Effect of the Aqueous Extract of Seeds of Casimiroa Edulis and Certain Drug Combinations on Cardiac Contractility, Phosphorilase$_a$ and Adenyl Cyclase Activation in Isolated Perfused Mammalian Heart

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Abstract: *Objective: To examine the hypotensive effect of the aqueous extract of the seeds of the plant casimiroa edulis on mammalian heart and vasculatiure. Materials and Methods: Studies were done on cardiac contractility, phosphorilase and adenyl cyclase in male Dunkin Hartley guinea-pigs weighing between 300-400 g. on perfused heart by the Langendorff technique. Results: Positive inotropic and chronotropic effects were observed mediated by cyclic 3', 5' - AMP, which were not abolished by beta adrenergic-blocking agents. These results further indicate that casimiroa edulis has the capacity to activate adenyl cyclase in particulate fractions of guinea-pig heart. Conclusion: These results suggest that there are separate adenyl cyclase systems for casimiroa edulis and nor epinephrine. However, definite proof would require physical separation of the two enzymes. The simultaneous use of H_1-and H_2-receptor agonists and antagonists thus provides a means for effective and selective inhibition of the cardiovascular actions of casimiroa edulis.*

Keywords: Casimiroa edulis, Adenyl cyclase, Mammalian heart

1. Introduction

Plants are described as the major source of medicines, not only as isolated active principle to be dispensed in standardized dosage forms but also as crude drugs [1]. Cardiovascular ailments particularly hypertension is a very widespread condition which is not strictly considered as an illness but if not countered, progressively causes damage to all tissues and loss in their functionality. For this reason the find of new antihypertensive agents is prominent and medicinal plants and their derivatives are valuable for the purpose.

Mode of action of chemical constituents of plants which are having the known effects on hemodynamic and other cardiac parameters is now established. Effects of leaf extract from Clerodendron Colebrookianum have been found to decrease blood pressure in dose-dependent manner [2]. Effects of extract from Cleroden trichotonum on blood pressure and renal function have been studied by Lu Gw et al [3]. Intravenous injections of gambirine have shown a dose related fall in both systolic and diastolic blood pressure as well as heart rate [4]. Intravenous injections of the active component of the extract of Salviae miltiorrhizae radix (Den-Shen) have shown decreased blood pressure in a dose-dependant manner in rats [5]. Yang et al [6], studied the cardiovascular effects of Dehydro evodiamine, an alkaloid isolated from Evodia ruteacarpajussieu both *in vivo & vitro* experiments. Pharmacodynamic studies on aqueous extract of the root of Polypodium vulgare have shown a positive inotropic and chronotropic effect on perfused frog heart by causing hypotension and tachycardia in anesthetized dogs [7].

In the present study we have tried to investigate the cardiovascular profile of the aqueous extracts of the seeds of Casimiroa-edulis Lia Llave et Lex, the "Zapote blano" (white Zapote) a tropical plant belongs to a species of tropical fruiting tree in the family Rutaceae, native to eastern Mexico and Central America used by Mexican herb medicine since remote times [8]. Regional Mexican studies on the pharmacological effects of the seed's aqueous extract have shown the hypotensive quality of the plant extract [9, 10].

This pioneer phytochemical work was later followed by studies on Casimiroa edulis [11, 12, 13, 14, 15, 16, 17, 18] trying to corroborate the hypnotic properties attributed to this sweet fruit.

Lozoya et al [19], Ortega et al [20], have confirmed the vigorous hypotensive effect produced by the aqueous and alcoholic extract from Casimiroa edulis seeds on cats, rabbits and dogs, together with the constrictor effect produced on the uterus by *in vitro* experiments on several animal species including human.

From the literature cited above, it is now clear that the seeds and leaves of the plant Casimiroa edulis have been used in Folk medicine as an hypotonic and sedative and more recently as an antihypertensive. Effects of aqueous extract of casimiroa edulis (Rutacea) on blood pressure and heart rate in albino rats has been reported by Garcia et al [21]. These studies demonstrated a rapid and transitory increase in blood pressure. The amplitude of the blood pressure rise was dose dependent.
Endothelium-dependent vasorelaxing activity of aqueous extracts of lyophilized seeds of Casimiroa edulis on rat

mesenteric arterial bed has been reported in the literature by Baisch et al [22].

Phytochemical studies have shown that the leaf essential oil of Casimiroa edulis was dominated by sesquiterpene hydrocarbons, predominately germacrene D (16-22%) and (E)-caryophyllene (16-17%), consistent with the traditional use of this plant as a sedative, sleep inducer and hypotensive [23].

The hexanic and methanolic extracts of Casimiroa edulis showed vasorelaxation in arterial tissues in rat precontracted by phenylephrine (0.5 μM); the extracts from seeds always caused a greater relaxation in comparison to those from leaves [24].

Recently age-dependent vasorelaxation of Casimiroa edulis and Casimiroa pubescens extracts in rat caudal artery in vitro has been reported, thus supporting the traditional use of Casimiroa decoctions as antihypertensive, also in elderly [25].

More recently Bertin et al [26] have reported that the phenolic compounds isolated from Casimiroa have shown vasorelaxation on rat arterial tissues although with different effectiveness thus suggesting its possible role against hypertension and vasculopathies.

Further more phenolic compounds from leaves of Casimiroa edulis showed adipogenesis activity as well [27].

In more than fifty years of active research around 20 different substances were obtained from the seeds, leaves and barks of Casimiroa edulis [28] and some of their structures were elucidated but the hypogenic active principle was never established and/or published.

As a first step in the characterization of the hypotensive activity of Casimiroa edulis, the present study has been carried out using animal model. Included in the study is the determination of the effects of the aqueous seed extract of casimiroa edulis, the part of the plant reported to be more active [29, 17, 18] on cardiac contractility, phosphorilase and adenyl cyclase in male Dunkin Hartley guinea-pigs which may be helpful for the identification of active compounds of the fraction of the crude extract for further pharmacological evaluation to determine its influence in diverse hemodynamic parameters.

2. Materials and Methods

Preparation of casimiroa edulis extract

The aqueous extract of casimiroa edulis seeds was prepared as described previously [17]. Briefly ripe fruits of casimiroa edulis were obtained from local markets and after being defatted, the dry powdered kernels were extracted successfully by maceration at room temperature with hexane, dichloromethane, 4:1 and 1:1 mixtures of dichloromethane-methanol, methanol and finally water. Organic solvents were eliminated in a rotatory evaporator; water was removed by lyophilization. For the pharmacological tests, the solid aqueous extract thus obtained (approximately yield: 5.5%) was dissolved in isotonic NaCl solution to a concentration of 100

Effects of the aqueous seed extract of casimiroa edulis were studies on cardiac contractility, phosphorilase and adenyl cyclase in male Dunkin Hartley guinea-pigs weighing between 300-400 g. Animals were injected with heparin sodium 8mg/kg, s.c.) 60 minutes prior to sacrifice. The animals were killed and the heart was rapidly removed and perfused by the Langendorff technique with chenoweth-koelle solution by the established method [30], modified by Bell et al [31] at a flow rate of 3.6 ml/min. The flow rate was maintained by a peristaltic pump. The perfusion solution was equilibrated with a 95% O2-5% CO2 gas mixture at 37 C0. Contractility was monitored by means of a Palmer clip placed in the apex of the heart and connected to a Grass force displacement transducer and recorded on a Grass model 7 polygraph. Diastolic tension was adjusted to 5 g. The hearts were allowed to equilibrate for 25 minutes. At this time two dose-response curves to casimiroa edulis were obtained by injecting the extract via a sidearm cannula. The hearts were then perfused with chenoweth-koelle buffer solution containing theophylline (10-3M). Following a 5 minute equilibration period the extract dose-response curves were repeated. The dose-response curves thus obtained were averaged and plotted as a percentage of the maximum response that could be obtained with casimiroa edulis.

For the phosphorilase experiments the hearts were perfused as noted above with either buffer or buffer plus 10-3M-theophylline. Extract was then injected via the sidearm cannula and the heart was frozen at the peak of the contractile response by means of a pair of Wollenberger tongs [32] previously chilled in a mixture of alcohol-dry ice. An 80 to 100 mg portion of the apex was cut away and phosphorylase activity measured in the direction of glycogen synthesis as previously described [33].

Since total enzyme activity did not change, all results are expressed as % phosphorylase a which is: [(enzyme activity without AMP)/ (enzyme activity with AMP)] X 100.

For adenyl cyclase experiments a washed particulate preparation of guinea pig heart was used as the source of the enzyme. Enzyme activity was determined by measuring the conversion of [14C]-ATP to [14C]-cyclic AMP as previously described [34]. The enzyme was prepared and activity measured on the same day. Enzyme activity is expressed as pmol cyclic AMP produced/4/min/mg protein. Protein was determined by the established method [35, 36].

Drugs and chemicals used were, 1-norepinephrine bitartrate (Winthrop Lab.; theophylline (Merck and Co.); heparin sodium (Nutritional Biochemicals Corp.); phenoxybenzamine (SK&F); tolazoline HCl (Ciba Pharm. Co.); sodium flouride (Mallinckrodt); diphenhydramine HCl (Parke Davis and Co.); tripelennamine HCl (Ciba Pharm. Co.); propranolol (Ayerst Lab. Inc.); 1-epinephrine bitartrate (Winthrop Lab.); imidazole (General Biochemicals); 1-histidine (sigma Chem. Co.); betazole HCl and 3-(B-aminoethyl)-1,2,4-triazole dihydrochloride (Eli Lilly and Co.).

3. Results

As shown in Figure.1, casimiroa edulis increased the contractility of the isolated perfused guinea pig heart. The

positive inotropic effect of extract was significantly enhanced (P<0.05) by theophylline in a concentration of either 10^{-4} or 10^{-3}M. Casimiroa edulis also increased the activity of phosphorylase$_a$ in the heart (Table.1). Enzyme activation by the casimiroa edulis was also enhanced by theophylline.

Casimiroa edulis also proved capable of stimulating cardiac adenyl cyclase as is shown in Figure 2, whereas epinephrine approximately doubled the enzyme activity.

When injected into the perfused guinea pig heart casimiroa edulis (0.8Ug) increased the force of contraction. Tripelennamine (3 x 10^{-6} M) did not decrease the casimiroa edulis effect. The higher concentration of tripelennamine (10^{-4} M) decreased the extract response. However, at this concentration, tripelennamine was cardiotoxic and could only be perfused through the heart for 4 minute. Longer persuasion times result in cardiac arrest. (Table 2). The effect of the antihistamine tripelennamine on casimiroa edulis induced activation of cardiac phosphorylase$a$ is demonstrated in Table 3. Again tripelennamine (3x 10^{-6} M) did not reduce the response while tripelennamine (10^{-4} M) significantly reduced the activation of the enzyme. Blockade of the extract response by the higher concentration of tripelennamine appears to be a relatively non-specific phenomenon since the activation of the enzyme by isoproterenol was also blocked by tripelennamine (10^{-4} M).

All remaining experiments were concerned with the study of various drugs on cardiac adenyl cyclase in an attempt to characterize the active site for casimiroa edulis and to compare these effects with those obtained in the whole heart. Two antihistamines, diphenhydramine and tripelennamine, were used as potential antagonists of the extract. Diphenhydramine (Table.4) only slightly decreased the stimulation of adenyl cyclase produced by 10^{-6}M extract when tested at a concentration of 10^{-6}M. Total blockade of the extract response was achieved with a concentration of 10^{-4} M diphenhydramine. Diphenhydramine (10^{-5} M) lowered the maximum response to the extract stimulation of adenyl cyclase (Figure.3). Similar results were obtained with tripelennamine except that there was absolutely no decrease in the extract response with 10^{-6} M-tripelennamine (Table.5). The apparent non-specificity of the blockade with these drugs at the higher concentration (10^{-4} M) is demonstrated in the experiments with propranolol (Table. 6). Propranolol produced a decrease in the casimiroa edulis response similar to that obtained with the antihistamines. Propranolol, however, proved very capable of specifically blocking the epinephrine stimulation of cardiac adenyl cyclase (Table.7). In this experiment propranolol (10^{-5} M) blocked the stimulation produced by epinephrine (10^{-4} M) 90% whereas tripelennamine (10^{-5} M) did not decrease the epinephrine response at all. The alpha--adrenergic blocking agents phenoxy-benzamine and tolazoline also did not decrease the casimiroa edulis response when used in a concentration of 10^{-6} M indicating that alpha-receptors are also not involved. None of the blocking agents tested in concentrations of 10^{-5} M affected the stimulation of the enzyme produced by sodium fluoride (10^{-2} M) (Table.8).

4. Discussion

The data presented demonstrate that, in the guinea-pig

Casimiroa edulis has a positive inotropic effect and can increase the activity of cardiac phosphorylase a in intact hearts. Casimiroa edulis can also increase the activity of cardiac adenyl cyclase in a washed particulate preparation from guinea-pig. Both the inotropic effect and the phosphorylase activating effect of extract were greatly enhanced by the theophylline. While these data do not prove a cause and effect relationship they are at least suggestive that Casimiroa edulis produces its effects on the heart by stimulating adenyl cyclase thus increasing the intracellular concentration of cyclic AMP as has been suggested by Lein et al [37].

The antihistamine tripelennamine did not prove to be an effective antagonist of either the mechanical or biochemical effects of Casimiroa edulis on the heart. An extremely high concentration (10^{-4}M) of antihistamine was effective in significantly decreasing the inotropic or phosphorylase activating effect of the extract. At this concentration tripelennamine was cardiotoxic and also blocked the effect of isoproterenol on the heart (Tables 5 and 6). The results support the work of Hattori et al [38] who suggested that the cardiac histamine receptor differed from other histamine receptors since it could not be readily blocked by antihistamines. Similar effects were noted when drug interactions were studied on cardiac adenyl cyclase. Low doses of antihistamines (10^{-6}M) or propranolol (10^{-6}M) had little or no effect on casimiroa edulis extract induced histamine-like-activation of the enzyme. With both the antihistamines and the B-blocker, however, higher concentrations (10^{-4}M) completely blocked the extract response. This latter concentration is cardiotoxic in intact hearts and again demonstrates the non-specificity of the blockade. Similar results have been reported in isolated heart preparations [39]. Phenoxybenzamine and tolazoline, both alpha-adrenergic blocking agents, also failed to decrease the Casimiroa edulis response. The blocking agents did not appear to affect stimulation of the enzyme by sodium fluoride (Table-8). This would suggest that their actions are confined to the regulatory subunit since fluoride is believed to activate the catalytic site only [40].

In the present study propranolol was found to be a selective inhibitor of the epinephrine stimulation of cardiac adenyl cyclase whereas the antihistamine antagonism appeared to be less specific. These data suggest that Casimiroa edulis and epinephrine can stimulate adenyl cyclase by interacting with separate receptors. Further experiments tended to support this hypothesis. Epinephrine and Casimiroa edulis when combined together at maximal stimulatory concentrations tended to activate adenyl cyclase to a slightly greater extent than did extract alone. One possible explanation for this discrepancy is that greater stimulation due to extract alone was found in the present study than was reported by Klein and Levey [41]. Therefore it is possible that a near maximal stimulation due to an interaction of Casimiroa edulis with the regulatory subunit of the enzyme prevented a greater interaction when the two agonists were combined. Nevertheless the conclusions from the data are the same as those of previous workers [41] i.e. that separate and distinct receptors exist for the two agonists. This hypothesis was further strengthened by the experiments using the histamine analogs betazole and the triazole derivative. The combination of either of these compounds with epinephrine resulted in an increase in enzyme activity over that obtained with either drug alone. In addition betazole decreased the

extract response to a value intermediate between that obtained with the Casimiroa edulis and betazole indicating competition for the same site. In our experiments none of the cardiac effects of the extract were antagonized well by tripelennamine. In the present study it was found that the diphenhydramine was not that specific in blocking the histamine like activation of adenyl cyclase produced by extract. While a concentration of 10^{-4}M did block the histamine response it also reduced epinephrine stimulation of the enzyme although to a lesser degree. Diphenhydramine (10^{-5}M) appeared to lower the maximum response to Casimiroa edulis more than shift the extract dose-response curve to the right. It was also demonstrated that at 10^{-4}M propranolol was capable of reducing the Casimiroa edulis response and further supports the hypothesis that the cardiac histamine receptor differs from other histamine receptors [42] and appears that there are at least three regulatory sites on the regulatory subunit of cardiac adenyl cyclase.

Activation of any one of the regulatory sites ultimately leads to activation of the catalytic site. It is postulated that activation of any of the regulatory sites must follow a final common pathway leading to an increase in catalytic site activity since combinations of norepinephrine and glucagon or histamine and epinephrine did not result in additive stimulation as reported previously [43].

Moura et al [44], however, were unable to demonstrate stimulation with thyroid hormone in enzyme obtained from rat heart. The tool to clarify the role of histaminergic effect of casimiroa edulis in the cardiovascular system are thus established to some extent and progress in this area can be anticipated with interest.

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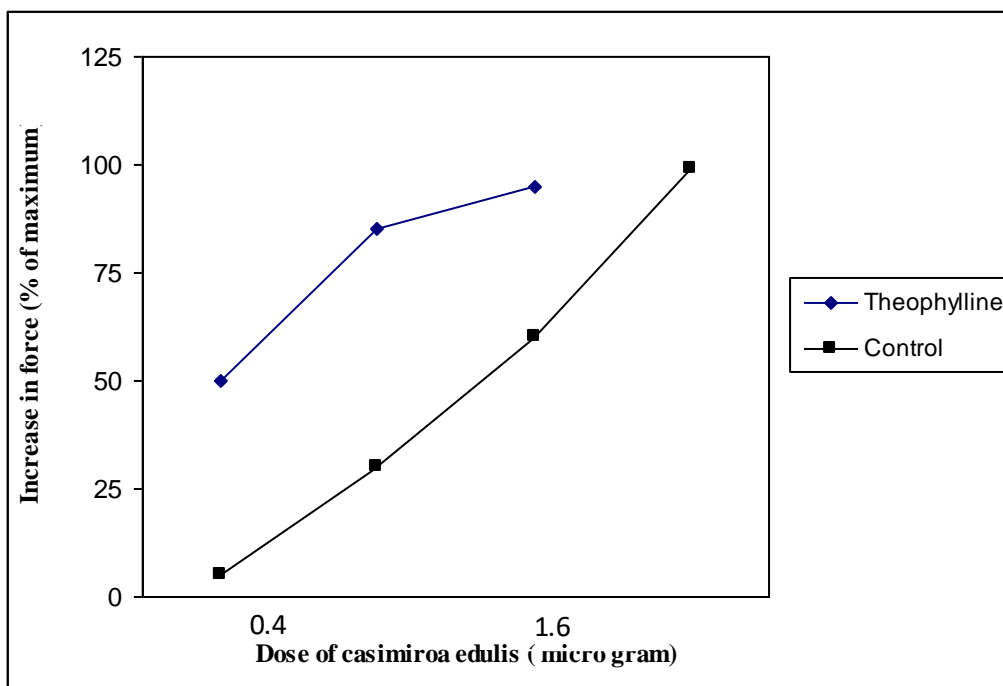
Figure 1A: Effect of casimiroa edulis on cardiac contractile force in the presence and absence of theophylline 10^{-3} M in guinea-pig hearts ($n = 50$). The increase in the force is expressed as a percentage of the maximum response obtained with casimiroa edulis. n represents sample number. The standard errors, in all cases were less than the area covered by the circle.

Figure 1B: Effect of casimiroa edulis on cardiac contractile force in the presence and absence of theophylline 10^{-4} M in guinea-pig hearts (*n= 50*). The increase in the force is expressed as a percentage of the maximum response obtained with casimiroa edulis. *n* represents sample number. The standard errors, in all cases were less than the area covered by the circle.
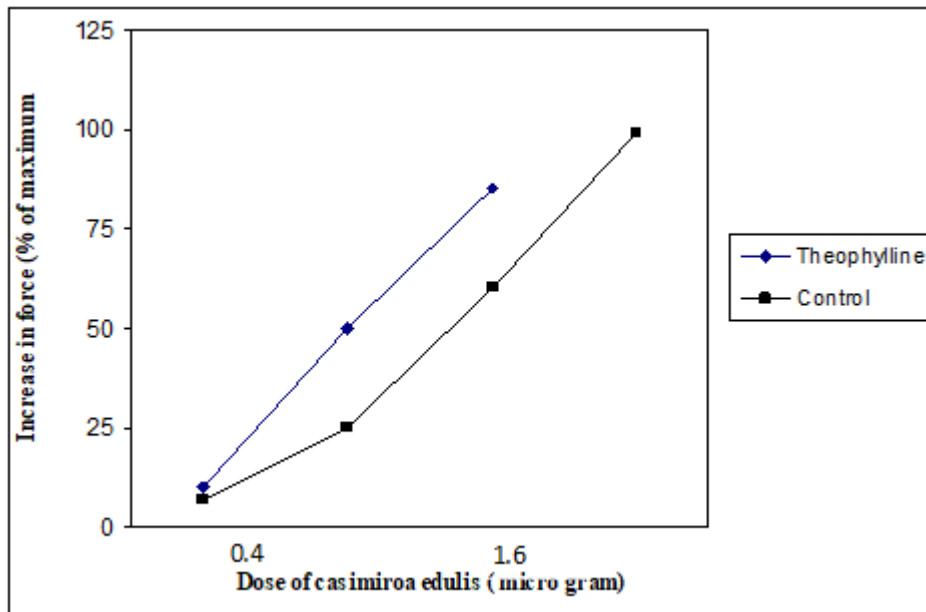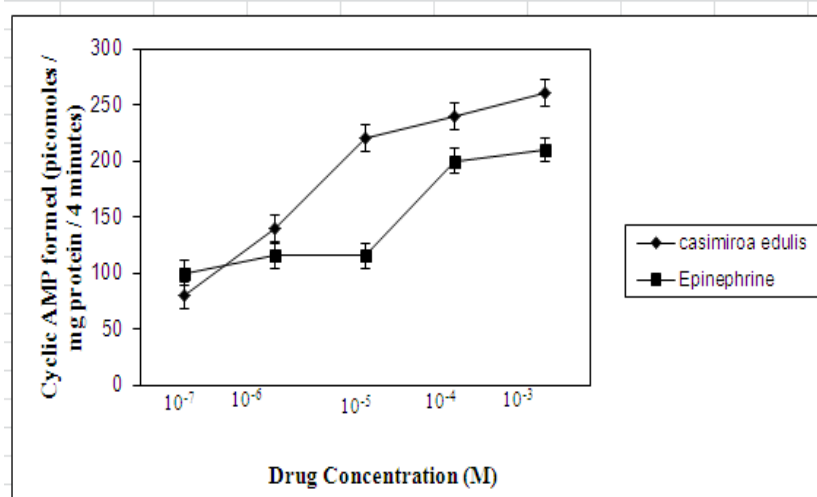

Figure 2: Dose response curves demonstrating the stimulation of guinea-pig heart adenyl cyclase by casimiroa edulis and epinephrine. (*n= 50*). *n* represents sample number. Vertical bars represent the SE.
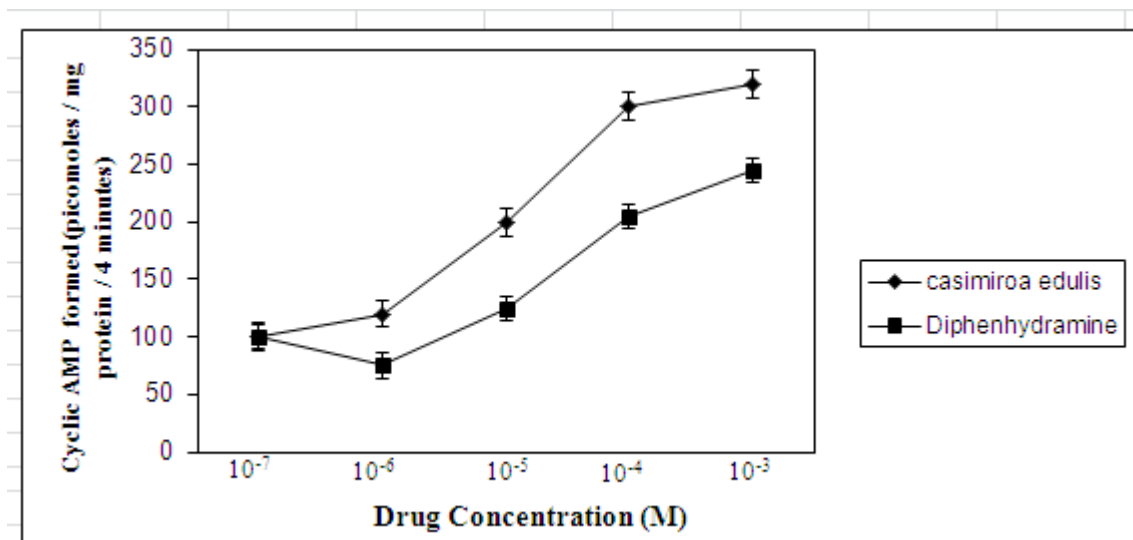

Figure 3: Dose response curves demonstrating the interaction between diphenhydramine (10^{-5}M) and casimiroa edulis on

cardiac adenyl cyclase in guinea-pig heart. ($n= 50$). n represents sample number. Vertical bars represent the SE.

Table 1: Effect of casimiroa edulis and theophylline on cardiac phosphorilase$_a$ in guinea-pig heart.
Values are Mean $\pm$ SE, (n = 50); n represents total number of samples

Dose of casimiroa edulis (µg)	% Phosphorilase$_a$ $\pm$ SE	
	Buffer perfused	Buffer perfused Plus Theophylline (10^{-3} M)
0	2.6 ± 1.3	9.7 ± 2.8 *
0.1	2.9 ± 1.4	26.3 ± 5.9 *
0.2	5.8 ± 1.4	45.8 ± 2.5 *
0.4	10.5 ± 1.5 **	44.8 ± 3.9 *
0.8	27.1± 4.8 **	-
1.6	32.1± 4.0 **	-

* Significantly greater than casimiroa edulis ($p < 0.05$)
** Significantly greater than no drug ($p < 0.5$)

Table 2: Effect of tripelennamine on casimiroa edulis-induced contractility in the isolated guinea-pig heart. Values are Mean $\pm$ SE, ($n = 50$); n represents total number of samples

Treatment	Increase in force (g) obtained with 0.8 µg casimiroa edulis
None	4.5 ± 0.4
Tripelennamine (3x 10^{-6}M)*	3.5 ± 0.4
Tripelennamine (10^{-4} M)**	1.8 ± 0.3***

*Perfused through the heart for 10 minutes.
** Perfused through the heart for 4 minutes.
*** Significantly less than no drug ($p < 0.05$)

Table 3: Effect of tripelennamine on drug-induced increase in cardiac phosphorilase$_a$ in the isolated guinea-pig heart. Values are Mean $\pm$ SE, ($n = 50$); n represents total number of samples.

Treatment	Phosphorilase$_a$
Saline	1.0 ± 1.1
Tripelennamine 10^{-4} M	3.9 ± 1.4
Casimiroa edulis 0.8 µg	29.1 ± 3.6
Casimiroa edulis 0.8 µg + Tripelennamine 10^{-4} M	4.8 ± 1.1*
Casimiroa edulis 0.8 µg + Tripelennamine 3x10^{-6} M	30.5 ± 3.6*
Isoproterenol 0.2 µg	31.7 ± 3.3
Isoproterenol 0.2 µg + Tripelennamine 10^{-4} M	0.0 ± 0.0*

*Significantly less than casimiroa edulis or isoproterenol alone. Tripelennamine perfused through the heart.

Table 4: Effect of casimiroa edulis and diphenhydramine on adenyl cyclase in isolated guinea-pig heart. Values are Mean $\pm$ SE, ($n = 50$); n represents total number of samples

Treatment	Experiment No. 1 Diphenhydramine (10^{-6}M) Mean Activity	Experiment No. 2 Diphenhydramine (10^{-6}) Mean Activity	Diphenhydramine (10^{-4}M) Mean Activity
Control	181± 5.0	102 ± 1.2	125± 2.0
Casimiroa edulis (10^{-6} M)	235 ±.6.0	150 ± 3.6	156 ± 3.2
Diphenhydramine	157 ± 2.0	105 ± 2.3	132 ± 8.2
Casimiroa edulis (10^{-6} M) + Diphenhydramine	191± 2.0	141± 4.1	113 ± 1.2

Adenyl cyclase activity expressed as picomoles of cyclic AMP formed / mg protein / 4.0 minutes.

Table 5: Effect of casimiroa edulis and tripelennamine on adenyl cyclase in isolated guinea-pig heart. Values are Mean $\pm$ SE, ($n = 50$); n represents total number of samples.

Treatment	Experiment No. 1 Tripelennamine(10^{-6}M) Mean Activity	Experiment No. 2 Tripelennamine (10^{-6}) Mean Activity	Tripelennamine (10^{-4}M) Mean Activity
Control	181± 5.0	102 ± 1.2	125 ± 2.0
Casimiroa edulis (10^{-6} M)	235 ±.6.0	150 ± 3.6	156 ± 3.2
Tripelennamine	151±5.0	106 ± 2.2	110 ± 4.1
Casimiroa edulis (10^{-6} M) + Tripelennamine	208 ± 2.8	154 ± 7.1	99 ± 1.3

Adenyl cyclase activity expressed as picomoles of cyclic AMP formed / mg protein / 4.0 minutes.

Table 6: Effect of casimiroa edulis and propranolol on adenyl cyclase in isolated guinea-pig heart
Values are Mean ± SE, ($n = 50$); n represents total number of samples.

Treatment	Experiment No. 1 Propranolol (10^{-6}M) Mean Activity	Experiment No. 2 Propranolol (10^{-6}) Mean Activity	Propranolol (10^{-4}M) Mean Activity
Control	181± 5.0	102 ± 1.2	125 ± 2.0
Casimiroa edulis (10^{-6} M)	235 ±.6.0	150 ± 3.6	156 ± 3.2
Propranolol	118 ± 3.0	100 ± 5.2	128 ± 4.1
Casimiroa edulis (10^{-6} M)+Propranolol	163 ± 9.8	163 ± 3.1	129 ± 7.1

Adenyl cyclase activity expressed as picomoles of cyclic AMP formed / mg protein / 4.0 minutes.

Table 7: Effect of epinephrine, propranolol and tripelennamine on adenyl cyclase in isolated guinea-pig heart. Values are Mean ± SE, ($n = 50$); n represents total number of samples

Treatment	Mean Activity
Control	111 ± 5.0
Epinephrine (10^{-4}M)	246 ± 9.1
Propranolol (10^{-5}M)	79 ± 1.0
Tripelennamine(10^{-5}M)	77± 7.2
Epinephrine (10^{-4}M) + Tripelennamine(10^{-5}M)	231 ± 8.0
Epinephrine (10^{-4}M) + Propranolol (10^{-5}M)	93 ± 6.2

Adenyl cyclase activity expressed as picomoles of cyclic AMP formed / mg protein / 4.0 minutes.

Table 8: Effect of blocking agents on sodium fluoride (NaF) stimulation of adenyl cyclase in isolated guinea-pig heart. Values are Mean ± SE, ($n = 50$); n represents total number of samples

Treatment	Mean Activity
None	142 ± 7.0
NaF (10^{-2} M)	723 ± 11
Tripelennamine(10^{-5}M) + NaF	715 ± 19
Diphenhydramine (10^{-5}M) + NaF	740 ± 15
Propranolol (10^{-5}M) + NaF	754 ± 18

Adenyl cyclase activity expressed as picomoles of cyclic AMP formed / mg protein / 4.0 minutes.