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Evaluation of toxicity of Bilsaan stem bark extracts in Swiss Albino mice

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ABSTRACT

Objective: *Sambucus nigra* commonly known as Bilsaan is used extensively in traditional medicine in Saudi Arabia to treat various types of illness. The establishment of safe dose of *S. nigra* is crucial while considering health-promoting benefits as exposure to excess amount may cause undesirable effects. The aim of the present study was to evaluate the acute oral toxicity of ethanolic (Eth) and methanolic (Meth) extracts from the bark of Bilsaan (Bil) in mice.

Materials and Methods: The acute oral toxicity study of Eth-Bil and Meth-Bil extracts was carried out by the administration of 10, 100, and 1000 mg/kg body weight to mice in the 1st phase. According to the observation of the 1st phase, the Eth-Bil extract was given 50, 100, 200, and 400 while Meth-Bil was administered 200, 400, 800, and 1600 mg/kg body weight.

Results: The LD_{50} of the *S. nigra* (Bilsaan) methanol and ethanol extracts in mice was determined to be 31.62 mg/kg body weight for both of the extracts as calculated by Lorke's method. The gross observation of systemic organs demonstrated 10–90% increase in the weight of organs of animals treated with increasing concentration of extracts in comparison to control. Similarly, the hematological analyses also showed abnormality and reduction in total leukocytes count exposed with the higher concentration of extracts.

Conclusions: The results indicate that the oral administration of Eth-Bil and Meth-Bil extracts produce a significant toxic effect in mice but support in the establishment of safe dose range, as the intake of high doses of *S. nigra* (Bilsaan) extracts may exhibit mild organ toxicity.

Keywords: Bilsaan, Acute toxicity, Extracts

Introduction

It is evident from several epidemiologic studies that fruits, vegetables, whole grains, seeds, and herbs contained within the diets may decrease the risk or delay the progression of certain ailments such as cancer, cardiovascular disease, and diabetes.[1] The idea of using natural foods to minimize the risk of various types of cancer dates back many decades.[2] It is believed that 33% of total mortality occurred due to cancer could be prevented by including the high amounts of natural foods in the diet.[3-6] Remarkably, nearly 50% of the drugs which made available in the market in the past 30 years were either derived directly from plants or chemically modified.[7]

The plant *Sumbucus nigra* called Elder or Bilsaan is one of the oldest traditional plants in Saudi Arabia that is consumed as medicine for the treatment of several diseases. It is grown in Makkah and commonly known as Bilsaan, Bishaam and Balsam Makkah in the Arab world. As mentioned in the Canon of Medicine, Bilsaan has anti-inflammatory and wound healing properties, dispels the clogs, congestion/phlegm in the chest. Besides, it can cure Sciatica, Epilepsy, headaches, and uterine associated illnesses improves the digestion and strengthens the liver as well.[8] Earlier, several studies were conducted on *S. nigra* that is found in Europe, has been shown to increase the tumor necrosis factor -α, Interleukin (IL)-1β, IL-6, and IL-8 in blood-derived monocytes.[9] Evidently, it has been reported to have antioxidant, anticonvulsant, antiviral, and antidiabetic potential as well.[10-15] However, no study has been done so far on *S. nigra* or Bilsaan which is found in Makkah region of Saudi Arabia in spite of health benefit effects and broad market of bilsaan oil.

Keeping the facts including this particular lack of research evidence into consideration, the study of bilsaan to explore as the natural drug may be the promising strategy as one of the major disadvantages of the chemically synthesized drug is toxicity to normal cells. However, the toxicity of dietary

constituents are not properly evaluated as there is general misconception that herbal medicines are devoid of adverse or toxic side effects. Therefore, before evaluating health-related benefits, the toxicity of extracts of bilsaan should be explored appropriately as of any synthetic drug.[16,17] Noticeably, no study has been done so far associated with the toxicity of *S. nigra*. The present study was aimed to evaluate the safety level of bilsaan extracts with acute toxicity assay in Swiss albino mice.

Materials and Methods

Tree materials

The stem barks of *S. nigra* were collected from local sources in Medina, Saudi Arabia. The dried stem barks of bilsaan tree were crushed into coarse powder using a homogenizer followed by kitchen blender machine and stored in airtight bottles.

Preparation of bilsaan crude extracts

The powdered stem barks for each extract were defatted using cyclohexane by mixing 1:3 ration followed by stirring for 3 h. The defatted powdered was dried completely and placed in thimber cups for extraction by Soxhlet hot extraction method separately. Finally, the extracts were evaporated under vacuum in rotary evaporator to remove the traces of methanol and ethanol.

Experimental animals

The study was conducted in forty-eight inbred male and female swiss albino mice in 1:1 ratio. The animals of both sexes were kept individually in autoclavable polypropylene cages (three mice per cage) throughout the acclimatization as well as experimental period. The ethical guidelines of Qassim University to handle the animals were followed throughout the study.

Acute toxicity study

The acute toxicity study was conducted in according to the standard method established by Lorke using twenty-seven animals for each extract in two phases (12 in first and 15 in second phase).[18]

In the first phase, twelve mice for each extract were divided into four groups of three mice each for the doses of 0, 10, 100, and 1000 mg/kg body weight (b.w). Groups 2, 3, and 4 animals were given 10, 100, and 1000 mg/kg body weight (bw.) In the second phase, additional precise doses of 0, 50, 100, 200, and 400 mg/kg bw for ethanolic (Eth)- Bilsaan (Bil) and 0, 200, 400, 800, and 1600 mg/kg b.w for methanolic (Meth)-Bil extracts were administered to three mice per dose group to further determine the correct LD_{50} value. All the animals included in the study were observed regularly from the day of the exposure for 2 weeks. At the end of 14 days, all surviving mice were sacrificed and then autopsy and histopathological studies were conducted after processing the liver tissues.

The LD_{50} was calculated by the formula:

$$LD_{50} = \sqrt{D_0 \times D_{100}}$$

D_0 = Highest dose that gave no mortality
D_{100} = Lowest dose that produced mortality.

Organs and body weight

All the vital organs such as heart, kidneys, liver, lung, and spleen were isolated, and all of the individual organs were weighed and compared between both treated and control groups.

Hematological analyses

In the present study, the mice blood was evaluated for total leukocyte count (TLC) and differential leukocyte count.

Histological examination

The liver tissues were fixed in 10% buffered formalin in labeled bottles and processed for histological examination. Tissues embedded in paraffin wax were sectioned 5 mm

Table 1: General appearance and behavioral observations of acute toxicity study for control and treated groups of Meth-Bil 1st phase. Animals

Dose (mg/kg b.w) / Observations	0	10	100	1000
Body weight	Normal	Decrease	Decrease	Slight decrease
Food intake	Normal	Normal	Normal	Normal
Urination	Normal	Normal	Normal	Normal
Eye color	Normal	Normal	One closed	Normal
General physique	Normal	Normal	Normal	Normal
Change in skin	Not effect	Hair loss	Not effect	Injury
Death	Alive	Alive	One dead	Two dead

Table 2: General appearance and behavioral observations of acute toxicity study for control and treated groups of Eth-Bil 1st phase

Doses (mg/kg b.w) / Observations	Control	10	100	1000
Body weight	Normal	Normal	Decrease	-
Food intake	Normal	Normal	Normal	-
Urination	Normal	Normal	Normal	-
Eye color	Normal	Normal	Abnormality	-
General physique	Normal	Normal	Normal	-
Change in skin	Not effect	Not effect	Not effect	-
Death	Alive	Alive	One dead	All dead

thick, stained with hematoxylin and eosin, mounted on glass slides and examined under a standard light microscope.

Flow-cytometric analysis of cell cycle

The blood samples were treated with ice-cold red blood cell lysed buffer for 10 min with gentle rocking followed by centrifugation at 250× g for 10 min. Then, the cells were re-suspended in sample buffer followed by the addition of ribonuclease (100 mg/ml) and incubated at 37°C for 30 min. Cells were centrifuged at 300× g and resuspended in 1 ml of sample buffer containing 50 mg/ml propidium iodide and further incubated for 40 min at 4°C. The cells were centrifuged, suspended in 500 ml of sample buffer and then analyzed (excluding debris) using a MacsQuant flow cytometer (Miltenyi Biotec).

Table 3: Acute toxicity of Meth-Bil and Eth-Bil

Extracts	Phase 1		Phase 2	
	Dose (mg/kg)	Mortality	Dose (mg/kg)	Mortality
Methanol extract	0	0/3	0	0/3
	10	0/3	200	0/3
	100	1/3	400	2/3
	1000	2/3	800	3/3
			1600	3/3
Ethanol extract	0	0/3	0	0/3
	10	0/3	50	1/3
	100	1/3	100	1/3
	1000	3/3	200	0/3
			400	2/3

Statistical analysis

Statistical analysis was performed as a mean of variance ± SEM ($n = 5$) followed by analysis of variance test using Sigma Stat, for multiple comparison test among the groups. A probability level of $P < 0.05$ was accepted statistically.

Results

Acute toxicity study and determination of LD_{50}

The observational data on the appearance and the general behavioral pattern of mice from the 1st phase study are shown in Tables 1 and 2. It exhibited reduction in the weight, injury and 66.66% mortality in the animals exposed with 100 mg/kg b.w. of Meth-Bil, while it was 33% mortality and reduction in weight with 1000 mg/kg b.w. Remarkably, all the animals died within 2 days after the exposure with 1000 mg/kg b.w of Eth-Bil while one died along with some abnormality in the eyes in 100 mg/kg b.w [Table 3].

According to the observational study and mortality rate, the doses for phase two were modified as 200, 400, 800, and 1600 mg/kg b.w for Meth-Bil while 50, 100, 200, and 400 mg/kg b.w for Eth-Bil. It was shown a decrease in body weight and lethargy followed by death in 1600 and 800 mg/kg b.w of Meth-Bil and abnormality in eyes color followed by 66% mortality in 400 mg/kg as well [Tables 3 and 4]. As shown in Tables 3 and 5, it was observed that 66% of animals died in 400 and 33% in 100 mg/kg b.w of Eth-Bil.

The mortality in various treated groups suggested the LD_{50} using the formula given above to be 140 mg/kg b.w for Meth-Bil as well as Eth-Bil both [Figure 1].

Table 4: General appearance and behavioral observations of acute toxicity study for control and treated groups of Meth-Bil 2nd phase

Doses (mg/kg b.w Observations	0	200	400	800	1600
Body weight	Normal	Increase	Increase	Increase	Decrease
Food intake	Normal	Normal	Normal	Normal	-
Urination	Normal	Normal	Normal	Normal	-
Eye color	Normal	Normal	Abnormality	Normal	-
General physique	Normal	Normal	Normal	-	Lethargy
Change in skin	Not effect	Not effect	Swelling	-	-
Death	Alive	Alive	Two dead	All dead	All dead

Table 5: General appearance and behavioral observations of acute toxicity study for control and treated groups of Eth-Bil 2nd phase

Observations	Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Body weight	Normal	Decrease	Normal	Normal	Normal
Food intake	Normal	Normal	Normal	Normal	Normal
Urination	Normal	Normal	Normal	Normal	Normal
Eye color	Normal	Dry eye	Normal	Normal	Normal
General physique	Normal	Normal	Normal	Normal	Normal
Change in skin	Not effect	Not effect	Not effect	Not effect	Not effect
Death	Alive	One dead	One dead	Alive	Two dead

Effect of bilsaan extracts on relative organ body weight

The gross observation of systemic organs demonstrated the 10–90% increase in the weight of organs of animals treated with various concentration of extracts in comparison to control [Table 6].

Effect of bilsaan extracts on hematological parameters

The hematological data showed a decrease in 3 and 4.5 million TLC in the animals exposed with 200 and 400 mg/kg b.w, respectively. The decrease in TLC was observed consistently as the mean was recorded 8×10^6, 6.7×10^6, 5.9×10^6, and 4.2×10^6 in the animals treated with 50, 100, 200, and 400 mg/kg b.w [Table 7] correspondingly. The ruptured cells were observed in the animals exposed with 200 and 400 mg/kg b.w. of Meth-Bil [Figure 2]. As depicted in Figure 3, ruptured cells as well as degranulation were documented in the animals exposed with 50, 100, and 200 mg/kg b.w of Eth-Bil. The vacuolization was also seen in the animals exposed with 100 mg/kg b.w [Figure 3b].

Effect of bilsaan extracts on liver

The histological analysis of liver tissues showed significant changes with a high concentration of extracts. As depicted in Figure 4b, it contains dilated portal triad with a large number of erythrocytes, with mild fatty degeneration in the animals treated with 200 mg/kg b.w Meth-Bil. The maximum dilation of the portal triad and large number of erythrocytes, with perivascular, is seen in the animals exposed with 400 mg/kg b.w Meth-Bil. The severe fatty degeneration and sinusoidal dilation were also reported [Figure 4c]. The liver section of the animals exposed with 50, 100, and 200 mg/kg b.w Eth-Bil showed dilation of the portal triad, central vein, and congested area, with perivascular leukocytes [Figure 5b-d]. The severe fatty degeneration and sinusoidal dilation in the animals treated with 50 mg/kg b.w. are shown in Figure 5b.

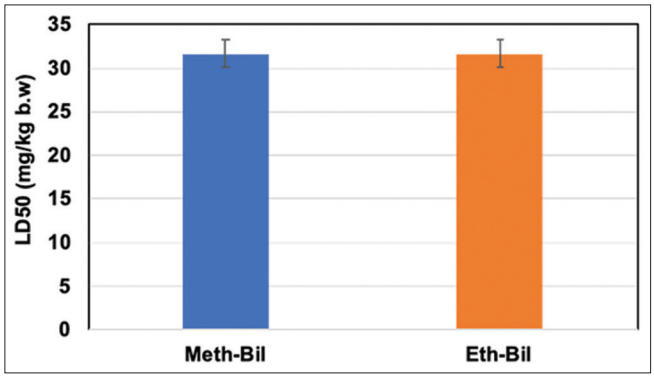

Figure 1: LD_{50} of Meth-Bil and Eth-Bil after the observational study of both phases

Effect of bilsaan extracts on cell cycle

As shown in cell cycle analysis using flow cytometry 71.33% cells in G1, 12.33% in S and 16.34 % in G2/M phase were analyzed in control mice [Figures 6a and 7a]. In the animals exposed with 200 mg/kg b.w Meth-Bil, more than 25% shifted to G1 phase as 10.5% and 15% from S and G2/M phase, respectively [Figure 6b]. Remarkably, this shift extended to another peak of 8.66% SubG1 phase that is a clear sign of toxicity along with 85.17%, 2.16, and 4.01 of G1, S, and G2/M, respectively, in the animals exposed with 400 mg/kg b.w of Meth-Bil [Figure 6c]. Similarly, the total cell shifted to G1 ~98% in the animals were given the dose of 100 and 200 mg/kg b.w of ethanol extracts [Figure 7c].

Discussion

In recent years, herbal products have been shown the widespread attention to fight against several diseases. As there is wrong perception for all the dietary constituents for not having any adverse or toxic effects, the toxicity of plant medicines are not properly evaluated. Therefore, the toxicity of extracts and dietary constituents should be explored appropriately as of any synthetic drug.[16,17] It plays a crucial role in the establishment of safe dose level of these herbal remedies if they are evaluated to have potential into pharmacological products. The present study was aimed to evaluate the methanolic (Meth) and ethanolic (Eth) extracts of bilsaan for toxicity study and to establish the safe dose range that could be used for further studies. Noticeably, it is the first *in vivo* study of bilsaan till date irrespective of extensive use of this ancient plant in certain ailments. As per the chemical labeling and classification of acute systemic

Table 6: Effect of exposure of Meth-Bil and Eth-Bil on average organ weight(g) of mice

Dose (mg/kg b.w)	Meth-Bil 0	Meth-Bil 200	Meth-Bil 400	Eth-Bil 0	Eth-Bil 50	Eth-Bil 100	Eth-Bil 200	Eth-Bil 400
Organs	Weight (in g)							
Heart	0.15±0.010	0.16±0.003	0.17±0.019	0.15±0.010	0.15±0.03	0.16±0.011	0.17±0.012	0.2±0.011
Liver	1.33±0.042	1.43±0.052	1.61±0.033	1.33±0.042	1.43±0.038	1.61±0.024	1.73±0.072	2.21±0.016
Kidneys	0.33±0.030	0.34±0.024	0.36±0.030	0.33±0.030	0.33±0.033	0.34±0.011	0.36±0.031	0.4±0.023
Spleen	0.18±0.027	0.2±0.031	0.24±0.019	0.18±0.027	0.18±0.02	0.2±0.035	0.24±0.02	0.35±0.031
Lungs	0.14±0.013	0.2±0.017	0.24±0.009	0.14±0.013	0.17±0.009	0.2±0.041	0.2±0.011	0.29±0.013

Values are expressed as mean ± SEM. $P > 0.05$ when compared to control group.

Table 7: Effect of exposure of Meth-Bil and Eth-Bil on total leukocyte counts (TLC) in the surviving animals of phase 2

Dose (mg/kg b.w)	TLC
Met-Bil	
0	9.1×10^6/ml
200	6×10^6/ml
400	4.5×10^6/ml
Eth-Bil	
0	9.1×10^6/ml
50	8×10^6/ml
100	6.7×10^6/ml
200	5.9×10^6/ml
400	4.2×10^6/ml

Values are expressed as mean ± SEM. $P > 0.05$ when compared to control group.

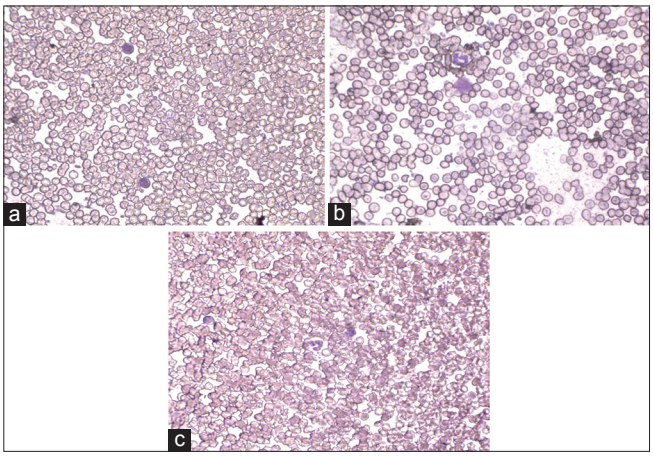

Figure 2: Effect of Meth-Bil exposure (a) Vehicle control (b) 200 (c) 400 mg/kg b.w surviving animals of phase 2 on the leukocytes qualitatively (Leishman stain, ×400)

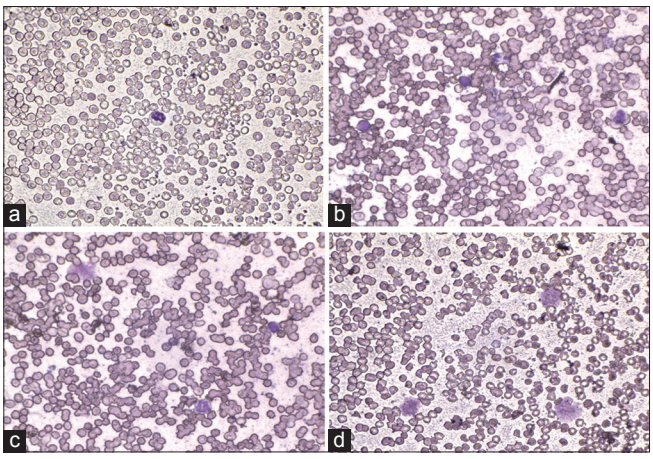

Figure 3: Effect Eth-Bil (a) vehicle control (b) 50 (c) 100 (d) 200 mg/kg b.w surviving animals of phase 2 on the leukocytes qualitatively (Leishman stain, ×400)

toxicity given by Organization for Economic Cooperation and Development (OECD), the calculated LD_{50} values (31.62 mg/kg b.w) for both Meth-Bil and Eth-Bil extracts

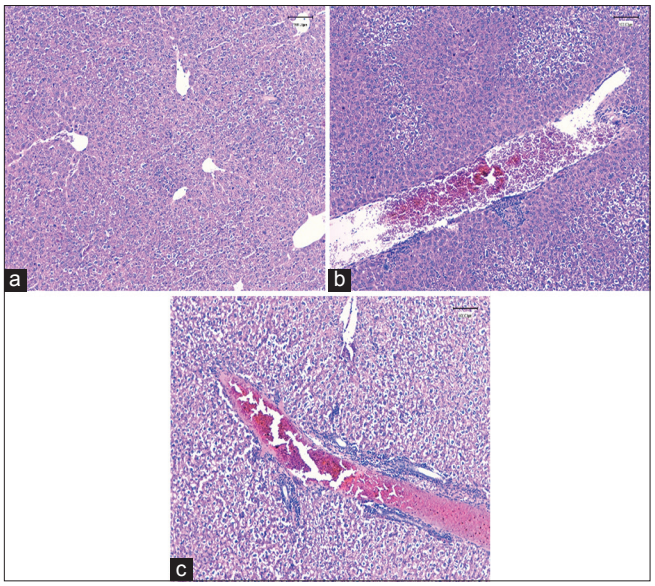

Figure 4: Effect of Meth-Bil exposure (a) vehicle control (b) 200 (c) 400 mg/kg b.w surviving animals of phase 2 on liver tissues (hematoxylin and eosin, ×100)

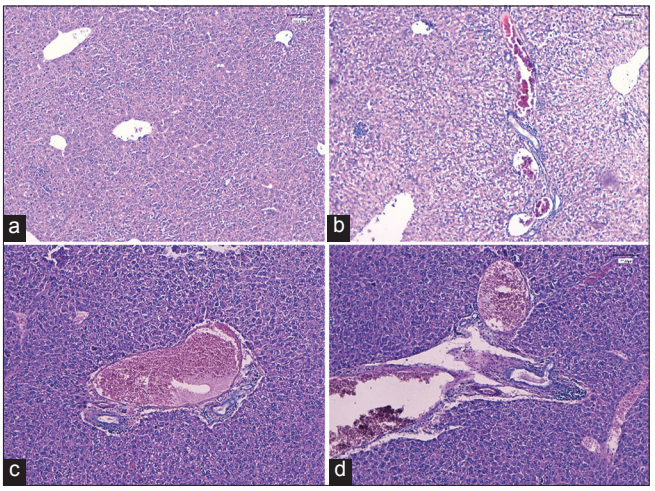

Figure 5: Effect Eth-Bil (a) vehicle control (b) 50 (c) 100 (d) 200 mg/kg b.w surviving animals of phase 2 on liver tissues (hematoxylin and eosin, ×100)

would be classified in category 3.[19] In the present study, cell cycle analysis was also performed to determine whether the observed toxicity is related to cell cycle arrest at any phase. The quantitative data of flow cytometry revealed that all the surviving animals exposed to toxic doses underwent significant cell cycle shift from S and G2/M to G1.

The data are very consistent with all the parameters included in the study. The bilsaan can be a promising agent in pharmaceutics, however sub-acute toxicity yet to be established using the present study as reference. Although shifting in the peaks of phase show toxicity, exact information regarding the variation in the phases can be confirmed by molecular pathway associated with phases and sub-phases of the cell cycle.

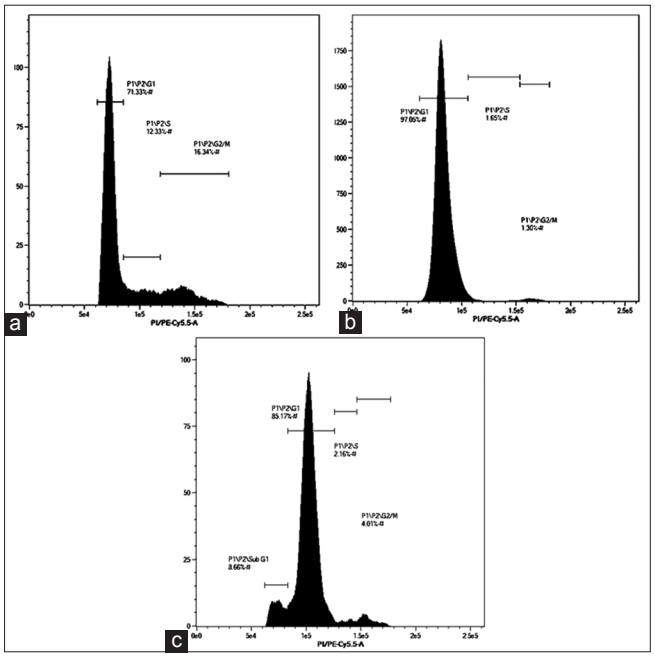

Figure 6: Effect of Meth-Bil exposure (a) vehicle control (b) 200 (c) 400 mg/kg b.w surviving animals of phase 2 on various phases of cell cycle

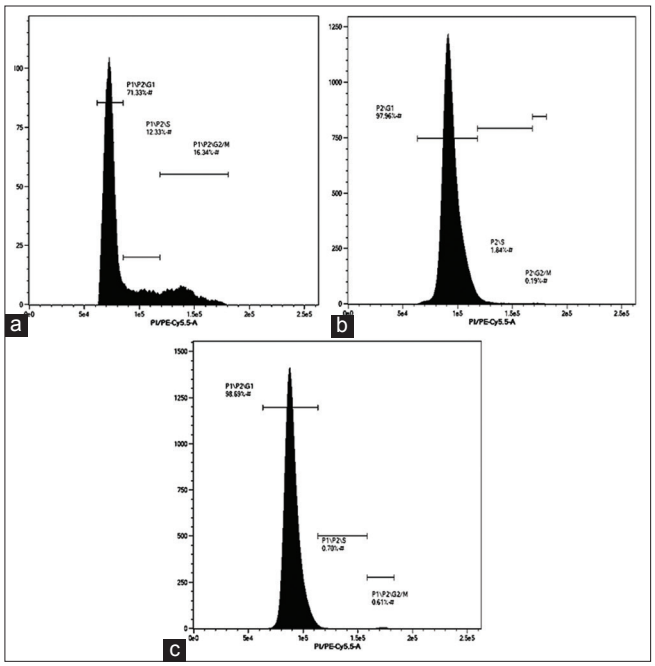

Figure 7: Effect of Eth-Bil exposure (a) vehicle control (b) 100 (c) 200 mg/kg b.w surviving animals of phase 2 on various phases of cell cycle

Conclusions

The present study provides significant information of bilsaan extracts associated with toxicity and safety dose level. It showed a certain level of toxicity within and above LD_{50} which is useful for any future *in vivo* and clinical study of this traditional medicine. However, further investigations including sub-acute toxicity are required to determine the safety level of doses on the animal fetus, pregnancy, and their reproductive capacity.

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