



Original Article

Effects of *Carthamus tinctorius* L. solvent extracts on anti-proliferation of human colon cancer (SW 620 cell line) via apoptosis and the growth promotion of lymphocytes

Teerakul Arpornsawan^{*1}, Sawang Petvises¹, Arthid Thim-uam², Atirada Boondech², and Sittiruk Roytrakul³

¹ Department of Medical Technology, Faculty of Allied Health Sciences,
Thammasat University, Pathum Thani, 12120 Thailand.

² Department of Biochemistry, Faculty of Medical Sciences,
Naresuan University, Phitsanulok, 65000 Thailand.

³ Genome Institute, National Center for Genetic Engineering and Biotechnology,
113 Thailand Science Park, Klong Luang, Pathum Thani, 12120 Thailand.

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Abstract

The *in vitro* effects of dichloromethane, methanol and hexane extracts of *Carthamus tinctorius* on caspase-dependent anti-tumor activity against human colon carcinoma SW620 cell lines were investigated by MTT assay, Annexin V staining and real-time RT-PCR. In addition, the immunomodulatory activity of each solvent extract was examined by determination of lymphocyte proliferation using flow cytometry. Only dichloromethane extract of *C. tinctorius* exhibited the inhibitory effect on growth of SW620 cells with IC₅₀ of 0.15 mg/ml, respectively, in comparison to the Hep2 (0.5 mg/ml) and control BHK cells (0.6 mg/ml). Moreover, it was associated with up-regulation of caspase 3, 7 and 9 and down regulation of Bcl2 transcripts in treated SW620 cell. The dichloromethane extract showed the highest stimulatory effect on the lymphocyte proliferation with an increase of 8±1.6 fold, followed by the methanol and hexane extract with increases of 12±1.1 and 14±1.6 fold, respectively.

Keywords: anti-proliferative effect, *Carthamus tinctorius* L, human colon carcinoma (SW620 cell line), apoptotic pathway, caspase, lymphocyte proliferation

1. Introduction

Safflower (*Carthamus tinctorius* L.) or Dok-Kam-Phoi has been used in folk remedies in many countries such as Thailand, China, India, Japan and Korea for treatments of gynecological diseases, heart diseases and inflammation. The active ingredients from safflower extract that have been reported so far are serotonin derivatives (Zhang *et al.*, 1997), flavonoids (Yaginuma *et al.*, 2003), kinobione A (Kanehira

et al., 2003), hydroxy safflor yellow A (Zhu *et al.*, 2003), erythro-alkane-6, 8-diols (Yasukawa *et al.*, 1996), and tinctormine (Meselhy *et al.*, 1993). These ingredients have been shown to contain pharmacological properties such as protection of bone (Kim *et al.*, 2002), an effect on lipid metabolism (Ihara *et al.*, 1998), anti-oxidation (Yaginuma *et al.*, 2003; Kanehira *et al.*, 2003; Lee *et al.*, 2002), anti-hypertension (Liu *et al.*, 1992), inhibition of proinflammatory cytokine production (Takii *et al.*, 2003), anti-inflammatory effect (Akihisa *et al.*, 1996), inhibition of tumor, and inhibition of tyrosinase enzyme (Loo *et al.*, 2004; Kanehira *et al.*, 2003).

Nowadays, the anti-proliferative effects on many cancer cell lines by several Thai herbs have been reported.

* Corresponding author.

Email address: teerakul@tu.ac.th

A compound (Zhu-xiang) from herbal extracts containing ginseng and safflower has been shown to inhibit proliferation of MDA-MB-231, breast cancer cells by induction of apoptosis and the effect was greater than those of commonly used cytotoxic drugs (Loo *et al.*, 2004). An apoptotic cascade induced in SW620 human colonic carcinoma cells has been reported (Chinery *et al.*, 1997; Heerdt *et al.*, 1994; 1996; 1998; Bordonaro *et al.*, 1999; Polyak *et al.*, 1997). This study was therefore aimed to determine comparative effects of dichloromethane, methanol and hexane extracts of *C. tinctorius* to human colon carcinoma SW 620 and laryngeal carcinoma Hep2 cell lines and to explore the mechanism contributing to these effects especially in terms of induction of apoptosis and proliferation of lymphocyte.

2. Material and Methods

2.1 Preparation of the crude extracts of *C. tinctorius*

The flowers of *C. tinctorius* were collected from plants and crude dichloromethane, hexane and methanol extracts were prepared as previously described by Arpornsawan *et al.* (2010).

2.2 *In vitro* cytotoxicity assay by MTT assay

Human laryngeal carcinoma (Hep2) cell lines, human colon carcinoma (SW620) cell lines and baby hamster kidney (BHK) cell lines were cultured at 37°C in a 5%CO₂ incubator, in RPMI 1640 (Sigma), supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml) and streptomycin (100 µg/ml). To determine the cytotoxicity, cells were treated with crude extracts from *C. tinctorius* at the indicated concentration for 24 hours. The cytotoxic activity was assessed by a rapid colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) bromide as previously described by Arpornsawan *et al.* (2006).

2.3 Fluorescent microscopic analysis of apoptosis by Annexin-V staining

Programmed cell death was determined using two methods. First, the presence of phosphatidylserine on the outer leaflet of apoptotic membranes was detected using an Annexin V (sc-4252 AK) apoptosis detection kit (Santa Cruz Biotechnology, California, USA). Second, the presence of apoptotic DNA breaks was assessed using propidium iodide (PI) staining. Briefly, one million SW620 cell lines were treated with 0.15 mg/ml of the dichloromethane extract of *C. tinctorius* overnight. Negative control was cells incubated without plant extract. Cells were washed and incubated with Annexin V FITC and propidium iodide (PI) for 30 minutes at room temperature in the dark. The stained cells were visualized under a fluorescence microscope (Olympus IX71 with a DP70 camera, Tokyo, Japan).

2.4 Determination of Caspase gene expression by real time-PCR

Total RNA was extracted from SW620 cells treated with 0.15 mg/ml of dichloromethane fraction for 2, 6, 9 and 18 hours using TRI reagent® (Molecular Research Center, Inc.). Total RNA from control or treated cell line was reverse transcribed using oligo(dT)20 primer and ImProm-II Reverse transcription system (Promega, USA). The synthesis of the first-strand cDNA was run at 42°C for 60 min. The SYBR Green real-time RT-PCR was performed using iCycler iQ Real-time PCR System (Bio-Rad). The experimental cycling profile was set as recommended in the manufacturer's manual for QuantiTect SYBR Green PCR kit (QIAGEN). The targeted gene was then analyzed using human EF1a as an internal control. Control expression levels were included in the calculation model to standardize each reaction run with respect to RNA integrity, RT efficiency and cDNA sample loading variations based on the method described by Pfaffl *et al.*, 2001. The primers used are shown in Table 1.

Table 1. Primer pairs used for real time PCR reaction

Target gene	Forward and reverse primer 5' _ 3'	Reference or Accession number
Caspase 3	TGT TTG TGT GCT TCT GAG CC CAC GCC ATG TCA TCA TCA AC	NM032991.2
Caspase 7	CCA ATA AAG GAT TTG AC AG CC GC AT CT GTG TC AT TG AT GG	NM_033338.4
Caspase 8	GAT CAA GCC CCA CGA TG AC CCT GTC CAT CAG TGC CAT AG	NM_001228.3
Caspase 9	CAT TTC ATG GTG GAG GTG AAG GGG AAC TGC AGG TGG CTG	NM_032996.1
Human EF1-alpha	GGG TCA GAT TTC TTG AT GG CTG AAG CGG CTG GCC AAG	NM_001961.3

2.5 Isolation of peripheral blood mononuclear cells (PBMC)

All procedures were performed in accordance with the guidelines of WMA Declaration of Helsinki -Ethical principles for Medical Research involving human subjects and the study protocol was approved by a local Ethics Committee. Heparinized blood samples were collected from 3 normal subjects after informed consent. PBMC were isolated by density gradient centrifugation with Isoprep (Robin Scientific, USA) as previously described. The isolated PBMC were cultured at 37°C in a 5% CO₂ incubator, in XVIVO-10 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Invitrogen, New York, NY, USA), 2mM-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

2.6 In vitro $\gamma\delta$ T lymphocytes proliferation

The 2x10⁵ cells/mL of PBMC were cultured in XVIVO-10 medium in the presence of 10 µg/ml of pamidronate and 100 IU/mL of IL-2. Both pamidronate and IL-2 were maintained throughout the experiments. The 100 µg/ml of three fractions of *C. tinctorius* (methanol, hexane, dichloromethane) with 5 µg/ml of pamidronate and 50 IU/mL of IL-2 were added to the test experiments for comparison. All culture cells were collected on day 10 for phenotypic analysis by flow cytometry.

2.7 Phenotypic analysis

Stimulated cells were stained with CD⁴⁵-PerCP, CD³-FITC, CD⁴-FITC, CD²⁵-PE, CD⁵⁶-PE, V γ 9-FITC, and V δ 2-FITC. All antibodies were purchased from Coulter-Immunotech. Isotype control, mouse IgG1 and IgG2 were included. Stained cells were analyzed by FACScan (Becton Dickinson) using the CellQuest software program.

3. Results

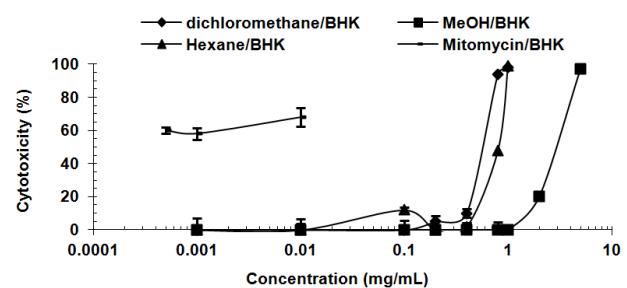
3.1 Crude extracts from dichloromethane fraction could elicit cytotoxicity in human colon cell lines but not other cell types at the same concentration

BHK, Hep2 and SW620 cell lines were incubated at various concentrations of each solvent extract of *C. tinctorius*. All of the solvent extracts were able to mediate cytotoxicity in all cell lines, albeit with different doses. The cytotoxicity patterns were similar between all three extracts in which the higher concentration of the extract, the higher cytotoxicity (Figure 1A-C). Among three safflower extracts, the methanol extract gave the lowest cytotoxicity. Both dichloromethane and hexane extracts were 100 % cytotoxic to normal and the carcinoma cell lines tested at 1 mg/ml. However, at the concentration of 0.2 mg/ml, the dichloromethane extract showed 67% cytotoxicity to SW620 cell lines while gave less than 10% of killing to BHK and Hep2 cells, indicating the activity of this extract as an inhibitory agent to colon carcinoma cell lines but not to other cell types.

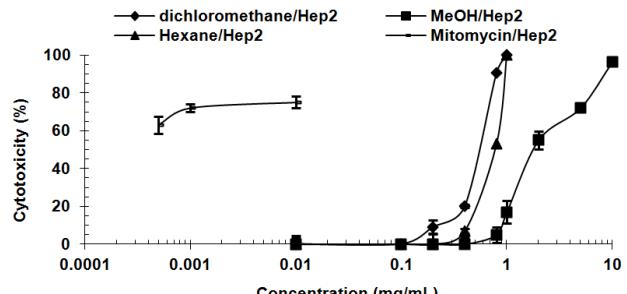
3.2 Crude extract in dichloromethane fractions induced apoptosis in SW620 cell lines

To determine whether the cytotoxicity properties of dichloromethane extract of the safflower are involved with apoptosis, SW620 cells were treated with 0.15 mg/ml of the dichloromethane extract of *C. tinctorius* (IC₅₀) for 24 hours prior to incubating with Annexin V and propidium iodide (PI). The apoptotic cells as determined by AnnexinV positive cells were visualized using fluorescent microscopy. As shown

A



B



C

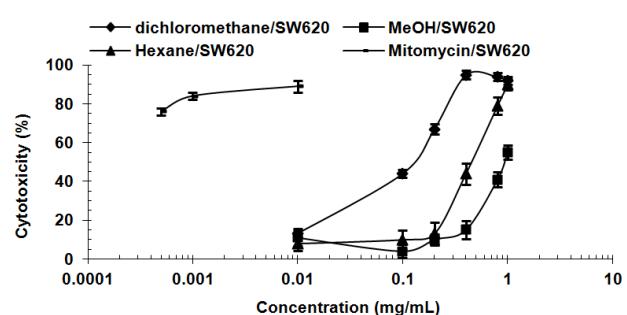


Figure 1. *In vitro* cytotoxic activity of the crude extract of *C. tinctorius* flowers in BHK cell line (A), Hep2 cell line (B) and SW620 cell line (C). Crude extracts in hexane, dichloromethane and methanol fractions were incubated with the target cells at the indicated concentrations. Cytotoxicity was determined using MTT assay as described in the test. Individual lines represent the average of % cytotoxicity plus/minus standard deviation (SD) from four independent experiments.

in Figure 2, marked increase of apoptosis in the dichloromethane extract-treated cell lines (70%) was observed. These results indicate that apoptosis may contribute to the cytotoxicity mediated by dichloromethane extract of the safflower in the SW620 cell lines.

3.3 The apoptosis of the safflower dichloromethane extract-treated SW620 cell lines were involved with caspase activation

To dissect the apoptosis mechanism that the dichloromethane extract of *C. tinctorius* L induced on human colon cancer cells, caspase activation is of interest as it is the key component in mediating apoptosis. SW620 cells were treated with dichloromethane extract and the cells were collected at various time points. mRNA expression levels of caspases 3, 7, 8 and 9 were determined using quantitative real-time RT-PCR. Interestingly, the level of caspases 3, 7 and 9 mRNA were up-regulated and reached the maximum at 6 hours before gradually declined (Figure 3). Expression of caspase 3 mRNA was the highest while caspase 8 mRNA showed no difference between the treated- and control cell lines.

3.4 Solvent extracts of *C. tinctorius* L had no anti-proliferation effect to gdT lymphocytes

To determine the effect of *C. tinctorius* on the proliferation of $\gamma\delta$ T lymphocytes *in vitro*, PBMC from three normal volunteers were isolated and cultured in conditions that promote expansion of $\gamma\delta$ T lymphocytes (complete medium and 10 μ g/ml of pamidronate and 100 IU/ml of IL-2). Each solvent extract of the safflower was then added and the cells were cultured for a further 10 days. Cells were harvested, counted and stained with a set of antibody for phenotypic analysis. The expansion of 3 subsets of T lymphocytes, $\gamma\delta$ T lymphocytes, CD3 $^+$ CD56 $^+$ T lymphocytes and CD4 $^+$ CD25 $^+$ T lymphocytes was determined. There were no significant differences in the expansion of individual populations either in the presence or in the absence of each solvent extract. The total number of control cells increased 10-fold with the median of 20.7×10^5 cells/ml (range: 15.4-21.6). The expansions of cells treated with extract of *C. tinctorius* in methanol, hexane, and dichloromethane were 8-fold with the median of 16.4×10^5 cells/ml (range: 13.6-18.0), 12-fold with the median of 24.9×10^5 cells/ml (range: 12.2-26.8) and 14-fold with the median of 28.5×10^5 cells/ml (range: 25.1-31.3) respectively (Table 2).

The distribution patterns between V γ 982, the CD3 $^+$ CD56 $^+$, and CD4 $^+$ CD25 $^+$ T lymphocytes upon stimulation with various solvents were slightly different. Incubation with the methanol extract gave slightly lower number of V γ 982 and CD3 $^+$ CD56 $^+$ T lymphocytes (Figure 4). However, incubation with dichloromethane fractions gave a similar pattern to the control cells in which the largest proportion was V γ 982 T lymphocytes followed by CD3 $^+$ CD56 $^+$ and CD4 $^+$ CD25 $^+$ T cells, respectively.

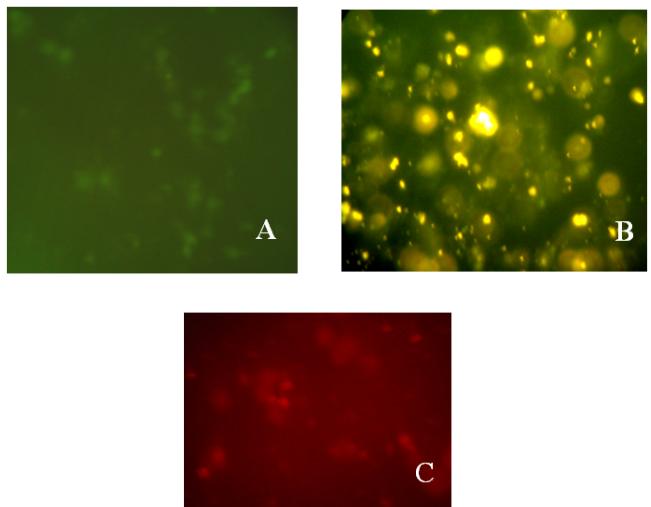


Figure 2. Fluorescence microscopic analysis of apoptosis by Annexin V/PI staining. SW 620 cells were cultured in the presence of the dichloromethane extract of *C. tinctorius* at fifty percent of inhibition concentration (IC_{50}) for overnight. Cells were stained with Annexin V and counterstained with propidium iodide (PI). A field from control SW 620 cells is shown in (A). In dichloromethane extract treated SW 620 cells, the uptake of Annexin V was demonstrated (B). Cells with nuclear morphology consistent with apoptosis are shown in (C).

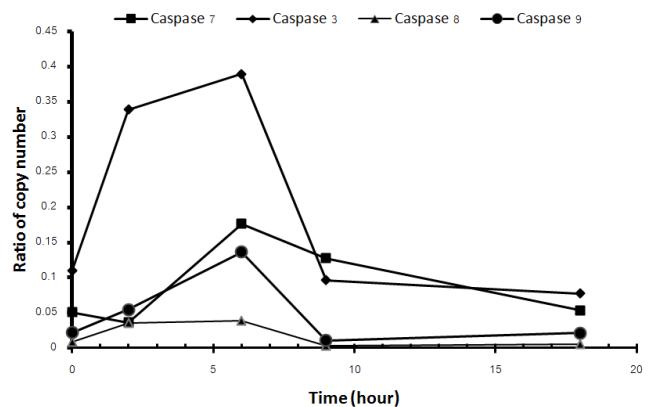


Figure 3. Pro-apoptotic effect of on human colon cancer cells. RT-PCR analysis of the expression of caspase 3, caspase 7, caspase 8, and caspase 9 mRNA in SW620 cell line incubated with the dichloromethane extract at 2, 6, 9, 18 hour.

4. Discussion

In the present study, we have shown that the crude extract of *C. tinctorius* in three types of solvent, dichloromethane, hexane and methanol displayed cytotoxicity against both normal and cancerous cell lines. The crude extract in dichloromethane fraction displayed the interesting property of being able to induce cytotoxicity in human colon carci-

Table 2. Effects of *C. tinctorius* extract on the proliferation of PBMC in the study of gd T lymphocytes expansion.

Experiment settings	Initial PBMC plated	Median of total cell (cells/ml) days 10 (x10 ⁵ cells/ml)	Fold increase numbers collected at
Cocktail of 10 ug/ml of pamidronate and 100 IU/mL of IL-2	2.0x10 ⁵	20.7 (range; 15.4-21.6)	10±2.4
Cocktail of 5 ug/ml of pamidronate and 50 IU/mL of IL-2 plus 100 ug/ml of <i>C. tinctorius</i> methanol extract	2.0x10 ⁵	16.4 (range; 13.6-18.0)	8±1.6
Cocktail of 5 ug/ml of pamidronate and 50 IU/mL of IL-2 plus 100 ug/ml of <i>C. tinctorius</i> Hexane extract	2.0x10 ⁵	24.9 (range; 12.2-26.8)	12±1.1
Cocktail of 5 ug/ml of pamidronate and 50 IU/mL of IL-2 plus 100 ug/ml of <i>C. tinctorius</i> Dichloromethane extract	2.0x10 ⁵	28.5 (range; 25.1-31.3)	14±1.6

Results are presented as mean ± SEM of three experiments carried out in duplicate.

noma cell lines but not other types of cancer cells at the indicated concentration.

Pathogenesis of cancer may be caused by failure of cells to undergo apoptotic cell death. Therefore, treatment with natural products that induces apoptosis of cancer cell with less toxicity to normal cells would be a promising anti-cancer strategy (Tan *et al.*, 2009). A variety of phytochemical agents such as resveratrol, curcumin, sulforaphane, gingerol, indole-3 carbinol, withanolide and catechins can induce apoptosis in various human cancer cells (Khan *et al.*, 2008; Khan *et al.*, 2010; Nibili *et al.*, 2009; Martin *et al.*, 2006).

Microscopic analysis of Annexin V/PI staining showed that dichloromethane extract of *C. tinctorius* induced apoptosis in the SW620 cell lines. This supports the previous finding that the safflower oil was found to dramatically inhibit the growth of the HT-29 malignant human colon cell line (Salerno *et al.*, 1991). In addition, the safflower oil selectively inhibited malignant melanoma growth over normal melanocytes *in vitro* (Smith *et al.*, 1992). The apoptogenic molecules released from mitochondria to the cytosol trigger the activation of caspases and subsequent cell death (Ly *et al.*, 2003). Apoptotic cell death can be triggered either through receptor (extrinsic)-mediated pathway via caspase-8 or the mitochondrial (intrinsic)-mediated pathway via caspase-9 (Mishra and Kumar 2005). Both of these mechanisms will merge and lead to the activation of the downstream caspase-3, 6 and 7 (Hengartner, 2000). The present study demonstrated that treatment with *C. tinctorius* fraction increased mRNA level of caspases 3, 7 and 9. These results suggest that induction of apoptosis is mediated through the intrinsic pathway. An intracellular caspase-8 plays a role in communicating with the death domain of cell membrane receptors. However, *C. tinctorius* extract showed no effect on caspase 8 mRNA suggesting that apoptosis is not mediated through the extrin-

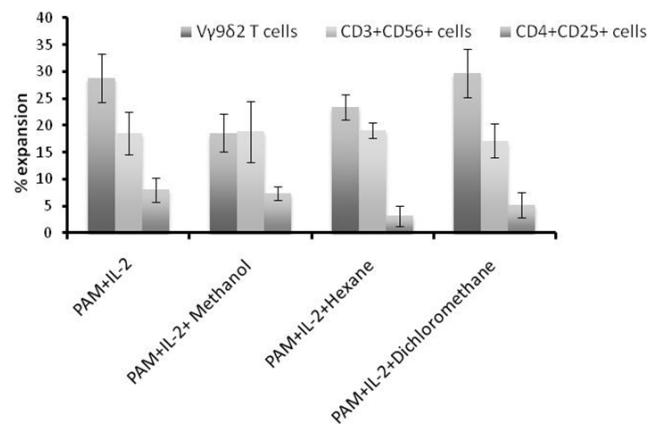


Figure 4. The population of V γ 9 δ 2 T lymphocytes, CD 3^+ CD 56^+ , and CD 4^+ CD 25^+ T cells expanded in culture using pamidronate and IL-2 supplement with various extracts of *C. tinctorius*. PBMC were isolated and cultured in conditions that promoted expansion of the presence of $\gamma\delta$ T lymphocytes as described in the test. Results are presented as mean±SEM of three experiments carried out in duplicate.

Table 3. IC₅₀ values (mg/mL) of the crude extract of *C. tinctorius* flowers in BHK, Hep2 and SW620 cell lines.

Cell lines	% cytotoxicity IC ₅₀ (mg/mL)		
	Dichloromethane	Methanol	Hexane
BHK	0.6	3	0.8
Hep2	0.5	1.5	0.7
SW620	0.15	1	0.4

sic pathway. Resveratrol and diallyl disulfide are examples of natural compounds that mediate apoptosis via the intrinsic pathway (Pratheeshkumar *et al.*, 2010; Zhang *et al.*, 2012).

The dichloromethane extract showed high cytotoxicity to SW620 cell lines indicating signaling pathways leading to apoptosis differ from one cell type to another. Expression of death receptors may be down-regulated or absent in BHK and Hep2 cells. Signaling to cell death in BHK and Hep2 cells may be inhibited by an increase in anti-apoptotic molecules or by a decrease or defective function in proapoptotic proteins (Fulda and Debatin, 2006).

The apoptotic pathway in leukocytes can be activated by external stimuli that trigger surface receptors (Schulze-Osthoff *et al.*, 1998; Suda *et al.*, 1997), antitumor agents (Nagami *et al.*, 2002) and cytotoxic compounds (Lemaire *et al.*, 1999). Interestingly, the solvent extracts of *C. tinctorius* had no anti-proliferative effect on 3 subsets of T lymphocytes which are the key players in eradicating the tumor cells *in vivo*. However, our results do not rule out the possibility that other death receptor systems are activated by dichloromethane extract of *C. tinctorius*.

5. Conclusions

The apoptotic pathway in association with immune-stimulating activity appears to be activated by dichloromethane extract of *C. tinctorius* in SW620 cells. This *C. tinctorius* extract can be further explored for its therapeutic potential in the *in vivo* chemoprevention of cancers.

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