



Antimutagenic activity of compounds isolated from *Ajuga bracteosa* Wall ex. Benth against EMS induced mutagenicity in mice

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ABSTRACT

Ajuga bracteosa Wall ex. Benth. (Lamiaceae) has been reported to possess many biological activities including antibacterial, antifungal, antispasmodic and antioxidant activity but there is no report as such on its mutagenic and/or anti-mutagenic activity. The aim of the present study was to isolate compounds from the methanol extract of the aerial parts of *Ajuga bracteosa* and determine their anti-mutagenic activity against the mutagen, EMS in animal model mice. The study was undertaken in order to corroborate the traditional use of the plant *Ajuga bracteosa*. The compounds were isolated from the methanol extract of the aerial parts of *Ajuga bracteosa* using silica gel column chromatography. Structural elucidation of the isolated compounds was done using spectral data analysis and comparison with literature. High performance liquid chromatography (HPLC) was used for the qualitative and quantitative determination of the isolated compounds in the crude methanol extract. The isolated compounds and standard drug were evaluated *in vivo* for antimutagenic activity against EMS induced mutagenicity taking mice as model organism by micronucleus and chromosomal aberration tests. Four major compounds were identified as 1) 14, 15-dihydroajugapitin 2) β -Sitosterol 3) Stigmasterol and 4) 8-O-acetylharpagide. A quick and sensitive HPLC method was developed for qualitative and quantitative determination of three isolated marker compounds from *Ajuga bracteosa*. 14, 15-dihydroajugapitin reduced the micronuclei by 85.10%, followed by β -Sitosterol (72.3%) while as 8-O-acetylharpagide reduced the micronuclei by 46%. It is therefore evident from the present study that the plant contains rich source of anticancer and antimutagenic drugs.

1. Introduction

Among the many mortality cases, cancer is one of the major causes of deaths throughout the world [1]. It has also been projected that, the number of new cancer cases and deaths will double from 14 million to 26 million and 8.2 million to 17 million by 2030, respectively [2]. The synthetic chemotherapeutic agents currently used for treating cancer have not succeeded in fulfilling the expectations despite huge amount of cost in their development. Hence, it is the need of the hour to develop new, healthy, more effective and affordable drugs to treat cancer [3].

Ajuga bracteosa Wall ex. Benth. is a perennial erect, ascending hairy herb, often prostrate with oblanceolate or sub-spathulate leaves and grows upto 5–50 cm tall. It is distributed in subtropical and temperate regions Bhutan, Pakistan, Afghanistan, China, Malaysia at an altitude of

1300 m asl. In India, it abounds in western Himalaya, plains of Punjab, upper Gangetic Plains of India [4] and in Kashmir at an altitude of 1300 m [5]. It is found along roadsides, open slopes, and rock crevices [6]. The aim of the present study was to isolate important bioactive compounds from the methanol extract of the aerial parts of *Ajuga bracteosa* and determine their antimutagenic activity against EMS induced mutagenicity in mice. Thus the present study was undertaken to confirm the traditional use of *Ajuga bracteosa*.

2. Material and methods

2.1. Plant material

After seeking permission from the state forest department, the plant

Abbreviations: DDMP, 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyran-4-one; 5HMF, 5-(hydroxymethyl)-2-furan carboxaldehyde; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; LD₅₀, Lethal dose at which mortality is 50%; mg/kgbw, milligram per kilogram of body weight

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material was collected for the study. Only minimum quantity of sample for sole research purposes was collected. The plant material of *Ajuga bracteosa* was collected from Sinthan Top (12, 500 ft) area of Kashmir Valley, India at an altitude of 3, 800 m (latitude 33°34' N and 75°30' E). The collection was made in the month of July 2014. The plant material was identified with the help of taxonomists at Centre for Biodiversity and Taxonomy (CBT), Department of Botany, University of Kashmir. A voucher specimen no. KASH- KU/BAW- 780 was deposited in the Department's herbarium. The plant name has been verified with www.theplantlist.org.

2.2. Extraction and isolation

The plant material was properly washed, shade dried and pulverized into powder using a mechanical grinder. The powdered material was extracted with methanol using the cold maceration technique. The extracts obtained were kept at 4 °C for further use.

A portion (30 g) of the methanol extract was dissolved in minimum amount of dichloromethane (DCM) and adsorbed on silica gel, air dried and chromatographed over silica gel (60–120 mesh). The column was eluted with petroleum ether: ethyl acetate in the ratio of 1:1 to afford compound 1. The same column then eluted with pure DCM yielded compound 2. Finally, the column was further eluted with a step gradient mixture of DCM/methanol in the ratio of 97:3, 96:4, 90:10 and 85:15 to afford compounds 3 and 4.

2.3. High-performance liquid chromatography (HPLC) analysis

Liquid chromatography separation was performed using Nexera UHPLC composed of quaternary pump, prominence degassing unit, Autosampler, Column oven and Diode Array Detector (DAD). Chromatographic separation was carried out using Enable RP C18 Column (250mm × 4.5 mm, 5 µm) at 25 °C. Elution was performed at a flow rate of 0.6 ml/min and the injection volume was 5 µl. The injection volume was kept constant at 5 µl for the extract as well as the standards. Proper dilutions were made to the extract to keep it in the range of LOD and LOQ. Solvents used were 0.1% formic acid in water (A) and methanol (B). All solvents were filtered through a 0.45 µm nylon filter after ultrasonic degassing. Isocratic flow of 80:20 A: B was used for analysis. The chromatograms were recorded at 278 nm and data was acquired using Lab solutions software. For the purpose of quantitative analysis, the extract was analysed under the conditions described above. Compounds were run at four different concentrations and found to be linear in the range with a correlation coefficient (r^2) of 0.999–0.997. The estimation of the compounds content in the extract was performed using linear regression analysis. The standard solution was prepared by dissolving 1 mg of the compound in 1 ml of methanol.

2.4. Antimutagenic activity of isolated compounds

2.4.1. Experimental animals

Both the sexes of albino mice, Balb/c strain useful for research in cancer and immunology, age of 6 weeks, weighing 25–35 g were obtained from the Indian Institute of Integrative Medicine (IIM), Canal Road Jammu-India, kept in plastic cages in an animal room under controlled conditions of temperature ($22 \pm 2^\circ\text{C}$), humidity ($55 \pm 10\%$), 12 h 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.

2.4.2. Treatment protocol

The mice were divided into 7 groups, with 5 animals per group. EMS was used to induce mutations. Just before use, the EMS was diluted in normal saline. The exposure route was by gavage (1/4th of LD₅₀ of EMS;

117.5 mg/kgbw). Mice in group 1 served as negative control, group 2 served as positive control (EMS for 24 h), compounds 1, 2, 3 and 4 were given to other four groups simultaneously after EMS. The effect of compounds was compared with the available drug Paclitaxel. The mice were killed by cervical dislocation on 16th day for evaluation of micronucleus and chromosomal aberrations.

2.4.3. The micronucleus test

The method of MacGregor et al. was used for micronucleus test. Mice were sacrificed by cervical dislocation. Slides were prepared with blood collected from the jugular vein. The slides were air-dried, fixed in absolute methanol, stained in 10% Giemsa and then coded for blind analysis. Two thousand polychromatic erythrocytes (PCE) were analysed per group. The proportion of PCE and normochromatic erythrocytes (NCE) in 2000 erythrocytes/group was calculated, and was evaluated by scoring the slides under oil immersion at 100x using Olympus BX 50 microscope (Tokyo, Japan).

2.4.4. Chromosomal aberration

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 min. After incubation, centrifugation at 800 rpm for 4 min 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.

3. Results

3.1. Phytochemical composition

A total of four known phytochemical compounds 1) 14, 15-dihydroajugapitin 2) β - Sitosterol 3) Stigmasterol and 4) 8-O-acetylharpagide] were isolated from the methanol extract of aerial parts of *Ajuga bracteosa*. The structures of the isolated compounds were established using various spectroscopic techniques and direct comparison with literature.

3.2. HPLC analysis

A simple and sensitive HPLC method was developed for identification of the standard compounds isolated from the methanol extract of *Ajuga bracteosa*. The chromatographic separation of these compounds was achieved in less than 40 min with retention time 10.3, 16.06, 26.04 and 30.04 respectively. Fig. 1 shows the chromatograms obtained from methanol extract and standards isolated from the aerial parts of *Ajuga bracteosa*. The peaks corresponding to the individual compounds are symmetrical and well resolved from other co-extracted material. The external standard method was used for the quantification process. Quantification of the compounds by HPLC-DAD showed their content to be 1.6 µg (14, 15-dihydroajugapitin), 0.35 µg (β - Sitosterol), 0.53 µg (Stigmasterol) and 0.85 µg (8-o-acetylharpagide) per gram of the crude methanol extract.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the equations: $\text{LOD} = 3.3 \sigma/S$ and $\text{LOQ} = 10 \sigma/s$ where σ and S represent the standard deviation of response and the slope of the calibration curve respectively. Limit of detection represents the lowest concentration of the analyte in a sample that can be detected by HPLC under the developed method while as limit of quantification represents the lowest concentration that can be quantified under the operating conditions. The data of retention time, regression equation, correlation coefficient (r^2), linear range as well as limits of detection (LOD) and quantification (LOQ) of each compound are summarized in

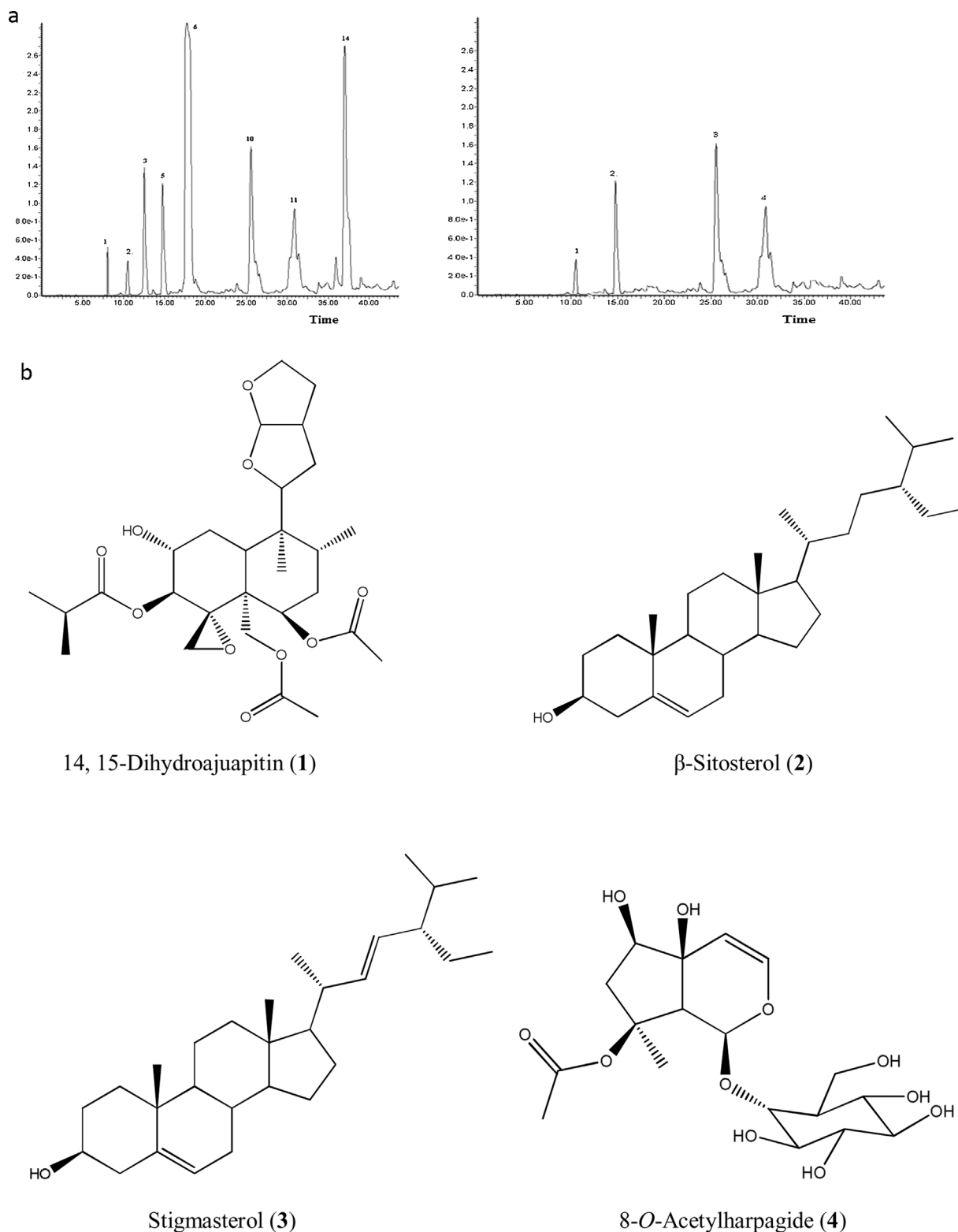


Fig. 1. A) HPLC chromatogram of the methanol extract solution of the aerial parts of *Ajuga bracteosa* B) HPLC chromatogram of the standards isolated from the aerial parts of *Ajuga bracteosa*.

Table 1.

3.3. Micronucleus test

According to MN testing, mice treated with compound 1 showed maximum antimutagenic activity, followed by compound 2 and 3 with MN frequencies of 3.1 ± 0.22 , 3.7 ± 0.23 and 4.25 ± 0.29

respectively as compared with the positive control (7.1 ± 0.79). Compound 1 i.e. 14, 15-dihydroajuapitin reduced the MN levels by 85.10%, followed by compound 2 i.e. β - Sitosterol while as compound 4 i.e. 8-O-acetylharpagide reduced the MN level by 46% (Table 2).

Table 1Retention time, regression equation, correlation coefficient (r^2), linear range and limits of detection (LOD) and quantification (LOQ) for isolated standards from *Ajuga bracteosa*.

Compound name	Retention time	Regression equation	r 2	Linear range ($\mu\text{g/ml}$)	LOD ($\mu\text{g/ml}$)	LOQ ($\mu\text{g/ml}$)
14, 15-dihydroajugapitin	10.3	58402.3*x-2305.79	0.998	1–8	0.135	3.95
β - Sitosterol	16.06	2091.01*x + 1131.76	0.998	2–16	0.167	0.63
Stigmasterol	26.04	8117.48*x + 2279.86	0.997	10–80	3.87	17. 65
8-O-acetylharpagide	30.04	103248*x + 1468.14	0.997	0.5–4	0.162	0.51

Table 2Frequency profile of micronuclei and chromosomal aberrations induced alone by ethyl methanesulphonate and simultaneous exposure of compound 1, 2, 3 and 4 to evaluate antimutagenicity in *Mus musculus*.

Groups	MN test			CA Test		
	Total No. of cells analysed	Frequency of MN	% Reduction	Total No. of cells analysed	Total aberrations	% Reduction
Negative control	2000	2.40 \pm 0.12		500	11	
Positive control	2000	7.10 \pm 0.79		500	129	
EMS + Compound 1(100 mg/kgbw)	2000	3.10 \pm 0.17	85.10 ^{***}	500	30	83.8 ^{***}
EMS + Compound 2 (100 mg/kgbw)	2000	3.70 \pm 0.20	72.3 ^{**}	500	41	74.5 ^{**}
EMS + Compound 3 (100 mg/kgbw)	2000	4.25 \pm 0.23	60 ^{**}	500	58	60.1 ^{**}
EMS + Compound 4 (100 mg/kgbw)	2000	4.90 \pm 0.29	46 [*]	500	84	38.1 [*]
Paclitaxel (100 mg/kgbw)	2000	2.80 \pm 0.11	91 ^{***}	500	21	91.53 ^{***}

NC: Negative control (distilled water), PC: Positive control [Ethyl methane sulfonate (EMS) 117.5 mg/kgbw; dose is 1/4th LD₅₀], Values with different asterisks (^{*} p < .05: significant, ^{**} p < .01: highly significant, ^{***} p < .001: extremely significant) differ significantly from the positive control (Mann-Whitney U test).

3.4. Chromosomal aberrations

Compound 1 i.e. 14, 15-dihydroajugapitin reduced the CA levels by 83%, followed by compound 2 i.e. β - Sitosterol (72%), while as compound 4 i.e. 8-O-acetylharpagide reduced the CA level by 38% (Table 2).

4. Discussion

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 [7]. Many compounds have been isolated and from various natural sources and studied for antimutagenic activity e. g. 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), 5-(hydroxymethyl)-2-furan carboxaldehyde (5HMF) and hexadecanoic acid, methyl ester. All these compounds are likely to possess potent antimutagenic activity. DDMP isolated from onion in one of the previous studies have modulated the activity of NF- κ B (nuclear factor kappa-light-chain enhancer of activated B cells) thereby inducing the apoptotic cell death of cancer cells [8]. Recently, it was isolated and identified as a potent antioxidant from *Pyrus pyrifolia* Nakai [9], supporting some recent studies which showed a strong correlation between antioxidant and anticancer activity [10–12].

An important compound 5- (hydroxymethyl)-2-furan carboxaldehyde (5-HMF) was also isolated from various plants and was tested for various important biological activities like antioxidant, uterotonic, anti-platelet aggregation and radical scavenging activity [13,14].

The β -sitosterol is known to be effective against a number of cancers like human breast cancer [15], colon carcinoma [16] and prostatic cancer [17]. The β -sitosterol also inhibits the proliferation of breast cancer cells in a dose dependent manner [18]. The authors revealed that a higher caspase activity after adding β -sitosterol to the cell line resulted in caspase-induced apoptosis. Besides, the compound also showed antiproliferative and apoptosis activities in human leukemic cells by activating caspase-3 and Bax/Bcl-2 ratio [19].

Stigmasterol is known to possess many important biological activities like antihypercholesterolemic, antimutagenic, antileishmanial, antimalarial, antitrypanosomal, platelet aggregation inhibitor and antiviral [20]. Previous *in vitro* studies confirm that plant extracts rich in

stigmasterol and β -sitosterol are cytotoxic against HepG2 (liver), Caco-2 (colon) and MDA-MB-231 (breast) cancer cell lines [21,22], thus suggesting that these compounds are effective drugs for carcinogenesis.

5. Conclusion

Based on the above results it can be concluded that the compounds isolated from the methanolic extract of *Ajuga bracteosa* are having potential to reduce the antimutagenic effects of any mutagenic agent. These isolated compounds might prove very useful in the formation of anticancer drugs.

Conflict of interest

The authors declare that there are no conflicts of interest.

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