

Spasmolytic effect of *Vitis vinifera* leaf extract on rat colon

*Gharib Naseri M.K., Zarei M., Amiri O.

Department of Physiology, School of Medicine, Ahwaz Jundishapur University, Ahwaz, Iran

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ABSTRACT

Vitis vinifera (grape) leaf has been used traditionally to treat diarrhea and its extract induces relaxation in rat aorta and uterus. The aim of present study was to investigate the effect of grape leaf hydroalcoholic extract (GLHE) on rat colon contractions induced by some spasmogens. A piece of distal colon from male adult Wistar rats were dissected and mounted in an organ bath containing Tyrode solution and colon contractions recorded by an isotonic transducer under 1g resting tension. The GLHE (0.5- 4 mg/ml) reduced the contractions induced by KCl (60 mM), BaCl₂ (4 mM), acetylcholine (1 μM) dose-dependently (P<0.001). The spasmolytic effect of GLHE on ACh-induced contraction was unaffected by propranolol (1 μM), phentolamine (1 μM), L-NAME (300 μM), and naloxone (1 μM). In Ca²⁺-free but rich in KCl (120 mM) Tyrode solution, cumulative concentrations of CaCl₂ induced colon contractions which, were inhibited by the extract. Glibenclamide (3 μM) had no effect on the extract spasmolytic activity, but tetraethylammonium (5 mM) contracted the pre-relaxed colon induced by the extract. Results suggest that the grape leaf hydroalcoholic extract spasmolytic effect is due to the blockade of the voltage dependent calcium channels and activation of Ca²⁺-operated potassium channels.

Keywords: *Vitis vinifera*, Antispasmodic, Rat, Colon.

INTRODUCTION

Vitis vinifera (grape) is a perennial woody vine native to Asia which was introduced in Europe and other continents (1). In Iran, grape leaves are used in a traditional food (vine leaf dolma) and for treatment of diarrhea and bleeding (1). The procyanidins are the most important constituents of grape (2). Grape seed extract has been reported to reduce blood lipids in hyperlipidemic rabbits (3) and its procyanidins have demonstrated vasorelaxant effects in human aorta (4). It is reported that the vasorelaxant effect is mediated by NO and cGMP synthesis (5). Grape leaves with antioxidant activity (6) have been reported to treat chronic venous insufficiency in human (7) and nephrotoxicosis induced by citrinin (8). It has also been demonstrated that the grape leaf hydro-alcoholic extract (GLHE) induces spasmolytic effect on rat uterus precontracted by oxytocin (9) and the same extract induces vasorelaxant effect on rat isolated aorta. The latter effect was dependent on integrity of endothelium and NO and cGMP productions (10). The aim of the present study was to investigate the effect of GLHE on isolated rat colon contractility and mechanism(s) which are involved in this action.

MATERIALS AND METHODS

Extract preparation

The fresh and healthy leaves of grape (*Vitis vinifera* v. askari) were collected in April 2005 from Ahwaz Jundishapur University of Medical Sciences (AJUMS) campus, identified by Dr. Siahpoosh from Department of Pharmacognosy, Faculty of Pharmacy. A voucher specimen was deposited in the herbarium of the department of Pharmacognosy under number A06390001M. The leaves were dried under shade and powdered. The powder was mixed with 70% alcohol (10g: 46 ml) for 72 h at room temperature. The mixture was filtered through filter paper (Whatman No.1). Evaporation of solvent of the filtrate gave a dark green powder yield 19% w/w with respect to the dry starting crude material which was stored at 4 °C until be used.

Animals and colon preparation

Adult male Wistar rats (200-250 g) from AJUMS animal facility were used. Procedures involving animals and their care were conducted in compliance with Committee of Ethics in Research of the AJUMS. Animals were housed in cages (5 per cage) at 20-24 °C and 12/12 light/dark cycle and were allowed free access to food and water.

Animals were housed in cages with mesh bottom to prevent coprophagy and deprived from food but not water 24 h prior of the experiment. They were anaesthetized by diethyl ether and after laparotomy, a piece of the distal colon (15 mm) was dissected and the intraluminal content was flushed out with cooled oxygenated Tyrode solution. The tissue was mounted, under 1 g tension, between two stainless steel hooks in a 10 ml organ bath containing air-bubbled Tyrode solution (37 °C, pH 7.4). The lower hook was fixed at the bottom of the organ bath and the upper one was connected to an isotonic transducer (Harvard Transducer, UK). A Universal Harvard Oscillograph (UK) was used to record the colon contractions. The equilibrium period was 60 min in which the organ bath solution was refreshed at 15 min intervals.

Solutions and chemicals

The composition of Tyrode solution (mmol) was as follow: NaCl (136), KCl (2.7), CaCl₂ (1.8), MgCl₂ (1.8), NaH₂PO₄ (0.3), NaHCO₃ (12) and glucose (5.6). All solutes were purchased from Merck (Germany), L-NAME, propranolol, acetylcholine, glibenclamide and tetraethylammonium were from Sigma (USA), phentolamine was from Novartis (USA) and naloxone hydrochloride was from Tolidaru (Iran).

Procedures

The contractility and reproducibility of contraction was evaluated by introducing the tissue in KCl (60 mM) for 5-10 min. Three times after refreshing the bath solution and at least 15 min after rest, KCl was again added to the bath and when the plateau was achieved, the extract was applied to the tissue cumulatively at 0.5-4 mg/ml. The higher concentration of the extract was used when a new plateau achieved. The same procedures were carried out with BaCl₂ (4 mM) and acetylcholine (ACh, 1 μM). Each tissue was only used for one of the spasmogens. To study the involvement of adrenergic (α and β), and opioid receptors, NO and potassium channels, the spasmolytic effect of extract (0.5 mg/ml) on ACh (1 μM)-induced contraction were recorded and then the same procedure was repeated 20 min after incubation of tissue with phentolamine, propranolol, naloxone and glibenclamide in separate protocols. The interval of these two protocols was at least 30 min accompanied with refreshing the bath solution for several times. To study the involvement of calcium-operated potassium channel, extract (1 mg/ml) was added to KCl- precontracted colon and then tetraethylammonium (TEA) applied to the bath. To show the role of extracellular calcium, in Ca²⁺-free but rich in potassium (120 mM) Tyrode solution, the

colon was contracted by applying calcium chloride at 0.225-3.6 mM cumulatively. The same procedure was repeated in the presence of extract (0.5 mg/ml).

Data and statistical analyses

Changes in colon contractility (%) were calculated and presented as mean±SEM. Student's *t*-test and ANOVA were used for group's comparison. *P*<0.05 was considered to be significant.

RESULTS

Effect of extract on colon contraction induced by KCl, BaCl₂ and ACh

Cumulative concentrations of extract (0.5, 1, 2 and 4 mg/ml) reduced the colon contraction induced by KCl (60 mM), BaCl₂ (4 mM) and ACh (1 μM) in a dose dependent manner (ANOVA, *P*<0.001; *n*=8 for each group). The spasmolytic effect of extract on ACh-induced contraction was greater than other spasmogens (Fig. 1).

Spasmolytic effect of extract in the presence of L-NAME

The spasmolytic activity of extract (0.5 mg/ml) on ACh-induced colon contraction was unaffected by L-NAME (300 μM, 30 min; *n*=7) as a nitric oxide synthase inhibitor (Table 1).

Spasmolytic effect of extract in the presence of phentolamine and propranolol

Phentolamine (1 μM, 30 min; *n*=7) and propranolol (1 μM, 30 min; *n*=7) as non-selective α- and β-adrenoceptor antagonist, respectively, did not alter the spasmolytic activity of the extract (0.5 mg/ml) on ACh (1 μM)-induced colon contraction (Table 1).

Spasmolytic effect of extract in the presence of naloxone

Naloxone (1 μM, 30 min) as a non-selective opioid receptor antagonist not only did not reduce the antispasmodic activity of the extract (0.5 mg/ml) on ACh (1 μM)- induced colon contractions, but rather increased (*P*<0.05) the extract activity (*n*=7).

The role of extracellular calcium on the extract spasmolytic effect

In Ca²⁺-free but rich in potassium (120 mM) Tyrode solution, cumulative concentrations of CaCl₂ (0.225, 0.45, 0.9, 1.8, 2.7 and 3.6 mM) induced colon contraction dose-dependently.

The role of potassium channels in the GLHE spasmolytic activity

(ANOVA, *P*<0.001; *n*=7). After 3 min exposure to GLHE (0.5 mg/ml), the colon contractions for

Table 1. Effect of *Vitis vinifera* leaf extract (0.5 mg/ml) on ACh-induced rat colon contraction in the absence and in the presence of adrenergic (α and β), opioid receptor antagonist and nitric oxide synthase inhibitor.

A	B	C	B	D	B	E	A	F
100	58.2	66.1	61.0	59.3	25.9	27.3	58.8	39.8
± 0.0	$\pm 6.6^*$	$\pm 6.4^*$	$\pm 6.1^*$	$\pm 6.6^*$	$\pm 8.1^*$	$\pm 8.5^*$	$\pm 5.6^*$	$\pm 8.3^{*#}$
(n=7)	(n=7)	(n=7)	(n=7)	(n=7)	(n=7)	(n=7)	(n=8)	(n=8)

A: Ch ; B: Ach+E ; C: L+ACh+E ; D: Pro+Ach+E ; E: Ph+Ach+E ; F: N+Ach+E

all concentrations of CaCl_2 , as shown in Fig. 2, were reduced significantly ($P < 0.001$).

As shown in Fig. 3, the spasmolytic effect of the GLHE (0.5 mg/ml) on ACh-induced colon 1 μM contraction was unaffected by glibenclamide as an ATP-activated potassium channel (3 μM , 30 min; n=7). However, The GLHE inhibitory effect on KCl (60 mM)-induced contraction was reduced by

washing the tissue, the response of tissue to the spasmogens returned to the normal level, which suggests that effects are membrane mediated. The elevation of intracellular calcium concentration is a main factor for smooth muscle contraction (11). The main route of this elevation is calcium influx via the calcium channels and the other route is promotion of calcium release from reticulum

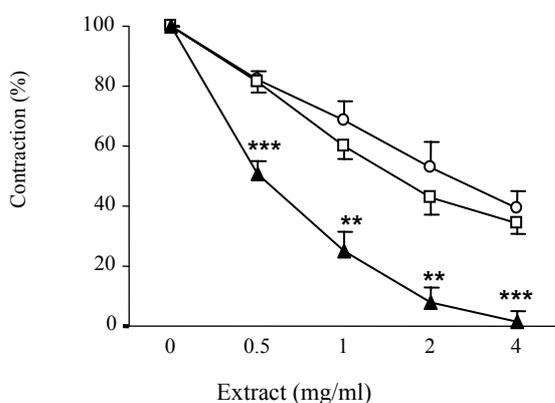


Figure 1. Spasmolytic effect of cumulative concentrations of grape leaf extract (0.5, 1, 2 and 4 mg/ml) on KCl- (\circ ; 60 mM), BaCl_2 - (\square ; 4 mM) and ACh (\blacktriangle ; 1 μM)-induced contractions in rat isolated colon. The inhibitory effect of extract on these three spasmogens are dose dependent (ANOVA, $P < 0.0001$, n=8) and on ACh-induced contraction is greater than other spasmogens (** $P < 0.001$, *** $P < 0.0001$).

tetraethylammonium (TEA, 5 μM) as a calcium-operated potassium channel i.e. the relaxed colon was contracted by application of TEA ($P < 0.001$; n=7). However, as shown in Fig. 4, the contraction induced by TEA was not as great as it was initially caused by KCl ($P < 0.01$).

DISCUSSION

This study showed that grape leaf hydroalcoholic extract is a potent relaxation-producing mixture, which reduced contractions induced by a variety of agents known to produce contractions under experimental conditions in rat colon. It reduced KCl-induced contractions, a calcium channel mediated spasmogens, as well as those produced by acetylcholine, a receptor mediated agent, and BaCl_2 a non-selective smooth muscle agonist. Consistent with previous studies (9, 10) following

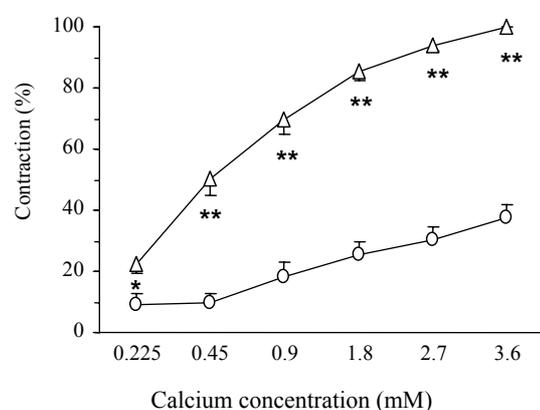
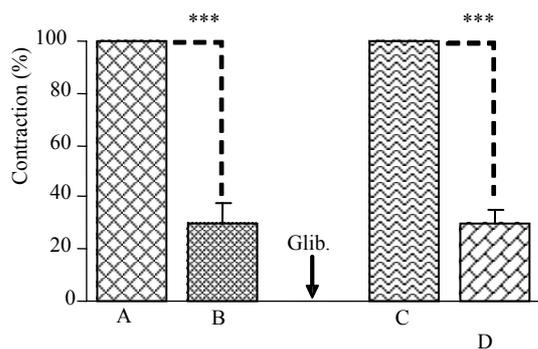


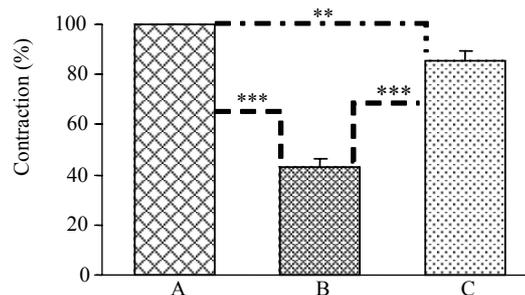
Figure 2. Spasmogenic effect of cumulative concentrations of calcium on rat colon in Ca^{2+} -free with rich potassium Tyrode solution. This spasmogenic effect is evaluated in the absence (\triangle) and in the presence (\circ) of grape leaf extract (0.5 mg/ml). The calcium spasmogenic activity is reduced by the extract (n=7, * $P < 0.05$ and ** $P < 0.001$).

sarcoplasmic. However the former route is more important in colon contraction (12). The L-type voltage dependent calcium channels (VDCCs) which are identified in colon smooth muscle cells (13), are activated by depolarization induced by high potassium in extracellular fluid. BaCl_2 causes contraction by blocking potassium channels (14) and by promoting Ca^{2+} release from intracellular pool (15). On the other hand, ACh activates M_2 muscarinic receptors to open non-selective cation channels and L-type VDCCs (16) and also elevates inositol triphosphate (IP_3) production, which causes releasing of Ca^{2+} from sarcoplasmic reticulum (17). The potent inhibitory effect of GLHE on ACh-induced contraction can not be due to the direct effect of extract on muscarinic receptors since it is reported that the ACh and smooth muscle (20) but our results showed that



A: ACh ; B: A+Ext. ; C: Glib.+A ; D: C+Ext.

Figure 3. Effect of glibenclamide (30 min) on spasmolytic activity of the grape leaf extract by ACh-induced contraction in rat colon. Figure shows that the inhibitory effect of extract is unaffected by glibenclamide (***) $P < 0.001$; $n = 7$). ACh; acetylcholine (1 μ M), Ext.; Extract (0.5 mg/ml), Glib.; Glibenclamide (3 μ M).



A: KCl ; B: A+Ext. ; D: B+TEA

Figure 4. Effect of grape leaf extract on KCl-induced colon contraction and the effect of TEA on the extract inhibitory activity. The inhibitory effect of the extract has been reduced by TEA although the spasmogenic effect of KCl is not returned completely (** $P < 0.01$, *** $P < 0.001$; $n = 7$). Ext.; Extract (1 mg/ml), TEA; Tetraethylammonium (5 mM), KCl (60 mM).

GLHE induce vasorelaxation in rat aorta pre-contracted by phenylephrine. Atropine reversed the vasorelaxatory effect of ACh but not the GLHE vasorelaxatory activity (10). Therefore it may be concluded, that the extract prevents the consequent events of binding the ACh to its receptors i.e. elevation of $[Ca^{2+}]_i$. It has been suggested that those substances which inhibit the KCl-induced contraction in smooth muscle probably induce their effects by blocking VDCCs and preventing Ca^{2+} influx (18, 19). Our results showed that the spasmolytic effect of extract was reduced by lowering extracellular calcium, which indicates that the inhibitory effect of extract requires calcium. The ineffectiveness of phentolamine and propranolol on spasmolytic effect of extract indicate that α - and β -adrenoceptors are not involved in this action. The β -adrenoceptors involvement in uterus relaxation induced by the GLHE also was not supported (9). It has been reported that NO relaxes GI tract the spasmolytic effect of GLHE was unaffected by L-NAME as a nitric oxide synthase inhibitor. Therefore, involvement of NO in vasorelaxatory effect of the extract (10) on colon is not the case. The opioid receptors activation inhibits the GI motility and naloxone antagonized this activity (21). However naloxone even enhanced the extract potency. Therefore, the involvement of these receptors in the spasmolytic activity of extract was not supported. Activation of potassium channels induces hyperpolarization and inhibits contraction. Our results demonstrated that the spasmolytic

effect of extract was unaffected by glibenclamide (as an ATP-operated potassium channel) and therefore, it may be concluded that the GLHE inhibitory activity is not mediated through these channels. On the other hand, the relaxatory effect of extract was reduced significantly by tetraethylammonium (TEA, as a calcium-operated potassium channel) which indicates the involvement of calcium-operated potassium channel. It has been reported that grape seed procyanidins induces vasorelaxatory effect in rat aorta. This effect was inhibited by TEA (22) and therefore this report is consistent with our results, although, different parts of this plant were used. Although it has been reported that TEA is a non-selective K^+ channels blocker, results of some investigations has shown that TEA blocks the Ca^{2+} -operated potassium channels (23, 24). The ineffectiveness of glibenclamide and the effectiveness of TEA on GLHE spasmolytic activities on the other hand support the involvement of Ca^{2+} -operated potassium channels.

In conclusion, this study demonstrated that the grape leaf hydroalcoholic extract induces spasmolytic effect in the isolated rat colon through VDCCs and also activation of Ca^{2+} -operated potassium channels. These results are consistent with traditional usage of the grape leaf for treatment of diarrhea.

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