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In vivo Antioxidant and Potential Antitumor Activity of Aqueous Ethanol Extract of Leaves of *Senna alata* (L.) Roxb (Ceasalpiniaceae) on Bearing Carcinomatous Cells

[1]C.A. Pieme, [1]V.N. Penlap, [2]B. Nkegoum and [3]J. Ngogang
[1]Microbiology and Pharmacology Laboratory, Department of Biochemistry,
Faculty of Sciences, University of Yaounde I, P.O. Box 812, Cameroon
[2]Pathology Laboratory, Faculty of Medicine and Biomedical Sciences,
University of Yaounde I, P.O. Box 2787, Cameroon
[3]Medical Biochemistry Laboratory, Faculty of Medicine and Biomedical Sciences,
University of Yaounde I, P.O. Box 2787, Cameroon

Abstract: The study was designed to investigate the subacute toxicity, *in vivo* antioxidant and antitumor activity of aqueous ethanol extract of *Senna alata* on bearing carcinomaous cells. The results of the evaluation of the toxicity on albinos Wistars rats showed no death of rats and the increase of their weight after 26 days of administration of the extract. The liver enzymes activity alanine amino transferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALP) did not varied significantly as well as the concentration of creatinine of the treated rats both in the liver homogenate and serum compare to the control. The study was extended to the evaluation of *in vivo* antitumor activity of extract of *S. alata* on bearing carcinomatous cells on Nude mice. The results showed that after treatment with the extract at 100 and 200 mg kg^{-1} body weight, the levels of MDA decreased significantly (3.44±0.76-1.97±0.48) while the concentration of glutathione and the activities of CAT and SOD increased significantly. The results suggest that the aqueous ethanol extract of *S. alata* is not toxic and exhibits significant antitumor and antioxidant effects on bearing carcinomatous cells.

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

INTRODUCTION

It is known that under conditions of oxidative stress, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in the human body in higher quantity. The reactive species as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), nitrogen oxide ($NO^{\bullet}$), peroxynitrite ($ONOO^{\bullet}$) and hypochlorous acid (HOCl) are normal products of human organ. Oxygen derived free radicals, such as the superoxide anion and hydroxyl radical are cytotoxic and promote tissue injury (Peterhans, 1997). In excess, ROS/RNS can be harmful in the body and cause severe damages which are associated with the development of cancer, cardiovascular disease, cataract, neurological disorders and lung disease (Vaya and Aviram, 2001). Oxygen free radicals may attack lipids and DNA giving rise to a large number of damaged products (Imlay and Linn, 1998). Superoxide, the most important source of initiating radical *in vivo* is produced in mitochondria during electron chain transfer. ROS induced DNA damage as the reactions of free radical with DNA include strain breaks, base modification and DNA-protein crosslink (Vaya and Aviram, 2001). Antioxidants act as a major defence against radical mediated toxicity by protecting the damages caused by free radicals (Nayana and Janardhanan, 2000). The antioxidant enzymatic defence is a very important source to neutralize the oxygen free radical mediated tissue injury (Probhu *et al*., 2006). 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 E (Vaya and Aviram, 2001).

Antioxidants function as inhibitors at initiation and promotion/propagation/transformation stages of tumor promotion/carcinogenesis and protect cells against oxidative damage (Bagchi *et al*., 2004). For the few years, there is an increasing interest in the protection effect of vegetable, fruits and medicinal herbs for the prevention from free-radical mediated diseases (Myagmar and Aniya, 2000). Recent chemical investigations of herbs have revealed the presence of antioxidant compounds as flavonoids, tannins and polyphenols (Myagmar *et al*., 2004). Furthermore although medicinal plants are used as antioxidants in traditional medicine, their claimed

Corresponding Author: C.A. Pieme, Microbiology and Pharmacology Laboratory, Department of Biochemistry, Faculty of Sciences, University of Yaounde I, P.O. Box 812, Cameroon Tel: (00237) 74551871

therapeutic properties could be due, in part, to their capacity for scavenging oxygen free radicals. Several anti-inflammatory, digestive, antinecrotic, neuroprotective and hepatoprotctive drugs recently been shown to have an antioxidant and/or radical scavenging mechanism as a part of their activity (Myagmar *et al*., 2004). In searching for novel natural antioxidant, some plants recently have been extensively studied for their antioxidant and radical scavenging compounds. *Senna alata* (L.) Roxb. (Caesalpipiaceae) is found in different areas of Africa. The plant in used in Cameroon for the treatment of constipation, gastroenteritis, jaundice, intestinal helminthiasis, Eczemia, thyphoenteritis, ringwoun and food poison (Adjanohoum *et al*., 1991). Its leaf is also credited for the treatment of haemorrhoids, constipation, inguinal hernia, intestinal parasitosis, blennorrhagia, skin infections, syphilis and diabetes in Nigeria (Igoli *et al*., 2004). Many healers in different part of Africa have been used the leaves of *S. alata* for a long time to the treat of tinea versicolor and ringworm infections and other diseases (Kochar, 1981).

The EtOH extract of *S. alata* have been also reported to inhibit the growth of *D. congolensis* while its aqueous-MeOH extract have shown higher antifungal on fungi (*Microsporum canis*, *Blastomyces dermatitidis*, *Trichophyton mentagrophyte*, *Candida albicans*, *Aspergillus flavus*) and weak inhibition property on bacteria (*Dermatophilus congolensis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Corynebacterium parvum*, *Actinomycesbovis*, *Nocardia asteroids*, *Clostridium septicum*, *Bacillus pumilus* (Ali-Emmanuel *et al*., 2003; Makinde *et al*., 2007). Some studies also reported weak antifungal activity of aqueous-EtOH, HCl, EtOH, lyophilized and aqueous extracts of leaves of *S. alata* on dermatophytes and *C. albicans*. Recently, our study demonstrated the potential antitumor activity of aqueous-EtOH extract of the leaves *S. alata* using *Agrobacterium tumefaciens* (*A. tumefaciens*) in the potato disc tumor bioassay method (Pieme *et al*., 2005). The results of this test were confirmed by the anti-proliferative activity of the extract of *S. alata* on Leukemia L1210 cells. Despite several interesting therapeutic effects and the popular used of this plant, no *in vivo* antioxidant and antitumor study has been reported. This study is therefore carried out to evaluate *in vivo* the antioxidant and antitumor activity of the aqueous-EtOH extract of leaves of *S. alata*.

MATERIALS AND METHODS

Plant material: Fresh leaves of *S. alata* 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 1871/YA.

Preparation of the extract: The collected plant material was dried at room temperature (30±3°C), pulverized and finely sieved. The powder obtained (250 g) was macerated in 1000 mL of a mixture of ethanol/water (4:1 v/v) for 36 h. The extract 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 45 g of a green blue pasty aqueous-ethanol extract obtained was stored at 5°C.

Toxicity studies

Animals: Male albino rats Wistar strain weighing 102-134 g were obtained from the animal laboratory of the Biochemistry Department of the University of Yaoundé I, 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 (16-18 h) 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.

Sub acute toxicity: The rats were divided into three groups of 10 rats each (5 males and 5 females) and received by intra-gastric gavages the plant extract at 500 and 1000 mg kg^{-1} body weight or distilled water (control) every 48 h for 26 days. During the experimental period, morphological, behavioural and toxic symptoms of the animal were also observed and the animals were weighed, food and water intake were monitored every 48 h.

After 26 days, all surviving animals were given no food overnight, they were sacrificed by decapitation and the blood was immediately collected, centrifuged at 3000 rpm for 10 min for the preparation of the serum. The serum obtained was used for the analysis of biochemical index. The liver was excised, rinsed in ice-normal solution followed by cold 0.1 M Tris-HCl (pH 7.5), blot dried and weighed. A 20% (w/v) homogenate was prepared in the 0.1 M Tris-HCl buffer and was used for biochemical analysis.

Biochemical determinations: The serum was analysed to evaluate the liver enzyme and some biochemical index such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Bessey *et al*., 1946; Lowry *et al*., 1954), creatinine (Bartels *et al*., 1972) and protein (Gornall *et al*., 1949). The same parameters were also assayed with the 20% liver homogenates except the creatinine.

Antitumor activity

Animal: 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 were given sterilized water.

Tumor cells: 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 MEM medium into the incubator 5% CO_2 at 37°C for 48 h. After 48 h, 10 mL of carcinomatous cells culture were centrifuged at 3000 prm for 5 min and the pellet washed twice with sterilised NaCl 0.9%.

Experimental protocol: Male Nude albinos mice were divided into 4 groups of 6 animals each. The 1 group (normal) did not received carcinomatous cells and extract, the 2 group (control) received 0.2 mL of the carcinomatous cells but not extract, the groups 3 and 4 (treated groups) received the same volume of suspension of carcinomatous cells and 100 and 200 mg kg^{-1} body weight of the extract. The aqueous-EtOH extract of *S. alata* was dissolved into the sterile distilled water and directly used in the assay. The cells were collected and suspended in the sterilised NaCl 0.9%. The viable cells were counted (Methylène blue) under light microscope and were adjusted at 2×10^5 cells mL^{-1}. 0.2 mL of 2×10^5 cells mL^{-1} were injected intraperitoneally to all the groups of mice except group 1. Immediately mice of the groups 3 and 4 were treated *per os* with the extract with 100 and 200 mg kg^{-1} of body weight every day for 26 days. At the end of the experiment, the mice were sacrificed, their blood were collected for the preparation of hemolysate while the liver help to 20% liver homogenate.

Estimation of *in vivo* antioxidant: The blood collected served to the preparation of plasma. After this, the buffy coat was removed and packed cells (RBCs) were washed twice 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 and used to measure the activities of enzymatic antioxidants superoxide dismutase (SOD) (Misra and Frdovich, 1972) and catalase (CAT) (Sinha, 1972) and protein (Bradford, 1976). A 20% (w/v) homogenate of the mice was prepared in the 0.1 M Tris-HCl (pH 7.5) buffer and used for the determination of SOD, CAT, Glutathione (Ellman, 1959), Malondialdheyde (Wilbur *et al.*, 1949) and protein (Bradford, 1976).

Phytochemical analysis: The phytochemical analysis of aqueous-EtOH extract of *S. alata* carried out to check bioactive components such as polyphenols, flavonoids, tannins saponins and alkaloids (Harborne, 1998; Odebiyi and Sofowora, 1978; Treasese and Evans, 1989).

Statistical analysis: 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 Keurls test served to locate these differences. $p<0.05$ was considered as the level of statistical significance and the statistical package used was SPSS 10.1.

RESULTS

Toxicity studies: The results indicate that no abnormal symptoms and no death of rats after treatment with aqueous-EtOH of leaves of *S. alata*. The weight gained of rats treated with 500 mg kg^{-1} of extract was higher than those of the control groups and 1000 mg kg^{-1} (Fig. 1). The results showed not significant variation of the AST and ALT activities as well as other parameters tested both in

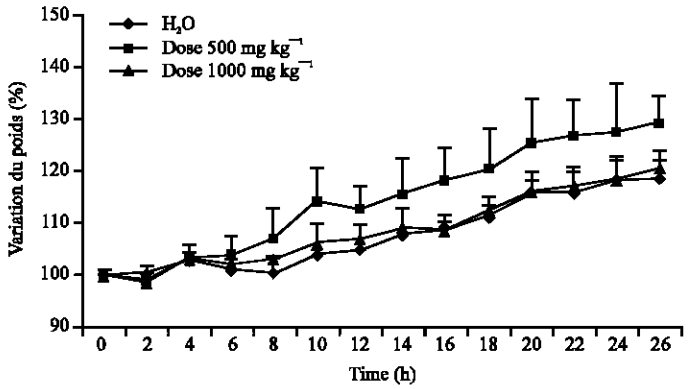

Fig. 1: Evolution of relative weights of rats during the study of acute toxicity of aqueous-EtOH extract of *S. alata* (n = 5)

Table 1: Blood and liver biochemical indices of rats during the study of subacute toxicity of aqueous-EtOH extract of *S alata* after 26 days

Biochemical's parameters	Control	500 mg kg^{-1}	1000 mg kg^{-1}
Serum			
ASAT (UI L^{-1})	83.63±0.06	83.66±0.12	83.69±0.05
ALAT (UI L^{-1})	39.93±0.29	40.05±0.19	40.06±0.10
PAL (UI L^{-1})	46.34±2.51	46.72±2.40	47.49±1.00
Protein (mg L^{-1})	0.17±0.01	0.16±0.02	0.16±0.20
Creatinine (mg L^{-1})	1.61±0.12	1.58±0.04	1.63±0.47
Liver homogenate			
ASAT (UI L^{-1})	84.06±0.22	83.73±0.27	83.99±0.29
ALAT (UI L^{-1})	68.42±3.43	67.03±3.00	65.23±4.65
PAL (UI L^{-1})	21.74±0.20	22.85±1.12	23.78±2.44
Protein (mg mL^{-1})	0.08±0.01	0.09±0.01	0.10±0.02
Glutathione (μM mg^{-1} of protein)	18.37±2.96	17.99±2.29	18.45±2.83

Values are expressed as mean±SD (n = 5)

Table 2: Blood and liver biochemical indices during the study of antitumor activity of the aqueous-EtOH extract of *S. alata*

Biochemical's parameters	Normal	Control	100 mg kg^{-1}	200 mg kg^{-1}
Serum				
SOD (UI mg^{-1} of protein)	16.11±1.40*	14.33±0.43	16.50±0.39*	17.56±1.41*
CAT (μM (min mg)$^{-1}$ of protein)	35.05±2.67	33.06±2.50	34.31±0.81	36.98±2.67*
Protein (mg mL^{-1})	0.72±0.07	0.69±0.02	0.71±0.01	0.69±0.04
Liver homogenate				
SOD (UI mg^{-1} of protein)	23.54±0.37*	21.24±0.16	22.23±0.38*	24.69±0.84*
CAT (μM (min mg^{-1} of protein)	50.36±1.27*	47.94±0.25	48.43±0.12	51.16±0.45*
MDA (μM mg^{-1} of protein)	1.76±0.03	3.44±0.76*	2.57±0.21*	1.97±0.48*
Glutathione (μM mg^{-1} of protein)	1.63±0.28	2.12±0.41*	1.85±0.04	2.23±0.20*
Protein (mg mL^{-1})	0.48±0.00	0.50±0.01*	0.49±0.00*	0.48±0.01

Values are expressed as mean±SD (n = 6). *Significant $p<0.01$

the serum and liver homogenate after 26 days of treatment with the aqueous-EtOH extract compare with the control. According to this result, the aqueous-EtOH extract of *S. alata* showed no toxic effects (Table 1).

Antitumor effect of aqueous-EtOH extract of *S. alata* on SOD and CAT levels: The effect of aqueous-EtOH extract of *S. alata* on parameters involved in the antitumor activity have been studied. The results showed a significant ($p<0.01$) reduction of the activity of SOD and CAT of the group who received only carcinomatous cells compare to the control group. After treatment with the aqueous-EtOH extract of *S. alata* at 100 and 200 mg kg^{-1}, the activities of SOD and CAT of the serum and liver homogenate increased significantly ($p<0.01$) compare to the control group (Table 2).

Antitumor effect of aqueous-EtOH extract of *S. alata* on malondialdheyde (MDA) and glutathione (GHS) levels: In present study, the levels of the MDA and GHS significantly increased in the control group compare with the normal one. After administration of the extract at 100 and 200 mg kg^{-1}, significantly reduction of MDA levels was noted while the reduced GHS increased.

Phytochemical analysis: The results of phytochemical analysis of aqueous-EtOH extract of *S. alata* indicate the presence of tannins, polyphenols, steroids, glycosides, flavonoids, anthraquinone and saponins.

DISCUSSION

The present investigation was carried out to evaluate the toxicity, the *in vivo* antioxidant and potential antitumor activity of aqueous EtOH extract of *S. alata* 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. Accordingly, the assessment of the level of AST, ALT and ALP provides a good and simple tool to measure the protective activity of the target drug against the hepatic damage of the target compounds (Hewawasam *et al*., 2004). 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 kidneys (McIntyre and Rosalki, 1991). The level of ALT 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 hepatotoxicty of aqueous-EtOH extract of leaves of *S. alata*. ALP is an enzyme associated with the biliary tract but not specific to it, since ALP can be detected in bone and the placenta. The activity of ALP is elevated after a biliary tract damage or inflammation. Thus extract of *S. alata* did not cause an obstruction of biliary tract, its damage or its inflammation during the study of the toxicity. No significant increase of the creatinine levels were noted at 1000 mg kg^{-1} indicating

that the extract did not affect the kidney function. In cancer chemotherapy, the major problem is the control of the reactive oxygen species (ROS) generated by exogenous and endogenous system (Vaya and Aviram, 2001). 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 the reactive oxygen species and the prevention of cancer disease. Glutathione which is a potent inhibitor of neoplastic process plays a key role in the endogenous antioxidant system. It is found in particularly high concentration in the liver and it is known to have an important function in the protective process (Yerra *et al*., 2005). In our study a significant increase in glutathione content was observed in the tissues of the treated group compared to the control and normal groups. According to the result the extract of *S. alata* have protective activity effect.

Similar results was observed by Raghavan *et al*. (2006). GSH is a nonenzymic mode of defence against free radicals. *In vitro*, GSH can react with OH^-, hypochlorous acid (HOCl), peroxylnitrite, carbon centred radicals and singlet oxygen yielding thiyl radicals (GS^{--}), which in turn can generate superoxide radicals (Raghavan *et al*., 2006). Hence, SOD might cooperate with GSH in helping remove free radicals *in vivo* (Halliwell, 1999). The extent of lipid peroxidation is measured through malondialdehyde activity (MDA), a pro-oxidant factor that determines the oxidative damage. MDA, the end product of lipid peroxidation, was reported to be higher in cancer tissues than healthy organ (Yagi, 1991). In this study, the level of MDA which is reported to be higher in the control group of mice than other groups. This indicates that the tissues were subjected to increased oxidative stress. The administration of aqueous-EtOH extract of *S. alata* reduced significantly the level of MDA. This phenomenon can be attributed to different bioactive compounds present in the extract such as tannins, flavonoids and polyphenol. Polyphenol have been reported to reduce lipid peroxidation by free radical scavenging and antioxidant activity (Sasikumar and Devi, 2000). The activities of SOD and CAT are involved in the clearance of superoxide (O_2^-) and hydrogen peroxide (H_2O_2). SOD catalysed the diminution of superoxide into H_2O_2, which can be eliminated by glutathione peroxidase and/or catalase (Rushmore and Picket, 1993).

A significant decrease of the activities of SOD and CAT were noted in this study to a group of mice who received only carcinomatous cells compared with the normal. After the administration of aqueous-EtOH extract of *S. alata* at 100 and 200 mg kg^{-1} significantly increase of SOD and CAT activities in dose dependant manner were found. The reduction of the activities of SOD and CAT in this group may be attributed to the lost of Mn^{2+} or Zn^{2+} containing in the SOD (Metin *et al*., 2003). The inhibition of SOD and CAT activities is a result of tumor growth (Yagi, 1987). A hypothesis have been put forward that the inhibition of tumor cell growth can be attributed to the increment in the steady state levels of hydrogen peroxide as a result of the increased dismuting activity of manganese superoxide dismutase (Chung *et al*., 2004). It have been reported that several extracts derived from plant are useful in the prevention and treatment of cancer diseases due to their antioxidants properties. The screening of aqueous-EtOH extracts of *S. alata* revealed the presence of several groups of compounds such as tanins, polyphenols, steroids, glycosides, flavonoids, triterpens and saponins. These bioactive compounds have been reported to possess antioxidant, antitumor activities either by induction of apoptosis, cytotoxicity or other mechanisms of action involved in the cancer therapy (Amit *et al*., 2001). In this study the antioxidant activity of the extract of *S. alata* probably implies several groups of compounds with different action mechanism.

The bioactive compounds found in the extract of *S. alata* could directly eliminate free radicals, or increase the activity of superoixde dismutase by electron transfer (Chung *et al*., 2004). These antioxidant properties of aqueous-EtOH extract of *S. alata* might have anticancer activity on bearing carcinomatous cells.

CONCLUSION

In conclusion, the present study demonstrates that the aqueous-EtOH extract of *S. alata* is not toxic. Its decreased lipids peroxidation and there by augmented the endogenous antioxidant enzymes in the liver and hemolysate. All these parameters suggest that the aqueous-EtOH extract of *S. alata* exhibits potential *in vivo* antitumor and antioxidant activities. Further research is going on for bio-guide fractionation and evaluation of its apoptosis on the other type of cancer cells.

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