

Studies on Hepatoprotective, Antispasmodic and Calcium Antagonist Activities of the Aqueous-methanol Extract of *Achillea millefolium*

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The crude extract of *Achillea millefolium* (Am.Cr) was studied for its possible hepatoprotective effect against D-galactosamine (D-GalN) and lipopolysaccharide (LPS) induced hepatitis in mice and antispasmodic effect in isolated gut preparations to rationalize some of the folklore uses. Co-administration of D-GalN (700 mg/kg) and LPS (25 µg/kg) produced 100% mortality in mice. Pre-treatment of animals with Am.Cr (300 mg/kg) reduced the mortality to 40%. Co-administration of D-GalN (700 mg/kg) and LPS (1 µg/kg) significantly raised the plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels compared with values in the control group ($p < 0.05$). Pre-treatment of mice with Am.Cr (150–600 mg/kg) significantly prevented the toxins induced rise in plasma ALT and AST ($p < 0.05$). The hepatoprotective effect of Am.Cr was further verified by histopathology of the liver, which showed improved architecture, absence of parenchymal congestion, decreased cellular swelling and apoptotic cells, compared with the toxin group of animals. In isolated rabbit jejunum preparations, Am.Cr caused a concentration-dependent (0.3–10 mg/mL) relaxation of both spontaneous and K⁺-induced contractions as well as shifting the Ca⁺⁺ concentration-response curves (CRCs) to the right, similar to that caused by verapamil. These results indicate that the crude extract of *Achillea millefolium* exhibits a hepatoprotective effect, which may be partly attributed to its observed calcium channel blocking activity. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: *Achillea millefolium*; hepatoprotective; antispasmodic; calcium antagonist.

INTRODUCTION

Achillea millefolium Linn. (Asteraceae) is a perennial herb, commonly found in northern areas of Pakistan. The aerial parts of the plant are used either whole or separately for medicinal purposes. The plant has a folklore reputation as being diaphoretic, stimulant, antipyretic, analgesic, antiinflammatory, antispasmodic, carminative and antihelmentic (Baquar, 1989). It has been used in various liver diseases either alone or as a part of a compound formulation (Said, 1982). It is one of the constituents in two very commonly available herbal preparations for liver disorders in Pakistan, namely 'Jigarine' and 'Liv-52'. However, the plant has not been studied widely, in particular, for its effectiveness in hepatic and gastrointestinal disorders. This study was conducted to evaluate the hepatoprotective and antispasmodic activities of this plant to provide a scientific basis for some of its traditional uses.

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MATERIALS AND METHODS

Plant material. Aerial parts of *Achillea millefolium* were purchased from a well-known local herbalist. The plant was identified by Mr Afzal Rizvi, a taxonomist at the Hamdard University and a voucher specimen (AM-PL-03-02-43) has been submitted to the herbarium of the Department of Biological and Biomedical Sciences, the Aga Khan University, Karachi.

Preparation of crude extract. The plant materials were cleaned of adulterants, crushed into small pieces and soaked in a 70% aqueous-methanol solution in a large container for 3 days with occasional shaking. It was then filtered through clean cotton cloth and the filtrate thus obtained was stored in clean, dark glass bottles at 4 °C. This procedure was repeated twice and the combined filtrate was evaporated on a rotary evaporator under reduced pressure to a thick semi-solid paste, i.e. the crude extract (Am.Cr), yielding approximately 18%. Am.Cr was solubilized both in saline and distilled water for use in *in vivo* and *in vitro* experiments, respectively.

Drugs and animals. D-Galactosamine (D-GalN), lipopolysaccharide (LPS) (*E. coli* 055:B5), acetylcholine chloride, potassium chloride and verapamil hydrochloride

were purchased from Sigma Chemicals Co, St Louis, MO, USA. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) estimation kits were purchased from Randox, UK.

Balb-C mice (20–25 g) and rabbits (1.0–1.5 kg) of local breed, either sex were used for this study. The animals were housed at the Animal House of the Aga Khan University, maintained at 23–25 °C and were given a standard diet and tap water. Mice were kept in plastic cages (47 × 34 × 18 cm), 5–7 animals per cage, lined with sawdust renewed after 48 h. Rabbits had free access to water, but food was withdrawn 24 h prior to experiment and killed by a blow on the back of the head. The experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (1996) and approved by the Ethical Committee of the Aga Khan University.

Survival study. The animals were divided into three groups of five mice each. Group 1 received normal saline (0.01 mL/kg). Group 2 (toxin group) received normal saline (0.01 mL/kg) followed after 1 h, by 700 mg/kg D-GalN and 25 µg/kg LPS. Group 3 (treatment group) received plant extract (300 mg/kg) followed, after 1 h, by 700 mg/kg D-GalN and 25 µg/kg LPS. Extract, toxins and normal saline were administered intraperitoneally. Any change in the behavior was observed for 6 h and mortality was observed after 24 h.

Induction of hepatic injury. Hepatic injury was induced by intraperitoneal co-administration of D-GalN and LPS at doses of 700 mg/kg and 1 µg/kg, respectively (Tiegs *et al.*, 1989). Control animals received normal saline i.p. at a dose of 0.01 mL/kg.

Estimation of hepatic injury. Hepatic damage was estimated by measuring the plasma levels of ALT, AST and concomitant changes in liver histopathology. Eight hours after the administration of toxins, the mice were killed by cervical dislocation. Blood was collected in sterilized and heparinized syringes by direct cardiac puncture. The samples obtained were centrifuged at 14 000 rpm for 10 min in an Eppendorf centrifuge. Plasma thus obtained was used for the estimation of ALT and AST levels spectrophotometrically (Rietman and Frankel, 1957) using Randox diagnostic kits. The excised liver was placed in a glass bottle containing 10% buffered formal saline (isotonic formalin solution) that was changed after 12 h to remove any residual blood. The tissue was then dehydrated in increasing concentrations of alcohol, cleared with xylene, impregnated and embedded in paraffin to form a block. Approximately 5 µm sections were cut from the tissue block, fixed on glass slides, stained with hematoxylin and eosin. The slides thus prepared were examined under a light microscope for evidence of hepatic damage. Hepatic damage was considered to occur when there was disruption of the parenchyma architecture with congestion, presence of cellular swelling and apoptotic cells.

Hepatoprotective study. The animals were divided into six groups of seven mice each. Group 1 received normal saline (0.01 mL/kg). Group 2 received normal

saline followed, after 1 h, by toxins (D-GalN 700 mg/kg and LPS 1 µg/kg). Groups 3, 4 and 5 received Am.Cr at doses of 150 mg/kg, 300 mg/kg and 600 mg/kg, respectively, 1 h before the co-administration of toxins. Extract, toxins and vehicle (normal saline) were administered intraperitoneally.

Acute toxicity study. *Achillea millefolium* was administered to a group of ten mice at a dose of 3 g/kg intraperitoneally. The animals were kept under constant observation for 6 h to note any behavioral change and mortality was recorded after 24 h.

Isolated tissue experiments. The spasmolytic activity of the plant material was studied using isolated rabbit jejunum as described previously (Gilani *et al.*, 1994). Respective segments of 2 cm length were suspended in a 10 mL tissue bath containing Tyrode's solution, maintained at 37 °C and aerated with a mixture of 95% oxygen and 5% carbon dioxide. The composition of the Tyrode's solution in mM was: KCl 2.68, NaCl 136.9, MgCl₂ 1.05, NaHCO₃ 11.90, NaH₂PO₄ 0.42, CaCl₂ 1.8 and glucose 5.55. Intestinal responses were recorded isotonicly using BioScience transducers and an oscillograph. Each tissue was allowed to equilibrate for at least 30 min before the addition of any drug.

Determination of calcium antagonist activity. K⁺ was used to depolarize the intestinal preparations (Farre *et al.*, 1991). High K⁺ (80 mM) was added to the tissue bath, which produced a sustained contraction. Samples were then added in a cumulative fashion to obtain concentration-dependent inhibitory responses. The relaxation of intestinal preparations, pre-contracted with K⁺ (80 mM) was expressed as the percent of the control response mediated by K⁺.

To confirm the calcium antagonist activity of test substances, the tissue was allowed to stabilize in normal Tyrode's solution, which was then replaced with Ca⁺⁺-free Tyrode's solution containing EDTA (0.1 mM) for 30 min in order to remove calcium from the tissues. This solution was further replaced with K⁺-rich and Ca⁺⁺-free Tyrode's solution, having the following composition (mM): KCl 50, NaCl 91.04, MgCl₂ 1.05, NaHCO₃ 11.90, NaH₂PO₄ 0.42, glucose 5.55 and EDTA 0.1. Following an incubation period of 30 min, control concentration-response curves (CRCs) of Ca⁺⁺ were obtained. When the control CRCs of Ca⁺⁺ were found to be superimposable (usually after two cycles), the tissue was pretreated with the plant extract for 60 min to test the possible calcium channel blocking effect. The CRCs of Ca⁺⁺ were reconstructed in the presence of different concentrations of the test material.

Statistical analysis. Mortality and survival are given as a percentage. Plasma ALT and AST are given as mean ± standard deviation and the mean of two groups were compared by Student's *t*-test with a value of *p* < 0.05 considered as significant. The percent hepatoprotection or percent reduction was calculated as follows:

$$100 - \left(\frac{\text{Treatment group mean} - \text{control mean/toxin group mean}}{\text{control mean}} \times 100 \right)$$

RESULTS

Survival study

Co-administration of D-GalN 700 mg/kg and LPS 25 µg/kg intraperitoneally produced 100% mortality in a group of mice. Pre-treatment of animals with Am.Cr (300 mg/kg) reduced the mortality to 40%.

Hepatoprotective activity

Plasma levels of ALT and AST in the control group of mice were found to be 292 ± 39 and 542 ± 158 IU/L (Table 1). Co-administration of toxins raised the plasma levels of ALT and AST to 2344 ± 131 and 2211 ± 254 IU/L in group 2 respectively, which were significantly higher than the control group ($p < 0.05$). The plasma ALT and AST levels (IU/L) in mice pre-treated with the plant extract (150 mg/kg) were found to be 1815 ± 121 and 1722 ± 158 in group 3, 1637 ± 193 and 1662 ± 199 in group 4 (300 mg/kg) and 1455 ± 195 and 1452 ± 232 in group 5 (600 mg/kg), respectively. These values were significantly lower ($p < 0.05$) than those in the toxin group and showed dose dependent improvement.

The liver histopathology of the toxin group showed a marked disruption in the liver parenchyma, cellular swelling and congestion with severe apoptosis (>10 apoptotic cells per high power field, in 10 or more fields) and occasional necrotic foci (Fig. 1B) compared with the control (Fig. 1A). Treatment groups showed an absence of congestion and focal necrosis with a dose-dependent improvement in cellular swelling, architecture and the number of apoptotic cells (Fig. 1C, 1D and 1E).

Acute toxicity study

Achillea millefolium administered at a dose of 3 g/kg intraperitoneally showed no apparent change in behavior after 6 h or mortality after 24 h.

Calcium antagonistic activity

Achillea millefolium caused a dose-dependent (0.3–10 mg/mL) inhibition of spontaneous and K⁺ (80 mM)-

induced contractions of isolated rabbit jejunum preparations (Fig. 2A). Incubating the intestinal preparations with Am.Cr (0.3–1.0 mg/mL) shifted the Ca⁺⁺ CRCs to the right (Fig. 3A). These effects were comparable to that produced by verapamil as shown in Fig. 2B and 3B.

DISCUSSION

D-GalN and LPS induced hepatitis in mice is a commonly used test model that mimics the sequence of events in human hepatitis (Hishinuma *et al.*, 1990). The magnitude of damage can be assessed conveniently by measuring ALT and AST levels released into the blood and by microscopic examination of the concomitant histopathological changes (Beckingham and Ryder, 2002). LPS activates liver Kupffer cells, releases several inflammatory mediators such as tumor necrosis factor alpha (TNF-α), nitric oxide (NO), prostaglandins, leukotrienes and interleukins, causing hepatocellular damage and apoptosis, while D-GalN specifically inhibits hepatic protein synthesis by depleting cellular stores of uridine nucleotides (Keppler *et al.*, 1970). When co-administered with LPS, D-GalN inhibits the hepatic cytoprotective protein synthesis, thus potentiating the hepatotoxic effects of LPS, evident from the increased plasma level of ALT, AST and concomitant histopathological changes. The plant extract significantly decreased the ALT and AST levels in treated animal groups compared with the control, suggesting the hepatoprotective effect of *Achillea millefolium* extract which was confirmed by the liver histopathological observation of treated animals.

Kupffer cells synthesize inflammatory mediators by mechanisms that are partly dependent upon increased intracellular calcium (Hsuan *et al.*, 1999). Furthermore, the pathological role of increased intracellular calcium ions is also well established. Within the hepatocytes, excessive cytosolic calcium ions alters the plasma membrane integrity of the cell and its organelle, damages DNA and degrades essential proteins by activating phospholipases, endonucleases and proteases (Schanne *et al.*, 1979). In addition, excessive intra-mitochondrial accumulation of calcium causes a release of cytochrome c through the mitochondrial permeability transition pore, which is one of the major events in initiating apoptosis (Szewczyk and Wojtczak, 2002).

Table 1. Effect of multiple dose pre-treatment of *Achillea millefolium* extract on toxins induced hepatitis in mice

Group	Treatment	ALT (IU/L)	AST (IU/L)
1	Normal saline (0.01 mL/kg)	292 ± 39	542 ± 158
2	Normal saline (0.01 mL/kg)	2344 ± 131 ^a	2211 ± 254 ^a
3	<i>Achillea millefolium</i> (150 mg/kg)	1815 ± 121 ^b (25.78%)	1722 ± 158 ^b (29.30%)
4	<i>Achillea millefolium</i> (300 mg/kg)	1637 ± 193 ^b (34.46%)	1662 ± 199 ^b (32.90%)
5	<i>Achillea millefolium</i> (600 mg/kg)	1455 ± 195 ^b (43.32%)	1452 ± 232 ^b (45.48%)

^a $p < 0.05$, group 1 vs 2.

^b $p < 0.05$, group 2 vs 3, 4 and 5.

values represent mean ± SD of the seven determinations, with % reduction given in parenthesis beneath each value. Toxins, extract and vehicle were administered intraperitoneally.

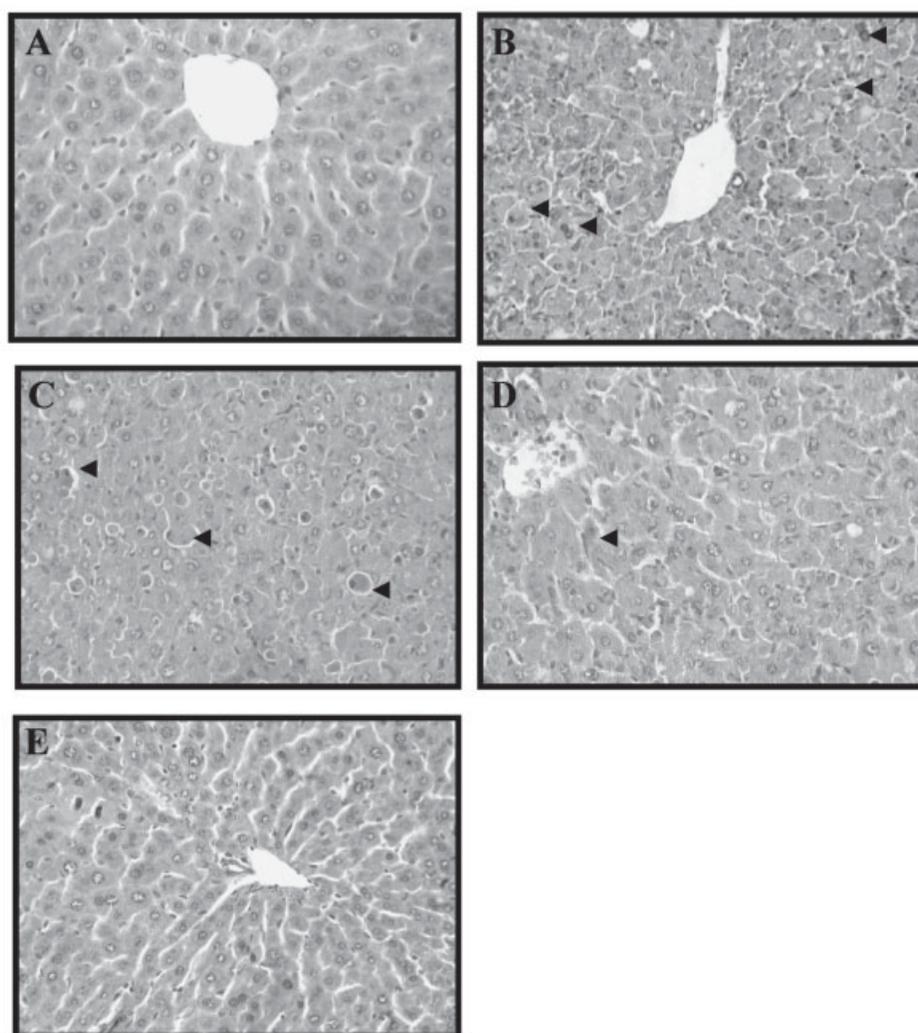


Figure 1. Representative microphotographs (40× magnification, hematoxylin and eosin staining) showing effects of *Achillea millefolium* extract on toxin-induced histopathological changes in mouse liver. The control group (A) showed normal parenchyma architecture and sinusoidal spaces with no apoptosis and congestion. The toxin group (B) showed disturbed architecture with marked cellular swelling, loss of cell adhesion and many apoptotic cells (arrow heads). The pre-treatment groups (C, D and E) showed improved architecture, less marked cellular swelling and apoptosis with increasing dose of treatment.

In isolated tissue experiments, the plant extract caused inhibition of spontaneous and high K^+ -induced contractions of rabbit jejunum preparations, thus showing that the antispasmodic action is mediated through a calcium antagonist effect, which was confirmed when the *Achillea millefolium* extract caused a concentration-dependent rightward shift in the Ca^{++} CRCs, similar to that caused by verapamil, a standard calcium channel blocker (Fleckenstein, 1977). This finding explains one of the multiple mechanisms by which the extract might have produced hepatoprotection, as calcium channel blockers have been shown to decrease the production of macrophage-related cytokines and lipid mediators (Szabo *et al.*, 1993; Molle *et al.*, 2000) and thus protects against their deleterious effects. The inner mitochondrial membrane contains binding sites

for calcium channel blockers (Zernig *et al.*, 1990) and they have been shown to inhibit calcium influx, preventing cellular damage.

In conclusion, an aqueous-methanol extract of *Achillea millefolium* showed hepatoprotective activity against D-GalN/LPS induced hepatitis in mice, as well as an antispasmodic effect mediated through calcium channel blockade. These findings provide a scientific basis for its traditional use as a hepatoprotective and antispasmodic agent.

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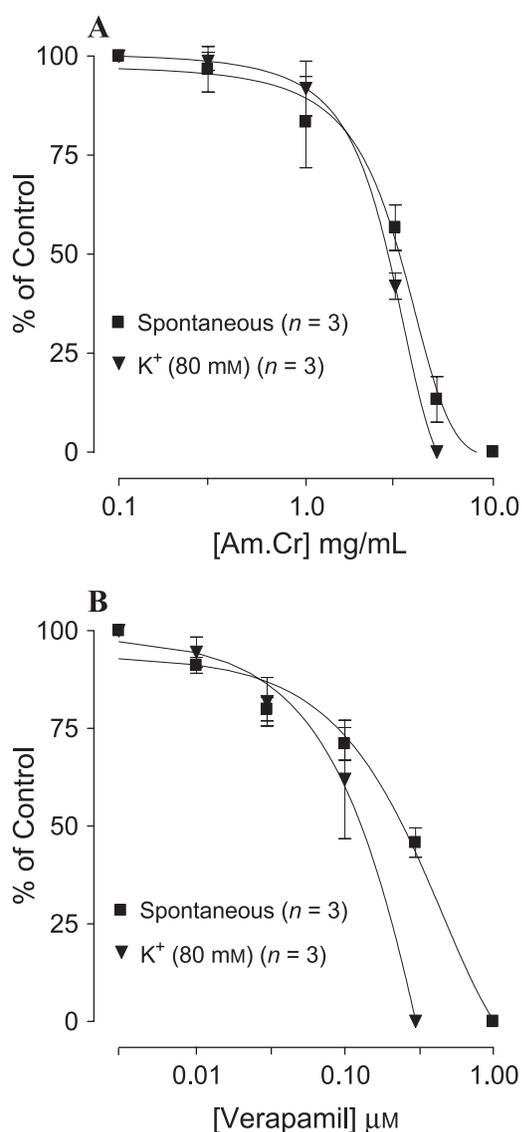


Figure 2. Concentration-dependent inhibitory effect of (A) crude extract of *Achillea millefolium* (Am.Cr) and (B) verapamil on spontaneous and K^+ (80 mM)-induced contractions in isolated rabbit jejunum preparations.

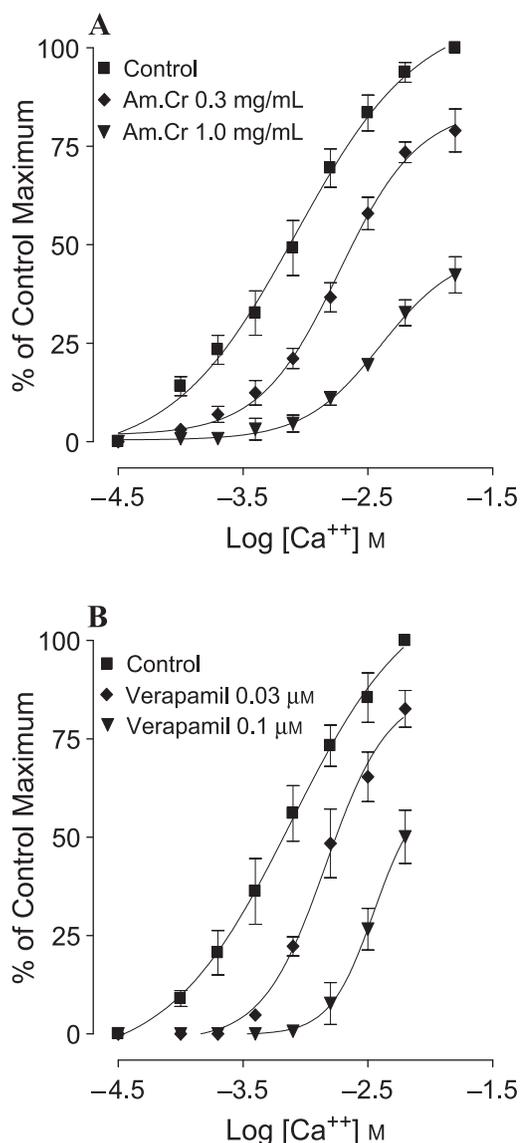


Figure 3. Concentration-response curves of Ca^{++} in the absence and presence of increasing doses of (A) crude extract of *Achillea millefolium* (Am.Cr) and (B) verapamil in isolated rabbit jejunum preparations.

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