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EFFECT OF PHYSOSTIGMINE ON CARDIOTONIC ACTIVITY OF PAROTOID GLAND SECRETION OF *BUFO MELANOSTICTUS* (SCHNEIDER) ON ISOLATED HEART OF FROG

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ABSTRACT

The present investigation was carried out to study the cardiotonic activity of parotoid gland secretion of common Indian Toad *Bufo melanostictus* on isolated perfused frog heart (*Rana tigrina*). The cardiotonic activity of parotoid gland secretion of *B. melanostictus* in normal as well as when induced with physostigmine (eserine) (an organophosphate) was studied. The cardiotonic activity was studied through Syme's technique; digoxin and propranolol were used as standard drug and β-blocker respectively to characterize the effects on the receptors. The isolated perfused and hypodynamic heart of frog showed a dose dependent positive ionotropic effects. Cardiac stimulant activities were exhibited by the parotoid gland secretion on frog's heart. Propranolol was unable to block the effect of physostigmine on toad parotoid gland secretion.Thus, the present investigation reports that the parotoid gland secretion increased the force of contraction, heart beat and cardiac output in perfused frog's heart, whereas, there was no change on hypodynamic heart, indicating that there may be existence of two components, one with β–receptor stimulating activity and other acting directly on the frog's heart (independent of β_1-adrenoreceptors).

KEY WORDS: *Bufo melanostictus*, *Rana tigrina*, Cardiotonic activity, Parotoid gland secretion, Physostigmine.

INTRODUCTION

Amphibians are treated as bioindicators of aquatic and terrestrial ecosystems by means of their sensitivity towards environmental changes and are the most threatened and rapidly declining vertebrate groups disappearing from different habitats on a global scale. This decline is mainly because of extensive use of agrochemicals especially the pesticides. Amphibian skin is morphologically, biochemically and physiologically a complex organ possessing a wide range of functions necessary for their survival. Amphibians secrete a wide range of substances belonging to many chemical classes with varied pharmacological activities. Many of these compounds could serve the frog in defense as chemical irritants secondary to a physiological role.

In recent years there has been an increased interest in search of new pharmaceutical compounds of natural origin has intensified and been extended to include sources other than plant material[1]. The alarm/defense secretions of Amphibians also exhibit a number of pharmacological activities and quite a good number of pharmacologically active substances like pahutoxin [2], bufotenin and bufotoxins[3], biogenic amines[4] , bioactive peptides[5,6,7] have been isolated in purified form from the skins of amphibians.

Amphibian skin plays an important role in defense mechanism and is characterized by the presence of cutaneous glands namely mucous glands secreting mucus known to be glycoproteinaceous, functioning as a lubricant and also necessary for cutaneous respiration and granular glands (poison glands) which secrete serous that protects the skin from mechanical damages, prevent microbial settlement on the skin and also provide protection from predators such as birds, mammals, snakes and crocodiles[.8,9,10,11].

The parotoid gland of the *Bufo* species has been known to secrete venomous secretions rich in several bio active compounds like bufotenins, bufalins and bufotoxins[3] and were used as drugs in the treatment of heamorrhage of gums, sinusitis and other systemic illnesses, including cardiac disorders by Chinese and Japanese physiscians in folk medicine and is very popular with a name of “Chan Su” [12,13] . The toad venom especially bufotenin secreted by these glands was a subject of mythological, pharmacological and medicinal practices for centuries [14].

In view of the importance of bioactive compounds present in secretions of parotoid glands to pharmacology, especially to heart physiology, an investigation was carried out on the effect of parotoid gland secretion of toad *Bufo melanostictus*, on isolated hearts of frog.

MATERIALS AND METHODS

Studies on Heart beat

The toads (7cm to 10 cm in length, weighed about 50 to 70 gm.) were collected from the vicinity of Kakatiya University hostel buildings, Warangal, Telangana State, India. The parotoid glands were gently pressed with the help of sterile forceps to release the secretions according to Meyer and Linde[15]and these secretions were collected into ice-jacketed containers. The secretions were weighed to nearest milligram. They were stored in cool conditions until further analysis. The secretion and the glands were weighed to the nearest milligram and were homogenized (10%) in cold 0.9% of normal saline or frog Ringer's solution. The secretions were vortexed for 5 minutes and centrifuged at 2000 rpm for 20 minutes. The freshly collected compounds were dissolved in normal saline/frog ringer's solution to study the effect on isolated hearts of freshly sacrified *Rana tigrina*. The isolated heart mounted on to Syme's cannula [16] was used for the study.

Preparation of isolated hearts for experiment:

Frog (*Rana tigrina*) was stunned by head-blow using a steel rod and pithed. The skin and abdomen were cut and opened. The pectoral girdle was cut using a bone cutter and pericardium was cut and removed. Syme`s cannula was connected to the reservoir of frog ringers solution and introduced was immediately introduced into the sinus venosus of the heart through posterior venacava after pericardium and the connecting blood vessels were removed and the heart was removed from the animal and mounted on to the stand. The heart was covered with thin layer of cotton and was wetted continuously with frog ringer's solution to prevent the drying of the tissue. The heart was connected to the starting lever, which was in turn connected to kymograph drum for recording the heart beat. The flow of ringer's solution into Syme's cannula was maintained by fixing a glass tube into the cork fixed to the reservoir (Marriott bottle) tightly. The heart was allowed to stabilize and when the heart rate and cardiac output were stabilized, the recordings made on a slowly rotating drum to which sooted kymograph paper was affixed. The effect of toad parotoid gland secretion *per se* (in ringer) were studied on isolated perfused frog hearts. The parameters studied included the force of contraction, heart rate and cardiac output. The volume of frog ringer's solution rate and cardiac output. The volume of frog Ringer's solution coming out of the heart per minute (Cardiac output) was measured by collecting the solution into a measuring jar. The heart rate was measured by counting the number of heart beats per minute. Minimum 5 min time was allowed between the additions of parotoid exudates *per se* Combined the paragraph (in

Ringer) and its fraction. When blocker was used, it was diluted with known amount of the physiological solution in syringe itself and added slowly.

The heart rate (HR), cardiac output (CO) and force of contraction were the parameters used for the study. The solutions of parotoid gland secretion *per se* and the extracted fractions were prepared in frog ringer's solution. No suspending agents were used. The heart was constantly moistened with frog's ringer solutions from time to time.

Hypo Dynamic Frog's Heart

An isolated frog heart preparation as described under Syme's technique was set up Meyer and Linde[15]. Instead of one reservoir, two reservoirs each for ½ calcium and full calcium were used. The levels in the reservoir maintained constantly, which was tested by connecting each of the reservoirs to the Syme's cannula.

Experiments were conducted by rendering the frog hypodynamic heart by letting into heart, frog ringer's containing ½ calcium from another reservoir through syme's cannula. Force of contraction was monitored to give half the magnitude of normal force of contraction. The effects of parotoid gland secretion on hypo dynamic heart were determined by using frog ringer's solution with ½ calcium concentration. Propranolol, the non specific β-blocker was used to characterize the effects on the receptors.

Studies were conducted on 6 animals and each time collections from individual toads were used to see the reproducibility of the effects. Samples were given as spot dose into Syme's canula through a tuberculin syringe. The heart rate and the cardiac output were measured simultaneously. Dose-dependent effects of the parotoid gland secretion were noted to get optimum results.

Preparation of digoxin solution

The marketed digoxin test samples (Sunpharma Ltd.) were obtained from local market. Various different dilutions were made with distilled water and labeled as follows, B1- 25 µg/ml**,** B2- 50 µg/ml. above prepared samples were evaluated for their cardiotonic activity and treated as standard.

Heading : Composition and preparation of hypodynamic ringer's solution.

Hypodynamic ringer solution was prepared by using standard method[17]

S. No.	Ingredients	Quantity
1	Sodium Chloride (NaCl)	6.5 gm
2	Potassium Chloride (KCl)	0.14 gm
3	Calcium Chloride ($CaCl_2$)	0.03 gm
4	Sodium bicarbonate ($NaHCO_3$)	0.2 gm
5	Glucose	2.0 gm
6	Distilled Water	1000 ml

Preparation of OP Compound concentrations for induction to study Pharmacological studies [18]

To observe the pharmacological effects of parotoid gland secretion after exposure to OP compound, ($2X10^{-5}M$)) concentration of Physostigmine was used. The OP compound concentrations and normal saline were induced sub-cutaneously into parotoid gland contra laterally. The *in vivo* effects of Physostigmine, on cardiotonic activity of parotoid gland secretion were noticed after 4 hours time interval.

RESULTS AND DISCUSSION

The normal parotoid gland secretion i.e., without the induction of Physostigmine showed dose dependent changes on hypodynamic heart as well as on isolated heart of frog in all parameters such as heart rate (HR), cardiac output (CO) and percentage of increase in force of contraction (% IFC). The normal parotoid gland secretion showed dose dependent changes, and the results were observed as 4 beats/min (HR), 10 ml/min (CO). At 0.1 ml (50µg) dose, the heart rate was noted as 40 beats/min (HR), a cardiac output of 12 ml/min (CO) and with an increase of 50% in force of contraction, while at 0.2 ml (100µg) dose, the results obtained were 38 beats/min (HR), 15 ml/min (CO) and 70% of increase in force of contraction (% IFC). At 0.3 ml (150µg) dose, results were 36 beats/min (HR), 16 ml/min (CO) and 90% of increase in force of contraction, while at the standard digoxin dose (1000ng), the results were 38 beats/min (HR), 16 ml/min (CO) and 30% increase in force of contraction. At the maximum concentration of propranolol and the maximum dose of parotoid gland secretion i.e., at 0.3 ml (150µg), the results obtained were 38 beats/min (HR), 14 ml/min (CO) and 20% increase in force of contraction which revealed that the Propranolol was unable to block the response of parotoid exudates *per se* (in Ringer) totally.

Effect of Physostigmine

The physostigmine (OP) induced parotoid gland exudates revealed that the cardiac output and increase in force of contraction (the positive inotropic effect) *per se* (in Ringer) increased on isolated heart and hypodynamic heart (**Table, Figure and Graph. 2**) compared to non induced parotoid gland exudates (**Table, Figure and Graph. 1**).

On hypodynamic heart, physostigmine induced parotoid gland secretion has showed the dose dependent changes and results were observed as 52 beats/min (HR), 10 ml/min (CO), physostigmine induced parotoid gland secretions showed dose dependent changes at concentration 0.1 ml (50µg) and results were 49 beats/min (HR), 13 ml/min (CO) and 40% increase in force of contraction (% IFC) and at concentration 0.2 ml (100µg), results were found to be 46 beats/min (HR), 15 ml/min (CO) and 60% increase in force of contraction (% IFC), whereas at concentration 0.3 ml (150µg) results were observed as 44 beats/min (HR), 16 ml/min (CO) and 80% increase in force of contraction (% IFC). The standard digoxin (400ng) results were 42 beats/min (HR), 14 ml/min (CO) and 10% increase in force of contraction (% IFC). In the presence of Propranolol and at the maximum dose of physostigmine induced parotoid gland secretion i.e., at 0.3 ml (150µg), results were found to be 49 beats/min (HR), 13 ml/min (CO) and 30% increase in force of contraction (% IFC) and revealed that the Propranolol was not able to block the response of parotoid exudates *per se* (in Ringer) totally **(Figure, Table and Graph-2)**.

Table-1 Effect of parotoid gland secretion on isolated frog heart (the parameters include the force of contraction, heart rate and cardiac output.)

Dose	**Result**			
On Normal Heart	HR (beats/min)	CO (ml/min)	Increase in force of contraction (in mm)	% Increase in force of contraction
Normal	48	12.5	-	-
0.1 ml(50 µg)	50	13	5	50
0.2 ml(100 µg)	52	13	3	30
0.3 ml(150 µg)	54	13	2	20
Digoxin 400 ng	48	12	0.5	5
Digoxin 1000 ng	48	14	1	10
On Hypodynamic heart				
Normal	42	10	-	-
0.1 ml(50 µg)	40	12	5	50
0.2 ml(100 µg)	38	15	7	70
0.3 ml(150 µg)	36	16	9	90
Digoxin 1000 ng	38	16	3	30
PP+0.3ml(150 µg)	38	14	2	20
Normal	42	11	-	-

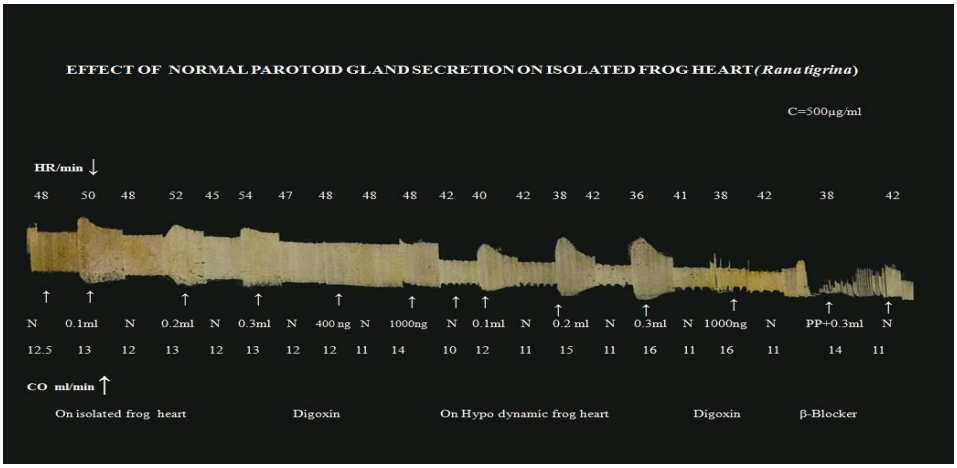
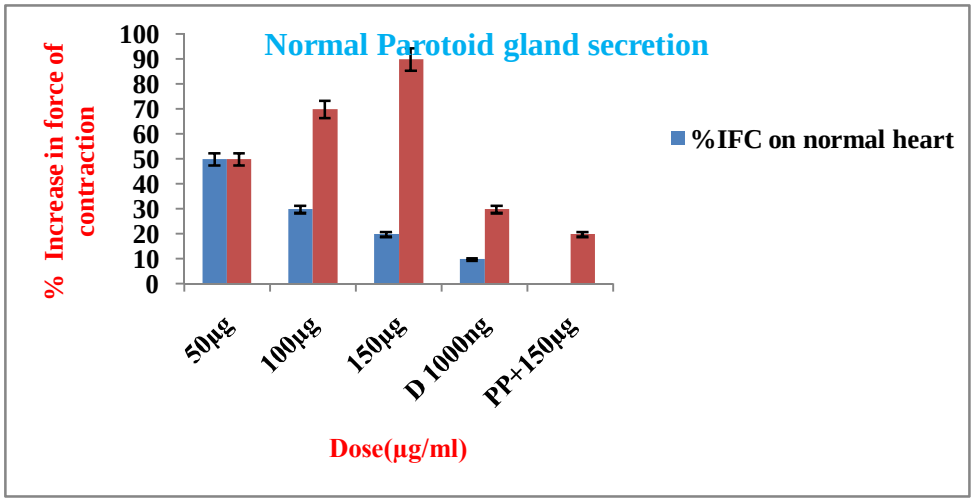


Figure-1 Effect of parotoid gland secretion on isolated frog heart (the parameters includes the force of contraction, heart rate and cardiac output).



Graph-1 Effect of parotoid gland secretion on isolated frog heart elicited a dose dependent increase in force of contraction (positive inotropic effect).

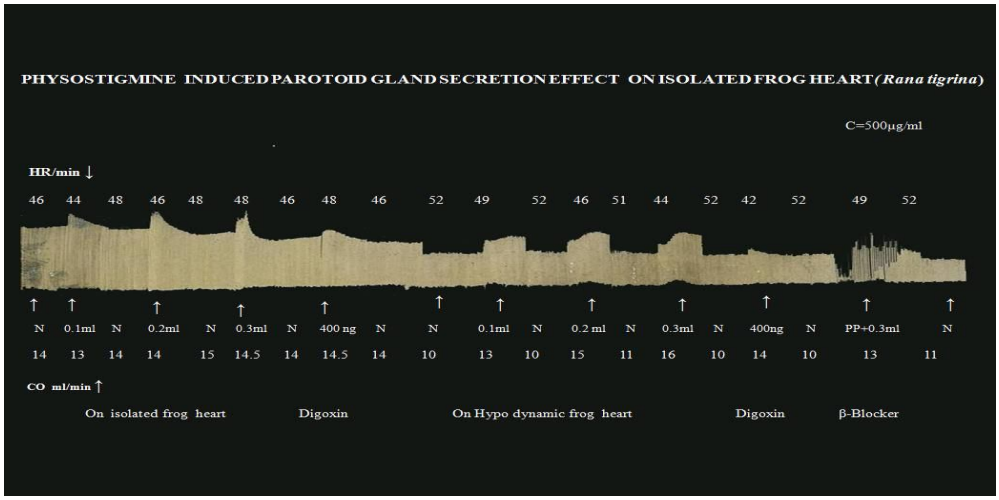


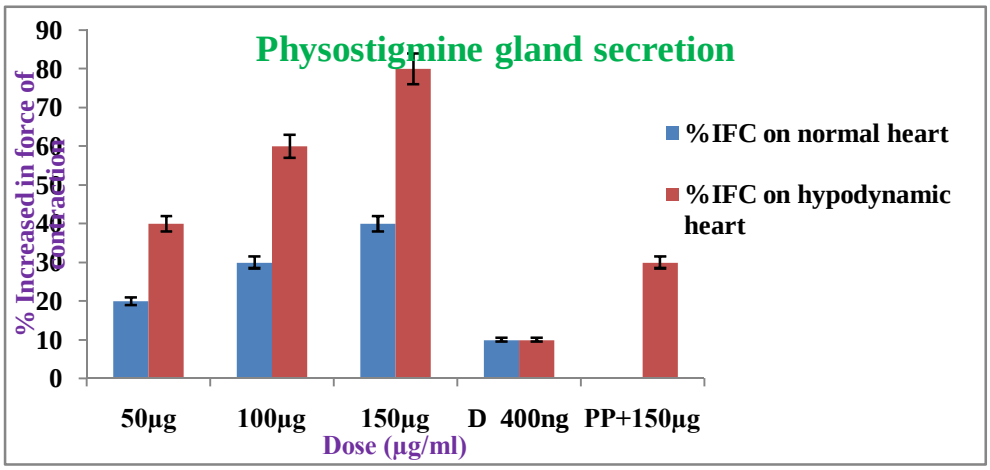
Figure-2 Effect of Physostigmine induced parotoid gland secretion on isolated frog heart (the parameters includes the force of contraction, heart rate and cardiac output).

Table-2 Effect of Physostigmine induced parotoid gland secretion on isolated frog heart elicited a dose dependent increase in force of contraction (positive ionotropic effect).

Dose	Result			
On Normal Heart	**HR (beats/min)**	**CO (ml/min)**	**Increase in force of contraction (in mm)**	**% Increase in force of contraction**
Normal	46	14	-	-
0.1 ml(50 µg)	44	13	2	20
0.2 ml(100 µg)	46	14	3	30
0.3 ml(150 µg)	48	14.5	4	40
Digoxin 400 ng	48	14.5	1	10
On Hypodynamic heart				
Normal	52	10	-	-
0.1 ml(50 µg)	49	13	4	40
0.2 ml(100 µg)	46	15	6	60
0.3 ml(150 µg)	44	16	8	80
Digoxin 400 ng	42	14	1	10
PP+0.3ml(150 µg)	49	13	3	30
Normal	52	11	-	-

The bufotenins, bufalins, bufotoxins of toads [3] and bufalins present as free genins or as conjugates of steroids in these secretions were reported to be similar to digitalis in their action. Further studies of [19]Thomos, 1996 suggested that cardiotonic compounds affect the force of contraction without affecting the heart rate significantly. The results obtained in the present investigation are consistent with above observations. The parotoid gland secretion exhibited the cardiac stimulant activities [20, 21, 22].

The OP compounds like physostigmine could not inhibit the cardiotonic activity of parotoid gland exudates but can inhibit the level of biochemical constituents and esterase activities [23].



Graph -2 Effect of Physostigmine induced parotoid gland secretion on isolated frog heart elicited a dose dependent increase in force of contraction (positive ionotropic effect).

It has been reported that amphibian skin is a rich source of bioactive compounds with potential therapeutic activities such as antibacterial, antifungal, antiprotozoal, antineoplastic, antidiabetic, analgesic and also sleep inducing[12] and proved to be useful in treatment of various ailments encountered during cardiac arrest and also in neurological disorders[24] .

The vertebrates, including humans are sensitive towards bufonid poisons[25,26] which possess cardiotonic, convulsive and also hallucinogenic activities[27]. As the cardiac disorders are of serious medical concern and are increasing throughout the world [28],various drugs used to treat congestive heart failure have several side effects**,** hence there is a need of development of pharmacologically and therapeutically significant drugs of biological origin without side effects.

Thus, from our present investigation it is reported that the parotoid gland secretion of toad *B. melanostictus* exhibited positive ionotrophic effects on isolated heart of frog *R. tigrina* which is evidenced through the kymograph including different parameters such as heart beat rate, cardiac output and force of contraction and hence can be used as a potent source of medicine and employed in the treatment of cardiac disorders.

CONCLUSION

It can be concluded from our present investigation that the parotoid gland secretion being rich in bioactive components showed cardiotonic activity on isolated frog's heart, this is a preliminary study and requires studies to evaluate traditional use and also to isolate the active chemical constituents which are responsible for the cardiotonic activity as well as to determine the possible mechanism of action.

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