Antioxidative effects of the methanolic extract of *Hibiscus asper* leaves in mice

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Abstract

While *Hibiscus asper* Hook.f. (Malvaceae) is a traditional herb largely used in the tropical regions of Africa as a vegetable, potent sedative, tonic and restorative, anti-inflammatory and antidepressive drug, there is very few scientific data concerning its efficacy. This study was undertaken in order to evaluate the possible antioxidative effects of the methanolic extract of *H. asper* leaves in laboratory mice. The antioxidant activity of the methanolic extract of *H. asper* leaves (100 mg/kg, 200 mg/kg, 400 mg/kg and 800 mg/kg) was assessed through the determination of two antioxidant enzymes: superoxide dismutase (SOD) and glutathione peroxidase (GPX), as well as a lipid peroxidation marker (malondialdehyde - MDA) in adult male and female Swiss albino mice. In both laboratory mice’s sexes, methanolic extract of *H. asper* leaves showed potent antioxidant activities. Chronic administration of the methanolic extract (daily, for 3 weeks) significantly increased antioxidant enzyme activities (SOD and GPX) and reduced lipid peroxidation (MDA level) in mice temporal lobe homogenates, suggested antioxidant activity.

These results confirm the traditional claims and provide promising baseline information for the potential use of the methanolic extract of *H. asper* leaves as an antioxidant agent in the fight against brain oxidative stress.

**Keywords:** *Hibiscus asper*, extract, antioxidant, mice.
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**Introduction**

Oxidative stress is the condition arising from the imbalance between toxic reactive oxygen species (ROS) and antioxidant systems [1]. Various tissues have different susceptibilities to oxidative stress [2]. Brain is particularly more vulnerable to oxidative damage due to relatively low levels of antioxidants, high levels of polyunsaturated fatty acids, high metal content and oxygen utilization [3, 4]. In this way, lately there has been an increased interest in the relevance of the oxidative stress processes in various neuropsychiatric disorders, as our group has also previously demonstrated [5-9].

On the other hand, herbal therapies can be considered as alternative or complementary medicine. In the search for new molecules useful for the treatment of neurological disorders, worldwide medicinal plant research has progressed constantly, demonstrating the pharmacological effectiveness of different plant species in a variety of animals models [10]. This is reflected in the large number of herbal medicines whose psychotherapeutic potential has been assessed in a variety of animal models. These studies have provided useful information for the development of new pharmacotherapies from medicinal plants and for the new isolated active phytoconstituents.

*Hibiscus asper* Hook.f. (Malvaceae) is an important medicinal plant widely distributed throughout tropical Africa and Madagascar. This species belongs to the genus *Hibiscus* represented by 250 species and characterized by the presence of biological active compounds like flavonoids, phenolic acids, and polysaccharides [11]. In the western region of the Africa, this plant is widely used by the traditional practitioners for the treatment of inflammation, anemia, jaundice, leucorrhoea, poison antidote, depression and dysmenorrhea [12]. In the Western Region of Cameroon the leaves are used as a potent sedative, tonic and restorative. It is also used to treat male infertility and skin infection [13]. Furthermore, recently, we have demonstrated that the methanolic extract of *H. asper* leaves possesses a wide spectrum of biological activities, including *in vitro* antioxidant activity, anxiolytic and antidepressant actions, as well as positive effects on spatial memory formation in Parkinson’s disease animal model, 6-OHDA-lesioned rats. Moreover, we also have demonstrated in the same model that the methanolic extract of *H. asper* leaves may act as a possible antioxidant agent and could confer neuroprotection against the underlying dopaminergic neuron degeneration in the 6-OHDA-lesion rodent model of Parkinson’s disease [14].

In this way, the objective of the present work was to analyze and confirm the possible antioxidant effects of the methanolic extract of *H. asper* leaves in the hippocampus of the normal laboratory mice.

**Materials and Methods**

**Plant material and plant extract**

*H. asper* leaves were collected in Fotouni (Western Region, Cameroon) in May 2010 and identified by Dr. Focho Derreck, Department of Plant Biology (University of Dschang, Cameroon). Voucher specimen was deposited in the Cameroon National Herbarium, Yaoundé, under the number Lucha034 for ready reference. Basically, the preparation of plant extracts was the same as previously described [16]. The leaves of *H. asper* were dried under shade and pulverized. 100 g of the pulverized leaves were macerated in 1L of 90% methanol for 5 days at room temperature (25°C). It was later filtered, and the solvents were separated from the residues by gravity filtration and then evaporated in vacuum. The yield of 12.5 g of crude organic extract was 12.5%.
Animals

30 male and 30 female Swiss albino mice weighing 31-37g (for the male) and 23-35 (for the female) were obtained from the Department of Pharmacodynamic and Clinical Pharmacy, “Gr.T.Popa” University of Medicine and Pharmacy, Iasi, Romania. They were housed in polypropylene cages in the Laboratory of Animal Physiology of the Department of Biology, Alexandru Ioan Cuza University of Iasi, under standard environmental conditions of temperature at 22 ± 1ºC, a 12 h dark-light cycle and allowed free access to water and standard pellet diet throughout the study. The animals were acclimatized for one week before the experiment. Mice were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare from Romania and all procedures were in compliance with the European Council Directive of 24 November 1986 (86/609/EEC). This study was approved by the local Ethic Committee and also, efforts were made to minimize animal suffering and to reduce the number of animal used.

Drug administration

The laboratory mice of both sexes were divided into 5 groups (6 animals per group): (1) Control group (received saline treatment); (2) 4 experimental groups received the methanolic extract of H. asper leaves treatment (100 mg/kg, 200 mg/kg, 400 mg/kg and 800 mg/kg). The methanolic extract of H. asper leaves dissolved in saline was administered orally daily, for 3 weeks, using a mouse gavage feeding needle. Control animals received 0.9% saline solution. In all groups the given volume was 0.1 ml/10 g body weight.

Biochemical parameter assay

3 weeks after the methanolic extract of H. asper leaves administration, all mice were deeply anesthetized (50 mg/kg sodium pentobarbital i.p., Sigma) and were decapitated and their whole brains were removed. The hippocampi were collected. Each of hippocampal samples were weighted and homogenized (1:10) with Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in ice-cold 0.1M potassium phosphate buffer (pH 7.4), 1.15% KCl. The homogenate was centrifuged (15 min at 3000 rpm) and the supernatant was used for assays of SOD, GPX specific activities and MDA level.

Determination of SOD

The activity of superoxide dismutase (SOD) was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 1.5 mL reaction mixture contained 100 mM TRIS/HCl (pH 7.8), 75 mM NBT, 2 µM riboflavin, 6 mM EDTA, and 200 µL of supernatant. Monitorising the increase in absorbance at 560 nm followed the production of blue formazan. One unit of SOD is defined as the quantity required to inhibit the rate of NBT reduction by 50% [15].

Determination of GPX

Glutathione peroxidase (GPX) activity was analyzed with a spectrophotometric assay. A reaction mixture consisting of 1mL of 0.4M phosphate buffer (pH 7.0) containing 0.4mM EDTA, 1mL of 5mM NaN₃, 1mL of 4mM GSH, and 0.2 mL of supernatant had been preincubated at 37°C for 5 min. Then 1mL of 4mM H₂O₂ had been added and incubated at 37°C for further 5 min. The excess amount of GSH was quantified by the DTNB method [16]. One unit of GPX is defined as the amount of enzyme required to oxidize 1 nmol GSH/min.

Determination of MDA

Malondialdehyde (MDA), which is a measure of lipid peroxidation, was spectrophotometrically measured by using the thiobarbituric acid assay [17]. 200 µL of supernatant was added and briefly mixed with 1 mL of 50% trichloroacetic acid in 0.1M HCl and 1 mL of 26 mM thiobarbituric acid. After vortex mixing, samples were maintained at 95°C for 20 min. Afterwards, samples were centrifuged at 3000 rpm for 10 min and supernatants were read at 532 nm. A calibration curve was constructed using MDA as standard and the results were expressed as nmol/mg protein.
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**Estimation of protein concentration**

Protein was measured using the dye binding method of Bradford [18], and bovine serum albumin (BSA) was used as a standard.

**Statistical analysis**

All results are expressed as mean ± S.E.M. Statistical analyses were performed using one-way analysis of variance (ANOVA). Significant differences were determined by Tukey’s *post hoc* test. *F* values for which *p*<0.05 were regarded as statistically significant. Pearson’s correlation coefficient and regression analysis was used to evaluate the connection between the antioxidant defence and lipid peroxidation.

**Results**

**Effect of *Hibiscus asper* extract on SOD and GPX activities**

Biochemical analyses showed a significant increase of the main enzymatic antioxidant defences (SOD and GPX specific activities) estimated in the hippocampal homogenates of both male and female groups treated with the methanolic extract of *H. asper* leaves, suggesting that this plant extract has strong antioxidant proprieties.

Figure 1A shows a significant increase of the SOD specific activity (*F*(2,27)=6.32, *p*<0.001) in male groups treated with the methanolic extract of *H. asper* leaves compared to the control group. Also, Tukey’s *post hoc* analyses revealed significant statistical differences between the control and the *H. asper* (200 mg/kg) groups (**p**=0.005), the control and the *H. asper* (400 mg/kg) groups (**p**=0.01), the control and the *H. asper* (800 mg/kg) groups (**p**=0.01), the *H. asper* (100 mg/kg) and the *H. asper* (400 mg/kg) groups (**p**=0.01) and the *H. asper* (100 mg/kg) and the *H. asper* (800 mg/kg) groups (**p**=0.01) for SOD specific activity (Figure 1A).

Figure 1B shows a significant increase of the SOD specific activity (*F*(2,27)=6.29, **p**<0.001) in female groups treated with the methanolic extract of *H. asper* leaves compared to the control group. Additionally, Tukey’s *post hoc* analyses revealed significant statistical differences between the control and the *H. asper* (100 mg/kg) groups (**p**=0.0001), the control and the *H. asper* (200 mg/kg) groups (**p**= 0.01), the control and the *H. asper* (400 mg/kg) groups (**p**=0.007), the control and the *H. asper* (800 mg/kg) groups (**p**=0.001) and the *H. asper* (100 mg/kg) and the *H. asper* (800 mg/kg) groups (**p**=0.01) for SOD specific activity (Figure 1B).

![Figure 1A](image1.png)

**Figure 1.** Effects of the methanolic extract of *H. asper* leaves (100, 200, 400 and 800 mg/kg) on SOD specific activity in male (A) and female (B) mice hippocampal homogenates. Values are means ± S.E.M. (n=6 animals per group).

We also observed a significant increase of the GPX specific activity (*F*(2,27)=4.23, **p**<0.01) (Figure 2A) in male groups treated the methanolic extract of *H. asper* leaves compared to the control group. Also, Tukey’s *post hoc* analyses revealed significant statistical
differences between control and *H. asper* (100 mg/kg) groups (p=0.001) and control and *H. asper* (400 mg/kg) groups (p=0.01) for GPX specific activity (Figure 2A).

In addition, a significant increase of the GPX specific activity (F(2,27)=3.54, p<0.01) (Figure 2B) in the female groups treated the methanolic extract of *H. asper* leaves compared to the control group. Also, post hoc analyses revealed significant statistical differences between the control and the *H. asper* (100 mg/kg) groups (p=0.01), the control and the *H. asper* (400 mg/kg) groups (p=0.01), the control and the *H. asper* (800 mg/kg) groups (p=0.01), the *H. asper* (100 mg/kg) and the *H. asper* (200 mg/kg) groups (p=0.001), the *H. asper* (200 mg/kg) and the *H. asper* (400 mg/kg) groups (p=0.01) and the *H. asper* (200 mg/kg) and the *H. asper* (800 mg/kg) groups (p=0.01) for GPX activity (Figure 2B).

**Figure 2.** Effects of the methanolic extract of *H. asper* leaves (100, 200, 400 and 800 mg/kg) on GPX specific activity in male (A) and female (B) mice hippocampal homogenates. Values are means ± S.E.M. (n=6 animals per group).

**Effect of Hibiscus asper extract MDA level**

Male mice groups treated with the methanolic extract of *H. asper* leaves exhibited attenuation in lipid peroxidation, indicated by a significant decrease of MDA level (F(2,27)=22.07, p<0.0001) (Figure 3A) estimated in the hippocampal homogenates compared to the control group. Also, Tukey’s post hoc analyses revealed significant statistical differences between the control and the *H. asper* (100 mg/kg) groups (p=0.001), the control and the *H. asper* (200 mg/kg) groups (p=0.001), the control and the *H. asper* (400 mg/kg) groups (p=0.001), the control and the *H. asper* (800 mg/kg) groups (p=0.001), the *H. asper* (100 mg/kg) and the *H. asper* (800 mg/kg) groups (p=0.05), the *H. asper* (200 mg/kg) and the *H. asper* (800 mg/kg) groups (p=0.001) and the *H. asper* (400 mg/kg) and the *H. asper* (800 mg/kg) groups (p=0.01) for MDA level (Figure 3A).

In addition, female mice groups treated with the methanolic extract of *Hibiscus asper* leaves also exhibited attenuation in lipid peroxidation, indicated by a significant decrease of MDA level (F(2,27)=15.66, p<0.0001) (Figure 3B) estimated in the hippocampal homogenates compared to control group. Also, Tukey’s post hoc analyses revealed significant statistical differences between the control and the *H. asper* (100 mg/kg) groups (p=0.05), the control and the *H. asper* (200 mg/kg) groups (p=0.001), the control and the *H. asper* (400 mg/kg) groups (p=0.01), the control and the *H. asper* (800 mg/kg) groups (p=0.001), the *H. asper* (100 mg/kg) and the *H. asper* (800 mg/kg) groups (p=0.001), the *H. asper* (200 mg/kg) and the *H. asper* (800 mg/kg) groups (p=0.001) and the *H. asper* (400 mg/kg) and the *H. asper* (800 mg/kg) groups (p=0.01) for MDA level (Figure 3B).

These results support the hypothesis that the methanolic extract of *H. asper* leaves may have induced a decrease in neuronal oxidative stress in both male and female mice groups.
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![Figure 3](image)

**Figure 3.** Effects of the methanolic extract of *H. asper* leaves (100, 200, 400 and 800 mg/kg) on MDA level in male (A) and female (B) mice hippocampal homogenates. Values are means ± S.E.M. (n=6 animals per group).

Additionally, a significant positive correlation between GPX vs. MDA (n=30, r=0.612, p=0.004) (Figure 4) was evidenced by determination of the linear regression in male groups exposed to the methanolic extract of *H. asper* leaves. Still, no significant correlation between SOD vs. MDA (n=30, r=-0.361, p=0.118) was observed.

In female mice groups, no significant correlations between SOD vs. MDA (n=30, r=-0.183, p=0.439) and GPX vs. MDA (n=30, r=0.400, p=0.081) was evidenced by determination of the linear regression.

![Figure 4](image)

**Figure 4.** Correlation between GPX vs. MDA in the control group and in the methanolic extract-treated groups.

**Discussion**

In the present paper we confirmed the possible antioxidant status for the methanolic extract of *H. asper* leaves in the hippocampi of laboratory mice, the most susceptible brain area to the effects of the oxidative stress [19]. These effects were mainly expressed through a general increase in the specific activities of both antioxidant enzymes determined here (SOD and GPX), as well as a decrease of the central lipid peroxidation processes, demonstrated by the decreased levels of MDA, as compared to control mice.

As previously mentioned [14], our preliminary phytochemical screening revealed the presence of flavonoids, polyphenols and saponines in the methanolic extract of this plant. In this way, SOD, which is the first line of defense against oxidative stress development, is increasing its activity, acting on the superoxides radicals [1]. Additionally, the increased specific activity of GPX could be explained by the possible increased levels of its substrate.
glutathione (GSH), which was previously reported to be stimulated by the flavonoids (the largest group of polyphenols present in many plants, known to promote a number of physiological benefits, especially in cognitive function and memory impairment [20] and also antioxidant activity), through transactivation of the gamma-glutamylcysteine synthetase catalytical subunit promoter [21].

In this way, the increased activity of SOD results in a decrease production of ROS, as we previously mentioned. After that, the resulting hydrogen peroxide is degraded by GPX in its high concentrations. As described, since GPX activity is dependent on its substrate GSH, this enzyme is also offering a strong antioxidant protection, considering that GPX is functioning optimally in the stimulated GSH concentrations.

The explaining mechanism for these aspects consists in the fact that SOD and GPX are critical antioxidant enzymes that act cooperatively at different sites in the metabolic pathway of free radicals, and increased levels of both these enzymes offer an optimal protection against oxidative stress [2].

In this way, superoxide radical, spontaneously or with the catalyses of SOD, is converted to hydrogen peroxide, which is then educed to water and oxygen molecules by the catalyses of GPX. The findings of the present study suggest that increased SOD and GPX activity are decreasing reactive oxygen species production.

In this way, this might explain the decreased lipid peroxidation processes, which are mainly expressed through the reduced MDA levels. These aspects are also confirmed by the significant correlations which were found between the specific activity of GPX and the decreased levels of MDA from the hippocampus (Fig. 4).

The aforementioned results could be extremely important, considering that currently the exact mechanism which results in the generation of various neuropsychiatric disorders are not fully understood.

Regarding the effects of the different doses of extract which we used in the present protocol, we observed that the dose of 100 mg/kg resulted in a significant increase of both antioxidant enzymes (SOD and GPX) in the females, as compared to all other doses (Fig. 1B and Fig. 2B). On the other side, in the male groups both antioxidant enzymes were significantly increased in the 200 mg/kg, 400 mg/kg and 800 mg/kg doses, when compared to the smaller dose of 100 mg/kg (Fig. 1A and Fig. 2A). Additionally, in the case of the lipid peroxidation marker MDA, we could observe a significant decrease of the MDA levels especially in the 800 mg/kg dose, as compared to all other doses used, in both sexes this time (Fig. 3A and Fig. 3B).

Additionally, some other experiments regarding the possible effects of the methanolic extract of *H. asper* leaves on some additional animal models are undergoing now in our lab.

Conclusions

We showed the possible antioxidant effect for the methanolic extract of *H. asper* leaves in the hippocampi of the laboratory mice. These effects were mainly expressed through a general increase in the specific activities of both antioxidant enzymes determined here (SOD and GPX), as well as a decrease of the MDA concentration, as a marker of the lipid peroxidation processes.

References

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