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Sedative effect of vapor inhalation of essential oil from *Heracleum afghanicum* Kitamura seeds

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Heracleum afghanicum (Apiaceae) is a perennial plant indigenous to Afghanistan. Phytochemical and pharmacological analyses of *H. afghanicum* seeds essential oil were carried out to investigate its possible sedative effects on mice spontaneous locomotor activity. The essential oil was analyzed by gas chromatography (GC) and GC/mass spectrometry (GC/MS), and thirty-three constituents were identified. Hexyl butyrate (34.3%) and octyl acetate (21.1%) were found as its principal constituents. The sedative effect of *H. afghanicum* essential oil was confirmed using an open field test with ddY mice. The essential oil significantly decreased the locomotor activity of mice, suggesting its sedative effect. Hexyl butyrate and octyl acetate were found to be responsible for the sedative activity of *H. afghanicum* seeds essential oil.

Keywords: *Heracleum afghanicum*; Apiaceae; essential oil composition; vapor inhalation; sedative effect; aliphatic esters; hexyl butyrate; octyl acetate

Introduction

Essential oils are used for pharmaceutics, for flavoring, or as starting materials for the synthesis of other compounds (1). The medicinal use of essential oils began in ancient Egypt and has continued to the present day. Essential oils are believed to be very important for their effectiveness in treating various illnesses but the lack of a scientific basis for their use remains an obstacle (2). Aromatherapy, which uses essential oils for various treatments, has attracted much attention as an alternative medicine, and the inhalation of essential oils is one of these treatments.

The genus *Heracleum* includes approximately seventy species distributed all around the world (3). Many kinds of secondary metabolites such as anthraquinones, coumarins and flavonoids have been isolated from several species of this genus and their structures were identified (4). The essential oil compositions of these species have been studied and many volatile components such as monoterpenes, oxygenated monoterpenes, sesquiterpenes and different aliphatic esters were reported (4–13). *Heracleum afghanicum* (locally known as Balderghan) is a perennial plant endemic to Afghanistan. Its seeds are used as a spice, its leaves are used as a pain killer and anti-fever treatment, and the young stems are edible. The antimicrobial, anticonvulsant, analgesic and anti-inflammatory effects of other members of *Heracleum* such as *H. persicum* have been reported in recent studies (4–7). However, scientific studies of the phytochemical and pharmacological aspects of *H. afghanicum* have not been undertaken. The objective of this study was to identify the chemical composition of the essential oil of this plant, to investigate its possible effects on locomotor activity in mice and to analyze the active components of the essential oil. In this paper, the chemical composition and sedative activity of vapor inhalation of essential oil from *H. afghanicum* seeds are being reported for the first time.

Materials and methods

Materials

Seeds of *H. afghanicum* were collected at Syakhark in Darrah-i-Fringel (Ghorband district, Parwan province, Afghanistan) in July 2008 and air dried. The plant was identified by Mr Kh. A. Yarmal, a professor of botany, Faculty of Science Kabul University and three voucher specimens (Nos. 4968, 4969 and 4970) were deposited in the herbarium of Experimental Station for Medicinal Plants, Graduate School of Pharmaceutical Sciences, Kyoto University, and one specimen (No. 100) in the herbarium of the Faculty of Sciences, Kabul University. Lavender oil (Nacalai Tesque Co., Ltd.) and benzylacetone (Tokyo Kasei) were used as positive controls. Triethyl citrate (TEC; Merck), an odorless solvent, was used for dissolving the fragrant components. Hexyl butyrate, octyl acetate, octyl butyrate and butyl butyrate

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were purchased from Wako. All chemicals used in this study were of the highest grade available.

Isolation of essential oil and fractionation

The essential oil of *H. afghanicum* was prepared by distillation of seeds for 2 hours using an apparatus designated in Japanese Pharmacopeia (JP XV) and captured in hexane. The pale yellow essential oil obtained was dried over anhydrous sodium sulfate and stored in sealed vials at 4°C before analysis. The essential oil was subjected to silica gel column chromatography for fractionation. The column was eluted with hexane-acetone (3:1) to give fractions 1–2, and then washed with absolute acetone to give fraction 3.

Animals

Male four-week old ddY mice were purchased from Japan SLC (Shizuoka, Japan). The animals were housed in colony cages with a 12-hour light/dark cycle at 25±2°C and relative humidity of 50±10% with free access to food and water before being used for experiments. All experiments were conducted between 10:00 and 17:00 hours under the same conditions. Animal experiments were designed following recommendations by Animal Research Committee of Kyoto University, Kyoto, Japan (approval number 2010-23). Experimental procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan (2006).

Evaluation of spontaneous motor activity

The sedative effects of *H. afghanicum* essential oil were evaluated on mice spontaneous motor activity in an open-field test. Doses administered were expressed as milligrams of the essential oil in 400 µL of TEC per cage. Four pieces of filter-paper soaked with essential oil dissolved in TEC were placed in the four corners of the inner walls of the glass cage (W 60 cm × L 30 cm × H 34 cm) using adhesive tape, so that the vapor pervaded the cage by natural diffusion. Sixty minutes after charging the sample, a mouse was placed in the center of the cage and was monitored by a video camera for 60 minutes. The frequency of each mouse crossing the lines drawn on the floor of the cage (at 10-cm intervals) was counted every 5 minutes for 60 minutes. The area under the curve (AUC), which represented total locomotor activity, was calculated by trapezoidal rule as described by Takemoto et al. (8).

Qualitative and quantitative analyses of essential oil

The qualitative analysis of essential oil was carried out on a Hewlett Packard 5890 series gas chromatograph connected to AUTOMASS 50 (JEOL) with operation conditions as follows; column: fused silica capillary column, TC-wax (HP), 60 m × 0.25 mm × 0.25 µm; column temperature program: 40–130°C increasing at a rate of 2°C/minute, holding at 130°C for 25 minutes, 130–140°C at 2°C/minute, holding at 140°C for 15 minutes, 140–200°C at 15°C/minute, ending at 200°C for 30 minutes; injector temperature: 180°C; carrier gas: helium, 25 cm/second; column head pressure: 100 kPa; ionization energy: 70 eV; injection volume: 1.0 µL; MS interface temperature: 150°C; MS mode: electron impact (EI); detector voltage 0.4 kV; mass range: 35–300 u; scan speed: 300 u/second.

Quantitative analysis was carried out on Hitachi G-5000 equipped with a flame ionization detector with the following conditions; column: fused silica capillary column, TC-wax (HP), 60 m × 0.25 mm × 0.25 µm; column temperature: same as GC/MS; injector: 180°C, detector: 210°C, flame ionization detector (FID); carrier gas: helium, 0.8 mL/minute; split ratio: 33:1; column head pressure: 200 kPa; injection volume: 1 µL. Relative retention indices of constituents were determined using *n*-alkanes as standards. Chemical compounds were identified by NIST 02 database matching or by comparison of their retention time and mass spectra with authentic samples, and confirmed by comparison of their retention indices either with those of authentic compounds or with data in literature (14). The quantitative analysis was achieved with an FID.

Statistics of data

Data were expressed as mean value ± standard error of mean (SEM). Statistical analyses were done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using GraphPad Instat (GraphPad Software, San Diego, CA, USA). A probability level of $p<0.05$ was interpreted to be statistically significant.

Results and discussion

Analysis of essential oil

The seeds of *H. afghanicum* afforded 1.5% (v/w) essential oil with a pale yellow color and sharp characteristic odor. Table 1 shows the thirty-three constituents identified in the essential oil listed in order of their elution from the TC-wax column. It was found that *H. afghanicum* essential oil consists mainly of aliphatic esters. Hexyl butyrate (34.3%) and octyl acetate (21.1%) were found to be the principal constituents of the essential oil. These two compounds had also been reported as

Table 1. Chemical composition of essential oil from *Heracleum afghanicum* seeds.

Compounds	RI[a]	95% RI[b] range in reference	Peak area (%)	Compounds	RI	95% RI range (let.)	Peak area (%)
Isopropyl isobutyrate	980		2.1	*E*-5-Decenyl acetate	1512		3.4
Isopropyl 2-methylbutanoate	1053		2.5	*cis*-4-Decenal	1536		t
Isopropyl 3-methylbutanoate	1077		1.9	Octyl isobutyrate	1545		1.5
Butyl butyrate	1221		2.5	**Linalool**	**1558**	**1506–1571**	1.0
Isopropyl 3-methyl-2-butanoate	1235		0.7	**1-Octanol**	**1567**	**1513–1584**	3.6
Terpinene	1238		0.7	1,4-Octadiene	1598		t
Isopentyl butyrate	1264		t	*cis*-5-Octen-1-ol	1611		0.6
Hexyl acetate	1276		2.5	Hexyl caproate	1613		1.5
Octanal	**1288**	**1259–1315**	t	Octyl butyrate	1620		6.9
Unknown	1299		0.7	Octyl 2-methylbutyrate	1624		t
Hexyl isobutyrate	1344		0.7	Octyl isovalerate	1630		1.7
n-Hexanol	**1362**	**1314–1386**	2.3	1-Pentylallyl butyrate	1653		1.0
Hexyl butyrate	1419		34.3	*E*-4-Tridecen-1-yl acetate	1699		t
Hexyl 2-methylbutyrate	1428		1.1	Octyl hexanoate	1813		0.6
Hexyl isovalerate	1444		t	Anethol	1839		2.3
Octyl acetate	1479		21.1	Unknown	1876		1.5
Botanoic acid, 4 hexenyl ester	1489		t	Octanoic acid	1962		0.7
Decanal	**1505**	**1448–1525**	0.8				

Notes: [a]Retention indices. [b]Reference 14. Compounds listed in order of their elution from TC-wax column. t, trace.

the principal components of *H. persicum* seed essential oil (9). The main compounds of fractions 1, 2 and 3 were hexyl butyrate and octyl acetate, octanol and octanoic acid, respectively.

Effect of H. afghanicum essential oil on mice locomotor activity

The results of the administration of the whole essential oil are shown in Figure 1. The essential oil was administered to mice by inhalation of doses ranging from 0.004 to 4 mg. Sedative activity was observed within a dose range of 0.004 to 4 mg and the strongest effect was observed at a dose of 0.4 mg, which calmed mice after 10 minutes. The AUC values of treated groups were significantly smaller than the control and the decrease in locomotor activity produced by doses of 0.4 and 4 mg were statistically significant (Figure 1a, $p<0.01$, $p<0.05$). This suggested the potential sedative activity of *H. afghanicum* essential oil.

Effects of fractions on locomotor activity of mice

The fractions were individually administered to mice by inhalation at doses of 0.004, 0.04 and 0.4 mg. Among the fractions, fraction 1 showed sedative activity, which significantly decreased the locomotor activity of mice and the strongest effect were observed at a dose of 0.04 mg. The AUC values of treated groups with fraction 1 were significantly smaller than the control and the decrease in locomotor activity shown by 0.04 mg was statistically significant (Figure 2a, $p<0.05$). Fractions 2 (Figure 2b) and 3 (Figure 2c) were not significantly effective. This suggested that Fraction 1 contained the active ingredients of the essential oil and it was then analyzed for its chemical composition using GC/MS. The results revealed that hexyl butyrate and octyl acetate were the main compounds of fraction 1, along with other minor constituents such as octyl butyrate and *E*-5-decenyl acetate.

Effects of hexyl butyrate, octyl acetate and octyl butyrate on the locomotor activity of mice

The main compounds of fraction 1, hexyl butyrate, octyl acetate and octyl butyrate were assayed for their sedative activity. Hexyl butyrate was administered to mice at doses of 0.04, 0.4 and 4 mg. It significantly decreased the locomotor activity of mice in a dose-dependent manner. The strongest effects were observed at doses of 0.4 and 4 mg, which sedated mice after 10 minutes of administration. The AUC values of the 0.04, 0.4 and 4 mg treated groups were 66, 42.7 and 53% of the control, respectively, suggesting the strong sedative activity of this compound (Figure 3a, $p<0.01$, $p<0.05$). Octyl acetate also significantly decreased the locomotor activity of mice and the effective dose was

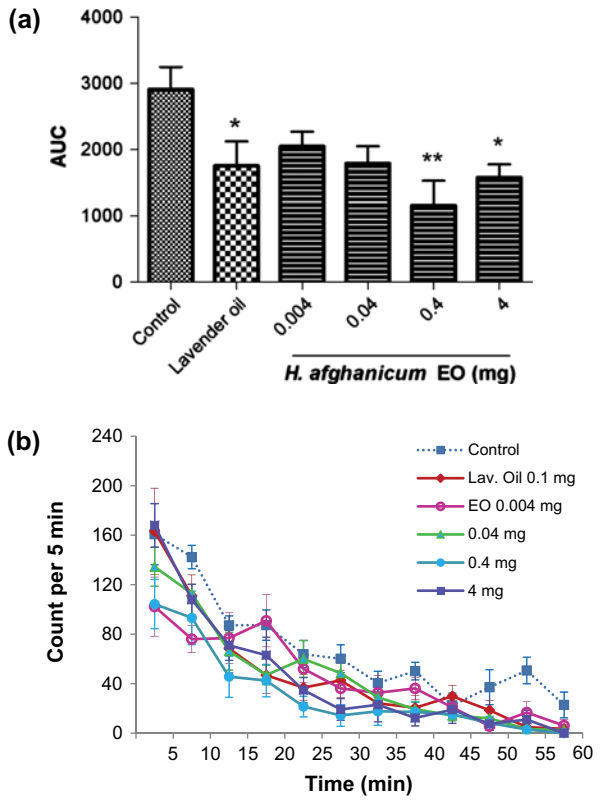

Figure 1. Total spontaneous motor activity (a) and locomotor activity transition (b) of mice that received vehicle (triethyl citrate 400 µL), lavender oil (0.1 mg) and *Heracleum afghanicum* oil (0.004, 0.04, 0.4 and 4 mg). Data are shown as means ± SEM of six mice. Statistical differences vs the control group were calculated using analysis of variance (ANOVA), followed by Dunnett's test. $^{*}p<0.05$, $^{**}p<0.01$.

4 mg, whereas the 40-mg dose caused abnormal actions such as excess excretion. The AUC values of the treated groups with octyl acetate were 75.4%, 63.2%, 41.5% and 80% of the control, respectively (Figure 3b, $p<0.01$). However, octyl butyrate did not alter the locomotor activity of mice, and the AUC values for treated groups with this compound were 92%, 82.5% and 103% of the control, respectively (Figure 3c). Results from these experiment confirmed that hexyl butyrate and octyl acetate were responsible for the sedative activity of fraction 1. In addition, it was observed that the effect of hexyl butyrate on locomotor activity of mice was more potent than octyl acetate. These two compounds have linear structures and same number of carbons but differ in their function group, suggesting that the butyrate function may be important for the sedative activity of these compounds.

Comparison of the effects of octyl butyrate and butyl butyrate with hexyl butyrate

It was observed that hexyl butyrate decreased the locomotor activity of mice, whereas octyl butyrate, which differed from hexyl butyrate only in carbon chain length, did not alter the locomotor activity of the mice. The structure–activity relationship of the aliphatic esters present in *H. afghanicum* essential oil was thus investigated. Figure. 4 shows a comparison of the effects of butyl butyrate, hexyl butyrate and octyl butyrate on the locomotor activities of mice. Hexyl butyrate (C=10) and butyl butyrate (C=8) showed sedative activity, while octyl butyrate (C=12) was not effective. In addition, the sedative effect of hexyl butyrate was stronger than that of butyl butyrate. These three compounds structurally differ only in carbon chain length. It is thought that the length of carbon chain may affect sedative activity of these aliphatic esters. However, the discrepancy between the sedative activity of hexyl butyrate and butyl butyrate and their carbon chain length suggests that carbon chain length alone may not account for the sedative activities of these compounds. It may be due to differences in their physical properties such as vapor pressure and lipophilicity, in addition to carbon chain length. It has been reported that each increase in chain length of two carbons resulted in a decrease in vapor pressure at 20°C by a factor of four, as described by Jocelyn et al. (15). Also, hexyl butyrate (log P=3.28) is more lipophilic than butyl butyrate (log P=2.39) and may easily penetrate the cell membrane. It is possible that the sedative effects of the aliphatic esters of *H. afghanicum* may be influenced by carbon chain length, vapor pressure and lipophilicity.

The effective doses of hexyl butyrate and octyl acetate in this study were in the range of 0.4–4 mg. According to the literature, the oral toxicity (LD_{50}) of these compounds are 5000 and 3000 mg/kg, respectively (16, 17). This indicates that our applied doses are much smaller than the toxic doses and the observed effects may not be a result of toxic effects.

The numbers of patients with psychiatric disease such as insomnia, anxiety, depression and Alzheimer's disease are increasing (18). Current therapeutic goals in the treatment of psychiatric diseases are to improve the quality of life, normalize mood, increase awareness of personal pleasures and interests, and reverse functional and social disabilities. The currently available synthetic drugs used for these purposes sometimes have severe adverse effects and even addiction or dependency (18, 19). Therefore, there is a need for more effective and safer drugs. Thus essential oils exhibiting anti anxiety and sedative effects are very important. In the present study, *H. afghanicum* essential oil exhibited significant sedative activity using a vapor inhalation system, which is rather safe and acceptable for patients, especially children and elderly people.

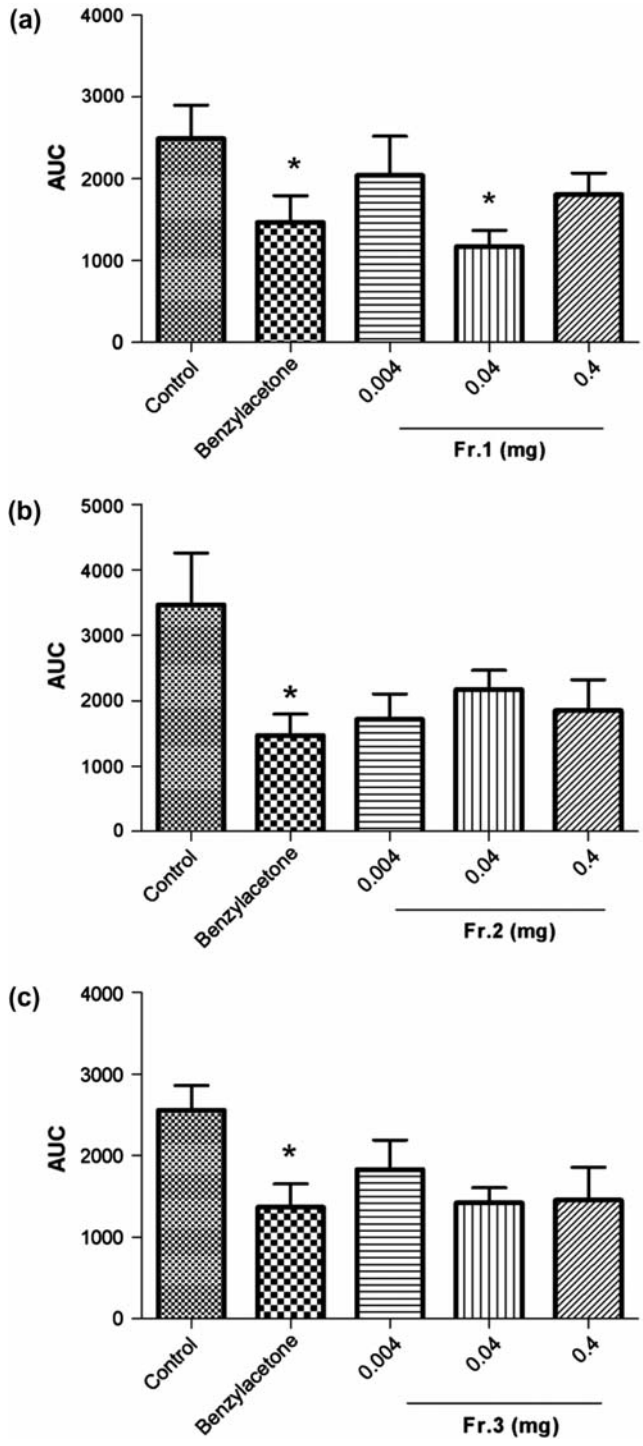

Figure 2. Total spontaneous motor activity of mice treated with vehicle (triethyl citrate 400 µl), benzylacetone (0.4 mg), fraction 1, 2 or 3 (a, b and c, respectively). Data are shown as the means ± SEM of six mice. Statistical differences vs the control group were calculated using analysis of variance (ANOVA), followed by Dunnett's test. $^{*}p<0.05$, $^{**}p<0.01$.

Conclusion

This study revealed that *H. afghanicum* essential oil consists mainly of aliphatic esters with hexyl butyrate and octyl acetate as principal compounds. The essential oil significantly decreased the locomotor activity of mice at 0.4 mg, suggesting the potential sedative activity of this oil. Hexyl butyrate is shown to be the main active compound of *H. afghanicum* essential oil.

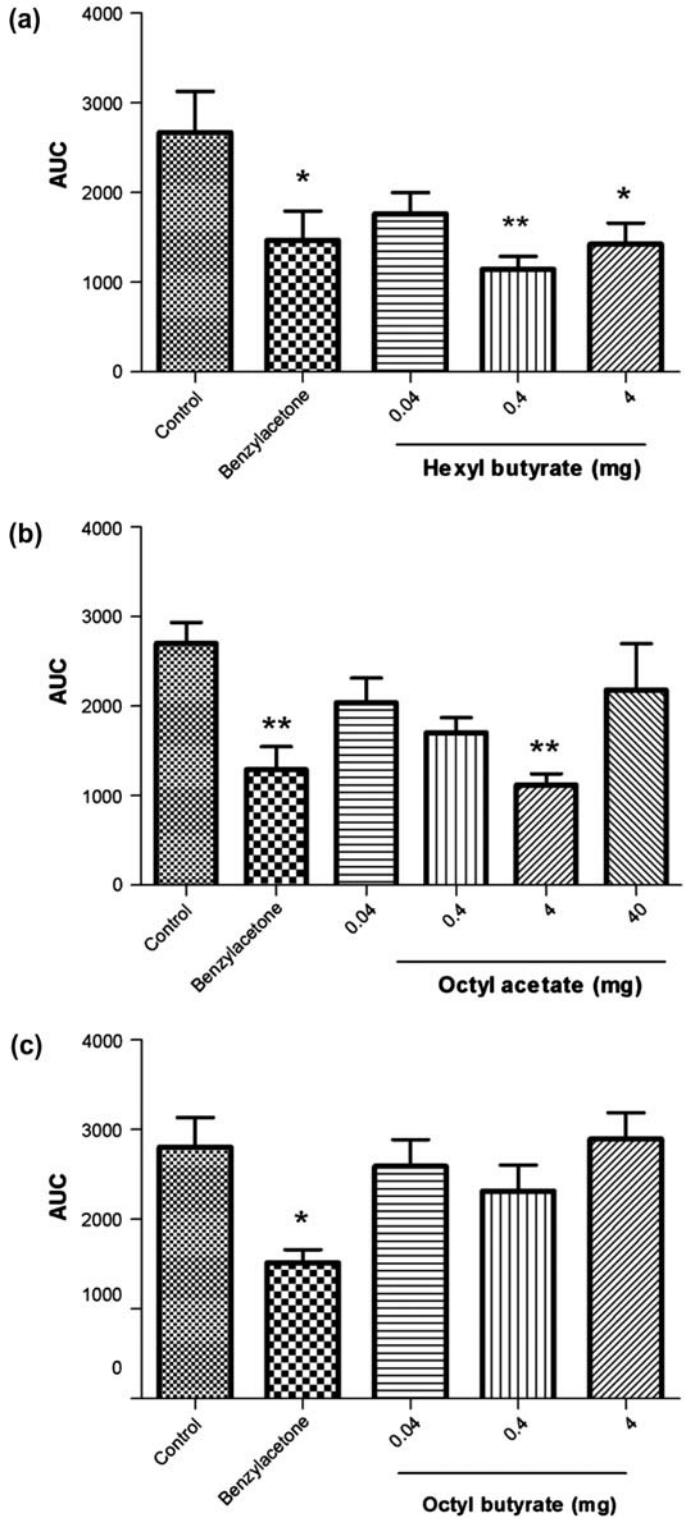

Figure 3. Total spontaneous motor activity of mice treated with vehicle (triethyl citrate 400 µl), benzylacetone (0.4 mg), hexyl butyrate (a), octyl acetate (b) or octyl butyrate (c) Data are shown as the means ± SEM of five mice. Statistical differences vs the control group were calculated using analysis of variance (ANOVA), followed by Dunnett's test. $^{*}p<0.05$, $^{**}p<0.01$.

Moreover, the results of this study revealed that hexyl butyrate was more potent then octyl acetate, suggesting that the butyrate function may be very important in the sedative activity of aliphatic esters. However, further studies are required in order to clarify the mechanism of action as well as to investigate the structure–activity

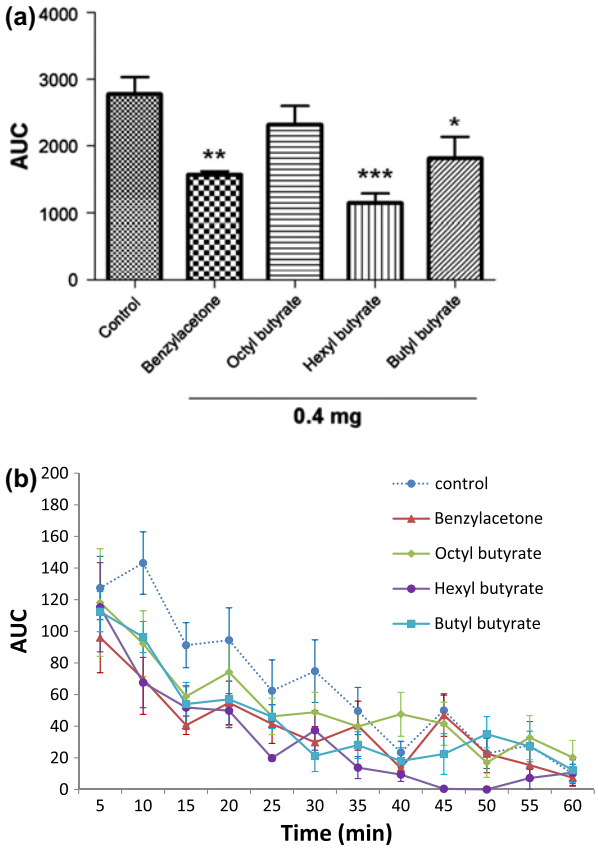

Figure 4. Total spontaneous motor activity (a) and locomotor activity transition (b) of mice that received vehicle (triethyl citrate 400µl), benzylacetone, octyl butyrate, hexyl butyrate and butyl butyrate (0.4 mg). Data are shown as means ±SEM of five mice ($^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs control group).

relationship. In this paper, the phytochemical and pharmacological activity of *H. afghanicum* seeds essential oil is being reported for the first time.

Acknowledgements

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