

Potent Cytotoxic Activity of *Saururus cernuus* Extract on Human Colon and Breast Carcinoma Cultures under Normoxic Conditions

RAMESH B. BADISA¹, VEERA L. D. BADISA², EVAN H. WALKER³ and LEKAN M. LATINWO²

¹College of Pharmacy and Pharmaceutical Sciences and

²Department of Biology, Florida Agricultural and Mechanical University, Tallahassee, Florida;

³Walker Cancer Research Institute, Inc., 1634 Metropolitan Blvd, Tallahassee, Florida, U.S.A.

Abstract. Background: *Saururus cernuus* (Sc) is a small plant, used for the treatment of various human inflammations. The present study aimed at investigating the cytotoxic potential of the methanolic extract of this plant against brine shrimp larvae and human carcinoma cells at normoxic conditions. Materials and Methods: The *in vivo* lethality test was evaluated at various doses against brine shrimp larvae at different time periods. Similarly, the extract was tested for 48 h at various concentrations against human CL-18 and MDA-MB-231 carcinoma cell lines and the toxicity was evaluated using the dye binding crystal-violet assay method. Results: In the shrimp assay, the extract was very active, with ED_{50} values ranging from 1.83 ± 0.2 to 2.79 ± 0.2 $\mu\text{g/ml}$ at various incubation periods. The extract was also very potent in human CL-18 and MDA-MB-231 cultures with LD_{50} values of 1.9 ± 0.17 and 0.26 ± 0.03 $\mu\text{g/ml}$, respectively. Conclusion: The results of this study indicate that Sc extract contains very stable, potent anticancer compounds, which gain access into the cells quickly and kill carcinoma cells and shrimp larvae at normoxic conditions.

The *Saururus cernuus* (Sc) plant belongs to family Saururaceae. This family has four genera and six species, which are natives of North America and Asia. Sc was used as a folk remedy by Native Americans, as well as by early colonists (1). It is a small weed, and grows to a height of about one or two feet in ditches, swamps or marshes, distributed throughout the Eastern half of the US (2). This

plant, which is commonly known as lizard's tail and breast weed, is used for treatment of breast, kidney, bladder, etc. inflammations (1). Most recently, some compounds from this plant were shown to be selective inhibitors of hypoxia-inducible factor-1 (HIF-1) in a T47D human breast tumor cell line (3) under *in vitro* conditions. Hypoxic regions in the solid tumors are the areas where little or no oxygen and little nutrients are available. This situation enables these cells to synthesize abundant quantities of HIF-1 as the means of adaptation and survival. Although inhibition of HIF-1 synthesis was shown to reduce the tumor volume in animal studies (4), its degradation at normoxic conditions, prevailing on the periphery of solid tumors, was also reported (5). Cells at normoxic regions would, therefore, unlikely be affected by HIF-1 inhibitors. This results in the survival of tumor cells that could not only become drug resistant to future treatments, but could also accelerate tumors to metastatic stage (6). Hence, it is highly important that the chemotherapeutic drugs should destroy the tumor cells completely both under hypoxic and normoxic conditions, typically present in all solid tumors.

While the cytotoxicity of the *chinensis* species of *Saururus* under normoxic conditions (7, 8) and the inhibitory nature of pure compounds from the *cernuus* species under hypoxic conditions (3) were known, a review of the literature revealed that no studies addressed the effect of the *cernuus* species on various human colon and breast carcinoma cell lines under normoxic conditions. Since cells at hypoxic and normoxic conditions differ in their sensitivities to various compounds due to expression of certain specific genes (9-11), it would be of interest to study the cytotoxic nature of Sc extract on human cancer cells under normoxic conditions *in vitro*. Therefore, our primary aim was to evaluate the cytotoxic potential of the extract prepared from Sc on two different human carcinoma cell lines. The potency of the material was also evaluated on brine shrimp larvae under normoxic conditions.

Correspondence to: L.M. Latinwo, Ph.D., Department of Biology, College of Arts and Sciences, Florida A&M University, Tallahassee, FL-32307, U.S.A. Tel: +850 599 3907, Fax: +850 561 2996, e-mail: lekan.latinwo@famu.edu

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Materials and Methods

Chemicals. Trypsin, minimum essential medium (α modification), antibiotic-antimycotic solution (Ab/Am), bovine insulin and human recombinant insulin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum, RPMI-1640 (modified), penicillin, streptomycin and L-glutamine were purchased from Media Tech (Herndon, VA, USA). Cosmic calf serum was purchased from Hyclone Lab Inc. (Logan, UT, USA), and gentamicin reagent solution was purchased from Invitrogen (Carlsbad, CA, USA). All other routine chemicals were of analytical grade.

Plant material and preparation of extract. A new batch of whole plants of *Sc* were collected in the vicinity of Gulf specimen Marine Laboratories, Inc., Panacea, Florida in April, 2005. The dried material was steeped in 100% methanol for a week in the dark, and the crude extract was prepared as per the method described earlier (12). To the known amount of the crude residue, 100% dimethyl sulfoxide (DMSO) was added to make 250 mg/ml stock (w/v). A working stock of the extracts in DMSO was also prepared at 25 mg/ml. Both stocks were stored at 4°C until further use for evaluations.

In vivo lethality test by brine shrimp bioassay. In this technique, the *in vivo* potency of the extract was evaluated against shrimp larvae. For this purpose, the brine shrimp cysts or eggs (*Artemia salina*) were rehydrated in a tank containing artificial seawater, prepared with 1.9% salt mixture (Instant Ocean, Aquarium Systems, Inc.) in distilled water under constant illumination at room temperature (22-28°C). The assay was performed in triplicate vials at various doses as described by Meyer *et al.* (13) and modified by McLaughlin (14). The shrimp larvae in artificial seawater and DMSO in artificial seawater served as controls. The final concentration of DMSO in each vial during treatments did not exceed 0.4% (v/v). In some cases, parallel studies were carried out in the dark to check the effect of light on the potency of the extract. No external source of food was added to shrimp larvae, as they can survive on the yolk source alone for 72 h. At the end of the incubation periods, the surviving larvae per dose were counted and utilized for analysis.

Cell cultures and maintenance. Human colon adenocarcinoma (CL-187) and estrogen-independent human breast carcinoma (MDA-MB-231) cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and were used in the present investigation. These cell lines were maintained as described earlier (15, 20).

Treatments with the extracts. The cytotoxic studies with cell cultures were done in polystyrene, flat bottom 96-well microtiter plates as described earlier (16). In some experiments, the extract was adjusted to pH 5.0 and incubated at 37°C for 48 h in order to study the effect of pH, before testing at various concentrations. Similarly, the stability of the extract was determined by testing the old preparations of the extract on the cultures. In all cases, the culture plates were incubated for 48 h continuously without further renewal of growth media in a 5% CO₂ at 37°C, with the plates capped in the normal fashion. All studies were repeated at least twice (n=6 to 9). After 48 h of exposure, the cytotoxicity of the

extracts was evaluated by dye uptake assay using crystal violet as described previously (17).

Statistical analysis. The experimental results were presented as mean \pm SD. The data were analyzed for significance by single way ANOVA and then compared by Dunnett's multiple comparison tests, using GraphPad Prism Software, Version 3.00 (San Diego, CA, USA). The test values of $p<0.05$ and $p<0.01$ were considered significant and highly significant, respectively.

Results

Toxicity of *Sc* extract to shrimp larvae. The *in vivo* brine shrimp assay is an inexpensive benchtop assay mainly used to evaluate the potency of various kinds of extracts (13,16-20). The interaction between *Sc* and brine shrimps was not evaluated previously. Initially, when the extract was tested at 1 mg/ml, all shrimp larvae were found dead within 2 h. Further evaluation at lower doses, *i.e.* 0.5, 0.25, 0.125, 0.0625 and 0.01 mg/ml was still found to be very potent and all larvae were dead within 2 h at all doses. Therefore, the assay was repeated at 0.5, 1.0, 1.5, 2.0 and 2.5 μ g/ml and the live shrimp larvae were counted regularly at 1, 2, 3, 4, 20 and 24-h incubation periods. It was found that no deaths occurred at any dose within the first hour of the treatment, except that the larvae from 1.5 to 2.5 μ g/ml treatments became weak in comparison to controls. These observations may imply that various potent compounds in the extract entered quickly into the larvae. It was further observed that the number of shrimp deaths increased from 1.5 μ g/ml onwards with the increase in incubation period (TI). At the 2.5 μ g/ml treatment, more shrimps died than at the 1.0 μ g/ml treatment, indicating a gradual increase in mortality of shrimp larvae with increasing dose of the crude extract. Interestingly, no shrimp larvae were found dead at 0.5 μ g/ml even after 24 h exposure with the extract. However, these larvae appeared weak with increasing incubation period. The ED₅₀ values were determined at every time-point and are presented in TII. As speculated, the ED₅₀ values decreased from 2.79 \pm 0.2 to 1.83 \pm 0.2 μ g/ml as the incubation period increased, revealing the potent nature of the extract. The fact that the potent activity of the extract was not affected when the vials were incubated in the dark indicated that no photochemical products were formed from the extract due to continuous illumination of vials during incubation periods at room temperature (data not shown).

Cytotoxicity of *Sc* extract to the cell cultures. Based on the high activity on the shrimp larvae, the *in vitro* cytotoxic property of the *Sc* methanolic extract was evaluated on CL-187 and MDA-MB-231 carcinoma cultures under regular normoxic conditions in an incubator with 5% CO₂ in air at 37°C. Initially, the cultures were tested at a fixed concentration of 0.1 mg/ml for 48 h continuous exposure to

Table I. One-way ANOVA for shrimp deaths (%) at various incubation periods when treated with *Sc* crude extract. Shrimp death (%).

Conc. (µg/ml)	1 h	2 h	3 h	4 h	20 h	24 h	ANOVA F ratio
0.5	0	0	0	0	0	0	-
1.0	0	0	3.3±5.7	3.3±5.7	6.6±11.5	6.6±5.7	0.6857
1.5	0	6.6±5.7	10*±0	10*±0	43.3**±5.7	43.3**±5.7	66.93
2.0	0	33.3**±5.7	33.3**±5.7	46.6**±5.7	53.3*±5.7	56.6**±10	21.04
2.5	0	40**±0	50**±10	56.6**±5.5	66.6**±5.7	70**±10	44.2
ANOVA F ratio	-	83.0	18.95	104.2	55.14	71.08	-

Each value represents the mean percent±SD (n=3). * Significant at $p < 0.05$; **highly significant at $p < 0.01$ by Dunnett's multiple comparison test.

the extract, where 100% cell death in both cultures was observed. Further evaluation of the extract at 10.0, 25.0, 50.0 and 75.0 µg/ml also resulted in 100% cell death, revealing the highly cytotoxic nature of the extract. Based on these observations, the CL-187 cell line was subsequently tested at 1.0, 2.5, 5.0, 7.5 and 10.0 µg/ml, while MDA-MB-231 was tested at 0.1, 0.25, 0.5, 0.75 and 1.0 µg/ml.

It was found that the extract showed high cytotoxic activity against the CL-187 and MDA-MB-231 cell lines with LD₅₀ values of 1.9±0.17 µg/ml (F1) and 0.26±0.03 µg/ml (F2), respectively. As per the standard procedure, the crude extract with an LD₅₀ less than 20 µg/ml was considered active (21). The extract was found to be more than 10- fold more active than the compounds, compared with reference to this standard procedure. Therefore, the plant material was considered to be highly potent in both human carcinoma cell lines. It was shown that the pH of human tumors was lower than that of the normal tissue (22). In order to study the effect of pH on the potency of the extract, the extract was incubated at pH 5.0 for 48 h at 37°C. Interestingly, the level of potency of the extract to these human cultures was not affected (Data not shown). Similarly, the prolonged storage (>2 years) of the extract in DMSO at 4°C did not alter the extent of potency (data not shown). Both these observations indicate the high stability and potency of the extract.

Discussion

In the course of the shrimp assay, it was observed that addition of the extract to a sea salt solution at doses 0.5 mg/ml or higher resulted in precipitation in the vial. Since no precipitation occurred upon addition of only DMSO to the control vials (maximum concentration = 0.4% final, v/v), it is clear that the precipitation resulted from the extract. The precipitates sometimes attached to the shrimp larvae, impeding their free swimming, leading to the settling of larvae at the bottom of the vial. The studies at lower concentrations (1.5 to 2.5 µg/ml), where no precipitation was formed, demonstrated that shrimp death was very

Table II. The effective dose values of *Sc* crude extract on shrimp larvae at various incubation periods.

Incubation (hours)	ED ₅₀ (µg/ml)	95% confidence interval
1	0.00	-
2	2.79±0.2	4-9
3	2.58 ±1.3	4-7
4	2.29±0.2	3-14
20	1.88±0.3	2-6
24	1.83±0.2	2-7

The data of live shrimp larvae were analyzed by Finney Computer program in order to determine the effective dose values (ED₅₀). Each value is the mean±SD (n=3).

clearly due to certain cytotoxic compounds of the extract rather than to the precipitates. As *Sc* extract was shown to contain a variety of lignans and neolignans (23), the potent nature of extract in the present study may be attributed to some of these chemicals.

Based on the observation that shrimps were killed at lower concentrations (1.0 to 2.5 µg/ml, Table I) together with the very low ED₅₀ values (2.79±0.2 to 1.83±0.2 µg/ml, Table II) on shrimp larvae, the extract of *Sc* is rated very potent. In view of these interesting results, the extract was tested for cytotoxicity against CL-187 and MDA-MB-231 carcinoma cell lines and was found to be very active, with LD₅₀ values of 1.9±0.17 and 0.26±0.03 µg/ml, respectively (Figures 1 and 2). The observation that cell cultures in the present study did not have similar LD₅₀ values with the same extract of *Sc* may imply that various components in the extract interacted with one or more target sites in these cells. Similar observations were previously reported on different cell cultures treated with a single test material (24).

It is evident from the LD₅₀ values that the estrogen-independent MDA-MB-231 cell line responded very well with this extract, with potency more than seven times greater than the LD₅₀ values of the CL-187 cell line. As cell culture studies were conducted under normoxic conditions, these data suggest that the mechanism of cell death in these

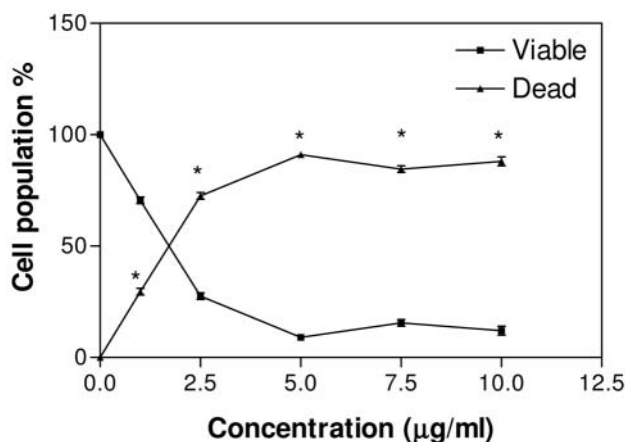


Figure 1. Cytotoxic effect of *Sc* crude extract on the human colon adenocarcinoma cell line CL-187 under normoxic conditions. The cells were treated at 1.0, 2.5, 5.0, 7.5 and 10.0 µg/ml for 48 h in 5% CO₂ in air at 37°C in an incubator and were evaluated for cytotoxicity by the crystal violet method. Results represent the mean±S.D of three experiments (n=9, *p<0.01, significantly different from control). The lethal dose of extract that killed 50% cells (LD₅₀) was determined from the point where the curves crossed (1.9±0.17 µg/ml).

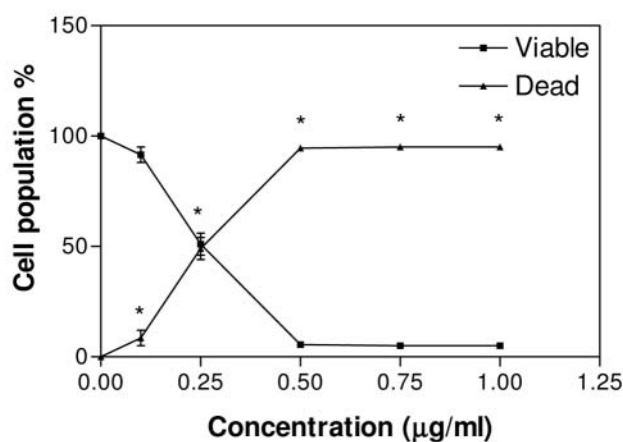


Figure 2. Cytotoxic effect of *Sc* crude extract on the human breast carcinoma MDA-MB-231 cell line under normoxic conditions. The cells were treated at 0.1, 0.25, 0.5, 0.75 and 1.0 µg/ml (n=6) for 48 h in 5% CO₂ in air at 37°C in an incubator and were evaluated for cytotoxicity by the crystal violet method. Results represent the mean±S.D of two experiments (n=6, *p<0.01, significantly different from control). The lethal dose of extract that killed 50% cells (LD₅₀) was determined from the point where the curves crossed (0.26±0.03 µg/ml).

cultures is not through the inhibition of HIF-1 factors, which are synthesized under hypoxic conditions. This indicates clearly that the target sites of the active compounds of the extract in cells under normoxic conditions are different than those under hypoxic conditions.

A comparison of the results indicated that there was a high degree of correlation of cytotoxic activities between shrimp larvae assay (Table II) and the cell culture assay (Figures 1 and 2), in agreement with previous reports (17, 25). Incidentally, the extract exhibited almost the same level of potency on the shrimp (ED₅₀: 1.83±0.2 µg/ml, Table II) and the CL-187 cell line (LD₅₀: 1.9±0.17 µg/ml, Figure 1).

Conclusion

One of the important criteria for a therapeutic drug for disseminated cancer is that the drug should have, not only the capacity to gain access into the tumor cells quickly, but it should also possess the ability to destroy all tumor cells at both normoxic and hypoxic regions of tumors. The present *in vivo* and *in vitro* studies demonstrated the ability of potent compounds in the extract to gain access into the cells quickly. Our study, along with earlier reports, also clearly demonstrated that *Sc* extract contains certain compounds with very high cytotoxic activity against various human carcinoma cell lines at hypoxic and normoxic states under *in vitro* conditions. The potency of the compounds in the extract was not affected either by low pH or by prolonged

storage at 4°C. Since this plant was used as a folk remedy for various diseases including certain type of tumors (26), it would be interesting to investigate to what extent the potent nature of this extract, can be extrapolated *in vivo* in animal models. Studies are currently under way.

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References

- Phares DL: *Saururus cernuus*. Am J Pharmacol 39: 468, 1867.
- Hoyer MV, Canfield DE, Horsburgh CA *et al*: Florida freshwater plants. In: A Handbook of Common Aquatic Plants in Florida Lakes, University of Florida, Institute of Food and Agricultural Sciences, Gainesville, Florida, p. 172, 1996.
- Hodges TW, Hossain CF, Kim YP *et al*: Molecular targeted antitumor agents: The *Saururus cernuus* dineolignans manassantin B and 4-O-demethylmanassantin B are potent inhibitors of hypoxia activated HIF-1. J Nat Prod 67: 767-771, 2004.
- Jiang BH, Agani F, Passaniti A *et al*: V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. Cancer Res 57: 5328-5335, 1997.
- Huang LE, Gu J, Schau M *et al*: Regulation of hypoxia-inducible factor 1α is mediated by an O₂ dependent degradation domain *via* the ubiquitin proteasome pathway. Proc Natl Acad Sci USA 95: 7987-7992, 1998.

- 6 Hockel M, Schlenger K, Aral B *et al*: Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 56: 4509-4515, 1996.
- 7 Sung SH and Kim YC: Hepatoprotective diastereomeric from *Saururus chinensis* herbs. *J Nat Prod* 63: 1019-1021, 2000.
- 8 Rho MC, Kwon OE, Kim K *et al*: Inhibitory effects of manassantin A and B isolated from the roots of *Saururus chinensis* on PMA-induced ICAM-1 expression. *Planta Med* 69: 1147-1149, 2003.
- 9 Rice GC, Hoy C and Schimke RT: Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. *Proc Natl Acad Sci USA* 83: 5978-5982, 1986.
- 10 Maxwell PH, Dachs GU, Gleadle JM *et al*: Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci USA* 94(15): 8104-8109, 1997.
- 11 Liu H, Savaraj N, Priebe W *et al*: Hypoxia increases tumor cell sensitivity to glycolytic inhibitors: a strategy for solid tumor therapy (Model C). *Biochem Pharmacol* 64: 1745-1751, 2002.
- 12 Chaudhuri SK, Badisa R B, Pilarinou E *et al*: *Licamichauxii* A and B acids – two Ent-kaurene diterpenoids from *Licania michauxii*. *Nat Prod Lett* 16(1): 39-45, 2002.
- 13 Meyer BN, Ferrign NR, Putnam JE *et al*: Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med* 45: 31-34, 1982.
- 14 McLaughlin JL: The brine shrimp lethality bioassay. In: *Methods in Plant Biochemistry*. Hostettmann K (ed.). London, Academic Press, pp. 8-10, 1991.
- 15 Badisa RB, Chaudhuri SK, Pilarinou E *et al*: *Licania michauxii* prance root extract induces hsp 70 mRNA and necrotic cell death in cultured hepatoma and colon carcinoma cell lines. *Cancer Lett* 149(1-2): 61-68, 2000.
- 16 Couladis M, Badisa RB, Baziou P *et al*: Antioxidant and cytotoxic activities of hypericum species on brine shrimps and human cancer cell lines. *Phytotherapy Res* 16: 719-722, 2002.
- 17 Badisa R B, Tzakou O, Couladis M *et al*: Cytotoxic activities of some Greek Labiatae herbs. *Phytotherapy Res* 17: 472-476, 2003.
- 18 Badisa RB, Couladis M, Tsortanidou V *et al*: Pharmacological activities of some *Argyranthemum* species growing in the Canary Islands. *Phytotherapy Res* 18(9): 763-767, 2004a.
- 19 Badisa RB, Tzakou O, Couladis M *et al*: Cytotoxic activities of *Salvia* plants of Labiatae family. *Pharmaceut Biol* 42(8): 640-645, 2004c.
- 20 Badisa RB, Lambert TA, Ikediobi CO *et al*: Selective anticancer activity of pure *Licamichauxii*-B acid in culture cell lines. *Pharmaceut Biol* 44(2): 141-145, 2006.
- 21 Likhitwitayawuid K, Angerhofer CK, Chai H *et al*: Cytotoxic and antimalarial alkaloids from the bulbs of *Crinum amabile*. *J Nat Prod* 56: 1331-1338, 1993.
- 22 Meyer KA, Kammerling EM, Amtman L *et al*: pH studies of malignant tissues in human beings. *Cancer Res* 8: 513-518, 1948.
- 23 Rao KV and Alvarez FM: Chemistry of *Saururus cernuus*. I. Saucernetin, a new neolignan. *J Nat Prod* 45: 393-397, 1982.
- 24 Badisa RB, Badisa VLD, Watson DW *et al*: Cytotoxic activities of Snow Lotus filtrates against human cancer cell lines, and origin of active factors. *Pharmaceut Biol* 42(2): 117-124, 2004b.
- 25 Anderson JE, Goetz CM and McLaughlin JL: A blind comparison of simple bench-top bioassays and human tumor cell cytotoxicities as antitumor prescreens. *Phytochem Anal* 2: 107-111, 1991.
- 26 Hartwell JL: Plants used against cancer: a survey. *Lloydia* 34: 204-255, 1971.

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