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Xanthomicrol is the main cytotoxic component of *Dracocephalum kotschyii* and a potential anti-cancer agent

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Abstract

Spinal-Z, a methanolic mixture of dried powdered seeds of *Peganum harmala* Linn. and leaf of *Dracocephalum kotschyii* Boiss. is an Iranian ethno-medical remedy. It has been used for the treatment of various types of cancer for many years. To evaluate the use of Spinal-Z in treatment of cancer, we examined its effects against a panel of malignant cell lines and tumors induced in mice.

The in vitro antiproliferative activities of Spinal-Z, the seed extract of *P. harmala* and the leaf extract of *D. kotschyii* were determined using the MTT assay. The concentration of the agent required to inhibit cell growth by 50% (IC_{50}) was estimated. In addition, the anti-tumor activities of the remedy and its constituents were investigated.

Viability of cells treated with Spinal-Z and its components decreased in a dose dependent manner. Spinal-Z and its components showed cytotoxic effects against all cell lines tested. The leaf extract of *D. kotschyii* showed a greater preferential cytotoxic effect than the seed extract of *P. harmala* and Spinal-Z, on all cell lines tested. Harmine showed cytotoxicity against HL60 and K562 cell lines. This could explain the cytotoxic effect of *P. harmala* on these cells. The leaf extract of *D. kotschyii* was able to inhibit tumor proliferation in mice. The active ingredient in the leaf extract of *D. kotschyii* appears to be a flavone identified as xanthomicrol. Xanthomicrol was able to inhibit proliferation of a number of malignant cells. The cytotoxic effects of xanthomicrol were more selective towards malignant cells than doxorubicin.

Keywords: Spinal-Z; *Peganum harmala*; *Dracocephalum kotschyii*; Harmine; MTT; Xanthomicrol

1. Introduction

Agents that preferentially inhibit proliferation of neoplastic cells may offer means of developing drugs effective against cancer in human. One of the strategies for finding such agents is screening plant material, especially those which have been reported to have anti-cancer, anti-inflammatory, anti-fungal or anti-bacterial effects (Cordell et al, 1991; Cox and Balick, 1994; Wu et al., 2002). Spinal-Z, is a traditional Iranian anticancer remedy (Sobhani et al., 2002). It was used by traditional healers as a plant concoction for the treatment of many forms of cancer in humans. These traditional healers believed that the concoction was especially effective against leukemia and GI tract malignancy. Spinal-Z is an extract consisting of two plants: *Peganum harmala* Linn. seeds and *Dracocephalum kotschyii* Boiss leaves (80:20% w/w) (Sobhani et al., 2002). *P. harmala*, a member of the family Zygophyllaceae, is a poisonous plant that is native to Iran (Sobhani et al., 2002). Many different pharmacological effects have been attributed to

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P. harmala. Some of the reported pharmacological effects of *P. harmala* may be attributed to its β-carboline alkaloid content, mainly harmine and harmaline. These alkaloids are both monoamine oxidase inhibitors (Kim et al., 1997) and spasmolytic (Shi et al., 2001). Harmine the major β-carboline alkaloid in *P. harmala* extract is a DNA topoisomerase type I inhibitor (Sobhani et al., 2002), specific cyclin-dependent kinases inhibitor (Song et al., 2004), antileishmania (Di Giorgio et al., 2004), genotoxic (Boeira et al., 2001, 2002) and recombinogenic (Boeira et al., 2002) agent. It has shown cytotoxic activity against a series of tumor cell lines (Lamchouri et al., 2000). Although a large number of studies on the pharmacological effects of *P. harmala* and its components have been reported (Beutler et al., 1993; Lamchouri et al., 1999; Mahmoudian et al., 2002), there is little information available on the pharmacological effects of the leaf extract of *D. kotschyii*. *D. kotschyii*, a member of Labiatae family, is a native Iranian plant (Ghahreman, 1987). Its leaf extract has been reported to have immunomodulatory (Amirghofran et al., 2000) and antihyperlipidemic (Ebrahim Sajjadi et al., 1998) effects. In this study, the cytotoxic effects of Spinal-Z are compared with the effects of its components, in particular *D. kotschyii*, against a number of cell lines. Also, the anti-tumor effect of Spinal-Z and its constituents are examined in mice inoculated with cancer cells. In addition an attempt is made to compare the in vitro and in vivo effect of these plant extracts with known anti-tumor compounds: Doxorubicin and Camptothecin.

2. Results

2.1. In vitro cytotoxicity tests

The cytotoxicity of Spinal-Z, the leaf extract of *D. kotschyii*, the seed extracts of *P. harmala* and its major β-carboline alkaloid harmine were examined in several human tumor cell lines (HL60, K562, Hela, KB, A549, Saos-2, A2780-CP, A2780-S, MCF-7, A375 and A172). HFFF-P16 cells were used as non-malignant control. The IC_{50} values against each cell line are presented in Table 1. Also, a cytotoxic index was calculated for each agent as IC_{50} of test compound against HFFF-P16 divided by its IC_{50} against cancer cell line (Fig. 1). This cytotoxic index could give an indication of the preferential effects of the test compound on cancer cell lines compared with non-malignant cells. In all the malignant cell lines tested, Spinal-Z showed cytotoxic effects (Table 1). The cytotoxic indices for Spinal-Z against A2780-S, HL60, K562 and Saos-2 cells suggested that this remedy had preferential cytotoxicity against these cell lines (Fig. 1). Of all three agents used *D. kotschyii* showed the greatest absolute and preferential cytotoxicity in most cell lines tested (Fig. 1). The data obtained in the current study confirmed the cytotoxic effects of *P. harmala* seed extract. In all cell lines tested *P. harmala* seed extract had greater cytotoxic effect compared to Spinal-Z (Table 1). However in most cell lines, the cytotoxic indices for *P. harmala* seed extract and Spinal-Z were similar (Fig. 1).

Table 1
The cytotoxic effect of Spinal-Z, *Peganum harmala* seed extract and *Dracocephalum kotschyii* leaf extract against a panel of human cell lines

Cell line	*Dracocephalum kotschyii*	*Peganum harmala*	Spinal-Z
A172	6.8 ± 0.9	27.1 ± 1.0	NM
A2780-s	7.5 ± 1.0	26.4 ± 1.1	36.1 ± 1.0
HL60	16.3 ± 1.0	24.5 ± 1.0	66.7 ± 1.0
KB	18.1 ± 1.2	174.4 ± 1.3	441.2 ± 2.3
K562	26.9 ± 3.2	19.4 ± 1.4	64.7 ± 1.95
MCF-7	41.7 ± 3.9	70.1 ± 1.0	162.3 ± 2.2
Saos-2	51.8 ± 1.1	84.4 ± 1.5	80.6 ± 1.3
Hela	57.7 ± 0.9	59.1 ± 1.0	106.1 ± 2.1
A2780-cp	78.1 ± 3.7	60.8 ± 1.9	129.4 ± 1.7
A549	87.8 ± 1.7	NM	NM
A375	100.3 ± 1.5	NM	NM
HFFF-P16	331 ± 1.2	182.9 ± 1.2	444 ± 2.3

IC_{50} value was calculated by measuring cell proliferation using the MTT assay and is defined as the drug concentration (μg/mL) causing a 50% inhibition of cell growth.
Data represent mean values ± SD for three independent experiments.
NM: not measured.

In order to further delineate the preferential cytotoxic effects of the agents used, the mean IC_{50} of the agent against all cell lines was compared with the IC_{50} value of the agent against each cell line (Fig. 2). A172, A278-S and HL60 cell lines exhibited the greatest sensitivity to *D. kotschyii* extract and the KB cell line was relatively much more sensitive to the *D. kotschyii* extract than to the *P. harmala* extract or Spinal-Z.

IC_{50} values obtained for harmine, the major β-carboline alkaloid found in *P. harmala* extract against HL60 and K562 cell lines were found to be 1.7 ± 0.1 and 1.9 ± 0.3 μg/mL, respectively.

In order to evaluate the contribution of *D. kotschyii* and *P. harmala* towards the cytotoxic effects of Spinal-Z, mixtures of various proportions of the two extracts were prepared (Table 2) and their cytotoxicity was evaluated against HL60 cell line. A mixture containing 60% *D. kotschyii* extract showed the smallest IC_{50} and therefore the greatest effectiveness against HL60 cells.

2.2. In vivo anti-tumor properties

The anti-tumor activities of the extracts and doxorubicin (DOX) (as positive control) were studied by treating mice, inoculated with WEHI 164 cells, with the test mixtures. In cell culture studies the IC_{50} values for Spinal-Z, *D. kotschyii* (DK), and *P. harmala* (PH)

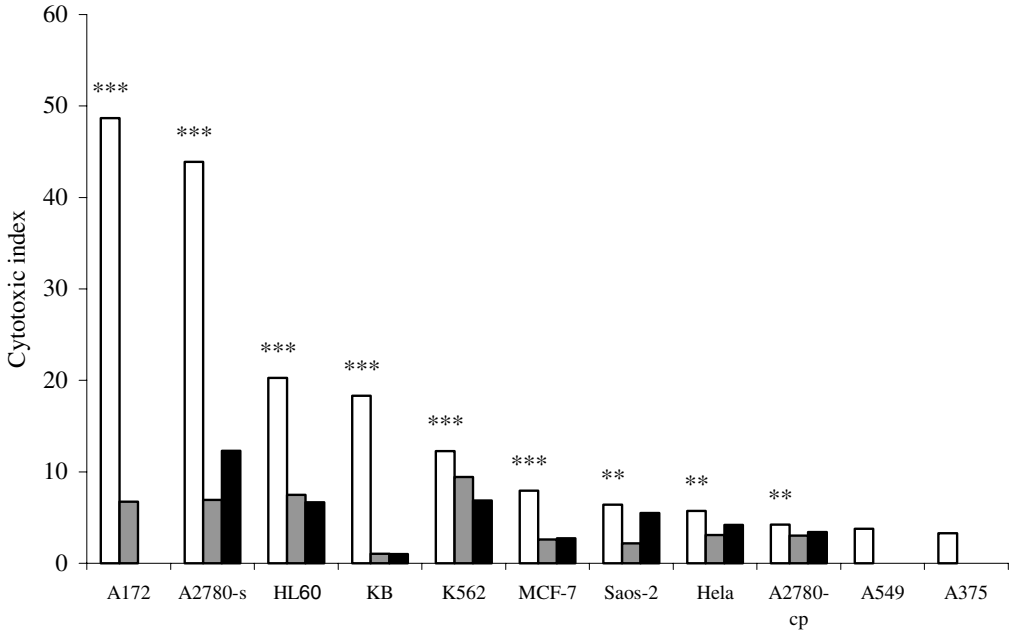

Fig. 1. The cytotoxic indices of Spinal-Z (■), *Dracocephalum kotschyii* leaf extract (□) and *Peganum harmala* seed extract (▩) on a panel of cell lines. In all cell lines tested, the cytotoxic index of *Dracocephalum kotschyii* was statistically different from *Peganum harmala* and Spinal-Z ($^{***}p < 0.001$, $^{**}p < 0.01$).

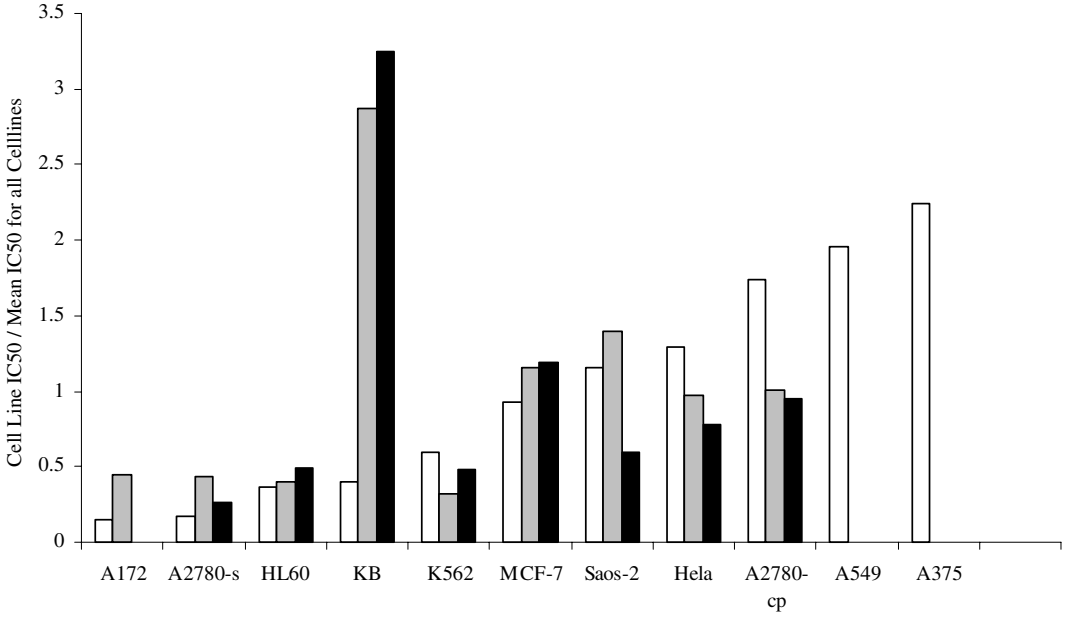

Fig. 2. Differential cytotoxic effects of Spinal-Z (■), *Dracocephalum kotschyii* leaf extract (□) and *Peganum harmala* seed extract (▩) on a panel of cell lines. The graph displays the ratio of mean IC_{50} value of the test compound for the particular cell line and the IC_{50} value of the test compound against all cell lines tested.

against WEHI 164 cells were determined to be 76.1 ± 0.14, 26.5 ± 0.16 and 54.2 ± 0.22 μg/mL, respectively.

The changes in the volume of tumors induced in mice were calculated (Fig. 3). Compared to the control group, both DK and DOX groups showed a statistically significant slower rate of tumor volume growth 12 days after inoculation. The final tumor volume, determined on the last day, was statistically smaller in both DK and DOX groups compared to the control group ($p < 0.001$). In addition, the tumor volume in animals treated with DK was smaller than the group receiving DOX after day 12. This difference was statistically significant ($p < 0.001$).

Table 2
Effects of *Dracocephalum kotschyii* content upon the cytotoxicity of in-lab prepared "Spinal-Z" remedy

	Spinal-Z	*Peganum harmala*:*Dracocephalum kotschyii* content (% w/w)						
		100: 0	0:100	20:80	40:60	50:50	60:40	80:20
IC_{50} (μg/mL)	66 ± 1	24 ± 2	16.3 ± 2	22.6 ± 2	12.5 ± 1	49.0 ± 3	27.7 ± 2	17 ± 1

IC_{50} value was calculated by measuring HL60 cell proliferation using the MTT assay and is defined as the drug concentration (μg/mL) causing a 50% inhibition of cell growth.
Data represent mean values ± SD for three independent experiments.

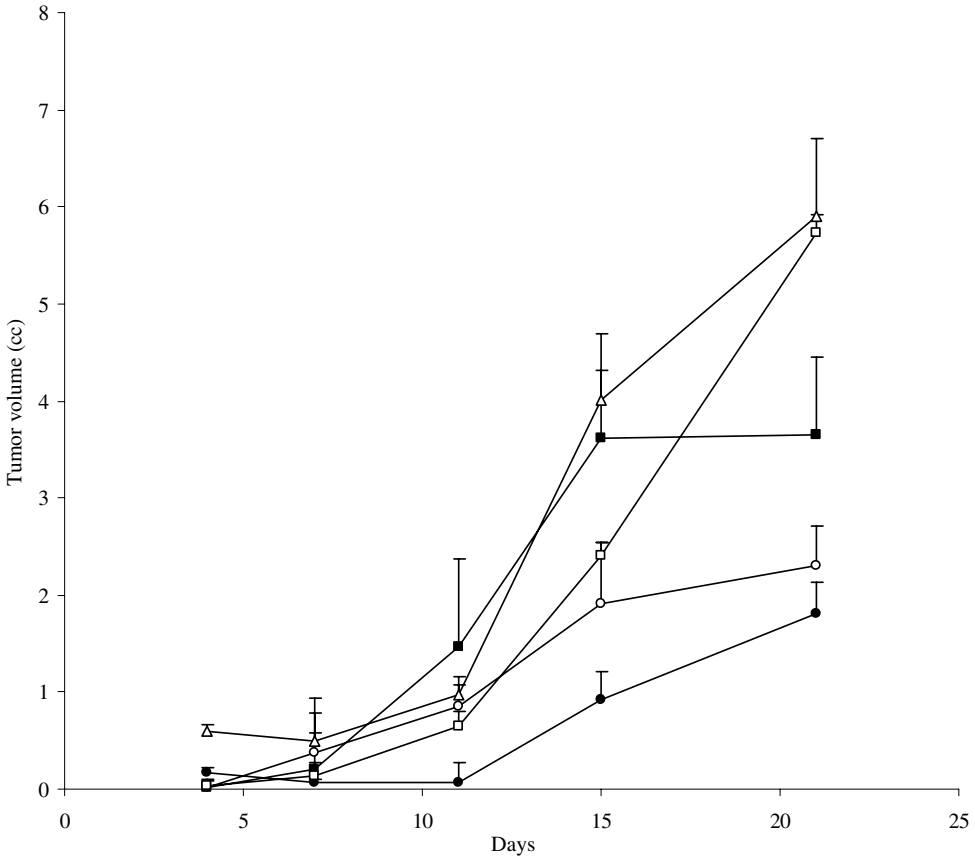

Fig. 3. Effects of Spinal-Z and its components on solid tumor volumes induced in BALB/c mice. Five groups of six BALB/c mice were injected subcutaneously with WEHI 164 cells. The animals were injected with (○) Doxorubicin, (●) *Dracocephalum kotschyii* leaf extract, (□) Spinal-Z, (△) *Peganum harmala* seed extract or (■) vehicle. The volume of the tumor was measured every 4 days. Values are means ± SD, $n = 6$.

Although up to day 12, tumor volume in mice receiving *P. harmala* was smaller than the control group, from day 15, the trend was reversed. The effect of Spinal-Z on tumor volume resembled the effects of *P. harmala*.

The weights of tumors after excision (Table 3) were in agreement with the calculations of the tumor volumes on day 24.

The changes in mice body weights were recorded (Fig. 4). Animals receiving *D. kotschyii* and DOX started to gain weight from day 7, while the body weight of control animals or mice receiving Spinal-Z remained more or less unchanged throughout the treatment. The animals receiving *P. harmala*, showed a decrease in body weight from day 12 onward. The change in body weight on day 24 compared to day 0, was used to calculate BWL (Table 4). Compared to the control group, the mice receiving *D. kotschyii*, Spinal-Z or DOX, showed a negative BWL, indicating weight gain. The weight gain in mice receiving Spinal-Z was statistically negligible, while the weight gain in mice receiving *D. kotschyii* or DOX was statistically greater than the control group ($p < 0.001$). Animals receiving *P. harmala* showed a statistically greater BWL compared to the control group. In addition, the D/U ratio was highest in the animals receiving *P. harmala* and lowest in the mice receiving *D. kotschyii* or DOX (Table 4).

Results from histological study of the tumor tissues after hematoxylin–eosin staining are presented in

Table 3
Effects of Spinal-Z and its constituents upon tumor growth in mice inoculated with WEHI-164 cells

Group	The weight of tumor (g)
Control	2.5 ± 0.26
Doxorubicin	1.6 ± 0.47**
Spinal-Z	2.8 ± 0.41ns
Peganum harmala	3.8 ± 0.78**
Dracocephalum kotschyii	1.4 ± 0.34**

Five groups of six BALB/c mice were injected subcutaneously with WEHI 164 cells. The animals were injected with the plant extract, doxorubicin or vehicle. On day 24, animals were killed and tumors were removed and weighed. The weight of tumors for animals in test groups were compared with those of the control group.
ns = not statistically significant.
Values are means ± SD, $n = 6$.
** $p < 0.01$.

Table 4
Effects of Spinal-Z and its constituents on the intensity of the side effects in and survival of tumor bearing mice

	BWL	Average life span	% ILS	D/U
Control	0.045	22.7 ± 2.1	–	1/6
Doxorubicin	−11.64	25 ± 0.01*	10.06	0/6
Spinal-Z	−1.864	23.3 ± 2.1ns	2.73	1/6
Peganum harmala	19.532	18 ± 2.28***	−18.55	4/6
Dracocephalum kotschyii	−10.22	25 ± 0.01*	10.06	0/6

Body weight loss (BWL) was calculated as $(1 - BWn/BWs) \times 100$ where BWn is the body weight on day n and BWs is the body weight on day zero.
Increase in life span (ILS) was calculated as $T - C/C \times 100$, where T is the average number of days the animal in each group survived and C is the average number of days the animals in control group survived.
D/U: Number of toxic deaths/total number of mice.
ns = not statistically significant.
Values are means ± SD, $n = 6$.
* $p < 0.05$.
*** $p < 0.001$.

Table 5. There was no statistically significant difference between the percent death observed in the tumor tissues from the DK, PH or Spinal-Z groups while all three groups were statistically different from the control group ($p < 0.001$). The ratio of apoptosis to necrosis was highest in the DK group and lowest in the DOX group.

2.3. Isolation and identification of the active ingredient

The methanolic extract of *D. kotschyii* was subjected to TLC. 13 fractions were obtained and tested for cytotoxic effects on HL60 cell line. Fraction 10 showed cytotoxic effects. This fraction was further purified using semi-preparative HPLC which yielded 9 peaks (Fig. 5). Peak IV, with a retention time of 41 min exhibited cytotoxic activity against HL60 cell line. The structure of peak IV was determined using spectral analysis:

5-Hydroxy-2-(4-hydroxyphenyl)-6,7,8–trimethoxy-4H-chromene-4-one.

Yellow powder, m.p: 227–230 °C, IR (KBr) (νmax, cm^{-1}): 1691 (C=O) Ms, m/z (%): 345 ($m^+ + 1$, 15), 344 (M+, 30), 329 (20), 301 (15), 284 (5), 211 (20). Anal. Calc. for $C_{18}H_{16}O_7$ (344.8): C, 62.64; H, 4.64; CH30, 26.97%. Found: C, 62.7; H, 4.71; CH3O, 27.07% ^{1}H NMR (500.1 MHz, $CDCl_3$): $\delta = 3.96$, 3.98, and 4.12 (9H, 3 *s*, 3 OMe), 5.63 (1H, *brs*, OH), 6.60 (1H, *s*, CH), 7.00 (2H, *d*, $^3J_{HH} = 8.4$ Hz, $CH_{3'}$), 7.85 (2H, *d*,

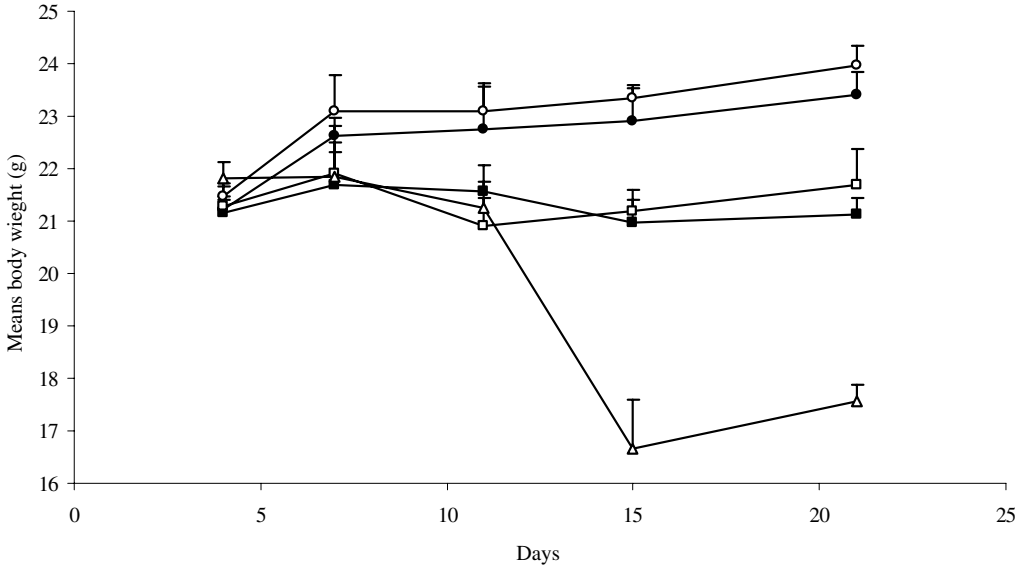

Fig. 4. Effects of Spinal-Z and its components on body weight of mice inoculated with WEHI-164 cells. The animals were injected with (○) Doxorubicin, (●) *Dracocephalum kotschyii* leaf extract, (□) Spinal-Z, (△) *Peganum harmala* seed extract or (■) vehicle. The animal body weight was measured every 4 days. Values are means ± SD, $n = 6$.

Table 5
Effect of Spinal-Z and its components on cell death induced in WEHI-164 solid tumors

Group	% Total cell death	Ratio apoptosis to necrosis
Control	15 ± 1	0.33 ± 0.1
Doxorubicin	32 ± 1.3	0.48 ± 0.03**
Spinal-Z	58 ± 3.2	0.05 ± 0.01***
Peganum harmala	66 ± 7.1	1.04 ± 0.22***
Dracocephalum kotschyii	60 ± 3.1	1.86 ± 0.20***

Five groups of six BALB/c mice were injected subcutaneously with WEHI 164 cells. The animals were injected with the plant extract, doxorubicin or vehicle. On day 24, animals were killed and tumors were removed and weighed. Tumor tissues were stained using hemotoxylin–eosin and examined microscopically for cell death. %Total cell death and ratio of apoptotic to necrotic cell death for each group were compared with the control group.
Values are means ± SD, $n = 6$.
** $p < 0.01$.
*** $p < 0.001$.

$^3J_{HH} = 8.4$ Hz, CH_2), 12.55 (1H, *s*, OH). ^{13}C NMR (125.7 MHz, DMSO-d_6): $\delta = 60.04$, 61.96, and 62.40 (3 OMe), 103.12 (C_3), 106.82 (C_{10}), 116.65 ($CH_{3',5'}$), 121.50 ($C_{1'}$), 128.97 ($CH_{2',6'}$), 133.15 (C_8), 136.191 (C_6), 145.69 (C_7), 149.021 (C_9), 152.99 (C_5), 162.05 ($C_{4'}$), 164.8 (C_2) and 183.03 (C_4).

The trivial name of this compound is xanthomicrol and the semi-systematic name is 5,4′-dihydroxy-6,7,8-trimethoxyflavone (Fig. 6) (Stout and Stout, 1961; Grayer et al., 2001; Jamzad et al., 2003). The cytotoxicity of xanthomicrol obtained from methanolic DK extract was investigated against a panel of cell lines (Table 6). Xanthomicrol showed a much larger IC_{50} value compared to doxorubicin in all cell lines tested. However, the cytotoxicity index of xanthomicrol against all cell lines tested was significantly greater than doxorubicin ($p < 0.001$).

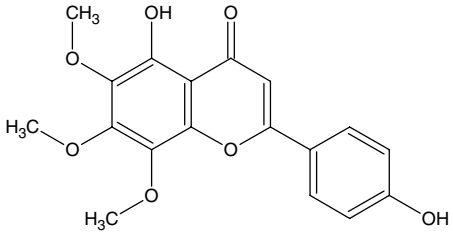

Fig. 6. Structure of Xanthomicrol.

The mechanism of the cytotoxic effect of xanthomicrol was studied using fluorescent microscopy. Xanthomicrol induced a concentration dependent total and apoptotic cell death (Fig. 7). Necrotic cell death induced by xanthomicrol, however, reached a plateau at a concentration of 50 ng/mL. Camptothecin too induced a concentration dependent total cell death. However at higher concentrations, the contribution of necrotic cell death towards total cell death was much greater with camptothecin than xanthomicrol. Camptothecin was more effective in inducing total cell death at 100 and 250 ng/mL, while at lower concentrations (12.5 and 50 ng/mL) xanthomicrol produced greater cell death.

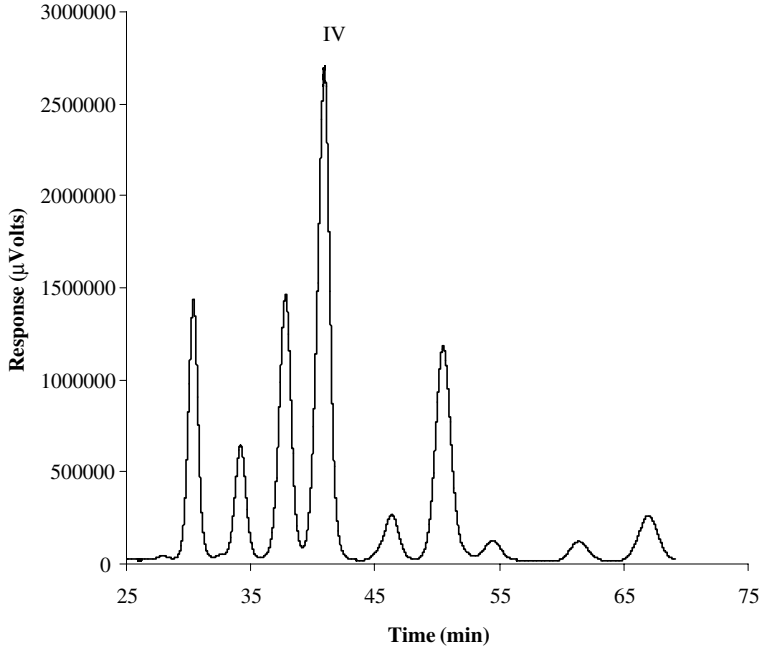

Fig. 5. The HPLC chromatogram of Plate Layer Chromatography fraction 10 of DK methanolic extract. Peak IV with a retention time of 40 min contained the maximum cytotoxic activity against HL 60 cells. Chromatography conditions: Column: Nucleosil 100–10, 25 cm × 16 mm. Mobile phase: Acetonitrile: 0.05 M HCl (75:25 v:v) pumped at 10 mL/min. Detection: 220 nm.

Table 6
The cytotoxic effect of xanthomicrol and doxorubicin against a panel of human cell lines

	IC_{50} (μg/mL)		Cytotoxicity index	
	Xanthomicrol	Doxorubicin	Xanthomicrol	Doxorubicin
HL60	0.88 ± 0.04	0.04 ± 0.02	15.76	0.42
K562	1.69 ± 0.03	0.11 ± 0.05	8.2	0.18
Saos-2	0.75 ± 0.09	0.02 ± 0.01	18.5	1.1
A2780-cp	1.56 ± 0.04	0.06 ± 0.02	8.9	0.32
A2780-s	0.89 ± 0.06	0.06 ± 0.01	15.5	0.32
HFFF-P16	13.8 ± 0.09	0.018 ± 0.01	–	–

IC_{50} value was calculated by measuring cell proliferation using the MTT assay and is defined as the drug concentration (μg/mL) causing a 50% inhibition of cell growth. Cytotoxicity index was calculated by dividing the IC_{50} of the test compound against HFFF-P16 cells by the IC_{50} of the test compound against the particular cell line.
Data represent mean values ± SD for three independent experiments.

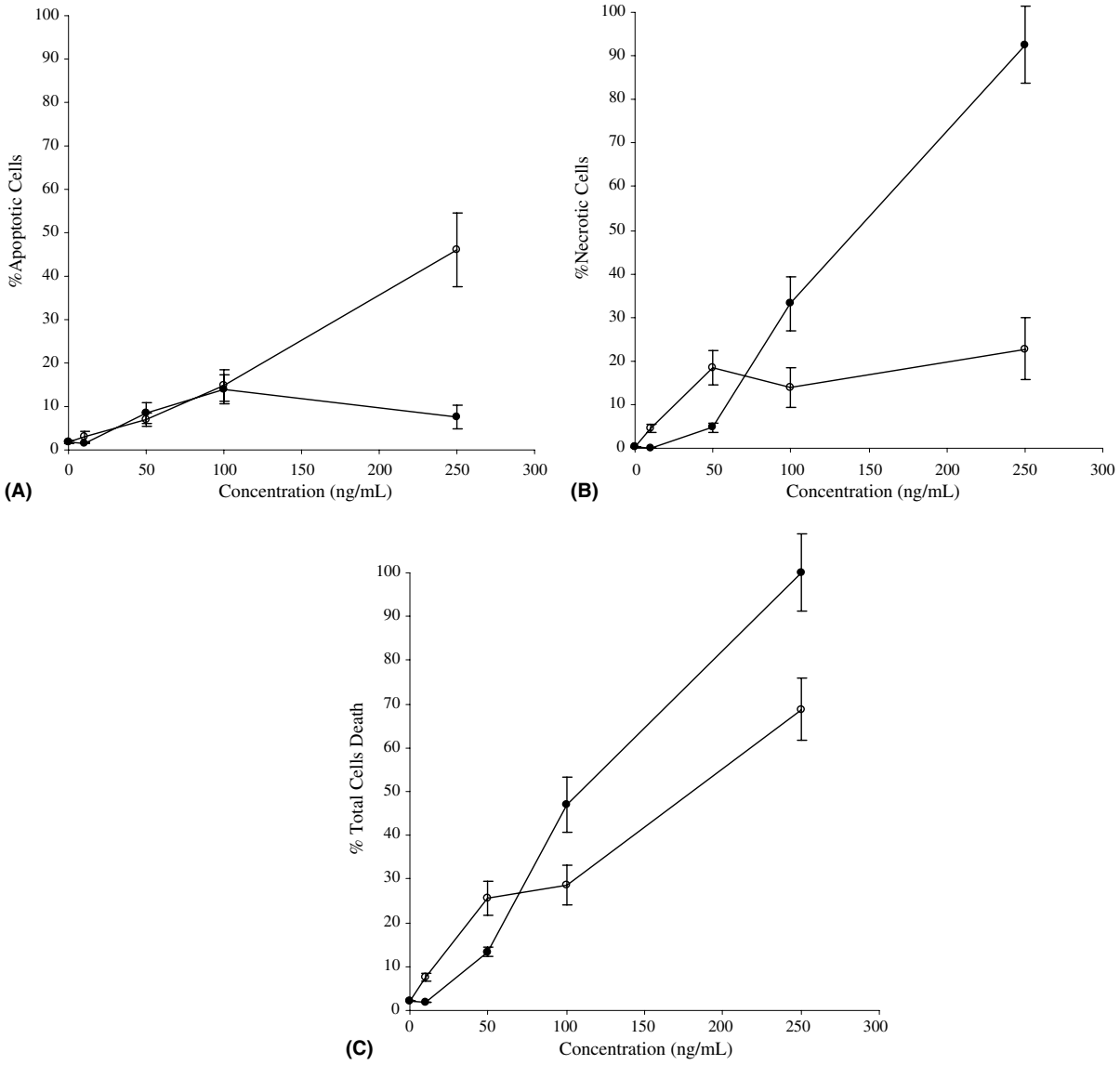

Fig. 7. Effects of xanthomicrol (○) and camptothecin (●) on apoptotic (A), necrotic (B) and total cell death (C) induced in cultured HL60 cells. HL60 cells were treated with the drug for 4h and stained immediately with acridine orange and propodium iodide. The stained cells were examined using fluorescence microscopy. Values are means ± SD, $n = 6$.

3. Discussion

In this work, the cytotoxic and anti-tumor effects of Spinal-Z, an Iranian ethnobotanical anti-cancer remedy, and its constituents were studied. In vitro cell proliferation inhibition test using MTT viability assay confirmed that both Spinal-Z and its constituents (the leaf extract of *D. kotschyii* and the seed extract of *P. harmala*) have cytotoxic activity against all cell lines tested. Comparison of the cytotoxic indices (Fig. 1) and differential cytotoxicity (Fig. 2) calculated for Spinal-Z, suggests that this cytotoxic effect is preferentially exerted against cancer cell lines. Both herbal constituents of Spinal-Z showed cytotoxicity in in vitro tests. The absolute cytotoxicity of Spinal-Z against most cell lines tested, resembled the cytotoxicity profile of *P. harmala* extract more than the profile obtained with *D. kotschyii* extract. Spinal-Z is an extract of 80% w/w *P. harmala* powdered seeds and 20% w/w *D. kotschyii* powdered leaves. It is thus expected that the effects of Spinal-Z should resemble those of its main component i.e. *P. harmala* seed extract. This view is confirmed by strong similarity between the cytotoxic indices and differential cytotoxicity of Spinal-Z and *P. harmala* seed extract.

Our results suggested that *D. kotschyii* leaf extract is the more potent constituent of Spinal-Z. Compared to *P. harmala* seed extract, *D. kotschyii* had a smaller IC_{50} value against A172, A2780-s, HL60, KB, MCF-7 and Saos-2 cells. Also, *D. kotschyii* extract was less toxic towards HFFF-P16 cells compared to *P. harmala* extract. The obtained data indicated that a 80:20 w/w mixture of *P. harmala*:*D. kotschyii* extracts may not be optimal. When the cytotoxic effects of mixtures of different proportions of *P. harmala* seed extract and *D. kotschyii* leaf extract against HL60 cell line were investigated (Table 2), it was found that a 40:60 w/w mixture of *P. harmala*:*D. kotschyii* extracts had the smallest IC_{50} value. However, the difference between the IC_{50} values of different proportion mixtures was not very large. An anomaly observed was the 3-fold difference between the IC_{50} values of the 80:20 mixture of *P. harmala*:*D. kotschyii* prepared in the lab and Spinal-Z (commercial product). During the preparation of Spinal-Z, a mixture of 80:20 *P. harmala* seed and *D. kotschyii* leaf are refluxed together, while in the lab, a mixture of 80% *P. harmal* a seed extract and 20% *D. kotschyii* leaf extract were prepared. The different preparation procedures might explain the difference observed between the cytotoxic effects of Spinal-Z and the 80:20 mixtures of *P. harmala* and *D. kotschyii* extracts.

P. harmala had previously been shown to have cytotoxic effects (Lamchouri et al., 2000). In this work, the cytotoxic effects of P. *harmala* on three previously unreported cell lines (K562, HL60, and A172) were demonstrated. This work also confirmed the idea that cytotoxic effects of *P. harmala* seed extract could be attributed to its harmine contents (Sandler et al., 2002). The in vitro experiments carried out in this study, confirmed the effectiveness of Spinal-Z and its components as cytotoxic agents against a panel of cell lines. To investigate the anti-tumor properties of this remedy, effects of Spinal-Z and its components on tumors induced in BALB/c mice (using murine fibrosarcoma WEHI-164 cell line) were investigated. In order to determine the effectiveness of the agent, the volume of tumor was estimated every four days. Also to assess the degree of side-effects induced by the agent, changes in the body weight was determined every four days, and in order to show the overall effectiveness of the treatment, the life spans of the animals were calculated. After the animals were killed and the tumors were excised and weighed, the tumor tissue were fixed and stained for microscopic examination. As a positive control, doxorubicin was used.

The results from in vivo experiments suggest that compared with the clinically used DOX dose regimen, *D. kotschyii* leaf extract was more effective in inhibiting tumor growth rate. Animals receiving DOX or *D. kotschyii* leaf extract showed no loss of body weight, suggesting minimal toxicity and/or side-effects associated with the treatment. The average final tumor weight in animals receiving DOX or *D. kotschyii* leaf extract was similar and significantly smaller than control. Average life span, increase in span of life and the D/U ratio was the same for the animals receiving DOX or *D. kotschyii* leaf extract.

Animals receiving *P. harmala* seed extract scored badly on all criteria. They had a large tumor volume growth rate, the largest BWL, the shortest span of life and therefore a negative increase in life span. Also, more animals in this group died during the experiment than those belonging to the control group. The animals receiving Spinal-Z, were only marginally better than the control group on BWL and average life span. The tumor volume growth rate in the Spinal-Z group was faster than the control group during the last few days of the experiment and the final tumor weight was larger than the control, although this was not statistically significant.

Microscopic examination of the hemotoxylin–eosin stained tumor tissues revealed that the total tumor cell death in animals receiving the leaf extract of *D. kotschyii* was higher than the group receiving DOX. Also, the leaf extract of *D. kotschyii* produced a much greater ratio of apoptosis/necrosis than DOX treatment. Although compared to the *D. kotschyii* group, the absolute cell death was greater in the *P. harmala* group; the apoptosis/necrosis ratio was significantly lower in the latter.

The results obtained thus far suggested that: Spinal-Z and its components (the leaf extract of *D. kotschyii* and the seed extract of *P. harmala*) were cytotoxic. *D. kotschyii* leaf extract acted more selectively on the cancer cells compared to normal cells, than Spinal-Z or *P. harmala* seed extract. From an in vivo point of view,

D. kotschyii leaf extract would probably make a better anti-tumor remedy than either Spinal-Z or *P. harmala* seed extract. This was mainly because the animals receiving *D. kotschyii* showed less side-effects (as represented by BWL and D/U) and slower tumor volume growth rates than those receiving *P. harmala* seed extract or Spinal-Z. Hence, *D. kotschyii* leaf extract appeared to act effectively and selectively as an anti-tumor remedy and thus the isolation and identification of the active component would be desirable.

In order to produce sufficient amounts of the active component for spectral identification rapidly, a combination of TLC and HPLC was used. TLC was able to separated *D. kotschyii* leaf extract into 13 fractions, one of which (fraction 10) contained cytotoxic activity. This fraction was separated into 9 peaks using reverse phase HPLC. Of the 9 peaks, peak 4 had cytotoxic properties. This peak was identified as xanthomicrol.

The cytotoxic effects of xanthomicrol against a number of cell lines were investigated. Although in all cell lines tested, xanthomicrol had a much larger IC_{50} value than DOX, the cytotoxic index calculated, was much greater for xanthomicrol than for DOX. Thus one might conclude that although larger amounts of xanthomicrol are needed to reach the tumor cells, the compound produced far less damage to non-malignant cells than DOX and would probably produce less side-effect. Results from fluorescent staining of HL60 cells treated with xanthomicrol suggest that at all concentrations used, major proportion of cell death induced by this compound is apoptotic. This is in contrast with camptothecin which only at lower concentrations caused apoptotic cell death and at higher concentrations caused mainly necrotic cell death.

Xanthomicrol was isolated from *Yerba Beuna* (*Satureja douglasii*) by Power and Salway and its structure was refined Stout and Stout (1961) . Since its first isolation, this flavone has been demonstrated to exist in many different plants including the genus *Ocimum* (fam. Labiatae) (Grayer et al., 2001), *Brickellia paniculata* (fam. Compositae) (Meckes et al., 2002) and more recently in *D. kotschyii* by Jamzad et al. (2003). Meckes et al demonstrated that xanthomicrol was able to inhibit acetylcholine, histamine and Ca^{2+} induced spasms of the guinea pig ileum (Meckes et al., 2002). They also showed xanthomicrol to be anti-inflammatory (Meckes et al., 2002).

In the present study, we were able to demonstrate that xanthomicrol has cytotoxic and anti-tumor effect and is probably the active ingredient in *D. kotschyii* leaf extract.

4. Conclusion

Spinal-Z and its major component, *P. harmala* seed extract, are cytotoxic. But neither appears to be clinically valuable anti-tumor agent in mice inoculated with WEHI-164 cells. *D. kotschyii* leaf extract, however, is both preferentially cytotoxic against malignant cells in vitro and has beneficial effects as an anti-tumor preparation in mice inoculated with WEHI-164 cells. The cytotoxic/anti-tumor component in the *D. kotschyii* leaf extract appears to be xanthomicrol. Clearly, further investigation on the mechanism of action and in vivo anti-tumor effects of xanthomicrol are needed to demonstrate whether or not xanthomicrol is likely to be a clinically significant anti-tumor agent.

5. Experimental

5.1. Materials

RPMI-1640 medium, FBS (Fetal bovine serum), penicillin, streptomycin, harmine · HCl, MTT {3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide}, dimethylsulfoxide (DMSO), propidium iodide (PI), acridine orange (AO), hematoxylin, eosin and camptothecin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cell lines (K562: Human chronic myeloid leukemia, HL60: Human promyelotic leukemia, Hela: Human cervix carcinoma, KB: Human oral epithelial carcinoma, A 549: Human lung carcinoma, Saos-2: Human osteogenic sarcoma, A2780-CP: Human ovarian carcinoma resistant to cisplatin, A2780-S: Human ovarian carcinoma sensitive to cispaltin, MCF-7: Human breast adenocarcinoma, A375: Human malignant melanoma, A172: Human glioblastoma, and HFFF-P16: Human fetal foreskin fibroblast) were obtained from Pasteur institute, Tehran, Iran. Commercial Spinal-Z remedy was a gift from Darou Pakhsh Pharmaceutical Company and contained the dried methanolic extract of an 80:20 mixture of dried powdered seeds of *P. harmala* and leaves of *D. kotschyii*. Doxorubicin was obtained from TEMAD pharmaceutical company, Tehran, Iran.

5.2. Animals

Male 6-week-old BALB/c mice were purchased from Pasteur Institute (Tehran, Iran). The animals were kept 6 in a cage and had free access to food pellets and water. All procedures involving animals were approved by and carried out under the supervision of the Razi Institute's Ethic Committee.

5.3. Preparation of plant material

5.3.1. Preparation of *P. harmala* and *D. kotschyii* methanolic extracts

The leaves of *D. kotschyii* and seeds of *P. harmala* were collected from Esfahan and Tehran provinces,

respectively, and identified by Dr. G. Amin, the head of herbarium at the school of pharmacy, Tehran University. Voucher specimens of *P. harmala* (6536 TEH) and *D. kotschyii* (6537 TEH) have been deposited in the Herbarium of Department of Pharmacognosy, School of Pharmacy, University of Tehran.

Hundred grams of dried pulverized plant material (leaves of *D. kotschyii* or seeds of *P. harmala*) were refluxed with 300 mL methanol for 1h. The suspension was filtered. The resulting extract was dried *in vacuo* and stored at −80 °C until needed.

5.3.2. Isolation and purification of the active component in *D. kotschyii* leaf extract

In order to purify the active ingredient of *D. kotschyii*, a combination of preparative TLC and HPLC were used. After fractionation of the methanolic extract by TLC, the most active fraction was subjected to semi preparative HPLC analysis as described below. Hundred and fifty milligrams of methanolic *D. kotschyii* extract was dissolved in 5 mL Methanol. The solution was applied to a silica coated glass plate (20 cm × 20 cm) as a strip, 2 cm from the plate edge. The plate was developed in a TLC tank using chloroform: methanol: 14 M NH_4OH (15:4:1). Thirteen bands were separated on the plate. Each band was scraped off the plate and the components were eluted from silica using 100 mL methanol. The 13 methanolic fractions obtained were dried in vacuo and dissolved in DMSO for subsequent MTT assay.

In order to obtain enough material for subsequent HLPC purification and spectral analysis, 15 g of *D. kotschyii* methanolic extract were fractionated as described above. Hundred milligrams of fraction 10 from TLC analysis ($R_f = 0.69$) was dissolved in 10 mL mobile phase. To remove particulate matter, the mixture was centrifuged at 20000*g* for 10 min. The clear supernatant was subjected to semi-preparative HPLC. The chromatographic system comprised of: Two Wellchrom Knaur k-1001 HPLC Pumps, a Linear 200 UVIS UV/Visible detector and a Macherey-Nagel Ecoprep HPLC column (Nucleosil 100-10, 25 cm × 16 mm). Mobile phase Acetonitrile: 0.05 M HCl (75:25) was pumped at 10 mL/min (each pump set at 5 mL/min). Detection was at 220 nm. 0.4 mL sample was loaded into the column during each run. Peaks were collected, dried in vacuo and assayed for cytotoxic effects. The HPLC purification was repeated to obtain about 15 mg of each purified compound. The structure of the purified compound with highest cytotoxic activity was determined using spectral analysis.

5.3.3. Preparation of test solutions for in vitro cytotoxicity assay

Spinal-Z, the in-house prepared *P. harmala* seed or *D. kotschyii* leaf extracts were dissolved in DMSO at a concentration of 200 mg/mL. After filtration through a 0.2 μm Porafil filter (Macherey-Nagel GmbH & Co. KG Germany), the solutions were diluted to the desired concentrations using RPMI-1640 medium.

For the study of the combinational effects of the two plant extracts, 200 mg/mL filtered stock solutions were combined to yield 80:20, 60:40, 50:50, 40:60 and 20:80 mixtures of *P. harmala* seed and *D. kotschyii* leaf extracts.

Harmine was dissolved in DMSO at 1 mg/mL. 1 mg of TLC fraction was dissolved in 1 mL DMSO. The solution was filtered through a 0.2 μm Porafil filter and diluted to the required concentration using RPMI-1640 medium.

1 mg purified compound obtained from semi-preparative HPLC was dissolved in 1 mL DMSO and diluted to the required concentrations using RPMI-1640 medium.

5.4. In vitro cytotoxicity assay

5.4.1. MTT assay

MTT assay (Mosmann, 1983) was used to measure cytotoxicity of Spinal-Z and its components. Each cell line was maintained and treated in suspension in RPMI-1640 medium supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. Cells were grown under an atmosphere of 5% CO_2/95% air at 37 °C. These cells were then plated in 96 well plates (5×10^3 cells/well) and incubated under 5% CO_2/95% air at 37 °C for 24 h. The cells were treated with the test compounds for 72 h. After addition of MTT (10 μL/well, 5 mg/mL in phosphate-buffered saline), the plates were incubated for 4 h under 5% CO_2/95% air at 37 °C. Oxidized dye was dissolved by addition of 100 μL DMSO per well. The absorbance was measured at 570 nm using a Dynex MRX micro plate reader.

5.4.2. Fluorescence staining

HL60 cell line was maintained and treated in suspension in RPMI-1640 medium supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. Cells were grown under an atmosphere of 5% CO_2/95% air at 37 °C. Cells were plated in 6 well plates (1×10^6 cells/well) and incubated under 5% CO_2/95% air at 37 °C for 24 h. Cells were treated with the test compound or camptothecin, both dissolved in DMSO, for 4 h. A manual apoptosis assay was performed by using a dual staining solution containing 10 μg/mL acridine orange (AO, Sigma) and 50 μg/mL propidium iodide (PI, Sigma) in phosphate buffered saline (PBS) (Simpson et al., 1999). A 100 μL volume of HL60 cell suspension was mixed 1:1 with the dual staining solution. The resulting mixture was loaded onto a glass slide and examined immediately at 200× magnification, using an Olympus IX-71 inverted fluorescence microscope equipped with a U-MWB2 fluorescence mirror unit.

Three separate counts were performed for each slide. Top, middle and bottom regions of slide, each containing 100 cells, gave a total of 300 counted cells per sampling point. Viability results are presented as a percentage of the total number of cells counted. No attempts were made to distinguish between late and early apoptotic cells. Cells were categorized into groups by virtue of their color and chromatin morphology. Green cells with dispersed chromatin were designated as viable. Green cells with condensed chromatin or red cells with condensed chromatin fell into the apoptotic category, while those red cells with dispersed chromatin were designated to be necrotic.

5.4.3. In vivo anti-tumor assay

Five groups of six BALB/c mice were injected subcutaneously in the right flank with 1.5×10^6 WEHI 164 fibrosarcoma cells (Wilson et al., 1989). One day later mice were injected intraperitoneally, everyday for 3 weeks, with 30 μL of DMSO (Control group), 30 μL 30 mg/kg Spinal-Z (SZ group), 30 μL 6 mg/kg *D. kotschyii* leaf extract (DK group), or 30 μL 24 mg/kg *P.harmala* seed extracts (PH group). The mice in the last group (DOX group) were injected with 30 μL 2 mg/kg doxorubicin on days 3 and 17 after subcutaneous infiltration of WEHI 164 cells (Wilson et al., 1989). At four day intervals, the animals were weighed and the tumor size was estimated by measuring the diameters of the elliptical tumors in two perpendicular dimensions using vernier calipers. The volume of tumor was calculated using the formula: $4\pi r_1^2 r_2/3$, where r_1 and r_2 were the short and long tumor radii, respectively (Rajeshkumar et al., 2002). In order to evaluate intensity of the side-effects of the treatment, body weight loss (BWL) and the ratio of animals which died during the course of experiment to the number of animals used (D/U) in the test were determined (Kumazawa et al., 1998). BWL was calculated as $(1 - \mathrm{BW}n/\mathrm{BWs}) \times 100$, where BW$n$ is the body weight at day n and BWs is the body weight at day zero. Increase in life span (ILS) was calculated as $T - C/C \times 100$, where T is the average number of days the animal in each group survived and C is the average number of days the animals in control group survived (Kruczynski et al., 1998; Kumazawa et al., 1998).

On day 24, the animals were killed by cervical dislocation and tumors were removed and weighed. The excised tissues were fixed in 10% formalin for 24 h and subsequently embedded in paraffin. Five micrometer sections were prepared and stained by standard hematoxylin–eosin method (Will et al., 1994). The sections were studied microscopically for the presence of dense chromatin bodies – representative of apoptosis and occurrence of necrotic cell death. The pathologist examining the sections was unaware of the animal group each section belonged to.

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