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Partly Purified Latex Containing Cardiac Glycosides from Laticiferous Plants as Inhibitors of Calf Brain Na^+, K^+-ATPase

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ABSTRACT. The main objective of this work was to evaluate the pharmacodynamic action of naturally occurring cardiac glycosides (CGs) on synaptosomal Na^+, K^+-ATPase of calf-brain. CGs from lattices of *Calotropis procera* and *Nerium oleander* were determined quantitatively as digoxin equivalent. Total CGs content present in both plants was determined quantitatively by the 2, 2', 4, 4'-tetra-nitrodiphenyl (TNDP) reagent. The total CGs content was equivalent to 68 and 1169 mg digoxin/g of dried partly purified latex (PPL), respectively. Both plant latex containing CGs showed potent enzymic inhibition as compared to ouabain, a standard Na^+, K^+-ATPase inhibitor. Lineweaver-Burk plots of the *in vitro* inhibition of Na^+, K^+-ATPase revealed that; (1) Na^+, K^+-ATPase activation by ATP was inhibited by ouabain, *C. procera* and *N. oleander* latices; (2) ouabain and *C. procera* latex (PPL) produced a similar inhibitory pattern with a significant decrease in the apparent maximum velocity (V_{max}) while the Michaelis-Menten constant (K_m) values remained almost constant; (3) *N. oleander* latex (PPL) produced a different inhibitory pattern with a significant decrease in both V_{max} and K_m values. These results of substrate and inhibitor kinetic studies of the neural enzyme, revealed the existence of at least two active sites, one for the substrate (ATP) and the other for glycoside binding which confirms previous studies. The Lineweaver-Burk analysis of the mode of inhibition indicated two types of reversible inhibition; (1) noncompetitive inhibition with regard to ouabain and *C. procera* latex (PPL); (2) uncompetitive inhibition with regard to *N. oleander* latex(PPL). These results are of utmost importance to understand the mode of enzymic inhibition for the development of heart drugs from natural digitalis-like substances.

Introduction

Saudi Arabia, despite its relative dryness, is still rich in its plant community. Among the most famous endogenous plants, which are widely distributed is *Calotropis procera (C. procera)* (Usher in Arabic), in addition to the other ornamental plant *Nerium oleander (N. oleander)* (Nerium or Dafla in Arabic) (Al-Robai *et al.*, 1998; Danish *et al.*, 1991; Markov *et al.*, 1999 and Sciber *et al.*,1982). The latex (milk white) of this plant contains several toxic compounds known collectively as cardiac glycosides (CGs) (Sciber *et al.*, 1982) which act as specific inhibitors of Na^+, K^+-ATPase and hence, together whith other extracts and the plant itself, are toxic to both vertebrates and invertebrates (Al-Robai *et al* ., 1993b).

CGs are very interesting compounds to study, since some of them showed other clinical importance. For example, some CGs showed anticancer properties (Haux, 1999), antiviral and antifungal activities (Takechi *et al.*, 1999), anti-inflammatory and analgesic effects (Kumar & Basu, 1994, Al-Robai *et al.*, 1998).

Extensive work has been done on the chemistry and pharmacology of toxic principles of both plants, yet work on their inhihitory action on Na^+, K^+-ATPase is very limited (Al-Robai *et al.*, 1993a,b and Al-Robai, 1997).

Materials and Methods

Collection of plant latex

Latex (milky sap) was collected between mid-September 1999 and end of April 2000 at 11.30 am (Al-Robai *et al.*,1998) from the stem after cutting at the tip, leaf-stem juncture, and/ or pod-stem juncture of wild plant growing within the premises of King Abdulaziz University campus in Jeddah-Saudi Arabia. Several samples were colleeted from both plants (*Calotropis procera* and *Nerium oleander*). The milky sap samples were put in 250 ml conical flasks surrounded by crushed ice. As the crude latex contains an inert eoagulum of white clastie material (Atal & Sethi, 1962), it had to be purified partially by centrifugation in a cooled centrifuge (0-4 C°) at 1500xg for 15 min and the filtration of the supernatant through four layers of cheese cloth were followed.

Quantitative determination of cardiac glycosides

Partially purified samples were kept frozen at (-20 C°) then thawed and dried for 16-24 hr at 60 C°, and allowed to come to room temperature in a desiccator. Dried samples were then crushed with a mortar and pestle. Samples of the remaining powder (0.2 or 0.5 g) were extracted with (95%) ethanol (10 or 25 ml) for 1 hr at 70-78 C° in a shaking water-bath. The tubes containing the ethanol solution were vigorously agitated at 5 min intervals till they cooled to room temp. and were then allowed to stand for 20 min (Seiber *et al.*, 1982).

Total CGs content in plant latex was determined by a spectrophotometric method (spectroassay) using 2, 2', 4, 4'-tetra-nitrodiphenyl (TNDP) reagent (Brower *et al.*, 1972). One ml aliquots of the plant latex extract in ethanol was used after constructing standard curve between the various concenrration of digoxin (3.2-50 µg/ml) to be read at absorbance of 575nm.

Synaptosomal preparation

Crude synaptosomes were prepared from a fresh calf forebrain obtained from a local slaughter-house (Booth & Clark, 1979). Hind-brain region was dissected (Cerbellum) and homogenized by hand (20 strokes with a Teflon-glass tissue grinder) in 10 volumes of ice-cold (0.32M) sucrose solution. The homogenate was centrifuged at 500xg for 10 min. The resulting supernatant was subsequently centrifuged at 12,000xg for 20 min to yield the crude synaptosomal pellet, which was used as the source of neural Na^+, K^+-ATPase.

Although, synaptosomal preparation contains more than one type of ATPase such as Na^+, K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase, the main type found possessing the highest enzymatic activity in synaptosomes in particular, is Na^+, K^+-ATPase (Booth and Clark, 1979). Also, since none of the ATPses, except Na^+, K^+-ATPase is inhibited by CGs,

it could be assumed that the total ATPase activity of synaptosomes represents Na^+, K^+-ATPase activity.

Although, the actual synaptosomal Na^+, K^+-ATPase is less than the total ATPase activity, the assumption of expressing the enzymic activity as Na^+, K^+-ATPase as previously mentioned, will not alter the over-all picture of the extent of inhibition by latex contaning CGs and its mode of action.

Na^+, K^+-ATPase assay

The Na^+, K^+-ATPase activity was quantified by measuring the release of Pi from ATP (Serrano, 1978). The quantity of the Pi in the assay was determined spectrophotometrically against a standard curve derived from a solution of known Pi concentration (Fiske & Subbarow, 1925).

Results

The Amount of Total CGs in Calotropis procera Latex Extract

Total CGs content present in *C. procera,* as determined quantitatively by the TNDP method, was found to be equivalent to 15.00 ± 0.004 µg digoxin/222.22µg of dried latex (PPL). This indicates that each gram of dry latex contains 68 mg digoxin equivalent. The concentration of total CGs is estimated to eonstitute about 6.8% based on dry weight of the latex (PPL) (Fig. 1).

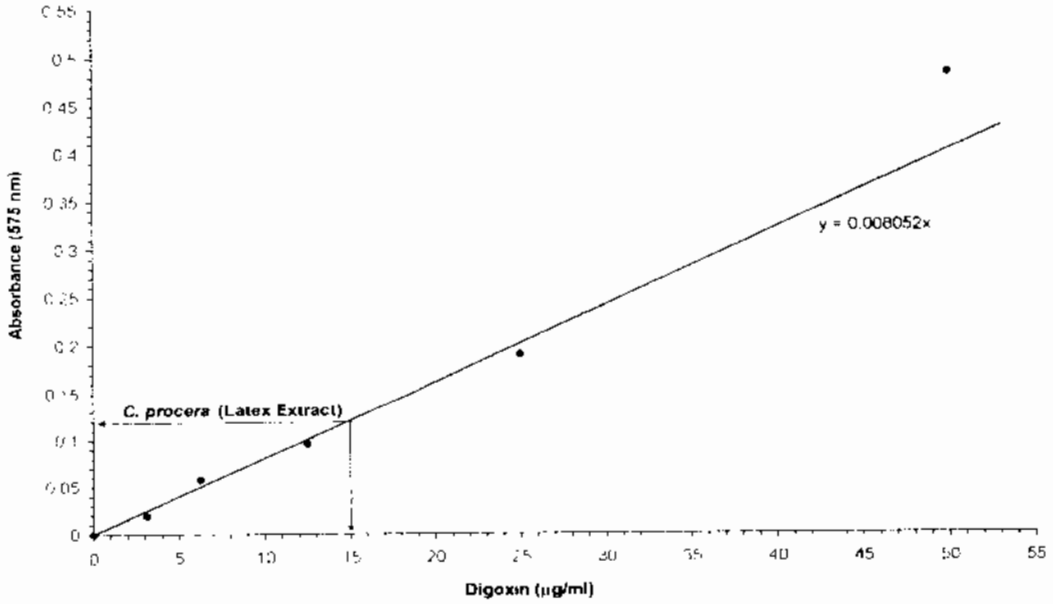

Fig. (1). The Amount of Digoxin Equivalent in 1 ml *C. procera* Latex (PPL) Diluted 1:100.

The Amount of total CGs in Nerium oleander Latex Extract

Total CGs content present in *N. oleander*, as determined quantitatively by the TNDP method, was found to be equivalent to 40.90 ± 0.015µg digoxin/34.96 µg of dried latex (PPL). This indieates that each gram of dry latex contains 1169mg digoxin quivalent. The concentration of total CGs is estimated to constitute about almost 100% based on dry weight of the latex (PPL) (Fig. 2).

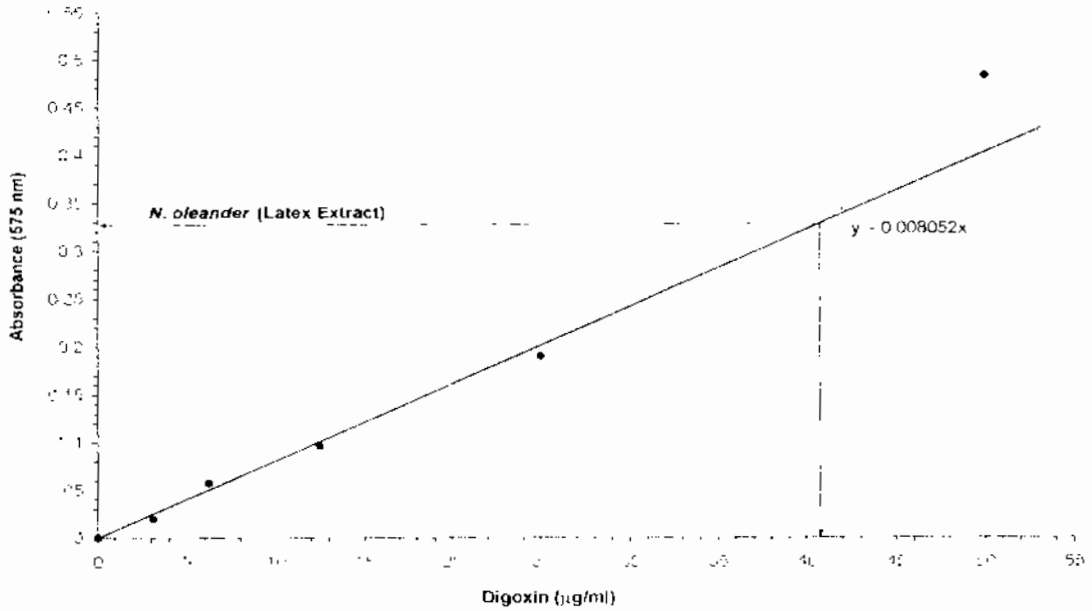

Fig. (2). The Amonnt of Digoxin Equivalent in 1ml *N. oleander* Latex (PPL) Diluted 1:500.

Effect of the Substrate Concentration [S] on the Specific Na^+, K^+-ATPase Activity

Different ATP concentrations showed a typical sigmoidal curve when plotted against the specific Na^+, K^+-ATPase activity (Fig. 3). A range of ATP concentration between 0.33 to 6.59mM showed a maximnm enzyme activity of 14.54 ± 0.26 mg Pi/mg protein/min (153.06 ± 2.73 µmol Pi/mg protein/min) when ATP concentration reached 6.59mM. A double reciprocal plot was constructed between ATP concentration 1/[S] and specific Na^+, K^+-ATPase activity (1/v) (Fig. 4). The maximum velocity (V_{max}) was found to be 15.62 mg Pi/mg protein/min (164.40 µmol Pi/mg protein/min) and Michalis-Menten constant (K_m) was 0.42mM. The slope of the line, which equals to K_m/V_{max}, was found to be 0.027 (Fig. 4).

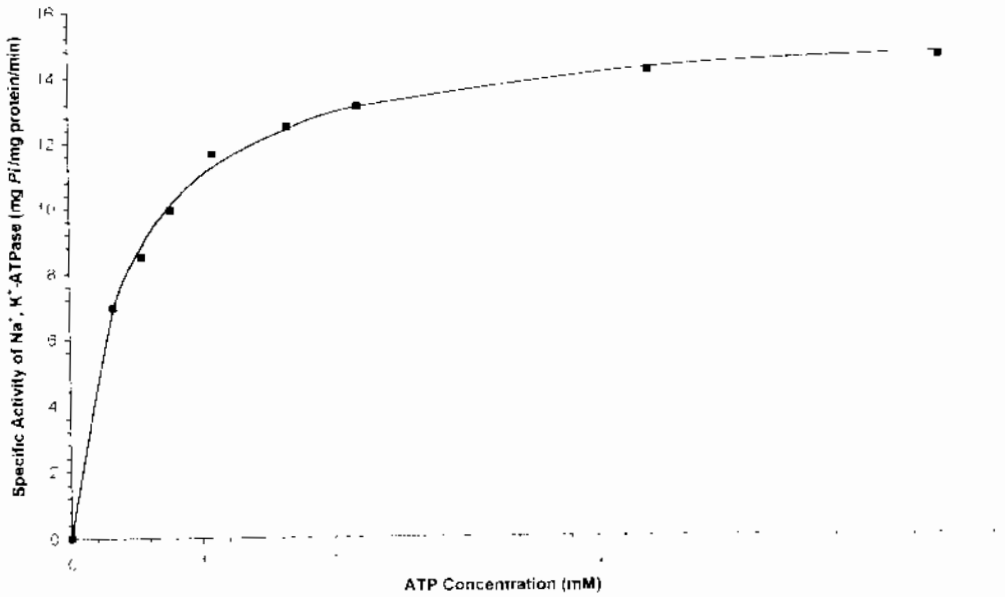

Fig. (3). Effect of ATP Concentration on Specific Na^+, K^+-ATPase Activity.

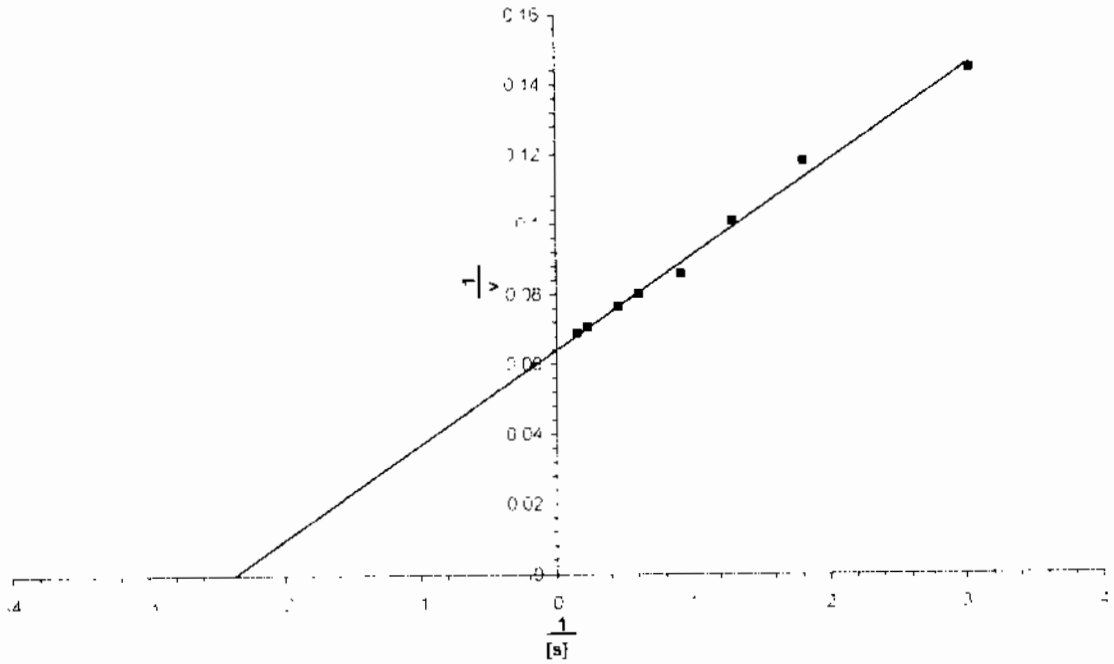

Fig. (4). Effect of ATP Concentration on Specific Na^+, K^+-ATPase Activity (Lineweaver – Burk Plot).

Effect of Incubation Time [T] on Specific Na^+, K^+-ATPase Activity.

The effect of different incubation times (5, 10, 15, 20 and 25 min) on specific Na^+, K^+-ATPase activity was conducted (Fig. 5). Progressive increase in specific Na^+, K^+-ATPase activity was seen as incubation time increases. The best incubation time to an optimum maximal activity was 10 min, and this time obtained was fixed consequently throughout the study.

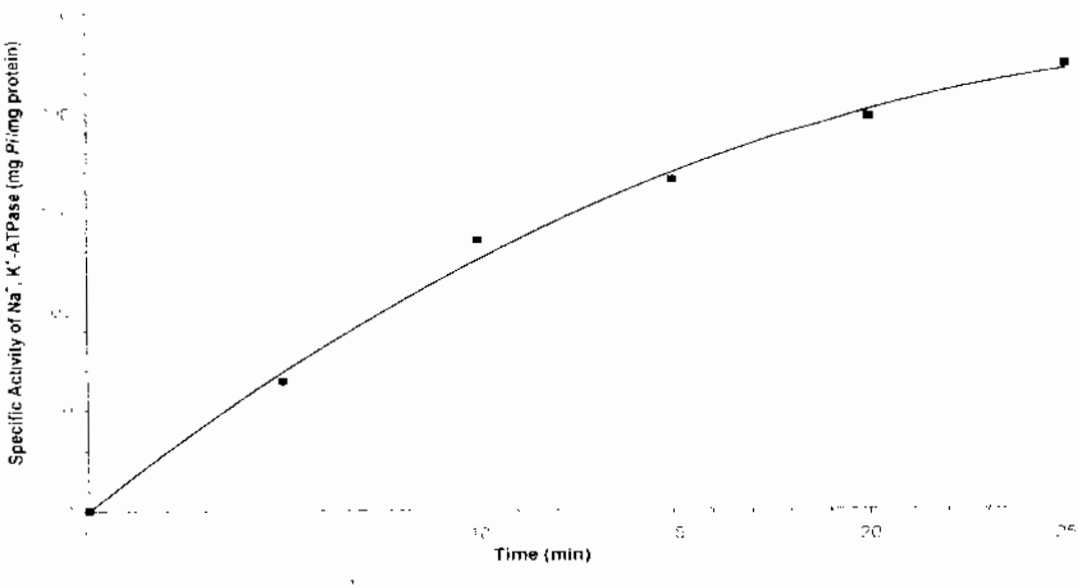

Fig. (5). Effect of Incubation Time on Specific Na^+, K^+-ATPase Activity (Undiluted Enzyme).

Effect of the Enzyme Concentration [E] on Specific Na^+, K^+-ATPase activity

Five different enzyme concentrations [E] were used to determine the optimum [E] that should be used to conduct this study. Various enzyme dilutions were used ranging from undiluted enzyme preparation (synaptosomal protein fraction) to 1:2, 1:4, 1:6 and 1:8 dilutions (Fig. 6). There was a progressive increase in specific Na^+, K^+-ATPase activity as the concentration of the enzyme protein concentration, in the reaction medium, increases. High enzymatic activity was obtained when the undiluted synaptosomal enzyme was used directly. Therefore, subsequent enzyme assays were conducted by using the undiluted enzyme preparation.

Effect of Ouabain Inhibitor [I] on Specific Activity of Na^+, K^+-ATPase

Different ATP concentrations were used to determine the specific Na^+, K^+-ATPase activity in the absence and presence of ouabain inhibitor [I] at two concentrations (100 and 200 µg) (Fig. 7). Three distinct sigmoidal curves were obtained when ATP concentrations [S] were plotted against specific Na^+,K^+-ATPase activity (v) (Fig. 7). When a double reciprocal plot was constricted between 1/[S] and (1/v) (Fig. 8) three straight lines were obtained with varying slopes. The slope of the line (K_m/V_{max}) in the absence of ouabain was 0.027 whereas the slopes with ouabain, at 100 and 200 µg, were 0.038 and 0.076, respectively (Fig. 8). The K_m value of the control experiment (in the absence of ouabain) was 0.42mM and V_{max} was 15.62 mg Pi/mg protein/min (164.40 µmol Pi/mg protein/min). It should also be noted that in the presence of ouabain the K_m value remains unchanged 0.41 and 0.44 at [I] of 100 and 200 µg respectively.

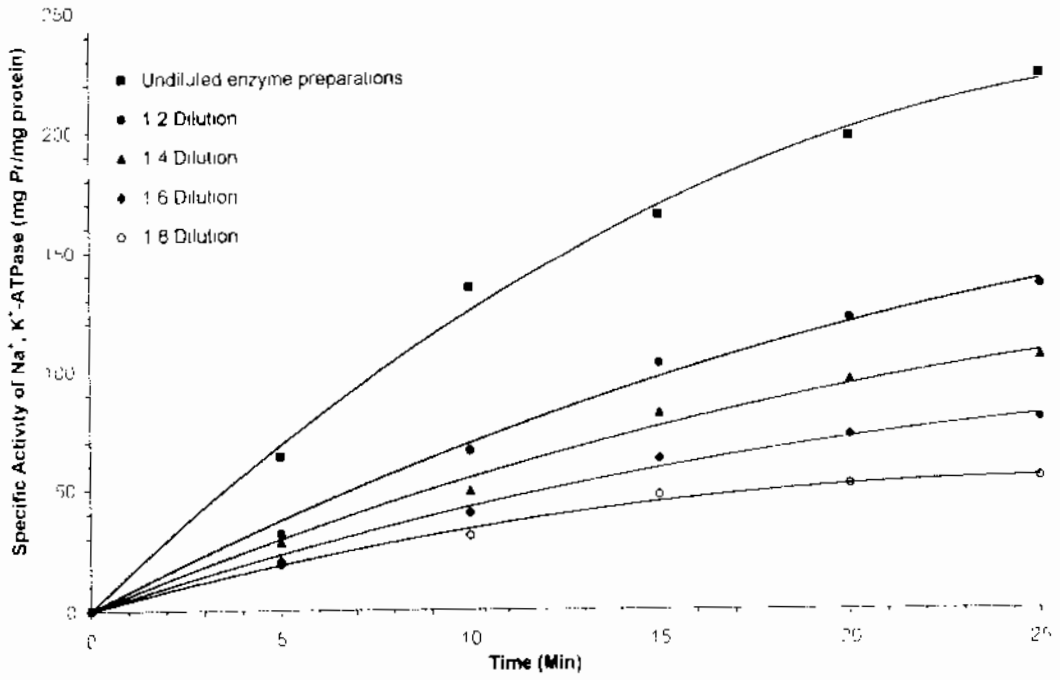

Fig. (6). Effect of Incubation Time on Specific Na^+, K^+-ATPase Activity of Diluted and Undiluted Enzyme Preparations.

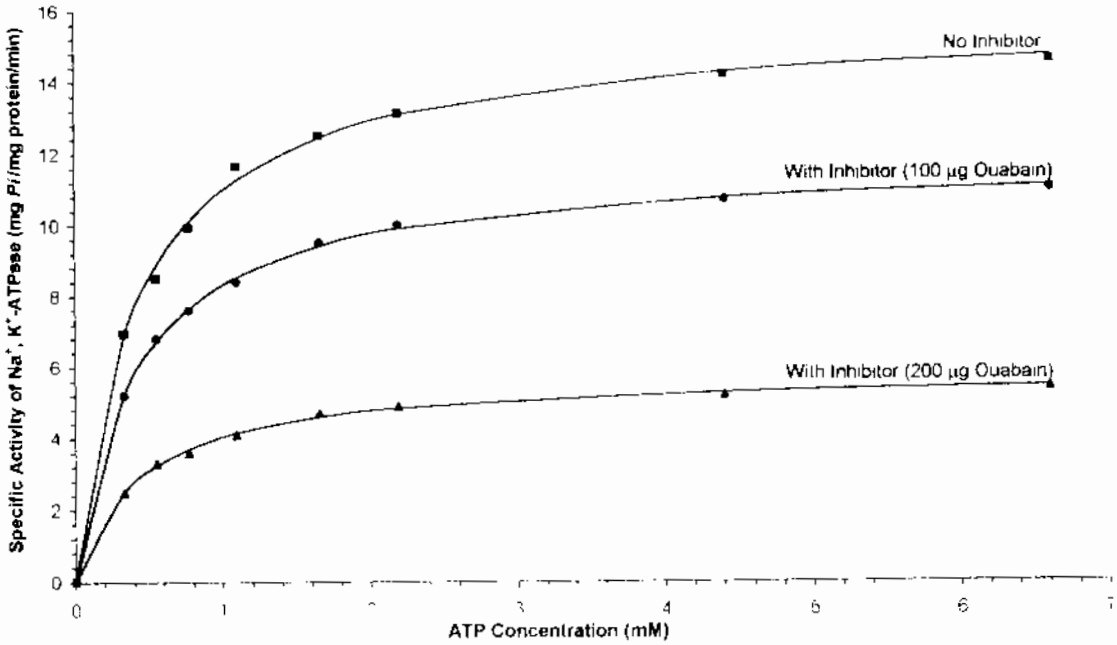

Fig. (7). Effect of ATP Concentration on Specific Na^+, K^+-ATPase Activity in Presence of Ouabain Inhibitor.

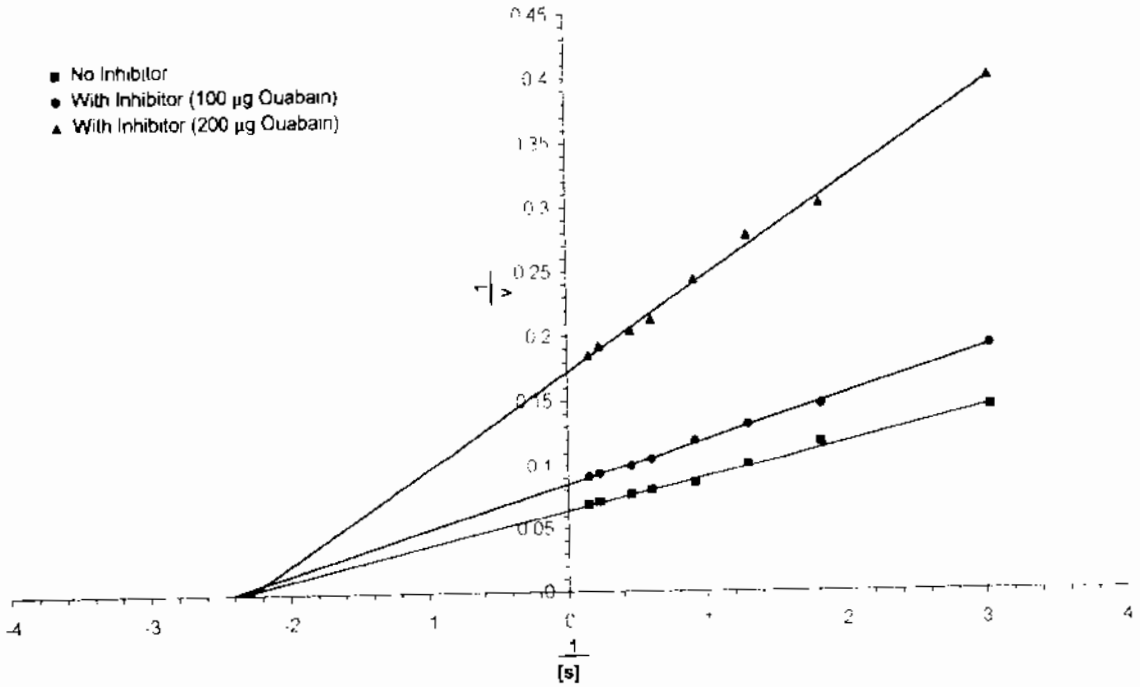

Fig. (8). Effect of ATP Concentration on Specific Na^+, K^+-ATPase Activity in Presence of Ouabain Inhibitor (lineweaver-Burk Plot).

However, the V_{max} value decreased significantly to 11.67 and 5.80 mg Pi/mg protein/min (123.83 and 61.05 µmol Pi/mg protein/min) at [I] concentrations of 100 and 200 µg respectively (Fig. 7).

The percentage inhibition of specific Na^+, K^+-ATPase activity was calculated by considering the specific Na^+, K^+-ATPase activity without the inhibitor as 100% (153.06 ± 2.73 µmol Pi/mg protein/min) (Fig. 3). At concentrations of 100 and 200 µg ouabain, the specific Na^+, K^+-ATPase activity was inhibited by 75.91% ± 2.19 and 36.9% ± 1.12 respectively (Fig. 7).

Effect of Partly purified Calotropis procera Latex on Specific Activity of Na^+, K^+-ATPuse

Different ATP concentrations were used to determine the Na^+, K^+-ATPase inhibition in the presence and absence of *C. procera* latex (PPL) [I] at two concentrations 5.3 and 10.6 µg digoxin equivalent of *C. procera* latex (PPL). Three distinct sigmoidal curves were obtained when ATP concentrations [S] were plotted againsr specific Na^+, K^+-ATPase activity (v) (Fig. 9). When a double reciprocal plot was constricted between 1/[S] and (1/v) three straight lines were obtained with varying slopes (Fig. 10). The slope of the line (K_m/V_{max}) in the absence of *C. procera* latex (PPL) was 0.027 whereas the slopes with *C. procera* latex (PPL), at 5.3 and 10.6 µg digoxin equivalent, were 0.0907 and 0.1279 respectively (Fig. 10). The K_m value of the control experiment (in the absence of *C. procera* latex (PPL)) was 0.42mM and V_{max} was 15.62 mg Pi/mg protein/min (164.40 µmol Pi/mg protein/min). In the presence of *C. procera* latex (PPL) the K_m value remains unchanged 0.43 and 0.42 at [I] of 5.3 and 10.6µg digoxin equivalent of *C. procera* latex (PPL) respectively. However, the V_{max} value decreased significantly 4.74 and 3.32mg Pi/mg protein/min (49.94 and 34.98 µmol Pi/mg protein/min) at [I] of 5.3 and 10.6 µg digoxin equivalent *C. procera* latex (PPL) respectively (Fig. 10).

The percentage inhibition of specific Na^+,K^+-ATPase activity was calculated by considering the specific Na^+,K^+-ATPase activity without the inbihitor as 100% (153.06 ± 2.73 µmol Pi/mg protein/min) (Fig. 3). At concentrations of 5.3 and 10.6 µg digoxin equivalent of *C. procera* latex (PPL), the specific Na^+,K^+-ATPase activity was C. *procera* latex (PPL), the specific Na^+,K^+-ATPase activity was inhibited by 30.26 ± 0.52 and 21.77% ± 1 respectively (Fig. 10).

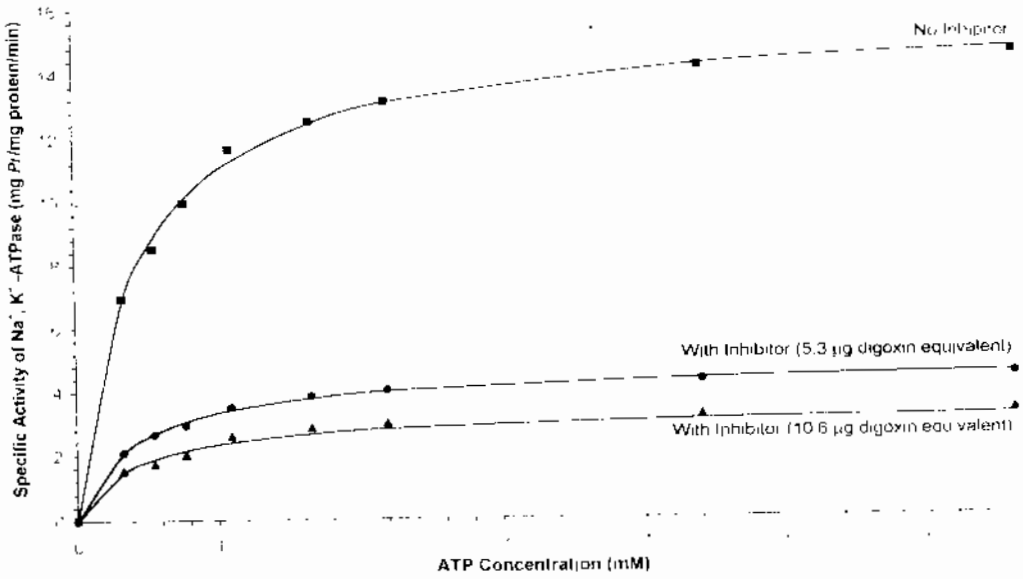

Fig. (9). Effect of ATP Concentration on Specific Na^+, K^+-ATPase Activity in Presence of *C. Procera* Latex (PPL) Inhibitor.

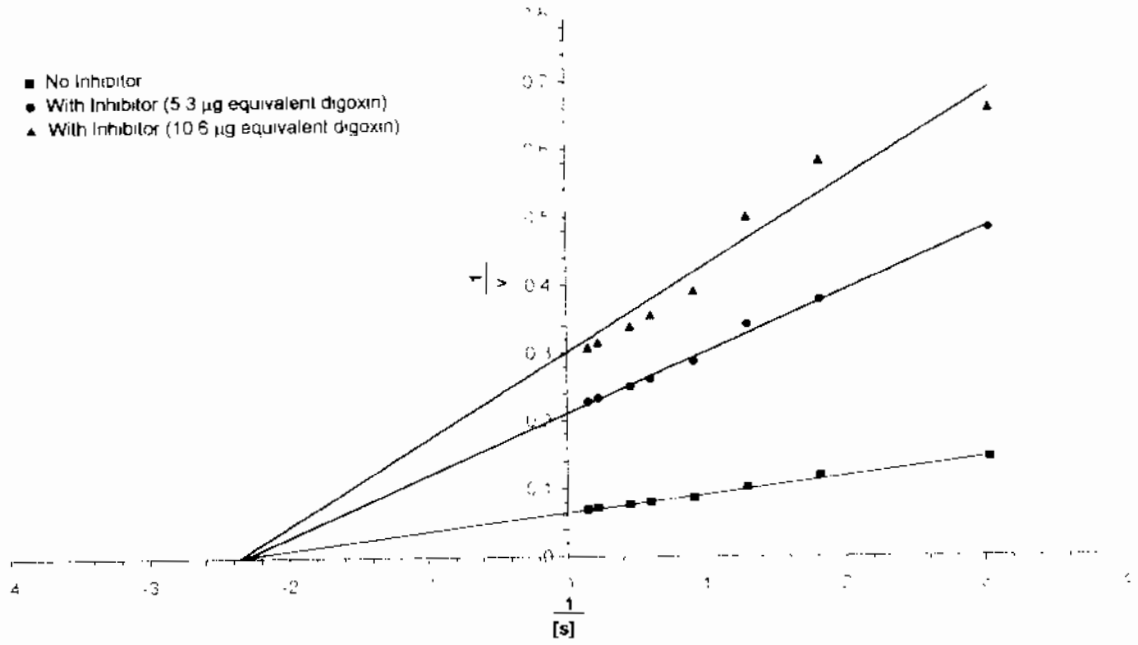

Fig. (10). Effect of ATP Concentration on Specific Na^+, K^+-ATPase Activity in Presence of *C. Procera* Latex (PPL) Inhibitor (lineweaver-Burk Plot).

Effect of Partly Purified Nerium Oleander Latex (PPL) on Specific Activity of Na^+, K^+-ATPase

Different ATP concentrations were used to measure specific Na^+, K^+-ATPase activity in the presence and absence of *N. oleander* latex (PPL) [I] at two concentrations of 257.06 and 514.13 µg digoxin equivalent of *N. oleander* latex (PPL). Three distinct sigmoidal curves were obtained when ATP concentrations [S] were plotted against specific Na^+, K^+-ATPase activity (v) (Fig. 11). When a double reciprocal plot was constricted between 1/[S] and (1/v) three straight lines were obtained with varying slopes (Fig. 12). The slope of the line (K_m/V_{max}) in the absence of *N. oleander* latex (PPL) was 0.027 whereas the slopes with *N. oleander* latex (PPL), at 257.06 and 514.13 µg digoxin equivalent, were 0.041 and 0.044, respectively. The K_m value of the control experiment (in the absence of *N. oleander* latex extract (PPL)) was 0.42mM and V_{max} was 15.62 mg Pi/mg protein/min (164.40 µmol Pi/mg protein/min). In the presence of *N. oleander* latex (PPL) the K_m value decreased significantly to 0.2 and 0.17mM at [I] of 257.06 and 514.13 µg digoxin equivalent of *N. oleander* latex (PPL) respectively. However, the V_{max} value decreased significantly 4.94 and 3.92 (52.01 and 41.66 µmol Pi/mg protein/min) at [I] of 257.06 and 514.13 µg digoxin equivalent *N. oleander* latex (PPL) respectively (Fig. 12).

The percentage inhibition of specific Na^+, K^+ ATPase activity was calculated by considering the specific Na^+, K^+-ATPase activity without the inhibitor as 100% (153.06 ± 2.73 µmol Pi/mg protein/min) (Fig. 3). At concentrations of 257.06 and 514.13 µg digoxin equivalent of *N. oleander* latex (PPL), the specific Na^+, K^+-ATPase activity was inhibited by 36.94% ± 4.44 and 30.39% ± 4.34 respectively (Table 4, Fig. 12).

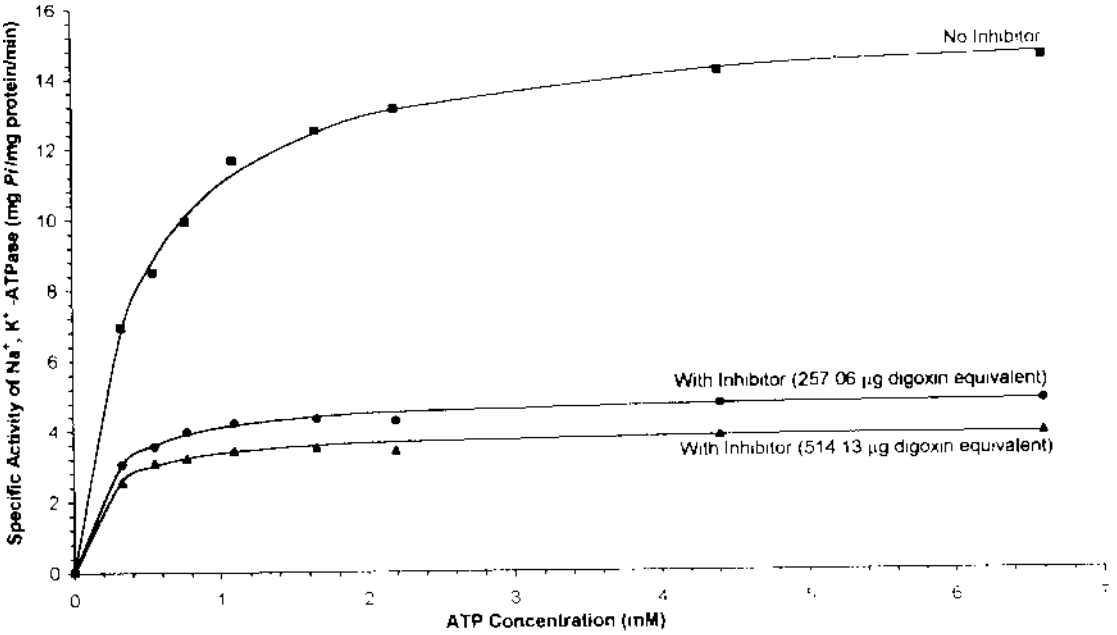

Fig. (11). Effect of ATP Concentration on Specific Na^+, K^--ATPase Aetivity in Presenee of *N. oleander* Latex (PPL) Inhibitor.

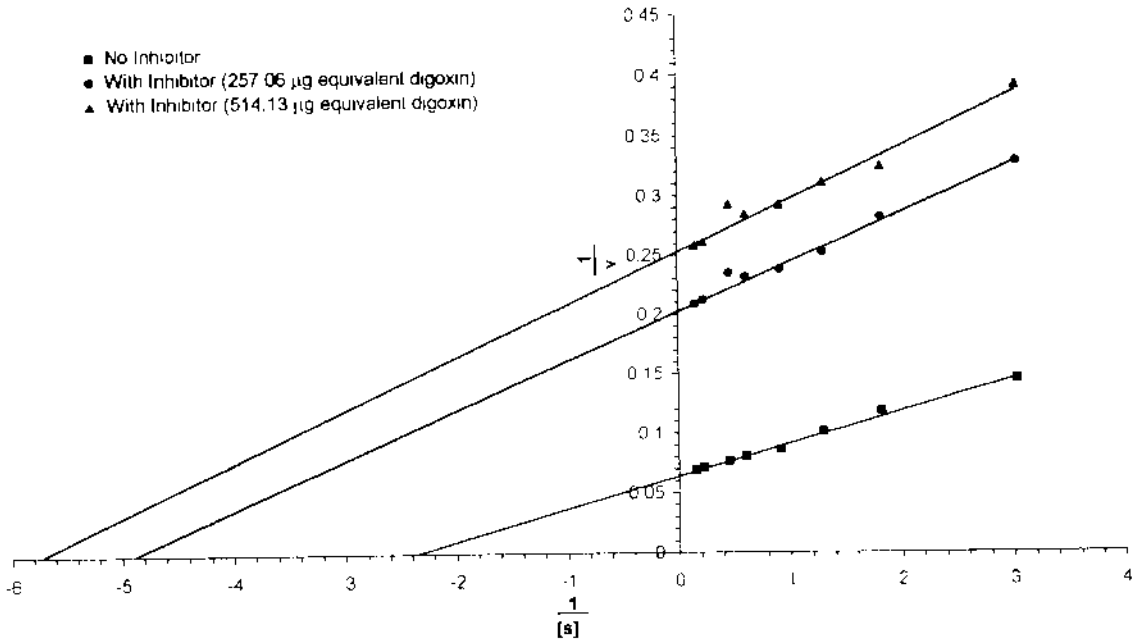

Fig. (12). Effect of ATP Concentration on Speeific Na^+, K^--ATPase Aetivity in Presence of *N. oleander* Latex (PPL) Inhibitor (Lineweaver-Bork Plot).

A Comparison Between the Potency of Inhibition Among the three CC.

A comparison between the kinetic values obtained for the (ouabain, *C. procera* and *N. oleander* latex (PPL)) is shown in the K_m values of specific Na^+, K^+-ATPase activity toward its ATP substrate which remained unchanged in the absence of any inhibitor 0.42mM.

It remained also unchanged in the presence of ouabain 0.42mM at 100 µg or 0.171µmol and 0.44 at 200µg or 0.342 µmol respectively and in the presence of *C. procera* latex (PPL) 0.43mM at 5.3 µg equivalent digoxin and 0.42mM at 10.6 µg equivalent digoxin respectively. On the other hand, the V_{max} values of ouabain and *C. procera* latex (PPL) were decreased significantly as compared to control.

N. oleander latex (PPL) however, behaved differently as for its mode of inhibition action. Thus, the K_m and V_{max} values were decreased signifieantly as compared to values of the control.

The slopes of the four lines were significantly different indicating different degrees of inhibition. The most potent inhibitor of Na^+, K^+-ATPase activity was *C. procera* (PPL) (slope= 0.091-0.128) followed by *N. oleander* (PPL) (slope 0.041-0.044) then the least was ouabain (slope 0.035-0.075).

These results indicate that within the three inhibitors, there are two types of inhibition involved. Ouabain and *C. procera* latex (PPL) inhibit Na^+, K^+-ATPase by mode of noncompetitive inhibition, whereas *N. oleander* latex (PPL) inhibits the enzyme by mode of uncompetitive inhibition.

Discussion

The results of the present study showed that the total amount of CGs in *C. procera* latex is high and is comparable with other previous results (Table 1). It is interesting to note that CGs content of latex of various parts of *C. procera* fluetuated seasonally as well as on hourly bases during the same day (Blum, 1981; Seiber *et al.*, 1982; Danish *et al.*, 1991 and Al-Robai *et al.*, 1998). The fluctuation of CGs content seems to be correlated with the various numbers of insect feeding on the plant (Al-Robai *et al.*, 1997). Moreover, CGs contents of the *C. procera* latex may also be influeneed by ecological faetors (Mooney & Chu, 1974; Nelson *et al.*, 1981; and Johnson *et al.*, 1984) such as temperature, humidity and rainfall under whieh the plant is growing (Cooper-Driver *et al.*, 1977; Mckey *et al.*, 1978; and Linocolin and Mooney, 1984). The lowest CGs concentration in the plant was in summer, a season of very high temperature in Jeddah, Saudi Arabia and the highest was in winter-spring (Al-Robai *et al.*, 1998).

The present results demonstrate also that CGs content of latex of *Nerium oleander* is higher than was reported earlier (Pearn, 1987 and ReynoldS, 1989). It is possible that CGs content of the *N. oleander* latex is influenced by several ecological factors such as temperature, humidity and rain-fall. The physiological action of oleander CGs is similar to that of the classie digitalis-like CGs, i.e. inhibition of Na^+, K^+-ATPase (Langford & Boor, 1996). The main poisonous principle and the most studied CG from this plant is oleandrin (Laborde, 1997). The concentration of oleandrin in the plant tissues is approximately 0.08% (Schvartsman, 1979). It is insoluble in water; and has littlc resistanee to light but heat-stable (Pearn, 1987 & Reynolds, 1989).

Table (1). The Amount of total CGs in Latex of *C. procera*.

REF. (YEAR)	METHOD USED TO DETERMINE CGS	AMOUNT OF CGS
Seiber *et al.*, (1982)	TNDP as a reagent and digitoxin as standard	231-161 mg equivalent digitoxin /g dry weight
Danish *et al.*, (1991)	TNDP as a reagent and digitoxin as standard	10 mg equivalent digitoxin /g dry weight
Al-Robai *et al.*, (1998)	TNDP as a reagent and ouabain as standard	Varies according to the season and ranges from 6.3 27 mg equivalent ouabain /g dry weight
Present study (2002)	TNDP as a reagent and digoxin as standard	68 mg equivalent digoxin/g dry weight

The neural or synaptosomal Na^+, K^+-ATPase is involved in the restoration of ions equilibria of Na^+ and K^+ ions through the synaptic membrane. Synaptosomal preparation of Na^+, K^+-ATPase usually contains other membrane-bound ATPases, but the main type present in the highest concentration is Na^+, K^+-ATPase (Booth & Clark, 1979). Ouabain inhibits only Na^+, K^+-ATPase activity where other ATPases remain uninhibited (Arnaiz, 1992). Synaptosomal membrane Na^+, K^+-ATPase is known to exist in three isoforms. The third isoform was suggested when using a crude rat-brain synaptosomes. This isoform was not sensitive to ouabain inhibition at low concentrations (10^{-11} to 10^{-7} M). Higher concentrations of ouabain (10^{-6} to 10^{-3}) however, resulted in a biphasic inhibition. It was suggested that the new isoform represents an inactive enzyme form of brain Na^+, K^+-ATPase which is present under resting condition (Foley & Linnoila, 1993).

In light of these results, it is known that several natural and synthetic compounds inhibit selectively Na^+, K^+-ATPase activity. Among them, digitoxin, ouabain, dehydroouabain, cassaine, N-ethylmaleimide, p-hydroxy-mercuribenzoate, ethacrynic acid and many others (Frick, 1976). Among these specific inhibitors also, lithium, vanadium, some neurotransmitters, endogenous CGs (digitalis-like compounds) many plant and animal CGs and some insecticides.

In conclusion, CGs present in two latex (PPL) from *C. procera* and *N. oleander* behaved differently as inhibitors of neural or synaptosomal Na^+, K^+-ATPase. The kinetics of such inhibition revealed a dose-dependent effect and the Linewaever-Burk plots indicate that both ouabain and *C. procera* latex contaning CGs act as noncompetitive inhibitors by lowering V_{max} significantly without altering the K_m values. *N. oleander* latex on the other hand, exhibited an uncompetitive mode of inhibition by lowering both V_{max} and K_m values significantly. The potency of the inhibitory action on neural Na^+, K^+-ATPase was in the descending order; *C. procera* then *N. oleander* (PPL) and the least was ouabain. These results should be of utmost importance in understanding the mode of action of CGs and its pharmacodynamics behavior in living cells.

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الجليكوسيدات القلبية من النباتات اللبنية كمثبطات للصوديوم _بوتاسيوم أتيبيز في مخ العجل

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المستخلص. يهدف هذا البحث إلى تقييم بعض الخصائص الصيدلانية للمادة اللبنية المحتوية على الجليكوسيدات القلبية من نباتين محليين تحتوي أنسجتهما على سائل لبني، وهما نبات العشر أو العشار، ونبات الدفله أو وردة الحمار. ولقد استخدمنا إنزيم الصوديوم ، بوتاسيوم – أتيبيز المعزول من الأجسام المشبكية العصبية لمخ العجل، كنظام كيموحيوي مستهدف لدراسة تأثيرات هذه المركبات الطبيعية. و باستخدام طريقة سبكتروفوتومترية تعتمد على وجود رابع نيترو ثنائي الفينيل كمادة كاشفة، و الدايجوكسين كمادة قياسية. تم تقدير المحتوى الكلي للجليكوسيدات القلبية في المادة اللبنية في كل من العشر و الدفله بنحو ٦٨ و١١٦٩ ملي جرام مكافئ للدايجوكسين/جرام وزن جاف، على التوالي. كلا النوعين من الجليكوسيدات القلبية في المادة اللبنية للنباتين له تأثيرات تثبيطية قوية على الإنزيم مقارنة بتأثير الوابين، المثبط التقليدي المعروف لهذا الإنزيم. ومن رسم علاقة لينويفر ــ بيرك المعروفة (برسم ثنائي التبادل)، لتجارب التثبيط الإنزيمي و التي أجريت في أنبوب الاختبار لوحظ التالي: (١) أن إنزيم الصوديوم ، بوتاسيوم ــ أتيبيز يتم تنشيطه بمادة التفاعل الطبيعية (الأدينوسين ثلاثي الفوسفات)، بينما يتم تثبيطه بكل من الوابين والمادة اللبنية المحتوية على الجليكوسيدات القلبية لكل من النباتين. (٢) أن كلاً من الوابين ومستخلص المادة اللبنية المحتوية على الجليكوسيدات القلبية من نبات العشر يتشابهان في نوعية التثبيط الذي تحدثه على الإنزيم حيث أدى كلاهما إلى حدوث انخفاض معنوي ملحوظ في قيمة الســـرعة القصوى لنشاطية الأنزيم (V_{max}) في حين أن قيمة ثابت مايكل (K_m) لـــم تتغير. (٣) أحدث مستخلص المادة اللبنية المحتوي على الجليكوسيدات القلبية من نبات الدفله تثبيطاً بطريقة مختلفة عن الوابين ومستخلص العشر، حيث أحدث تثبيطاً معنوياً ملحوظا في قيمتي السرعة القصوى (V_{max}) وثابت مايكل (K_m). و من الرسم ثنائي التبادل تبين وجود نوعين من التثبيط: (أ) تثبيط غير تنافسي يحدثه كل من

الوابين و مستخلص المادة اللبنية لنبات العشر. (ب) تثبيط لا تنافسي يحدثه مستخلص المادة اللبنية لنبات الدفله. تعد هذه النتائج ذات أهمية كبيرة لمعرفة آليات عمل هذه المنتجات الطبيعية على الأنزيم الذي له صلة وثيقة بعمل عضلة القلب والعقاقير الطبية التي تعالج بعض الأمراض التي تصيبها.