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Biological Activities of Xanthatin from *Xanthium strumarium* Leaves

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The objective of the present work was to evaluate the biological activities of the major bioactive compound, xanthatin, and other compounds from *Xanthium strumarium* (Asteraceae) leaves. Inhibition of bloodstream forms of *Trypanosoma brucei brucei* and leukaemia HL-60 cell proliferation was assessed using resazurin as a vital stain. Xanthatin was found to be the major and most active compound against *T. b. brucei* with an IC_{50} value of 2.63 μg/mL and a selectivity index of 20. The possible mode of action of xanthatin was further evaluated. Xanthatin showed antiinflammatory activity by inhibiting both PGE_2 synthesis (24% inhibition) and 5-lipoxygenase activity (92% inhibition) at concentrations of 100 μg/mL and 97 μg/mL, respectively. Xanthatin exhibited weak irreversible inhibition of parasite specific trypanothione reductase. Unlike xanthatin, diminazene aceturate and ethidium bromide showed strong DNA intercalation with IC_{50} values of 26.04 μg/mL and 44.70 μg/mL, respectively. Substantial induction of caspase 3/7 activity in MIA PaCa-2 cells was observed after 6 h of treatment with 100 μg/mL of xanthatin. All these data taken together suggest that xanthatin exerts its biological activity by inducing apoptosis and inhibiting both PGE_2 synthesis and 5-lipoxygenase activity thereby avoiding unwanted inflammation commonly observed in diseases such as trypanosomiasis.

Keywords: in vitro; *Trypanosoma brucei brucei*; HL-60 cells; MIA PaCa-2 cells; xanthatin; *Xanthium strumarium*

INTRODUCTION

Xanthium strumarium L. (Asteraceae), locally called 'Bandaa' (Amharic) in Ethiopia, is an annual or a short-lived perennial that grows to 75 cm. It is a weed and is found throughout Ethiopia. It is native to Central or South America but has now a cosmopolitan distribution (Tadesse, 2004). In Ethiopia, the plant is used traditionally for the treatment of fungal infections of human skin (Wondimu *et al*., 2007). The leaf powder mixed with lime is usually applied on the skin against *Tinea versicolor* (Abebe *et al*., 2003). In the traditional folk medicine of many other countries, *X. strumarium* has been reported for the treatment of rhinitis, rheumatism, eczema, cancer, ulcer and malaria (Ma *et al*., 1998; Yin *et al*., 2005; Gautam *et al*., 2007).

A number of studies have been conducted to explain the putative traditional medicinal uses of *X. strumarium*. A methanol extract was shown to have *in vitro* antibacterial and antifungal activities (Jawad *et al*., 1988). Methanol–water and water extracts showed *in vitro* antiplasmodial activity (Tran *et al*., 2003). The methanol, ethanol, dichloromethane and chloroform extracts exhibited *in vitro* cytotoxic activities against various cancer cell lines (Roussakis *et al*., 1994; Kim *et al*., 2003; Ramírez-Erosa *et al*., 2007). The 50% ethanol extract of the leaves of the plant was also reported to have antitrypanosomal effects both *in vitro* and *in vivo* (Talakal *et al*., 1995). The recent observation that mice infected with drug-resistant *Trypanosoma congolense* treated with an extract from the leaves of *Xanthium strumarium* survived (47.6 ± days) longer than mice treated with standard drug diminazene aceturate (27.6 ± days) (Nibret *et al*., 2007) prompted us to elucidate the bioactive agent responsible for the trypanocidal activity of *X. strumarium* and to obtain an insight into the putative target(s).

X. strumarium and other species of the genus *Xanthium* are known for the production of sesquiterpene lactones termed xanthanolides, which are responsible for most of the biological activities of *Xanthium* species. The interest in these compounds is high because of the historical discovery of artemisinin, an endoperoxide sesquiterpene lactone, from *Artemisia annua* for the treatment of malaria. In addition to sesquiterpene lactones (Malik *et al*., 1993), *X. strumarium* has been reported to contain carboxyatractyloside (Cole *et al*., 1980), caffeoylquinic acid (Agata *et al*., 1993) and thiazinediones (Han *et al*., 2006).

This paper reports the isolation of a sesquiterpene lactone, xanthatin and three other compounds from the leaves of *X. strumarium*. Xanthatin was found to be one of the major active compounds of the plant. In this study, the biological activities as well as the putative modes of action of xanthatin are reported in relation to its disease (trypanosomiasis) progression inhibitory activity.

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MATERIALS AND METHODS

Reagents. Fetal bovine serum, MEM, DMEM and RPMI 1640 media were purchased from Invitrogen, Karlsruhe, Germany. Diminazene aceturate, DNA-methyl green, NADPH, resazurin, sodium linoleate and soybean lipoxygenase, were from Sigma-Aldrich, Steinheim, Germany. Trypanothione disulfide and Apo Logix™ JC-1 kit were purchased from Bachem, Heidelberg, Germany and Bachem, UK, respectively.

Plant material. The plant was collected from Debre Markos, 300 km north of Addis Ababa, Ethiopia, in March 2006 by one of us (E.N.) from its natural habitat and was identified by Mr Melaku Wondafrash, Addis Ababa University. The plant specimen was deposited at the Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany under the accession number P7109 for further reference.

Preparation of plant crude extracts. The dried leaves (20 g) of the plant were ground and macerated in water, methanol and dichloromethane, respectively, and left on a shaker for two consecutive days. Then the methanol and dichloromethane extracts were filtered and evaporated to dryness under reduced pressure using a Rotavapor at 45 °C. The water extract was filtered and freeze-dried using a lyophilizer.

Isolation and characterization of compounds. As a preliminary assay, 10 g of the plant (*X. strumarium*) was extracted using three different solvents (dichloromethane, methanol and water) and tested against both types of cells (*T. b. brucei* and HL-60). Based on the results (methanol and dichloromethane extracts against trypanosomes) obtained, the profile of compounds was monitored on TLC plates and a prominent spot was observed in both dichloromethane and methanol extracts. Following this, the methanol extract, which was believed to be a 'total extract' that contained the active compound including others, was prepared as follows: 950 g of dried leaves of *X. strumarium* was macerated with methanol for 24 h and all soluble components of the leaves were extracted exhaustively three times. The extract was then chromatographed on a silica gel 60 and then the sub-fractions were tested for their biological activities. After testing some of the sub-fractions and profiling their compounds on TLC, all the sub-fractions were pooled together and the extract was further treated with activated charcoal to remove the chlorophyll. The extract with the charcoal was left to stand for 10 min, mixed very well and filtered three times using Whatman filter paper. The filtrate was then evaporated to dryness using a Rotavapor at 45 °C. A total of 70 g crude methanol extract was recovered. The extract was divided into two parts that were chromatographed on a silica gel 60 (Merck 0.063–0.200 mm) column on two different occasions. The gradient elution method was employed for collection of the fractions in the following order of polarity: cyclohexane (100%); cyclohexane/EtOAc (9:1); cyclohexane/EtOAc (8:2); cyclohexane/EtOAc (7:3); cyclohexane/EtOAc (5:5); EtOAc (100%); EtOAc/MeOH (9:1) and finally an increase in the polarity of EtOAc/MeOH. Every 10 min, the collected fractions were monitored on TLC for their compound profiles. Light yellow oil (9 mg; compound **1**), white needle crystal (80 mg; compound **2**), white amorphous powder (16 mg; compound **3**), white gray amorphous powder (18 mg; compound **4**) were sequentially isolated. The same procedure was employed to check the reproducibility of the method for the recovery of compound **2**. This compound was recovered successfully by recrystallizing in 100% cyclohexane on two different occasions.

Electron spray ionization-mass spectrometry (ESI-MS). Xanthatin, dissolved in methanol, was directly injected to a Bruker Esquire-LC ion trap mass spectrometer (Bruker-Daltonik GmbH, Bremen, Germany). The flow rate of the solvent was adjusted to 3 μL/min. The MS and ESI MS/MS spectra were then recorded.

Gas liquid chromatography-mass spectrometry (GLC-MS). The molecular weights of xanthatin, stigmasterol, squalene and β-sitosterol-O-glucoside were determined using GLC-MS. The analysis was carried out on a Hewlett-Packard gas chromatograph (GC 5890 II, Hewlett-Packard GmbH, Bad Homburg, Germany) equipped with OV-1 column (30 m × 0.25 mm × 0.25 μm) (Ohio Valley, Ohio, USA). The capillary column was directly coupled to a quadrupole mass spectrometer (SSQ 7000, Thermo-Finnigan, Bremen, Germany). The injector temperature was at 250 °C. Helium was used as a carrier gas and its flow rate was 2 mL/min. The EI-MS spectrum was recorded at an electron energy of 70 eV; ion source, 175 °C.

Nuclear magnetic resonance (NMR). The ^{1}H and ^{13}C spectra were recorded on a Varian 500 MHz AC NMR spectrometer. Two-dimensional NMR experiments (COSY; HSQC; HSQCAD; HMBC; APT) were performed and analysed for assignments of ^{1}H and ^{13}C signals. All the compounds were measured in deuterated solvents at 25 °C.

Cell cultures. Bloodstream forms of *Trypanosoma brucei brucei* TC221 cells, the causative agent of animal trypanosomiasis, were grown in Baltz medium (Baltz *et al.*, 1985) supplemented with 20% inactivated fetal bovine serum and 1% penicillin–streptomycin. The HL-60 cells (human myeloid cell line) and MIA PaCa-2 cells (pancreatic cancer cells) were grown in RPMI 1640 and DMEM, respectively, supplemented with 0.2 mM L-glutamine, 1% penicillin–streptomycin and 10% heat inactivated fetal bovine serum. The cell cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

Trypanocidal and cytotoxic activity assay. The assays were carried out as described previously (Nibret *et al.*, 2009). The extracts and pure compounds were dissolved in dimethyl sulfoxide (DMSO). The extracts were serially diluted with culture medium in two-fold fashion into seven different concentrations so as to attain final concentrations ranging from 3.91 to 250 μg/mL in 96-well plates. Each concentration of the test drug was done in triplicate and repeated twice. The solvent, DMSO, did not exceed 1.25% in the medium that contained the highest concentration of extract or compound tested. Wells containing only solvent and wells without solvent served as controls. Diminazene aceturate, the standard trypanocidal drug, was also included as a positive control.

Both *T. b. brucei* and HL-60 cells were seeded into 96-well plates at a density of 1×10^4 cells and the total final volume of each well was maintained at 200 μL. The cells were incubated with the test drugs for 48 h and the anti-trypanosomal activity and cytotoxicity of extracts were evaluated using resazurin as a cell proliferation indicator dye (Rolón *et al*., 2006). Briefly, 10 and 6 μL of resazurin (3 mM), respectively, were added to trypanosome and HL-60 cell cultures and the cultures were incubated with resazurin for 24 h and 6 h, respectively, before measuring the 96-well plates at 48 h of incubation. The absorbance of the plates was read in a Tecan® plate reader at dual wavelengths of 492 nm and 595 nm. The concentration at which 50% of the growth of cells was inhibited was calculated from the dose-response curve by linear interpolation taking two concentrations above and below 50% (Huber and Koella, 1993).

Mitochondrial membrane potential assay using flow cytometry. A change in mitochondrial membrane potential was assessed using Apo Logix™ JC-1 kit (Bachem, UK). In healthy cells, the dye (JC-1) accumulates and forms J-aggregates in the mitochondria and fluoresces as red, whereas in apoptotic cells, where the mitochondrial membrane potential is collapsed, the JC-1 cannot accumulate in the mitochondria and remains in the cytoplasm and fluoresces green. In this assay, untreated controls, xanthatin treated trypanosomes and positive controls were included. *Trypanosoma brucei brucei* cells were cultured in Baltz medium and were treated with 1 μg/mL and 2 μg/mL of xanthatin and incubated for 2 h. Cells for the positive control were treated with cycloheximide (2 μM). After 2 h of incubation, the cells were harvested and prepared for flow cytometry analysis following the procedures described by the manufacturer of JC-1 kit. Flow cytometry was performed on FACSCalibur (Becton Dickinson, Heidelberg, Germany). Red and green fluorescences were acquired through FL2 and FL1 bandpass filters, respectively.

Trypanothione reductase (TR) inhibition assay. Recombinant TR from *Trypanosoma cruzi* was prepared following published procedures (Sullivan and Walsh, 1991). The TR activity was measured spectrophotometrically (Hitachi Spectrometer, Model −150-20, Japan) at 25 °C in TR assay buffer (40 mM Hepes, 1 mM EDTA, pH 7.5) (Jockers-Scherübl *et al*., 1989). A stock solution of 4 mM inhibitor was prepared in DMSO. In a final volume of 1 mL TR assay buffer, the assays contained 5% DMSO, 100 μM NADPH, 12 μU TR, 43 or 108 μM trypanothione disulfide (TS_2) and varying concentrations of the inhibitor. The reaction was started by adding TS_2 and the decrease of absorption at 340 nm due to NADPH consumption was followed. Controls contained all components except the inhibitor.

Test of irreversible inhibition of TR by xanthatin. In a 50 μL reaction mixture, different concentrations of xanthatin (0.4 μM, 4 μM, 40 μM and 120 μM) were incubated with 35 μL of TR (3.5 U/mL) solution and 160 μM NADPH at room temperature for 60 min. At 10 min intervals, an aliquot of 5 μL was removed and used as an enzyme solution in the standard assay (100 μM NADPH, 108 μM of TS_2). For checking the stability of the reduced enzyme over the pre-incubation period, a control contained all components except the inhibitor. The third premix contained buffer, TR and inhibitor but no NADPH to check if also the oxidized enzyme can react irreversibly.

Prostaglandin E_2 assay. To determine whether prostaglandin E_2 (PGE_2) production was affected by xanthatin treatment, PGE_2 production in MIA PaCa-2 cells was assessed using a Monoclonal EIA Kit® (Cayman® Chemicals). After 24 h of incubation with 25 and 100 μg/mL of xanthatin, the cells were stimulated with 30 μM of arachidonic acid. After 15 min, culture supernatants were collected and centrifuged to remove debris. Prostaglandin levels were then determined by a competitive enzyme immunoassay using PGE_2 Monoclonal EIA Kit® (Basu *et al*., 2005). Inhibition of the prostaglandin E_2 formation was calculated relative to the blank control and NS-398 was used as a positive control.

5-Lipoxygenase inhibition. The inhibition of lipoxygenase by xanthatin was determined spectrophotometrically in the following conditions. One mL of 0.1 M potassium phosphate buffer pH 9.0 containing 10 μL enzyme soybean lipoxygenase, (7.9 U/mL stock solution) and 20 μL of various concentrations of xanthatin were incubated at room temperature for 10 min. The reaction was initiated by adding 25 μL of 62.5 μM sodium linoleate and the reaction was monitored at 234 nm at 10 s intervals in an LKB® Biochrom spectrophotometer. The initial reaction rates were determined from the slope of the straight line portion of the curve and inhibition of the enzyme activity was calculated by comparison with the negative control. Norhydroguaretic acid (NDGA) was used as a positive control.

DNA–methyl green assay. The possible interaction of xanthatin with DNA was evaluated following published procedures (Burres *et al*., 1992; Wink *et al*., 1998). Different concentrations of xanthatin and the standard drugs diminazene aceturate and ethidium bromide were dissolved in absolute ethanol and 100 μL was dispensed into 96-well microtiter plates. Afterwards, the solvent was removed under vacuum. After complete removal of the solvent, 200 μL of DNA–methyl green complex solution was added per well. The plates were incubated in the dark for 24 h and finally measured at 642 nm in a Safire2® plate reader. Wells containing only the DNA–methyl green complex served as negative controls. The auto-absorbance of the compounds was subtracted in the IC_{50} calculations.

Caspase activation assay. The ability of xanthatin to trigger caspase 3/7 activity in MIA PaCa-2 cancer cells was assessed by caspase-Glo™ 3/7 assay kit (Promega® Mannheim, Germany) (Youns *et al*., 2009). This test provides a proluminescent caspase-3/7 substrate, which contains the caspase-3 specific tetrapeptide sequence DEVD in a reagent optimized for cell lysis and for the determination of caspase activity. Cells cultured in DMEM were seeded in 96-well plates and were treated with 12.5, 25, 50 and 100 μg/mL of xanthatin. After 6 h treatment, 100 μL of caspase 3/7 reagent was added to each well, mixed and incubated for 30 min at room temperature. Luminescence was measured in a Mithras LB 940 instrument (Berthold Technologies, Bad Wildbad, Germany). The caspase activation was expressed as percentage of the untreated medium control.

RESULT

Isolation and identification of compounds

The isolated compounds **1**, **2**, **3** and **4** were identified to be squalene, xanthatin, stigmasterol and β-sitosterol-O-glucoside, respectively, by mass spectrometry and NMR (Fig. 1). Large quantities of xanthatin (compound **2**) were obtained by column chromatographic technique. Xanthatin ($C_{15}H_{18}O_3$) was analysed for its fragmentation pattern using ESI MS/MS and GLC-EI MS. In ESI MS positive mode, xanthatin showed a prominent pseudomolecular ion at *m*/*z* 247. In EI MS, xanthatin revealed a molecular ion at *m*/*z* 246.22; its molecular mass was calculated to be 246.31. The NMR spectra and MS spectrometric data of the isolated compounds were all in agreement with previously published data (Marco *et al*., 1993; Ali *et al*., 2002) and with characteristics of authentic compounds.

Trypanocidal and cytotoxic activities

The trypanocidal and cytotoxic activities of the four compounds isolated from the leaves of *X. strumarium* are summarized in Table 1. Xanthatin was found to be the most active trypanocidal agent with an IC_{50} value of 2.63 μg/mL. The selectivity index (SI) of 20 calculated for xanthatin was found to be much lower than that of the standard trypanocidal drug diminazene aceturate (SI > 1464), implicating that xanthatin exhibits cytotoxic effects on the human cell line, HL-60.

Xanthatin induces mitochondrial membrane potential ($\Delta\Psi_m$) reduction in *T. b. brucei*

A decline of mitochondrial membrane potential gives a clue for an early event in the process of cell death. This phenomenon was studied using membrane potential-sensitive probe JC-1, which forms monomers (green fluorescence) at low membrane potential and J-aggregates (red fluorescence) at higher potential. After 2 h of incubation with test drugs, a higher decrease in red fluorescence was observed in trypanosomes treated with 1 μg/mL and 2 μg/mL of xanthatin than in those trypanosomes in the negative control and positive control (cycloheximide treated cells) (Fig. 2).

Anti-trypanothione reductase (TR) activity of xanthatin

Trypanothione reductase that occurs exclusively in trypanosomatides is the key enzyme of the parasite thiol redox metabolism. Up to a concentration of 100 μM, xanthatin did not show any inhibition. In the second approach the enzyme was allowed to react with xanthatin in the presence or absence of NADPH. After different times, the remaining activity was measured in a standard assay. Both in the presence and absence of NADPH a weak inactivation was observed which may suggest unspecific reaction with Cys 52 in the active site. Incubation of the reduced enzyme with 4 μM of xanthatin for 1 h caused a 26% TR inhibition (Table 2). However, the degree of inactivation did not significantly increase at higher xanthatin concentrations, suggesting a rather unspecific interaction.

Xanthatin inhibits PGE_2 synthesis

To determine whether xanthatin is able to inhibit cyclooxygenase activity, the inhibition of PGE_2 synthesis by xanthatin was assessed in MIA PaCa-2. Treatment of the cells with 100 μg/mL of xanthatin for 48 h reduced the arachidonic acid stimulated PGE_2 secretion by 24% compared with the DMSO-treated controls. Treatment of cells with 25 μg/mL of the same compound also inhibited PGE_2 secretion by 22%. The standard drug, NS-398 at a concentration of 3.14 μg/mL inhibited the PGE_2 secretion by 64% (Fig. 3).

Xanthatin inhibits 5-lipoxygenase

Xanthatin inhibited 5-lipoxygenase activity in a dose-dependent manner (Fig. 4). A 92% inhibition was

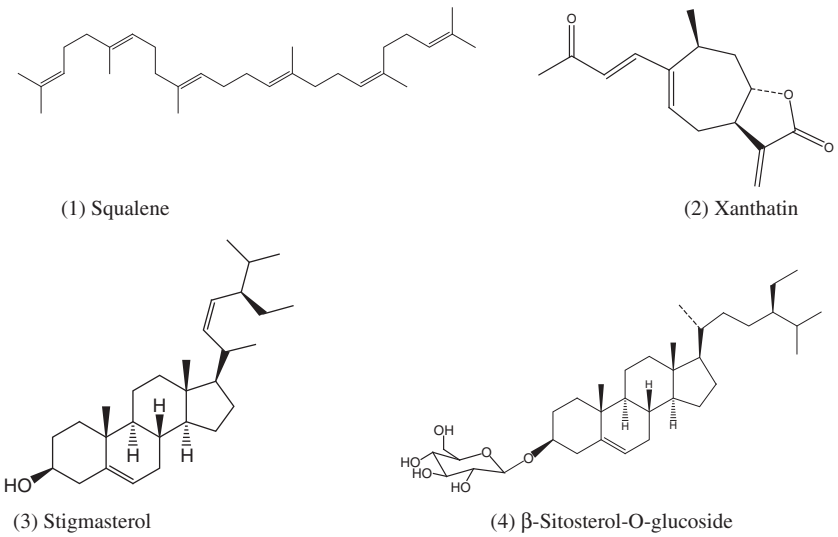

Figure 1. Chemical structures of compounds that were isolated from *X. strumarium* and tested for their trypanocidal and cytotoxic activities.

Table 1. Antitrypanosomal and cytotoxic activities of extracts and compounds from *X. strumarium* leaves

Extract/compound	IC_{50} (μg/mL)		Selectivity index (SI)
	T. b. brucei	HL-60	
Water extract	128.88	>250	>2
Methanol extract	10.86	>250	>23
Dichloromethane extract	3.82	>250	>65
Squalene (1)	4.90	7.09	1.5
Xanthatin (2)	2.63	52.50	20
Stigmasterol (3)	160.92	50.07	0.31
β-Sitosterol-O-glucoside (4)	19.61	24.91	1.3
Diminazene aceturate	0.088	>128.88	>1464

observed at a concentration of 97 μg/mL, which is the highest concentration of the test compound investigated in this assay. The IC_{50} value of the compound was found to be 61.95 μg/mL. In comparison, the IC_{50} value of the standard drug, NDGA, was calculated to be 0.72 μg/mL.

Xanthatin does not intercalate DNA

Xanthatin showed no intercalation of the DNA–methyl green complex up to the highest (250 μg/mL) concentration tested. The drugs, diminazene aceturate and ethidium bromide, however, showed strong interactions with DNA with IC_{50} values of 26.04 μg/mL and 44.70 μg/mL, respectively.

Xanthatin induces caspase 3/7 activation

MIA PaCa-2 cells treated with 12.5, 25, 50 and 100 μg/mL of xanthatin were evaluated for caspase 3/7 activation. Xanthatin induced a strong caspase 3/7 activity in a dose-dependent manner. The highest caspase 3/7 activity was observed after 6 h of treatment with 100 μg/mL of xanthatin (Fig. 5).

DISCUSSION

The bio-guided evaluation of methanol and dichloromethane extracts from *X. strumarium* revealed the presence of bioactive compounds in both crude extracts. Sub-fractions from the extracts had been tested for their biological activities and they were verified to contain the major compound, xanthatin. Other lipophilic compounds of the original extract were then excluded from the extract using activated charcoal so as to isolate xanthatin from this plant. Xanthatin showed the highest trypanocidal activity with a good selectivity index from the four compounds that were isolated and tested. Squalene also exhibited trypanocidal activity. Stigmasterol had the lowest trypanocidal activity, but β-sitosterol-O-glucoside, showed some activity. The sugar moiety attached to the sterol is probably responsible for the higher activity of β-sitosterol-O-glucoside compared with stigmasterol. The mode action of β-sitosterol-O-glucoside is apparently like saponin, which anchors the lipophilic moiety in the phospholipid bilayer of biomembrane and keeps out the hydrophilic part (sugar moiety) that interacts with other glycoprotein and glycolipid outside the cell membrane. This interaction will make the cells leaky and ultimately results in a cell death (Wink, 2001, 2006, 2008).

The presence of xanthatin in *Xanthium* species has been reported by various authors. The compound was shown to have cytoprotective activity against ulcers in rats that were induced by absolute ethanol (Favier *et al*., 2005). It was also shown to have *in vitro* anticancer activities in human colon (WiDr cells) and breast (MDA-MB-231) cancer cells (Ramírez-Erosa *et al*., 2007). In other studies, xanthatin showed bactericidal and fungicidal activities and its biological effects were correlated with the α-methylene-γ-lactone part of its chemical structure (Ginesta-Perls *et al*., 1994; Pinel *et al*., 2005). 8-Epi-xanthatin and 8-epi-xanthatin epoxide, which are the epimers of xanthatin, disturbed insect development (Kawazu *et al*., 1979), and showed antimalarial (Joshi *et al*., 1997), anti-farnesyltransferase and cytotoxic properties (Kim *et al*., 2003).

To determine the modes of action, xanthatin was tested against some molecular targets. Trypanothione reductase, a flavoenzyme that is found only in trypanosomatides but not in the mammalian cells, is one of the most attractive targets for drug development (Krauth-Siegel *et al*., 2005). As shown here, xanthatin did not compete for the active site. However, the inhibitor caused a time-dependent-inactivation of the reduced and oxidized form of the enzyme. However, this irreversible mode of interaction with trypanothione reductase is probably an unspecific reaction. This type of interaction might be attributed to the exomethylene part of xanthatin that enables its unspecific interaction,

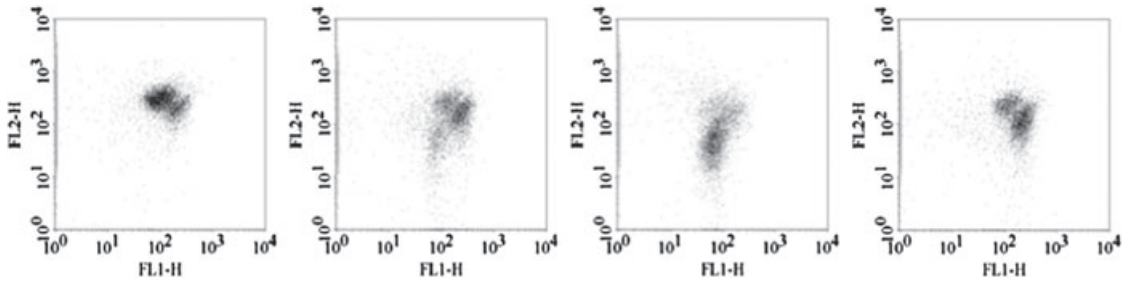

Figure 2. Xanthatin induced mitochondrial membrane potential ($\Delta\Psi_m$) reduction in *T. b. brucei* after 2 h of incubation with (B) 1 μg/mL and (C) 2 μg/mL of xanthatin. Cycloheximide (2 μM) was used as positive control. A plot of red (FL2) versus green (FL1) was analysed by flow cytometry and used as an estimate of $\Delta\Psi_m$.

Table 2. Irreversible inhibition of TR by xanthatin over time

Preincubation with ± NADPH		Inhibition (%) after incubation	
Inhibitor (μM)	Incubation time (min)	In the presence of NADPH	In the absence of NADPH
0.40	20	14	0
0.40	30	19	0
4	20	22	20
4	60	26	22
40	30	18	12
40	60	26	25
120	30	27	28
120	60	33	31

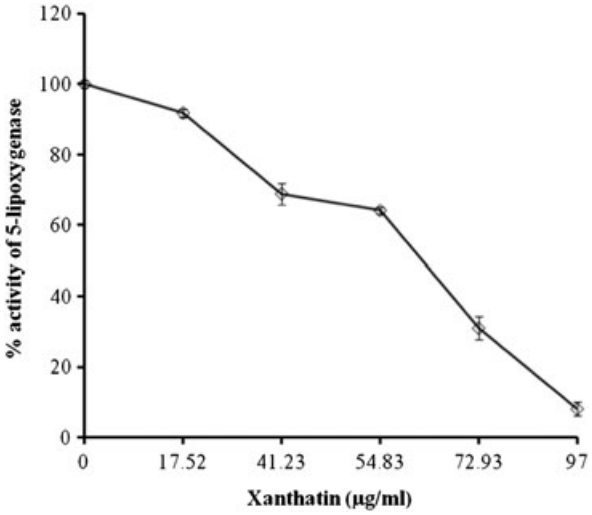

Figure 4. Inhibition of 5-lipoxygenase by xanthatin. The data are presented as mean ± SE.

e.g. covalent bonding, with any part of the enzyme (Wink, 2008). Although the sesquiterpene lactones with α-methylene-γ-lactone moiety are known to interact with thiol and amino groups, it seems that xanthatin did not strongly interact with cysteines of the active site of trypanothione reductase.

Prostaglandins and leukotrienes, which are produced by cyclooxygenase and lipoxygenase respectively, are the best known mediators in inflammatory processes. Both cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2) are involved in the production of PGE_2. Cyclo-oxygenase-2 (COX-2) upregulation is a common event in human cancer. Once the enzyme is induced, it plays a major role in the production of PGE_2 that is involved in tumorigenesis (Howe, 2007). COX-2 promotes cancer development by suppressing apoptosis, facilitating angiogenesis and enhancing the metastatic potential of cancer cells (Gupta and Dubois, 2001). In diseases such as trypanosomiasis where a sustained interplay between the parasite and the host is a common event, an occurrence of an inflammation process is unavoidable. Moreover, trypanosomes themselves produce prostaglandins and that may ultimately contribute to the overall pathology of the disease (Pentreath *et al.*, 1990; Kubata *et al.*, 2000). Xanthatin inhibited both cyclooxygenase and 5-lipoxygenase activity. Although xanthatin showed an antiinflammatory activity, this effect was not comparable with that of standard drugs that are known to inhibit the two key enzymes involved in the inflammation process. The prolonged survival of mice infected with drug-resistant *T. congolense* after *Xanthium strumarium* extract treatment (Nibret *et al.*, 2007) may be explained by (1) the low toxicity of the major bioactive compound, xanthatin, *in vivo* (Roussakis *et al.*, 1994), (2) the ability of xanthatin to inhibit the two key enzymes involved in an inflammation process, (3) moreover its direct effect in suppressing the parasitemia inside the host.

As shown here, xanthatin did not intercalate into DNA up to the highest concentration tested. It seems that the biological activity of the compound is a result of its effect on other molecular targets other than the DNA. The standard trypanocidal drugs (diminazene aceturate and ethidium bromide used in veterinary medicine), however, are famous for their ability to intercalate into DNA (Bailly *et al.*, 1992). The trypanocidal activity of these two drugs is due to their ability to

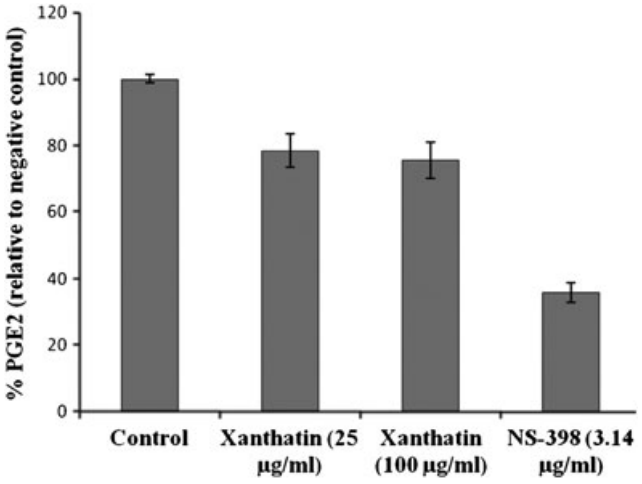

Figure 3. Inhibition of PGE_2 secretion from MIA PaCa-2 cells by xanthatin. NS-398 is the positive control. The data presented are mean value ± SE.

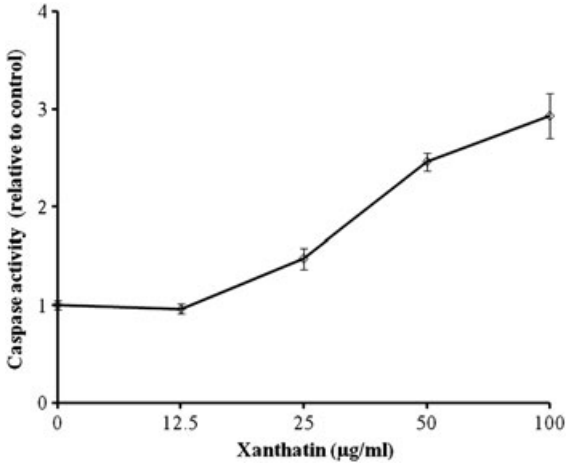

Figure 5. The effect of xanthatin in triggering 3/7 caspase activity in MIA PaCa-2 cells in relation to negative control. The data are presented as mean ± SE.

intercalate DNA causing mutation, interfering with the transcription and then ultimately killing the parasite (Wink, 2007).

One of the mechanisms by which some known anticancer agents kill cancerous cells is via apoptosis. In the present investigation, it was determined whether xanthatin induces apoptosis in MIA PaCa-2 cells as one of its modes of action for cytotoxic activity. The maximum caspase 3/7 activity was observed after 6 h of incubation with 100 μg/mL xanthatin. It seems that the induction of apoptosis was effected by activation of caspase 3, which is considered to be a final effector enzyme responsible for caspase-dependent cell death. DNA fragmentation, which is the hallmark of apoptosis, was also observed in human leukaemia cell line (HL-60) (data not shown). The sesquiterpene lactones of various types have been known to induce apoptosis in various cell lines. Sesquiterpene lactones trigger mitochondrial membrane transition, loss of mitochondrial membrane potential and release of pro-apoptotic mitochondrial proteins, e.g. cytochrome c and Smac, subsequently leading to caspase activation and apoptotic cell death (Zhang *et al*., 2005). Apoptosis inducing activity of xanthatin in the present study is in agreement with the results obtained from other sesquiterpene lactones having α-methylene-γ-lactone that were capable of inducing apoptosis in cancer cells with characteristic features of strong caspase-3 activity and phosphatidylserine exposure, among other effects (López-Antón *et al*., 2007; Ma *et al*., 2009). Xanthatin also caused a programmed cell death-like in trypanosomes as evidenced by a reduction in mitochondrial membrane potential (Rosenkranz and Wink, 2008).

In summary, xanthatin exerts its biological activity, among others, by inhibiting cyclooxygenases, 5-lipoxygenase and inducing apoptosis via caspase-dependent manner that resulted in a controlled cell death that avoided unwanted inflammation process that would otherwise aggravate the pathology that is commonly observed in diseases such as trypanosomiasis.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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