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# Research Article
# Anticancer Agents from *Xanthium strumarium* Fruits Against C6 Glioma Cells

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## Abstract

**Background and Objective:** Glioma is known to be highly resistant to radiotherapy and chemotherapy. Therefore, new therapeutic agents for glioma are being extensively researched. In this study, we aimed to investigate anti-tumoural effects of *Xanthium strumarium* L. (Asteraceae family) extracts and its pure constituents on C6 glioma cells. **Materials and Methods:** In this study, cytotoxicity, lipid peroxidation, apoptotic effect, phosphatidylserine externalization, cell cycle analysis, invasion, kinase activity, COX-2 expression and micronucleus tests were used. **Results:** As a result of IR, $^{1}$H-NMR, $^{13}$C-NMR, 1D and 2D NMR analysis, 5 known compounds were characterized as xanthinosin (1), stigmasterol (2), xanthatin (3), xanthinin (4) and xanthanol (5). Among them, the most cytotoxic xanthanolide was xanthinin (4) with an $IC_{50}$ value of 7.5 µM. It was thought that this effect may be due to the oxidative damage and protein kinase activity of this compound. While xanthinosin (1) and xanthanol (5) showed the selective cytotoxic effect on C6 glioma cells with low $IC_{50}$ values (22.46 and 40.12 µM, respectively), it was determined that their toxicity on Human Umbilical Vein Endothelial (HUVEC) cells was lower ($IC_{50}$ >75 µM). **Conclusion:** It was concluded that xanthanolides isolated from *X. strumarium* could be used as target molecules in future studies as chemotherapeutic agents on glioma.

Key words: *Xanthium strumarium*, cocklebur, xanthanolides, glioma, apoptosis, lipid peroxidation, radiotherapy

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Glioma is the name given to tumours around glial cells and usually occurs in the brain and spinal cord tissues. Glioma, the most common form of brain tumours, can be seen both supratentorially and infratentorial. An estimated 24,530 adults (13,840 men and 10,690 women) in the USA diagnosed with brain tumors. Brain tumors account for 85% to 90% of all primary central nervous system tumors[1]. Glioblastomas constitute about 57% of the average annual of all neuroepithelial tumors and about 48% of all malignant brain and central nervous system tumors[2]. Furthermore, most patients with malignant glioma cannot survive more than 1 year. Despite surgery, radiotherapy and chemotherapy, the success rate of these treatments is often low due to the lack of knowledge about the molecular mechanisms underlying tumour formation, invasiveness and tumour heterogeneity in glioma[3-5]. C6 rat glioma cells are morphologically very similar to human glioblastoma multiforme[3].

Since ancient times, natural products isolated from plants and animals have played a key role in the discovery of new drugs and the treatment of various diseases. Approximately 67% of anticancer drugs such as vinblastine, vincristine, paclitaxel (taxol) and docetaxel are plant-derived products[6]. In addition to their use as medicine, some natural products are also used as starting materials in the synthesis of some complex drugs. *Xanthium strumarium* L. (Syn. *X. brasilicum* Vell.) (Asteraceae), commonly known as cocklebur, is a 1 year plant and grows on river banks, road edges, irrigation ditches, rocky sediments and steppes[7]. *X. strumarium* is recognized by varies names such as Geshkay in Pakistan, Cang er zi in China and Woolgarie bur in North America and Pıtrak or Domuz pıtrağı in Anatolia[8]. It is in the first place in terms of the prevalence in agricultural areas with a rate of 90.57%[7]. The leaves of *X. strumarium* and *X. spinosum* are traditionally used as a diuretic, diaphoretic and sedative[8]. In the Muğla-Fethiye region of Turkey, powdered fruits of this species are added into coffee and anise to enrich the flavour of these beverages[8].

Sesquiterpenes, one of the plant secondary metabolite groups, have been extensively studied for their antitumour effects against various cancer cells. *Xanthium* species are very rich in guaien class sesquiterpene lactones, also known as xanthanolides[9-12]. A variety of xanthanolides including xanthinosin, xanthatin, xanthinin, xanthanol, 4-O-dihydroinusoniolide, deacetyl-xanthanol, 4-epixanthanol, 4-epiisoxanthanol, isoxanthanol, 8-epi-xanthatin and 2-hydroxy anthinosin have been isolated and characterized from the *Xanthium* species[9-12]. Since xanthanolides contain multiple chiral centres, they vary in their chemical structures and therefore most xanthanolides are generally diastereoisomers of each other[10,13]. Various extracts of *Xanthium* species and their xanthanolides have been reported to have *in vitro* cytotoxic activities on some cancer cells[9,12-19]. For instance, xanthatin, 4-epixanthanol, 4-epi-isoxanthanol and 2-hydroxyx anthinosin isolated from the chloroform extract of the leaves of *X. italicum* have been reported to show growth inhibitory effect on A431 (skin cancer), HeLa (cervix cancer) and MCF-7 (breast cancer) cells[12]. Xanthatin and xanthinosin isolated from *X. strumarium* were shown to have moderate to high cytotoxic activity on the WiDr ATCC, MB-231 ATCC and NCI-417 cells[13]. Furthermore, 8-epi-xanthatin and 8-epi-xanthatinepoxide from *X. strumarium* significantly reduced the *in vitro* proliferation of 5 human cancer cells known as A549 (non-small cell lung cancer), SK-OV-3 (ovary cancer), SK-MEL-2 (melanoma), XF498 (central nervous system cancer) and HCT-15 (colon cancer)[16]. In particular, the cytotoxic activities of xanthatin have been reported in various cancer cells such as MDA-MB-231 (breast cancer)[17], A549 (lung cancer)[18] and B16-F10 (murine melanoma)[19].

The study aimed to evaluate the cytotoxic activities of xanthanolides isolated from the prickly fruits of *X. strumarium* on C6 glioma and HUVEC cells by using several test systems such as lipid peroxidation levels, apoptotic DNA fragmentation, cell cycle analysis, *in vitro* invasion assay, *in vitro* kinase activity, immunocytochemical staining and micronucleus counting. To the best of our knowledge, there is no study in which the anticancer activity of metabolites isolated from *X. strumarium* was tested on C6 glioma cells. This study could be assumed as the 1st report on the anticancer activity of xanthanolides isolated from this species on the cells in question.

## MATERIALS AND METHODS

**Study area:** First part of this study about xanthanolides isolation and purification was carry out at Organic Chemistry Lab., Kilis 7 Aralik University, Kilis, Turkey from 2016-2018 and 2nd part about biological activity at Cell Culture Lab., Gaziantep University, Gaziantep, Turkey from 2018-2020.

**General:** NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer using $CDCl_3$, operating at 400 MHz and 100 MHz for $^1$H-NMR and $^{13}$C-NMR, respectively. Chemical shifts were recorded in $\delta$ (in ppm) downfield from TMS as an internal standard and coupling constants were presented in Hz. The FTIR spectra were recorded on a PerkinElmer Model 1600 FT-IR spectrophotometer (Massachusetts, USA). Melting

points were determined by using a Thermo Scientific 9200 (Waltham, MA, USA) apparatus. Optical rotation was measured on a Bellingham and Stanley ADP 220 polarimeter (Tunbridge Wells, UK) equipped with a sodium lamp and a 10 cm microcell with $CHCl_3$ as the solvent at 20°C. Column Chromatography (CC) was carried out over silica gel 60 (70-230 and 230-400 mesh, Merck), Thin-Layer Chromatography (TLC) were performed on silica gel 60 pre-coated plates, (F-254 (Merck). The spots on the TLC plates were visualized by UV254, UV366 and spraying with 1% vanillin-$H_2SO_4$ followed by heating (105°C).

**Plant sample and extraction:** The plant samples were collected from Kilis-Sogutludere location, Southeastern Anatolia region of Turkey, in November, 2015 by Assist. Prof. Bedrettin Selvi in Botany Department of Gaziosmanpaşa University, Tokat, Turkey (Voucher Number: GOPU 7953). The powdered fruits (1 g) were weighed and extracted individually in $CH_2Cl_2$, ethyl acetate, acetone and methanol (10 mL) to determine the appropriate organic solvent for extraction. The extracts were applied on preparative TLC plate using $CHCl_3$:$CH_3OH$ (9:1) and $CHCl_3$:ethyl acetate (9:1 and 8:2) mobile phases. According to the TLC chromatogram, the suitable organic solvents were determined as $CH_2Cl_2$ and acetone for extraction. The acetone was evaporated under reduced pressure and a dark brownish extract (63.66 g, 2.65% yield) was obtained.

**Isolation of metabolites from the *X. strumarium* fruits:** The concentrated acetone extract (60 g) was subjected to silica gel CC (450 g, 70-230 mesh) by using $CHCl_3$/EtOAc (8:2) eluent system. The fractions (50 mL each) were checked on silica gel TLC by using $CHCl_3$/EtOAc (8:2) and hexane/EtOAc (9:1 and 7.5:2.5) mobile phase systems and the fractions with the same Rf values were combined. Thus, 6 fractions, named as XSA (fractions 1-8, 4.16 g), XSB (fractions 9-25, 16.90 g), XSC (fractions 26-55, 4.61 g), XSD (fractions 56-99, 11.26 g), XSE (fractions 100-109, 6.30 g) and XSF (fractions 110-135, 8.35 g) were obtained. According to TLC analysis, the XSA fraction was determined to contain vegetable oil and chlorophyll, therefore, no chromatographic studies were performed on it. XSB fraction (16.90 g) was subjected to the silica gel CC (200 g, 70-230 mesh) by using $CHCl_3$/EtOAc (9.5:0.5 and 9:1) to obtain the compounds 1 (4.05 g), 2 (218 mg) and 3 (120 mg). The compound 3 (2.63 g) was also isolated from the XSC fraction (4.61 g) by silica gel CC (100 g, 70-230 mesh) eluted with $CHCl_3$/EtOAc (9:1) and was crystallized with hexane/EtOAc (6:4). The fraction XSD was found to contain a major component according to TLC analysis. To isolate this component, the fraction (11.26 g) was subjected to silica gel CC (160 g, 70-230 mesh) by using hexane/EtOAc mobile phase to obtain compound 4 (1.10 g). The compound 4 was crystallized with hexane/EtOAc (6:4). Compound 5 (575 mg) was isolated from the fraction XSE (6.30 g) by using silica gel CC (85 g, 230-400 mesh) eluted with hexane/EtOAc (6:4). The spectroscopic data of the isolated compounds were given in supplementary materials.

**Cytotoxicity studies:** HUVEC (ATCC CRL-1730) and C6 rat glioma cells (ATCC CCL-107) were commercially purchased from ATCC. HUVEC cells were selected as representative cells to evaluate angiogenesis of various formulations due to limited accumulation of active agents in the tumour and low drug permeability from vessels to brain tumour cells[20]. Cells were grown in DMEM (Pan Biotech) medium containing 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine at 37°C in a 5% $CO_2$ incubator[21]. To determine the cytotoxicity of the compounds on the cells, Methylthiazo Tetrazolium (MTT), which is an assay based on the colourimetric measurement of mitochondrial dehydrogenase enzyme activity in living cells, was applied[22]. Xanthinosin (1), stigmasterol (2), xanthatin (3), xanthinin (4) and xanthanol (5) were prepared as stock solutions (10 mM). Acetone and dichloromethane extracts were also prepared as stock solutions in DMSO (100 mg $mL^{-1}$). The solutions were stored at +4°C. C6 glioma and HUVEC cells were seeded into each well of the 96 well plates ($5 \times 10^3$ cells). The pure metabolites (6.25-100 µM) and the extracts (6.25-100 µg $mL^{-1}$) were added to the cells. These concentrations were determined taking into account the maximum doses of the drugs that can be administered to humans[23-25]. Cells were incubated for 24-48-72 hrs at 37°C in 5% $CO_2$. After these incubation periods, MTT was added. To dissolve the formazan crystals in water, SDS (10%) solution was added after 4 hrs. The absorbance of the cells was measured at 570 nm after 18 hrs. All experiments were performed in triplicate. The viability rates (%) of the cells were determined by using the following formula[26]:

$$\text{Percentage} = \frac{\text{Mean absorbance of sample} - \text{Mean absorbance of blank}}{\text{Mean absorbance of negative control} - \text{Mean absorbance of blank}} \times 100$$

$IC_{50}$ values of the extracts and pure metabolites were calculated using ED50 plus v1.0 software and further analysis were performed by using $IC_{50}$ values of the metabolites[27].

**Lipid peroxidation:** MDA levels of the C6 glioma and HUVEC cells were determined by using the methods described previously[26,28].

**Apoptotic DNA fragmentation assay:** Apoptosis can be visualized by standard agarose gel electrophoresis on the basis that a nuclear endonuclease creates a ladder pattern of 180-200 bp fragments on DNA. Therefore, in this study, the formation of a ladder in DNA by induction of apoptosis in C6 and HUVEC cells was investigated. Cells ($5\times10^4$) were seeded in 24 well plates, incubated at 37°C and maintained 5% $CO_2$ for 48 hrs. After the addition of *X. strumarium* extracts and metabolites at the same doses, the cells were incubated for 48 hrs. After incubation, the nuclear DNA of the cells was extracted and purified using QIAamp DNA Mini Kit (QIAGEN 51304, Manchester, England). DNA concentrations were estimated by measuring the absorbance at 260 nm. Oligonucleasomal fragmentation of DNA was run in 1.75% agarose (60 V, 3.5 hrs) and visualized under UV light.

***In vitro* kinase assays:** The $2\times10^6$ cells were seeded in 24 well plates, incubated at 37°C and maintained 5% $CO_2$ for 24 hrs. After the addition of *X. strumarium* extracts and metabolites, cells were incubated for 24 h at the same condition. Kinase activation in the cells with metabolites and extracts was analyzed by using the ADP sensor Universal Kinase Activity Assay Kit (BioVision, USA). As described earlier, cells were collected by trypsinization. The cell pellet (500 µL or ~4 volumes) was suspended and homogenized in the buffer on ice (10-50 passes) until efficient lysis was confirmed by viewing the cells under the microscope. Homogenized cells were diluted with PBS and dialyzed in dialysis tubing (8-10 kD mwco) (SpectraPor, Sigma Aldrich, USA). After 24 hrs, protein concentration in cell lysate was measured at 280 nm by a microliter plate photometer. To increase the kinase activity, 20 µL (1-100 ng) sample and 20 µL Kinase Assay Buffer were placed in each well and 1-2 µL 1 M DTT was added.

To obtain the ADP standard curve, serial dilutions of ADP standard and Kinase Assay Buffer were added into wells and absorption at 570 nm was read after incubation at room temperature for 2 hrs. Appropriate dilutions of the sample were tested to ensure that readings remained within the linear range of the standard curve. Kinase activity was calculated by using the formula given by kit instruction (ADP sensor Universal Kinase Activity Assay Kit, BioVision, USA) below:

$$\text{Sample kinase activity} = \frac{B}{C} \text{x } \Delta T = \text{pmol/min/ng} = \text{µmol/min/m} = \text{U mg}^{-1}$$

where, B was ADP of the standard curve (pmol), C was protein concentration (ng) and ΔT was reaction time.

For quantification of cellular total protein, a modified lowry assay for microplates was used[29].

**Immunocytochemical analysis:** Cellular COX-2 expression level in cells in the presence of extracts and metabolites was determined by using immunocytochemical staining according to the reference[30].

**Micronucleus (MN) assay:** The *in vitro* MN assay is a mutagenicity test used to detect chemicals that induced to form small DNA fragments binding membrane, micronucleus, in interphase cells. Micronucleus originates from chromosome fragments without a centromere or whole chromosomes that not immigrated with the other chromosomes in anaphase stage. Hence, this assay detect the effect of clastogenic and aneugenic chemicals. The MN test was performed following the guidelines for *in vitro* mammalian cell micronucleus testing with some modifications[31].

**Phosphatidylserine translocation (Annexin V-FITC):** Annexin V was used to detect apoptotic activity by confirming the externalization of phosphatidylserine residues in early apoptosis. Apoptosis detection Kit (BioVision, CA, USA) was used according to the manufacturer's instructions. Brie y, $5\times10^3$ cells were deposited in each well of 24-well microplate. After 24 hrs of growth, the extracts and xanthanolides were added for another 24 hrs. The cells were then harvested and washed with PBS solution and resuspended in binding buffer. The cells were stained with annexin V-FITC and PI for 5 min at room temperature in the dark. Finally, apoptotic cells were quantified by using flow cytometry (FACS Calibur, BD Biosciences, USA) and analyzed by using Muse Cell Analyzer (Merck, Germany).

**Cell cycle analysis:** The effect of the extracts and xanthanolides on the cell cycle was determined according to a method described previously[32].

***In vitro* invasion analysis:** A total of $2.5\times10^5$ cells treated with extracts and xanthanolides were seeded in a 24-well plate, cultured for 24 hrs at 37°C and maintained at 5% $CO_2$. Then, the cells were transferred on the top of the Matrigel-coated invasion chamber (24-well, 8 µm pore size) (BD Biosciences) in a serum-free medium. Then the medium containing serum was added to the lower chamber and

incubated for 16 hrs. Cells were washed twice in PBS and fixed with 3.7% PBS containing formaldehyde at room temperature for 2 min. After washing twice in PBS, pure methanol was added for permeabilization at room temperature for 20 min. The cells were stained with Giemsa for 15 min in a dark room. Non-invaded cells were removed from the inner part of the chamber by using a cotton swab and the rest of the cells were analyzed under an inverted microscope. Diff-Quick differential staining kit (Dade Behring, Inc., Newark, DE, USA) was purchased for fixation and staining of invaded cells[4].

**Statistical analysis:** Results were expressed as Mean±SD. Tukey's test was used to compare the differences between the average values of the groups. Statistical analysis was performed by using GraphPad Instat (v. 3.10) (GraphPad Software Inc., San Diego, CA, USA) and $p<0.05$ were considered to be statistically significant.

## RESULTS AND DISCUSSION

**Structure elucidation:** In the current study, 5 known compounds including 4 xanthanolides (1, 3, 4, 5) and a triterpene (2) were isolated from the acetone extract of common cocklebur fruits by using CC, TLC and crystallization methods. The chemical structures of the compounds were characterized by using UV, IR, $^{1}$H- and $^{13}$C-NMR spectroscopic methods (Fig. 1). The structures of the compounds were also confirmed by using 1D-NMR (DEPT) and 2D-NMR methods ($^{1}$H-$^{1}$H COSY, $^{1}$H-$^{13}$C COSY, HMBC and NOESY). The nuclear overhauser effects assigned between H-8/H-14, H-8/H-6b and H-9b/H-8 and between H-6a/H-7 and H-7/H9a in the NOESY spectra of the xanthinosin (1), xanthatin (3), xanthinin (4) and xanthanol (5), proved the *trans*-lactone ring junction and the β-oriented 14-methyl group. The chemical structures of the known compounds were also confirmed by comparison of their spectral data with those reported in the literature such as xanthinosin (1)[9,10,12,15,33], stigmasterol (2)[34,35], xanthatin (3)[9,10,12,15,33], xanthinin (4)[9,10,12,15,33] and xanthanol (5)[9,10,15,33] and long-range $^{1}$H-$^{13}$C correlations from their HMBC spectra (Fig. 2).

Sesquiterpenes, one of the plant secondary metabolite groups, have been extensively studied against various cancer cells due to their antitumour effects. *Xanthium* species, which were traditionally used in the treatment of various cancer types in different parts of the world are very rich in xanthanolides[9-12,16,33,36]. Since xanthanolides contain more than one chiral centre, they vary in their chemical structure and therefore most xanthanolites are usually diastereoisomers of each other[10,13]. Many xanthanolides including xanthinosin (1), xanthatin (3), xanthinin (4), xanthanol (5), 4-O-dihydroinusoniolide, deacetylxanthanol, 4-epixanthanol, 4-epiisoxanthanol, isoxanthanol, 8-epi-xanthatin and 2-hydroxyxanthinosin have been isolated and characterized from some *Xanthium* species[9-11,16,33]. In the current study, 4 xanthanolides, xanthinosin (1), xanthatin (3), xanthinin (4) and xanthanol (5) and a triterpenic compound, stigmasterol (2), were isolated from the acetone extract of the prickly fruits of *X. strumarium* by using CC, TLC and crystallization methods.

Table 1: $IC_{50}$ values of dichloromethane and acetone extracts of *X. strumarium* and five metabolites on C6 and HUVEC cell lines

| | $IC_{50}$ values | |
|---|---|---|
| Extracts and compounds | C6 | HUVEC |
| Dichloromethane ext. (μg mL$^{-1}$) | 25.56 | 20.53 |
| Acetone ext. (μg mL$^{-1}$) | 24.32 | 21.89 |
| Xanthinosin (1) (μM) | 22.46 | 74.65 |
| Stigmasterol (2) (μM) | 95.21 | >100 |
| Xanthatin (3) (μM) | 7.50 | 11.50 |
| Xanthinin (4) (μM) | 14.18 | 12.61 |
| Xanthanol (5) (μM) | 40.12 | 89.95 |

**Cytotoxicity results:** The MTT viability test was performed to determine the cytotoxic effects of the dichloromethane and acetone extracts of *X. strumarium* and 5 metabolites, xanthinosin (1), stigmasterol (2), xanthatin (3), xanthinin (4) and xanthanol (5) isolated from the acetone extract against C6 rat glioma and HUVEC cells. $IC_{50}$ values of the extracts and metabolites were calculated by using the ED50 plus v1.0 software (Table 1). The cytotoxic activities of the extracts and pure metabolites were shown in (Fig. 3a-g). As shown in the Figure, *X. strumarium* extracts inhibited the growth of C6 and HUVEC cells. Therefore, the plant's metabolites, xanthinosin (1), stigmasterol (2), xanthatin (3), xanthinin (4) and xanthanol (5), were isolated to significantly suppress or completely stop the proliferation of C6 and HUVEC cells (Fig. 3a-g). Except for stigmasterol (2), xanthanolides exhibited a significant inhibitory effect on cells even at low doses (at the concentrations of 6.25, 12.5 and 25 μM) (Fig. 3a-g). These results showed that xanthanolides were the potent cytotoxic compounds on C6 and HUVEC cells. On the other hand as can be seen from Fig. 3d, lower doses of stigmasterol (2) were found to be a weaker cytotoxic agent as compared to other xanthanolides. Cytotoxicity results showed that xanthinosin (1) and xanthanol (5) have selective cytotoxic activity on C6 glioma cells ($IC_{50}$: 22.46 μM and 40.12 μM, respectively), with less affecting the viability of HUVEC cells ($IC_{50}$: 74.65 μM and 89.95 μM, respectively) (Fig. 3c and g). These 2 xanthanolides

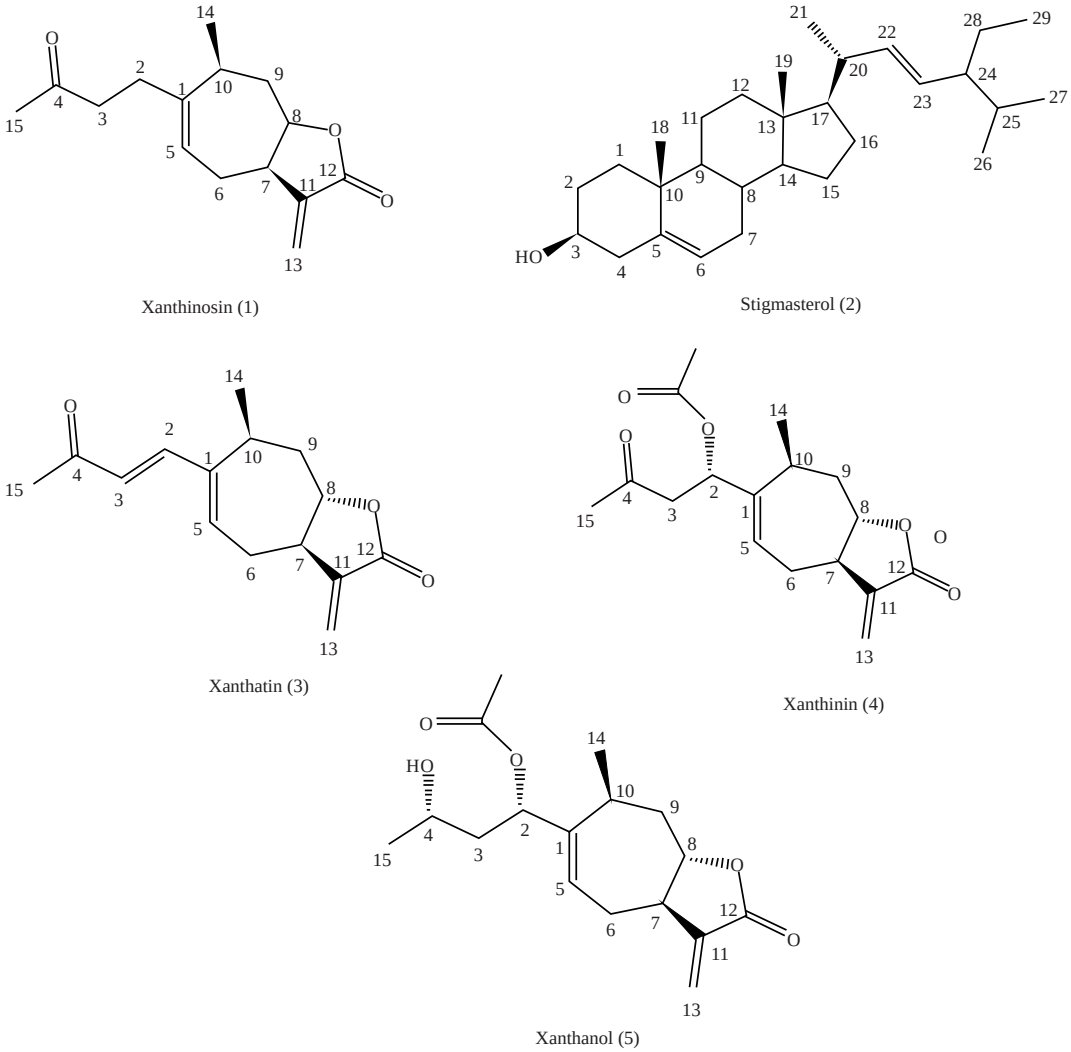


Fig. 1: Chemical structures of the *Xanthium strumarium* fruits metabolites

were thought to be potential substances with a high therapeutic index. Stigmasterol (2), a common steroid, caused a slight decrease in the viability of C6 cells ($IC_{50}$: 95.21 μM) but did not affect the viability of HUVEC cells ($IC_{50}$: >100 μM) (Fig. 3d). Other potential cytotoxic agents, xanthatin (3) ($IC_{50}$: 7.5-11.5 μM) and xanthinin (4) ($IC_{50}$: 12.61-14.18 μM), severely suppressed the viability of C6 glioma and HUVEC (Fig. 3e and f). In previous reports, xanthanolites isolated from *Xanthium* species were shown to be more active than extracts[12,16,17,19,33]. Our results consistent with these reports showed that dichloromethane and acetone extracts of *X. strumarium* exhibited significant inhibitory effects on the proliferation of C6 glioma and HUVEC cells (Fig. 3a and b). In addition to this, its pure metabolites, xanthinosin (1), xanthatin (3), xanthinin (4) and xanthanol (5) strongly inhibited the proliferation of C6 glioma cells. Additionally, xanthatin (3) and xanthinin (4) were found to be more active than the others. Our results also showed that xanthinosin (1) and xanthanol (5) exhibited a potent cytotoxic effect on C6 cells and vice versa on HUVEC cells. From these results, it can be concluded that the 4 pure metabolites [xanthinosin (1), xanthatin (3), xanthinin (4) and xanthanol (5)] acted as potent cytotoxic agents. It was thought that xanthinosin (1) and xanthanol (5), which exhibit selective cytotoxicity especially in C6 and HUVEC cells, can be used for cancer treatment as the alternative agents.

**Lipid peroxidation levels:** ROS are produced continuously as a result of aerobic respiration and oxidation of various substrates in living cells cause lipid peroxidation by attacking unsaturated fatty acids in tissues[37,38]. Lipid peroxidation is a well-defined mechanism of cellular damage in living

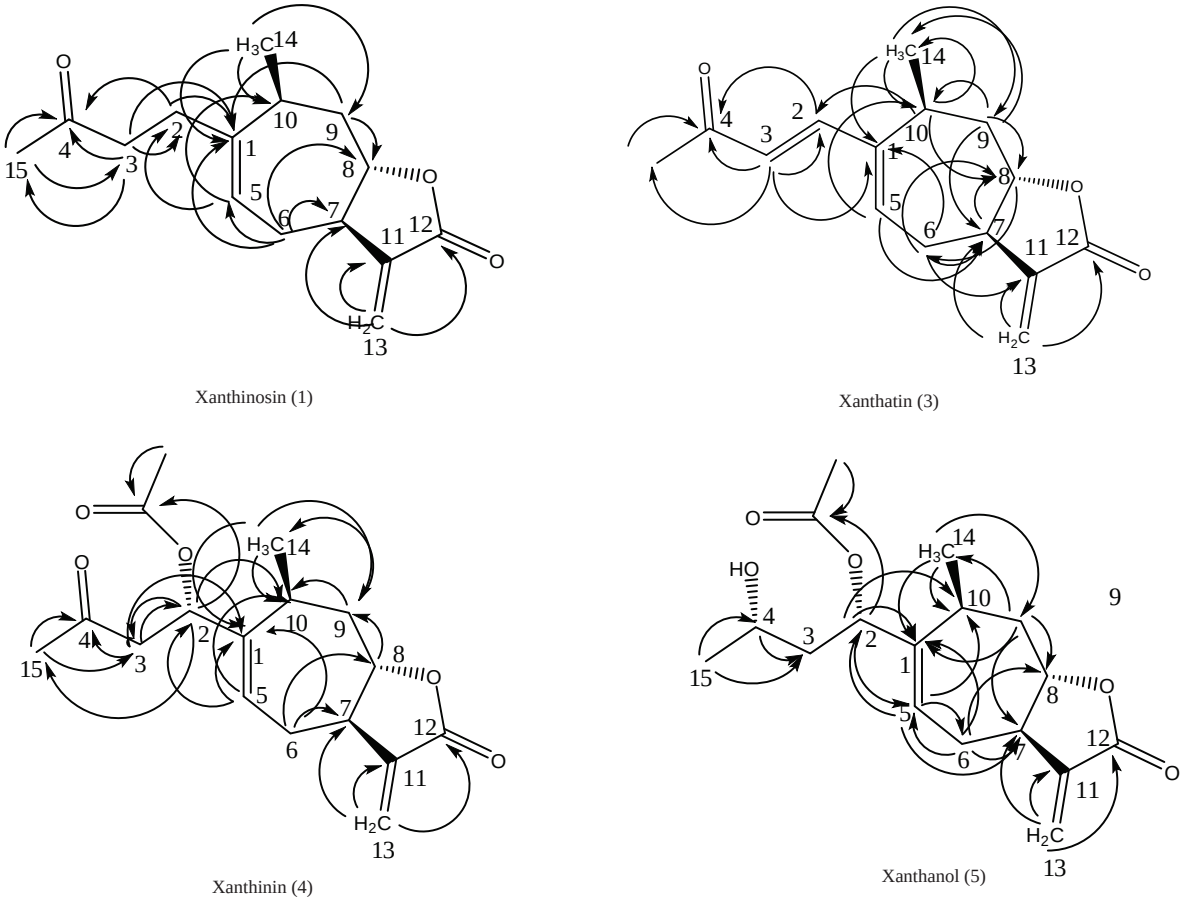


Fig. 2: HMBC correlations of the xanthanolides (H→C)

organisms and is an indicator of oxidative stress in cells and tissues. Lipid peroxides derived from polyunsaturated fatty acids are unstable and form a series of complex compounds containing reactive carbonyl compounds such as malondialdehyde (MDA). Some researchers have reported that increased reactive oxygen species that occurred as a result of lipid peroxidation in cancer cells may play a critical role in the progression of cancer[37,38]. Cells exposed to oxidative stress are more likely to be vulnerable to damage caused by ROS attacks arising from the exogenous agents[39]. Therefore, increasing ROS levels in cancer cells with different reagents is considered a treatment strategy for cancer[40,41]. In recent years, some experimental reports showed that chemotherapeutic anticancer agents were used as an important remedy to induce an increase in ROS production and ultimately apoptosis[42,43]. Therefore, levels of malondialdehyde (MDA), a lipid peroxidation product in C6 glioma and HUVEC cells, were measured to determine whether the extracts, pure compounds and the commercial anticancer agent 5-FU induced apoptotic activity by increasing oxidative stress. As shown in (Fig. 4a-b) the extracts and all compounds increased lipid peroxidation levels in C6 glioma and HUVEC cells in comparison to control. In particular, MDA levels were found to be statistically different in both cells treated with xanthatin (3) and xanthanol (5) ($p<0.05$ and $p<0.001$, respectively) (Fig. 4a-b). In addition, although xanthinosin (1) had an oxidative effect on C6 cells, it was found to be less oxidative on HUVEC cells ($p<0.01$). Oxidative stress levels in C6 glioma and HUVEC cells were found to be compatible with the cytotoxic activities of extracts and pure metabolites (Fig. 4a-b). These results show that cocklebur compounds exhibited cytotoxic activity due to their oxidative effects on C6 glioma cells.

**Apoptotic DNA fragmentation:** In recent years, some experimental reports have shown that ROS induction has been used by chemotherapeutic anticancer agents to induce apoptosis[33,41-43]. Therefore, DNA fragmentation analysis was performed according to $IC_{50}$ values of metabolites (cytotoxic dose and one more high dose alternatively) and extracts of *X. strumarium* fruits (Fig. 5a-b). As can be seen from Fig. 5a, the xanthatin (3), xanthanol (5) and acetone extract significantly caused DNA breakage but others broke DNA less

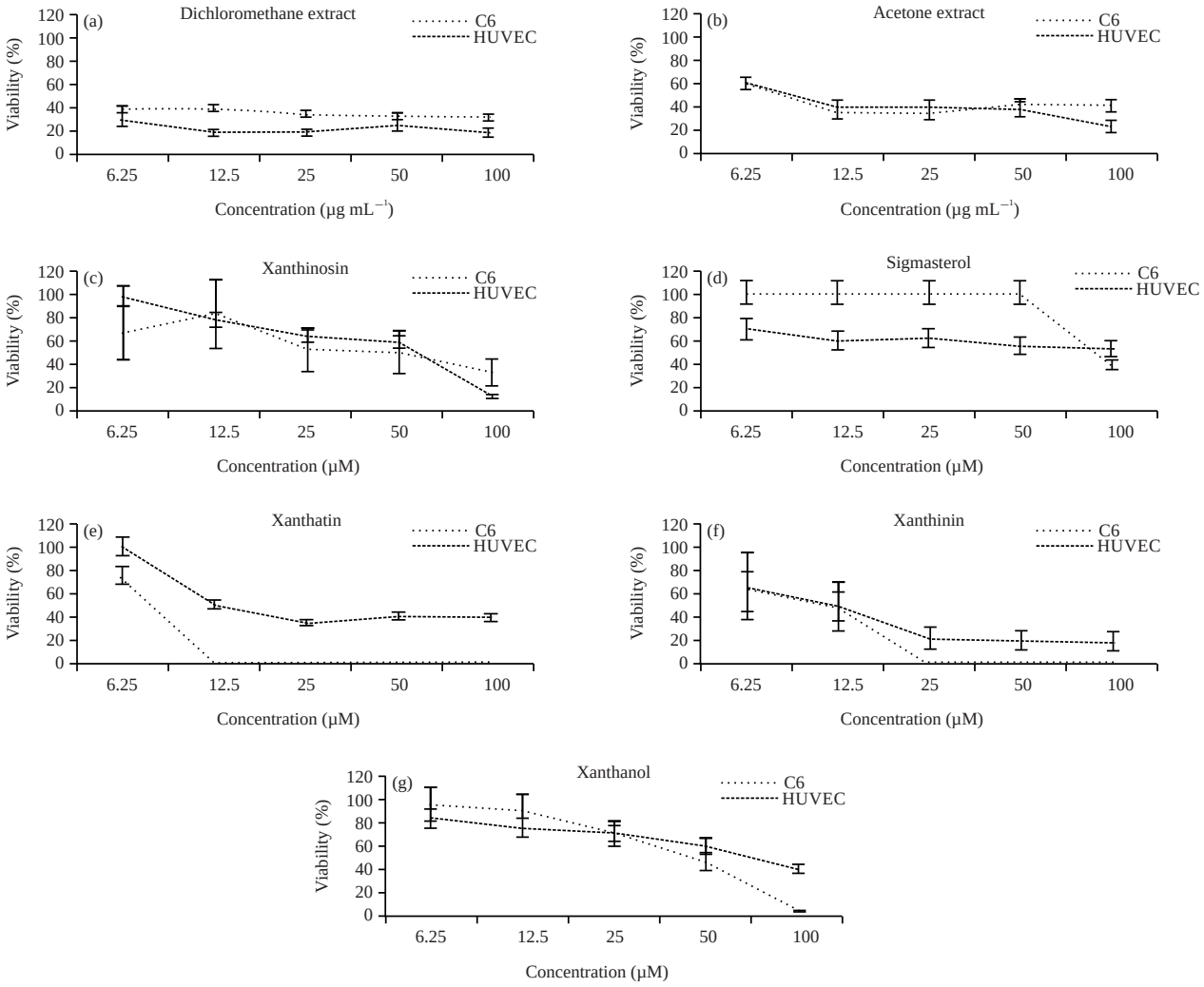


Fig. 3(a-g): Cytotoxic activities of the extracts and the compounds of *X. strumarium*. Cytotoxicity of (a) Dichloromethane extract, (b) Acetone extract, (c) Xanthinosin, (d) Stigmasterol, (e) Xanthatin, (f) Xanthinin and (g) Xanthanol

in C6 cells. However, among the metabolites, stigmasterol (2) led to the formation of breaks on DNA obviously, while the rest caused partial breaks except xanthinin (4). In addition, the extracts did not cause any break in the DNA of the HUVEC cells (Fig. 5b). These 180-200 bp fragments showed that xanthanolides had a selective apoptotic potential on C6 and HUVEC cells. High ROS production in C6 cells treated with the compounds was thought to be one of the mechanisms that induce apoptosis in cancer cells. Consistent with the previous results, treatment of cells with xanthatin (3) and xanthanol (5) resulted in oligonucleosomal DNA fragmentation in C6 glioma cells. Except for xanthinin (4), all metabolites caused DNA fragmentation in HUVEC cells. The formation of DNA breaks in the nucleosomes of the cells showed that these metabolites have cytotoxic effects that further increase apoptosis in these cells.

***In vitro* kinase activity:** Protein amount and kinase activities of C6 glioma and HUVEC cells were calculated based on BSA and ADP standard curves (Table 2). As shown in the table, all the pure metabolites reduced kinase activity in C6 glioma cells compared to the control. However, between treatments, xanthinosin (1), stigmasterol (2) and xanthanol (5) strongly reduced kinase activity in C6 cells compared to the control group. In contrast to C6 glioma cells, all treatments significantly increased the kinase activity in HUVEC cells in comparison to the control group ($p<0.05$). The effects of xanthinin (4) and xanthinosin (1) increasing kinase activity on HUVEC cells were found at higher levels ($p<0.05$). Protein kinases have been used as targets for developing new drugs for various diseases including cancer, inflammation, autoimmune diseases, heart disease and animal diseases[44,45]. Protein kinases also play a critical role in signalling pathways

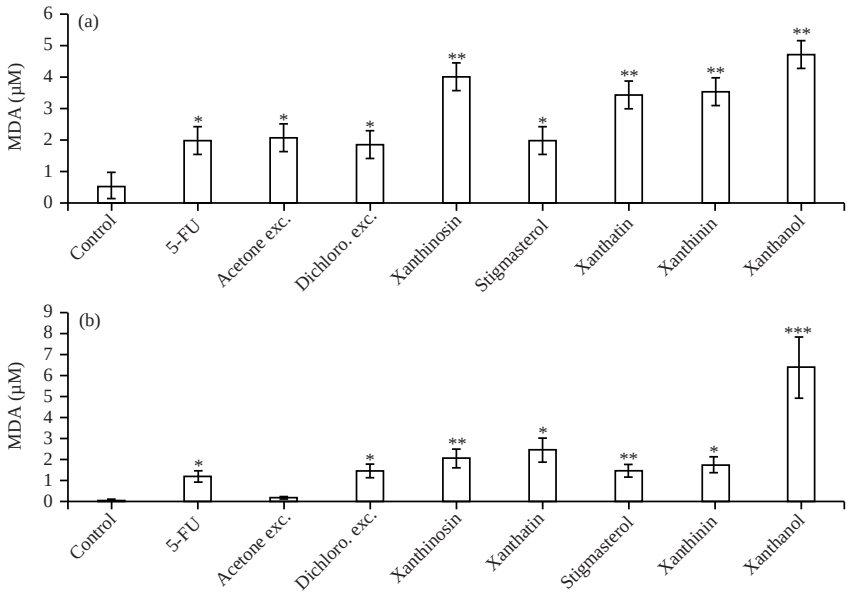


Fig. 4(a-b): MDA levels of the C6 glioma and HUVEC cells, MDA (µM) concentration in (a) C6 and (b) HUVEC
*Statistically significant at p<0.05, **p<0.01 and ***p<0.001 as compared to control groups

Table 2: Kinase enzyme activities in the C6 and HUVEC cells

| Treatments | OD (570 nm) | ADP (pmol) | Protein (ng) | Kinase activity (U mg$^{-1}$) |
|---|---|---|---|---|
| **C6 cells** | | | | |
| Control | 0.28 | 23.66 | 25.8 | 110.04 |
| Xanthinosin (1) | 0.30 | 24.64 | 62.0 | 47.69*** |
| Stigmasterol (2) | 0.28 | 23.28 | 56.1 | 49.80*** |
| Xanthatin (3) | 0.27 | 23.75 | 54.27 | 76.40** |
| Xanthinin (4) | 0.27 | 22.63 | 30.7 | 88.44* |
| Xanthanol (5) | 0.28 | 23.68 | 56.1 | 50.66*** |
| **HUVEC cells** | | | | |
| Control | 0.27 | 22.34 | 79.6 | 33.68 |
| Xanthinosin (1) | 0.27 | 22.35 | 52.1 | 51.48** |
| Stigmasterol (2) | 0.27 | 22.38 | 56.9 | 47.21** |
| Xanthatin (3) | 0.29 | 23.12 | 61.25 | 46.73** |
| Xanthinin (4) | 0.28 | 23.68 | 39.2 | 72.50*** |
| Xanthanol (5) | 0.27 | 22.52 | 69.9 | 38.66* |

*Significant at p<0.05, **p<0.01, ***p<0.001 as compared with control groups

involved in the development of cancer[44-46]. Continuous activation of protein kinases leads to uncontrolled cell proliferation, abnormal signal transduction and cell invasion, thus creating cells with oncogenic activity[44,47]. According to our results, total kinase activity was lower in C6 glioma cells treated with the cytotoxic compounds of cocklebur fruits compared to the control group. In particular, xanthinosin (1) ($p<0.001$), stigmasterol (2) ($p<0.001$) and xanthanol (5) had an inhibitory effect of $\geq$50% on kinase activity (Table 2). These results indicate that these compounds exhibited a cytotoxic effect on C6 cells by inhibiting protein kinase activity[39,42]. In contrast to C6 cells, kinase activity was found to be higher in HUVEC cells treated with cocklebur metabolites, especially xanthinosin (1) ($p<0.01$) and xanthinin (4) ($p<0.001$). Therefore, it was thought that compounds of cocklebur fruits analyzed in the current study were less cytotoxic to HUVEC cells than C6 glioma cells and could be used as selective anticancer agents against C6 glioma cells.

**Immunocytochemical staining of COX-2:** The level of COX-2 expression in the cells treated with the cocklebur compounds was assessed by performing immunocytochemical staining. The COX-2 expression intensity in the cells was evaluated by 2 different investigators and the cells were scored according to their dyeing intensity by scoring them as described in the experimental section (Table 3 and Fig. 6a-d). Our results showed that only stigmasterol (2) increased COX-2 expression in C6 glioma cells compared to control. In contrast to C6 glioma cells, COX-2 expression levels were found to be higher in those treated with all the compounds isolated from

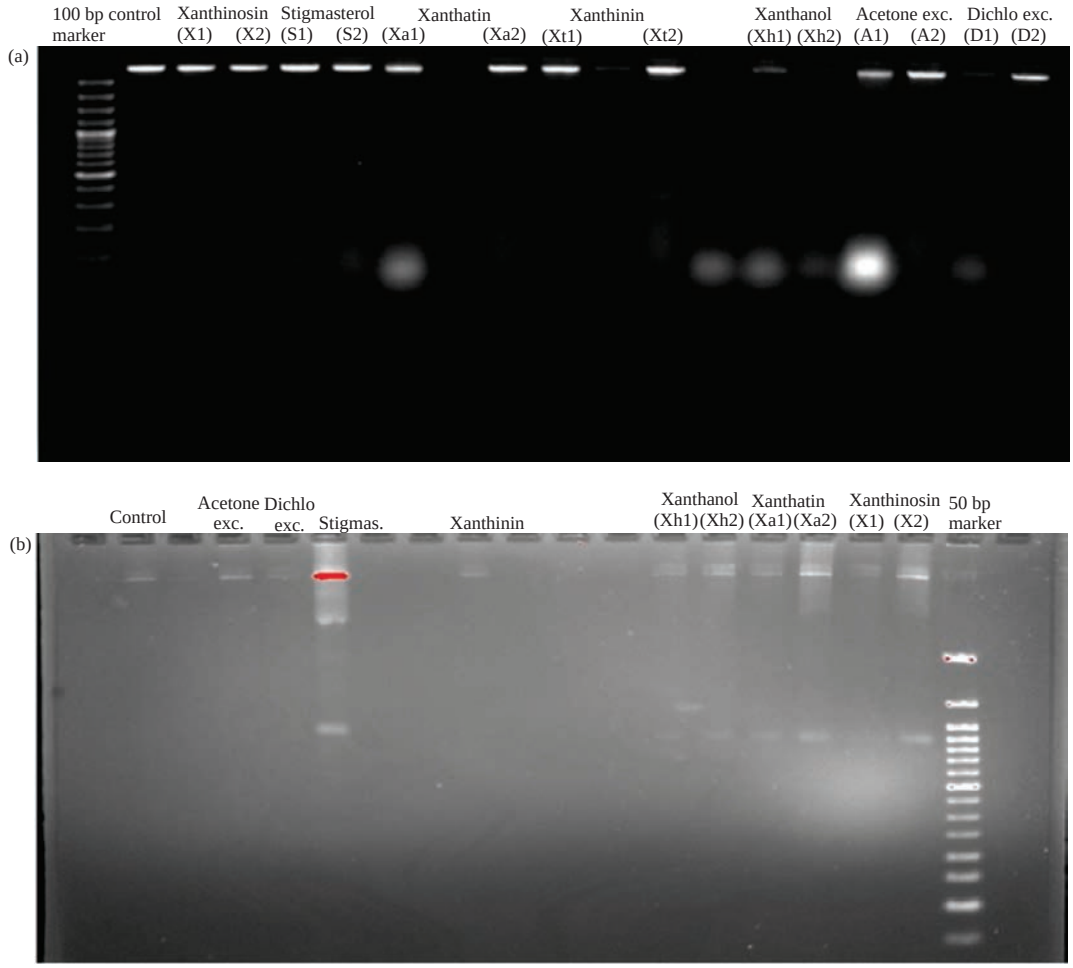


Fig. 5(a-b): DNA fragmentation (a) C6 and (b) HUVEC cells treated with *X. strumarium* and pure compounds
Xanthinosin (1), stigmasterol (2), xanthatin (3), xanthinin (4), xanthanol (5), dichloromethane extract, acetone extract

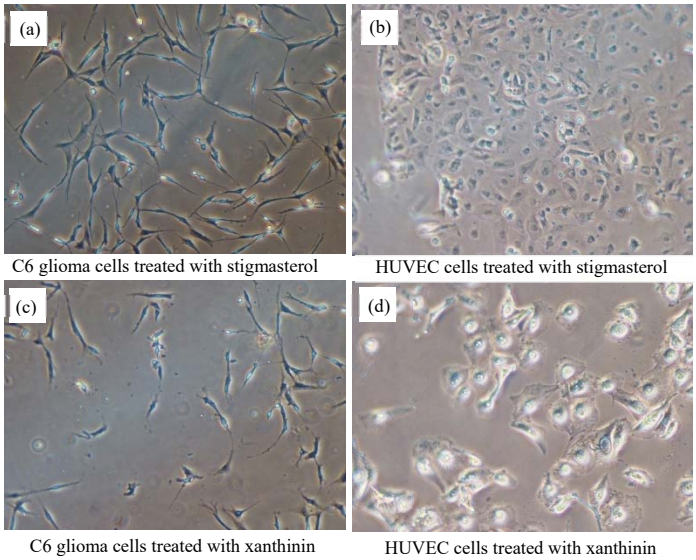


Fig. 6(a-d): Immunocytochemical COX-2 dyeing in C6 and HUVEC cells, (a) C6 with stigmasterol, (b) HUVEC with stigmasterol, (c) C6 with xanthinin and (d) HUVEC with xanthinin

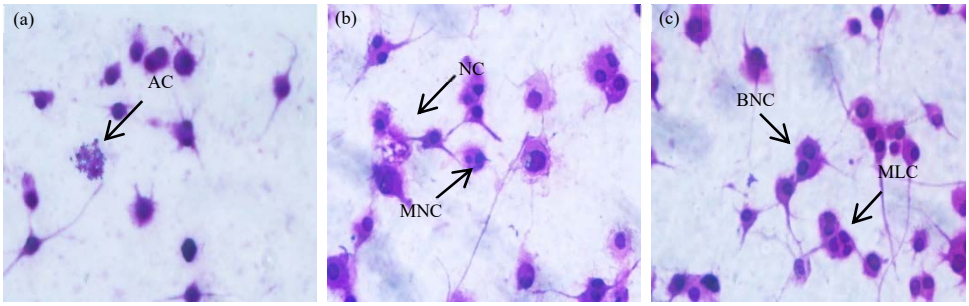


Fig. 7(a-c): Genotoxic effect of xanthanolides on cells, (a) C6 cells with xanthanol (5), (b) C6 cells treated stigmasterol (2) and (c) HUVEC cells treated xanthinin (4)
BNC: Binucleated cell, AC: Apoptotic cell, MNC: Micronucleated cell, NC: Necrotic cell and MLC: Multinucleated cell

Table 3: COX-2 expression severity in C6 glioma and HUVEC cells

| Treatments | C6 glioma | HUVEC |
|---|---|---|
| Control | + | + |
| Xanthinosin (1) | + | ++ |
| Stigmasterol (2) | ++ | +++ |
| Xanthatin (3) | + | ++ |
| Xanthinin (4) | + | +++ |
| Xanthanol (5) | + | +++ |

No staining (0), weak staining (+), medium staining (++) and strong staining (+++)

Table 4: Micronucleus count in C6 Glioma and HUVEC cells

| | Treatments | | | | | |
|---|---|---|---|---|---|---|
| Cell types | Xanthinosin (1) | Stigmasterol (2) | Xanthatin (3) | Xanthinin (4) | Xanthanol (5) | Control |
| **C6 cells** | | | | | | |
| Binucleated | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |
| Multinucleated | 92 | 73 | 62 | 98 | 75 | 49 |
| Micronucleated | 365 | 544 | 267 | 307 | 245 | 424 |
| Apoptotic | 5 | 17 | 14 | 23 | 25 | 15 |
| Necrotic | 11 | 35 | 20 | 11 | 17 | 32 |
| **HUVEC cells** | | | | | | |
| Binucleated | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |
| Multinucleated | 10 | 19 | 53 | 58 | 45 | 14 |
| Micronucleated | 380 | 257 | 221 | 299 | 223 | 130 |
| Apoptotic | 31 | 24 | 17 | 27 | 20 | 9 |
| Necrotic | 516 | 359 | 304 | 312 | 287 | 33 |

cocklebur fruits compared to HUVEC cells (Table 3). COX-2 has the functions of providing apoptosis resistance, facilitating metastasis and invasion and COX-2 expression is generally known to increase in pre-malignant and malignant processes of glioma cells. Our results showed that the compounds, except stigmasterol (2), did not affect COX-2 expression (Table 3). It has been reported that COX-2 is constitutively up-regulated in many human cancer types[48-50]. Studies in cancer cell cultures have shown that high COX-2 expression demonstrates tumourigenic potential by facilitating the migration and invasion of epithelial cells into the extracellular matrix and making the cells resistant to apoptosis[49-51]. These phenotypic changes can only be reversed with highly selective COX-2 inhibitors[48-50]. Furthermore, recent reports have shown that COX-2 plays a crucial role in angiogenesis associated with neoplastic tumour cells[49,50]. Elevated COX-2 expression is a characteristic feature of both pre-malignant and malignant stages of glioma[43-46]. In the present study, xanthinosin (1), xanthatin (3), xanthinin (4) and xanthanol (5) did not reduce COX-2 expression in C6 glioma cells compared to the control group. However, none of the metabolites except stigmasterol (2) up-regulated COX-2 expression in C6 cells. These findings suggest that this pathway cannot mediate the cytotoxic effects of these metabolites[48-51]. However, our results showed that all metabolites increased COX-2 expression in HUVEC cells.

**MN assay:** To determine genotoxicity in C6 glioma and HUVEC cells treated with the compounds, 1000 binuclear cells from each test group were counted and the number of micronuclei was determined. Furthermore, multinucleated, necrotic and apoptotic cells were counted (Table 4 and Fig. 7a-c). The

Table 5: Apoptotic cell counts (%) in C6 glioma and HUVEC

| | Xanthinosin (1) | Stigmasterol (2) | Xanthatin (3) | Xanthinin (4) | Xanthanol (5) | 5-FU | Control |
|---|---|---|---|---|---|---|---|
| **C6 cells** | | | | | | | |
| Living cells | 0.21 | 76.54 | 84.03 | 82.56 | 48.18 | 11.90 | 100.00 |
| Early apoptosis | 2.72 | 4.53 | 8.99 | 7.66 | 47.30 | 19.05 | 0.00 |
| late apoptosis (dead) | 96.86 | 18.49 | 5.36 | 6.08 | 4.04 | 61.90 | 0.00 |
| Total apoptosis | 99.58 | 23.02 | 14.35 | 13.74 | 51.34 | 80.95 | 0.00 |
| **HUVEC cells** | | | | | | | |
| Living cells | 92.28 | 52.86 | 56.47 | 64.94 | 53.00 | 22.59 | 76.28 |
| Early apoptosis | 3.71 | 14.05 | 11.32 | 10.31 | 20.59 | 58.16 | 12.01 |
| late apoptosis (dead) | 0.88 | 33.07 | 30.88 | 22.67 | 24.22 | 18.70 | 8.74 |
| Total apoptosis | 4.59 | 47.12 | 42.21 | 32.98 | 44.81 | 76.86 | 20.75 |

results showed that the numbers of micro-nucleated cells found in C6 glioma cells treated with stigmasterol (2) were as high as the control cells (Table 4). However, all compounds increased the numbers of micro-nucleated cells in HUVEC cells. Moreover, xanthinin (4) and xanthanol (5), which were potent cytotoxic compounds against C6 glioma cells, increased the numbers of apoptotic cells compared to the apoptotic cells in control, while xanthinosin (1) decreased the numbers of apoptotic cells. It was found that the numbers of multinucleated cells treated with the compounds were higher in C6 glioma cells than in control cells. These results showed that *Xanthium* phytochemicals were genotoxic agents against C6 glioma cells. On the other hand, in HUVEC cells, all treatments promoted a high degree of necrosis with apoptosis. MN is an extra-nuclear body that contains damaged chromosome fragments and/or whole chromosomes that are not incorporated into the nucleus after cell division. Accumulated DNA damage, chromosomal aberrations and the defects in cell repair system can form MN. A variety of genotoxic agents can induce MN formation leading to cell death, genomic imbalance or cancer development. In C6 glioma cells, all the metabolites, except stigmasterol (2), reduced the numbers of MN and thus enhanced DNA stability. The cocklebur metabolites reduced DNA damage in C6 glioma cells by 14-43% and the highest activity was exhibited by xanthanol (5) (43%) (Table 3). These results show that the elimination or reduction of the genotoxic effect of xanthanolides can be considered a positive indicator for their safe use as therapeutic agents. However, xanthanolides increased MN numbers in HUVEC cells compared to control. This increase in micronuclei after xanthanolide treatment can be considered as a side effect of the mechanism of action of drugs[52].

**Annexin V-FITC apoptosis analysis:** In the Annexin V method, apoptosis in cells treated with pure compounds was determined by evaluating the changes in the phosphatidylserine (PS) localization in the phospholipid membrane of the apoptotic cells. While PS is usually found in the inner membrane of the normal cells, in the apoptotic cells it is mainly found in the outer membrane. In the presence of $Ca^{+2}$, Annexin V, which is an anticoagulant molecule, binds to phosphatidylserine and thus early apoptotic cells can be marked. The ratio of apoptotic cells (%) in C6 glioma and HUVEC cells treated with pure metabolites were presented in Table 5 and apoptotic profiles of xanthanolides were shown in (Fig. 8 and 9a-e). 5-FU, a commercial chemotherapeutic drug, induced apoptosis in C6 and HUVEC cells by 80.95% and 76.86%, respectively compared to control group. However, xanthinosin (1) and xanthanol (5) caused apoptosis in C6 cells (Table 5). The results showed that xanthinosin (1) in particular is a potent apoptotic agent[53,54]. On the other hand, the apoptotic effects of the rest of the metabolites were found to be low. Unlike C6 glioma cells, xanthinosin (1) acted as a very weak apoptotic agent against HUVEC cells, while others caused apoptosis in HUVEC cells in the range of 32.98% and 47.12%. As can be seen from the results presented in Table 5, all compounds tested caused early and late apoptosis in both C6 glioma and HUVEC cells. One of the most interesting results of this study was that xanthinosin (1) acted as a selective apoptotic agent against C6 glioma cells with a rate of 99.58% apoptosis (Table 5). This metabolite caused a total of 4.59% apoptosis in HUVEC cells. Based on these results, the cytotoxic effects of these compounds could be attributed to their apoptotic properties.

**Cell cycle analysis:** C6 glioma and HUVEC cells were stained with PI and analyzed by using flow cytometry to determine whether the antiproliferative effects of pure metabolites were due to changes in the cell cycle[21,55]. Cell cycle phase distribution of nuclear DNA from C6 and HUVEC cells was calculated with Kaluza analysis software (BD Biosciences) in the presence and absence of cocklebur compounds (Fig. 10a-f and 11a-h). The flow cytometric analysis showed that xanthinin (4) and xanthanol (5) caused an increase in the number of cells having 2N by arresting the cell population in

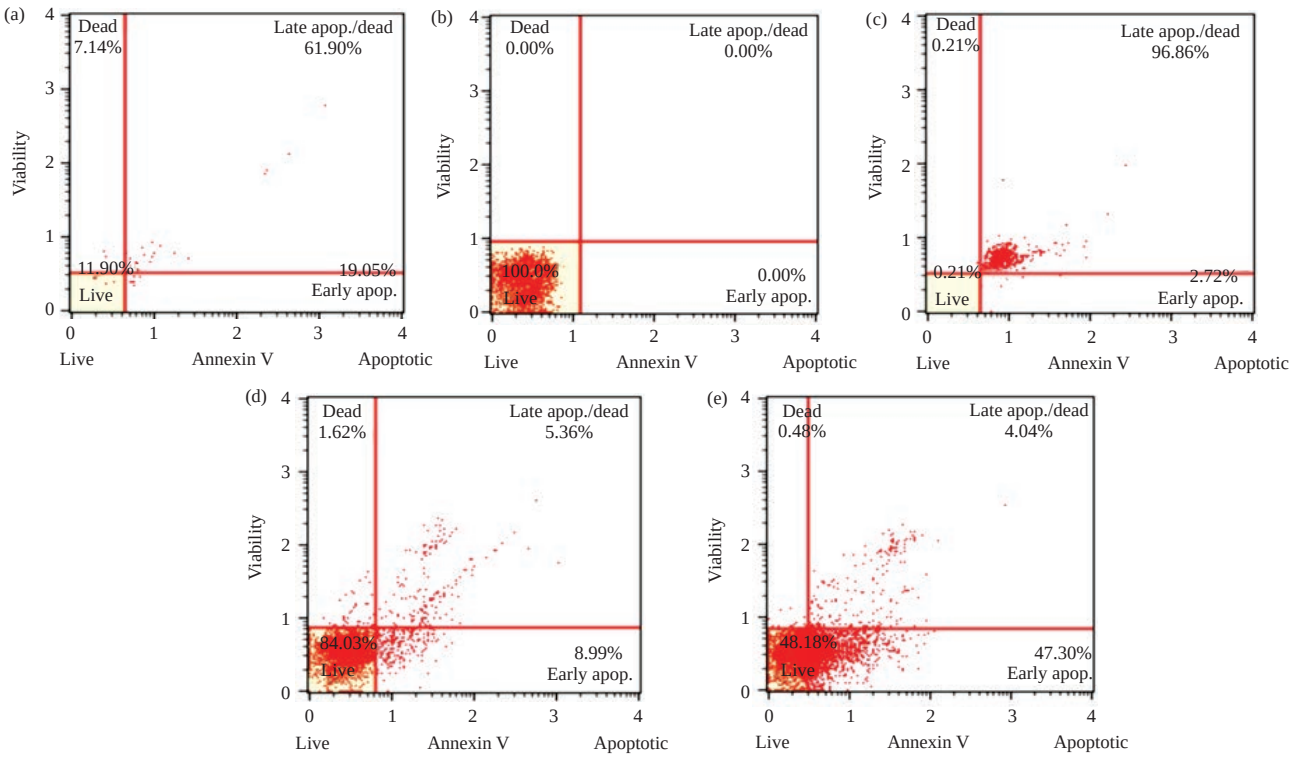


Fig. 8(a-e): Apoptosis profiles of C6 cells treated with some xanthanolides, (a) With 5'FU, (b) Control cells, (c) With xanthinosin, (d) With xanthinin and (e) With xanthanol

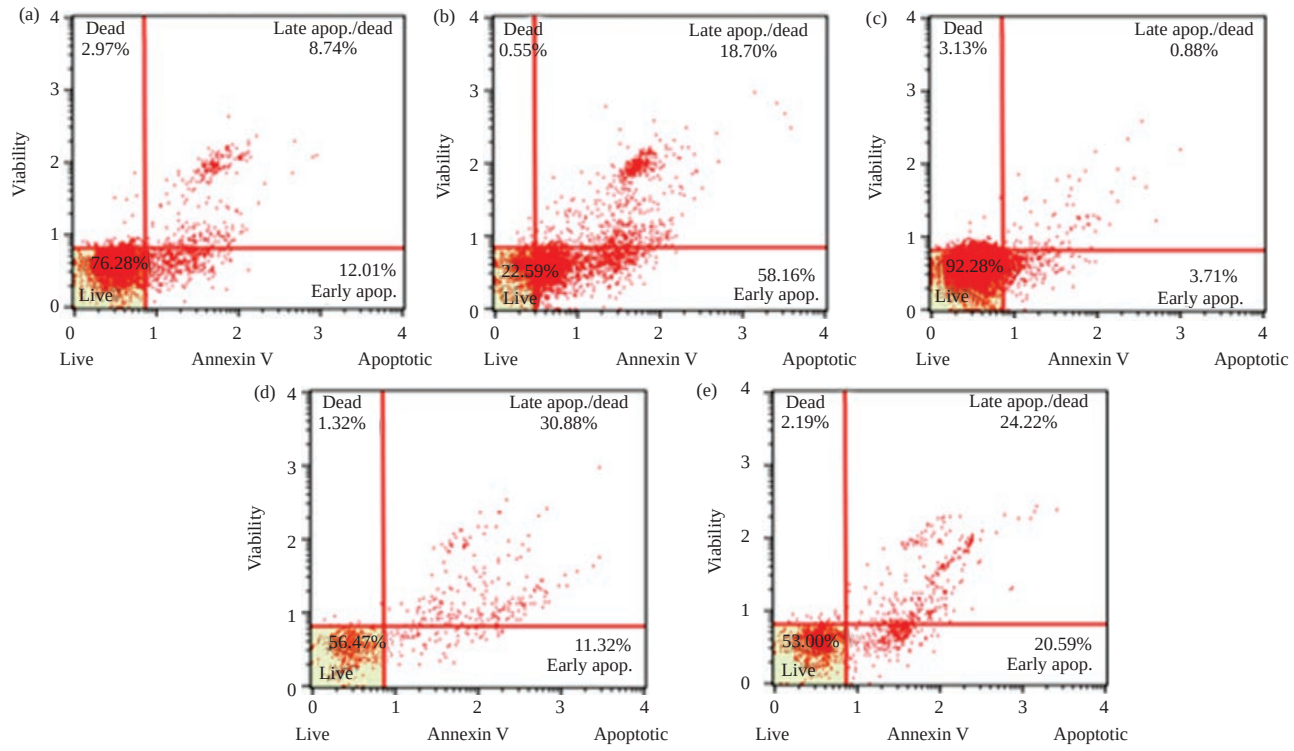


Fig. 9(a-e): Apoptosis profiles of HUVEC cells treated with some xanthanolides, (a) Control cells, (b) HUVEC cells with 5'FU, (c) With xanthinosin, (d) With xanthinin and (e) With xanthanol

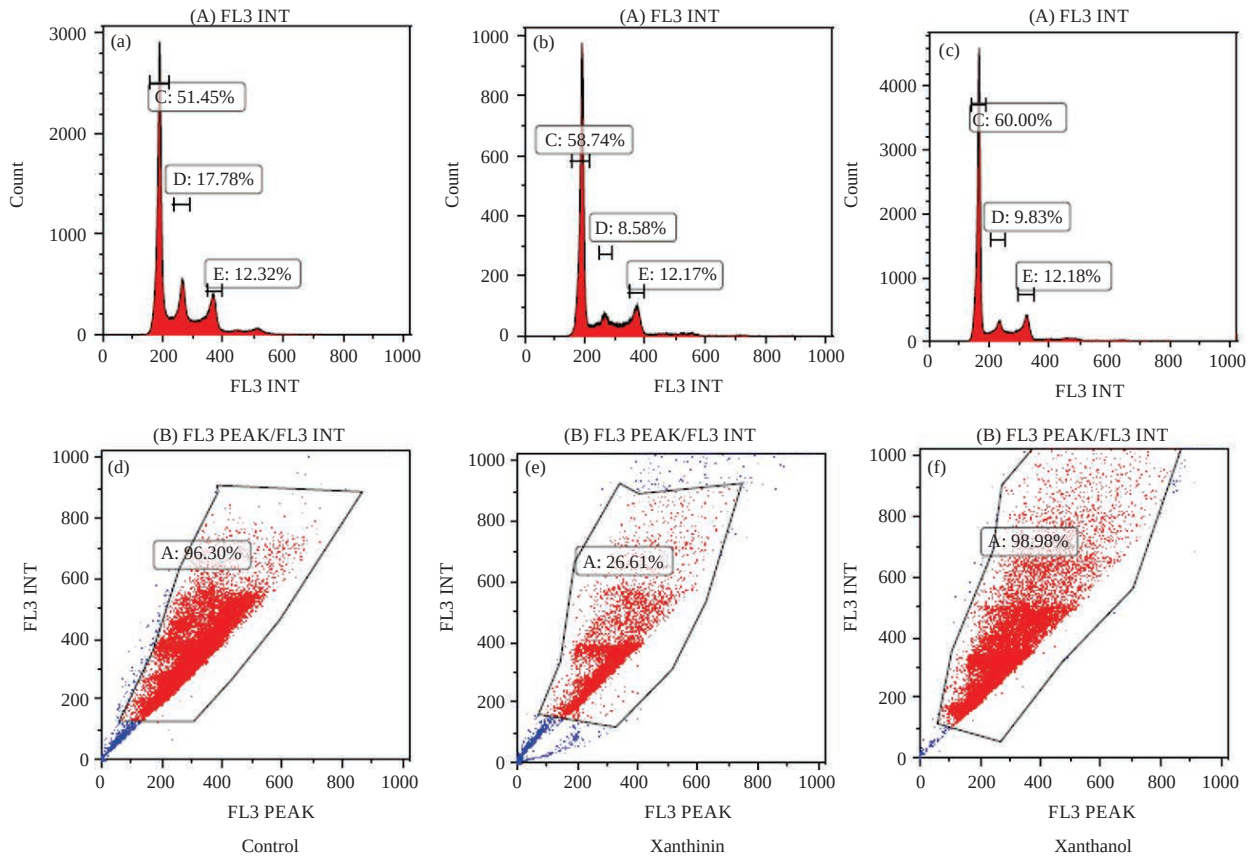


Fig. 10(a-f): Analysis of cell cycle progression from C6 cells, (a,d) Control cells, (b,e) C6 cells with xanthinin, (d,f) With xanthinol
Untreated control and treated cells with potential xanthanolides, xanthinin (4) and xanthanol (5)) C: G1 phase, D: S phase and E: G2 phase

the $G_0/G_1$ phase of the cell cycle of C6 cells. In addition, xanthinin (4) and xanthanol (5) caused a decrease in the number of cells in the S phase (Fig. 10e-f). These findings indicated that xanthinin (4) and xanthanol (5) inhibited C6 cell proliferation by inducing cell cycle arrest in $G_0/G_1$ phase. It was found that the rest of the metabolites did not affect the cell cycle compared to control. As seen in the effect of xanthinin (4) and xanthanol (5) on C6 cells, xanthinosin (1) showed a similar effect on the cell cycle of HUVEC cells. However, stigmasterol (2) and xanthinin (4) arrested cell populations in the S phase, causing an increase in the number of cells with 4N. Therefore, these results showed that xanthinosin (1) inhibited the growth of HUVEC cells by arresting the cell cycle in the G phase. However, stigmasterol (2) and xanthinin (4) inhibited the growth of these cells by arresting the cell cycle in the S phase (Fig. 11a-h).

***In vitro* invasion:** Glioma cells are highly capable of adhesion and invasion, therefore, it is important to uncover the underlying mechanisms. The prognosis of malignant glioma is closely related to its ability to easily penetrate into the parenchymal tissue[56]. The numbers of invasive C6 and HUVEC cells were given in (Fig. 12a-b). All applications of the metabolites were tested and 5-FU reduced the invasion of C6 and HUVEC cells compared to the control group. In particular, stigmasterol (2), xanthatin (3), xanthinin (4) and xanthanol (5) significantly inhibited invasion of C6 cells compared to control ($p<0.001$). Stigmasterol (2) and xanthanol (5) inhibited the invasion of more cells than 5-FU used as a positive control agent ($p<0.01$) (Fig. 12a). Furthermore as shown in Fig. 12b, all metabolites tested exhibited a higher inhibitory effect on the invasion of HUVEC cells ($p<0.001$).

As a result, isolated xanthanolides, xanthinosin (1), xanthatin (3), xanthinin (4) and xanthanol (5) and a triterpenic compound, stigmasterol (2) from cocklebur were detected as active metabolites for cells. Some of them exhibited selective activities between cancer and healthy cells. This situation has potent significance for therapeutic indexing. In this study, first active metabolites isolated, purified and characterized and then their potential biologic activities were showed and as finally pathways of their activities were searched. Although several researches related with some of these metabolites

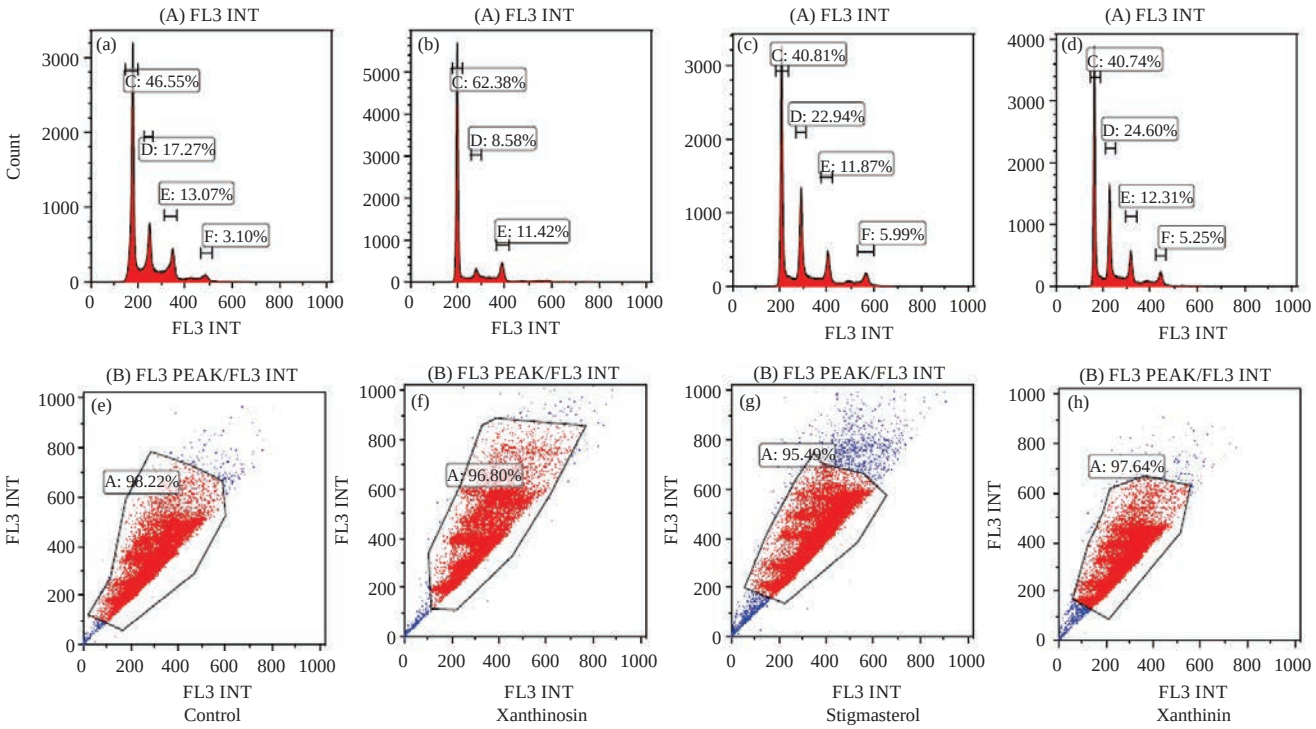


Fig. 11(a-h): Analysis of cell cycle progression from HUVEC cells, (a,e) Control cells, (b,f) HUVEC with xanthinosin, (c,g) With stigmasterol and (d,h) With xanthinin

Untreated control and treated cells treated with xanthinosin (1), stigmasterol (2) and xanthinin (4)), C: G1 phase, D: S phase, E: G2 phase and F: M phase

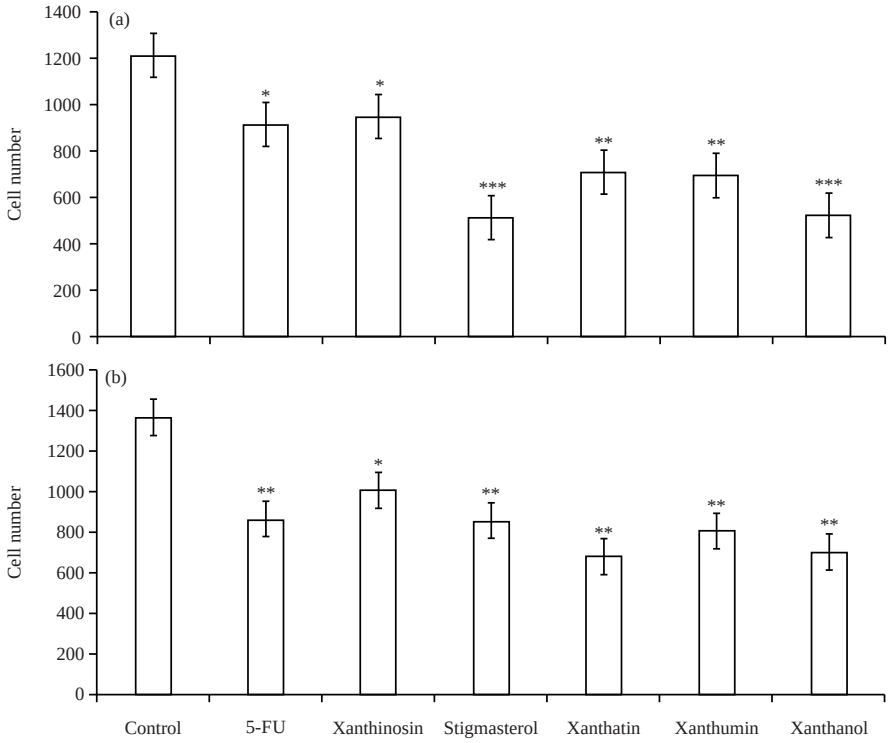


Fig. 12(a-b): Invasive (a) C6 and (b) HUVEC cells treated with the pure compounds isolated from *X. strumarium*

*Statistically significant at p<0.05, **p<0.01 and ***p<0.001 as compared to control groups

were found in literature, this study made more good for evidence related activities with pathways. And also, side effects of metabolites such as genotoxicity were investigated. However, our study has some limitations related with molecular interactions between these metabolites and target proteins and also with organism response to these agents. Hence, we recommend that it has been needed to further analyses *in silico* and *in vivo* for researching the capacities of being drug for cancer.

## CONCLUSION

The present results showed that acetone and dichloromethane extracts and pure metabolites isolated from acetone extracts of *X. strumarium* fruits exhibited quite different cytotoxicity patterns and mechanisms of action. These findings showed that plant extracts and metabolites purified from plants may exhibit different biological properties. Therefore, while research on the biological activity of plant extracts alone may have important results, the isolation and biological activities of pure plant metabolites are more important to science. In this study, 5 pure metabolites (one steroid and 4 xanthanolides) isolated from *X. strumarium* fruits were evaluated for their cytotoxic activities and mechanisms of action on C6 glioma cells. Our results showed that xanthinosin (1), xanthatin (3), xanthinin (4) and xanthanol (5) showed potent cytotoxic effects on C6 glioma cells and also xanthinosin (1) and xanthanol (5) had selective cytotoxicity. Based on the results obtained, it was thought that these compounds could be target molecules for future studies on C6 glioma.

## SIGNIFICANCE STATEMENT

The study discovers the pure metabolites with selective effects on cells obtained from *Xanthium strumarium* that can be beneficial for searching new agents for cancer therapy. This study is an original research the following xanthanolides are isolated, purified and characterized and are investigated cytotoxic effect with pathway prediction by the authors (Karagoz I.D., Cakir A., Ozaslan M., Kilic I.H., Tepe B., Akdogan E. and Kazaz C.) and of this article. Therefore this paper is obviously significant.

## ACKNOWLEDGMENT

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