

**IN VIVO ANTIOXIDANT AND POTENTIAL ANTITUMOR ACTIVITY
EXTRACT OF *LEEA GUINEENSIS* ROYEN EX. L. (LEEACEAE)
ON CARCINOMATOUS CELLS**

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Summary

The study was designed to investigate the subacute toxicity, *in vivo* antioxidant and antitumor activity of aqueous methanol extract of *Leea guineensis* (*Leeaceae*) (AMELG) on bearing carcinomatous cells. The results the toxicity study indicated no death of rats after 26 days of administration of the extract. At the concentrations of 500 mg/kg and 1000 mg/kg of the body weight, the liver enzymes activity alanine amino transferase (ALT) and aspartate amino transferase (AST) did not varied significantly as well as the level of glutathione. However, the concentration of creatinine and the activity of alkaline phosphatase (ALP) enzyme of the treated rats increased significantly from 57.26 to 62.33 (liver homogenate) and 89.87 to 92.52 (serum) compare to the control. The results of *in vivo* antioxidant activity revealed that the level of MDA decrease significantly ($3.01 \pm 0.05 - 2.17 \pm 0.48$) while the concentration of glutathione and the activities of CAT and SOD increased significantly after treatment with AMELG at 100 mg/kg and 200 mg/kg body weight. The results suggest that the aqueous methanol extract of *Leea guineensis* is not toxic and exhibits significant antioxidant and antitumor effects on bearing carcinomatous cells.

Key words: Antitumor, antioxidant, oxidative stress, carcinomatous cells

1. Introduction

It is recognized that under certain conditions of oxidative stress, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in the human body in higher quantity. ROS are yielded from the leakage of electron into oxygen from various systems in our body. Oxidation in biological systems causes damage to cell membranes, nucleoproteins, and cellular organelles triggering mutagenesis, carcinogenesis, and cardiovascular and other diseases¹. ROS are produced mainly endogenously as by-products of regular metabolism have been implicated in causation of the above conditions. The reactive species as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO \cdot), nitrogen oxide (NO \cdot), peroxyntirite ($ONOO_3$) and hypochlorous acid (HOCl) are normal products of human organ. In excess, they can be harmful in the body and induce severe damages which are associated with the development of cancer, ageing, cardiovascular disease, cataract, neurological disorders, lung disease other diseases^{2,3}. Nitric oxide (NO) reacts with molecular oxygen (O_2), superoxide (O_3^{2-}) and transition metals to yield nitrogen dioxide (NO_2), peroxyntirite ($ONOO_3$), and metal nitrosyl adducts, respectively, and each of these products has characteristic physiological and/or pathological roles⁴. Oxygen free radicals may attack lipids and DNA (strain breaks, base modification and DNA-protein crosslink) giving rise to a large number of damaged products^{2,5}. Superoxide, the most important source of initiating radical *in vivo*, is produced in mitochondria during electron chain transfer. Both forms of SOD catalyse the dismutation reaction of O_2^- ; however, in the presence of H_2O_2 , SOD can produce potentially damaging oxidants such as the hydroxyl (OH \cdot) radical, and high levels of SOD can be damaging to cells^{3,4}. In addition, reactive oxygen species induce some oxidative damage to biomolecules like lipids, nucleic acids, proteins and carbohydrates. As a result, ROS have been implicated in more than 100 diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, and cancer^{6,7,8}. All aerobic organisms have a natural defense system that combats ROS and thereby minimizes such oxidative damage. The antioxidant enzymatic defence is a very important source to neutralize the oxygen free radical mediated tissue injury⁹. Levels of O_3^{2-} are kept low in the cell by the enzyme superoxide dismutase (SOD), which dismutates O_3^{2-} to hydrogen peroxide (H_2O_2) and O_2 . Organs protect themselves from the toxicity of excess ROS/RNS in different ways including endogenous and exogenous antioxidants as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione, flavonoids, vitamins A and E².

Recently, interest has considerably greater in finding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity^{10,8}. For the few years, there is an increasing concern in the protection effect of vegetable, fruits and medicinal herbs for the prevention from free-radical mediated diseases¹¹. Recent chemical investigations of herbs have revealed the presence of antioxidant compounds as flavonoids, tannins and polyphenols¹². Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers^{13,8}. Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods^{14,8}. Hence, the studies on natural antioxidant have gained increasingly greater importance. The use of traditional medicine for the purpose is widespread and plants still present a large source of natural antioxidants drugs. Several anti-inflammatory, digestive, antinecrotic, neuroprotective and hepatoprotective drugs recently been shown to have an antioxidant and/or radical scavenging mechanism as part of their activity¹⁵. In searching for novel natural antioxidant, some plants have been extensively studied in the few years for their antioxidant and radical scavenging compounds.

Leea guineensis (Leeaceae) is an evergreen shrub or small tree native to tropical Africa. Leea's are vigorous growers and need quite a lot of space. Flowers are in much-branched cymes and are up to 8 inches across. This plant grows in the humid places found in the forest region and galleries through tropical Africa. Many tradi-practitioners and herbalists of Cameroon used the leaves of *L. guineensis* to pelvic inflammatory disease¹⁶. The *in vitro* antimicrobial and antitumor activity of *Leea guineensis* have been determined¹⁷. Despite these interesting therapeutic effects and the popular use of this plant, no *in vivo* antioxidant and antitumor study has been reported. This study is therefore carried out to evaluate *in vivo* toxicity, the antioxidant and antitumor activity of the aqueous-methanol extract of leaves of *L. guineensis* on nude mice and albinos rats.

2. Materials and Methods

2.1. Preparation of the extract and toxicity studies

Fresh leaves of *L. guineensis* were collected near the Eloundem mountain in Yaoundé, the capital city of Cameroon. The sample was identified at the National Herbarium and a voucher specimen was deposited there with the number 11971HNC. These leaves were dried at room temperature (30 ± 3 °C), pulverized. The powder obtained (250g) was macerated in 1000 ml of a mixture of methanol /water (4:1v/v) for 36 h. The extracted solution was filtered using Whatman filter paper N° 1 and concentrated in an air circulating oven at 54°C until total dryness. The experiment was repeated twice with the same powder and a total of 24 g of a green blue pasty extract obtained was stored at 5°C.

Male albino rats *Wistar* weighing 102 - 134 g were obtained from the animal laboratory of the Biochemistry Department of the Yaoundé I University, Cameroon. All the animals were kept in the house under environmental conditions (27 ± 2 °C). The animals had free access to water and a standard diet. For the study, they were deprived of food but not water for (16-18h) prior to administration of the extract. The principles of laboratory animal care were followed while the Department's ethical committee approved the use of the animals and the study design.

The rats were divided into three groups of 10 rats each (5 males and 5 females) and received by intra-gastric gavages 500 mg/kg, 1000 mg/kg body weight of extract or distilled water (control) every 48 h for 26 days. During the experimental period, morphological, behavioural and toxic symptoms were observed. At the same time the animals were weighed, food and water intake were also monitored. At the end of the experiment, all surviving animals were sacrificed by decapitation after suppression of food overnight. The blood sample was collected freshly in the dry non-heparinised centrifuge tubes. This blood was allowed to coagulate before centrifuged. The supernatant serum was picked up for analysis. The liver was excised, rinsed in ice-normal solution followed by cold 0.1 M Tris-HCl (pH 7.5). It was blotted and weighed. The 20% (w/v) homogenate was prepared in the 0.1 M Tris-HCl buffer and the supernatant was used for biochemical analysis.

The serum was employed for the determination of the level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT)¹⁸; alkaline phosphatase (ALP)^{19,20}; creatinine²¹ and protein²². The same parameters were also assayed with the 20% liver homogenates.

2. 2. Antitumor activity

2. 2. 1. Cell type and culture conditions

Carcinomatous cells were obtained into the anatomy and pathology Laboratory of the Faculty of Medicine and Medical Sciences of the University of Yaoundé I. The cells were maintained *in vitro* in the standard medium (MEM supplemented with 10% CSF) into the incubator 5 % CO₂ at 37 ° C for 48 h. After this period, 10 ml of suspension of carcinomatous cells culture was centrifuged at 3000 rpm for 5 min at 5 ° C and the pellet washed twice with sterilised NaCl 0.9 %.

2. 2. 2. Experimental Protocol

Male *nude* albinos mice of about 8 weeks of age with an average body weight of 21 ± 2 g were used for the experiment. They were fed with standard laboratory diet and have sterilized distilled water as a drink. These mice were divided into four groups of six animals each. The first group (normal) did not received carcinomatous cells and extract, the second group (control) received 0.2 ml of 2 X 10⁵ cells/ml of the line cells but not extract, the groups 3 and 4 were treated with the same volume of cell suspension and 100 mg/kg and 200 mg/kg b.w. of the extract *per os*. Before used, the cells collected were suspended in the sterilised NaCl 0.9 %. Their viability was check by counting viable cells (Methylène blue) under light microscope and the number was adjusted at 2 X 10⁵ cells/ml. 0.2 ml of 2 X 10⁵ cells/ml were injected intraperitoneally to all the groups of mice except group one. Immediately mice of the groups 3 and 4 were treated *per os* with the extract with 100 mg/kg and 200 mg/kg b.w. daily for 26 days. At the end of the experiment, all surviving mice were sacrificed, the blood was collected for the preparation of hemolysate while the liver was utilised for the 20 % liver homogenate. This was prepared exactly as described early in section 2.3.

After the preparation of plasma, the buffy coat of the blood was removed and packed cells (RBCs) were washed thrice with cold NaCl (0.9%). The RBCs lysate was prepared by lysing a known volume of RBCs with cold phosphate buffer pH 7.5. The hemolysate was separated by centrifuging at 3000 g for 10 min at 4 °C. The supernatant used for the determination of the enzyme activities of superoxide dismutase (SOD)²³ and catalase (CAT)²⁴ and also the protein concentration²⁵. The same parameters were determined with the 20 % (w/v) homogenate with the addition of Glutathione²⁶ and Malondialdheyde²⁷.

The qualitative phytochemical analysis was carried out to check up the bioactive components present in the extract such as polyphenols, flavonoids, tannins saponins and alkaloids^{28,29,30}.

The values were expressed as mean ± standard deviation (SD). Each value is a mean of five and six test. One-way analysis of variance (ANOVA) was used to determine the significant differences between parameters and the student-Newman Keurl's test served to locate these differences. $p < 0.05$ was considered as the level of statistical significant.

3. Results

The results indicate that no abnormal symptoms or death of rats were observed after treatment with AMELG. The entire animal gained the weight during the experiment.

However, the weight gained of rats treated with 500mg/kg b.w. was higher than the other after 4 days of treatment (figure 1). The evaluation of the liver function represented in the table 1 demonstrated not significant variation of the AST and ALT activities after 26 days of treatment in all groups of rats. However significant ($p < 0.05$) increased of the ALP activity and the creatinine level were noted in the treated groups compare to the control.

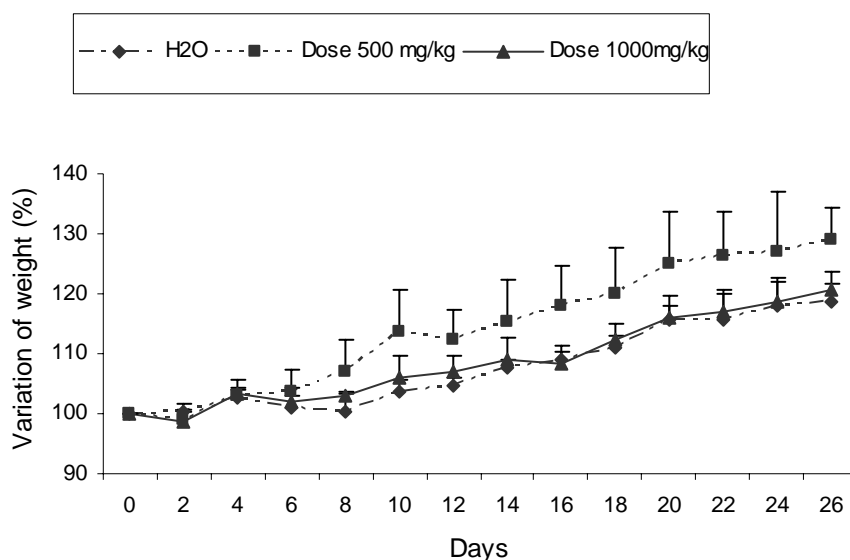


Figure 1: Evolution of the relative weigh of rats during the toxicity studies (n = 5).

Table1. Blood and liver biochemical indices of rats during the study of subacute toxicity of aqueous-methanol extract of *L. guineensis*.

Biochemical's parameters		Control	500 mg/kg	1000 mg/kg
Serum	ASAT (UI/L)	83,80 ± 0,33	83,62 ± 0,05	83,66 ± 0,02
	ALAT (UI/L)	39,38 ± 0,04	39,46 ± 0,13	39,49 ± 0,10
	PAL (UI/L)	89,87 ± 2,58 ^a	92,52 ± 1,29 ^b	90,58 ± 1,47 ^a
	Protein (mg/L)	0,15 ± 0,01	0,14 ± 0,02	0,14 ± 0,10
	Creatinine (mg/L)	1,62 ± 0,04 ^a	1,56 ± 0,07 ^a	1,74 ± 0,10 ^b
Liver homogenate	ASAT (UI/L)	84,92 ± 2,71	83,74 ± 0,12	83,65 ± 0,08
	ALAT (UI/L)	40,64 ± 0,11	40,77 ± 0,01	40,47 ± 0,39
	PAL (UI/L)	57,26 ± 4,82 ^a	60,66 ± 1,86 ^b	62,33 ± 6,08 ^c
	Protein (mg/ml)	0,025 ± 0,01	0,024 ± 0,02	0,023 ± 0,01

Values are expressed as mean ± SD (n = 5). Means with different superscript letter within each line are significantly different ($p < 0.05$).

The *in vivo* antioxidant proprieties of the AMELG were assayed by the determination of markers involved in the process such as SOD, CAT and GHS. The result was illustrated in the table 2. These results established the activity of SOD of the control group was reduced. After treatment with the AMELG at 200 mg/kg b.w., the activity of SOD increased significantly compare to the control and normal groups.

The same results showed that the activity of CAT of the control group was significantly reduced both in the serum and the liver homogenate compare to the normal group. The treatment with AMELG with 100 and 200 mg/kg increased significantly the level of CAT. The level of reduced GSH was significantly decreased in normal group. After the treatment with AMELG with 200 mg/kg b.w., the concentration of reduced GSH was found to be increased significantly. In contrary of GHS, the concentration of MDA was reduced significant after the administration of the extract. However, this concentration was increased in the control group compare to the normal one.

Table 2: Blood and liver biochemical indices during the study of antitumor activity of the aqueous-methanol extract of *L. guineensis*.

Biochemical's parameters		Normal	Control	100 mg/kg	200 mg/kg
Serum	SOD (UI/mg of protein)	16,11 ± 1,40	15,33 ± 0,43	16,23 ± 0,38	17,51 ± 1,50
	CAT(μM/min/mg of protein)	35,05 ± 2,67 ^a	33,06 ± 2,50 ^a	33,86 ± 1,32 ^a	39,04 ± 1,57 ^b
	Protein (mg/ml)	0,72 ± 0,07	0,69 ± 0,02	0,67 ± 0,04	0,68 ± 0,05
Liver homogenate	SOD (UI/mg of protein)	23,54 ± 0,37 ^c	21,24 ± 0,16 ^a	22,31 ± 0,20 ^b	22,70 ± 0,61 ^b
	CAT (μM/min/mg of protein)	50,36 ± 1,27 ^b	47,94 ± 0,25 ^a	48,83 ± 0,52 ^a	52,16 ± 0,45 ^b
	MDA (μM/mg of protein)	1,76 ± 0,03 ^a	3,44 ± 0,76 ^b	3,01 ± 0,05 ^b	2,17 ± 0,48 ^a
	Glutathion (μM/mg of protein)	1,63 ± 0,28 ^{ab}	2,12 ± 0,41 ^{bc}	1,26 ± 0,17 ^a	2,42 ± 0,39 ^c
	Protein (mg/ml)	0,48 ± 0,00 ^a	0,50 ± 0,01 ^c	0,49 ± 0,00 ^b	0,49 ± 0,00 ^b

Values are expressed as mean ± SD (n = 6). Means with different superscript letter within each line are significantly different ($p < 0.05$).

The results of phytochemical analysis of AMELG demonstrated the presence of tannins, polyphenols, steroids, alkaloids, glycosides, flavonoids, triterpens and saponins. These classes of molecules are involved in different manner against oxidation.

Discussion

The present investigation was carried out to evaluate the toxicity, the *in vivo* antioxidant and potential antitumor activity of aqueous methanol extract of *Leea guineensis* (AMELG) on bearing carcinomatous cells. For the toxicological study, alkaline phosphate (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzymes regularly used as markers of the hepatotoxicity. Our study revealed significant change on ALP which is not specific to hepatotoxicity. Inflammation of the hepatic cells results in elevation in the ALT and AST while the biliary tract cell injury leads predominantly in an elevation of the ALP³¹.

Although AST and ALT are found in the liver cells, AST is not specific to the liver function since it can be produced by heart, muscle tissue, pancreas, and kidneys³². The level of ALT is increased in conditions where the liver cells have been inflamed or undergone cell death. In the present study no abnormal elevation of the activities of ALT and AST were noted confirming the less or no hepatotoxicity effect of AMELG. ALP is an enzyme associated with the biliary tract but not specific to it, since ALP can be detected in bone and the placenta. Renal or intestinal damage can also cause the rise of ALP activity as well as the primary biliary cirrhosis, alcoholic hepatitis, gallstones in choledocholithiasis³². The activity of ALP is elevated after a biliary tract damage or inflammation. Thus AMELG may cause an obstruction of biliary tract, its damages or its inflammation during the study. The increase of the concentration of creatinine at 1000 mg/kg of body weight demonstrated that the extract metabolised is continuously eliminated without affected the function of the kidney.

In cancer chemotherapy, the major problem is the control of the reactive oxygen species (ROS) generated by exogenous and endogenous system². The antioxidant enzymatic system (SOD, CAT, glutathione peroxidase, glutathione-S-transferase) and non enzymatic system (Vitamins C and E, flavonoids, glutathione, uric acid and ceruloplasmin) are strongly involved in the regulation of ROS and the prevention of cancer disease. The anti-carcinogenic activity is due, in part, to the induction of the glutathione S-transferase and the quinone reductase gene through an antioxidant response element^{33,34}. Glutathione which is a potent inhibitor of neoplastic process plays a key role in the endogenous antioxidant system. It is found particularly in the liver at higher concentration and known to have an important function in the protective process³⁵. In our study, GSH level in the groups 3 & 4 increased significantly after administration of AMELG compare to the normal and control groups. This result permitted to suggest that AMELG possess a protective activity. Similar results was reported in several studies^{35,36}. *In vitro*, GSH can react with OH, hypochlorous acid (HOCl), peroxylnitrite, carbon centered radicals and singlet oxygen yielding thiyl radicals, which in turn can generate superoxide radicals. Hence, SOD might cooperate with GSH in helping remove free radicals *in vivo*^{37,38}. The extent of lipid peroxidation is measured through malondialdehyde activity (MDA), a pro-oxidant factor that determines the oxidative damage. ROS found in cancer tissues results in lipid peroxidation and subsequently to the increase of MDA level. MDA, the end product of lipid peroxidation, was reported to be higher in cancer tissues than in non diseased organ³⁹. The similar result was noted in our study. The level of MDA in our study was higher in the control than other groups of mice. This indicates that the tissues of these animals were subjected to increased oxidative stress or the initiation or/and progression of bearing of cancer disease. The administration of AMELG reduced significantly the level of MDA in the groups 3 and 4 compare to the control. This phenomenon could be attributed to different classes of compound present in the extract. The potent inhibition of lipid peroxidation as showed by the phenolic fraction and total extract may be due to the presence of chlorogenic acid. Several studies demonstrated that polyphenol can reduce lipid peroxidation by free radical scavenging and antioxidant activity⁴⁰. Furthermore, phenolic antioxidants exhibit antiinflammatory, anti-atherosclerotic, and anti-carcinogenic activities^{41,42}. The SOD and CAT are involved in the clearance of superoxide (O_2^-) and hydrogen peroxide (H_2O_2). SOD catalyses the diminution of superoxide into H_2O_2 , which can be eliminated by glutathione peroxidase and /or catalase⁴³. The decreased of the activities of SOD and CAT in the control group may be attributed to the lost of Mn^{2+} or Zn^{2+} containing in the SOD⁴⁴. The inhibition of SOD and CAT activities as the result of tumor growth was established³⁹. Similar results were observed in the control group confirming the increased of lipid peroxidation as a result of oxidation stress.

The administration of AMELG at 100 and 200 mg/kg b.w. increased significantly the activities of SOD and CAT in dose dependant manner. A hypothesis have been put forward that the inhibition of tumor cell growth can be attributed to the increase in the steady state levels of hydrogen peroxide as a result of the increased dismuting activity of manganese superoxide dismutase⁴⁵. Several extracts derived from plant are useful in the prevention and treatment of cancer diseases by induction of apoptosis⁴⁶. The antioxidant activity of AMELG probably implies several groups of compounds which owned different mechanism of action in the extract. Different classes of compounds such as tanins, polyphenols, steroids, alkaloids, glycosides, flavonoids, triterpens and saponins detected in AMELG have been reported to possess antitumor activity either by induction of apoptosis, cytotoxicity or other mechanism of action involved in the cancer therapy⁴⁶. Plant polyphenols are well known to show antioxidative activity and also to protect cultured cells from oxygen stress^{47,48}. The above mentioned ingredients discovered in the AMELG could directly eliminate free radicals, or increase the activity of superoxide dismutase by electron transfer⁴⁵. These antioxidant properties of AMELG might have anticancer activity on bearing carcinomatous cells.

In conclusion, the present study demonstrates that the aqueous methanol extract of *Leea guineensis* is not toxic. Its decreased lipid peroxidation and thereby augmented the endogenous antioxidant enzymes in the liver and haemolysed. All these parameters suggest that the aqueous methanol extract of *Leea guineensis* exhibits potential *in vivo* antitumor and antioxidant activity. Further study need to be carried out for biogued fractionation to identify the active molecule.

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