NATURAL DRUGS

PHYTOCHEMICAL ANALYSIS AND CARDIOTONIC ACTIVITY OF METHANOLIC EXTRACT OF RANUNCULUS MURICATUS LINN. IN ISOLATED RABBIT HEART

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Abstract: Ranunculus muricatus Linn. (RML) have been traditionally used for the treatment of various cardiovascular disorders. The aim of present study was to evaluate their cardiovascular effects in isolated perfused rabbit heart. The methanolic extract of RML was prepared by cold maceration process. The methanolic extract of RML (1 ng to 10 mg) was used to determine the percentage change in force of contraction (FC), heart rate (HR) and perfusion pressure (PP) by using Langendorff’s Perfused Heart Apparatus. The PP, FC and HR of isolated rabbit heart were measured by power lab data acquisition system. Moreover, phytochemical analysis and acute toxicity study were also performed. The methanolic extract at the doses from 1 ng to 10 mg exhibited a significant increase in perfusion pressure and force of contraction. Moreover, the crude extract of RML revealed a significant increase in heart rate at doses from 1 ng to 1 µg. The maximum rise in all the three parameters was observed at 1 µg and 1 ng, respectively. In another study, the methanolic extract was tested in the presence of propranolol and verapamil on isolated perfused rabbit heart. The study shown that the increase in HR and FC produced by the plant extracts was significantly reduced in the presence of propranolol whereas PP remained significantly raised even in the presence of propranolol. However, in the presence of verapamil, this increased PP was significantly reversed to a decrease while a significant positive inotropic and chronotropic effects were observed. It is concluded that the cardiotonic activity of methanolic extract of RML might be due to certain cardioactive chemical compounds. Further studies are needed to isolate these pharmacologically active phytochemical constituents and elucidate their exact mechanism of action.

Keywords: Ranunculus muricatus Linn., cardiotonic, rabbit, Langendorff’s isolated heart apparatus, phytochemical, acute toxicity

Cardiovascular diseases have been considered a severe health problem around the globe. The major risk factors for heart diseases include family history, sex, hypercholesterolemia, hypertension, obesity, and cigarette smoking. Most of these risk factors are prevalent in developing countries because of the absence of appropriate infrastructure. Therefore, these diseases have become a very common problem in the rich population of the developing countries (1). Pakistan is enriched with large variety of medicinal plants. According to a survey report more than 5000 species of plants with probable potential of pharmacological activities are distributed throughout Pakistan. Out of these 5000 species, about 600 to 700 species are being used by local people for many medicinal purposes (2). It is understood that synthetic medicines cause more side effects as compared to natural products; western countries are also shifting to natural products (3). Finding healing powers in plants is an ancient idea, in this respect herbs have been used for medical treatment since the beginning of human civilization (4).

The Ranunculaceae is a family with a large number of plants. It has 50 genera and 2000 species

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which are widely distributed throughout the northern hemisphere. It also found in southern temperate regions (5). One of Ranunculaceae important member is RML traditionally used to regulate normal heart beat, body temperature and also beneficial in fever and nausea (6).

MATERIAL AND METHODS

Plant material

RML was collected from marshy areas of Talash district Dir (lower) Khyber Pukhtoon Khwa (KPK) Pakistan in the month of May 2013. The plant was identified by Dr. Ali Hazrat (taxonomist) research officer of Shaheed Benazir Bhutto University, Sheringle Upper Dir, KPK, Pakistan. The specimen was deposited in the pharmacy department herbarium.

Plant extraction

After collection the plant was dried under shade at room temperature. The dried plants’ material was grounded into a coarse powder form by china herbal grinder. Methanolic extract of the plants was prepared using cold maceration process. RML material (12 kg) was soaked in methanol 5 liters and 8 liters for 72 hours at room temperature, respectively, with occasional shaking. After 3 days, these materials were filtered using muslin cloth and filter papers. The filtrates of extracts were evaporated under reduced pressure in rotary evaporator at 50°C. This methanolic extract was then air-dried to obtain a solid mass (7). The crude extract was greenish black in color and was also soluble in distilled water.

Animals and housing conditions

Rabbits (1000-1500 g) of either sex were used. All the animals were housed in controlled environment (23-25°C) at animal house of University of Sargodha, Sargodha. The study protocol was approved by the Institutional Animal Ethics Committee (IEC), Faculty of Pharmacy, University of Sargodha (Approval No. 40-B21 IEC UOS). Experiments comply with the declarations of National Research Council (8).

EXPERIMENTAL

Acute toxicity test

The objective of these experiments was to determine the LD₅₀ of the crude extract. For this purpose both male and female albino mice weighing 30-40 g were randomly divided into five groups of two animals each. Group I served as control and received normal saline (10 mL/kg) while other groups (Group 2, Group 3, Group 4 and Group 5) were given different doses (intraperitoneally) of crude extract of RML in an ascending order i.e., 100, 500, 1000, 1500 mg/kg, respectively. The mortality rate was observed for 24 h. Since no mortality occurred in any group treated with RML extract, so another five groups of mice were taken. They were again treated with the various doses of crude extract in an increasing order i.e., 2000, 2500, 3000, 3500, 4000 mg/kg, respectively. The highest dose, which did not kill any animals, and the lowest dose, which killed only one mouse, was noted. LD₅₀ was calculated from the geographic mean of these two doses (9).

Preliminary phytochemical test

The methanolic extract of RML was analyzed for the presence of different phytochemical constituents such as flavonoids, reducing sugars, tanins, phenolic compounds, saponins, alkaloids and cardiac glycosides by using standard methods (10).

Effects of crude extracts on various cardiac parameters by Langendorff method

The experiments were performed according to the method prescribed by Langendorff, 1895. A rabbit (n = 6) was injected with 1000 IU of heparin intravenously through the marginal ear vein, 30 min before dissection. Five minutes later, the rabbit was sacrificed; its heart was dissected out with about 1 cm of aorta attached, and transferred as quickly as possible into a Petri dish containing Krebs-Henseleit solution. The heart was then cleaned of any excessive tissue and mounted on the Langendorff’s apparatus containing Krebs-Henseleit solution maintained at 37°C. The aorta was tied to the glass cannula with a pressure transducer. A clip was attached to the apex of heart to measure the FC (g) by force-displacement transducer. Both the transducers were attached to the Power Lab data acquisition system and the recordings were measured using Chart 5.0 Pro software. The preparation was then allowed to equilibrate for 30 min before starting the experiment. After stabilization, different doses (1 ng, 10 ng, 100 ng, 1 µg, 10 µg, 100 µg, 1 mg and 10 mg/mL) of the extract were applied to assess various cardiac parameters (HR, FC and PP) with each heart serving as its own control. The drug in a fixed volume of 5 mL was injected with 5 mL syringe through a three way port. Each dose was first filtered with micro filter before injection. In order to elucidate the possible mechanism of action, the effect of selected dose of the extract was assessed both in the
absence and presence of propranolol (10⁻³ M) and verapamil (10⁻⁶ M) (7, 11).

**Statistical analysis**

The results were expressed as the mean ± standard error of the mean (SEM). Student’s t-test was applied; with p < 0.05 and p < 0.001 considered as significant and highly significant.

**RESULTS**

**Acute toxicity**

LD₅₀ of the RML was calculated from the data and was found to be 4000 mg/kg. The methanolic extract from 100 to 3500 mg/kg body weight did not produced significant changes in behavior, breathing, sensory and nervous system responses in mice.

**Preliminary phytochemical test**

Alkaloids, indole alkaloids, tannins, saponins, reducing sugars, cardiac glycosides, steroids and terpenoids were present in the plat as shown in Table 1.

**Effect of crude extract on various cardiac parameters**

The crude extract of RML in the doses from 1 ng to 10 mg exhibited a significant increase in PP and FC. The maximum (p < 0.001) increase in PP and inotropic effect was observed at 1 µg and 1 mg (83.5 ± 8.14 and 27.8 ± 0.08), respectively. The

![Graph showing the effect of crude extract on PP, FC, and HR](image1)

Figure 1. Effect of different doses of crude extract of RML on PP, FC and HR of isolated heart, where a = (p < 0.001), b = (p < 0.01), c = (p < 0.1), NS = Non-significant as compared to control

![Tracing showing the effect of crude extract on FC and PP](image2)

Normal 1 ng  Normal 1 µg

Tracing 1. Effect of crude extract of *Ranunculus maricatus* Linn. on FC and PP
crude extract at a dose from 1 ng to 1 µg produced a significant increase in chronotropic effect. The maximum increase (p < 0.001) in HR was observed at a dose of 10 ng (56.8 ± 6.18). It was interesting to note that at higher doses from 10 µg to 10 mg, a non-significant effect was produced. The remarkable effects in all the three parameters were observed at 1 ng (Fig. 1) (Tracing 1).

Effect of crude extract of RML on isolated heart in the presence of propranolol

The crude extract of RML at a dose of 1 ng, in the presence of propranolol 10⁻⁵ M exhibited a significant decrease in FC (-20.1 ± 0.23) and HR (-19.0% ± 6.96). However, there was not any reduction in PP in the presence of propranolol (Fig. 2).

Effect of crude extract of RML on isolated heart in the presence of verapamil

The crude extract of RML at a dose of 1 ng, in the presence of verapamil 10⁻⁶ M exhibited a significant reduction in PP (-8.12 ± 2.59) of the isolated heart. However, the increase in FC and HR was not reduced in the presence of verapamil (Fig. 3).

DISCUSSION

RML are important medicinal plants commonly found in Pakistan. They are of great utility and have been reported to be effective in the treatment of cough, fever, stomach ache, and various cardiac complications (6, 12).

<table>
<thead>
<tr>
<th>TEST</th>
<th>OBSERVATIONS</th>
<th>INFERENCE</th>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>Colored precipitate</td>
<td>+</td>
</tr>
<tr>
<td>Indole alkaloids</td>
<td>Change in coloration</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>Dark blue or greenish</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Persistent froth</td>
<td>-</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>Red precipitate formed</td>
<td>-</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>Reddish brown</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>Greenish/blue rings</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Reddish brown</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: + = present, - = absent

Figure 2. The effects of extract of *Ranunculus muricatus* Linn. on PP, FC and HR before and after the use of propranolol, where a = (p < 0.001) increased from the control before propranolol and A = (p < 0.001) increased as compared to the control after propranolol, b = (p < 0.05) increased from the control before propranolol, B = (p < 0.05) and C = (p < 0.001) decreased as compared to the control after propranolol.
The methanolic extract of RML was evaluated for its cardiovascular activities on isolated rabbit heart. The extract produced a significant cardiotonic effect. The crude extract of RML produced a maximum (p < 0.001) increase in FC, HR and PP at a dose of 1 µg and 1 ng, respectively. This cardiotonic activity of the crude extract might be due to the involvement of $\beta_1$ receptors or Ca$^{2+}$ channels. In order to investigate the possible role of $\beta_1$ receptors, both the extracts were tested in the presence of propranolol. The results revealed that the positive inotropic and chronotropic effects were significantly reversed to a decrease indicating an adrenaline like activity. These effects were in accordance with the prior studies (13).

The rise in PP in the presence of propranolol suggested that the constriction of coronary blood vessels might be due to the involvement of Ca$^{2+}$ channels or $\alpha$-receptors. In order to determine the possible role of Ca$^{2+}$ channels, verapamil was used to block these channels and then the crude extract was tested in the presence of verapamil. The extracts produced a significant reduction in PP, which was in agreement with previous studies (14). This effect might be due to the Ca$^{2+}$ entry through the voltage-dependent Ca$^{2+}$ channels. However, the role of $\alpha$-receptors should also be studied in order to determine the exact mechanism of action.

The preliminary phytochemical analysis has shown that the extract contains certain important chemical constituents that might be involved in an increase of all the three parameters. The methanolic extract of RML contains compounds such as alkaloids, flavonoids, cardiac glycosides, tannins, steroids and indole alkaloids. Previous studies have indicated that cardiac glycosides and steroids appear to be involved in a positive inotropic activity (15). It has been reported that certain alkaloids might be involved in the cardiotonic effect. Moreover, glycoalkaloids have also been known to exert their cardiotonic activities in isolated frog heart (16, 17). Cardiac glycosides, on the other hand, are known to work by inhibiting the Na+/K+ pump. This inhibition increases the level of Ca$^{2+}$ ions available for contraction of the heart muscle, which improves cardiac output and reduces distention of heart (18). Similarly, plant steroids are known to be effective for their cardiotonic activities (19). It has also been documented that tannins also appear to exert a significant cardiotonic activity in frog heart. They form insoluble calcium salts and the stimulant action depends on the presence of Ca$^{2+}$ in the perfusion fluid. It could be inferred from these studies that methanolic extract of RML possesses certain active phytochemical constituents that might be responsible for their cardiotonic activity in isolated rabbit heart. Moreover, tannins present in the extract might be involved in a rise in perfusion pressure as reported in previous studies (20).

CONCLUSION

It is concluded from the study that various biologically active compounds in the methanolic extracts of RML has produced positive inotropic and chronotropic effects on Langendorff’s isolated rab-
bit heart preparation. The results suggested that active principle(s) responsible for positive inotropic and chronotropic effects of RML extract act by β1 receptors and Ca2+ channels. However, further studies are required to isolate these pharmacologically active phytochemical constituents and elucidate their exact mechanism of action.

REFERENCES


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