Improved Up-and-Down Procedure for Acute Toxicity Measurement With Reliable LD50 Verified by Typical Toxic Alkaloids and Modified Karber Method

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Research Article

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Improved up-and-down procedure for acute toxicity measurement with reliable LD_{50} verified by typical toxic alkaloids and modified Karber method

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Abstract

Background: Up-and-down procedure (UDP) was recommended to replace traditional acute toxicity methods. However, it was limited due to the long experimental period (20 - 42 days). To improve UDP, an improved UDP method (iUDP) was developed by shortening observation time between sequence dosages. The aim of this study was to test the reliability of iUDP to provide a reliable method for the acute toxicity measurement of valuable or minor amount compounds.

Methods: Oral median lethal dosage (LD_{50}) of nicotine, sinomenine hydrochloride and berberine hydrochloride were measured both by iUDP and modified Karber method (mKM).

Results: LD_{50} of the three alkaloids measured by iUDP with 23 mice were 32.71 ± 7.46, 453.54 ± 104.59, 2954.93 ± 794.88 mg/kg, respectively. LD_{50} of the three alkaloids measured by mKM with 240 mice were 22.99 ± 3.01, 456.56 ± 53.38, 2825.53 ± 1212.92 mg/kg, respectively. The average time consumed by the two methods were 22 days and 14 days respectively. Total grams of the alkaloids used by the two methods were 0.0082 and 0.0673 (nicotine), 0.114 and 1.24 (sinomenine hydrochloride), 1.9 and 12.7 (berberine hydrochloride).

Conclusion: iUDP could replace mKM to detect acute toxicity of substances with comparable and reliable result. And it was suitable for valuable or minor amount substances.

Keywords: Acute toxicity; Improved up-and-down procedure; Median lethal dosage; Modified Karber method; Nicotine; Sinomenine hydrochloride; Berberine hydrochloride;

Background

Median lethal dosage (LD_{50}) was first proposed by J. W. Trevan in 1976 [1]. It is used to study acute toxicity and classify toxic substance [2]. The 95% confidence interval (95% CI, $\mu \pm \sigma$) is used to describe LD_{50} mean [3, 4]. Traditional acute toxicity methods to detect LD_{50} and 95% CI include Bliss method [5, 6], mKM [7, 8], arithmetical method of Reed and Muench [9], and Miller and Tainter method [10]. For one substance, 50~80 mice would be administrated to obtain LD_{50} and 95%CI in 14 days by traditional methods (a 14 day observation would carried on survival animals) [11, 12]. However, traditional acute toxicity methods violate animal rights and increase economic pressure [2, 13-15]. With 3Rs principles proposed (Reduction, Replacement, Refinement) [16, 17], up-and-down procedure (UDP) was advocated [14, 18]. In UDP, the dosage of $(N+1)^{th}$ would be determined by the poisoning symptoms of N^{th} animal after administration. Observed the N^{th} animal for 48 hours, if it died, the dosage of $(N+1)^{th}$ would be increased; Otherwise, dosage would be reduced. It is particularly time-consuming to test acute toxicity of one compound by UDP using 4 - 15 animals (Different toxicity compounds show different death and survival reversals, which may take 20 - 42 days, **Table1**). Analyzing 19160 journal articles on acute toxicity from January1986 to October 2020 by SCI Finder, we found that UDP was used just in 144 articles to test acute toxicity of substances (**Fig. 1**). Low precision and long period limit the popularity of UDP in acute toxicity study [19-21]. Recently, several studies have gradually increased animal numbers to improve the reliability of UDP [22-25]. In addition, Hiller, D.B. and Yu Y used UDP to detect drug intravenous toxicity and they increased mice at each dosage to improve precision of results [26, 27]. Sarah C. Finch used UDP to test acute toxicity of tetrodotoxin and tetrodotoxin–saxitoxin mixtures under different routes (i.p. and p.o.) [28]. However, more animals mean more substances would be consumed which is not friendly to valuable or minor amount compounds. In this research, reducing observation time between sequence dosages rather than increasing animal number is applied to improve UDP. Nicotine, sinomenine hydrochloride and berberine hydrochloride, the three known toxic compounds

are classic representatives of highly toxic, moderately toxic, and mildly toxic alkaloids. And they were poorly reported about oral acute toxicity of in mice [29, 30]. This study aimed to evaluate the feasibility and reliability of iUDP by comparing the LD_{50} of the three alkaloids tested both by iUDP and mKM.

Table 1. Comparison between UDP and traditional acute toxicity test methods

Method		Mice	Time (day)	Precision
UDP [31]		4~15	20~42	95% CI was wide, imprecise
Traditional acute toxicity methods	Bliss method [5]	~80	14	95% CI was narrow, precise
	mKM [32]	~80	14	95% CI was narrow, precise

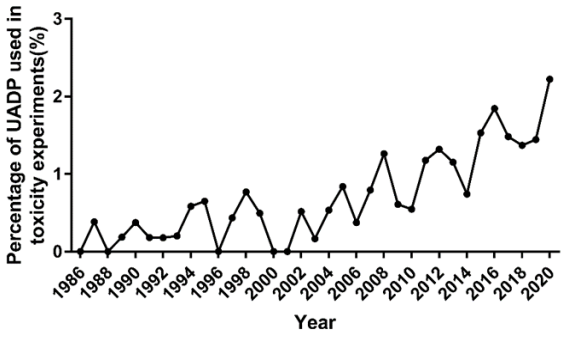


Fig.1. Percentage of UDP used in acute toxicity tests from January1986 to October 2020

Materials and Methods

Experimental animals

A total of 263 ICR female mice (7 ~ 8-week-old, 26 ~ 30g) were used. They were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were housed in individually ventilated cages and had free access to food and water. A 12h light/dark cycle was used in the room. The room temperature and humidity were 20 ~ 22℃, 50 ~ 70%, respectively. Before the start of the study, the animal experiments were approved by the Division of Animal Control and Inspection, Department of Food and Animal

Inspection and Control, Instituto para os Assuntos Cívicos e Municipais (IACM), Macao (AL020/DICV/SIS/2018).

In the experiment, each mouse was weighed and fasted 4 hours with drink water freely before administration. For oral administration of nicotine and sinomenine hydrochloride, 0.2ml was given for every 10 g of mice body weight. And 0.4ml of berberine hydrochloride was given for every 10 g of mice body weight. After administration, the mice were fasted for 1 hour with drink water freely. When the experiment was stopped, all the survived mice were humanely killed and necropsied after a 14-day observation. Observed and recorded the pathological changes of viscera.

Materials

Nicotine (purity > 99%, CAS: N3876-5ML) and berberine hydrochloride (purity > 99%, CAS: B3251) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Sinomenine hydrochloride (purity > 99%, CAS: Y1509004) was kindly provided by Hunan Zhengqing Pharmaceutical Group Limited (Huaihua, Hunan Province, China).

The acute toxicity assay of nicotine in mice by iUDP

According to previous literature results, nicotine was a highly toxic substance. Therefore, the estimated initial LD_{50} dosage was 20 mg/kg. Sigma was 0.2, slope was 5, and T was 1.6. Calculated the dosage by AOT425StatPgm. The sequential dosages were 2000, 1260, 800, 500, 320, 200, 126, 80, 50, 32, 20, 12.6, 8, 5, 3.2, 2 mg/kg. The first dosage of 12.6 mg/kg was given to the first mouse. Symptoms of poisoning were recorded within 24 hours. If it was survived, 20 mg/kg was given as the second dosage. If it died, 8 mg/kg was chosen. Follow the experimental sequence until the standard stopping rules appeared.

The acute toxicity assay of sinomenine hydrochloride in mice by iUDP

According to previous literature results, sinomenine hydrochloride was moderately toxic with a significant dosage-response relationship [30, 33]. Therefore, the estimated initial LD_{50} dosage was 175 mg/kg. Sigma was 0.2, slope was 5, and T was 1.6. Calculated the dosage by AOT425StatPgm. The sequential dosages were 2000, 1100, 700, 440, 280, 175, 110, 70, 44, 28, 17.5, 11, 7, 4.4, 2.8, 1.75 mg/kg. The first dosage of 175 mg/kg was given to the first mouse. Symptoms of poisoning were recorded within 24 hours. If it was survived, 280 mg/kg was given as the second dosage. If it died, 110 mg/kg was chosen. Follow the experimental sequence until the standard stopping rules appeared.

The acute toxicity assay ofberberine hydrochloride in mice by iUDP

According to previous literature results, berberine hydrochloride was a low or non-toxic compound. Therefore, the estimated initial LD_{50} dosage was 2500 mg/kg. Sigma was 0.5, slope was 2, and T was 3.16. Calculated the dosage by AOT425StatPgm. The sequential dosages were 5000, 2500, 790, 250, 79, 25, 7.9, 2.5, 0.79 mg/kg. The first dosage of 790 mg/kg was given to the first mouse. Symptoms of poisoning were recorded within 24 hours. If it was survived, 2500 mg/kg was given as the second dosage. If it died, 250 mg/kg was chosen. Follow the experimental sequence until the standard stopping rules appeared.

The acute toxicity assay of nicotine in mice by mKM

Twenty-four ICR female mice were randomly divided into 4 groups. The dosage ratio was 0.7, and oral dosage was 14, 20, 28.5, 40.8 mg/kg. The lowest dosage with 100% mortality (Dm = 40.8 mg/kg) and the highest dosage with 0% mortality (14 mg/kg) were obtained to provide references for subsequent experiments.

Fifty ICR female mice were randomly divided into 5 groups. The lowest and highest dosage were selected (16 mg/kg, 39.1 mg/kg, respectively). And 0.8 was chosen as the dosage ratio. After dosing, symptoms of poisoning, number of survival and dead mice were recorded. All mice were subjected to gross necropsy

The acute toxicity assay of sinomenine hydrochloride in mice by mKM

Twenty-four ICR female mice were randomly divided into 4 groups. The dosage ratio was 0.7, and oral dosage was 350, 500, 665, 715 mg/kg. Obtained the lowest dosage of 100% mortality (Dm = 665 mg/kg) and the highest dosage of 16% mortality (350 mg/kg). To obtain the highest dosage with 0% mortality (Dn), 300 mg/kg was added.

Fifty ICR female mice were randomly into 5 groups. The lowest and highest dosage were selected (300 mg/kg, 665 mg/kg, respectively). And 0.82 was chosen as the dosage ratio. After dosing, symptoms of poisoning, number of survival and dead mice were recorded. All mice were subjected to gross necropsy.

The acute toxicity assay of berberine hydrochloride in mice by mKM

Twenty-four ICR female mice were randomly divided into 4 groups. The dosage ratio was 0.5, and oral dosage was 1000, 2000, 4000, 8000 mg/kg. The lowest dosage with 90% mortality (8000 mg/kg) and the highest dosage with 16.7% mortality (1000 mg/kg) were obtained. Then 11428 (100% mortality) and 700 mg/kg (0% mortality) were carried out.

Fifty ICR female mice were randomly into 5 groups. The lowest and highest dosage were selected (703 mg/kg, 11250 mg/kg, respectively). And 0.5 was chosen as the dosage ratio. After dosing, symptoms of poisoning, number of survival and dead mice were recorded. All mice were subjected to gross necropsy.

Statistical Analyses

In iUDP, the dosage and numbers of all survival and dead mice were recorded. The computational formula as follows:

$$LD_{50} = \sum(Xi) / N + (A + C) * d/N, \quad (1)$$

$$SE = SD * \sqrt{(2/N)}, \quad (2)$$

Xi was the dosage level, N was the total number of animals, A and C values were obtained from Dixon's tables [30], which were obtained from the number of O and X in N trials. And d was lgDn minus lgD(n+1), SE was the standard error, SD was the standard deviation of all dosages in N trails.

In mKM, mortality rate of each group was calculated, and then values were substituted into formulas to obtain LD_{50} [34]. The computational formula as follows:

$$LgLD_{50} = LgDmax – (LgDN - LgD(N+1)) (\sum p -0.5), \quad (3)$$

$$SE_{50} = I*\sqrt{((\sum p-\sum p^2)/(n-1))}, \quad (4)$$

$$d = \pm 4.5 * LD_{50} * SE_{50}, \quad (5)$$

$$CI \text{ of } 95\% = LD_{50} \pm d, \quad (6)$$

m was $LgLD_{50}$, D was the dosage of each group, Dmax was maximum dosage level, DN was the dosage of N group, D(N + 1) was the dosage of (N + 1) group, p was the mortality of each group of animals, and d was the standard error (σ), I was LgDN minus LgD (N + 1), and n was the number of animals in each group.

Data of organ indexes were plotted in GraphPad Prism (7.0) using One-way ANOVA. And data were presented in mean ± SD, *P < 0.05 vs Normal, **P < 0.01 vs Normal.

Results

The LD_{50} and toxicity of nicotine in mice detected by iUDP

161 The result was calculated as follows according to the results of Table 2 and formula (1),
162 (2).

$$LD_{50} = 228.6 / 7 + (1.53 + 0.17) * 0.2 / 7 = 32.71,$$

$$SE = 13.96 * \sqrt{2/7} = 7.46,$$

163 Therefore, the LD_{50} for nicotine was 32.71 mg/kg and the 95% CI was [25.25, 40.17].
164 Compared to normal mice, lung in mice administrated with different dosage of nicotine
165 was enlarged (**Table 3**). There was a good dosage-effect relationship of nicotine on lung
166 injury in mice. As seen in Table 3, 20 and 32 mg/kg of nicotine increased lung weight in mice
167 ($P < 0.01$, $P < 0.01$, respectively). 50 mg/kg of nicotine significantly increased heart and lung
168 weight in mice ($P < 0.01$, $P < 0.01$). The organs of mice were shown in **Fig. 2**.

169 **Table 2.** Lethality and signs of toxicity of nicotine in mice tested by iUDP

Seq.	Dose (mg/kg)	Δm (g)	Short-term outcome	Symptoms	Pathology
1	12.6	1.1	O	Convulsive, weakness, recovered after 2h	No visible lesions were found in organs and tissues
2	20	1.5	O	Violently convulsive, recovered after 2h	Spleen was enlarged and in deep red color
3	32	1.4	O	Violently convulsive, weakness, recovered after 6h	Lung was enlarged and in deep red color
4	50	0.9	X	Violently convulsive, dead after 5min	Heart and lung were enlarged
5	32	1.1	O	Violently convulsive, weakness, recovered after 6h	Heart and lung were markedly enlarged
6	50	1.7	X	Violently convulsive, dead after 10min	Heart, liver and lung were enlarged
7	32	1.4	X	Violently convulsive, dead after 5min	Heart, liver and lung were enlarged

Stop criteria met: 3 reversals in 5 tests

Note: The sequence of outcomes: O for alive and X for dead.

Table 3. Effect of nicotine on organ indexes in ICR mice by iUDP

Dose (mg/kg)	Heart (%)	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)
0	0.466 ± 0.002	4.800 ±0.373	0.387 ±0.079	0.588 ±0.057	1.282 ±0.140
12.6	0.491	4.665	0.370	0.609	1.248
20	0.485	4.250	0.381	0.643**	1.185
32	0.474 ± 0.018	4.548 ±0.505	0.366 ±0.084	0.653 ± 0.056**	1.170 ± 0.058
50	0.581 ± 0.051**	5.123± 0.155	0.385 ± 0.063	0.702 ±0.015**	1.107±0.007

Note: *P < 0.05 vs Normal, **P < 0.01 vs Normal.

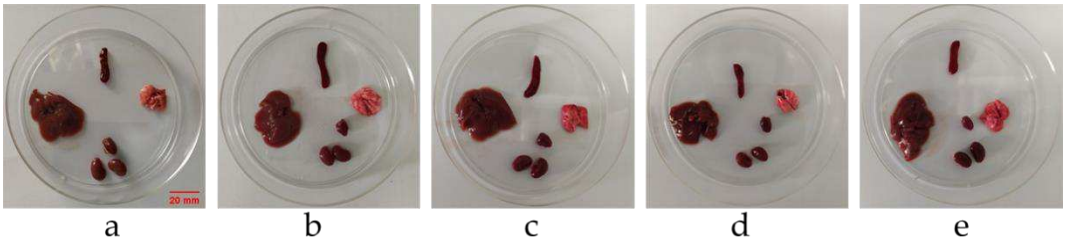


Fig. 2. Organs of mice administrated different dosage of nicotine by iUDP. (**a**) Control; (**b**) 12.6 mg/kg; (**c**) 20 mg/kg; (**d**) 32 mg/kg; (**e**) 50 mg/kg.

The LD_{50} and toxicity of sinomenine hydrochloride in mice detected by iUDP

The result was calculated as follows according to the results of **Table 4** and formula (1), (2).

$$LD_{50} = 3175/7 + (1.53 + 0.16) * 0.2 / 7 = 453.54,$$

$$SE = 195.67 * \sqrt{2/7} = 104.59,$$

Therefore, the LD_{50} of sinomenine hydrochloride was 453.54 mg/kg and the 95% CI was [349.0, 558.2].

Compared to normal mice, sinomenine hydrochloride has no effect on the organ indexes (**Table 5**). No visible lesions were found in organs and tissues in mice administrated with low dosage of sinomenine hydrochloride (**Fig. 3**).

Table 4. Lethality and signs of toxicity of mice after administration of sinomenine

185 hydrochloride by iUDP

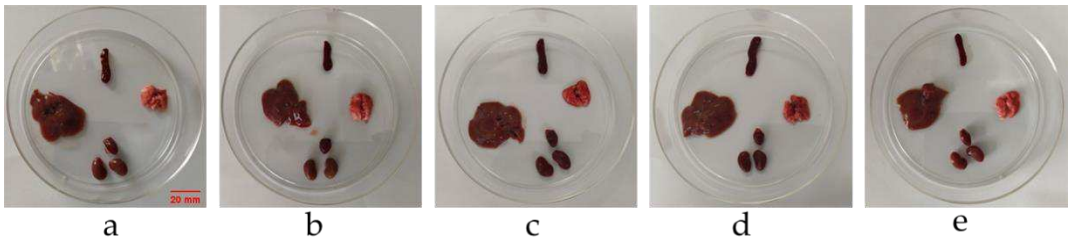
Seq.	Dosage (mg/kg)	Δm (g)	Short-term outcome	Symptoms	Pathology
1	175	1.1	O	Mild, shortness of breath, frightened, recovered after 2h	No visible lesions were found in organs
2	280	1.4	O	Shortness of breath, frightened, recovered after 5h	No visible lesions were found in organs
3	440	1.8	O	Tremor, breathlessness, and recovered after 2h	Liver were enlarged
4	700	1.3	X	Severe tremor, weakness, dead after 30min	Liver was enlarged
5	440	1.5	O	Mild tremor, weakness, and recovered after 2h	Liver and kidney were enlarged
6	700	0.9	X	Severe tremor, weakness, dead after 1h	Liver was enlarged
7	440	0.9	X	Breathlessness, tremor, and dead after 4h	Liver and kidney were enlarged
Stop criteria met: 5 reversals in 6 tests					

186 Note: The sequence of outcomes: O for alive and X for dead.

187 **Table 5.** Effect of sinomenine hydrochloride on organ indexes in ICR mice by iUDP

Dosage(mg/kg)	Heart (%)	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)
0	0.466 ±0.002	4.800 ±0.373	0.387 ±0.079	0.588 ±0.057	1.282 ±0.140
175	0.550	4.660	0.312	0.623	1.120
280	0.450	4.258	0.467	0.578	1.295
440	0.403 ± 0.012	4.382 ± 0.442	0.345 ± 0.082	0.519 ± 0.110	1.110 ± 0.035*
700	0.315 ± 0.065**	4.452 ± 0.486	0.293 ± 0.033**	0.566 ± 0.065	1.005 ± 0.085**

188 Note: *P < 0.05 vs Normal, **P < 0.01 vs Normal.



a b c d e

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Fig. 3. Organs of mice administrated different dosage of sinomenine hydrochloride by iUDP. (**a**) Control; (**b**) 175 mg/kg; (**c**) 280 mg/kg; (**d**) 440 mg/kg; (**e**) 700 mg/kg.

The LD_{50} and toxicity of berberine hydrochloride in mice detected by iUDP

The result was calculated as follows according to the results of **Table 6** and formula (1), (2).

$$LD_{50} = 26580/9 + (1.53 + 0.16) * 0.2 / 9 = 2954.93,$$

$$SE= 1686.29*\sqrt{(2 / 9)} = 794.88,$$

Therefore, the LD_{50} of berberine hydrochloride was 2954.93 mg/kg and the 95% CI was [2160.05, 3749.81].

Compared to normal mice, 5000 mg/kg of berberine hydrochloride increased spleen weight in mice ($P < 0.05$, **Table 7**). No visible lesions were found in organs and tissues in mice administrated with berberine hydrochloride (**Fig. 4**).

Table 6. Lethality and signs of toxicity of mice after administration of berberine hydrochloride by iUDP

Seq.	Dosage (mg/kg)	Δm (g)	Short-term outcome	Symptoms	Pathology
1	790	1.1	O	Reduced activity, recovered after 2h	No visible lesions were found in organs and tissues
2	2500	1.5	O	Reduced activity, recovered after 4.5h	No visible lesions were found in organs and tissues
3	5000	1.4	X	Reduced activity, weakness, dead after 10h	Liver was in deep red color
4	2500	0.9	O	Reduced activity, recovered after 4.5h	No visible lesions were found in organs and tissues

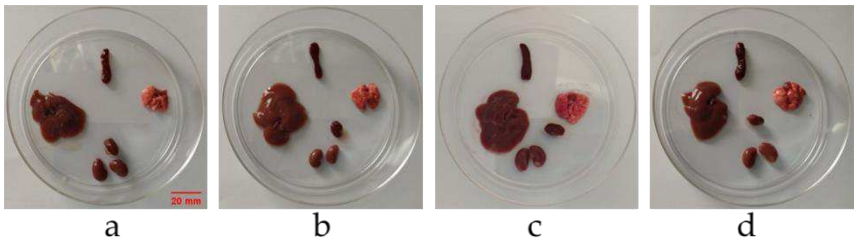
5	5000	1.1	X	Reduced activity, weakness, dead after 8h	Liver was in deep red color
6	2500	1.7	X	Reduced activity, dead after 16h	No visible lesions were found in organs and tissues
7	790	1.4	O	Reduced activity, recovered after 1h	No visible lesions were found in organs and tissues
8	2500	1.1	O	Reduced activity, recovered after 4h	No visible lesions were found in organs and tissues
9	5000	1.0	X	Reduced activity, weakness, and dead after 18h	Liver was in deep red color
		Stop criteria met: 3 reversals in 5 tests			

202 Note: The sequence of outcomes: O for alive and X for dead.

203 **Table 7.** Effect of berberine hydrochloride on organ indexes in ICR mice by iUDP

Dosage(mg/kg)	Heart (%)	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)
0	0.466 ±0.002	4.800 ±0.373	0.387 ±0.079	0.588 ±0.057	1.282 ±0.140
790	0.472 ± 0.028	4.602 ± 0.295	0.363 ± 0.063	0.580 ± 0.097	1.100 ± 0.100
2500	0.449 ± 0.045	4.472 ± 0.207	0.427 ± 0.096	0.627 ± 0.108	1.280 ± 0.073
5000	0.465 ± 0.039	4. 503 ± 0.200	0.426 ± 0.041*	0.598 ± 0.049	1.129 ± 0.068

204 Note: *P < 0.05 vs Normal, **P < 0.01 vs Normal.



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206 **Fig. 4.** Organs of mice administrated different dosage of berberine hydrochloride by iUDP.
207 (**a**) Control; (**b**) 790 mg/kg; (**c**) 2500 mg/kg; (**d**) 5000 mg/kg.

208 **The LD_{50} and toxicity of nicotine in mice detected by mKM**

209 The result was calculated as follows according to **Table 8** and formula (3, 4, 5, 6).

$$LgLD_{50} = lg39.1 – (lg20 – lg16) * [2.9 – 0.5] = 1.3616,$$

$$LD_{50} = 22.99,$$

$$SE_{50} = 0.096* \sqrt{((2.9-2.07)/ (10-1))} = 0.02915,$$

$$SE = \pm 4.5 * 22.99 * 0.02915 = 3.02,$$

210 Therefore, the LD_{50} of nicotine was 22.99 mg/kg and the 95% CI was [19.97, 26.01].

211 Compared to normal mice, 20 and 32 mg/kg of nicotine increased lung weight in mice
212 ($P < 0.05$, $P < 0.01$, respectively). 50 mg/kg of nicotine significantly increased heart and lung
213 weight in mice ($P < 0.01$, $P < 0.01$, **Table 9**). As seen in **Fig 5**, lung in mice administrated
214 with different dosage of nicotine were enlarged.

215 **Table 8.** Lethality and signs of toxicity of mice after administration of nicotine by mKM

Group	n	Dosage（mg/kg）	Morality（p）	p2	Pathology
1	10	16	0.2	0.04	No visible lesions were found in other organs and tissues.
2	10	20	0.3	0.09	Liver was enlarged and in deep red color
3	10	25	0.7	0.49	Liver was enlarged and in deep red color
4	10	31.25	0.8	0.64	Liver and kidney were enlarged and in deep red color
5	10	39.1	0.9	0.81	Liver and kidney were significantly enlarged and in deep red color

216 Note: The sequence of outcomes: O for alive and X for dead.

217 **Table 9.** Effect of different doses of nicotine on organ indexes in ICR mice by mKM

Dosage(mg/kg)	Hear (%)	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)

0	0.466 ± 0.002	4.800 ±0.373	0.387 ± 0.079	0.588 ± 0.057	1.282 ± 0.140
16	0.467 ± 0.023	4.667 ± 0.317	0.412 ± 0.066	0.603 ± 0.046	1.177 ± 0.075
20	0.482 ± 0.061	4.772 ± 0.476	0.468 ± 0.068	0.603 ± 0.081	1.220 ± 0.064
25	0.431 ± 0.002	4.825 ± 0.034	0.578 ± 0.154	0.665 ± 0.038*	1.211 ± 0.021
31.25	0.437±0.009	4.272± 0.363	0.423 ± 0.022	0.692 ± 0.058**	1.187± 0.052
39.10	0.490± 0.041	4.891± 0.105	0.391 ± 0.055	0.700 ± 0.020**	1.137 ± 0.09

Note: *P < 0.05 vs Normal, **P < 0.01 vs Normal.

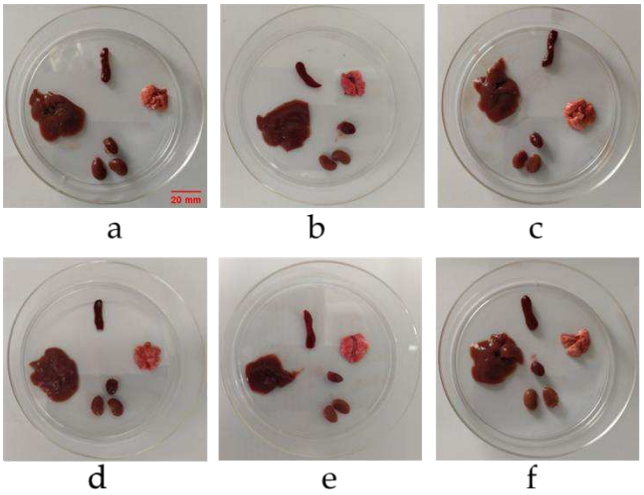


Fig. 5. Organs of mice administrated different dosage of nicotine by mKM. (**a**) Control; (**b**) 16 mg/kg; (**c**) 20 mg/kg; (**d**) 25 mg/kg; (**e**) 31.25 mg/kg; (**f**) 39.1 mg/kg.

The LD_{50} and toxicity of sinomenine hydrochloride in mice detected by mKM

The result was calculated as follows according to **Table 10** and formula (3, 4, 5, 6).

$$LgLD_{50} = lg\ 663 – (lg300 - lg365) * [2.3 – 0.5] = 2.66,$$

$$LD_{50} = 456.56,$$

$$SE_{50} = 0.09* \sqrt{((2.3-1.55) / (10-1))} = 0.02598,$$

$$SE = \pm 4.5 * 456.56 * 0.02598 = 53.38,$$

Therefore, the LD_{50} of sinomenine hydrochloride was 456.56 mg/kg and he 95% CI was [403.18, 509.94].

Compared to normal mice, the heart and kidney in mice administrated by 665 mg/kg of sinomenine hydrochloride were enlarged ($P < 0.05$, $P < 0.01$, respectively, **Table 11**). As seen in **Fig. 6**, no visible lesions were found in organs and tissues in mice administrated with sinomenine hydrochloride.

230 **Table 10.** Lethality and signs of toxicity of mice after administration of sinomenine
231 hydrochloride by mKM

Group	n	Dosage (mg/kg)	Morality (p)	p2	Pathology
1	10	300	0	0	No visible lesions were found in other organs and tissues
2	10	365	0.3	0.09	Liver was enlarged and in deep red color
3	10	446	0.4	0.16	Liver was enlarged and in deep red color
4	10	544	0.7	0.49	Liver and kidney were enlarged and in deep red color
5	10	663	0.9	0.81	Liver and kidney were significantly enlarged and in deep red color

232 **Table 11.** Effect of different doses of sinomenine hydrochloride on organ indexes in ICR
233 mice by mKM

Dosage(mg/kg)	Hear (%)	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)
0	0.466 ± 0.002	4.800 ±0.373	0.387 ± 0.079	0.588 ± 0.057	1.282 ± 0.140
300	0.494 ±0.091	4.948 ±0.500	0.404 ±0.085	0.571 ±0.109	1.217 ±0.184
365	0.454 ± 0.036	4.925 ± 0.298	0.393 ± 0.063	0.586 ± 0.092	1.101 ±0.104
446	0.403 ±0.012	4.382 ±0.442	0.335 ± 0.082	0.519 ± 0.110	1.210 ± 0.035
544	0.421 ±0.037	3.931± 0.240	0.327 ± 0.078	0.543 ± 0.022	1.109 ± 0.110*
663	0.345 ± 0.035**	4.327 ± 0.248	0.305 ± 0.021	0.554 ± 0.054	0.973 ± 0.063 **

234 Note: *P < 0.05 vs Normal, **P < 0.01 vs Normal.

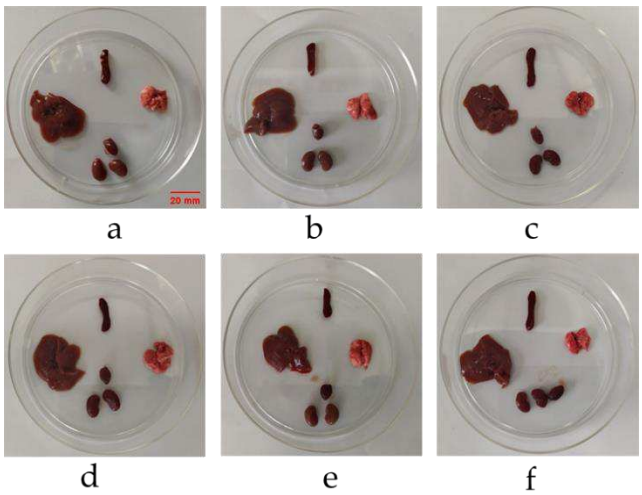


Fig. 6. Organs of mice administrated different dosage of sinomenine hydrochloride by mKM. (**a**) Control; (**b**) 300 mg/kg; (**c**) 365 mg/kg; (**d**) 446 mg/kg; (**e**) 544 mg/kg; (**f**) 663 mg/kg.

The LD_{50} and toxicity of berberine hydrochloride in mice detected by mKM

The result was calculated as follows according to **Table 12** and formula (3, 4, 5, 6).

$$LgLD_{50} = \lg 11250 - (\lg 1406 - \lg 703) * [2.5 - 0.5] = 3.4511,$$

$$LD_{50} = 2825.53,$$

$$SE_{50} = 0.3* \sqrt{((2.5-1.59)/(10-1))} = 0.09539,$$

$$SE = \pm 4.5 * 2825.53 * 0.09539 = 1212.92,$$

Therefore, the LD_{50} of berberine hydrochloride was 2825.53 mg/kg and the 95% CI was [1612.60, 4038.45].

Compared to normal mice, the liver, spleen and lung in mice administrated by 11250 mg/kg of berberine hydrochloride were enlarged ($P < 0.01$, $P < 0.01$, $P < 0.01$, **Table 13**). As seen in **Fig. 7**, the liver, spleen and lung in mice administrated with high dosages of sinomenine hydrochloride were enlarged.

Table 12. Lethality and signs of toxicity of mice after administration of berberine hydrochloride by mKM

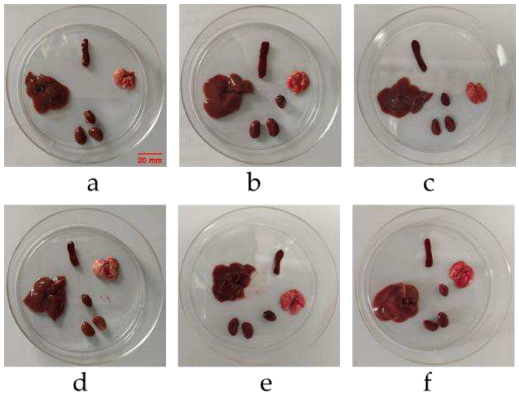
Group	n	Dosage（mg/kg）	Morality（p）	p2	Pathology

1	10	703	0.2	0.04	No visible lesions were found in other organs and tissues
2	10	1406	0.3	0.09	No visible lesions were found in other organs and tissues
3	10	2812	0.4	0.16	No visible lesions were found in other organs and tissues
4	10	5628	0.7	0.49	Lung were enlarged
5	10	11250	0.9	0.81	Liver and lung were enlarged, and spleen was reduced

248 **Table 13.** Effect of berberine hydrochloride on organ indexes in ICR mice by mKM

Dosage(mg/kg)	Hear (%)	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)
0	0.466 ±0.002	4.800 ±0.373	0.387 ±0.079	0.588 ±0.057	1.282 ±0.140
703	0.463± 0.018	5.010 ±0.558	0.406 ±0.092	0.553 ±0.069	1.227 ±0.203
1406	0.429 ±0.028	4.740 ±0.295	0.422 ±0.063	0.645 ±0.097	1.162 ±0.100
2812	0.454 ±0.017	4.453 ±0.242	0.398 ±0.075	0.667 ±0.031	1.198 ±0.131
5628	0.473 ±0.046	4.575 ±0.173	0.394 ±0.042	0.625 ±0.024	1.320 ±0.073
11250	0.442 ±0.053	5.877 ±0.309**	0.288 ±0.065**	0.697 ±0.090**	1.249 ±0.110

249 Note: *P < 0.05 vs Normal, **P < 0.01 vs Normal.



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251 **Fig. 7.** Organs of mice administrated different dosage of berberine hydrochloride by mKM.
252 (a) Control; (b) 703 mg/kg; (c) 1406 mg/kg; (d) 2812 mg/kg; (e) 5628 mg/kg; (f) 11250
253 mg/kg.

254 **Discussion**

In this study, nicotine, sinomenine hydrochloride and berberine hydrochloride were detected to obtained oral LD_{50} both by iUDP and mKM. According to toxicity categories in Classification Criteria for Acute Toxicity (**Table 14**) [35] and LD_{50} results (**Table 15**), the three alkaloids were divided into Category II (Highly toxic), III (Moderately toxic) and IV (Mildly toxic).

Oral LD_{50} is affected by many factors such as gender, age and fasting time, etc. [2]. Gender differences plays an important role in dosage-effect response [36, 37]. Females are more sensitive to compound than males [38]. It is recommended to use females for general acute toxicity studies [33]. Age, which is often poorly reported, affects the physiological state and sensitivity to substance [39]. Four to eight weeks mice (18 ~ 30g) are often used in toxicity tests [40-43]. It is indicated that ICR, KM, and BALB/c mice (26 ~ 30 g) under the state of 8 ~ 10 weeks are equivalent to the human adulthood [44]. To increase scientific validity and reduce experimental variability, the adult rodent animals are used in acute toxicity experiments [45]. In addition, the fasting status is often overlooked. It was reported that overnight-fasting affected the level of hormone and sensitivity of animals to drugs [46]. In this study, a 4h-fasting is recommended for mice.

There are two reasons to choose 24h as the observation interval. In the experiment, surviving mice returned to normal after 2 ~ 18 hours administration (**Table 2, 4, 6**). Nicotine (highly toxic), sinomenine hydrochloride (moderately toxic) have a fast poisoning reaction which would be relieve within 4-6 hours. But unknown chemicals may take a longer time to show its toxic reaction which is the same as berberine hydrochloride (8 – 18 hours). Second, individual differences lead to the differences between different methods [2, 47, 48]. To improve the repeatability of iUDP, the state of each animal should be as consistent as possible. It is best to fix the fasting start time and end time for each mouse. In this article, the mice were fasted daily from 9:00 am to 13:00 pm and the weight loss of each mouse was between 0.9 to 2.0 g.

In addition, the reliability and accuracy of iUDP could be improved by choosing appropriate initial dosage and slope. Initial dosage should be valued from all known toxicity information [49]. Slope of dosage response curve is a key regulator for sequential dosage. A

larger slope would bring a good 95%CI, which may lead to increase animal. A smaller slope would reduce the accuracy of 95%CI. Once the slope setting is not suitable, the entire experiment faced the risk of failure.

Table 14. Classification Criteria for Acute Toxicity [35]

Exposure route	Category I Very toxic	Category II Highly toxic	Category III Moderately toxic	Category IV Mildly toxic	Category V Practically non-toxic
Mice, oral (mg/kg)	<1	1~50	51~500	501~5000	5001~15000

Table 15. Comparison of acute toxicity results between iUDP and mKM in three alkaloids

Method	Compound	Category	Animals	Compound (g)	Expense (MOP)	Duration (Day)
iUDP	Nicotine	II	7	0.0082	1330	21
	Sinomenine hydrochloride	III	7	0.114	1330	21
	Berberine hydrochloride	IV	9	1.9	1900	24
mKM	Nicotine	II	74	0.0673	14060	14
	Sinomenine hydrochloride	III	80	1.24	15200	14
	Berberine hydrochloride	IV	86	12.7	16340	14

Conclusion

In light of experimental results, it may be concluded that iUDP is reliable to detect acute toxicity of unknown substances. And compared with traditional acute toxicity method, iUDP was more animal-friendly and economy which was suitable for valuable or minor amount substances.

Supplementary Materials:

Abbreviations

95% CI, 95% confidence interval; **iUDP,** improved up-and-down procedure; **LD_{50},** Median lethal dosage**; mKM**, modified Karber method;

Ethics approval and consent to participate

The animal experiments were approved by the Division of Animal Control and Inspection, Department of Food and Animal Inspection and Control, Instituto para os Assuntos Cívicos e Municipais (IACM), Macao (AL020/DICV/SIS/2018).

Consent for publication

All authors have read and agreed the published version of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no conflict of interest.

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Author Contributions

All the authors participated in development of the manuscript. The experiment design has mainly been developed by HZ and YYZ who also performed the statistical analyses and wrote the initial draft of the manuscript. The laboratory work was performed by YYZ, YFH and JL. All the authors have participated in the literature review and development of the manuscript and have approved the final version.

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Not applicable

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Figures

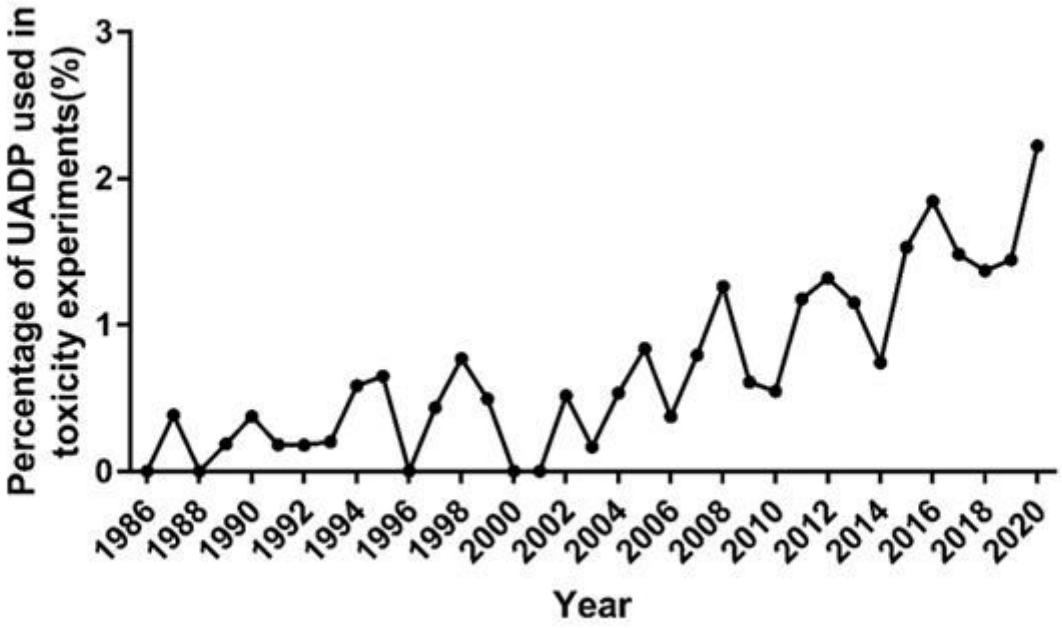

Figure 1

Percentage of UDP used in acute toxicity tests from January1986 to October 2020

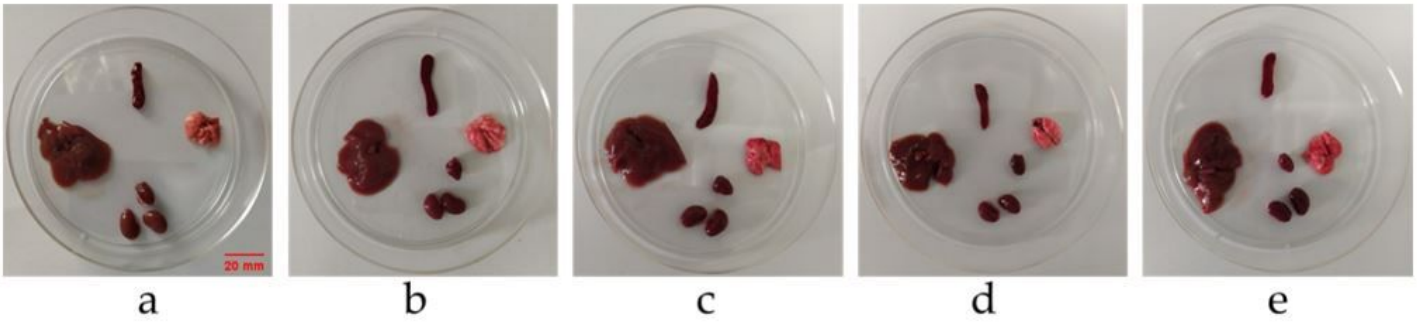

Figure 2

Organs of mice administrated different dosage of nicotine by iUDP. (a) Control; (b) 12.6 mg/kg; (c) 20 mg/kg; (d) 32 mg/kg; (e) 50 mg/kg.

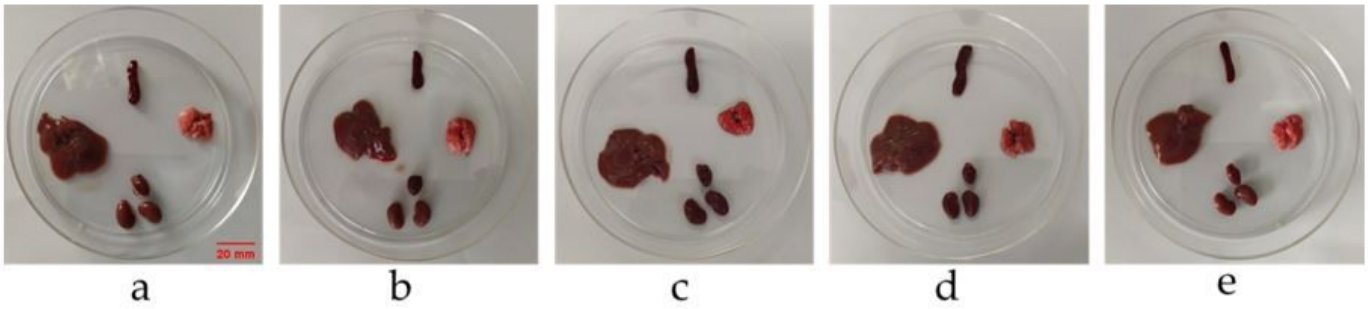

Figure 3

Organs of mice administrated different dosage of sinomenine hydrochloride by iUDP. (a) Control; (b) 175 mg/kg; (c) 280 mg/kg; (d) 440 mg/kg; (e) 700 mg/kg.

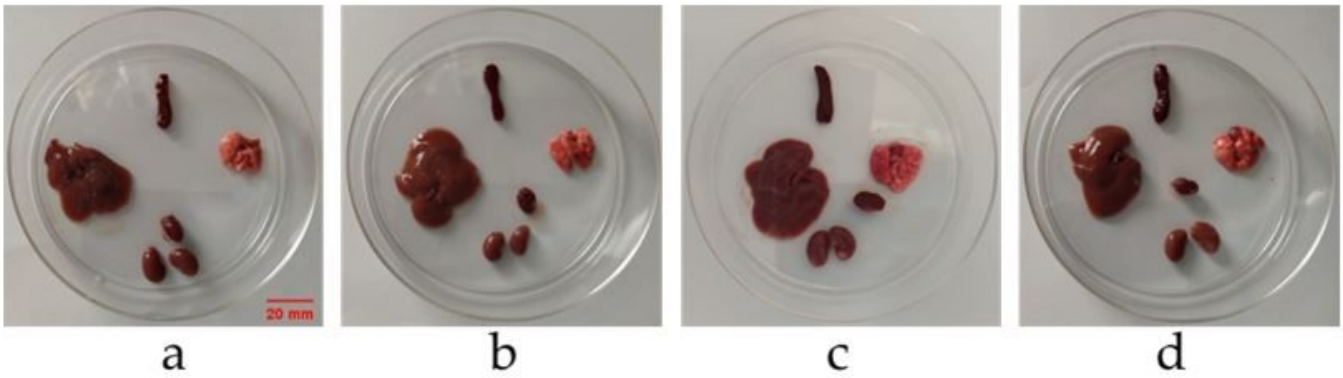

Figure 4

Organs of mice administrated different dosage of berberine hydrochloride by iUDP. (a) Control; (b) 790 mg/kg; (c) 2500 mg/kg; (d) 5000 mg/kg.

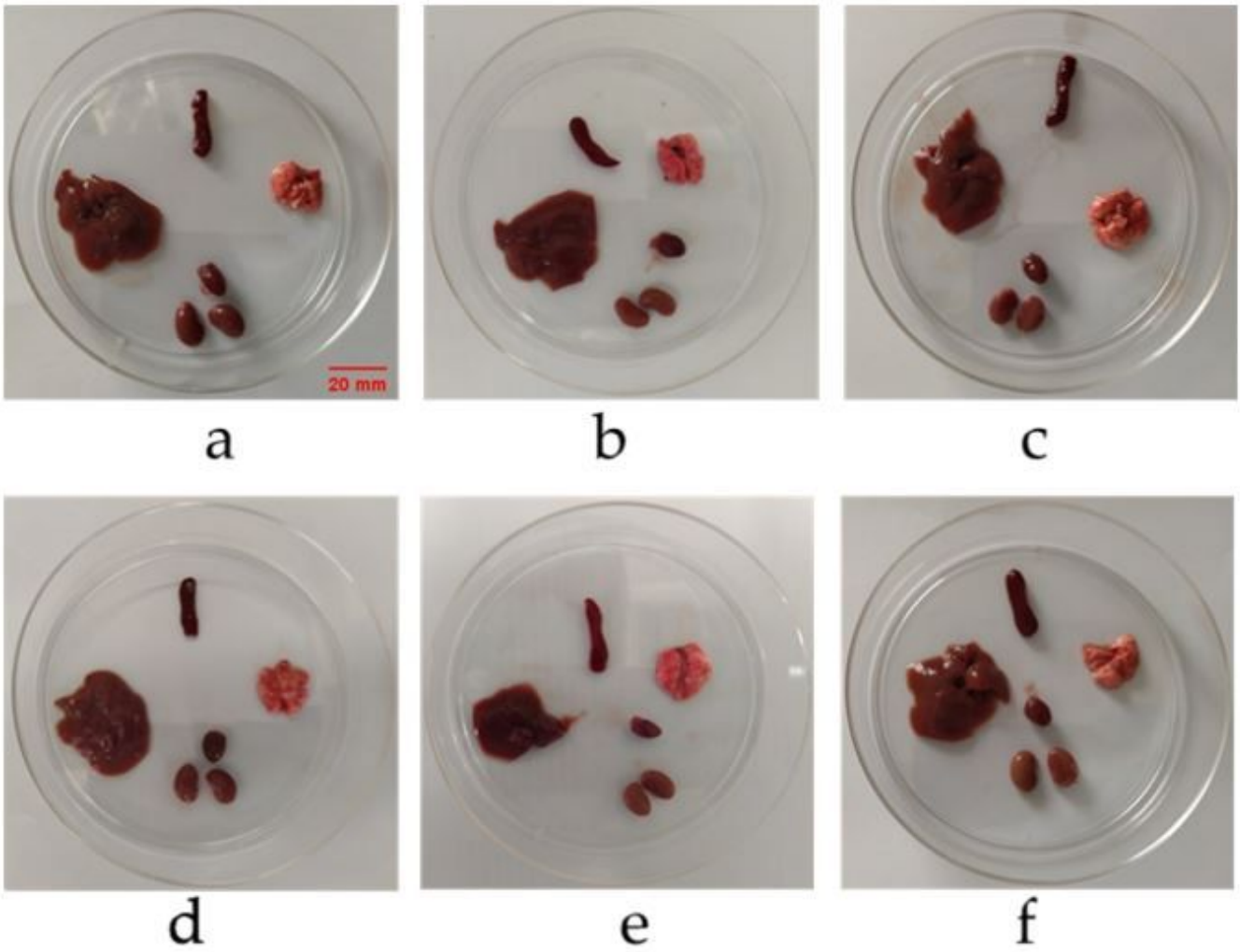

Figure 5

Organs of mice administrated different dosage of nicotine by mKM. (a) Control; (b) 16 mg/kg; (c) 20 mg/kg; (d) 25 mg/kg; (e) 31.25 mg/kg; (f) 39.1 mg/kg.

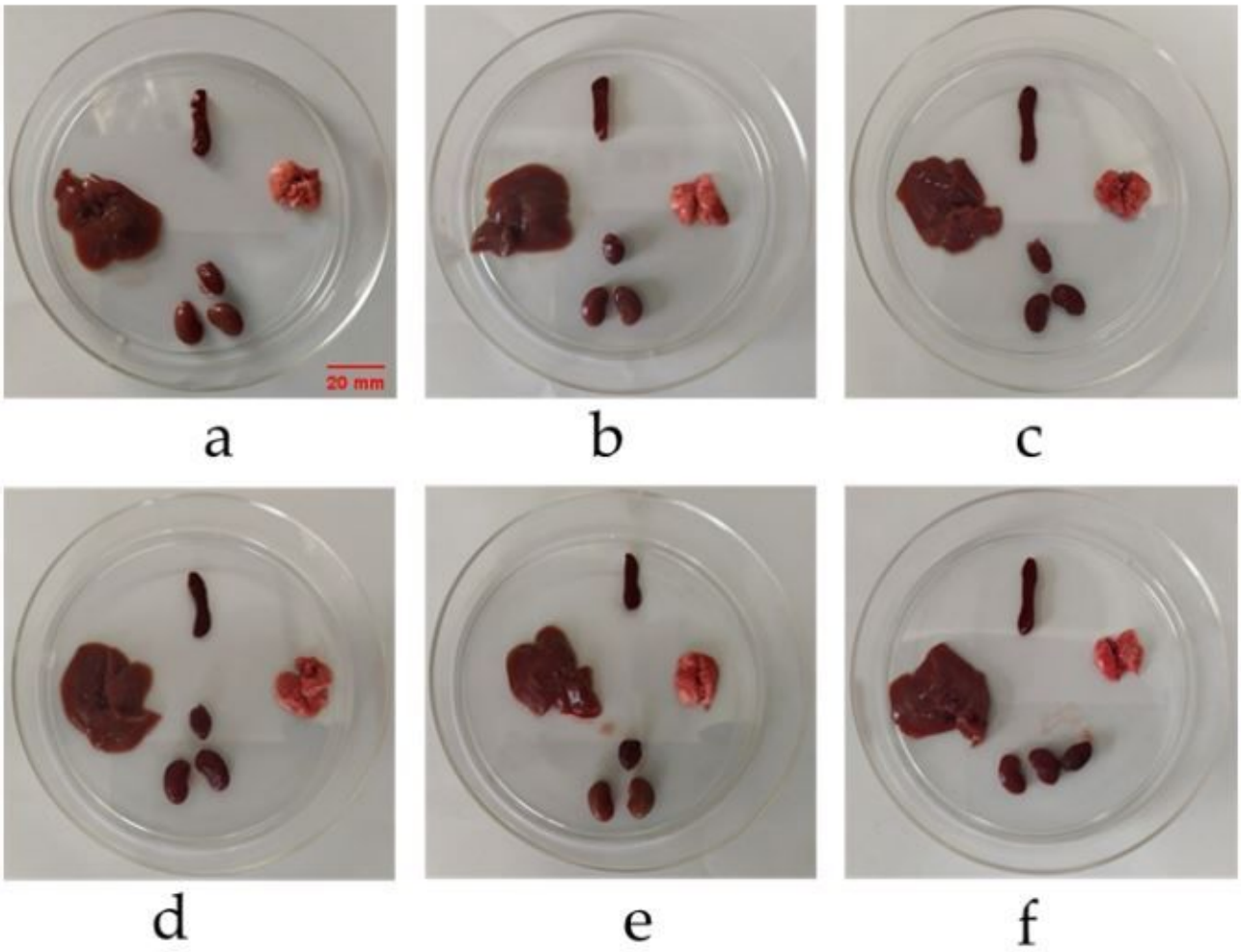

Figure 6

Organs of mice administrated different dosage of sinomenine hydrochloride by mKM. (a) Control; (b) 300 mg/kg; (c) 365 mg/kg; (d) 446 mg/kg; (e) 544 mg/kg; (f) 663 mg/kg.

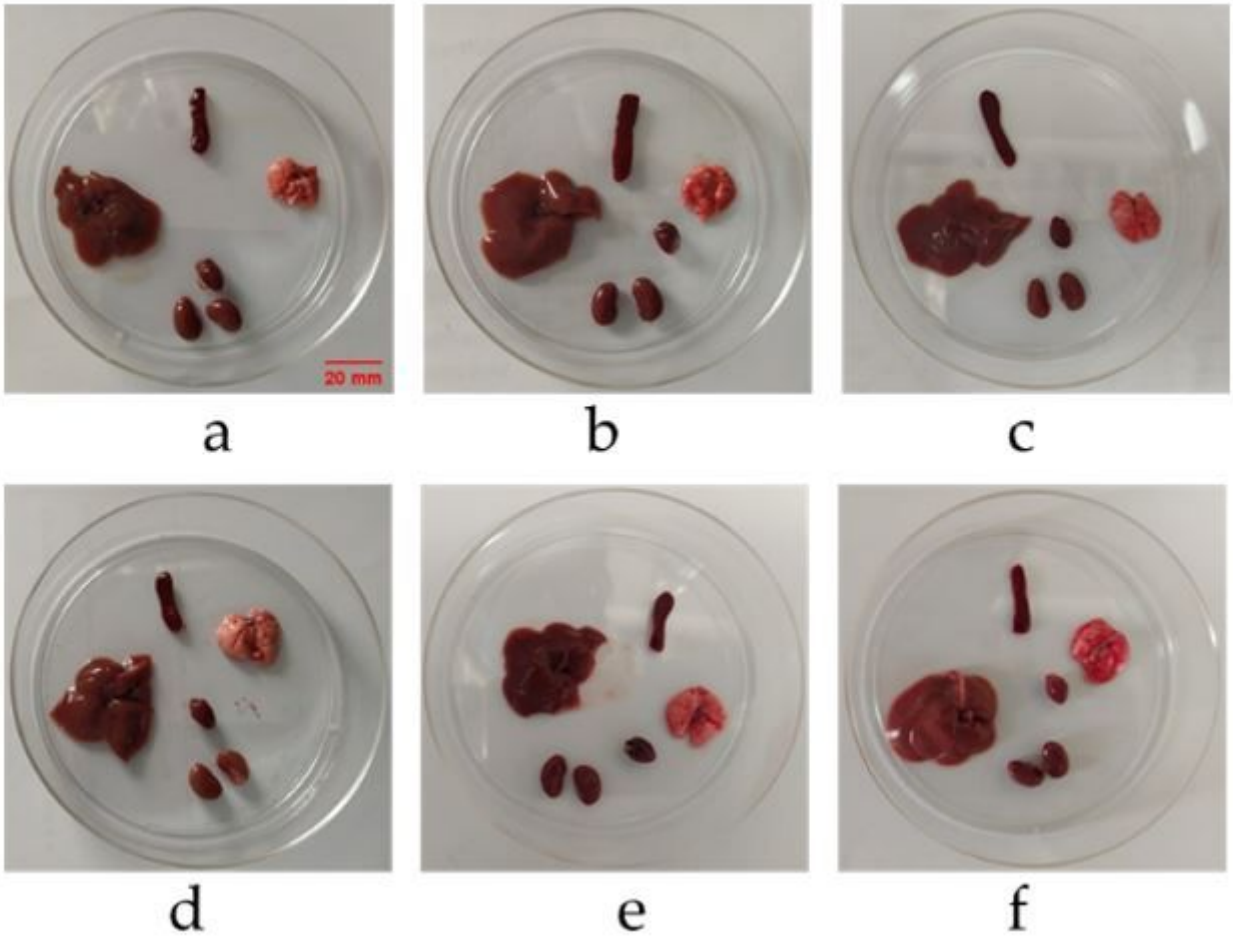

Figure 7

Organs of mice administrated different dosage of berberine hydrochloride by mKM. (a) Control; (b) 703 mg/kg; (c) 1406 mg/kg; (d) 2812 mg/kg; (e) 5628 mg/kg; (f) 11250 mg/kg.