Research Article

Evaluation of Mutagenic and Antimutagenic Activity of Aqueous Extract of *Ajuga bracteosa* Wall ex. Benth.

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Accepted on: 06-03-2016; **Finalized on:** 31-03-2016.

ABSTRACT

Since ancient times, traditional system of medicine from plant origin acts as the major remedy in the treatment of various diseases. The plants have been screened for various pharmaceutical agents and these remain an important source of new drugs. The aqueous extract of *Ajuga bracteosa* was evaluated for mutagenic and antimutagenic assay against mice pre-treated with 1/4th LD_{50} (117.5 mg/kgbw) of ethyl methanesulphonate by micronucleus and chromosomal aberration assay. Mice were treated with aqueous extract of *Ajuga bracteosa* (Ab-AE) (100, 200 300 & 400 mg/kgbw) for 15 days. Without the doses of EMS, no mutagenic effects were observed in blood and bone marrow samples of the mice. But Ab-AE showed antimutagenic effects on EMS induced mutagenicity in mice. It was observed that high doses of Ab-AE showed protective effects. The reduction profiles in the EMS induced MN at concentration of aqueous extract of *Ajuga bracteosa* (100, 200, 300 and 400 mg/kgbw) were estimated as 2.8 %, 6.3 %, 14.6 % and 23.8 % respectively. Thus, it can be concluded from the study that aqueous extract of *Ajuga bracteosa* exhibited no clastogenic effects but only possessing antimutagenic effects. This antimutagenic activity is an induction of its medicinal relevance.

Keywords: Ajuga, antimutagenicity, EMS, micronucleus, chromosomal aberrations, mice.

INTRODUCTION

Many indigenous plants used in traditional system of medicine are attributed with therapeutic properties[1]. The therapeutic properties of these plants are useful for healing and curing of human diseases because of the presence of phytochemical constituents[2-4]. These constituents include primary constituents like chlorophyll, proteins and common sugars and secondary constituent's viz. terpenoid, alkaloids and phenolic compounds[5]. Terpenoids and phenols exhibit important biological activities like anti-inflammatory, anticancer, anti-malarial, anti-viral, anti-bacterial activities and inhibition of cholesterol synthesis[6]. The people who live in upper reaches of Kashmir Himalaya use herbs for treatment of diseases[7]. The diversity of plant species of Kashmir Himalayas is a potential source of biologically active compounds; the effects on human health and genetic material are often unknown. Various chemicals such as antioxidants reduce the development of cancer by blocking genetic damage[8-10] and thus the field has gained much strength.

Ajuga bracteosa Wall ex. Benth. of family Lamiaceae is commonly known as 'Bungle' in English and 'Jan-i-adam' in Kashmiri. It is a perennial erect, ascending hairy herb, often prostrate with oblanceolate or sub-spathulate leaves and grows up to 5-50 cm tall. Its distribution extends from temperate regions of Western Himalayas viz., Kashmir, Pakistan, Afghanistan and China to Bhutan in Eastern Himalayas; Indian subtropical regions viz., plains of Punjab and upper Gangetic plains at an altitude of 1300 m[11,12] and in tropical regions of Malaysia. In Pakistan, it is found in northern hilly areas, where in local Hindi/Punjabi language it is called kori booti (means bitter herb) owing to its bitter taste. It is found along roadsides, open slopes, and rock crevices. The plant is used for the treatment of gout[13], rheumatism, palsy and amenorrhoea. Locally the leaves help in curing headache, pimples, measles, stomach acidity, burns, boils. It is effectively used against jaundice, hypertension, sore throat and as a blood purifier. Anti-inflammatory[14] and anti-cancerous[15] properties of *Ajuga bracteosa* have been reported. Investigators have also reported anti-malarial activities[16]. The present study was undertaken to screen the plant for its mutagenic and antimutagenic activity.

MATERIALS AND METHODS

Collection and air drying of plant material

Fig 1. Map of Jammu and Kashmir

Fig 1A. Satellite image of Collection Site (Sinthan Top)

Aerial parts of *Ajuga bracteosa* were collected from Sinthan Top area of District Anantnag (Fig. 1 and Fig. 1A) (Kashmir) in the month July, 2013. The plant was identified at the Centre of Biodiversity and Plant Taxonomy, Department of Botany, University of Kashmir, Srinagar, J & K and a voucher specimen (JKASH/CBT/226 Dated 08. 08. 2014) was deposited there. The parts were allowed to dry under shade (30 °C) for 8-10 days.

Preparation of extracts

After shade drying, the aerial parts were macerated to fine powder, 1 kg of leaves were extracted successively with hexane for defatenning and methanol for 16 h using Soxhlet apparatus. The extracts were filtered through a Buchner funnel using Whatman No. 1 filter paper, and all the extracts were concentrated to dryness under vacuum using a Heidolph rotary evaporator, yielding hexane, ethyl acetate, methanol and aqueous crude extracts of 65, 52, 46 and 36 g respectively. All the extracts were stored at 4°C in air tight glass bottles before use.

Phytochemical screening

Chemical tests were carried out on the extracts using standard procedures to identify the constituents[17–20].

Animals and treatment protocol

Both sex of albino mice, Balb/c strain useful for research in cancer and immunology, weighing 25-35 g were obtained from the Indian Institute of Integrative Medicine (IIM), Canal Road Jammu, kept in plastic cages in an experimental room under controlled conditions of temperature (22 ± 2°C), humidity (55 ± 10%), 12h light/dark cycles and access to food and water. They were randomized at the beginning of the experiment. The study design was approved by the Institutional Animal Ethical Committee, and the experiments undertaken in accordance with the ethical principles of the CPCSEA norms. The mice were divided into 8 groups, with 5 animals per group (Table 1). Ethyl methanesulfonate (EMS, Sigma Aldrich) was used to induce chromosomal aberrations for antimutagenic evaluation of aqueous extract of *Ajuga bracteosa*.

The micronucleus test

The method of MacGregor (1987) was used for micronucleus test. Mice were sacrificed by cervical dislocation. Blood was collected from the jugular vein and smears were made on pre-cleaned slides. The slides were air-dried, fixed in absolute methanol, stained with 10% Giemsa and then coded for blind analysis. One thousand polychromatic erythrocytes (PCE) were analysed per group. The proportion of PCE and normochromatic erythrocytes (NCE) in 200 erythrocytes/animal was calculated, to detect possible cytotoxic effects. The slides were scored blindly, using a light microscope with a 65x objective.

Chromosomal aberration assay

Mice were injected intraperitoneal with 0.5 ml of 0.06% colchicine and two hours later were sacrificed by cervical dislocation. Both the femurs were fleshed out from the muscles and kept in HBSS (Hank's balanced salt solution). The femurs were then rinsed with 3 ml 0.056% KCl solution in a centrifuge tube. The tube was then incubated at 37°C for 20 minutes. After incubation, centrifugation at 800 rpm for 4 minutes was carried out. Supernatant was discarded and fresh Carnoy's fixative was added (3:1 methanol: acetic acid). The process of centrifugation was repeated three times. Then slides were prepared, stained with 4% Giemsa, air dried and studied under compound microscope.

RESULTS AND DISCUSSION

Medicinal herbs possess therapeutic properties because of the presence of various bioactive constituents like alkaloids, phenolics, flavonoids, tannins, cardiac glycosides, terpenes, saponins, steroids etc. The phytochemical investigation of *Ajuga bracteosa* extracts in the present study revealed presence of different active ingredients (secondary plant metabolites) like flavonoids, phenolics, alkaloids, tannins, cardiac glycosides, terpenes, saponins, steroids, carbohydrates, amino acids and proteins as shown in Table 2.

The low frequency of micronucleated cells of blood cells of mice show no clastogenic effects of aqueous extract of *Ajuga bracteosa* (Ab-AE) at 100 and 400 mg/kgbw (Table 3). In other words, no statistically significant difference in the frequency of MN polychromatic erythrocytes (PCE) or the ratio of PCE to normochromatic erythrocytes (NCE), between the negative control and the groups that ingested extracts could be detected. The Ab-AE dose significantly decreases the frequency of EMS-induced MN PCE in mice at the doses of 100, 200, 300 and 400 mg/kgbw by 2.8 %, 6.3 %, 14.6 % and 23.8 % respectively (Table 3; Fig. 2). The number of cells with micronuclei also decreased with increase in the dose of the extract i.e., from 100 mg/kgbw to 400 mg/kgbw (Fig. 3).

Chromosomal aberration frequencies observed after various treatment schedules with EMS and different doses of Ab-AE is shown in Table 4. The EMS induced chromosomal aberrations were decreased by 25.39 %, 45.70 %, 57.42 % and 73.43 % at the doses of 100, 200, 300 and 400 mg/kgbw of the aqueous extract of *Ajuga bracteosa* (Fig. 4). EMS predominantly produced breaks, gaps, fragments and exchanges.

Important sources of new bioactive agents are the natural products. These natural products are obtained from medicinal herbs which are not only being used world-wide for the treatment of various diseases but also have great potential for providing novel drug leads with novel mechanism of action[21]. The biomarkers are important in understanding the role of both carcinogens and anticarcinogens in human cancer[22].

Table 1: Grouping, dose (distilled water, EMS and Ab-AE in concentrations of 100, 200, 300 and 400 mg/kgbw) and duration of experiment

Group	Dose	Purpose of group	Duration
Group 1	Distilled water	Negative control	15 days
Group 2	1/4th LD_{50} EMS	Positive control EMS	24 h
Group 3	Ab-AE 100 mg/kgbw	Positive control *Ajuga bracteosa*	24 h
Group 4	Ab-AE 400 mg/kgbw	Positive control *Ajuga bracteosa*	24 h
Group 5	Ab-AE 100 mg/kgbw + EMS	Treated Group	15 days
Group 6	Ab-AE 200 mg/kgbw + EMS	Treated Group	15 days
Group 7	Ab-AE 300 mg/kgbw + EMS	Treated Group	15 days
Group 8	Ab-AE 400 mg/kgbw + EMS	Treated Group	15 days

Ab-AE = Aqueous extract of *Ajuga bracteosa*

Table 2: Qualitative phytochemical screening of *Ajuga bracteosa*

Phytoconstituents	Test	Result
Alkaloids	Wagner's test	+ +
Phenolics	phenol test	+ +
Tannins	Ferric chloride test	+ +
Cardiac glycosides	Keller-Killani test	+ +
Terpenes	Salkwaski's test	+
Flavonoids	Shinoda's test	+ +
Saponins	Frothing test	+
Steroids	Libermann-Buchard's test	+
Carbohydrates	Molish test	+ +
Proteins	Biuret test	+
Polysterols	Salkowski's Test	+
Amino acids	Ninhydrin Test	+

(++) = strong presence, (+) = moderate presence

Table 3: Effects of Aqueous Extract of *Ajuga bracteosa* on MNPCE frequencies (mean ± SD) in mice, induced with ethyl methanesulfonate (EMS) 117.5 mg/kgbw (1/4th LD_{50})

	Treatment	Total No. of cells analysed per mice	No. of cells with micronuclei	% age of MN	% Reduction	P value
Group 1	Negative Control (Distilled water)	1000	2.35 ± 0.12			
Group 2	Positive control (EMS)	1000	7.23 ± 0.89			
Group 3	Ab- AE 100 mg/kg bw	1000	2.31 ± 0.10			
Group 4	Ab- AE 400 mg/kg bw	1010	2.29 ± 0.05			
Group 5	Ab- AE 100 mg/kg + EMS	1000	7.03 ± 0.71	97.2	2.8	0.05*
Group 6	Ab-AE 200 mg/kg + EMS	1000	6.78 ± 0.62	93.7	6.3	0.05*
Group 7	Ab-AE 300 mg/kg + EMS	1000	6.18 ± 0.52	85.4	14.6	0.05*
Group 8	Ab-AE 400 mg/kg + EMS	1000	5.51 ± 0.48	76.2	23.8	0.05*

Ab-AE = Aqueous extract of *Ajuga bracteosa*

Table 4: Frequency of Chromosomal aberrations observed after post-treatment with aqueous extract of *Ajuga bracteosa* in EMS treated mouse bone marrow cells

Treatments		Chromosomal Aberrations							
Concentration (mg/kgbw)	No. of cells	Rings	Fragments	Exchange	Breaks	Dicentrics	Gaps	Total Aberrations	%age of Aberrations
Distilled water	1004	2	6	-	15	-	-	23	2.29
EMS 117.5 mg/kg bw	1020	8	35	28	123	-	62	256	25.09
Ab-AE Alone 100 mg/kg bw	1015	3	6	-	15	-	-	24	2.36
Ab-AE Alone 400 mg/kg bw	1008	2	5	-	14	-	-	21	2.08
Ab-AE 100 mg/kg bw + EMS	1003	8	34	22	96	3	28	191	19.04
Ab-AE 200 mg/kg bw + EMS	1014	4	23	16	76	-	20	139	13.7
Ab-AE 300 mg/kg bw+ EMS	1009	4	20	15	50	1	19	109	10.80
Ab-AE 400 mg/kg bw + EMS	1015	2	14	12	26	-	14	68	6.69

Ab-AE= Aqueous extract of *Ajuga bracteosa*

For the positive use of natural products as therapeutic and chemopreventive agents, it is necessary to explore more thoroughly their real antimutagenic potential *in vivo* in connection with other antimutagenic factors. The antagonistic role of any natural compound against the genotoxic compounds was waiting to be unrevealed and the present study fills the lacunae in this field. The majority of higher plants contain a number of agents or phytoconstituents that are capable of causing mitigating effects to a number of mutagens[23]. As the antimutagenic studies are getting credence, there are many studies to show the rising trends of antimutagenicity. The plant *Arabidopsis thaliana* was utilized to screen the effects of various antimutagens (e.g. thiourea, cysteine, 9-hydroxyellipticine, phenolic agents) against chemically induced embryonic and chlorophyll mutation[24,25]. *Cymbopogon citratus* Stapf (Lemon grass) showed antimutagenic effects against mutagens in *Salmonella typhimurium* strains[26]. The mushroom, *Agaricus blazei* has protective effects against cyclophosphamide induced mutagenicity in mice. It was found that three different tea extracts of mushroom, significantly reduced the frequencies of MN in polychromatic erythrocytes and in reticulocytes[27].

The aqueous extracts of fermented and unfermented rooibos tea (*Aspalathus linearis*) and honey-bush tea (*Cyclopia intermedia*) possess antimutagenic activity against 2-acetylaminofluorine and aflatoxin B_1[28]. Vitamin C and E also significantly reduced the CA frequency in mouse bone marrow cells against rifampicin, an anti-tuberculosis drug[29]. The phytoconstituents from *Terminalia arjuna* suppressed the mutagenic effect of the aromatic amine, i.e., 2-aminofluorene (2-AF)[30]. The observed activity caused the inhibition of the metabolic activation of pro-mutagens. The extracts of *Acanthopanax divaricatus* were able to rapidly eliminate the mutagenic compounds from the cells before they induce the DNA damage[31]. In a similar study, it was observed that the methanol extracts of the lichens have antimutagenic effects against sodium azide[32].

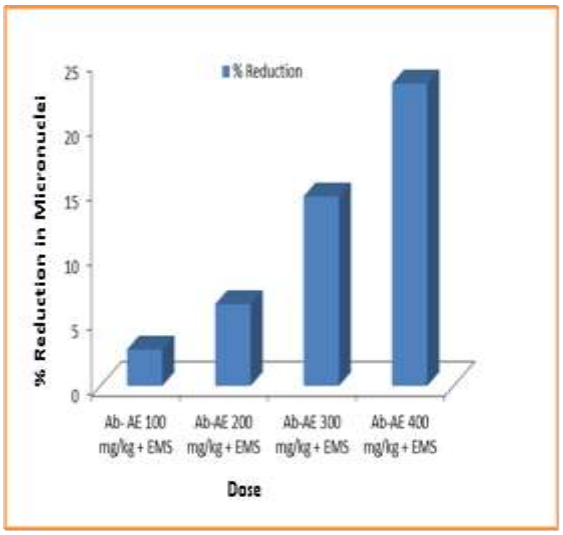

Fig. 2. % age reduction in EMS treated micronuclei with increase in concentration Aqueous extract of *Ajuga bracteosa*.

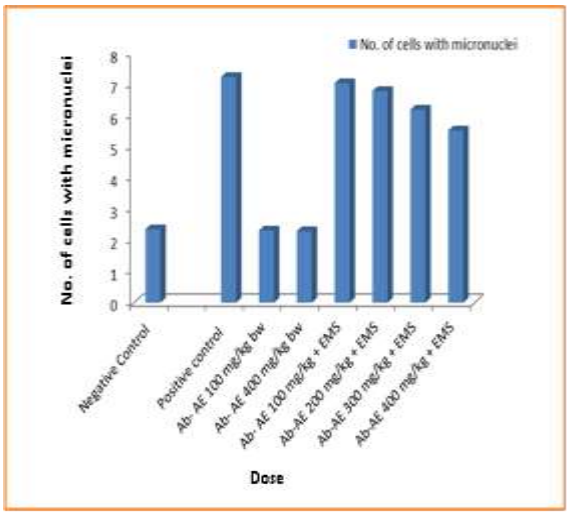

Fig. 3. Number of micronucleated cells in different groups of mice treated with EMS alone, EMS + Ab-AE and Ab-AE alone in different doses

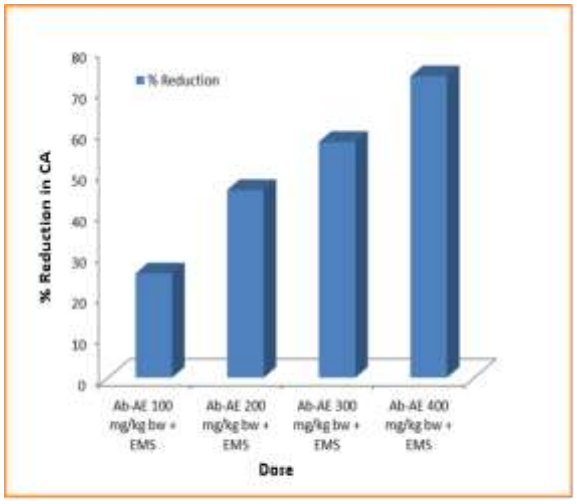

Fig. 4. Bar diagram showing the %age reduction in chromosomal aberrations (CA) induced by EMS following post-treatment with Aqueous extract of *Ajuga bracteosa* (Ab-AE)

The various plant extracts also possess antimutagenic properties against cyclophosphamide induced mutagenicity in mice[33]. In another study, it was found that the different extracts of *Dioscorea pentaphylla* significantly inhibited the effects of methyl methanesulphonate (MMS) induced mutagenicity[34]. They also found that the methanolic extract was highly antimutagenic in comparison to Petroleum ether and chloroform. The antimutagenic and anticancer activities of *Echinophora platyloba* DC was compared on acute promyelocytic leukemia cancer cells and found that the methanolic extract of this plant prevented the reverted mutations and the hindrance % was 93.4 % in antimutagenic test[35]. The leaves of *Myristica fragrans* (Houtt.) also possess antimutagenic activity against benzo[a]pyrene and cyclophosphamide induced mutagenicity in *Salmonella typhimurium* and *Mus musculus* and found that the aqueous extract significantly suppressed more than 50 % of the mutations in all the tested concentrations[36]. An edible wild plant, *Tragopogon longirostis* also possess various activities like antioxidant and antimutagenic and it was found that the ethanolic extract of its leaves exhibited antimutagenic properties at 2.5, 0.25, and 0.025 mg/plate concentrations[37]. The ethanolic extract of *Origanum vulgare* reduced the frequency of MN PCR from 10.52 ± 1.07 for CP to 2.17 ± 0.6 for the synergic test of CP and the ethanolic extract[38].

Ajuga bracteosa contain saponins which are known to produce inhibitory effect on inflammation[39]. Saponins have the property of precipitating and coagulating red blood cells. Some of the characteristics of saponins include formation of foams in aqueous solutions, hemolytic activity, cholesterol binding properties and bitterness[40]. Steroids, another important phytoconstituent present in *Ajuga bracteosa*, have been reported to possess antibacterial properties[41] and they are very important compounds especially due to their relationship with compounds such as sex hormones[42]. Alkaloids have been associated with medicinal uses for centuries. It has been reported that alkaloids possess analgesic[43], antispasmodic and antibacterial[44,45] properties. Glycosides are known to lower the blood pressure according to many reports[46]. Thus from the present study, it could be suggested that the identified phytoconstituents from *Ajuga bracteosa* make the plant valuable for bioactive compounds of sustainable medicine.

CONCLUSION

The medicinal plants are the source of the secondary metabolites i.e., alkaloids, flavonoids, terpenoids, phlobatannins and reducing sugars. Medicinal plants play a vital role in preventing various diseases. The antidiuretic, anti-inflammatory, antianalgesic, anticancer, anti-viral, anti-malarial, anti-bacterial and anti-fungal activities of the medicinal plants are due to the presence of the above mentioned secondary metabolites. Thus, *Ajuga bracteosa* can be used for discovering and screening of the phytochemical constituents which are very helpful for the manufacturing of new drugs. Thus we hope that the important phytochemical properties identified in this study in the local plant of Kashmir Himalaya will be helpful in copping different diseases of this particular region.

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Source of Support: Nil, **Conflict of Interest:** None.