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Neuroprotective Effects of *Khaya Anthotheca* (Welw.) C.DC (Meliaceae) Decoction on Neurodegeneration Induced by Estrogen Depletion in Rats

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Abstract

Background: Recently, we reported estrogen-like and anxiolytic effects of the decoction of *Khaya anthotheca* in ovariectomized rats. **Rationale:** The purpose of the present study was to assess the putative neuroprotective properties of the plant in ovariectomized rats. **Methods:** Thirty female Wistar rats were ovariectomized, while 6 were used as sham. After 14 days of endogenous hormonal decline, animals were randomly divided into six groups (n=6) and administered with distilled water, *K. anthotheca* decoction (125, 250 and 500 mg/kg doses) or estradiol valerate (1 mg/kg) for 28 days. Phytochemical analysis and antioxidant potential of the decoction were determined. Levels of oxidative stress biomarkers were determined in brain homogenates, while histopathological analysis was performed on brain sections, and expressions of neuroinflammation markers determined. **Results:** Polyphenols were detected in *K. anthotheca*, and the ferric reducing antioxidant power (FRAP) assay revealed antioxidant properties (635.50 ± 0.58 mg eq quercetin/g of dried decoction). Treatment with *K. anthotheca* decoction reduced MDA and increased GSH levels in brain homogenates ($p<0.01$). As estradiol valerate, the decoction, prevented neurodegeneration observed in cortices and hippocampi of untreated ovariectomized animals. **Conclusions:** Our results suggested that *K. anthotheca* is endowed with neuroprotective effects and warrant further studies, including other models of neurodegeneration and dementia.

Keywords: *Khaya anthotheca*, neuroprotection, antioxidative effects, ovariectomy, estrogen depletion, neurodegeneration, oxidative stress.

Abbreviations: BW: Body Weight; CA1: Cornu Ammonis 1; Casp. 3: Caspase 3; Caspase 3: Cysteine-aspartic protease type 3; E2V: Ovariectomized animals treated with estradiol valerate; Fas FAS: Fas cell surface dead receptor; FRAP: Ferric Reducing Antioxidant Power; GSH: Glutathione; H&E: Hematoxiline and Eosine; Iba-1: Ionized calcium-binding adapter molecule 1; *K. anthotheca*: *Khaya anthotheca*; KA 125: Ovariectomized animals treated with *Khaya anthotheca* decoction at doses 125 mg/kg; KA 250: Ovariectomized animals treated with *Khaya anthotheca* decoction at doses 250 mg/kg; KA 500: Ovariectomized animals treated with *Khaya anthotheca* decoction at doses 500 mg/kg; KA: Ovariectomized animals treated with *Khaya anthotheca* decoction; MDA: Malondialdehyde; NOVX: Sham operated animals treated with vehicle solution; OVX: Ovariectomized animals treated with vehicle solution

Introduction

The cortex, hippocampus, and amygdala are among the brain regions where estrogens may act to improve cognition, memory and mood. In these regions, estrogens have many beneficial effects, including neuroprotection, neurogenesis, as well as neurotrophic, antioxidant, and anti-inflammatory effects [1-2]. Estrogenic compounds induce these effects partly by decreasing the activity of activated brain macrophages [2-3]. These beneficial effects are lost at the menopause, as estrogen production stops. Unsurprisingly, estrogen depletion at the menopause was reported to associate with cognitive decline, depression, and anxiety [4], but also with several systemic detrimental alterations, including changes in plasmatic lipid fractions and enhanced oxidative activity [5-6].

In laboratory rodents, experimental menopause induced by ovariectomy is accompanied by oxidative damage in the brain [7]. Oxidative stress emerges due to the failure of the antioxidant defense mechanisms that are estrogen-dependent [8]. Oxidative stress is a hallmark of central nervous system diseases, particularly mood disorders and neurodegenerative diseases [9]. The impaired oxidant/antioxidant profile observed in menopause can be reversed by dietary polyphenols, underlining the potential benefits of these natural antioxidants [10-11]. Moreover, medicinal plants are a source of a wide variety of natural products with antioxidant properties [12-13].

Khaya anthotheca (Welw.) C.DC is a plant of Meliaceae family used in African traditional medicine to treat diseases helmenthiasis, malaria, gonorrhea, abdominal pain and migraine [14-15]. The *Khaya* genus is also used for the treatment of convulsion, fever, cough, stomach ache, rheumatism and dermatomycosis [16]. In Cameroon traditional medicine, the aqueous extract of *Khaya anthotheca* is empirically used to manage anxiety and to alleviate vaginal dryness in postmenopausal women [17]. We reported recently that *K. anthotheca* decoction displayed estrogen-like and anxiolytic effects following acute treatment [17] and ameliorate negative effects of vanadium inducing anxiety, memory loss and pathologies in the brain and ovary of mice [18]. To our knowledge, no study addressed the neuroprotective potential of *K. anthotheca* on menopausal model, despite the potent estrogen-like properties of this medicinal plant. Thus, in the present study we assessed the neuroprotective effects of *K. anthotheca* in ovariectomy-induced neurodegeneration context.

Material and Methods

Animals

Juvenile female Wistar rats (10-12 weeks old, 150 ± 10 g, n=36) were used in this study. Most animals were ovariectomized (n=30) while the remainder (n=6) were processed as Sham. The rats were bilaterally ovariectomized using the dorsal approach [19] under diazepam and ketamine anesthesia (10mg/kg and 50mg/kg BW; i.p., respectively). The animals were housed in an environmentally controlled room (temperature 25°C; humidity 50-80%; 12 h light–dark cycle). They had free access to a standard soy-free rat diet (SSniff GmbH, Soest, Germany) and had ad libitum access to tap water.

All procedures were performed in accordance with the European Community Guidelines for Laboratory Animal Use and Care (EEC Directive of 1986; 86/609/EEC).

Experimental design

On day 14 post surgery, animals were randomly divided in six groups (n=6 per group):

- Sham operated animals treated with vehicle solution (distilled water) (NOVX);
- Negative control group including ovariectomized animals treated with vehicle solution (OVX);
- Positive control group including ovariectomized animals treated with estradiol valerate (1 mg/kg BW, Progynova® 2 mg, DEL- PHARM, Lille, France) (E2V);
- And 3 groups made of animals treated with *K. anthotheca* decoction at doses 125 mg/kg BW (KA 125), 250 mg/kg BW (KA 250), and 500 mg/kg BW (KA 500).

All treatments were administered orally (2 mL/100 g). Body weight was determined weekly for 4-weeks. After 28-days of treatment, rats were fasted overnight (10–12h), then sacrificed under deep anesthesia (10 mg/kg BW diazepam and 50 mg/kg BW ketamine, i.p.). Brain was dissected out and weighed. Left hemispheres were processed for preparing homogenates. Right hemispheres were fixed in 10% formaldehyde and processed for histopathological studies.

Plant material processing

Samples of stem bark of *K. anthotheca* were collected in Mamougnam (District of Massangam, Noun Department, Cameroun) with the help of experts. The botanical samples were further identified at the National Herbarium of Cameroon (HNC) by comparing to available specimens and stored (voucher number 4230/HNC). The decoction was prepared as described previously [17].

Phytochemical screening and antioxidant potential analysis

Concentrations of various potentially bioactive phytochemicals, such as polyphenols and flavonoids were measured according to the methods described by Singelton *et al.* [20] and Zhishen *et al.* [21], respectively. The antioxidant potential of the decoction was determined using the ferric

reducing antioxidant power (FRAP) assay [22]. The tests were performed in triplicates (three-independent experiments).

Brain homogenate analysis and histopathological studies

Left hemispheres were processed to prepare homogenates (10% w/v in 0.1 M phosphate buffer, pH 7.4). The supernatant was collected after centrifugation and was used to estimate lipid peroxidation and reduced glutathione levels based on the estimation of their markers (MDA and GSH, respectively), according to methods described by Ohkawa *et al.* [23] and Ellman [24], respectively.

Right hemispheres were imbedded in paraffin and cut transversely (4 µm). Serial sections were processed either for H&E staining or immunohistochemical labelling using standard protocols and kit manufacturer's instructions (ABCAM, Cambridge, UK). Primary antibodies were goat anti-caspase 3 (marker of apoptosis), goat anti-Fas (marker of cell-cell-mediated neuronal death), and rabbit anti-iba1 (marker of activated microglia) (dilution 1:100, Santa Cruz Biotechnology, CA). The secondary antibodies, DAB, and the blocking solutions were provided by the kit manufacturer (ABCAM). All sections were counterstained with hematoxylin, except for iba1-labelled sections. Tris-EDTA buffer (1 mM EDTA solution, 10 mM Tris base, and 0.05% Tween 20 in distilled water, pH 9) was used for heat-induced antigen retrieval, while rinsing was performed using Tris-TBS buffer (0.1% Tween 20 in Tris-buffered saline, pH 7.6).

Statistical analysis

Statistical significance of differences between estradiol valerate/decoction-treated groups and OVX or Sham groups were determined using one-way ANOVA followed by Dunnett's post-hoc test. Statistical significance of differences between OVX and NOVX groups were determined using unpaired t-test (GraphPad Prism, version 5.03). A p-value <0.05 was considered significant. Data were expressed as mean ± S.E.M.

Results

Phytochemical analysis and FRAP antioxidant assay

Polyphenols were present in *K. anthotheca*, including flavonoids. The concentrations of these compounds are depicted in Table 1. The antioxidant activity of the extract was confirmed by the FRAP assay, which revealed a total antioxidant potential of 635.50 ± 0.58 mg eq quercetin/g of dried decoction (Table 1).

Effects of *Khaya anthotheca* on body weight

Animal's weight increased from treatment day 8 to treatment day 28 in control ovariectomized animals. OVX group displayed a gradual increase in body weight ($p<0.001$ from treatment day 28) compared to the sham group (Figure 1A). After 28-days of treatment, the decoction at all tested doses, induced a significant reduction of the body weight ($p<0.001$compared to OVX group) (Figure 1B). Comparable effects were observed in E2V group ($p<0.001$compared to OVX group).

Effects of *Khaya anthotheca* on oxidative stress markers

Table 2 depicts the effects of *K. anthotheca* treatment on MDA and GSH levels in brain homogenates. Increases in MDA ($p<0.01$) and decreases in GSH ($p<0.05$) levels were observed in OVX group compared to NOVX group. Compared to OVX group, rats treated with *K. anthotheca* decoction showed a significant reduction in MDA levels at all tested doses ($p<0.05$). Significant increases in GSH levels were observed only at the highest doses tested (250 mg/Kg, $p<0.05$; and 500 mg/Kg, $p<0.01$).

Effect of *Khaya anthotheca* on brain weight and tissue

A significant decrease in brain weight ($p<0.05$) was observed in untreated ovariectomized rats compared to NOVX group (Figure 2). However, no significant change in this parameter was observed following treatment with other substances.

Table 1: Phenolic content and in vitro antioxidant capacity of *Khaya anthotheca* extract.

Parameters	**Phenolic Concentration in *Khaya anthotheca* dried extract** (mg eq quercetin/g of dried extract)
Total polyphenols	275.13 ± 0.90
Total flavonoids	0.04 ± 0.00
	***In vitro* antioxidant capacity** (mg eq quercetin/g of dried extract)
Total antioxidant potential	635.50 ± 0.58

Data are mean ± SEM of triplicates from at least three-independent experiments.

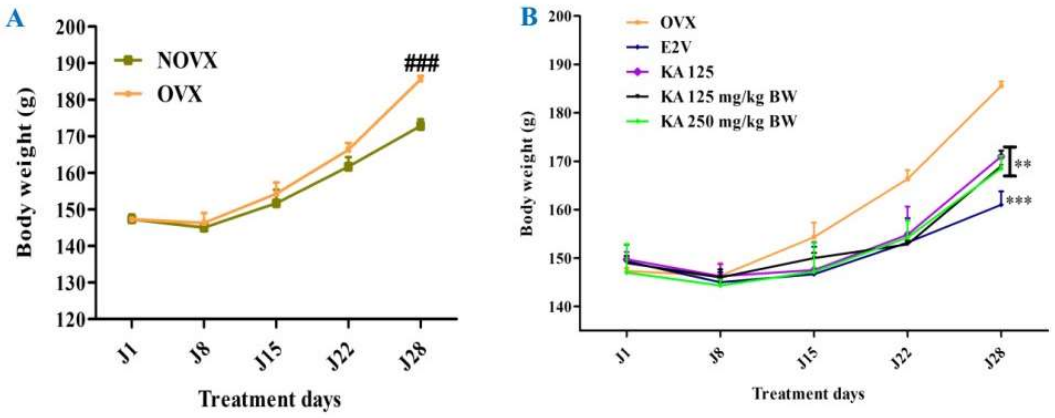

Figure 1: Effect of estrogen depletion (A) and *Khaya anthotheca* extract (B) on body weight after 28-days of treatments. Data are mean ± SEM, n=6 per group. NOVX: Sham operated animals treated with the vehicle; OVX: Ovariectomized animals treated with the vehicle; E2V: Ovariectomized animals treated with estradiol valerate at 1 mg/kg BW; KA: Ovariectomized animals treated with the aqueous extract of stem bark extract of *Khaya anthotheca* at doses of 125, 250 and 500 mg/kg BW. *** p<0.001 vs. OVX (one-way ANOVA followed by Dunnett's test). ### p<0.001 vs. NOVX (unpaired t-test).

Table 2: Oxidative stress status in whole brain after a 28-days of treatment with the aqueous extract of stem bark of *Khaya anthotheca* in ovariectomized Wistar rats**.**

Parameters	NOVX	OVX	E2V	Doses of KA (mg/kg BW)		
				125	250	500
MDA (µmol)	1.26 ± 0.07	1.49 ± 0.02##	1.23 ± 0.04**	1.33 ± 0.04*	1.31 ± 0.06*	1.26 ± 0.06**
GHS (µmol)	9.45 ± 0.32	8.35 ± 0.22#	8.16 ± 0.11αα	8.97 ± 0.30	9.25 ± 0.09*	9.34 ± 0.17**

Data are mean ± SEM, n = 6 per group. NOVX: Sham operated animals treated with the vehicle; OVX: ovariectomised animals treated with the vehicle; E2V: ovariectomized animals treated with estradiol valerate at 1 mg/kg BW; KA: ovariectomized animals treated with the aqueous extract of stem bark extract of *Khaya anthotheca* at the doses of 125, 250 and 500 mg/kg BW. * p<0.05, ** p<0.01 vs. OVX; αα p<0.01 vs. NOVX (one-way ANOVA followed by Dunnett's test). # p<0.05, ## p<0.01 vs. NOVX (unpaired t-test).

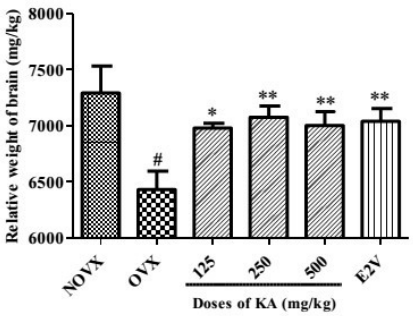

Figure 2: Effect of estrogen depletion and *Khaya anthotheca* extract on brain weight. Data are mean ± SEM, n=6 per group. NOVX: Sham operated animals treated with the vehicle; OVX: Ovariectomized animals treated with the vehicle; E2V: Ovariectomized animals treated with estradiol valerate at 1 mg/kg BW; KA: Ovariectomized animals treated with the aqueous extract of stem bark extract of *Khaya anthotheca* at the doses of 125, 250 and 500 mg/kg BW. ** p<0.01 vs. OVX (one-way ANOVA followed by Dunnett's test). #p<0.05 vs. NOVX (unpaired t-test).

Brain tissue observation revealed marked histopathological signs such as pericellular vacuolation, perivascular oedema. Indicators of apoptosis were also observed in pyramidal cells of the cortex (Figure 3B) and hippocampal CA1 layer (Figure 3J), including central chromatolysis, cell shrinkage, and cell vacuolation in ovariectomized animals. These signs were not observed in the sham group (Figure 3A), and were markedly improved by treatment with estradiol valerate (Figure 3C) or *K. anthotheca* decoction (Figure 3D).

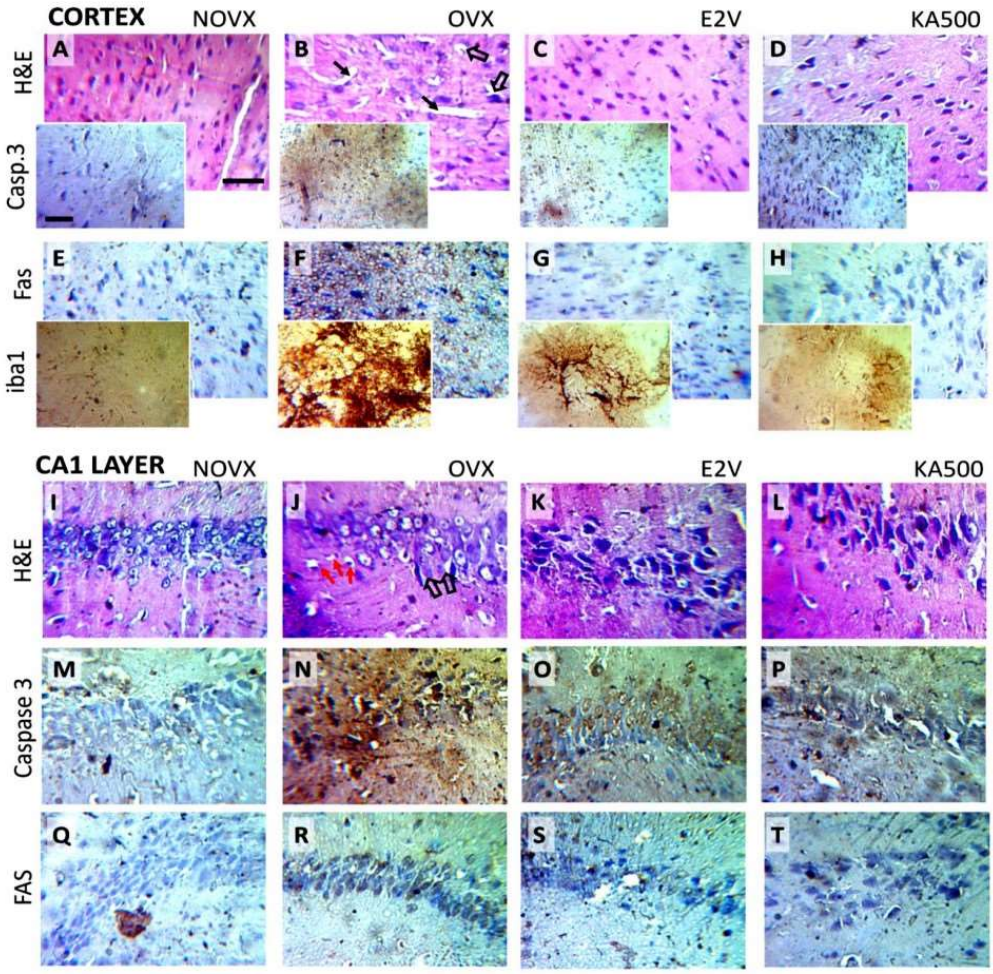

Figure 3: Effect of Khaya anthotheca extract on the microarchitecture of the brain cortex and hippocampus (CA1 layer). NOVX: Sham operated animals treated with the vehicle; OVX: Ovariectomized animals treated with the vehicle; E2V: Ovariectomized animals treated with estradiol valerate at 1 mg/kg; BW KA: Ovariectomized animals treated with the aqueous extract of stem bark extract of *Khaya anthotheca* at the doses of 500 mg/kg BW. Scale bar: 50 µm.

A-D. H&E stained brain cortices of representative sham animal (NOVX) (**A**), and ovariectomized animals administered with vehicle (OVX) (**B**), oestradiol valerate (E2V) (**C**), and *K. anthotheca* extract (KA) (**D**). Insets show the immunohistochemical expressions of caspase 3 in cortices of the same animals. Note that the fluid leakage (perivascular oedema) (black arrows), cell vacuolation (empty arrow) (**B**), and marked expression of caspase 3 (inset) in ovariectomized animals were improved in animals treated with oestradiol valerate (**C**), and *K. Anthotheca* (**D**).

E-H. Immunohistochemical expressions of Fas in representative animals of NOVX (**E**), OVX (**F**), E2V (**G**), and KA (**H**) groups. Insets show the immunohistochemical expressions of iba1 in cortices of the same animals. Note the marked expressions of Fas (**F**) and iba1 (inset) in ovariectomized animals.

I-L. H&E stained CA1 layer of hippocampi of representative animals of NOVX (**I**), OVX (**J**), E2V (**K**), and KA (**L**) groups. Note the pericellular vacuolation (empty arrow) and cell shrinkage (red arrow) (**J**) in ovariectomized animals.

M-T. Immunohistochemical expressions of caspase 3(**M-P**) and Fas (**Q-T**) in CA1 layers of representative animals of NOVX (**M, Q**), OVX (**N, R**), E2V (**O, S**), and KA (**P, T**) groups. Note the marked expressions in ovariectomized animals (**N, R**).

All the markers immunolabeled (caspase 3, Figures 3B, N; Fas and iba1) were over expressed in brains of ovariectomized animals (Figures 3F, and 3R), compared to sham group (Figures 3A, 3E, 3M and 3Q), and were markedly improved by treatments with estradiol valerate (Figure 3C, 3G, 3O and 3S) or *K. anthotheca* decoction (Figure 3D, 3H, 3P, 3T). The decoction effects increased with the dose (data not shown).

Discussion

In the present study, ovariectomized animals displayed significant body weight increase compared to sham group, 42-days after ovariectomy. This observation is in accordance with previous reports [25,26] and with observations in women undergoing natural menopause, where gradual loss of estrogen contributes to weight gain [27]. Estrogen deficiency results in the alteration of energy metabolism, which in turn results in abdominal fat increases [28,29]. Weight increase was not observed in ovariectomized animals treated with estradiol valerate or *K. anthotheca* decoction, suggesting that some constituents of this plant may have estrogen-like properties. These effects may be mediated partly by phytoconstituent families with well characterized phytoestrogen potential such as polyphenols, whose presence in *K. anthotheca* was revealed in the present study by phytochemical analyses.

Ovariectomy causes an oxidative stress deleterious for neurons in rats [7,30], as estrogen-mediated activation of cellular anti-oxidant systems is lacking [31]. In the present study, increase in the levels of lipid peroxidation marker MDA and decrease in the levels of intracellular radical scavenger GSH were observed in untreated ovariectomized rats compared to their sham counterparts. Treatment with *K. anthotheca* decoction improved (decreased) MDA levels, suggesting a mitigation LDL peroxidation [32]. In addition, *K. anthotheca* decoction increased GSH level, thus, probably mitigated the accumulation of hydroperoxides resulting from estrogen depletion [33,34]. Altogether, these observations suggest that *K. anthotheca* decoction is endowed with a strong antioxidant activity, further supporting the hypothesis of the presence of constituents with estrogen-like properties. The antioxidant properties of *K. anthotheca* were confirmed by ferric reducing antioxidant power (FRAP) assay. Polyphenols, a class of compounds reported antioxidant effects on brain tissue [35], was detected in *K. anthotheca* decoction in the present study.

Marked histopathological signs, including indicators of neuronal damage and loss, were observed in brain cortices and hippocampi (CA1 layer) of ovariectomized animals. As discussed above, these damages can be explained by oxidative stress. Like estradiol valerate, treatment with *K. anthotheca* decoction mitigated these signs, possibly due to estrogen-like effects [36]. In addition, we observed over expressions of markers of neuroinflammation in brains of untreated ovariectomized animals, but not in those treated with estradiol valerate or *K. anthotheca* decoction, suggesting a neuroprotective activity. Decreases in expressions of microglia inflammation marker iba1, of the marker of cell-cell-mediated neuronal death Fas, and of the apoptosis marker caspase 3 observed in this study may emerge from the well-established anti-inflammatory properties of estrogenic compounds [3,31,36]. On the same hand, a decrease in brain mass probably due to cell loss was observed in untreated ovariectomized rats at the end of the experiment, but not in their counterparts treated with *K. anthotheca* decoction, confirming neuroprotective effects.

Estrogen depletion at the menopause was reported to associate with cognitive decline [4]. Alzheimer's disease is the most common form of dementia and is characterized by a progressive decline in cognitive function [37]. In this study, the *K. anthotheca* extract shows neuroprotective properties effects on ovariectomized rat mediated partly by anti-apoptotic, anti-inflammatory and antioxidative effects. For that, it is important to evaluate other beneficial effects of the extract in other experimental models of neurodegeneration and dementia.

Conclusions

The present data shows a remarkable increase in the body weight, increased oxidative stress, and neuronal loss in Wistar rats 42-days after ovariectomy. These alterations were prevented or improved by subchronic treatment with *K. anthotheca* decoction, indicating that some constituents of *K. anthotheca* may have estrogen-like properties. The neuroprotective properties were mediated partly by anti-apoptotic, anti-inflammatory and antioxidative effects. These observations justify the traditional medicine used of this plant and warrant further studies, including in other experimental models of neurodegeneration and dementia.

Conflict of Interest

All the authors declare no conflicts of interest within this article.

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Authors' Contributions

ZGF conceived the study and performed the experiments, data collection and data analysis as well as the first draft manuscript writing. DS participated in the study conception, data analysis and manuscript proof reading. SEPF participated

in the experiments, data collection and data analysis as well as manuscript proof reading. PCA and BAY participated in the experiments, data collection and data analysis. ACF and DTRN participated in the experiments and data collection. ND participated in the study conception and supervision of data collection and analysis as well as the manuscript preparation.

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