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Neuroprotective effect of Baillonella toxisperma Pierre on the oxidative stress status in an experimental animal model of Alzheimer's disease

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ABSTRACT

Recent research findings have shown that neurodegenerative disorders like Alzheimer are partly caused by increased levels of reactive oxygen species. The present study aims to investigate the effect of *Baillonella toxisperma* on an oxidative stress status induced by aluminium chloride in female rats.

Hydroethanolic extract of *B. toxisperma* (HEEBT) was selected after evaluation of antioxidant potential using four methods notably TAC (total antioxidant capacity), scavenging of 1, 1-Diphenyl-2-Picrilhydrazyl (DPPH), hydroxyl radical (·OH) and inhibition of lipid peroxidation. It was used to evaluate neuroprotection in female albino wistar rats that were intoxicated with aluminium chloride.

Twenty-five rats were divided into five groups of five rats each; group 1: control group, group 2: $AlCl_3$ exclusively treated (32.5 mg/kgBW) and served as positive control; group 3, 4 and 5 were treated with $AlCl_3$ and either 150/300mg/kgBW of HEEBT or 100mg/kgBW vitamin E daily for three days 24H after administration of $AlCl_3$. After treatment, animals' blood and brain were collected. Hydroperoxide (ROOH), Malondialdehyde (MDA) and protein thiol levels were determined in brain homogenates while ALT, AST, protein and creatinine levels were evaluated in plasma and catalase in erythrocytes.

The *in vitro* antioxidant activity of the HEEBT was better than for ethanolic and aqueous extracts. Results of biochemical analysis of brains of female rats revealed that $AlCl_3$ significantly ($p<0,05$) increased MDA levels (3,97 vs 7.32 µM) and the activity of catalase (0.020 vs 0.096 mMH2O2/min/mg of proteins) in female rats. In contrast, female rats treated with HEE of *B. toxisperma* (150 and 300 mg/kg/day b.wt) showed a significant decrease ($p<0,05$) of MDA levels (4.43 and 4.58 µM) and the activity of catalase (0.045 and 0.054 mMH2O2/min/mg of proteins). The results obtained for toxicity markers (AST, ALT and creatinine) have the same profile.

Hydroethanolic extract of *B. toxisperma* have antioxidant and neuroprotective effects by scavenging free radical and protecting neurons cells against oxidative damage induced by aluminum chloride.

Keywords: oxidative stress; aluminum chloride; neurotoxicity; *Baillonella Toxisperma,* antioxidant status.

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Introduction

Oxidative stress corresponds to the disturbance of oxidative intracellular status[1] induced by the excessive production of free radicals or by the reduction of the capacity of the antioxidant defense system. Many factors such as metabolic perturbations, inflammation, physical agents, cytokines and exogenous oxidants can lead to the formation of free radicals. Free radicals and thus oxidative stress have been implicated in neurodegenerative diseases like Alzheimer disease.

Just one century after the discovery of Alzheimer disease, a form of senile insanity, there is already about 24 million people in the world with this neurodegenerative disease. This figure could reach 81 million by 2040 as a result of population aging[2]. Even if it is not directly linked to age, it touches about 3% of people older than 65 years and 20% of people after 85 years. These recent years, epidemiologists pull our attention to the role of antioxidants present in our diets and their implication in the prevention of diseases like Alzheimer[3]. Polyphenols, a powerful group of antioxidant molecules, are probably the most active molecules on these physiological disorders. Studies like those of the University of Bordeaux and that of PAQUID both in France have shown the beneficial effects of polyphenols in wines against Alzheimer. In these studies peoples who consumed wine had less risk (75% less) of developing Alzheimer. Furthermore, several in vitro and in vivo studies clearly reveal their numerous biological activities like anti-inflammatory, anti-hyperphosphorylation of tau, anti-amyloidogenic and antioxidant, all implicated in the physiopathology of Alzheimer disease.

It is also reported that following chronic exposure to aluminum, this compound accumulates in the hippocampus and induced the formation of β-amyloid plaques and neurofibrillar tangles like those found in Alzheimer's brain patients. Moreover, aluminium toxicity can induce oxidative damage through the potentiation of activity of Fe2+ and Fe3+ ions, impairment of mitochondrial electron transport chain and increased production of reactive oxygen species (ROS)[4]. The research of natural antioxidant, antilipid-peroxidative, antiinflammatory and thereby antiaging action is of interest.

Neuroprotective antioxidant substances could eliminate free radicals and also guarantee better reparation of neurons, so protecting them from toxic substances like aluminium chloride.

This leads us to the objective of this work which consisted in evaluating the possible beneficial effects of *Baillonella toxisperma*, a well known plant of African pharmacopeia, in protecting animal model mimicking Alzheimer disease after administration of AlCl3.

Methodology

Plant material

The Leaves and bark of B. toxisperma was collected in the East region of Cameroon precisely in Ngoumou, Mefou Division, Center Region, and Cameroon. The plant material was identified at the National Herbarium, Yaoundé, Cameroon and Voucher specimens were deposited under the number 63772YA. Bark Samples were cut into small pieces and dried then grinned into a fine powder. The obtained powder was used to prepare different extracts.

Preparation of extracts.

For each extract, 200g of powder was macerated for 48H in 1L of the corresponding solvent at room temperature. For the preparation of aqueous (AEBT), hydroethanolic (HEEBT) and ethanolic (EEBT) extracts, the solvents were respectively distilled water, 50% ethanol and 100% ethanol. The obtained filtrates were dried in an air drier at 50°C.

Quantitative phytochemical screening of extracts

Total polyphenol content

Polyphenol content was determined as described by Singleton and Rossi5. This method is based on the reduction of a phosphomolybdic-phosphotungstic chromogen by phenol moiety. The resulting colored complex formed absorbs light at 750 nm.

Flavonoid content

The method of Bahorun and collaborators6 using AlCl3 reaction and quercetine (dissolved in 2% ethanol) as standard was used.

Proanthocyan content

The method exploited here was that of Sun et al7 (1998) using cyanidric acid as standard.

Antioxidant screening of extracts of *B. toxisperma*

Total antioxidant capacity (TAC)

The method used was described by Prieto et al8, based on the reduction of molybdene MoVI into MoV in the presence of extracts to form a green complex (phosphate/MoV) in acid medium.

Scavenging of 1, 1-Diphenyl-2-Picrilhydrazyl (DPPH)

The DPPH• free radical is trapped by antioxidants to form a stable DPPH-H. The colour of the mixture of DPPH and extract changes from brown to yellow and absorbance is read at 517nm Katalinie et al.9. Ethanol was used as blank and the following formula was used to determine percentage inhibition:

% inhibition= (Absorbance (control)-Absorbance (sample))/Absorbance (control)x100

Scavenging of hydroxyl radical (·OH)

All prepared extracts of B. toxisperma were used to evaluate the scavenging of •OH. The capacity of extracts to scavenge this radical is based on Fenton's reaction by measuring the production of this radical and its effect on the oxidation and degradation of biological molecules like deoxyribose10.

Inhibition of ex-vivo lipid peroxidation

The method exploited here is that of Prassanth et al11 . Poly-unsaturated fatty acids contained in brain homogenates undergo peroxidation when put in the presence of iron. The generated malondialdehyde reacts with thiobarbituric acid to form a pink chromophore that absorbs at 532nm.

The brain homogenate used here was got from albino wistar rats after 12H of fasting. It was prepared at a concentration of 10% in potassium chloride then centrifugated at 1500rpm for 10min and the filtrate collected.

Protective effects of hydroethanolic extract of *Baillonella toxisperma* against aluminium chloride-induced neurotoxicity

Animals and treatment

Twenty-five female albinos' wistar rats weighing 230-250g were used for this experimentation that lasted 3 days. The animals were obtained from the animal house of the Department of Animal Biology of the University of Yaounde I, Cameroon. The acclimation period lasted one week. The animals had free access to standard diet and water. They were equally maintained at room temperature conditions through the experimental period. The rats were divided into 5 groups of 5 rats each. Except for the negative control group, all the other groups of rats received a single dose of 32.5mg/kgBW of aluminium chloride through intraperitoneal injection. The extract (treated groups), vitamin E (reference group) and 0.9% NaCl (control groups) were administered by oral gavages. The six groups were treated as follows;

Group 1 (Non-treated control, NC); 0.9% NaCl by oral gavage

Group 2 (Treated control, PC); 32.5mg/kgBW AlCl3+0.9% NaCl

Group 3 (BT150); 32.5mg/kgBW AlCl3+150mg/kgBW/day BT

Group 4 (BT300); 32.5mg/kgBW AlCl3+300mg/kgBW/day BT

Group 5 (Reference groups; VE100); 32.5mg/kgBW AlCl3+100mg/kgBW/day vitamin E

48H after the last treatment, the rats were sacrificed and their blood collected in EDTA tubes. Collected blood was centrifuged at 3400 rpm for 15 minutes; the supernatant was collected then stored at -20°C for further studies. For preparation of hemolysates of erythrocytes, after centrifuging total blood, 100 µl of pellets were pipetted into another tube then washed twice with 2 ml 0.9% NaCl and centrifuged at 3400 rpm for 10 min at room temperature. Hemolysates were got by adding 2ml of distilled water, and then they obtained supernatant was stored at -20 °C. Immediately after blood collection, the brain was collected for each rat then washed with 0.9% NaCl and weighed. They were immediately put on ice then grinned in normal saline and centrifuged. Homogenates of these organs were prepared at a concentration of 10% w/v 0.9% NaCl. The obtained filtrates were stored in ependorf tubes at -20°C.

Biochemical analyses

Protein concentration

The Biuret test12 was used to determine protein concentrations in plasma. In alkaline tartrate medium, proteins form a blue complex with a copper salt. The complex absorbs light at 540nm and the intensity of the coloration is proportional to the amount of proteins in solution. Bovine serum albumin was used as standard.

Plasma Aspartate amino transferase (AST) and Alanine Amino Transferase (ALT) Activity

In physiological enviroment, Aspartate amino transferase (AST) and Alanine amino transferase (ALT) catalyse the transfer of the amine group of aspartic acid and alanine to α-keto-glutarate to form glutamate and oxaloacetate and pyruvate respectively. Both pyruvate and oxaloacetate react with dinitrophenylhydrazone to give a complex that has its maximum absorbance at 505nm. Pyruvate was used as standard13. Both enzymes were measured in plasma of experimental rats.

Creatinine level

The method used for evaluation of plasma creatinine was that described by Bartels et al 14. In alkaline medium, creatinine forms an orange-yellow complex with picric acid. The intensity of the coloration is proportional to the concentration of creatinine in the medium.

Hydroxyl (OH) radical level

The method exploited here was that described by Jiang et al 15. This method is based on the principle that; in acid medium, the peroxide ion oxidizes Fe2+ into Fe3+ which reacts with xylenol orange to form a complex that absorbs at 560 nm. This parameter was measured in brain homogenates.

Protein thiol level

Protein thiols level in brain homogenates were measured according to the method described by Ellman16 in 1959 which is based on the fact that thiols carried by proteins are measured by following the evolution of the concentration of TNB (5-thio-(2-nitro-benzoic acid) which is a yellow complex formed by the reduction of DTNB (5,5-dithio-(2-nitro-benzoic acid) (Ellman's reagent) (412-415 nm).

Catalase (CAT) Activity

The activity of CAT was evaluated according to the method described by Sinha17 in erythrocytes based on the fact that the H2O2 remaining after the action of CAT combines with potassium dichromate to form an unstable blue-green complex which is later decolorized into a green complex on heating which absorbs light at a wavelength of 570nm.

Statistical analyses

The Statistical Package for Social Science (SPSS) 10.1 for Windows was used to analyze results and they were expressed as mean ± standard error. A one way ANOVA followed by the post hoc test of Tamhane and Duncan and Least Significance Difference (LSD) was exploited to determine difference between groups, results were considered significantly different when $p<0.05$. The Kolmogorov-Smirnov test was used to compare independent groups.

Results:

In this study, three systems of solvent were used to extract phytochemicals from BT. Table 1 below shows the extraction yield of each solvent. From these results we observe that the highest yield was with absolute ethanol and the lowest was with the aqueous extract.

Results of the phytochemical screening of prepared extracts are represented in Table 1 below. Even if the highest yield was not with HEEBT, the amount of polyphenols, flavonoids and proanthocyans was highest with this extract.

Figure 1 shows the TAC of extracts of B. toxisperma. All extracts had an antioxidant activity. HEEBT revealed the best activity (38,87±5,69 mg eq.Vit C/DM). The activity of the ethanolic and aqueous extracts were respectively 32,002 ± 5,697 and 14,655 ± 1,555 mgeq.Vit C/DM. They could be classified as follow; HEEBT>EEBT>AEBT.

The scavenging capacity of extracts and Vitamin C were compared by determining their IC50s (inhibition concentration 50). As can be observed in Figure 2, the hydroethanolic extract showed the lowest IC50 (10,02 µg/mL) followed by the ethanolic extract (68,04 µg/mL) and the aqueous extract (104,63 µg/mL). The IC50 of vitamin C was lowest than for all extracts of B. toxisperma (Vit C>HEEBT>EEBT>AEBT).

As concerns the scavenging of the hydroxyls radical, like for other radicals, the activities varied according to the extract (Figure 3). Here it is the ability of extracts to inhibit the oxidation of desoxiribose that was evaluated. Like for others, the IC50 of HEEBT was lowest compared to the other two extracts (5,625 mg/ml). The IC50 of vitamin E was highest than for all the extracts of (25,3 mg/ml). Classification of IC50 for all samples was as follow: HEEBT>AEBT>EEBT>Vit E.

Figure 4 represents the anti-radical activity of Vitamin E and extracts of B. toxisperma against lipid peroxidation in brain homogenates. The HEEBT revealed a more important inhibitory activity (IC50= 0,664 mg/mL) compared to AEBT (IC50= 2,737 mg/mL) and EEBT (IC50= 1,692 mg/mL) and practically equal to that of Vitamin E (IC50= 0,574 mg/mL). The classification of extract capacity in inhibiting lipid peroxidation in brain homogenates was as follows: Vit E>HEEBT>EEBT>AEBT.

Table 2 shows the effect of the administration of HEEBT 24H after treatment with aluminium chloride on oxidative stress markers levels in the brain. There was no significant difference in hydroperoxide levels between the group treated exclusively with aluminium chloride (PC) and the non-treated group (NC). The administration of HEEBT did not modified ROOH levels. This was not the case with vitamin E since rats supplemented with vitamin E showed an increased level ($p<0.05$). Compared to the NC, MDA level was found to be more important in the PC group even though not significant. Supplementation with the extract at both doses and with vitamin E reduced this level to concentrations even lower than for the negative control group.

The activity of catalase and the concentration of protein thiols in the brain of experimental rats are represented in Table 2. They show that AlCl3 induces a significant increase ($p<0.05$) of the activity of catalase compared to non-treated rats. Supplementation with extract and vitamin E, reduced activity to levels. Supplemented rats had catalase activities that were

not significantly different from non-treated rats even if higher. In regard to protein thiol results, no difference was observed between NC and PC, only the groups supplemented with BT increased this level, even though not significant.

Hepatic (AST and ALT) and renal (creatinine) toxic markers evaluated at the end of the experimentation, are represented in Table 3 below. It shows that, AlCl3 provokes a significant increase ($p<0.05$) in transaminase activities as well as a significant increase in creatinine level compared to the negative control group. Nevertheless, supplementation with B. toxisperma and vitamin E, tended to attenuate this toxicity by reducing the activities of transaminases and reducing plasma creatinine level.

DISCUSSION

Pathologies linked to aging like neurodegenerative diseases are associated to different cellular events amongst which reduction of mitochondrial functioning, neuronal apoptosis, protein aggregation and oxidative stress 18. This implies that antioxidants could play a beneficial role in these pathologies. It has been proposed that, they could act by protecting vulnerable neurons, by stimulating neuronal functioning, blood circulation in the brain and by favoring neurogenesis19.

Aluminium is a potential pro-oxidant that has been proven to increase peroxidative damage in the brain4. In this study we evaluated the protective effects of HEEBT against aluminium chloride induced neurotoxicity by evaluating some oxidative stress markers in the blood and the brain of rats. Our results show that, AlCl3 induced lipid peroxidation as MDA levels were found to be increased; similar results were got by Sadhana21. This increase suggests molecular damage induced by aluminum toxicity through overproduction of free radicals22. Treatment with HEE of B. toxisperma at 150 and 300mg/kg BW significantly reduced MDA levels compared to the group treated exclusively with AlCl3 suggesting that it protected neurons against free radicals and thus reducing peroxidative damage. This protection can be attributed to the antioxidant ability of B. toxisperma extract.

It's well described that Antioxidants can act in two major ways21: either by transfer of hydrogen atoms or by transfer of electrons. Therefore, for the in vitro evaluation of antioxidant activity of natural extracts, different methods have been developed.

Methods that are based on the transfer of hydrogen atoms measure the capacity of the antioxidant in donating a hydrogen atom to the radical while those that are based on electron transfer, measure the capacity of the extract to transfer an electron to any compound including metals, carbonyls and radicals. Based on physicochemical properties, the type of test used or the oxidative state of the substrate, it is recommended to use at least two methods to confirm an antioxidant activity21. This is why four complementary methods were chosen: TAC that quantifies the ability of all substances present in the extract to prevent oxidation (electron transfer), two tests evaluating the scavenging of free radicals (DPPH and OH) and also the inhibition of lipid peroxidation (hydrogen transfer).

The results revealed that all extracts showed good TAC, good scavenging activities that were comparable to vitamins C and E. These activities could be explained by the fact that most antioxidants amongst which polyphenols found in plants contain free hydroxyl moieties and double conjugate bonds able to provide hydrogen or electrons to radicals or metals in order to stabilize them22. The TAC of an extract is thus highly associated to its polyphenol content23. This could explain why HEEBT presented the best in vitro TAC.

Catalase is a crucial antioxidant enzyme that directly eliminates hydrogen peroxide. This enzymatic entity is present in mammalian peroxisomes and its activity is highest in red blood cells. Catalase decomposes H_2O_2 more efficiently at higher concentration compared to glutathion peroxidase (GPx) 24. This could explain why in this study no significant increase in protein thiol concentrations was observed but instead a significant increase in catalase activity. Probably, there was an important production of H_2O_2 induced by treatment with AlCl3. Studies have shown that the activity of catalase was directly linked to the concentration of $H_2O_2$25. This could explain the significant increase in its activity in AlCl3-exclusively treated rats as a response to the oxidative stress induced by AlCl3. Furthermore, numerous studies using animal models of Alzheimer's disease reviewed the benefic role of phenolic compounds such as curcumin in improving the amyloid, tau and redox status in Alzheimer's disease26. However, differential effects of toxic compounds on the levels of oxidative marker in the liver and brain might be explained by the fact that brain is affected in the final phase when using toxic compounds to mimic brain diseases while liver is affected in the primary phase27.

The reduction of the level of the activity of this enzyme in supplemented groups could be due to the fact that extracts and vitamin E protected cells against oxidative stress induced by aluminium.

In biological systems, due to its detoxification functions, hepatocytes are more vulnerable to toxic substances. Transaminases (AST and ALT) and plasma protein levels are known to be indicative of hepatic integrity. Sallam et al.28 reported that the accumulation of aluminium in the liver led to liver damage that is translated by an increase in the activity of enzymes like ALT and AST, alkaline phosphatase and plasma blood glucose levels. In this study, treatment with AlCl3 led to an increase in the levels of plasma transaminases compared to non-treated rats. Treatment with extracts (150 and 300 mg/kg P.C of BT) reduced these levels suggesting that they could protect liver cells against AlCl3 induced toxicity.

The kidney, like the liver, is implicated in the elimination of xenobiotics and is as well vulnerable to toxic substances. The results of this study suggest that treatment of rats with AlCl3 increases the level of plasma creatinine compared to those rats that received no treatment. The rats that received extracts plus AlCl3 showed lower creatinine levels compared to those that received AlCl3 exclusively. This suggests that, HEEBT could protect kidney cells from AlCl3 induced toxicity.

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Table 1: Amounts of polyphenols, total flavonoids and Proantocyanes of *B. toxisperma* extract

	Percentage yield (%)	**Total Polyphenol (mg eq.gallic acid/g DM)**	**Flavonoides (mg eq. quercetin/gDM)**	**Proanthocyanes (mg/eq. cyanidic ac/gDM)**
EA *B.toxisperma*	1.8	38.17±2.21	4.09±0.19	36.48±7.28
EE *B.toxisperma*	4.22	41.54±1.91	5.68±0.36	41.84±2.85
HEE *B.toxisperma*	3.2	65.99±4.14	4.66±0.07	75.49±2.63

Table 2: Effect of HEE of *B. toxisperma* on peroxidative lipid markers, protein thiol and erythrocyte catalase in brain homogenates of AlCl3 treated rats

Groups	**Hydroperoxides (µM)**	**MDA (µM)**	**Catalase (mMH_2O_2/min/mg protein**	**Protein thiols (µg/mg protein)**
CN	7,552±0,920	5,967±1,172	0.02 ± 0.00	0.31 ± 0.07
CP	7,210±0,414	6,308±1,633	0.09 ± 0.00*	0.31 ± 0.03
BT_{150}	7,265±0,255¥	4,437±3,000#	0.04 ± 0.01#	0.35 ± 0.15
BT_{300}	7,259±0,362¥	4,580±0,554#	0.05 ± 0.01#	0.44 ± 0.12
VE_{100}	8,295±0,324*#	4,631±0,652#	0.03 ± 0.00#	0.29 ± 0.07

** p <0,05*: *all groups compared to CN*; *#p<0,05*: *all groups compared to PC*; *¥p <0,05*: *all extract-treated groups compared to the vitamin E group.*

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Table 3: Effect of HEEBT on plasma protein and transaminases levels of aluminium chloride treated rats.

Groups	AST (UI/ml)	ALT (UI/ml)	Plasma protein level (mg/dl)	Creatinine (mg/dL)
CN	72.82±9.09	55.67±6.11	1.94±0.79	2.448±0.19
CP	94.06±11.73*	76.84±6.29*	2.29±0.26	3.922±0.49
BT_{150}	78.71±13.07#	56.28±4.31#	3.05±0.70*#¥	2.53±0.29
BT_{300}	79.62± 14.87#	52.43 ± 2.26#	2.88±0.15*#	2.27±0.01
VE_{100}	76.36±1.86#	60.13 ± 7.45#	1.65±0.13	2.29±0.18

** p <0,05: all groups compared to CN; #p<0,05: all groups compared to PC; ¥p <0,05: all extract-treated groups compared to the vitamin E group*

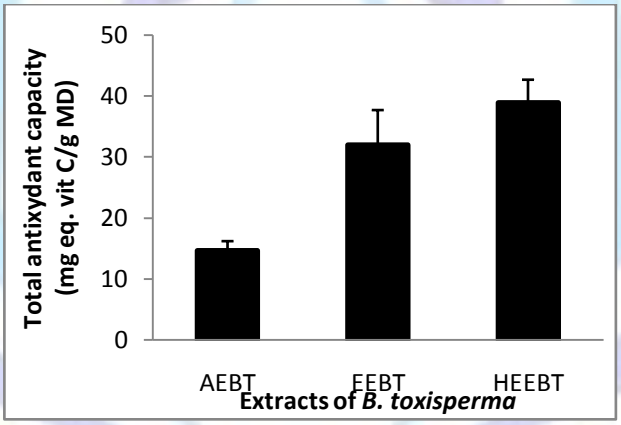

Figure 1: Total antioxydant capacity of aqueous, hydroethanolic and ethanolic extracts of *B. toxisperma*

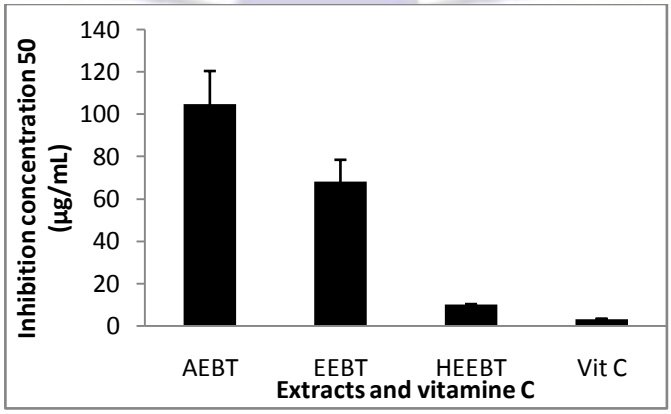

Figure 2: IC50s for the scavenging activity of extracts of *B. Toxisperma* and vitamine C of the DPPH free radical

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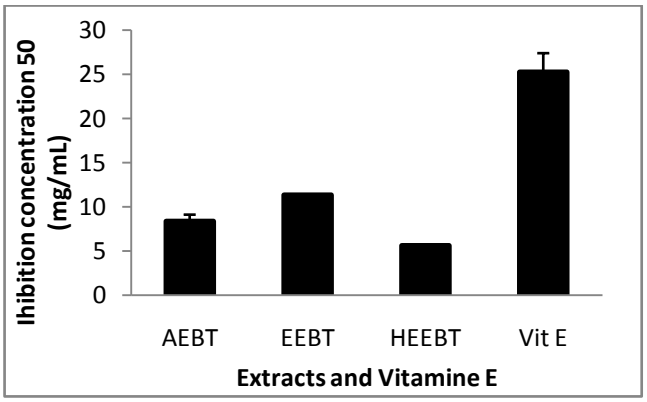

Figure 3: Inhibition concentration 50 for extracts of *B. toxisperma* and vitamine E for scavenging of the·OH radical

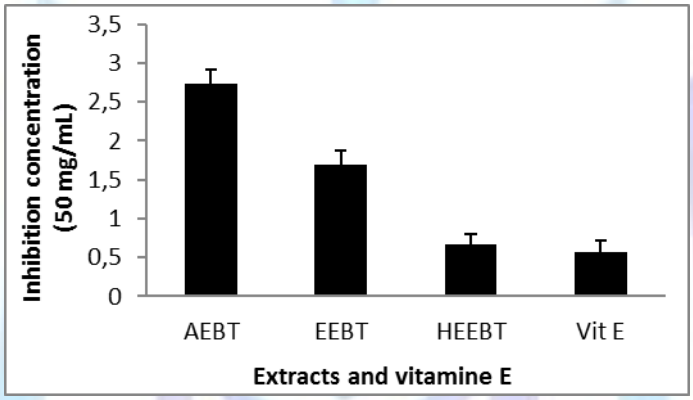

Figure 4: Inhibition of *ex-vivo* lipid peroxidaion in brain homogenates using Vitamine E and extracts of *B. Toxisperma*