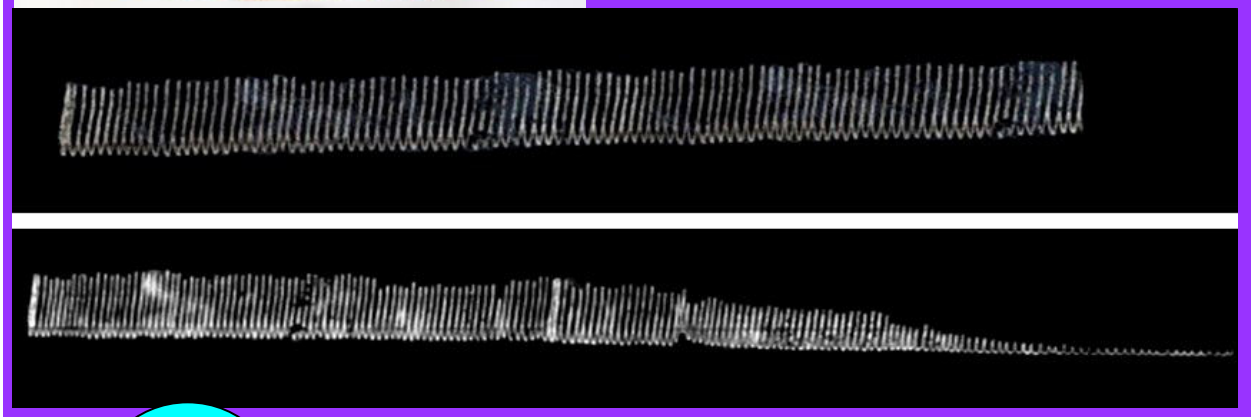

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Antimicrobial, anti-oxidant and calcium channel blocking activities of *Amberboa divaricata*

Shahid Muhammad Iqbal, Aamir Mushtaq and Qaiser Jabeen

Department of Pharmacy, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Bahawalpur 631 00, Pakistan.

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Abstract

Traditional healers in Pakistan use the herb *Amberboa divaricata* as tonic, aperiant, deobstruent, febrifuge, anti-diarrheal, antiperiodic, antipyretic, anti-cough and in skin disorders. *In vitro* tissue experiments were carried out on rabbit jejunum to elucidate the possible mechanism of its prescribed effects on gastrointestinal tract, while antibacterial and anti-oxidant experiments were performed to provide pharmacological evidence of its traditional use in skin disorders. The 70%methanolic crude extract of *A. divaricata* produced dose-dependent relaxation in isolated rabbit jejunum tissue in a concentration range of 0.1–3.0 mg/mL (n=5). Calcium response curves were constructed at concentration of 0.03 and 0.1 mg/mL (n=5), which produced rightward shift in a pattern similar to that of verapamil, confirming the calcium channel blocking activity. Agar disc diffusion assay at a concentration of 10 mg crude extract/disc showed clear zones of inhibition.

Introduction

The genus *Amberboa* belongs to family compositae and is represented by six species, one of which is *Amberboa divaricata*, an erect, stiff, dichotomously branched annual herb (Vardhana, 2008). Locally this plant is known as Birumdundi, Badaward or Daaba (Qureshi and Bhatti, 2008). Previous phytochemical investigation revealed that *A. divaricata* contains fructose, triacontane, campesterol, sigmasterol, sitosterol, jaceosidine, cynaropicrin, cycloartane type triterpenoids, guaianolides, sesquiterpenoids, lupeol, flavonol glycosides, green essential oils, acid resins, organic acids, gums, fatty matter and alkaloids (Ibrahim et al., 2010). Traditionally, this herb is used as tonic, aperient, deobstruent, febrifuge, anti-diarrheal, and anti-periodic, it is used in coughs, fever and general debility. It has cytotoxic and antibacterial properties. Seeds are antidote, astringent and resolvent (Bhattacharjee, 2005). For skin irritation plant is boiled in water and bath is taken. About two gram of plant is given in malaria and continued for three days to treat fever. For blood purification juice of fresh plant is used with black pepper (Qureshi and Bhatti, 2008). Beside a number of traditional uses, this herb was not previously evaluated pharmacologically to validate its use by traditional healers. So this study was designed to check its possible spasmolytic, antibacterial and anti-oxidant effects.

Materials and Methods

Plant material and extraction

The whole plant of *A. divaricata* was purchased from the local herbal market of Bahawalpur. It was authenticated by a botanist at Govt. S.E. College Bahawalpur, and sample of plant material was submitted at herbarium of pharmacology section Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Pakistan, having a voucher number AD-WP-03-10-004. The plant material (500 mg) was cleaned

from dirt and extraneous matter and then coarsely powdered. The powdered material was soaked in sufficient volume of 70%methanol with occasional stirring for three days, and then it was filtered initially through muslin cloth followed by filteration through Whatman qualitative grade 1 filter paper. The plant material was subjected to this procedure three times, the filtrate was collected, pooled together and evaporated at 40-50°C in rotary evaporator (Laborota 4000-efficient Heidolph, Germany). After the removal of solvent a thick semi solid mass of yellowish brown color was obtained with a percentage yield of about 11%. The crude extract was stored in air tight container at -20°C.

Chemicals and animals

All the chemicals used were of analytical grade, acetyl-choline chloride, atropine sulfate, verapamil hydrochloride, Folin-Ciocalteu reagent and DPPH were purchased from Sigma Chemicals Company, St. Louis, MO, USA. Sodium chloride, potassium chloride, magnesium chloride, calcium chloride, D-glucose, Sodium dihydrogen phosphate, sodium bi carbonate, EDTA, Mueller Hinton agar, nutrient broth and methanol were purchased from Merck, Germany. Animals used in this study like rabbits (1-1.5 kg), Swiss albino mice (20-25 g) were obtained from National Institute of Health Pakistan, and kept at animal house of Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur. The animals were allowed to feed and drink water but prior to experiment they were kept fasting for 24 hours. On the day of experiment rabbit was killed by a blow on back of the head and jejunum was excised out, which was immediately placed in Tyrode solution previously prepared, aerated and maintained at 37°C. Three microbial strains were used for antimicrobial activity, out of which two were gram positive i.e. *Staphylococcus aureus*, *Bacillus subtilis* and one was gram negative i.e. *Escherichia coli*. The bacterial strains were obtained from the Department of Biochemistry and Biotechnology, The Islamia University of Bahawalpur.

Preliminary phytochemical analysis

Phytochemical analysis of crude extract of *A. divaricata* (AD.Cr) was performed by methods as previously described (Usman et al., 2009). Briefly the presence of alkaloids was detected when orange precipitates appear -ed on treating with Dragendorff's reagent. Appearance of brownish green precipitates on treating with $FeCl_3$ indicate the presence of Tannins. Formation of persisted froth on vigorously shaking in water exhibited the presence of saponins. Appearance of cherry red color on performing modified Borntrager's test, reveal the presence of anthraquinones. The presence of glycosides was observed by performing Keller-killiani test. Flavonoids were detected by treating the aqueous solution of extract with KOH solution which results in dark yellow color and steroids were determined by mixing the extract with acetic acid and then addition of concentrated H_2SO_4 in ice cooled above solution which results in the formation of violet-blue color. Carbohydrates were determined by performing Molish test while Barfoed and Seliwanoff's test showed the presence of monosaccharides and keto-nes respectively. Appearance of red color on treating with acidified solution of phloroglucinol, indicate the presence of pentoses.

In vitro tissue experiments

In vitro tissue experiments were performed as previously described (Jabeen et al., 2009). Briefly several pieces of about 2 cm were cut from the excised jejunum and cleaned from the fatty matter, and then they were mounted in 50 mL tissue organ bath containing Tyrode solution maintained at 37°C. The tissue was continually aerated with carbogen gas (95% O_2 + 5% CO_2) and equilibrated for 30 min before addition of any drug or extract. Intestinal responses were recorded with the help of kymograph (MTA-786/1024). After 30 min of equilibration, jejunal tissue was stabilized with sub maximal concentration of acetyl-choline i.e. (0.3 µM). After stabilization intestinal tissue started to exhibit uniform spontaneous rhythmic contractions, and then the extract was added in cumulative manner to check its activity. The calcium channel blocking activity was explored by depolarizing the jejunal tissue with high K^+ (80 mM) as previously described (Farre et al., 1991). Addition of high K^+ caused sustained contraction in intestinal tissue, and then extract was added in cumulative manner to check its inhibitory effect on K^+ induced contractions. To confirm the calcium channel blocking activity of crude extract, tissue was stabilized in Tyrode solution. The Tyrode solution was then replaced with calcium free Tyrode solution that contained 0.1 mM EDTA, and tissue was allowed to stand for 30 min in order to remove the calcium from tissue. Then calcium free Tyrode solution was replaced with potassium rich and calcium free Tyrode solution containing 0.1 mM EDTA. The tissue was again allowed to stand for 30 min and then controlled calcium response curves were constructed and re constructed with Ca^{2+} until two super imposable curves are obtained. Then tissue was pretreated with different concentrations of plant extract for 60 min before constructing calcium response curves, and then compared with calcium response curve of verapamil a standard calcium channel blocker.

Antibacterial activity

The antibacterial activity of crude extract of *A. divaricata* was performed by methods with little modifications as previously described (Salama and Marraiki, 2010).

Agar disc diffusion assay- The antibacterial activity of crude extract of *A. divaricata* was determined by using

Mueller Hinton agar. Pre-adjusted bacterial culture of 0.5 Mcfarland standards was spread on 15 cm diameter petri plates, then sterile filter paper discs each containing 10mg of crude extract were placed. After placing the discs, plates were covered and incubated at 37°C for 24 hours. The antibacterial activity was determined by measuring the clear zone around the filter paper discs. Gentamicin and ampicillin discs were used as standards, and the experiment was carried out in triplicate.

Determination of MIC and MBC- Minimum inhibitory concentration was determined by microdilution assay as previously described (Sahin et al., 2003). The inoculums of bacteria were prepared and adjusted to 0.5 McFarland standards. The dried extract was reconstituted to the maximum concentration to be tested i.e. 20 mg/mL and then a serial 2-fold dilutions were made in a concentration ranging 10-0.078 mg/mL with sterile nutrient broth. Then 95 µL sterile nutrient broth and 5 µL of inocula were dispensed in sterile 96 well plates. A 100 µL of crude plant extract (20 mg/mL) was added into first well. Then 100 µL of each of serial dilutions were added to the next wells. The final volume in each well was 200 µL. Ampicillin/ Gentamicin were used as standard antibiotics, pure bacterial culture, nutrient broth served as positive and negative control respectively. One plate was used for each bacterial strain to prevent the cross contamination. Then sterile plate sealer was placed on each plate and incubated at 37°C for 24 hours. Microbial growth was observed and confirmed by plating 5 µL samples from clear wells on nutrient agar medium at 37°C for 24 hours. The MIC was described as minimum concentration of the crude extract which inhibited the growth of microorganism. The lowest concentration that showed no growth after this sub culture was taken as Minimum bactericidal concentration (Reddy et al., 2008). Each experiment was performed in triplicate.

Determination of total phenolic contents- Total phenolic contents of crude extract of *A. divaricata* were determined as described elsewhere (Ksouri et al., 2009). Briefly in a small test tube 125 µL of properly diluted crude extract was added to 500 µL of water and then 125 µL of Folin-Ciocalteu reagent was added and mixed. Then added 1250 µL of Na_2CO_3 (7%) and made-up the final volume to 3 mL with distilled water, and mixed it again thoroughly. After incubating for 90 min at 23°C in dark, the absorbance was taken at 760 nm. Total phenolic content of crude extract was determined by using standard gallic acid curve (0-500 µg/mL), and expressed as mg gallic acid equivalents per gram of dry weight of extract. The experiment was performed in triplicate.

Determination of total anti-oxidant capacity

The total anti-oxidant activity of the crude extract of *A. divaricata* was determined by the method as described elsewhere (Niciforovic, 2010). Briefly 0.3 mL of sample extract was mixed with 3 mL of reagent solution (4 mM ammonium molybdate 0.6 M sulfuric acid and 28 mM sodium phosphate), then incubated at 95°C for 90 min. The absorbance of the solution was measured at 695 nm after proper cooling of reaction mixture to room temper -ature. The total anti-oxidant capacity was expressed as milligrams of ascorbic acid per gram of the dry weight of extract. The experiment was performed in triplicate.

DPPH radical scavenging assay- DPPH radical scavenging assay was performed as previously described (Raja -Kannan et al., 2010), with little modifications. Briefly in a 96-well plate, 10 µL of test substance and 90 µL of 100 µM methanolic solution of diphenylpicryl-hydrazyl (DPPH) was added and mixed. The contents were incubated at room temperature for 30 min in the dark. Then absorbance was taken by using 96-well plate reader Synergy UT, Bioteck instrument USA at 517 nm. Quercitin was used as standard anti-oxidant. DPPH scavenging activity was determined by the given formula. The assay was performed in triplicate. %Scavenging activity = 100–[Absorbance of test compound/Absorbance of control] × 100

Acute toxicity testing

Acute toxicity test was performed on mice as previously described (Gilani et al., 2008). Animals were randomly assigned into four groups, and each group contained five mice. Group I, II, and III received the increasing doses of extract; i.e. 300, 1000 and 3000 mg/kg per oral respectively. Group IV served as negative control and received 10 mL/kg normal saline. The animals were kept under observation for 6 hours, allowed feed and drink water *ad libitum*. After 24 hours, they were observed for any lethality.

Statistical analysis

The statistical analysis was performed by using the software GraphPad Prism 5.01, while the data was expressed as mean of three experiments ± standard error of mean (n = no of experiments).

Results

The crude extract of *A. divaricata* was found to contain alkaloids, tannins, saponins, glycosides, flavonoids, steroidal compounds, carbohydrates, ketones, pentoses and soluble starch while coumarins and anthraquinones were absent.

The effect of crude extract of *A. divaricata* was checked on spontaneously contracting rabbit jejunal preparation in a concentration range of 0.1–3 mg/mL. It decreased spontaneous contractions of rabbit jejunum and on further increasing the concentration up to 3 mg/mL, it completely blocked the contractions as represented in

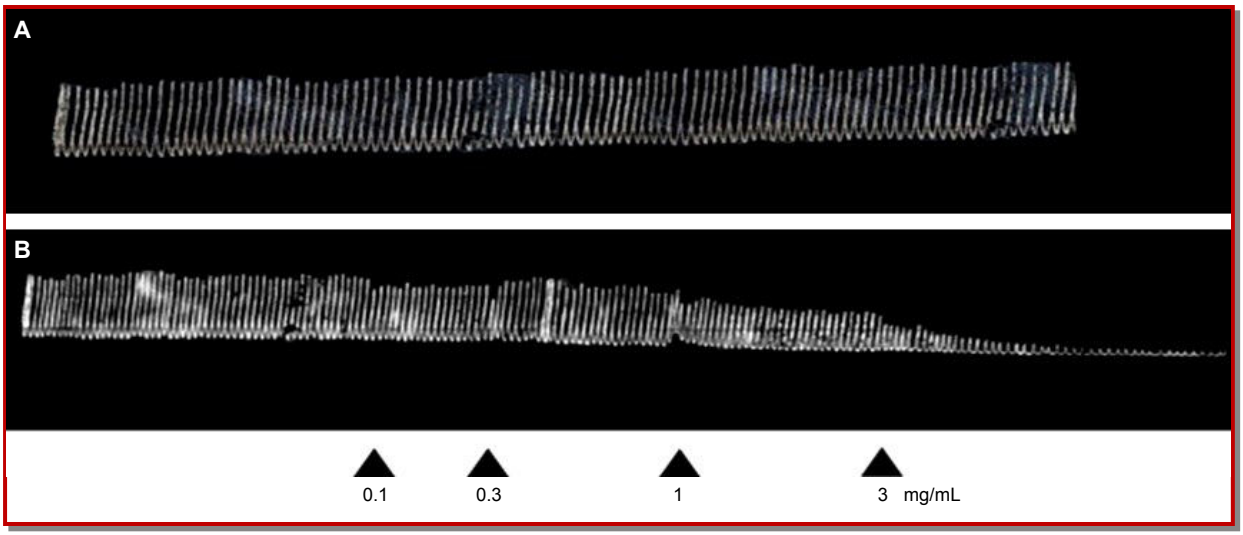

Figure 1: Representative tracing showing the (A) normal contractions of isolated rabbit jejunum tissue and (B) spasmolytic effect of crude extract of *A. divaricata* Ad.Cr when introduced in a cummulative manner

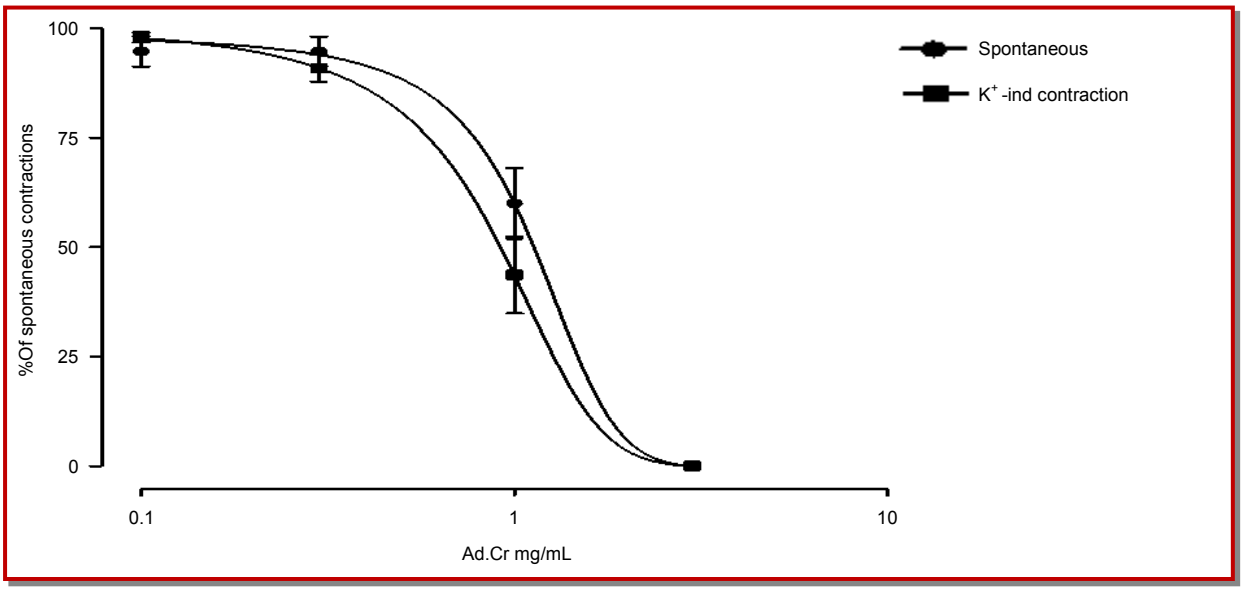

Figure 2: Concentration-dependent inhibitory effect of the crude extract of *A. divaricata* (Ad.Cr) on spontaneous and K^+-induced contractions in isolated rabbit jejunum preparations. Values shown are mean ± S.E.M. of 3-5 observations

Figure 1. The spasmolytic effect was concentration-dependent and tissue regained spontaneous contractions after 5-10 min of washing with Tyrode solution. The spasmolytic effect was further elaborated when crude extract of *A. divaricata* relaxed the pre-contracted rabbit jejunum with high K^+ i.e. (80 mM). Again the effect was concentration dependent and at concentration of 3 mg/mL, it completely relaxed the contracted tissue as shown in Figure 2. As these preliminary experiments performed on isolated rabbit jejunum tissue indicated the Ca^{+2} channel blocking activity of crude extract of *A. divaricata*, so calcium response curves were made in the presence and absence of crude extract of *A. divaricata*. The calcium response curves constructed at concentrations of 0.03 and 0.1 mg/mL showed right ward shift in a pattern similar to that of verapamil as shown in Figure 3, which confirms that the crude extract of *A. divaricata* contains calcium channel blocking activity.

The aqueous methanolic crude extract of *A. divaricata* (Ad.Cr) was checked at the concentration of 10 mg/disc for its antibacterial activity against three bacterial strains. The extract inhibited the growth of all three bacterial strains. The diameter of zone of inhibitions for *S. aureus*, *B. subtilis* and *E. coli* were 13.3 ± 0.6, 12.0 ± 1.0, 10.7 ± 1.2 mm respectively. Agar disc diffusion assay exhibited the sensitivity of all three strains against crude extract of *A. divaricata* as shown in Figure 4. So micro dilution assay was performed which revealed the MIC values for *B. subtilis* and *S. aureus* was 2.5 mg/mL, while for *E. coli* it was 5 mg/mL. Minimum bactericidal

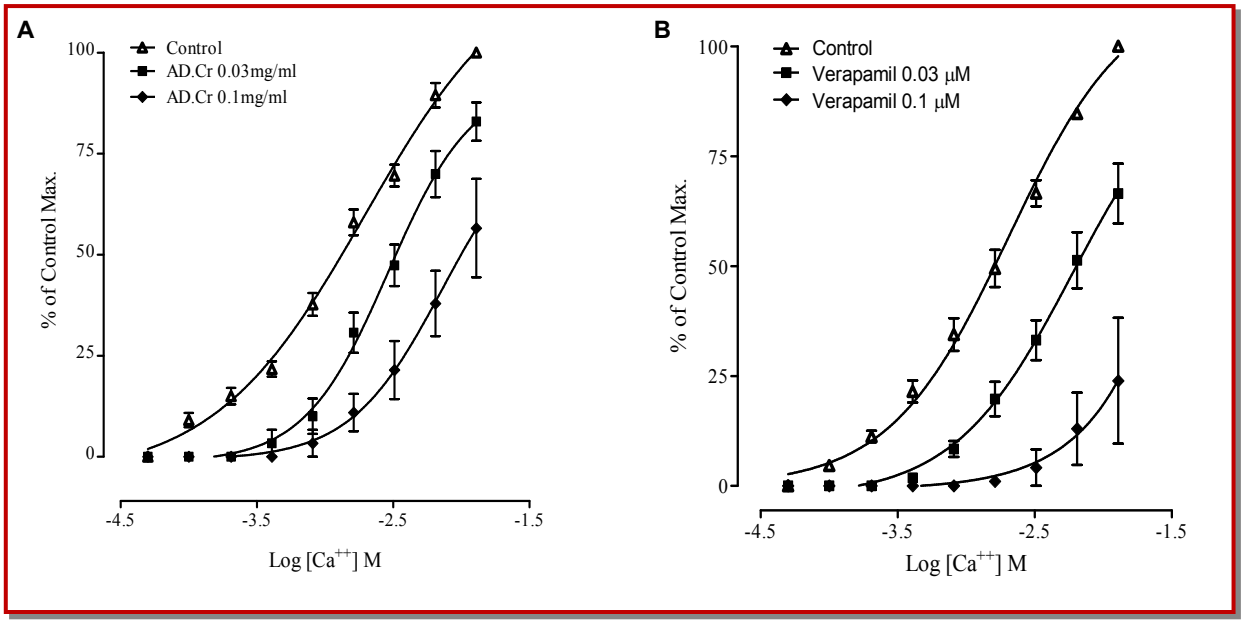

Figure 3: Concentration–response curves of Ca^{2+} in the absence and presence of different concentrations of (A) crude extract of *A. divaricata* (Ad.Cr). (B) Verapamil in isolated rabbit jejunum preparations. Values shown are mean ± S.E.M. of 4–5 observations

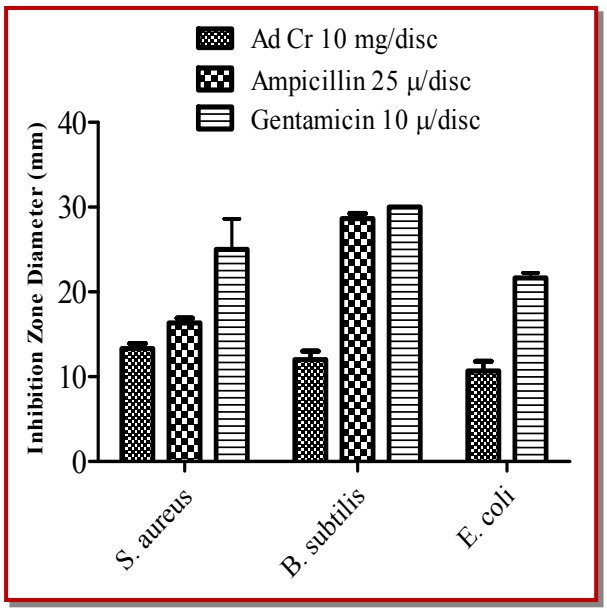

Figure 4: Comparison of Inhibition Zone Diameter against bacterial species by the crude extract of *A. divaricata* (Ad.Cr), ampicillin and gentamicin. Values are mean ± S.D. of three replicates

concentration is the minimum concentration that kills bacteria. The MBC values for all three strains were same i.e. 5 mg/mL.

Anti-oxidant capacity of *A. divaricata* crude extract was determined by calculating total phenolic contents, total anti-oxidant capacity and DPPH radical scavenging assay. The total phenolic contents, of *A. divaricata* crude extract were found to be 40.5 ± 2.5 (mg GAE/g DW), while the total anti-oxidant capacity was 22.4 ± 2.4 mg AAE/g DW, which showed its anti-oxidant potential. At the concentration of 0.1 mg/mL *A. divaricata* crude extract scavenged 31.4 ± 1.1% of DPPH radical, while at the same concentration quercetin caused 95.6 ± 0.02% inhibition as shown in Figure 5.

Acute toxicity test was performed on mice, and up to 3 g/kg, the crude extract of *A. divaricata* did not exhibited any adverse effect on the animals. So, the crude extract of *A. divaricata* was rendered safe up to the dose of 3 g/kg.

Discussion

Gastrointestinal motility is a complex physiological function. The two main determining factors of gastrointestinal motility are the smooth muscles and the enteric nervous system (Roman and Gonella, 1987). So, the enteric neurons and intestinal smooth muscle cells are two promising targets for drugs. The contraction of Smooth muscles basically depends on increased concen-tration of free cytosolic calcium, that may be either due to the extracellular entry of Ca^{2+} through calcium channels or by the release of Ca^{2+} ions from intracellular stores (Pietrobon et al., 1990). Ca^{2+} ions are continually exchanged between intracellular and extra-cellular Ca^{2+} stores which results in cyclic depolarization and repolarization of intestinal tissue that accounts for its involuntary contractions (Ali et al., 2009). Traditionally, the herb *A. divaricata* has been used in over-active disease of gastrointestinal tract such as abdominal spasm and diarrhea. The crude extract of *A. divaricata* exhibited spasmolytic effect on rabbit's jejunal preparation. A number of previous studies have men-

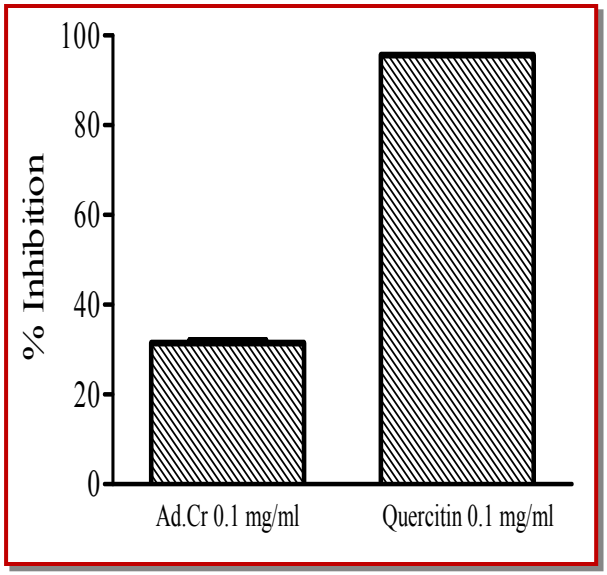

Figure 5: DPPH radical scavenging activities of *Amberboa divaricata* (Ad.Cr) and quercetin. Values are shown mean ± SEM of 3 values

tioned that spasmolytic effect of medicinal plants is usually due to calcium channel blockade (Gilani et al., 1999), so the crude extract of *A. divaricata* was checked on tissue preparation pretreated with high potassium, where it relaxed the contracted tissue. High concentration of K^+ in extracellular space acts as non-receptor spasmogen and depolarizes the smooth muscles followed by contraction (Karaki et al., 1997) in which voltage dependent Ca^{+2} channels are involved (Gharib et al., 2008). The phytochemical tests of the crude extract of *A. divaricata* showed that it contains various classes of bioactive compounds e.g. flavonoids, glycosides, saponins, steroids tannins and phenolic compounds. Previously it has been described that phenolic compounds and some flavonoids have effects on intestinal motility both *in vivo* and *in vitro* (Di carlo et al., 1999). Flavonoids such as quercitin has well documented antispasmodic effects on gastrointestinal smooth muscles and some researchers also conclude that this antispasmodic effect is related to calcium release from intracellular stores and/or interference with calcium entry through calcium channels (Capasso et al., 1991). A much recent study described that flavonoids contained in hexane extract of *Syzygium Samarangense* exhibited relaxant activity which was due to the blockade of calcium influx (Ghayur et al., 2006). So, it can be considered that the antispasmodic activity of *A. divaricata* may be due to its flavonoids and various phenolic compounds. Antimicro-bial agents can be derived from medicinal herbs and more than 1000 herbs have exhibited antimicrobial effects (Nychas, 1995). *A. divaricata* is used in skin irritations and as antimicrobial by traditional healers, so *A. divaricata* crude extract was evaluated against common bacterial species *E. coli, B. subtilis* and *S. aureus*. Plants are capable of synthesizing number of phenolic compounds and their derivatives which are considered as secondary metabolites (Schultes, 1978). Most of these secondary metabolites are part of plant defense mechanisms and protect them against the invasion of herbivores, insects and microorganisms. Phytochemicals, isolated from medicinal plants exhibit toxicity to microorganisms by different mechanisms. The mechanism underlying phenolic compound toxicity to microorganisms is thought to be mediated by enzyme inhibition possibly through interaction with sulfhydryl groups and with different proteins (Mason and Wasserman, 1987). Tannins are polymeric phenols which stimulate immune system and are considered to have wide range of anti infective activities (Haslam, 1996). They make complexes with proteins so they have ability to inactivate microbial enzymes, adhesion and transport proteins (Stern et al., 1996). Scalbert, listed 33 studies that described antimicrobial activities of tannins against fungi, bacteria and yeasts (Scalbert, 1991). Similarly flavonoids are the compounds which are synthesized in response to microbial infection in plants (Dixon et al., 1983), and are found to be effective against wide range of microorganisms (Cowan, 1999). They disrupt microbial membranes, make complex with bacterial cell wall, extracellular and soluble proteins (Tsuchiya et al., 1996). Phenolic compounds present in plants have gained considerable importance due to their anti-oxidant activities (Pan et al., 2008). Total phenolic contents of crude extract of *A. divaricata* exhibited its anti-oxidant potential, so the crude extract was subjected to further anti-oxidant assays. Phosphomolybdenum method was used to determine the total anti-oxidant capacity of *A. divaricata* which is a quantitative assay because it described the number of equivalents of ascorbic acid per gram of dry extract (Prieto et al., 1999). Plant extracts are frequently analyzed for their anti-oxidant activity by DPPH radical scavenging assay. DPPH is a stable free radical which interacts with compounds that can give an electron or a hydrogen atom and such compounds are considered as anti-oxidants (Singh et al., 2002).

Conclusion

From the results of different experiments performed on the crude extract of *A. divaricata* that it has spasmolytic, antibacterial and anti-oxidant effects, which provide a solid reason for its use in traditional medicine. These effects of crude extract are attributed due to the presence of different phytochemical compounds like flavonoids, tannins, saponins and different phenolic compounds. The spasmolytic activity provides rationale for its use in overactive bowl disorders while antibacterial and anti-oxidant properties give reason for its use in skin disorders.

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Author Info
Aamir Mushtaq (Principal contact)
e-mail: aamir_mushtaq@hotmail.com