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Chapter 5

In vivo anti prostate tumor potential of *Vernonia guineensis* Benth. (Asteraceae) tuber extract (VGDE) and the cytotoxicity of its major compound pentaisovaleryl sucrose

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Abstract

Vernonia guineensis Benth. (Asteraceae) root decoction is used in folk medicine in Cameroon to treat some ailments including prostate cancer. The aim of this study was to validate the claimed antiprostate cancer activity of *V. guineensis* Benth. *in vivo* and to investigate the cytotoxicity of a pentaisovaleryl sucrose isolated from *V. guineensis* on some cancer cell lines. A crude dichloromethane extract of *V. guineensis* (VGDE) was used for this study. For *in vivo* anti prostate cancer efficacy, nude mice (n=16) were injected sub cute with prostate cancer PC-3 cells. Upon the formation of the xenograft tumors, the mice were divided into two equal groups with approximately the same mean tumor volume per group. One group was treated with VGDE orally (500mg/kg) and the other with a vehicle control for 30 days. Body weight and tumor volumes were measured 2x a week and on the 33rd day, the mice were euthanized and tumors harvested and weighed. For the cytotoxicity study, the WST-1 assay was used to determine the activity of pentaisovaleryl sucrose previously isolated from VGDE. The cancer cell lines used in the cytotoxicity study included: breast, colon, leukaemia, lung, melanoma, ovarian and prostate. Prostate cancer (PC-3) xenograft tumors treated with VGDE showed a significant decrease in tumor size (P = 0.0295) compared to control. Pentaisovaleryl sucrose also demonstrated cytotoxicity against various cancer cell lines with IC_{50} values as follows: MDA- MD-231- 6.66 µM; MCF-7 – 7.50 µM; HCT116-14.12 µM; A549- 5.76 µM; HL60 – 6.43 µM; A375 – 8.64 µM; OVCAR3 – 9.53 µM; Capan1 – 7.13 µM; Mia-Paca 6.47 µM. VGDE does possess *in vivo* activity against prostate tumor and has potential for development into a natural product for the treatment of prostate cancer. This study thus provides preliminary validation for the folk use of *V. guineensis* against prostate conditions. Further *in vivo* studies are however required to confirm

these results and to understand the mechanism of action of VGDE and the *in vivo* efficacy of pentaisovaleryl sucrose.

5.1 Introduction

Prostate cancer is common in men worldwide and it is the second most diagnosed cancer and sixth leading cause of cancer deaths amongst men (Ferlay et al., 2010; Jemal et al., 2011). Prostate cancer was the third most important cancer in males in Sub-Saharan Africa in 2002 with 26,800 new cases accounting for 10.6% of all reported cancer incidences amongst men (Lingwood et al., 2008). Developing countries especially in Africa with much less access to modern cancer diagnostic and treatment facilities are projected to account for most of the new cancer diagnosis by 2020 (Jemal et al., 2012; Parkin et al., 2008). The search for alternative and affordable treatments is thus of great significance and medicinal plants present a great opportunity for future research on new anticancer agents.

Vernonia guineensis Benth. (Asteraceae) has been reported to be used in ethnomedicine to manage prostate related problems(Emmanuel, 2010) . Plants of the *Vernonia* genus are widely used in ethnomedicine and some have exhibited interesting activity in different bioassays (Toyang and Verpoorte, 2013). In a recent study, the crude extract of the root tubers of *V. guineensis* and pentaisovaleryl sucrose isolated from this plant exhibited *in vitro* cytotoxicity and clonogenic activity against the PC-3 and DU145 prostate cancer cell lines as well as *ex-vivo* antiangiogenic activity (Toyang et al., 2012a). The carrot-like tubers of *V. guineensis* are commonly used in ethnomedicine as an adaptogen to combat stress, as a stimulant, as a anthelmintic, an aphrodisiac, an antidote to poison, to treat malaria and jaundice (Iwu, 1993; Tchinda et al., 2002). The plant extracts have demonstrated bioactivity in antimicrobial,

anthelmintic, antitrypanosomal, cytotoxicity and antiplasmodial assays (Donfack et al., 2012; Tchinda et al., 2002; Toyang et al., 2012b; Toyang et al., 2013b; Toyang et al., 2013a).

Based on the results of the preliminary results in *in vitro* cytotoxic activity tests of *V. guineensis* and pentaisovalery sucrose on prostate cancer cell lines, this study was set up to evaluate the *in vivo* efficacy of the dichloromethane extract on prostate cancer xenografts.

5.2. **Materials and methods**

5.2.1. Plant collection. The plant material was collected as previously reported (Toyang et al., 2012a). Briefly, the tubers of *Vernonia guineensis* Var. *cameroonica* C. D. Adams were collected at Baicham, Boyo, Division of the North West Region of Cameroon in 2009. The sample was authenticated at the Limbe Botanic Garden, South West Region Cameroon and a voucher specimen No: SCA 12431 was deposited at the Limbe Botanic Garden Herbarium.

5.2.2. Extraction and isolation

The extraction was carried out as previously reported (Toyang et al., 2012a). Briefly, 1 kg of *V. guineensis* tuber powder was extracted twice with 4 L of dichloromethane. The filtrate was dried *in vacuo* in a rotavapor to give 87 g of a brownish oily residue which was cytotoxic to PC-3 prostate cancer cell line (IC_{50}=67.3 µg/ml).

The pentaisovaleryl sucrose (Fig. 4.1) used in this study was made available from a previous isolation reported in chapter 4 of this thesis (Toyang et al., 2012a).

5.2.3. Cell culture and injection

For the *in vivo* study, prostate cancer (PC-3) cells were maintained in minimum essential media (MEM) supplemented with 10% fetal calf serum (FCS), 20 mM l-glutamine, 2% penicillin–streptomycin, and 0.2% gentamicin until they were ready for injection into mice.

For the *in vitro* study, nine cancer cell lines (Breast –MDA-MB-231, Breast-MCF-7, Colon-HCT-116, Leukaemia-HL-60, Lung-A549, Melanoma-A375, Ovarian-OVCAR3, Pancreatic-Mia-Paca, Prostate cancer – CAPAN-1) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were maintained in minimum essential media (MEM) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 2% penicillin–streptomycin, and 0.2% gentamicin or in RPMI 1640 medium supplemented with 10% FBS and 1% L-glutamine.

5.2.4 Anti-proliferation study

The inhibitory efficacy of pentaisovaleryl sucrose (Figure 4.1) previously isolated from *V. guineensis* was investigated *in-vitro* using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche) colorimetric assay as described in chapter 4 of this thesis (Ngamwongsatit et al., 2008). Briefly, on the day the experiment is initiated, cells are trypsinized and plated into 96 well plates in 50µl of media. Test compounds are added approximately 18 hours after plating. Cells are plated at a density so that 72 hours post drug addition, the cells are in log phase (500-2000 cells/well). The compounds are solubilized in DMSO at a concentration of 100 mM, aliquoted and stored at -20oC. Following drug addition, the cells are allowed to proliferate for 72 hours. The experiment is terminated using WST-1 (Roche) 10µl per well and absorbance is read at 450 nm/690 nm. The effect of drugs on growth

was assessed as percent of cell viability. The IC_{50} values were determined from the compound dose versus control growth curves using Graphpad Prism software. All experiments were carried out at least in duplicate and the mean results determined.

5.2.5 Drug preparation and treatment

The dried crude dichloromethane extract of *V. guineensis* was formulated in DMSO, Tween 80 and normal saline (2:2:6). The drug concentration was 50mg/ml and each mouse administered

0.2ml for mouse weighing 20g giving a dose of 500mg/kg. The vehicle was prepared using the same diluent concentrations without the drug.

5.2.6 *In vivo* MTD study

All mice used were maintained in a pathogen-free environment in the Institute of Human Virology Animal Facility at the University of Maryland, School of Medicine in accordance with the Institutional Animal Care and Use Committee (*IACUC*) guidelines on the handling of research animals. To determine the maximum tolerable dose (MTD) for the experiment, 12 nude mice were divided into 3 equal groups. Two of the groups were respectively treated with

500mg/kg and 1,000mg/kg orally using a crude VGDE extract daily for 5 days and the third group was treated with the vehicle control. None of the animals died or showed any signs of adverse reaction to the drug or vehicle during the 5 days of the treatment. It was thus determined that it was safe to use any of the two concentrations in the efficacy study.

5.2.7 *In vivo* efficacy study

All mice were maintained as described above. Twenty two nu/nu (NIH) mice, 4-6 weeks old were inoculated with $3x10^6$ PC-3 prostate cancer cells in 33% matrigel/67% media with no FCS. Mice were monitored daily for tumor growth. When tumors reached ~100 mm3, the mice were divided into 2 groups (n= 8) so that the mean tumor volume was similar. Dosing was initiated on

day of sorting (day 1). Mice were dosed every day for a total of 30 days orally. The body weight of mice, were taken alongside tumor volumes 2x a week and the tumor volumes were determined using the following formula:

L x W2/2 (L=tumor length; W= tumor width)

Mice were euthanized by CO_2 asphyxiation on the 33rd day and tumors collected and weighed. Major organs including heart, liver, lungs, kidneys and pancreas were collected and examined for any abnormalities.

5.2.8 Statistical analysis

The antiproliferation assay experiments were run at least in duplicate with each experimental concentration duplicated during each run. The average mean of the different assays were calculated and the data represented as mean ± SEM. For the *in vivo* study, statistical analysis was carried out by analysis of variance (ANOVA) followed by Unpaired t-test analysis. $P < 0.05$ was considered as indicative of significance, as compared to the control group. All data were analyzed in Graphpad Prism (Graphpad Software, La Jolla, CA) software.

5.3.0 Results

5.3.1 Effect of VGDE on PC-3 tumor xenograft

VGDE extract demonstrated a significant reduction in tumor volume (P= 0.0295) compared to the control. Figure 5.1 presents a graph comparing tumor weights at the end of the experiment while Figure 5.2 presents the tracking of tumor progression during the 33 day trial. Organ examination did not reveal any abnormalities or lesions in the mice. Figure 5.3 presents the comparative antiproliferation activity of VGDE and its major compound pentaisovaleryl sucrose against the PC-3 cell line.

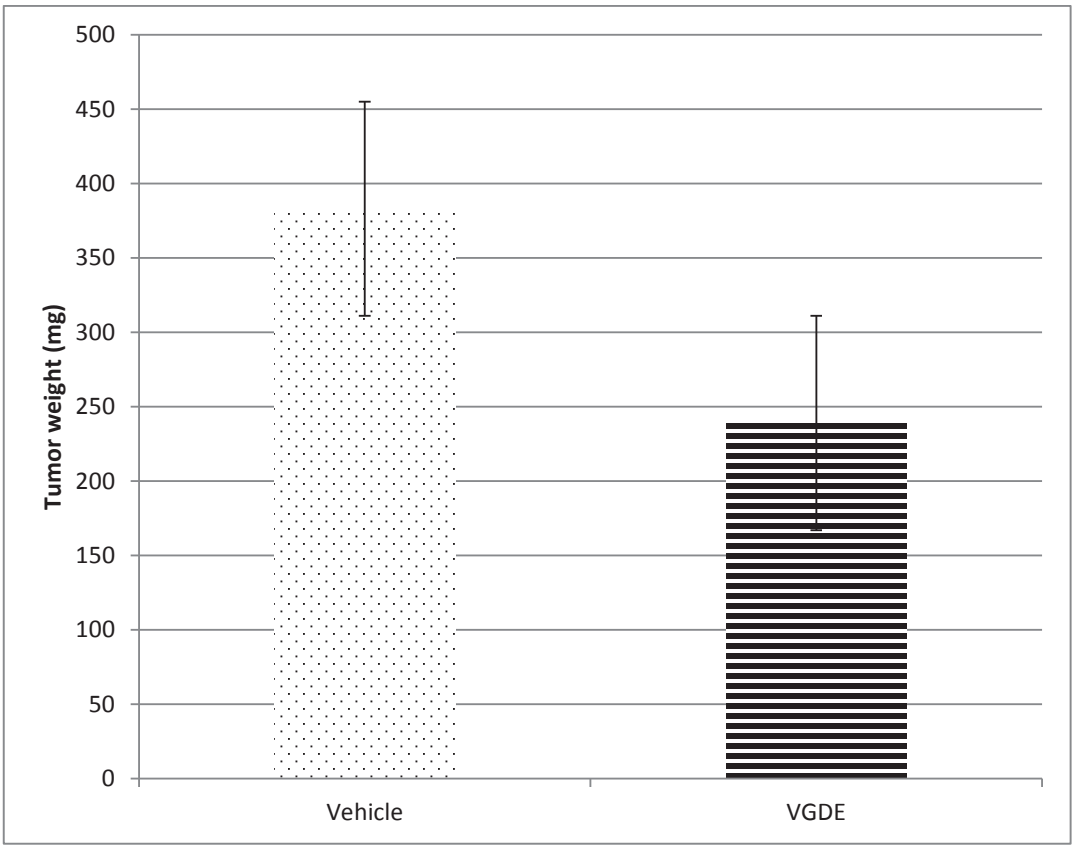

Fig 5.1. Nude mice (n=8) implanted with PC-3 cells ($3x10^6$/mouse) by sub cutaneous injection were treated for 30 days orally with VGDE (500mg/kg). Treatment started approximately 10 days after cell injection with the average tumor volume of about $100mm^3$ for the treatment and control groups. The mice were euthanized on the 33rd day and tumors collected and weighed. Treatment with VGDE resulted in a 37.59% reduction in tumor weight compared to the control group.

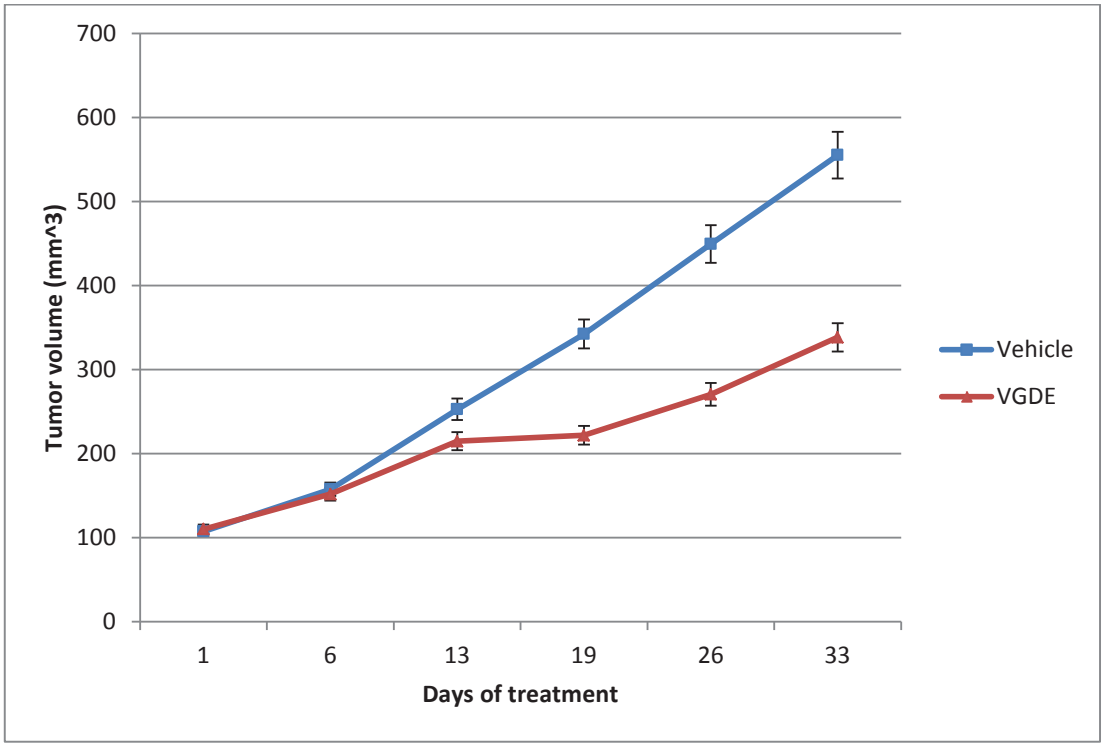

Fig. 5.2. Nude mice (n=8) implanted with PC-3 cells ($3x10^6$/mouse) by sub cutaneous injection were treated for 30 days orally with VGDE (500mg/kg). Treatment started approximately 10 days after cell injection with the average tumor volume of about $100mm^3$ for the treatment and control groups. Tumor volumes 2x a week and the tumor volumes were determined using the formula L x $W^2/2$ (L=tumor length; W= tumor width). VGDE extract demonstrated a significant reduction in tumor volume (P= 0.0295) compared to the control.

5.3.2 Effect of VGDE on body weight

VGDE extract at 500mg/kg for 30 days did cause a slight drop in the body weight of mice in the treatment group while there was a slight gain in control group. Figure 5.3 presents the body weight change measurements during the 30 day trial.

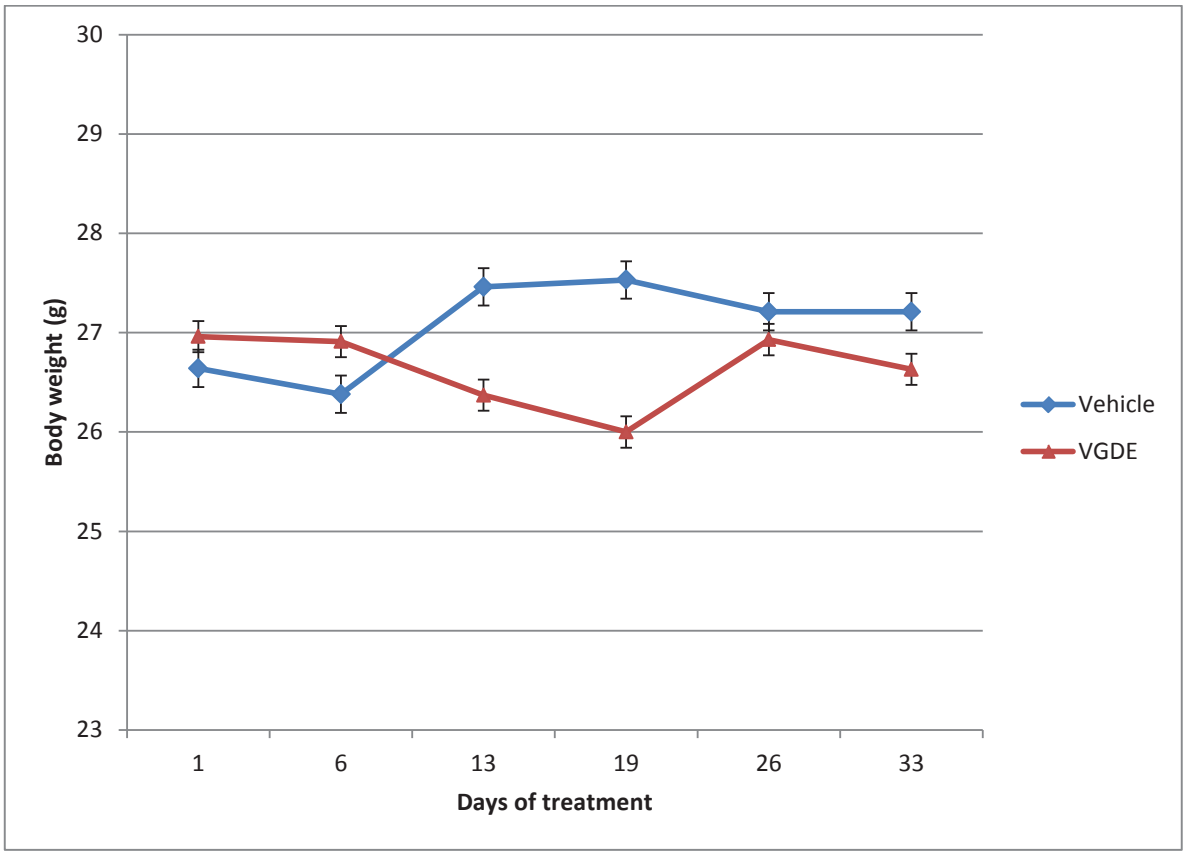

Fig. 5.3. Nude mice of age 4-6 weeks and weighing 20-30 g were used in the experiment. The body weight of mice was recorded 2x a week. VGDE extract at 500mg/kg for 30 days caused a slight drop (1.22%) in the body weight of mice in the treatment group compared to the weight of the same mice at the start of the experiment. The mice in the control group experienced a small gain (2.14%) in body weight compared to the weight at the start of the experiment.

5.3.3 Anti proliferation assay

The results of the anti proliferation activity of pentaisovaleryl sucrose (**4-I**) conducted against 9 cancer cell lines utilizing the WST-1 cell proliferation reagent are presented in Table 5.1. The compound showed activity against all 9 cell lines with the most potent activity observed against the lung cancer cell line (A549) with IC_{50} of 5.00 μM while it was least active against the colon cancer cell line (HCT-116) with IC_{50} of 14.12 μM. The IC_{50} values of pentaisovaleryl against the other cell lines were less than 10 μM.

Table 5.1. *In vitro* cytotoxicity activity of pentaisovaleryl sucrose (**4.1**) against selected human cancer cell lines in the WST-1 antiproliferation assay.

Cancer cell line	**IC_{50} (μM)**
Breast –MDA-MD-231	6.66±1.17
Breast - MCF-7	7.51±1.84
Colon-HCT - 116	14.12±6.16
Leukaemia - HL-60	6.39±0.37
Lung – A549	5.00±0.01
Human melanoma – A375	8.64±1.56
Ovarian – OVCAR3	9.53±3.76
Pancreas – Mia-Paca	6.47±0.96
Prostate – Capan1	7.13±0.09

5.4 **Discussion**

The results of the present investigation demonstrate the *in vivo* antitumor activity of the dichloromethane root extract of *V. guineensis* against the PC-3 prostate tumor. This study provides preliminary validation for the use of preparations of this plant in folk medicine to manage prostate related problems (Emmanuel, 2010). The absence of any noticeable signs of toxicity in the treated mice is also an indication that the VGDE extract can be considered to be reasonably safe. This is confirmed by the fact that there was statistically no significant difference ($P > 0.05$) in the body weight of the treated versus the untreated group as well as the absence of any negative morphological changes in the organs of all the mice in both groups. The results of this study also suggest that the pentaisovaleryl sucrose previously obtained from the dichloromethane extract of *V. guineensis* through bioactivity guided isolation with activity against 3 prostate cancer cell lines (Toyang et al., 2012a) may also be responsible for the *in vivo* activity observed. By in vitro comparison, the bioactive pentaisovaleryl sucrose isolated from VGDE is at least 10x more active than the parent extract (Figure 5.4).

Pentaisovaleryl sucrose demonstrated cytotoxic activity against the 9 cancer cell lines tested (Table 5.1). The lung cancer cell line A549 was most sensitive while the colon cancer cell line HCT-116 was least sensitive to Pentaisovaleryl sucrose. The cytotoxic effect of Pentaisovaleryl sucrose against other cell lines and its Multi Drug Resistant (MDR) modulating effect have previously been reported (Murakami et al., 2002; Toyang et al., 2012a). Sucrose esters are generally known to exhibit diverse behavior and are used mainly in the food and cosmetic industry as emulsifiers, antibacterial agents, crystallization inhibitors, and as permeation enhancers (Csóka et al., 2007; Garti et al., 2000; Mutoh et al., 2007). Due to their good solubilizing properties, there is an interest to use sucrose esters as pharmaceutical excipients for nasal drug delivery amongst other applications (Kürti et al., 2012). Apart from reported cytotoxic

and antibacterial activity of sucrose esters, studies have also shown that sucrose ester have anti-inflammatory properties with potency levels reaching those of indomethacin (Pérez-Castorena et al., 2010). Sucrose esters have also found use as insecticides and are thought to contribute to the insecticidal properties associated with tobacco leaves (Chortyk et al., 1996; Simonovska et al., 2006). The most interesting finding supporting the outcome of this study is that investigations have shown that tobacco leaf surface sucrose esters and some synthetic disaccharide esters have potential to be used in cancer prevention (Okabe et al., 1999). This supports our claim that pentaisovaleryl sucrose may indeed be responsible for antitumor activity demonstrated by VGDE.

5.5. Conclusion

This study confirms that the dichloromethane root extract of *V. guineensis* does possess *in vivo* activity against prostate tumors and has potential for development into a natural product for the treatment of prostate cancer. Further studies are however required to confirm these results as well as the *in vivo* efficacy of pentaisovaleryl sucrose (**4-I**) found in this extract and its mechanism of action.