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Research Paper:

Evaluation of the Extracts From Rhizomes of *Polygonum bistorta* for the Median Lethal Dosages in Swiss Albino Mice

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ABSTRACT

Background: *Polygonum bistorta* has been used as a remedy for jaundice, smallpox, pimples, measles, cholera, diarrhoea, dysentery, expelling worms, insect stings and snakebites. In this study, the crude extract from *P. bistorta* and two fractions viz. hexane and chloroform obtained from the crude extract were studied for their median Lethal Dosages (LD_{50}) in Swiss albino mice.

Methods: Powdered rhizomes of *P. bistorta* was macerated with chloroform and the crude extract was dissolved in a solvent mixture of methanol/water (95:5). The mixture was then subjected to solvent-solvent partition, first with hexane followed by chloroform. The crude extract and the hexane and chloroform fractions were evaluated for their LD_{50} in Swiss albino mice of both sexes.

Results: The LD_{50} of the crude extract and the hexane and chloroform fractions were determined to be 142.82, 200 and 200.17mg per kg of the mice body weight, respectively.

Conclusion: The LD_{50} values of the crude extract and the hexane and chloroform fractions from *P. bistorta* were determined. The crude extract of *P. bistorta* had greater lethality than the hexane and chloroform fractions. This is the first report on the LD_{50} values of Swiss albino mice for *P. bistorta*.

Keywords: *Polygonaceae,* Mortality, Mice, Toxicity, Rhizome extracts, Polygonum bistorta

Introduction

Known by other names, such as Bistort and Snakeroot, *Polygonum bistorta* (*P. bistorta*) belongs to the Polygonaceae family [1-3]. This plant has been used in traditional Indian, Chinese and Japanese medicine as a remedy for a number of conditions, such as measles, jaundice, smallpox, pimples, cholera, dysentery, worms, insect stings and snakebites [3]. It has also been used as an astringent [1, 2] and applied topically to wounds to stop bleeding. The roots and leaves of *P. bistorta* have been used as food ingredients in Europe and America [4, 5]. The anticancer [2], anti-inflammatory [6], antibacterial [7, 8], antifungal [9] and antioxidant activities [9, 10] of the *P. bistorta* extracts have previously been reported. However, to the best of our knowledge, the median lethal dosage (LD_{50}) of *P. bistorta* has not been reported previously. The objective of this study was to determine the LD_{50} of the crude extract and the hexane and chloroform fractions of *P. bistorta* in Swiss albino mice. The

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results are reported in this article. This is the first report on LD_{50} values of *P. bistorta* extracts.

Materials and Methods

Plant materials: Twelve grams of dried *P. bistorta* was obtained from a local market in Singapore. A voucher specimen viz. KMano PB2003 was issued at the Herbarium, Department of Biological Sciences, National University of Singapore (NUS).

Extraction procedures: The powdered plant materials were macerated with chloroform. Approximately, 250g of the brown residue from the chloroform crude extract was obtained after the removal of the solvent under vacuum. Approximately, 50g of the crude extract was kept separately for the MTT cytotoxicity assay and lethal dosage (LD_{50}) analyses. The remaining crude extract (200g) was dissolved in water/methanol (95:5 v/v) solvent mixture. The mixture was subjected to solvent-solvent partition, first with hexane and then with chloroform. Approximately, 117 and 82 grams of the brown residues of hexane and chloroform fractions, respectively, were obtained after the removal of the solvents under vacuum. For the remaining methanol/water fraction, much of the solvent was removed under vacuum followed by freeze-drying to eliminate the solvent's remnants. One gram of the yellowish-brown residue of methanol/water fraction was obtained. The crude extract, and the hexane and chloroform fractions were evaluated for their LD_{50} values. However, we did not evaluate the LD_{50} of the methanol/water fraction due its low quantity.

Solvents: The analytical reagent grades of hexane, chloroform and methanol were obtained from Sigma-Aldrich (St. Louise, MS, USA). The analytical grade of Dimethyl Sulphoxide (DMSO) was purchased from Merck Chemicals GmbH (Darmstadt, Germany). Deionized water was used for solvent-solvent partition.

Animal grouping & experimental procedures: In this study, 16 Swiss albino mice of both sexes, each weighing about 30g, were used and divided into four groups of four mice each. They were kept in the laboratory to get accustomed to the experimental environment. The crude extract, and the hexane and chloroform fractions, respectively, were given to the first three groups of rats with the fourth group being the controls. Solutions of 150 and 200mg of the crude extract, and the hexane and chloroform fractions per kg body weight of the mice were prepared separately in 10mL of 100% DMSO. These solutions were injected Intraperitoneally (IP) into the mice in groups 1, 2 and 3. The mice in group 4 (controls) received only 10 mL of 100% DMSO. The mice in all groups received normal diet. The general behaviors of the mice were observed for two weeks as per previously described procedures [11, 12]. On day 15, the mice were sacrificed by cervical dislocation and were used for further analyses.

Determination of the median Lethal Dosage (LD_{50}): The dose that caused the death of 50% of the experimental mice was defined as LD_{50}. Substances with LD_{50} values ranging between 1 and 5,000mg/kg of the mice's body weight was considered as practically important. The LD_{50} values <1 and 5,000mg/kg were regarded as very toxic and practically not important in this study [13, 14]. The LD_{50} of the crude extract, and the hexane and chloroform fractions of *P. bistorta* were determined based on the previously described procedures [13-15]. Finney's method of transformation of percentage mortalities to probits and the plot of probits (in ordinate) versus log doses (in abscissa) were used to determine LD_{50} [16-18]. Probit is the quantile function associated with the standard normal distribution.

Results

Animal behavior over time: The observed behavioral changes of the mice after IP injection of 150mg of the crude extracts, and the hexane and chloroform fractions over the 15 days of experiment are summarized in Table 1. The result indicated that the LD_{50} for the crude extract was ~150mg/kg, and for both the hexane and chloroform fractions was >150mg/kg (Table 1).

Since, the LD_{50} for both the hexane and chloroform fractions was >50 mg/kg, we repeated the experiment in order to establish the exact LD_{50} accurately. However, this time the mice in Groups 1, 2 and 3 received 200mg of each of the extracts per kg of the body weight. The results are presented in Table 2, indicating that the LD_{50} for the crude extract and the hexane and chloroform fractions were <200, >200 and ~200mg/kg, respectively. Therefore, the LD_{50} for the crude extract was established more realistically at ~150mg/kg, while the values for the hexane and chloroform fractions remained unchanged (Tables 1 & 2).

Determination of LD_{50}: The injected and log doses, number of dead mice, percent mortalities for each of the doses, and the probits are summarised in Table 3. The percent mortalities for each dose were transformed to probits based on Finney's method [16-18] (Table 4). The standard errors for the LD_{50} values were obtained using the Equation 1 [17, 18]:

1: SE of $LD_{50} = (\text{Log } LD_{84} - \text{Log} LD_{16})/2N$.

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Table 1. The observed behaviours of mice injected with 150mg/kg of the assigned extract

Day	Group 1 (Crude Extract)	Group 2 (Hexane Extract)	Group 3 (Chloroform Extract)	Group 4 (Controls)
1	All mice were sedated with slow respiration and arrested locomotion.	All mice were sedated with slow respiration and arrested locomotion.	All mice were sedated with slow respiration and arrested locomotion.	No abnormal behaviours noted in the mice.
2	Two mice died. The living mice were inactive and dull. The locomotion improved slightly without gains in body weight.	All mice lived but were inactive and dull. The locomotion improved slightly without gains in the body weight.	One mouse died. The living mice were inactive and dull. The locomotion improved slightly without gains in the body weight.	No abnormal behaviours noted in the mice. The body weight increased slightly.
3	The mice gained weights and recovered with significant improvement in locomotion.	All mice gained weight and recovered with significant improvement in locomotion.	The mice gained weight and recovered with significant improvement in locomotion.	No abnormal behaviours. Body weight increased.
4	The mice recovered greatly and gained weight. The locomotion & behaviours were normal.	The mice recovered greatly and gained weight. The locomotion & behaviours were normal.	The mice recovered greatly and gained weight. The locomotion & behaviours were normal.	No abnormal behaviours noted. They gained body weight.
5-14	The mice recovered fully with progressive gains in the body weight. They walked normally.	The mice recovered fully with progressive gains in the body weight. They walked normally.	The mice recovered fully with progressive gains in the body weight. They walked normally.	No abnormal behaviours noted They walked normally.
15	The mice appeared normal and were sacrificed.	The mice appeared normal and were sacrificed.	The mice appeared normal and were sacrificed.	The normal mice were sacrificed.

The crude extract, the hexane or chloroform extract was administered IP to mice in 10mL 100% DMSO; The control group received 10 mL 100% DMSO; N= 4 mice per group.

Crude extract: The plot of log doses vs probits of the crude extract of *P. bistorta* is presented in Figure 1. The LD_{50} value of the crude extract was calculated by extrapolation from the graph of the linear regression (Figure 1). The partial responses were obtained from the two experimental doses [15]. Also, the dose corresponding to probit 5 was the LD_{50} value of the crude extract (Figure 1). The probits of LD84 and LD16 of the crude extract were found to be 5.99 and 4.01, respectively, which were rounded-off to 6 and 4 (Table 2). Based on the graph, the log doses for the probits 6 and 4 were found to be 2.38 and 1.93, respectively, with the antilogs being 239.55

Table 2. Observed behaviours of mice in 200mg/kg of the body weight

Day	Group 1 (Crude extract)	Group 2 (Hexane fraction)	Group 3 (Chloroform fraction)	Group 4 (Control)
1	All mice were sedated with slow respiration and arrested locomotion.	All mice were sedated with slow respiration and arrested locomotion.	All mice were sedated with slow respiration and arrested locomotion.	No abnormal behaviour noted.
2	Three mice died. The living mice were inactive and dull. The locomotion improved slightly without appreciable gains in body weight.	All mice lived but were less active and very dull. The locomotion improved slightly without appreciable gain in body weight.	Two mice died. The living mice were inactive and dull. The locomotion improved slightly without appreciable gains in body weight.	No abnormal behaviour noted but gained slight body weight.
3	The living mouse recovered and gained weight. Their locomotion improved significantly.	All mice recovered and gained weight. Their locomotion improved significantly.	The living mice recovered and gained weight. Their locomotion improved significantly.	No abnormal behaviour noted but gained slight body weight.
4	The mice gained weight and recovered significantly. Their locomotive appeared normal.	All mice gained weight and recovered significantly. Their locomotive appeared normal.	The mice gained weight and recovered significantly. Their locomotive appeared normal.	No abnormal behaviour noted but gained body weight.
5 to 14	The living mice recovered fully and the body weight gained gradually. The locomotion turned normal.	The living mice recovered fully and the body weight gained gradually. The locomotion turned normal.	The living mice recovered fully and the body weight gained gradually. The locomotion turned normal.	No abnormal behaviour noted. The body weight gained gradually.
15	The mice appeared normal and were sacrificed.	The mice appeared normal and were sacrificed.	The mice appeared normal and were sacrificed.	The normal mice were sacrificed.

The crude extract, the hexane or chloroform extract was administered IP to mice in 10mL 100% DMSO;

The control group received 10 mL 100% DMSO; N= 4 mice per group.

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Table 3. Determination of lethal doses of extract and fractions of *P. bistorta*

Extract/Fractions		Dose (mg/kg)	Log Dose	Dead Mice (Number)	% Dead	Probits
Group 1	Crude extract	150	2.17	2	50	5.00
	Crude extract	200	2.30	3	75	5.67
Group 2	Hexane fraction	150	2.17	0	0	3.47[a]
	Hexane fraction	200	2.30	0	0	3.47[a]
Group 3	Chloroform fraction	150	2.17	1	25	4.33
	Chloroform fraction	200	2.30	2	50	5.00

[a] Corrected probits have been used only in the cases of zero and 100% mortalities [16].

The formula for 0% mortality= (0.25/n) x 10; For 100% mortality= (n-0.25/n) x 100 [16]

and 85.13, respectively. Substituting these values in approximate standard error equations resulted in a SE of 54.59. Therefore, the LD_{50} of the crude extract was 142.82±54.59 mg/kg based on the IP administration of the extract (Figure 1).

Chloroform extract: The plot of log doses versus probits for the chloroform fraction of *P. bistorta* is presented in Figure 2. The probits of LD84 and LD16 of the crude extract were 5.99 and 4.01, rounded-off to 6 and 4, respectively (Table 2). From the graph, the log doses for these were 2.50 and 2.10, respectively, and their antilogs were 315.14 and 127.15, respectively. Substituting these values for the approximate standard error equation resulted in a standard error of 66.47. Therefore, the LD_{50} of the chloroform extract was 200.17±66.47mg/kg when administered intraperitoneally (Figure 2).

Hexane extract: The hexane extract showed no mortality at either 150 or 200mg/kg. Supposedly, the hexane fraction may have an LD_{50} value >200 mg/kg in mice. For the same reason, it was impossible to calculate its exact LD_{50} from the graph since the plot of log doses versus probits requires at least partial responses [15]. Therefore, it was necessary to repeat the experiment with higher dosage of the hexane extract to determine the accurate LD_{50} value. However, we did not conduct further experiments since this in vivo study on mice required significantly larger quantity of the extract, which was not available. The methanol/water fraction was not investigated for its LD_{50} due to its low quantity available. We had to use large amounts of the crude extract, and the hexane and chloroform fractions for their MTT cytotoxic screening [1, 2] against murine and human cancer cell lines in addition to this in vivo study. The result of MTT

Table 4. Finney's method of transformation of mortality percentage to probits [17]

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.87	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

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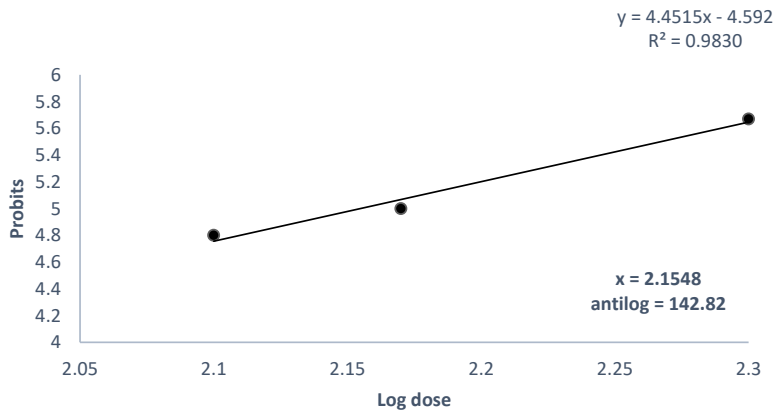

Figure 1. The plot of log doses versus probits of the crude extract of *P. bistorta*

cytotoxicity screening was already published [1, 2]. The cytotoxicity-guided fractionation of the hexane and chloroform fractions by chromatography resulted in several sub-fractions and the isolation of both known and new secondary metabolites [1, 2].

Discussion

In this study, the crude and the hexane and chloroform extracts of *P. bistorta* were evaluated for their LD_{50} values. Our study revealed that the crude extract might have contained all of the toxic ingredients. The successive extracts' fractionation with hexane and chloroform might have distributed it's the toxic ingredients. For the same reason, the crude extract showed an LD_{50} value of 142.82±54.59 mg/kg compared to the hexane and chloroform fractions. The hexane fraction might have contained mostly non-polar ingredients and the toxic effect might have been less than those of the extracts of the crude and chloroform fractions, showing a higher LD_{50} value >200mg/kg. On the other hand, the chloroform extract might have contained mostly the polar ingredients, hence the toxic effect being between those of the crude and hexane extracts (200.17±66.47mg/kg).

The pharmacological and biological activities of the various fractions of *P. bistorta* extracts have previously been reported. Its aqueous ethanolic extract has shown strong anti-inflammatory effect in experimental rats [6], with the active ingredients known as friedelinol and alnusenone (5-glutinen-3-1) [19]. The aqueous extract of *P. bistorta* has inhibited the mutagenicity of tryptophane pyrolysis product 1 (Trp-P-1) [20]. The cytotoxic activity of the extracts of the crude, hexane and chloroform fractions have been described previously [2]. Also, the antioxidant, antifungal and antibacterial activities of the extracts of *P. bistorta* have been reported previously [7-10]. Recently, the modulation of proteostasis by ROS-induced endoplasmic reticulum stress in human hepatoma cells by aqueous extract of *P. bistorta* has been reported [21]. The active ingredients of *P. bistorta* have been identified to be steroids, triterpenes, cycloartane-type triterpenes [1, 2, 22], phenolics [23, 24], tannins [8] and flavonoids [25].

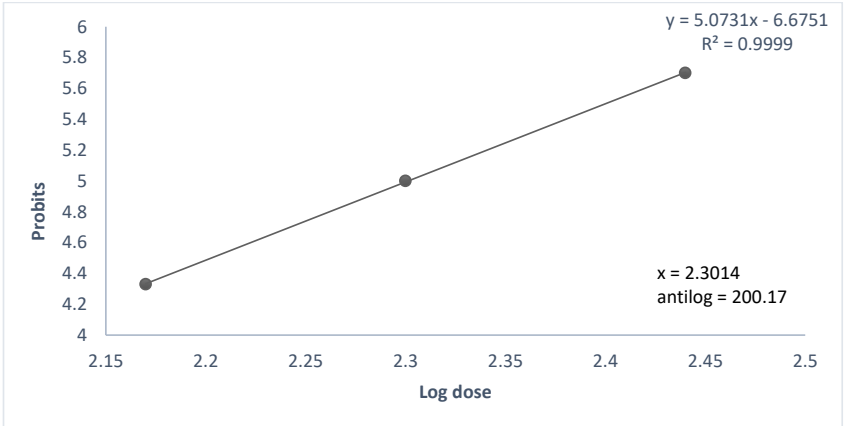

Figure 2. The plot of log doses versus probits of chloroform extract of *P. bistorta*

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Conclusions

This study evaluated the extracts of the crude, hexane and chloroform fractions of *P. bistorta* for their LD_{50} values in Swiss albino mice. The LD_{50} were found to be 142.82, >200 and 200.17mg/ kg of the mice body weight, respectively. To the best of the authors' knowledge, this is the first report of its kind on the LD_{50} values of *P. bistorta* plant in Swiss albino mice.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the National University of Singapore (NUS).

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This study was extracted from the the PhD. dissertation of the first author in the Department of Chemistry, Faculty of Science, National University of Singapore.

Conflict of interest

The authors declared no conflict of interest.

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