

# *In vitro* cytotoxicity analysis of *Zizyphus spina-christi* stem bark extract on human cancer cell lines

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## Abstract:

*Zizyphus spina-christi* (Rhamnaceae family) is an edible plant used in folk medicine. Therefore, it is of interest to report the cytotoxic effects of *Z. spina-christi* bark crude extract on human cell lines. Crude ethanol extract of *Z. spina-christi* bark was fractionated with increasing polarity (diethyl ether, chloroform, ethyl acetate and butanol fractions). The fractions were examined for their cytotoxicity against human colon cancer (HCT-116 and CACO-2), cervical cancer (HeLa and HEP-2), lung carcinoma (A-549), hepatocellular carcinoma (HepG-2), breast cancer (MCF-7) and prostate cancer (PC-3) cell lines using viability assay. Diethyl ether fraction of *Z. spina-christi* showed the highest cytotoxic effects among the four extracts of *Z. spina-christi*. The IC<sub>50</sub> of diethyl ether fraction was 7.14, 11.2, 11.6, 15.4, 39.8, 42.2, 84.2 and 153.8 µg/ml on HepG-2, A-549, CACO-2, HCT-116, MCF-7, PC-3, HeLa, and HEP-2 cell lines, respectively. Data shows that the diethyl ether fraction of *Z. spina-christi* showed effective cytotoxic effects in colon, lung and hepatocellular cancer cell lines.

**Keywords:** Anticancer; cytotoxic; phytochemicals; natural remedies, *Zizyphus spina-christi*

**Background:**

Resistance to chemotherapeutic agents, and adverse side effects of radiation are problems in clinical cancer treatment [1]. Drugs inspired by the natural products constitute many approved drugs for cancer as many as 247 drugs approved from 1981 to 2019 [2]. Therefore, it is of interest to report the cytotoxic effects of *Z. spina-christi* bark crude extract on human cell lines. It is commonly grown in desert areas with deficient rainfall, especially in the middle-east [3]. *Z. spina-christi* possesses significant amounts of diverse phytochemicals, most importantly flavonoids, saponins, tannins and triterpenes [4]. The bark of *Z. spina-christi* has potent biological activities such as antinociceptive [5], antidiarrheal [6] and antimicrobial [7] activities. The preliminary studies on the leaves of *Z. spina-christi* showed the potential cytotoxic effects against HeLa and MDA-MB-468 tumor cells [8]. We have reported the cytotoxic effects of the *Z. spina-christi* stem bark extract *in vitro* [9]. Phytochemical analysis of stem bark of *Z. spina-christi* indicates the presence of tannins, flavonoids, terpenoids, saponin glycosides and alkaloids [9]. Moreover, *in vivo* studies in rats showed that *Z. spina-christi* could inhibit the early stage of colon carcinogenesis [10]. However, investigations on the cytotoxic activity of *Z. spina-christi* were limited. Therefore, it is of interest to document the *in vitro* cytotoxicity analysis of *Zizyphus spina-christi* stem bark extract on human cancer cell lines.

**Materials and methods:****Chemicals:**

Ethanol, diethyl ether, chloroform, ethyl acetate, butanol, dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum, Dulbecco's Modified Eagle Medium (DMEM), HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)) buffer solution, L-glutamine, gentamycin, trypsin-EDTA were purchased from Lonza, Basel, Switzerland. All other chemicals and solvents used in this study were of analytical grade.

**Fractionation of *Z. spina-christi* bark extract:**

Fresh bark of the *Z. spina-christi* was collected from the Hail region, Saudi Arabia. The plant was identified by Dr. Sherif Sayed Sharawy and the specimen was deposited in the Herbarium, Department of Biology, College of Sciences, University of Ha'il. The air-dried bark of *Z. spina-christi* was repeatedly extracted (3 times) at room temperature with absolute ethanol by percolation till complete exhaustion. The ethanol extract was filtered and solvents were evaporated under reduced pressure. The ethanol extract was then fractionated by successive extraction with the solvents of increasing polarity viz. diethyl ether, chloroform, ethyl acetate and n-butanol.

The fractions were filtrated, and solvents were evaporated under reduced pressure.

**Mammalian cell lines:**

CACO-2 cells (human intestinal carcinoma ATCC® HTB-37™), HCT-116 cells (human colon carcinoma ATCC® CCL-247™), PC-3 cells (human prostate carcinoma ATCC® CRL-1435™), HeLa cells (human cervical carcinoma ATCC® CCL-2™), A-549 cells (lung carcinoma ATCC® CCL-185™), HepG-2 (hepatocellular carcinoma ATCC® HB-8065™), MCF-7 (breast cancer cell line ATCC® HTB-22™) and HEp-2 cells (cervical carcinoma ATCC® CCL-23™) were purchased from American Type Culture Collection (ATCC), USA.

**Cell line propagation:**

The cells were propagated in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin. All cells will be maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> and were sub-cultured two times a week.

**Cytotoxicity evaluation using viability assay:**

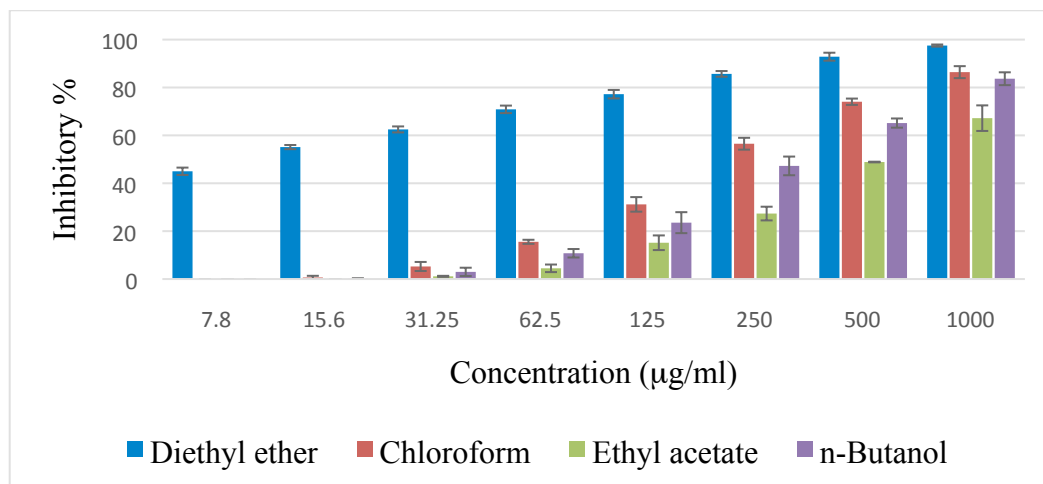
For cytotoxicity assay, the cells were incubated with different concentrations of the fractions of *Z. spina-christi* bark extract for 48 h. After the incubation period, the viable cells yield will be determined by the colorimetric method [11]. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the *Z. spina-christi* fractions. All experiments were carried out in triplicate. The cell cytotoxic effects of *Z. spina-christi* fractions were calculated. The optical density was measured (SunRise microplate reader, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as  $[(OD_t/OD_c)] \times 100\%$  where OD<sub>t</sub> is the mean optical density of treated wells (that was treated with *Z. spina-christi* fractions) and OD<sub>c</sub> is the mean optical density of untreated control wells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified *Z. spina-christi* fraction.

**The half-maximal inhibitory concentration (IC<sub>50</sub>):**

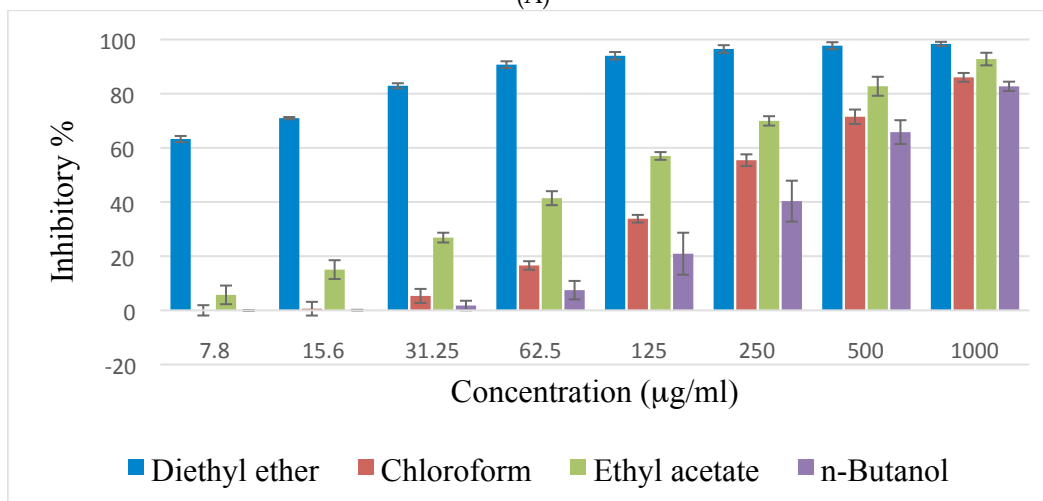
The concentration of *Z. spina-christi* fractions required to cause cytotoxic effects in 50% of cancer cells (LD<sub>50</sub>) will be estimated from graphic plots of the dose-response curve for each concentration using Graphpad Prism software (San Diego, CA, USA).

**Statistical analysis:**

The obtained results were analyzed using SPSS, IBM, Armonk, New York, United States to obtain the standard deviation (SD). The results were expressed as mean ± SD.



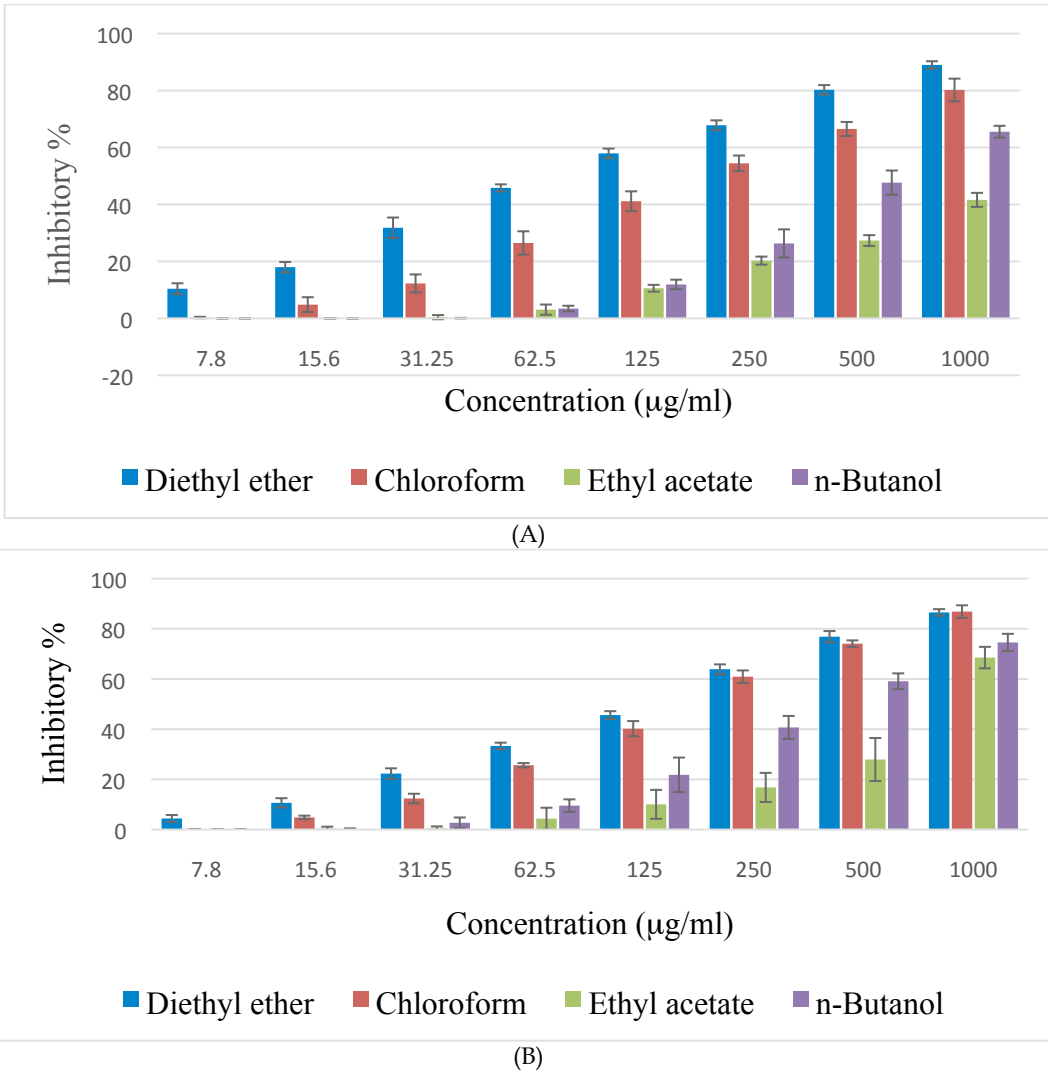
(A)



(B)

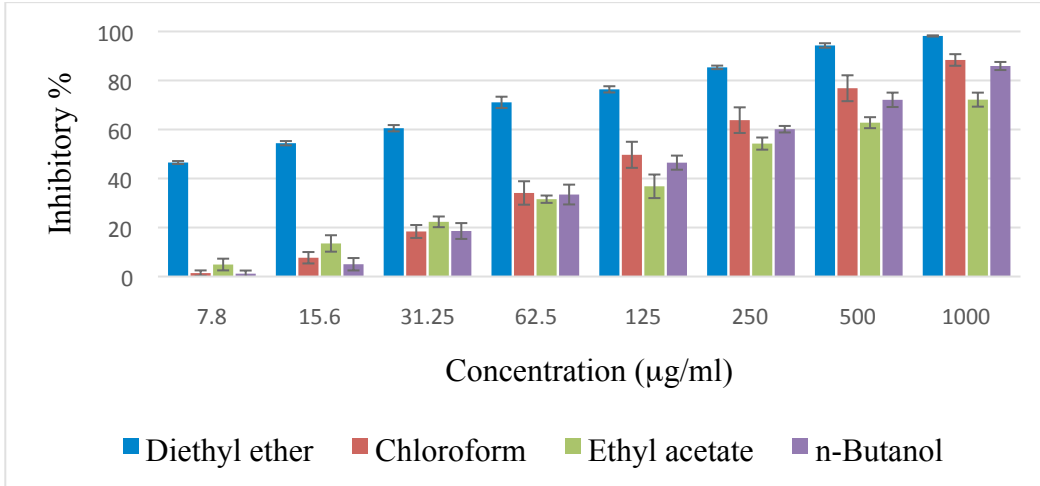
**Figure 1:** Cytotoxic effects of various fractions of *Z. spina-christi* bark against human colon carcinoma cell lines (A) CACO-2 and (B)HCT-116.

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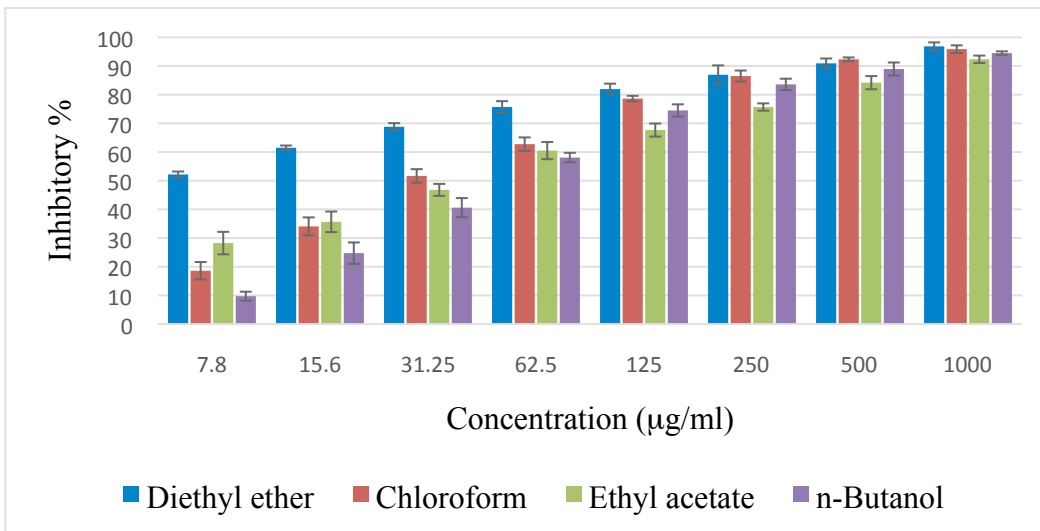


**Figure 2:** Cytotoxic effects of various fractions of *Z. spina-christi* bark extract against human cervical carcinoma cell lines (A) HeLa and (B) HEP-2.

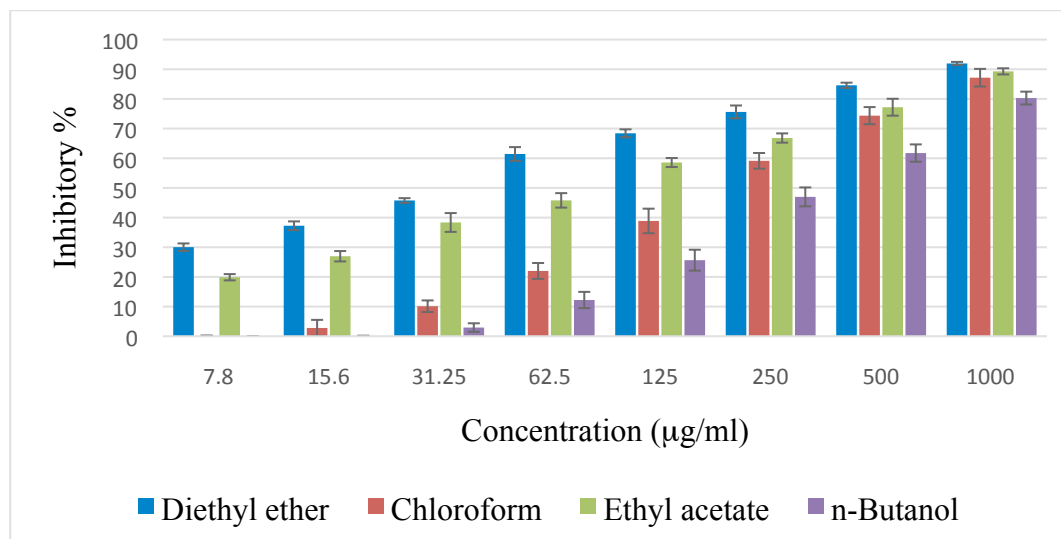
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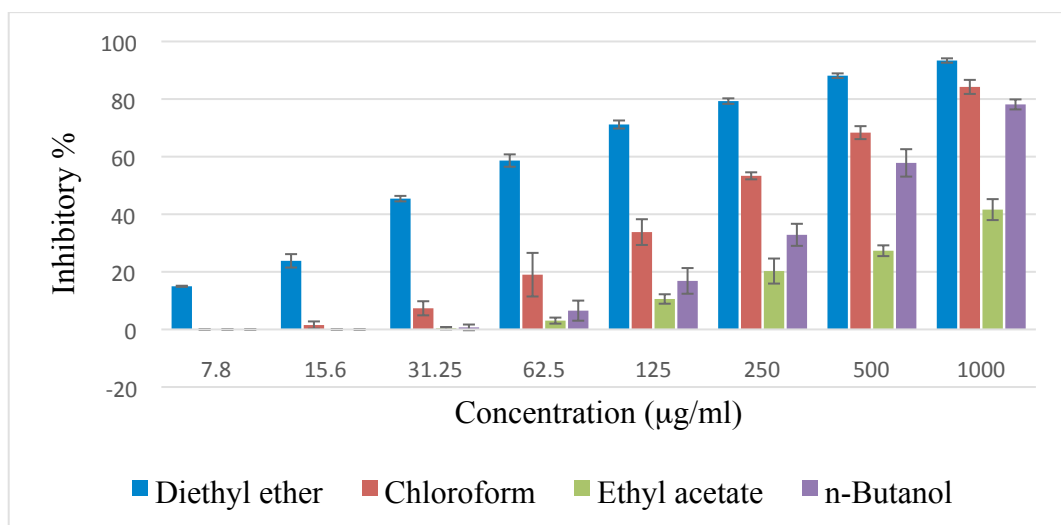
**Figure 3:** Cytotoxic effects of various fractions of *Z. spina-christi* bark extract against human lung cancer cell lines (A-549).



**Figure 4:** Cytotoxic effects of various fractions of *Z. spina-christi* bark extract against human hepatocarcinoma cell lines (HepG-2).



**Figure 5:** Cytotoxic effects of various fractions of *Z. spina-christi* bark extract against human hepatocarcinoma cell lines (HepG-2).



**Figure 6:** Cytotoxic effects of various fractions of *Z. spina-christi* bark extract against human prostate carcinoma cell lines (PC-3).

## Results:

### Cytotoxicity of *Z. spina-christi* against colon cancer cell lines (CACO-2 and HCT-116):

The control cells showed high proliferation that has been taken as 100% viability (0% cytotoxicity). As shown in **Figure 1**, the diethyl ether fraction of *Z. spina-christi* extract showed the highest dose-dependent (7.8 - 1000 mg/ml) cytotoxic effect against CACO-2 and

HCT-116 cell lines. This fraction showed more than 90% inhibitory activity against HCT-116 from the concentration of 62.5 mg/ml. Other fractions showed considerable effect only at concentrations more than 31.25 mg/ml. The second-highest cytotoxic activity was shown by the chloroform extract against CACO-1, and by the ethyl acetate fraction against HCT-116.

### Cytotoxicity of *Z. spina-christi* against cervical cancer cell lines (HeLa and HEP-2):

Diethyl ether fraction of *Z. spina-christi* extract showed the highest cytotoxic effects against the cervical cancer cell lines at all the tested concentrations in a dose-dependent manner (7.8 - 1000 mg/ml) (Figure 2). Other fractions also showed considerable effects at higher concentrations. The chloroform fraction showed the second-highest cytotoxicity effect against HeLa and HEP-2. In addition, chloroform fraction exhibited a comparable effect as that of diethyl ether fraction against HEP-2 at higher concentrations.

### Cytotoxicity of *Z. spina-christi* against lung cancer cell lines (A-549):

As shown in Figure 3, the diethyl ether fraction of *Z. spina-christi* extract showed a potent cytotoxic effect against the A-549 cell line even at low concentration (>40% of inhibitory activity at 7.8 mg/ml). All the fractions showed considerable cytotoxic activity at higher concentrations. Among the four fractions, chloroform fraction showed the second-highest activity, especially from the concentrations >62.5 mg/ml.

### Cytotoxicity of *Z. spina-christi* against hepatocarcinoma cell lines (HepG-2):

The diethyl ether fraction of *Z. spina-christi* extract showed a potent cytotoxic effect (>50% inhibition) against HepG-2 cell line from the concentration of 7.8 mg/ml (Figure 4). All the fractions showed a dose-dependent increase in cytotoxicity. Although the ethyl acetate fraction is effective than the chloroform fraction at low concentration (7.8 mg/ml), overall, the chloroform extract showed the second-highest cytotoxic effect.

### Cytotoxicity of *Z. spina-christi* against breast cancer cell lines (MCF-7):

The diethyl ether and ethyl acetate fractions of *Z. spina-christi* extract showed a cytotoxic effect against the MCF-7 cell line from

the concentration of 7.8 mg/ml. This fraction showed a dose-dependent increase in the cytotoxicity with about 80% of cytotoxic effect at 1000 mg/ml (Figure 5). The chloroform and butanol fractions showed cytotoxic effects from the concentration of 15.6 and 31.2 mg/ml, respectively.

### Cytotoxicity of *Z. spina-christi* against prostate cancer cell lines (PC-3):

The diethyl ether fraction of *Z. spina-christi* extract showed a potent cytotoxic effect against PC-3 cell line from the concentration of 7.8 mg/ml. This fraction showed a dose-dependent increase in the cytotoxicity with more than 90% of cytotoxic effect at 1000 mg/ml (Figure 6). The chloroform fraction showed considerable cytotoxic effect from the concentration of 15.6 mg/ml, whereas other fractions showed considerable effects only at concentrations of more than 62.5 mg/ml.

### IC50 of fractions of *Z. spina-christi* extract against various cell lines:

The IC50 values of diethyl ether fraction is lower than other fractions against all the tested cell lines (Table 1). The IC50 values of diethyl ether fraction are less than 10 mg/ml against HepG-2, less than 20 mg/ml against A-549, CACO-2 and HCT-116, less than 50 mg/ml against MCF-7 and PC-2, and more than 100 mg/ml against HEP-2. The diethyl ether fraction is highly active against HepG-2 (IC50=7.1 mg/ml) and least active against HEP-2 (IC50=153.8 mg/ml). The cytotoxic potency of diethyl ether fraction against various cell lines is descended in the following order: HepG-2 > A-549 > CACO-2 > HCT-116 > MCF-7 > PC-3 > HeLa > HEP-2. Ethyl acetate fraction showed cytotoxic effects with the IC50 value of less than 100 mg/ml against A-549, HepG-2, HCT-116, whereas chloroform and butanol fractions showed cytotoxic effects with the IC50 value less than 100 mg/ml only against HepG-2.

**Table 1.** Comparison of IC50 of various fractions of *Z. spina-christi* bark extract and vinblastine sulfate against various cell lines (CACO-2, HCT-116, HeLa, HEP-2, A-549, HepG-2, MCF-7 and PC-3)

Cell lines	Fractions of <i>Z. spina-christi</i> bark extract (mg/ml)				Vinblastine sulfate
	Diethyl ether	Chloroform	Ethyl acetate	n-Butanol	
CACO-2	11.6 ± 0.61	217.9 ± 5.50	532.4 ± 7.32	288.4 ± 43.32	50.3 ± 9.10
HCT-116	15.4 ± 0.27	143.4 ± 18.01	97.9 ± 8.64	344.9 ± 23.73	2.0 ± 0.36
HeLa	84.2 ± 7.32	208.2 ± 22.29	> 1000	565.4 ± 88.80	6.1 ± 0.92
HEP-2	153.8 ± 5.04	184.0 ± 14.81	765.9 ± 62.39	376.4 ± 44.8	10.2 ± 1.45
A-549	11.3 ± 0.73	127.9 ± 36.70	22.07 ± 9.20	157.3 ± 18.70	2.84 ± 0.32
HepG-2	7.1 ± 0.35	29.7 ± 1.66	47.4 ± 2.48	48.0 ± 2.35	3.68 ± 0.17
MCF-7	39.8 ± 1.96	193.6 ± 16.37	82.1 ± 9.8	301.0 ± 42.4	3.78 ± 0.54
PC-3	42.2 ± 3.12	228.6 ± 8.66	565.8 ± 48.15	421.6 ± 37.54	34.5 ± 7.42

**Discussion:**

Identification of new chemical entities through the biological activity-guided fractionation of plant materials is a novel approach to integrated drug discovery [12]. The phytochemical screening of ethanolic extract of *Z. spina-christi* bark showed the presence of tannins, flavonoids, terpenoids, saponin glycosides and alkaloids in the stem bark [9]. Because of the existence of bioactive compounds in the ethanolic extract, a preliminary study was conducted to elucidate the cytotoxic effect of *Z. spina-christi* bark extract using two human cancer cell lines, HCT-116 and MCH-7 [9]. Once the extract is found to be biologically active, the next recommended step is to proceed with fractionation of the extract using solvents with different polarities [13]. To further understand the cytotoxic effect of *Z. spina-christi* bark, four fractions were isolated from the ethanolic extract of *Z. spina-christi* bark based on polarity and tested for their cytotoxic effects against eight different human cancer cell lines.

Diethyl ether fraction of *Z. spina-christi* bark extract exhibited remarkable cytotoxic activity against the cell lines (with values of IC<sub>50</sub> 7.1, 11.3, 11.6, 15.4, 39.8, 42.2, 84.2 and 153.8 µg/ml against HepG-2, A-549, CACO-2, HCT-116, MCF-7, PC-3, HeLa, and HEp-2 cell lines, respectively). The fraction with low polarity (diethyl ether fraction) showed the highest cytotoxic effects than the fractions with high polarity (chloroform, ethyl acetate and butanol fractions). Comparing the IC<sub>50</sub> of all the fractions against cancer cell lines revealed that diethyl ether extract has multi-fold potent cytotoxic activity compared to the second-highly active fraction (1 fold to 18 fold). The lower IC<sub>50</sub> values represent the greater cytotoxic activity, whereas the higher the IC<sub>50</sub> values represent the lower cytotoxic activity. It was reported that tradecene, hexadecanoic acid, ethyl ester (ethyl palmitate) and ethyl linoleate are found only in diethyl ether fraction, not in other fractions of *Z. spina-christi* bark extract [14]. One or a few of the unique bioactive compounds present in the diethyl ether fraction may be responsible for their potent cytotoxic effects compared to other fractions.

Hexadecanoic acid (palmitic acid) has a potent cytotoxic effect against human leukemic cells *in vitro* and antitumor activity *in vivo*. It is reported that hexadecanoic acid exhibited the cytotoxic effect by selectively inhibiting DNA topoisomerase I in the tumor cells [15]. Docking study confirmed the interaction between hexadecanoic acid and DNA topoisomerase I. Thus, the presence of hexadecanoic acid and ethyl ester (ethyl palmitate) in the diethyl ether fraction may be responsible for preventing cell proliferation. Chloroform fraction of *Z. spina-christi* bark extract exhibited the second-highest cytotoxic activity (after diethyl ether fraction) against the five cell lines (with the IC<sub>50</sub> of 217.9, 208.2, 184.0, 29.7

and 228.2 µg/ml against CACO-2, HeLa, HEp-2, HepG-2 and PC-3, respectively). Betulin (a triterpene) was present in chloroform extract in large quantities as compared with other fractions. Betulin has shown anticancer and chemopreventive potentials against several established cancer cell lines, as well as primary tumor cell cultures *in vitro* and *in vivo* [16]. Betulin treatment can induce the expression of some cellular proteins indirectly involved in apoptosis. Betulin has the potential to selectively inhibit the activity of DNA topoisomerases II, without any influence on DNA topoisomerases I. Betulinic acid, a derivative of betulin, is a well-known inhibitor of other cancerous tumors, including human colon carcinoma and human prostate adenocarcinoma [17]. Either ethyl acetate fraction (against CACO-2, HeLa, HEp-2 and PC-3 cell lines) or butanol fraction (against HCT-116, A-549, HepG-2 and MCF-7 cell lines) showed the least cytotoxic activity among the four fractions of *Z. spina-christi* bark extract. On the other hand, ethyl acetate fraction showed a considerable cytotoxic effect against HCT-116, A-549 and MCF-7 than the chloroform and butanol fractions (but less effective than diethyl ether fraction). This selectivity of ethyl acetate fraction should be considered for further studies. When comparing the effect of highly-active diethyl ether fraction on various human cancer cell lines, this fraction showed potent cytotoxic activity against intestinal and colon (CACO-2 and HCT-116), lung (A-549) and liver (HepG-2) cancer cell lines. Interestingly, diethyl ether fraction showed a better cytotoxic effect against CACO-2 than the standard drug, vinblastine sulfate. As colon cancer is the most common form of fatal cancer worldwide, further isolation of bioactive from diethyl ether fraction is warranted for potential drug development.

**Conclusions:**

The fractions of ethanolic extract of *Z. spina-christi* bark showed cytotoxic effects on various human cancer cell lines (colon, cervical, lung, liver, breast and prostate cancer cell lines). The cytotoxic effects of fractions against CACO-2, HeLa, HEp-2, HepG-2 and PC-3 are in the order of diethyl ether >> chloroform > butanol > ethyl acetate. The cytotoxic effect of fractions against HCT-116, A-549 and MCF-7 are in the order of diethyl ether >> ethyl acetate > chloroform > butanol. The diethyl ether fraction of ethanolic extract of *Z. spina-christi* showed the highest activity compared to other highly-polar fractions (chloroform, ethyl acetate, and n-butanol fractions) for further consideration in cancer drug discovery.

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**Conflict of interest:**

The authors declare that there is no conflict of interest regarding the publication of this paper.

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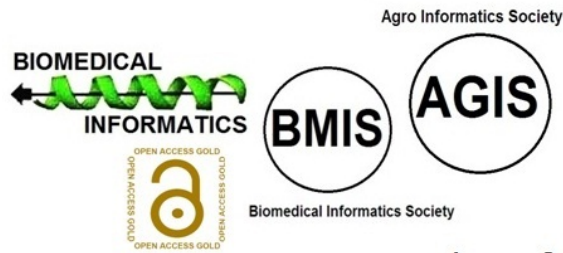
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