IN VITRO: EFFECT OF INCREASING CONCENTRATIONS OF PHOSPHODIESTERASE INHIBITORS VERSUS CASIMIROA EDULIS ON CYCLIC NUCLEOTIDES LEVELS IN RAT MYOCARDIAL STRIP

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ABSTRACT:
In vitro effects of increasing concentrations of PDE5 inhibitor sildenafil citrate versus seed extract of the plant casimiroa edulis in the presence of a selective inhibitor of PDE3 milrinone, widely used as a positive inotropic drug in the treatment of congestive heart failure were studied on the accumulation of cyclic nucleotides cGMP and cAMP in isolated rat myocardial tissue strips by established radioimmunoassay methods. These results revealed no significant increase in cGMP levels in response to 0.05, and 0.1 µM sildenafil or casimiroa edulis, however ten minutes of exposure of the tissue preparation to 1.0 µM sildenafil and 10 µM casimiroa seed extract resulted in a significant increase in cGMP levels (p<0.005). On the other hand 10 minutes of incubation with 0.05 µM concentrations of both sildenafil and casimiroa edulis resulted in a significant increase (p<0.005) in tissue cAMP with no further significant increase up to 10 µM concentration when compared with the control values. cGMP levels remained unaffected by all the concentrations of milrinone. In contrast, milrinone produced significant increase in cAMP levels at all concentrations in the cardiac strips.

A comparison between the increasing concentrations of sildenafil citrate and casimiroa edulis on the accumulation of cGMP/cAMP levels in isolated rat myocardial strips although showed a similar pattern; however in all the cases as expected, sildenafil produced greater activity than the casimiroa edulis. These results suggest the possibility of a similar mode of action of casimiroa edulis and sildenafil citrate on the accumulation of cyclic nucleotides in rat myocardial strips. PDE5 inhibition and poor cellular accumulation of cGMP induced by higher concentrations of sildenafil and comparatively higher concentrations of casimiroa edulis seed extract in rat myocardium and greater accumulation of cAMP induced by 0.05 µM and higher concentrations for both sildenafil and casimiroa edulis probably underlies the cardio protective effect of these compounds. In conclusion the present study suggests that both sildenafil and casimiroa edulis possesses a cardio-protective effect thus providing preliminary evidence that the aqueous seed extract of casimiroa edulis may be used as an alternative drug therapy to restore cardiac ailments.

Keywords: Casimiroa edulis; Sildenafil citrate; cGMP/cAMP levels, Rat myocardium.

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 for the population of developing countries (Phillipson, 1981, Bannerman et al., 1983, Aftab et al., 1996).

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Systemic evaluation of plants of medicinal value therefore becomes a major concern (Gil et al., 1995).

In the present study an attempt has been made to investigate the cardiovascular profiles of the aqueous extracts of the seeds of casimiroa-edulis Lia Llave et Lex, the "Zapote blano" (white Zapote) a tropical plant used by Mexican herb medicine since remote times (Power et al., 1911) as the "sleep-producer-fruit" when infusions of leaves or seeds were administered.

A number of previous chemical investigations of the seeds of casimiroa edulis have been reported causing a lowering of blood pressure in animals suggesting that pharmacological activity of casimiroa edulis seeds may be attributable to the presence of N\(^\alpha\), N\(^\alpha\) dimethylhistamine (Major, 1968., Murphy, 1968).

Initial report (Lozoya et al., 1977) 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. More recently, the hypotensive principle has been isolated and its structure elucidated, corroborating the original and consistent use of this plant (Ortega, et al., 1978). The main use among the population is always related to cardiac or cardiovascular diseases (Magos et al., 1999).

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. This compound illicit effect, including hypotension, indistinguishable from those of histamine (Lozoya et al., 1978; Garcia et al., 1994). The fall in blood pressure induced by histamine is characteristically transient (Gil et al., 1995; Godlewski et al., 1997), where as that caused by casimiroa edulis is remarkably long lasting (Lozoya et al., 1978; Garcia et al., 1994; Gil et al., 1995; Baish et al., 2004; Maiti et al., 2007), and could thus be attributed instead of any of the various imidazoles derivatives found in the plant. The pharmacology of these constituents has not been determined in detail, but their histamine like activity is not improbable, in view of their structural similarity with the biogenic amine.

Similar to the hypotensive nature of casimiroa edulis, is the compound sildenafil citrate (Viagra) a selective vasodilator that prolongs the action of cyclic guanosine monophosphate (cGMP), the primary mediator of vasodilatation in the corpus cavernosum of penis by selectively inhibiting cGMP-specific phosphodiesterase type-5 (PDE5) facilitating smooth muscle relaxation and the flow of blood (Goldstein, 1998). These effects occur to some extent in other portions of the cardiovascular system as well (Weishaar, 1987).

Sildenafil was originally designed to take advantage of the arterial dilation of PDE-5 in the coronary arteries, allaying the symptoms of angina. Indeed, coronary artery dilation was identified in dogs, rabbits and hypertensive rats (Kloner et al., 1999). In a similar manner to casimiroa edulis (Baish et al., 2004, Maiti et al., 2007), sildenafil citrate shows transient decrease in blood pressure followed by a change in pulse rate or amplitude in all the dose ranges (ACC/AHA, 1999).

Enzymes in the PDE family catalyze the hydrolysis of the intracellular signaling molecules cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which is the second messenger of nitric oxide (NO) and a principal mediator of smooth muscle relaxation and vasodilation. In addition to the effects of the NO-cGMP signaling pathway on the cavernosal smooth muscle, clinical findings have suggested that the vascular tone in the pulmonary, coronary, and other vascular tissues expressed by PDE-5 is the emergence of the novel therapeutic indications for sildenafil over a range of cardiovascular conditions that are well-established risk factors.
(Ravipati et al., 2007).

The pharmacologic target of sildenafil action, PDE-5, hydrolyse cyclic nucleotides (cNMPs) such as cGMP. Cellular cNMPs are regulated by a balance between their synthesis by respective cyclic enzymes and their degradation by PDEs, thus achieving alteration of cAMP and cGMP levels in response to different physiological conditions (Khairallah et al., 2008).

Keeping in view the similarity between the mode of action of casimiroa edulis and sildenafil citrate, as a potent peripheral vasodilator and as a positive inotropic agent both in vivo and vitro, present study has been designed to determine the effects of both the compounds on the in vitro formation of cGMP and cAMP in isolated cardiac muscle. This would benefit exploring the tremendous untapped potential of new drug development from plants for complementary medical practice.

MATERIALS AND METHODS

Preparation of the Casimiroa edulis Extract

The aqueous extract of casimiroa edulis seeds was prepared as described previously (Gil et al., 1995; Baish et al., 2004). Briefly, 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 mg/ml.

Tissue Preparation and Cyclic Nucleotides Assays

White albino male rats, age 20 weeks, weighing 300-350 grams/rat, were obtained from the institutional animal house facility and were hosted singly in separate standard cages at standard laboratory environment (22-24°C room temperature, 60-70% humidity, 12 hour light-dark cycle, free access to solid pellet diet). Overnight starved rats were sacrificed and the heart was excised immediately and weighed to calculate the ratio of heart weight to body weight (heart coefficient). Apical myocardium strips were placed in a chilled, organ protective solution (Custodiol-Alsbach, Germany) before muscle strip preparation.

Muscle strips were allowed to equilibrate for 60 minutes in 2-ml reaction vials containing modified Krebs-Henseleit buffer solution prepared with distilled deionized (Millipore) water (Peter et al., 2000) at 37°C equilibrated with 5% CO2 in oxygen, at 5 ml/minute using a pulsative flow pump. Cardiac strips were then exposed to sildenafil citrate as the standard solution (Pfizer, USA and milrinone (Sanofi-Winthrop Germany) at the concentrations of 0.05, 0.1, 1.0 and 10 µM for 10 minutes. Milrinone was used as a reference agent, a selective inhibitor of PDE3 that is widely used as a positive inotropic drug in the treatment of congestive heart failure (Mathias et al., 2007). Same procedure was repeated with the seed extract of the compound casimiroa edulis used as the experimental reagent. To terminate the reaction, the tissue was rapidly frozen in liquid nitrogen. The tissue was homogenized in the frozen state and cNMPs were extracted using 70% ethanol. After centrifugation at 3000g for 10 minutes at 4°C, the ethanolic phase was removed and lyophilized, and the remaining dry particulate fraction was resuspended in 50 mM sodium acetate buffer. Aliquotes of the samples were acetylated and assayed for cAMP and cGMP contents by specific radioimmunoassay using 125IcAMP and 125IJcGMP (Amersham Pharmacia Biotech. Germany). The protein content of particular fractions was determined using the Pierce BCA Protein Assay (Pierce Chemicals, Rockford, 111).

Statistical analysis was done using Gosset t test. A probability value less than 0.05 was considered significant. All data are given in picomoles of cNMP per milligram protein as the mean standard deviation of the mean. All stock solutions were prepared using ethanol and further diluted with saline or Krebs solution.
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considerable effect of the appropriate concentrations of ethanol on tissue cNMP contents was found.

RESULTS

The data for the effect of increasing concentrations of PDE5 inhibitor sildenafil citrate in relation to the reference agent PDE3 inhibitor milrinone (0.05, 0.1, 1.0 and 10 µM) on the accumulation of cGMP levels in isolated rat myocardial strips for 10 male rats and in 10 age-matched controls is shown in (Figure 1). No significant increase was
observed in cGMP levels in response to 0.05, and 0.1 µM sildenafil treated rat strips compared with the values obtained by the control samples, however ten minutes of exposure of the tissue preparation to 1.0 µM sildenafil resulted in an overall fourfold increase in cGMP levels. These values were statistically significant (p<0.005). Increasing the concentration of sildenafil to 10 µM resulted in only slight, but non significant increase in cGMP level compared to 1.0 µM sildenafil. In contrast, cGMP levels in the cardiac strips remained unaffected by all the concentrations of milrinone (Figure 1).
Effect of increasing concentrations of casimiroa edulis shown in figure 2 indicated no change in cGMP levels at the concentrations of 0.05 and 0.1µM, however ten minutes of exposure of the tissue preparation to 1.0 µM casimiroa edulis resulted in a slight but statistically significant increase (p<0.05) in cGMP level which was more significantly increased (p<0.005) at 10µM casimiroa edulis concentration when compared with the values obtained by the control samples. In all the experiments cGMP levels in the cardiac strips remained unaffected by all the concentrations of milrinone (Figure 2).

10 minutes of incubation with 0.05, 0.1 and 1.0 µM concentrations of both sildenafil and milrinone resulted in about fourfold significant increase (p<0.005) in tissue cAMP levels when compared with the control levels, however no
significant increase in cAMP levels were observed at the higher concentration of 10 µM for both the compounds when compared with the previous concentrations (Figure 3). Almost all the similar cAMP levels were observed when the cardiac tissue strips were incubated at 0.05, 0.1, 1.0 and 10 µM concentrations of casimiroa edulis and milrinone (Figure 4).

A comparison between the increasing concentrations of sildenafil citrate versus casimiroa edulis on the accumulation of cGMP/cAMP levels in isolated rat myocardial strips (Figures 5 & 6) revealed a significant increase (p<0.005) for sildenafil citrate at the concentration of 1.0 µM and 0.05 µM respectively which remained unaffected at the higher concentration of 10 µM. Unlike sildenafil citrate, casimiroa edulis showed slight increase at the concentration of 1.0 µM for cGMP and 0.05 µM for cAMP levels, although at 10.0 µM concentration, casimiroa edulis showed a significant increase in cGMP level, and 0.1 µM for cAMP level, almost equal to sildenafil (1.0 µM and 0.05 µM concentration for cGMP and cAMP respectively). Although a similar pattern of the increasing levels of cGMP and cAMP was observed between sildenafil and casimiroa edulis in tissue strips, however in all the cases as expected, sildenafil produced greater activity than the casimiroa edulis extract. These results suggest the possibility of a similar mode of action of casimiroa edulis and sildenafil citrate on the accumulation of cyclic nucleotides in rat myocardial strips.

DISCUSSION

Levels of cGMP in various smooth muscles are tightly regulated by several cyclic nucleotide phosphodiesterase enzymes (PDEs) that catalyze cGMP degradation and terminate this second messenger signal (Nausch et al., 2008). cGMP that was identified almost 40 years ago, is generated from GTP either by soluble guanyl cyclase or particulate guanyl cyclase. The former is activated by nitric oxide, where as the later binds a family of natriuretic peptides consisting of atrial, brain, and C-type natriuretic peptides (Feil et al., 2003). On the other hand, various phosphodiesterases regulate cGMP catabolism, including PDE-5A, PDE-6, PDE-9A, PDE-10A, and PDE-11A according to their tissue specificity (Traverse et al., 2000; Senzaki et al., 2001). Among these, PDE-5A is the most widely studied, and its inhibition is a primary mechanism for efficacy of sildenafil in erectile dysfunction (Andersson, 2001).

In non stimulated hearts, cGMP has been suggested to augment contractile function at low concentrations, likely via cross-talk with cAMP-dependent signaling, inhibiting PDE-3 and degradation of cAMP (Vila-Petroff et al., 1999). At higher concentrations, cGMP has negative inotropic effect by antagonizing cAMP via protein kinase G in mammals (Vila-Petroff et al., 1999).

The mammalian heart was one of the first tissues subjected to basic research on the regulatory significance of PDE isoenzymes and the action of selective PDE inhibitors. Thus, cardiac muscle has become a well characterized model in this field. Presence of PDE isoenzymes 1, 2, and 3 in the cardiac muscles has been reported (Wallis et al., 1999). PDE5 has recently been detected in cardiac and penile arterial tissues (Sanchez et al., 2008). Comparative studies have shown that only inhibitors of cGMP-inhibited PDE3, such as amrinone and milrinone, appear to have an inotropic effect in heart tissue (Conti et al., 1999). Thus PDE3 seems to be the most relevant isoforms in regulating the contractile activity of cardiac muscle.

The co-expression of PDE3 and PDE5 in several tissues may lead to the potential to target PDE3 activity by a drug that appears to be an inhibitor of PDE5 in cell-free system. Thus, an increase in cGMP by inhibition of PDE5 may exert an inhibitory effect on PDE3, resulting in an elevation of cellular cAMP by increasing the cardiac contractility. Sildenafil citrate (Viagra, Pfizer) is a highly selective inhibitor of PDE5 that potentiates the activity of cGMP in the corpus cavernosum, thereby augmenting vasodilator activity of neuronally mediated nitric oxide production (Nausch et al., 2008).
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Sildenafil has also been demonstrated to increase the cGMP levels and cause smooth muscle relaxation in the isolated segments of epicardial coronary artery. Similar to the mode of action of sildenafil is the seed extract of the plant casimiroa edulis which has established smooth muscle relaxation, hypotensive and hypnotic effects (Lozoya, 1980; Maiti et al., 2007). In the present study perhaps for the first time I have tried to establish the course of cGMP and cAMP in isolated rat myocardial strip in the presence of PDE5 inhibitor sildenafil in comparison with the seed extract of the plant casimiroa edulis and PDE3 inhibitor milrinone at various concentrations. Previous studies have shown that inhibition of PDE5 in the myocardium enhanced coronary blood flow during exercise-induced ischemia, blunted cardiac stimulation by dobutamine and reduced contractility of adrenergically stimulated papillary muscle (Traverse et al., 2000; Senzaki et al., 2001). In accordance with this, in our results PDE5 inhibition by sildenafil and cellular accumulation of cGMP induced by higher concentrations of sildenafil and comparatively more higher concentrations of casimiroa edulis seed extract in rat myocardium probably underlies the cardio protective effect of these compounds. These results have shown greater accumulation of cAMP induced by 0.05 µM and higher concentrations for both sildenafil and casimiroa edulis and a poor effect of sildenafil and casimiroa edulis on cGMP accumulation in rat myocardial strips. Similar suggestions have been made previously (Kukreja, 2007).

In conclusion, the present study suggests that both sildenafil and casimiroa edulis possesses a cardio protective effect. Inhibition of cGMP degradation by these compounds with a consequent accumulation of these signaling molecules may act as a negative regulator against cardiac ailments in-vitro, however further clinical studies are required to understand the downstream molecular mechanisms of such effects. Further more the present study aims at reporting and rationalizing the traditional uses of pharmacologically active natural products to explore the tremendous untapped potential of new drug development from plants.

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