

Acute toxicity and antispasmodic activities of *Achillea wilhelmsii* C. Koch

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Abstract: Since *Achillea wilhelmsii* is used as antispasmodic in traditional medicine, we conducted our current work to investigate its rationale on scientific grounds. Acute toxicity studies of crude methanol extract of *Achillea wilhelmsii* (Aw. CMeOH) is also performed. Effect of Aw. CMeOH and its fractions were tested on isolated sections of rabbits' jejunum at test concentrations 0.01, 0.03, 1.0, 3.0, 5.0 and 10mg/ml. The test extracts, in similar concentrations, were also tested on KCl-induced contractions. Calcium chloride curves were constructed for those fractions which relaxed KCl induced contractions in the absence and presence of the test samples to investigate its possible mode of action through calcium channels. Aw. CMeOH tested positive for flavonoids, saponins, tannins, glycosides, terpenoids, sterols, phenols, carbohydrates and proteins. LD₅₀ for acute toxicity studies is 2707±12.6 mg/kg. Mean EC₅₀ values for Aw. CMeOH on spontaneous and KCl-induced contractions are 3.41±0.18 (2.56-3.8, n=6) and 0.68±0.05 (0.6-0.85, n=6) mg/ml, respectively. Respective EC₅₀ values for *n*-hexane fraction on spontaneous and KCl-induced contractions are 3.06±0.08 (2.8-3.3, n=6) and 1.68±0.8 (1.4-1.9, n=6) mg/ml, respectively. Corresponding EC₅₀ (mg/ml) values for chloroformic, ethylacetate and aqueous fractions of *Achillea wilhelmsii* on spontaneous rabbits' jejunum preparations are 4.8±0.2 (4.41-5.63, n=6), 5.07±0.15 (4.7-5.58, n=6) and 5.2±0.13 (4.91-5.64, n=4), respectively. Constructing calcium chloride curves, in the presence of 0.1 mg/ml of Aw. CMeOH, mean EC₅₀ value (log molar [Ca⁺⁺]) is -1.98±0.03 (-1.89-2.05, n=6) vs. control EC₅₀ (log molar [Ca⁺⁺]) -2.41±0.02 (-2.32-2.44, n=6). Mean EC₅₀ value (log molar [Ca⁺⁺]) for 0.3 mg/ml *n*-hexane fraction is -1.76±0.05 (-1.70 -1.93, n=6) vs. control EC₅₀ (log molar [Ca⁺⁺]) value -2.18±0.07 (-2.0-2.46, n=6). While in the presence of chloroformic fraction (3 mg/ml), mean EC₅₀ (log molar [Ca⁺⁺]) value is -2.4±0.1 (-2.78 -2.9, n=6) vs. control EC₅₀ (log molar [Ca⁺⁺]) value -2.70±0.05 (-2.5-2.8, n=6). Mean EC₅₀ value (log molar [Ca⁺⁺]) for ethyl acetate fraction (1 mg/ml) is -1.94±0.07 (-1.75-2.05, n=6) vs. control EC₅₀ (log molar [Ca⁺⁺]) value -2.69±0.04 (-2.57-2.79, n=6). Mean EC₅₀ (log molar [Ca⁺⁺]) value for residual aqueous fraction (3 mg/ml) is -1.8±0.3 (-1.71-1.84, n=6) vs. control EC₅₀ (log molar [Ca⁺⁺]) -2.6±0.04 (-2.59-2.76, n=6). Whereas, the verapamil (0.1μM) EC₅₀ value (log molar [Ca⁺⁺]) is -1.7±0.1 (-1.6-1.8, n=6) vs. control EC₅₀ value (log molar [Ca⁺⁺]) -2.4±0.09 (-2.3-2.47, n=6). The present research work confirms that the intestinal relaxation effect of *Achillea wilhelmsii* is supporting its traditional use as antispasmodic. The plant species can be a source for calcium antagonist(s), which can preferably be isolated from *n*-hexane fraction.

Keywords: *Achillea wilhelmsii*; acute toxicity; antispasmodic; calcium chloride curves; verapamil.

INTRODUCTION

Achillea wilhelmsii (Local name: Zawal) belongs to family Asteraceae, which is one of the largest family of vascular plants, distributed throughout the world (Saeidna *et al.*, 2011; Yang and Hseih, 2006). One of the most important genera of this family is *Achillea* (Yarrow) (Javidnia *et al.*, 2004), which contains around 130 species in Europe, Asia and North America (Amjad *et al.*, 2011).

Achillea refers to the Achilles (Greek word meaning "hero") who used yarrow to treat the soldiers' wounds during battles (Saeidna *et al.*, 2011). Traditionally, various species of genus *Achillea* are reported to have anti-inflammatory, general tonic, diaphoretic, diuretic, emmenagogue and antispasmodic activities

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(Zargari, 1996; Saeidnia *et al.*, 2005). In Turkey, its herbal tea is used for flatulence and abdominal pains (Honda *et al.*, 1996). Other *Achillea* species were also previously studied. Thus aqueous extract of *A. kellalensis* has shown to be effective in wound healing (Pirbalouti *et al.*, 2010). *A. santolina* have showed antidiabetic activity (Yazdanparast *et al.*, 2007), while essential oils of *A. ligustica* have shown antimicrobial activity (Maggi *et al.*, 2009). Anti-spasmodic flavonoids were isolated from the *A. millefolium* L (Falk *et al.*, 1975). Hydroalcoholic extract of *A. millefolium* have antimotility effect on isolated guinea-pigs' ileum (Babaei *et al.*, 2007). Other species of *Achillea* have shown antispasmodic effects due to the presence of flavonoids. Quercetin, galangin and eupatilin are commonly found in *Achillea*, which have relaxation effects on ileum (Hammad and Abdalla, 1997).

Chemical components of *Achillea wilhelmsii* contain flavonoids, alkaloids, carvacrol, cineol, borneol, linalool, α - and β -pinene, borneol, camphor, rutin, caryophyllene, thujene, sesquiterpenoids and monoterpenoids (Javidnia *et al.*, 2004; Niazmand *et al.*, 2010). The plant has shown antihyperlipidemic and antihypertensive properties (Asgary *et al.*, 2000). The flowers powder is sprinkled on wound for wound healing while the decoction of plant is abortifacient (Ozgen *et al.*, 2012). We have recently reported that the crude saponins of *Achillea wilhelmsii* have cytotoxic and anthelmintic activities (Ali *et al.*, 2011). Its decoction is used for stomach pain, fever, motion of children and jaundice (Tareen *et al.*, 2010). Young shoots are used as green tea especially for stomach disorders (Ali and Qaiser, 2009). Based on the reported literature for *Achillea wilhelmsii*, which is used as antispasmodic in traditional medicine, we conducted our current work to investigate its rationale on scientific grounds (Tareen *et al.*, 2010; Ali and Qaiser, 2009). In addition to know about its phytochemistry, other objective was to know fraction(s) in which antispasmodic constituents are concentrated. Acute toxicity of the extract is also performed.

METHODS

Drugs and animals

Analytical grade chemicals were used throughout these experiments. Acetylcholine was purchased from BDH, Poole, England, which was used for the maintenance of tissues at quiescent doses. Rest of the chemicals were of E Merck grade, Germany. Tests solutions were prepared in distilled water at time of experiments. Rabbits of either sex (average weight= 1.9 kg) were purchased from a local market. They were bred at the "Animal House" of University of Malakand. The animals had free access to water. The animals were starved 24 hours prior to start of experiments. Ethical Committee of the Department of Pharmacy, University of Malakand endorsed the experimental protocols, and ensured its compliance with provisions of the "Animal Bye-Laws 2008, Scientific Procedures Issue-I" of the University of Malakand.

Data recording

A force Transducer (MLT 0210/A Pan Lab) was used to record the intestinal recordings, connected with Power lab (Model No: 4/25 T) ADInstruments, Australia. Bridge Pod Amplifier connected with the Power lab was used for amplification of the intestinal responses. Setting parameters were in range of "20 mv, Low pass 5Hz \times 10 gain (input 1) and @ 40/S".

Interpretation of data and statistical analysis

Chart 5 supplied with the power Lab was used to interpret the data. *P* value equal to or less than 0.05 was considered as statistically significant. Microsoft XL sheet was used to calculate mean values. Graph Pad prism was used to

calculate mean, SEM and draw curves for possible EC₅₀ shift.

Collection, authentication, extraction and fractionation of the plant materials

The aerial parts of the plant were collected from the local market of board bazar of Peshawar district, Khyber Pakhtunkhwa, Pakistan. Plant was identified by Professor Dr. Jehandar Shah, plant taxonomist Shaheed Benazir Bhutto University, Pakistan. A voucher specimen designated "AW-2009" has been submitted to the herbarium of the University of Malakand. The materials were washed with distilled water. After shade drying, the materials were grinded. 3.0 kg of the powdered materials were macerated with commercial grade methanol (6 liters) for 5-7 days. The menstrum was filtered through an ordinary filter paper. We repeated the process three times. The filtrates were combined and evaporated to a semisolid brownish extract (400.0 g) free of methanol using a rotary evaporator at 40°C. A portion of Aw. CMeOH was reserved for pharmacological screenings. Rest of the extract was suspended in distilled water for fractionation. The brownish extract (300.0 g) was dissolved in 500 ml distilled water and was successively fractionated with (300 ml of each solvent, three times each) *n*-hexane, chloroform and ethyl acetate. Following the said procedure, each fraction yielded corresponding fractions upon evaporation as *n*-hexane (Aw. *n*-hex) 40.0g, chloroform (Aw. Cl) 90.0 g, ethyl acetate (Aw. EtOAc) 120.0 g, and residual aqueous fraction (Aw. Aq) 50.0/g.

Preliminary phytochemical screenings

Preliminary phytochemical screening of the plant materials was performed according to reported procedures for the presence of alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids, quinones, streols, phenols, carbohydrates and proteins (Aduragbenro *et al.*, 2009; Kokate *et al.*, 1994).

Acute toxicity studies

Acute toxicity studies were performed for Aw. CMeOH. The test sample was administered intraperitoneally (i.p) as per Lorke method (1983) (Akuodor *et al.*, 2011). Mice of either sex were starved overnight. In the first stage, 3 groups, each of 6 mice, were treated with crude methanol extract at test doses of 10, 100 and 1000 mg/kg (i.p). In the second stage of acute toxicity screening, 4 groups of 6 mice each were further treated with 1500, 2000, 2500 and 3000 mg/kg. The experimental animals were continuously observed for twenty four hours. Death(s) in each group was recorded within 24 hours for calculation of LD₅₀ values.

Effects on spontaneous rabbits' jejunum preparations and KCl induced contractions

Rabbits of either sex (average weight 1.9 \pm 0.3 kg) were sacrificed. Their abdomens were surgically opened. Pieces of jejunums were removed and kept in Tyrode's solution

aerated with carbogen gas (95% oxygen: 5 carbon dioxide mixture). Constituents and concentrations (mM) used in Tyrode's solution were: "KCl 2.68, NaCl 136.9, MgCl₂ 1.05, NaHCO₃ 11.90, NaH₂ PO₄ 0.42, CaCl₂ 1.8 and glucose 5.55". Mesentery was removed from the intestinal tissues. Preparations of about 1.5 cm lengths were mounted in 10 ml tissue bath containing Tyrode's solution at control temperature 37±1°C. About 20 minutes were given to stabilize the tissues. After stabilization, Aw. CMeOH and its various fractions *n*-hex, Aw. Cl, Aw. EtOAc and Aw. Aq were tested at concentrations 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 5.0 and 10.0 mg/ml for possible relaxant effects (Gilani *et al.*, 2005; Ali *et al.*, 2010; Ali *et al.*, 2011).

Similarly, sustained contractions were produced by 80 mM solution of KCl in the rabbits' jejunum. The test extracts were tried at concentrations 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 5.0 and 10.0 mg/ml for possible relaxant effects to explain its mode of action.

Effects on calcium chloride curves in the rabbits' jejunal preparations

The tissues were successively decalcified in K-Normal Tyrode's solution and K Rich Tyrode's solution, thereafter. We stabilized tissues in normal Tyrode's solution for at least 30 minutes. Constituents and concentration (mM) of K Rich Tyrode's solution was: KCl 50, NaCl 91.04, MgCl₂ 1.05, NaHCO₃ 11.90, NaH₂ PO₄ 0.42, glucose 5.55 and EDTA 0.1. Control calcium chloride curves (in absence of test fractions) were constructed in decalcified tissues. Then known concentrations of the extracts were applied to the decalcified tissues. Following one hour incubation period, calcium chloride curves were constructed in the tissues with similar concentrations of log molar calcium (-4 to -1.6). Curves were constructed as per cent of control maximum. Similarly, in other series of experiments, curves were constructed for calcium chloride in presence and absence of known concentrations of verapamil, a standard calcium channel blocker. Their EC₅₀ values were compared with respective controls. The right shift in EC₅₀ was also compared with effects of verapamil, a voltage gated calcium channels inhibitor (Gilani *et al.*, 2005; Ali *et al.*, 2010; Ali *et al.*, 2011).

RESULTS

Upon preliminary phytochemical screenings, methanol extract tested positive for flavonoids, saponins, tannins, glycosides, terpenoids, sterols, phenols, carbohydrates and proteins. However, it tested negative for the presence of alkaloids and quinones (table 1). Results of acute toxicity studies are summarized in table 2. In the first stage of acute toxicity screening, all the test animals were alive meaning that the methanol extract was safe in the test concentrations. However, in the second stage of the

acute toxicity screenings, animals of group 1 survived. Two animals died in group 2 and four died in group 3. Whereas, all test animals died in group 4 at concentration 3000 mg/kg. Their per cent lethality is expressed in fig. 1 with LD₅₀ 2707.6±12.6 mg/kg. Effects of various test extracts on spontaneous rabbits' jejunal preparations and KCl-induced contractions are shown in fig. 2. According to fig. 2A, Aw.CMeOH caused relaxation of the spontaneous rabbits' jejunal preparations with EC₅₀ value 3.41±0.18 (2.56-3.8, n=6) mg/ml. Mean EC₅₀ value for effects on KCl-induced contractions are 0.68±0.05 (0.6-0.85, n=6) mg/ml. Respective EC₅₀ values for effects of *n*-hexane fraction on spontaneous and KCl-induced contractions are 3.06±0.08 (2.8-3.3, n=6) mg/ml and 1.68±0.8 (1.4-1.9, n=6) mg/ml, respectively (fig. 2B). This suggests that relaxing constituents were more concentrated in *n*-hexane fraction. Corresponding EC₅₀ values for chloroformic, ethylacetate and aqueous fractions of *Achillea wilhelmsii* on spontaneous rabbits' jejunum preparations are 4.8±0.2 (4.41-5.63, n=6), 5.07±0.15 (4.7-5.58, n=6) and 5.2±0.13 (4.91-5.64, n=4), respectively (fig. 2C, fig. 2D and fig. 2 E, respectively). The calcium chloride curves in the absence and presence of the test extracts are summarized in fig. 3. According to fig. 3A, in the presence of 0.1 mg/ml of Aw. CMeOH, mean EC₅₀ value (log molar [Ca⁺⁺]) is -1.98±0.03 (-1.89 - 2.05, n=6) vs. control EC₅₀ value -2.41±0.02 (-2.32 - 2.44, n=6). Mean EC₅₀ value for 0.3 mg/ml *n*-hexane fraction is -1.76±0.05 (-1.70-1.93, n=6) vs. control EC₅₀ value -2.18±0.07 (-2.0 - 2.46, n=6). While in the presence of chloroformic fraction (3mg/ml), mean EC₅₀ value is -2.4±0.1 (-2.78-2.9, n=6) vs. control EC₅₀ value -2.70±0.05 (-2.5-2.8, n=6). Mean EC₅₀ value for ethyl acetate fraction (1mg/ml) is -1.94±0.07 (-1.75-2.05, n=6) vs. control EC₅₀ value -2.69±0.04 (-2.57-2.79, n=6). Mean EC₅₀ value for residual aqueous fraction (3 mg/ml) is -1.8±0.3 (-1.71-1.84, n=6) vs. control EC₅₀ value -2.6±0.04 (-2.59-2.76, n=6). Whereas, the verapamil (0.1µM) EC₅₀ value is -1.7±0.1 (-1.6 - 1.8, n=6) vs. control EC₅₀ value -2.4±0.09 (-2.3 - 2.47, n=6).

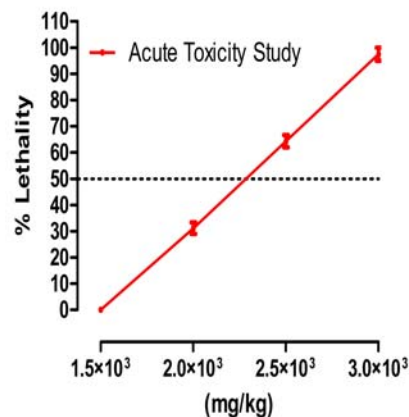


Fig. 1: Acute toxicity study of crude methanolic extract of *Achillea wilhelmsii* in mice.

DISCUSSION

As the plant species tested positive for presence of flavonoids, saponins, tannins, glycosides, terpenoids, sterols, phenols, carbohydrates and proteins, hence its pharmacological activities may be attributed to these groups of phytochemicals, which warrant for its isolation. LD₅₀ for the crude methanol extract is 2307.6±12.6 mg/kg. According to table 1, which demonstrates that plant's extract is safe in test dose up to 1500 mg/kg. Tests conducted on rabbits' jejunal preparations at concentrations within safe range of dose (table 1) showed that crude methanolic extract relaxed the spontaneous and KCl-induced contractions at low EC₅₀ values, which suggest that the relaxing constituents were concentrated more in the *n*-hexane fraction. Interestingly, 100% relaxing effects of *n*-hexane, chloroformic, ethylacetate and residual aqueous fraction occurred as the concentration of the test samples increased, suggesting that the relaxing constituents were shared in these test fractions. Therefore, all the fractions can be sources of relaxing constituents.

Table 1: Phytochemical screening of crude methanol extract of *Achillea wilhelmsii*

S. No	Class of Phytochemicals	Results
1	Alkaloids	□
2	Flavonoids	+
3	Saponins	+
4	Tannins	+
5	Glycosides	+
6	Carbohydrates	+
7	Quinones	-
8	Proteins	+
9	Sterols	+
10	Phenols	+
11	Terpenoids	+

However, 100% relaxing effects, on KCl-induced contractions, were observed in crude methanolic and *n*-hexane fraction that suggests the presence of calcium antagonists preferably in the *n*-hexane fractions and crude methanol. While rest of the fractions could not relax the KCl-induced contractions to level where we would

have drawn the EC₅₀ values (fig. 1) and therefore, suggests that some other mechanisms are also involved in the relaxing of tissues that warrant further work to elucidate its possible mechanisms. Like this may be through the histaminergic receptors or through the release of bound calcium (Ali and Shah, 2010).

It is noteworthy that the contractile effects in the intestine are due to free cytosolic calcium levels that enter the sarcoplasmic reticulum through voltage gated channels (Gilani *et al.*, 2005; Ali *et al.*, 2010; Ali *et al.*, 2011; Ali and Shah, 2010; Ahmad *et al.*, 2009; Ali and Shah, 2011a; Ali and Shah, 2011b; Ali *et al.*, 2011). More, exchange of calcium between intracellular and extracellular stores of calcium is routed through voltage gated channels that help in regulating spontaneous responses. Hence, it is postulated that the relaxing phenomenon may be through the calcium channels as high molar KCl-induced contractions are usually, though not necessarily, through the calcium channels (Gilani *et al.*, 2005; Ali *et al.*, 2010; Ali *et al.*, 2011; Ali and Shah, 2010; Ahmad *et al.*, 2009; Ali and Shah, 2011a; Ali and Shah, 2011b). Therefore, to further investigate the plant's mode of action, calcium chloride curves were constructed in the absence and presence of the test samples (fig. 3). The crude methanolic extract (0.1mg/ml) produced sufficient right shift in the EC₅₀ value (log molar [Ca⁺⁺]) = -1.98±0.03 vs. control EC₅₀ value = -2.41±0.02 (*P*<0.0001) (fig. 3 A). Same is true for *n*-hexane fraction at concentration of 0.3 mg/ml (fig. 3 B) that explains its potent effect at low concentration. Since, other fractions could relaxed the KCl-induced contractions by 30-35% of control maximum (figs. 2 C, D and E), hence it deduced that the relaxing mechanism do not follow the calcium pathways and hence warrants further work. Building of calcium chloride curves were based on the 30-35% relaxing effect of control maximum for these fractions with possible effect at higher concentrations. That is why, right shift was observed at higher concentration of these fractions like for chloroformic fraction (3 mg/ml, fig. 3 C), ethyl acetate fraction (1mg/ml, fig. 3 D) and residual aqueous fraction (3mg/ml, fig. 3 E). Such a shift might indicate that some of the calcium channels were blocked and calcium could not enter the cell through voltage sensitive L-type channels [29-30]. Similarly, the calcium curves constructed for verapamil (0.1/μM) gave mean EC₅₀

Table 2: Results of acute toxicity studies of crude methanol extract of *Achillea wilhelmsii* in mice (n=6 for each group).

1 st stage	Dose (mg/kg)			
	Group 1 (10 mg)	Group 2 (100 mg)	Group 3 (1000 mg)	
	All alive	All alive	All alive	
2 nd stage	Group 1 (1500 mg)	Group 2 (2000 mg)	Group 3 (2500 mg)	Group 4 (3000 mg)
	All alive	2 died	4 died	All died

value- 1.7 ± 0.1 vs. control EC_{50} value- 2.4 ± 0.09 ($P < 0.0004$, fig. 3 F). The right shift derived from the test samples resembled the right shift observed on the curves of verapamil, a standard calcium channel blocker (Ali and Shah, 2010; Ahmad et al., 2009). Therefore, we conclude that the mode of jejunal relaxation observed might be mediated through voltage sensitive calcium channels. More, it is postulated that the relaxant effect of *Achillea wilhelmsii* may be attributed to the phytochemical constituents such as saponins, flavonoids, tannins, sterols and triterpenes present in the plant as similar type of work in medicinal plants with similar phytochemistry have been reported to have antispasmodic activity (Cortes, 2006). Hence, *Achillea wilhelmsii* is useful species for isolation of medicinal bioactive molecules particularly for calcium antagonist(s) preferably from the *n*-hexane fraction.

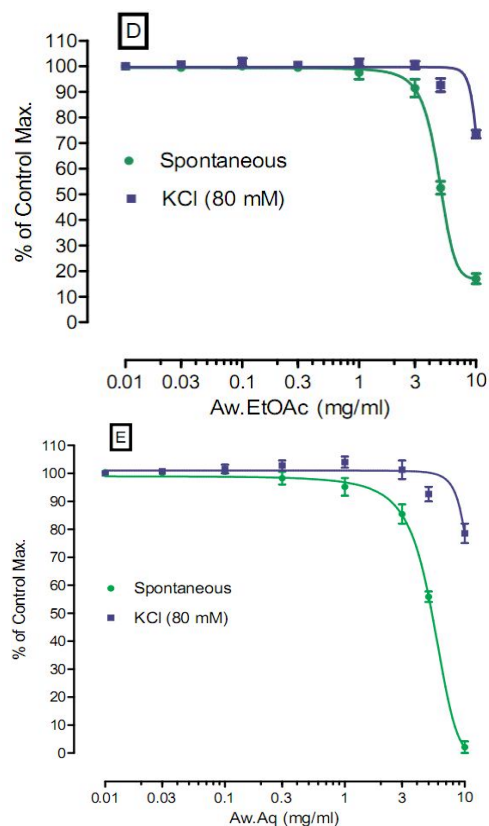
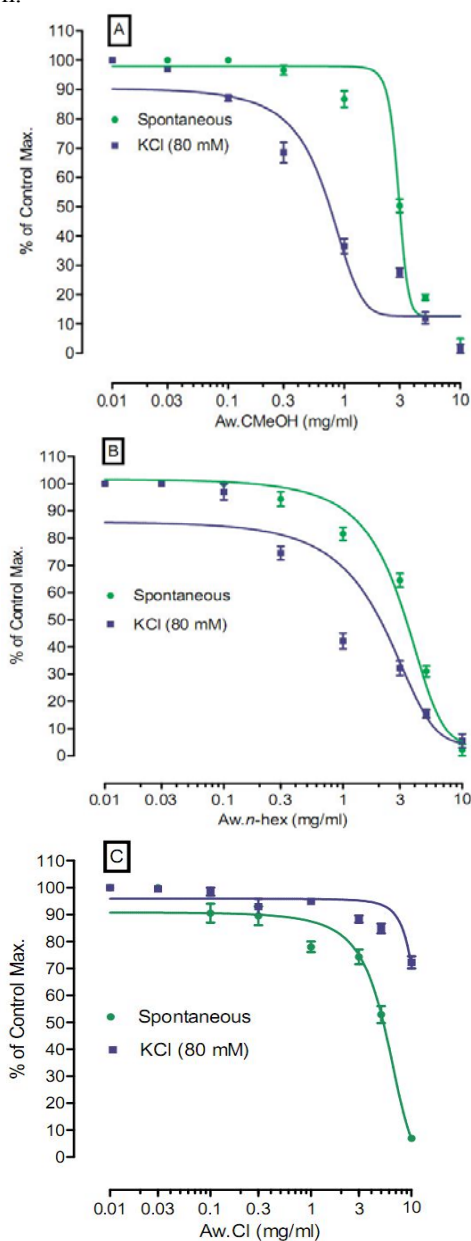


Fig. 2: The effects of crude methanolic extract of *Achillea wilhelmsii* and its fractions on spontaneous and KCl-induced contractions (Values are mean \pm SEM, $n=6$, $P < 0.05$).

CONCLUSIONS

Based on the current work, we conclude that the intestinal relaxation effect of *Achillea wilhelmsii* is supporting its traditional use as antispasmodic and the calcium antagonists can preferably be isolated preferably from *n*-hexane fraction.

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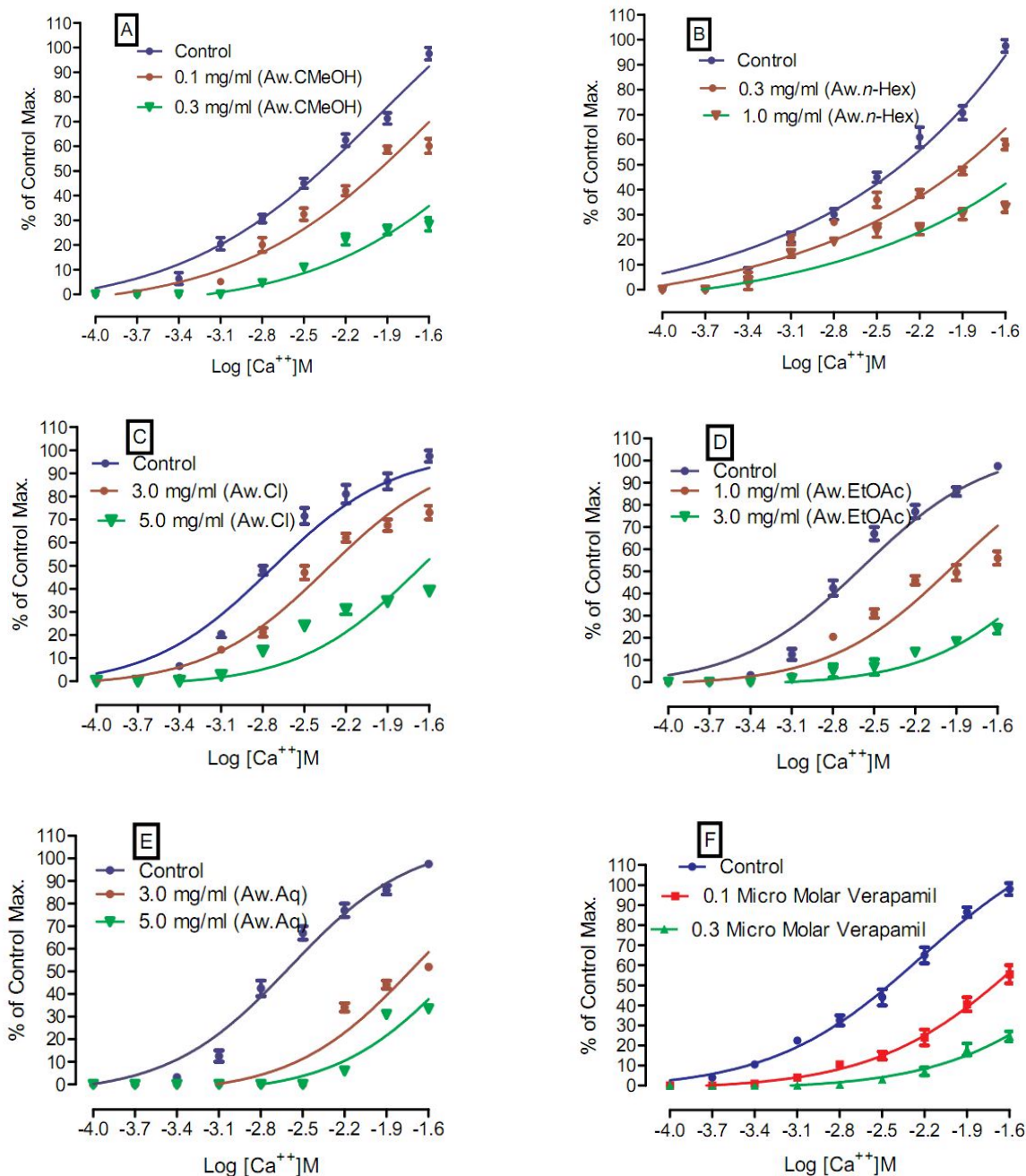


Fig. 3: Calcium chloride curves in the absence and presence of *Achillea wilhelmsii* (fig. 3 A-E). Calcium chloride curves in the absence and presence of Verapamil (fig. 3F). Values represent the mean \pm SEM, n=6. $P < 0.05$)

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