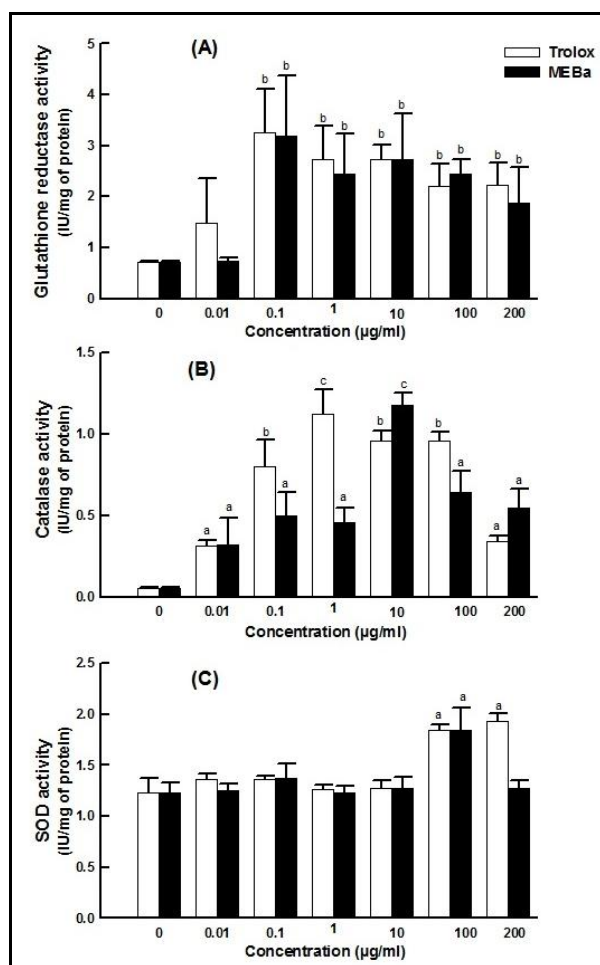


Figure 1. Effect of the methanol extract of *Basella alba* and trolox on the activity of glutathione reductase (A), catalase (B) and superoxide dismutase (SOD) (C). Data represent mean \pm SEM of 3 independent experiences. Values differ from the control (concentration 0 µg/ml) at ^aP < 0.05, ^bP < 0.01, and ^cP < 0.001 (Student-Newman-Keuls test).

DISCUSSION

The powerful oxidants including superoxide anions, hydroxyl radicals and hydrogen peroxide are known as reactive oxygen species (ROS) [25]. At high concentrations, they induce oxidative stress, a deleterious process that can damage all cell structures. They trigger lipid peroxidation, leading to decrease of membrane fluidity, increase of the leakiness, and damage membrane proteins, inactivating receptors, enzymes, and ion channels, *etc.* Peroxidation of polyunsaturated fatty acids also generates free radicals [1, 26]. Therefore, antioxidants are needed in different compartments of the body such as the circulating system, the cell cytoplasm where most cellular activities occur, and across the blood-brain-barrier and central nervous system. The body antioxidant pool can be supplied by diet including plants. Interestingly, many medicinal plants have shown notable antioxidant properties and plant derived products are largely used as antioxidants [5].

In this study, MEBa was screened for its antioxidant activity in the presence of a reference antioxidant compound, namely trolox, an α -tocopherol (vitamin E) analogue. Results showed that MEBa is more efficient than trolox in reducing the ferric iron. This result is similar to those reported by Gulcin *et al* [27] and Huda-Faujan *et al* [28], who demonstrated antioxidant activity on *Pimpinella anisum* seeds extracts and five Malaysian medicinal plants. The ability to reduce Fe(III) may be attributed to hydrogen donation from phenolic compounds [29], which were present in MEBa. In addition to its phenol content, MEBa also contains carotenoids and ascorbic acid that have proven antioxidant properties [12, 13]. The EC₅₀ values of MEBa on DPPH radical, and inhibition of β -carotene oxidation were higher when compared to trolox. However, a dose-dependent effect of the extract suggests its antioxidant activity. Similar EC₅₀ values were reported on the extracts from the plants *Herniaria glabra*, *Persica vulgaris*, *Trigonella foenum*, *Elettaria cardamomum* and *Piper nigrum* [6, 30].

The presence of phenols and other antioxidant compounds may also support the inhibitory effect of MEBa on lipid peroxidation, as demonstrated in this study. Donfack *et al* [31] also reported the inhibiting activity of fractions from *Erythrina senegalensis* on microsomal lipid peroxydation. Membrane lipids present in subcellular organelles are highly susceptible to free radical damage. Lipids, when reacting with free radicals, can undergo a highly damaging chain reaction of peroxidation, leading to both direct and indirect effects. The damage caused by lipid peroxidation is highly detrimental to cell function [5]. MEBa may thus be considered as an interesting candidate to be used in prevention of biomolecule peroxidation, especially lipid peroxidation.

Antioxidant enzymes, including SOD, catalase, and glutathione peroxidase/reductase, convert ROS into nonreactive oxygen molecules [26]. SOD is a well-known antioxidant enzyme which converts the superoxide radical to hydrogen peroxide (H₂O₂) [32]. It is an important enzyme that maintains normal physiological conditions in living cells, and helps the cells to cope with oxidative stress [33]. In fact, SOD spontaneously dismutates the superoxide anion (O₂^{•-}) to form O₂ and H₂O₂, and mainly acts by quenching of the radical molecule [34]. The H₂O₂ is further converted into oxygen and water by the enzyme catalase [35]. Another antioxidant enzyme, GR (NADPH:oxidized-glutathione oxidoreductase), catalyzes NADPH-dependent reaction leading to the regeneration of GSH that is needed for glutathione peroxidase action [36]. In this study, both catalase and SOD enzymes showed biphasic pattern activity in the presence of MEBa or the reference compound trolox.

This is consistent with the regulation of enzyme activity by some effectors [37]. Similar observation was made with angiotensin II on Na⁺/K⁺-ATPase activity on renal proximal tubules [38]. In addition to the effect on SOD and catalase, MEBa stimulated GR. Stimulation of these antioxidant enzymes was also reported by Hamden *et al* [39] in testes of rats treated with *Peganum harmala* extract. This stimulatory effect may be attributed to the protective ability of different antioxidant compounds found in *Basella alba*.

The activity of MEBa on the inhibition of β -carotene oxidation (β -CLAMS) and synthetic free radicals (DPPH, FRAP) supported its antioxidant potential, and is consistent with findings on testicular homogenates, according to which the extract induces/stimulates antioxidant enzyme activities. MEBa thus possesses both ROS scavenging capacity and antioxidant enzymes stimulatory property.

Altogether, our results demonstrated antioxidant properties of MEBa and provided data in favor of this plant as an antioxidant source. Multiple biological effects of *Basella alba* reported earlier, such as improvement of male virility and fertility, could therefore be partly attributed to its antioxidant activity.

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