

3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene a novel compound isolated from *Pycnocycla spinosa* extract with potent anti-spasmodic and antidiarrheal properties

H. Sadraei^{1,*}, M. Ghanadian², G. Asghari² and R. Sharifian¹

¹Department of Pharmacology and Toxicology, Isfahan Pharmaceutical Sciences Research Centre, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

²Department of Pharmacognosy, Isfahan Pharmaceutical Sciences Research Centre, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

Abstract

Bioassay monitoring of hydroalcoholic extract from the aerial part of *Pycnocycla spinosa* revealed that it contains components with spasmolytic activity *in vitro*. In addition, *P. spinosa* extract at oral dose of 1-5 mg/kg inhibits diarrhoea in animal models. Pharmacological screening of pure compounds isolated from *P. spinosa* hydroalcoholic extract led to the identification of 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene (PABEM) which is a new diterpene. In this research, we have investigated antispasmodic and antidiarrheal effects of PABEM for comparison with *P. spinosa* extract. Aerial parts of *P. spinosa* were extracted with ethanol. For antispasmodic studies, rat isolated ileum was suspended in Tyrode's solution in an organ bath. The ileum was contracted by acetylcholine (ACh, 0.5 μ M), serotonin (5-HT, 5 μ M) or electrical field stimulation (EFS). *P. spinosa* extract in a concentration dependent manner (10-640 μ g/ml) inhibited ileum contractions induced by ACh, 5-HT or EFS. The new compound isolated from *P. spinosa* extract "PABEM" in a similar manner inhibited the contractile response to ACh, 5-HT and EFS. However, the inhibitory effects of PABEM were observed at much lower bath concentrations. The relaxation effect of PABEM was started at 40 ng/ml bath concentration and with 2.5 μ g/ml PABEM in the bath, the contractile responses of ileum were completely abolished. Both hydroalcoholic extract of *P. spinosa* and PABEM reduced intestinal meal transit and castor oil and MgSO₄ induced diarrhoea in mice. However, PABEM was about 10 times more potent than its parent extract. This research shows that PABEM is probably the main component responsible for antispasmodic and antidiarrheal actions of *P. spinosa* extract.

Keywords: 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene; *Pycnocycla spinosa*; Antispasmodic; Antidiarrheal

INTRODUCTION

Pycnocycla spinosa Decne. exBoiss. is a wild plant growing in many parts of Iran (1,2). *P. spinosa* extract has shown to have both antispasmodic activity *in vitro* (3-7) and antidiarrhoeal activity *in vivo* (3,8,9). In addition, pharmacological dose of *P. spinosa* extract is very similar to the doses of loperamide, propantheline and dicyclomine for inhibition of gut motility (8,9). *P. spinosa* extract contains flavonoids, saponins and alkaloids-like components (10). Various separation techniques have been used for the isolation of pharmacologically active

substances of *P. spinosa* extract (11,12). Application of column chromatography technique was more successful in the separation of the active fractions (12). Further attempts resulted in identification of a number of bioactive substances from the active fractions of *P. spinosa* extract. These include isovanillin, isoacetovanillon (7,9) and 6-(4-hydroxy-3-methoxyphenyl)-hexanoic acid (13-14). All these substances possess antispasmodic activity and inhibits diarrhoea induced by castor oil and sulphate magnesium and reduces ileum charcoal meal transit (9,13). Furthermore, they inhibited ileum contractions induced by serotonin (5-HT), acetylcholine

*Corresponding author: H. Sadraei
Tel: 0098 31 3792 2608, Fax: 0098 31 36680011
Email: sadraei@pharm.mui.ac.ir

(ACh) or neuronal stimulation (7,13). However, potencies of these compounds are very close to the hydroalcoholic extract of *P. spinosa* (7,9,13). Therefore, other components must be responsible for antispasmodic and antidiarrheal activities of the extract. Further screening of the *P. spinosa* extract led to the separation of another potent bioactive compound (14). This compound which is a diterpene identified as 3,7,10,14,15-pentaacetyl- 5 -butanoyl- 13,17 -epoxy- 8 -myrsinene (PABEM) (14). The objective of this research was to evaluate antidiarrheal and antispasmodic potential of this new compound for comparison with the hydroalcoholic extract of *P. spinosa*.

MATERIALS AND METHODS

In flowering season of the plant (June 2012), the aerial parts of *P. spinosa* were collected from Isfahan University campus located in the base of the Sofah mountain in south of Isfahan city, Iran. The plant was identified as *Pycnocycla spinosa* Decne. exBoiss. var. *spinosa* in Biology Department of Isfahan University. A voucher specimen of the plant (A24) is deposited in the herbarium of the Faculty of Pharmacy and Pharmaceutical Sciences at the Isfahan University of Medical Sciences (Iran). The plant materials were dried in shade and powdered using electrical miller (Moulinex, France). The total extract was obtained by maceration (15). The active fractions were separated by column chromatography and identified as described before (12,14).

Drugs and solutions

Tyrode's solution with the following composition NaCl, 136.9; KCl, 2.68; CaCl₂, 1.8; MgCl₂, 1.05; NaHCO₃, 11.9; NaH₂PO₄, 0.42 and glucose 5.55 (mM) was made up in distilled water. ACh (100 mM, Sigma, Germany) and 5-HT (1 mM, Sigma, Germany) stock solutions were prepared in distilled water.

The extract and PABEM stock solutions were prepared in dimethyl sulphoxide (DMSO) for *in vitro* study and in alcohol for *in vivo* studies. Further serial dilutions were prepared in either DMSO or distilled water as appropriate.

MgSO₄ was prepared as 10% solution in distilled water. Charchol (3%) and tragacanth powder (5%) suspension were prepared in distilled water. Unless stated otherwise, all the chemicals were from Merck (Germany).

In vitro studies

Male Wistar rats (200-220 g) were killed according to the internationally accepted National Research Council Guide for the Care and Use of laboratory animals as recommended by the University authorities (16). Longitudinal strips of ileum were taken and placed in oxygenated- Tyrode's solution. Each strip was suspended in an organ bath under 1 g weight tension. The bathing fluid was Tyrode's solution which was gassed continuously with oxygen at 37 °C. The ileum tension was monitored with an isotonic transducer and displayed on a pen recorder device (Harvard, England).

All drugs were added directly into the bath. Tissue contraction was induced by ACh, 5-HT or electrical field stimulation (EFS) as described before (7). The selected concentrations of drugs were chosen following a series of pilot tests. Ileum contraction was measured from the baseline and expressed as percentage of initial response prior to the addition of testing agent. The IC₅₀ value (inhibitory drug concentration causing 50% of maximum response) was determined by plotting a full concentration response curve for each tissue.

In vivo studies

Male albino mice (25-30 g), bred in School of Pharmacy and Pharmaceutical Sciences (Isfahan University of Medical Sciences, Iran) animal house were kept at room temperature. The animals were fasted overnight prior to the experiments with free access to water. All animals were handled in accordance with the internationally accepted principles for laboratory animal use and care, as recommended by the University authorities (16).

In this study antidiarrheal effect of PABEM (isolated from *P. spinosa* extract) was assessed on castor oil and MgSO₄ induced diarrhoea and compared with that of hydroalcoholic extract. In addition, effect of this substance on

ileum movement was evaluated by charcoal meal transit test as described before (8,9). For each group, 10 mice were used (in total 27 groups). Number of wet defecation over the course of study was used for the assessment of diarrhoea index. Ileum transit was expressed as percentage of charcoal moved from pylorus to the caecum relative to the whole length of the ileum.

Statistical Analysis

Values are presented as mean \pm standard error of mean (SEM). Statistical analysis was performed using *Student's t*-test and/or one way analysis of variance (ANOVA) as appropriate. SigmaPlot computer program (version 11) was used for plotting the graphs and performing statistical analysis.

RESULTS

In vitro studies

ACh and 5-HT caused a rapid contraction in strips of the rat ileum suspended in Tyrode's solution, while EFS produced a biphasic response as described before (17). Hydroalcoholic extracts of *P. spinosa* inhibited ACh induced contraction in a concentration dependent manner (Fig. 1). The inhibitory concentrations causing 50% of maximum response (IC_{50}) was $139 \pm 17 \mu\text{g/ml}$ ($n=6$).

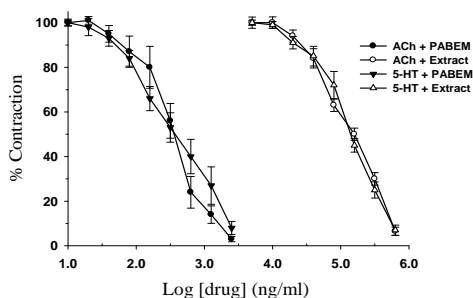


Fig. 1. Log concentration-inhibition response curve of *Pycnocyclus spinosa* hydroalcoholic extract and 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene (PABEM) on tension development to acetylcholine (ACh, 2 μM) and 5-hydroxytryptamine (5-HT, 2 μM) in isolated ileum of rat. Ordinate scale: ileum contractile response expressed as percent of initial control response. Abscissa scale: \log_{10} concentration of drugs. The points are mean and the vertical bars show the SEM ($n=6$).

PABEM (5 ng/ml to 2.5 $\mu\text{g/ml}$) also caused concentration dependent relaxation of rat ileum contraction induced by ACh (Fig. 1). The inhibitory effect was started at 40 ng/ml bath concentration and with 2.5 $\mu\text{g/ml}$ PABEM in the bath, 96% of the contractile response was attenuated. The IC_{50} value of PABEM on ACh induced contraction was calculated to be $425 \pm 95 \text{ ng/ml}$ ($0.64 \pm 0.13 \mu\text{M}$, $n=6$). After washing, the contractile response to ACh was restored. At similar concentration ranges, *P. spinosa* extract and PABEM in a similar manner inhibited the contraction induced by 5-HT (5 μM , Fig. 1) with IC_{50} values of $164 \pm 20 \mu\text{g/ml}$ and $417 \pm 95 \text{ ng/ml}$ ($0.61 \pm 0.12 \mu\text{M}$, $n=5$) respectively.

Addition of hydroalcoholic extracts of *P. spinosa* (10 $\mu\text{g/ml}$ to 640 $\mu\text{g/ml}$) into the bath resulted in inhibition of contractions induced by EFS with IC_{50} values of $73 \pm 13 \mu\text{g/ml}$ (EFS-1, Fig. 2) and $76 \pm 18 \mu\text{g/ml}$ (EFS-2, Fig. 2). PABEM at much lower concentration (20 ng/ml–2.5 $\mu\text{g/ml}$) attenuated both EFS-1 and EFS-2 responses with IC_{50} values of $266 \pm 69 \text{ ng/ml}$ ($0.39 \pm 0.09 \mu\text{M}$, Fig. 2) and $118 \pm 35 \text{ ng/ml}$ ($0.18 \pm 0.05 \mu\text{M}$, Fig. 2) respectively. At 2.5 $\mu\text{g/ml}$ bath concentration, the contractile responses to EFS were abolished.

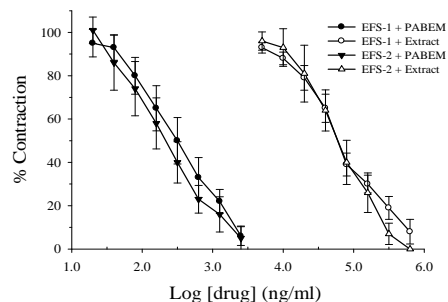


Fig. 2. Log concentration-inhibition response curve of *Pycnocyclus spinosa* hydroalcoholic extract and 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene (PABEM) on tension development to first (EFS-1) and second (EFS-2) contractile responses to electrical field stimulation (6V, 50 Hz, 1s duration) in isolated ileum of rat. Ordinate scale: ileum contractile response expressed as percent of initial control response. Abscissa scale: \log_{10} concentration of drugs. The points are mean and the vertical bars show the SEM ($n=6$).

In the vehicle-treated (DMSO 0.05-1.6%) time-matched control groups (ACh, 5-HT or EFS), there was no statistically significant changes in contractile tissue responses over the course of studies (ANOVA).

In vivo studies

Both hydroalcoholic extracts of *P. spinosa* and PABEM delayed the induction of diarrhoea (Table 1) and reduced the incidence of diarrhoea in mice in comparison with the control group. *P. spinosa* extract at doses of 5 mg/kg and 10 mg/kg reduced the incidence of castor oil induced diarrhoea by 36% and 80%, respectively (Fig. 3). PABEM at doses of 100 µg/kg and 500 µg/kg inhibited castor oil induced diarrhoea by 55% and 73%, respectively (Fig. 4). Oral dose of 2 mg/kg of PABEM had no further significant inhibitor effect when compared with dose of 500 µg/kg (Fig. 3). Loperamide with dose of 2 mg/kg

inhibited castor oil and MgSO₄ induced diarrhoea by 80% and 73% respectively in comparison with vehicle treated control groups (Fig. 3 and Fig. 4). The relative inhibitory effect of *P. spinosa* extract and PABEM at above doses, on MgSO₄ induced diarrhoea was similar to that of castor oil (Fig. 4). In addition, all these agents reduced small intestinal transit time of charcoal meal (Fig. 5). In the vehicle treated control group in 45 min, the charcoal meal move to 94% of the whole length of ileum.

PABEM reduced movement of charcoal meal in small intestine by 48% and 86% with oral doses of 500 µg/kg and 2 mg/kg, respectively (Fig. 5). *P. spinosa* extract with doses of 5 mg/kg and 10 mg/kg reduced the charcoal transit by 40% and 86% respectively, while loperamide at the dose of 2 mg/kg inhibited charcoal movement by 93% in comparison with the control groups (Fig. 5).

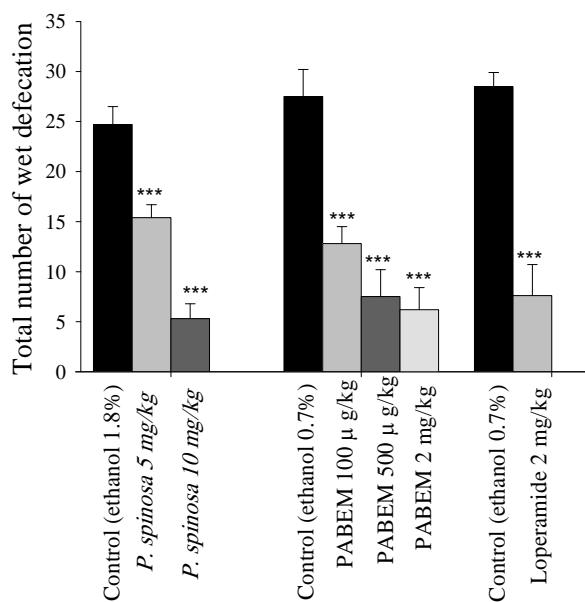


Fig. 3. Antidiarrhoeal activity of *Pycnocyclus spinosa* hydroalcoholic extract, 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene (PABEM) and loperamide on mice with MgSO₄ (0.5 ml, 10% oral solution) induced diarrhoea. Incident of diarrhoea were assessed as number of wet defecation following drug administration. The control groups were treated with the corresponding vehicle (ethanol) respectively. Data are mean ± SEM (n=10) for each group. *** $P < 0.001$ in comparison with corresponding vehicle treated control group (*Student's t*-test).

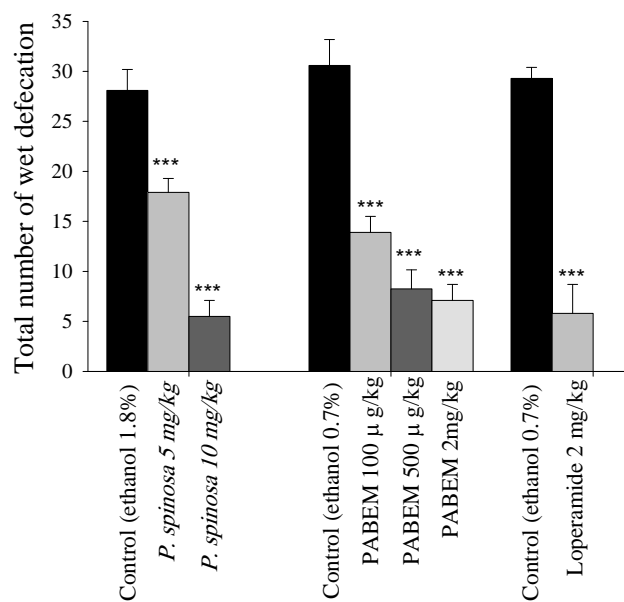


Fig. 4. Antidiarrhoeal activity of *Pycnocyclus spinosa* hydroalcoholic extract, 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene (ETAPM) and loperamide on mice with castor oil (0.5 ml, orally) induced diarrhoea. Incident of diarrhoea were assessed as number of wet defecation following castor oil administration. The control groups were treated with the corresponding vehicle (ethanol) respectively. Data are mean ± SEM (n=10) for each group. *** $P < 0.001$ in comparison with corresponding vehicle treated control group (*Student's t*-test).

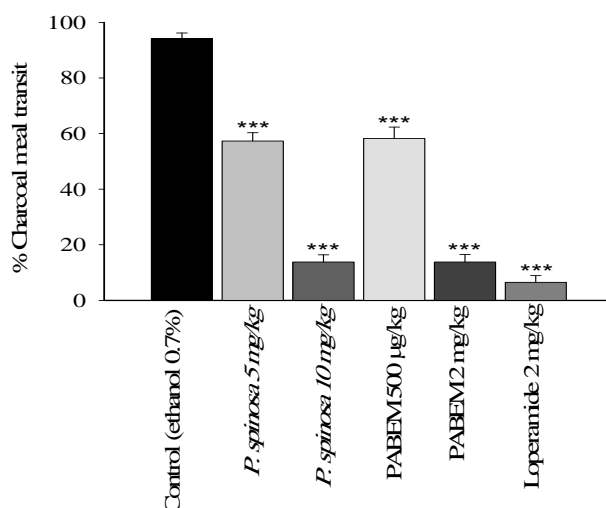


Fig. 5. Effect of *Pycnocyclus spinosa* hydroalcoholic extract, 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene (PABEM) and loperamide on small intestinal transit (0.5 ml of charcoal meal). Gastrointestinal transit was expressed as the percentage of distance that charcoal moved relative to whole length of small intestine over 45 min. Data are mean \pm SEM (n=10) for each group. *** P <0.001 in comparison with corresponding vehicle treated control group (*Student's t*-test).

Table 1. Time of induction of diarrhea after administration of laxatives in control and treatment groups.

Treatment	Delay in induction of diarrhea (min)	
	MgSO ₄ (0.5 ml of 10%)	Castor oil (0.5 ml)
Control (0.7% ethanol)	70 \pm 4.8	67 \pm 5.6
<i>P. spinosa</i> (5 mg/kg)	78 \pm 4.1	74 \pm 5.1
<i>P. spinosa</i> (10 mg/kg)	125 \pm 4.9***	122 \pm 2.7***
Control (1.75% ethanol)	70 \pm 5.1	66 \pm 5.1
PABEM (100 µg/kg)	115 \pm 4.1***	108 \pm 4.8***
PABEM (500 µg/kg)	129 \pm 4.5***	129 \pm 4.2***
PABEM (2 mg/kg)	159 \pm 3.7***	156 \pm 4.6***
Control (0.7%)	68 \pm 5.5	65 \pm 5.8
Loperamide (2 mg/kg)	164 \pm 4.1***	151 \pm 5.7***

Values are expressed as mean \pm SEM. for each group (n=10). *** P <0.001 in comparison with corresponding control (*Student's t*-test). PABEM;3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene.

DISCUSSION

P. spinosa extract has antispasmodic activities in several smooth muscle tissues including rat ileum, bladder and uterus (3-6). Four substances have been identified as active component of *P. spinosa* extract. These include isovanilline, 6-(4-hydroxy-3-methoxyphenyl)-hexanoic acid (HMPHA), isoacetovanillone and PABEM (14). All these compounds have antispasmodic activity on KCl induced contraction of rat ileum (14). Furthermore, compounds such as isovanilline, isoacetovanillone and HMPHA also inhibit

contraction induced by 5-HT and neuronal stimulation (EFS) (7,13).

The present work was conducted to assess the inhibitory effect of another component of *P. spinosa* extract "PABEM" on drugs and electrically evoked contraction of ileum smooth muscle preparations. Pre-incubation of rat isolated ileum with PABEM (20 ng/ml up to 2.5 µg/ml) for 15 min, concentration dependently inhibited ACh, 5-HT and EFS induced contractions. In the isolated ileum, *P. spinosa* extract also produced concentration dependent inhibitory effect of responses to ACh, 5-HT and EFS but at much higher

concentrations (20-640 µg/ml). In fact, PABEM was about 300 fold more potent than *P. spinosa* extract, in inhibiting ACh, 5-HT and EFS responses. Furthermore when compared at IC₅₀ levels, PABEM was 100 times more potent than isovanilline, acetoisovanilline (7) or HMPHA (13) in inhibiting 5-HT and EFS contraction in rat ileum. On the other hand, isovanilline and isoacetovanilone was found out to be a weak inhibitor of ACh induced contraction of rat ileum (7). Therefore, it is likely that PABEM has a major contribution to the inhibitory effect of *P. spinosa* extract. Restoration of the contractile response, following washing the tissue, indicated that the inhibitory effects were reversible.

Antispasmodic activity of PABEM could be responsible for the reduction of charcoal meal transit time, as well as, its anti-diarrhoeal activities. PABEM again at lower doses inhibited ileum transit and diarrhoea induced by castor oil and MgSO₄. Comparison of dose-effects clearly shows that anti-diarrheal and intestinal motility inhibition of PABEM with dose of 2 mg/kg is about the same as 10 mg/kg *P. spinosa* extract (Figs. 3, 4 and 5). Inhibition of MgSO₄ induced diarrhoea may indicate that PABEM may also reduce electrolyte secretion as MgSO₄ is an osmotic laxative (18).

These results demonstrate that both *P. spinosa* extract and PABEM of the extract have direct inhibitory effect on rat ileum smooth muscles both *in vitro* and *in vivo*. Comparisons of the effective doses and concentrations support the concept that PABEM is the most likely component responsible for pharmacological activity of *P. spinosa* extract. Additionally, this substance inhibits small intestine peristaltic movements *in vivo* and shows antidiarrheal effect similar to that of loperamide.

CONCLUSION

In conclusion, these results have shown that PABEM probably is the main pharmacologically active component of *P. spinosa* extract and is responsible for its antispasmodic and antidiarrheal activities of the extract. Therefore, PABEM can be used as

a standard component of the extract for further drug design and development. Furthermore, pure substance of this new compound can be developed as a new antispasmodic drug for the treatment of motility disorders of the gut.

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