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ANXIOLYTIC CONSTITUENTS FROM *STELLARIA MEDIA* LINN

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ABSTRACT

Bioactivity guided fractionation of the anxiolytic methanol extract has led to the isolation of two compounds — A1 and A2. The effects of both the compounds in five assays predictive of anxiolytic activity in mice of either sex were studied, with diazepam as anxiolytic control. Structures of A1 and A2 characterized by IR, ^{1}H NMR, ^{13}C NMR, MS techniques were found to be 6-methylheptyl-3′-hydroxy-2′-methylpropanoate and 2,2,4-trimethyloctan-3-one. Both these new compounds (ester and ketone), isolated from *Stellaria media* were found to be responsible for the anxiolytic activity of the plant.

KEYWORDS: *Stellaria media*, Bioactivity guided fractionation, Anxiolytic.

1. INTRODUCTION

Stellaria media L. (Caryophyllaceae) or chickweed is a winter annual herb.[1] It has been used traditionally in the treatment of mental tension and inflammations of the digestive, renal, respiratory and reproductive tracts.[2,3] The plant is employed in plasters used for broken bones and in the external treatment of various kinds of itching skin conditions.[4] Phytoconstituents reported from the plant include phenolic acids, flavonoids,[5] C-glycosyl flavones,[6] triterpenoid saponins,[7] a pentasaccharide,[8] lipids,[9] and aqueous constituents.[10]

Earlier studies carried out by the authors revealed that of the four extracts - petroleum ether, chloroform, methanol and water, prepared from *S. media* aerial parts, only the methanol extract exhibited anxiolytic activity.[11] Ethyl acetate fraction (EAF_5) derived from the bioactive methanol extract of *S. media* has been observed to exhibit significant anxiolytic.

activity.[12] An attempt has been made to isolate and identify the bioactive phytomoieties liable for the anxiolytic effects of *S. media* by resorting to bioactivity directed fractionation and chromatographic procedures.

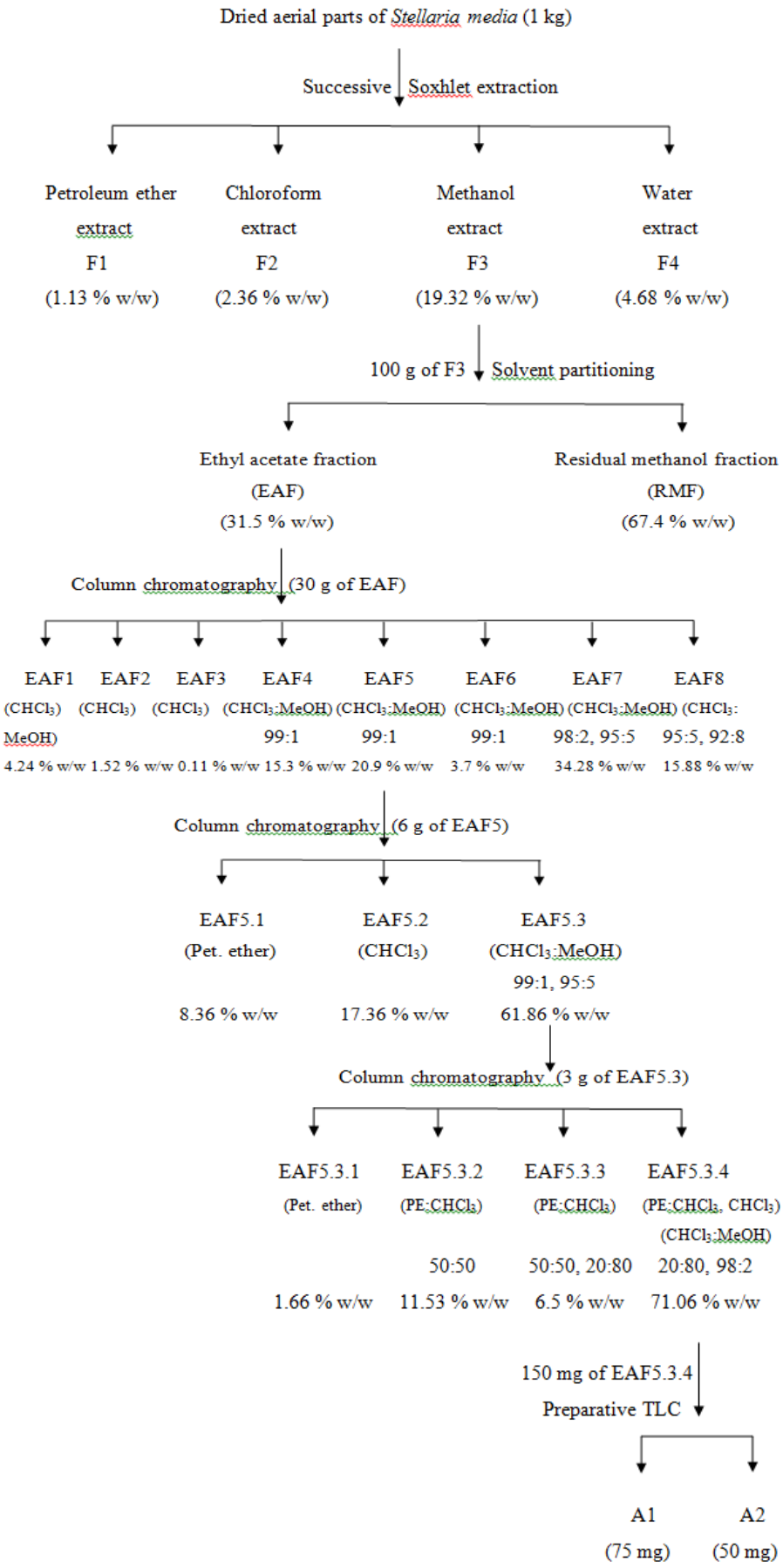
2. MATERIALS AND METHODS

2.1. Plant material

Aerial parts of *S. media* were collected from around the U.I.P.S. building, Panjab University, Chandigarh in February 2009. The identity of the plant was confirmed by Dr. H.B. Singh, Head, Raw Materials, Herbarium & Museum at the National Institute of Science Communication and Information Resources, (NISCAIR, CSIR), New Delhi 110 067. A voucher specimen no: NISCAIR/RHMD/Consult/2008-09/1170/202 is deposited in the same herbarium.

2.2. Test material

Bioactive EAF5.3.4 was obtained from the methanol extract of *S. media* after bioactivity guided fractionation (Scheme 1 depicted below). Subfraction EAF5.3.4 was then subjected to preparative TLC in order to separate bioactive constituents.



Scheme 1: Bioactivity guided separation of anxiolytic fraction from *Stellaria media*.

2.3. Animals

Laca mice of either sex, weighing 20-24 g were procured from the Animal House, Panjab University, Chandigarh. The mice were maintained on standard laboratory feed and water *ad libitum*. The animals were fasted 18 h prior to the biological study. The experiments were conducted in a semi sound proof laboratory. The biological studies were carried out as per the guidelines of the Institutional Ethical Committee (Reg. no. 1451/CAH) of Department of Pharmaceutical Sciences, Panjab University, Chandigarh.

2.4. Preparative TLC

Preparative thin layer chromatography was performed using 20 × 20 cm glass plates coated with 0.5 mm silica gel G (E. Merck).

2.5. Elevated plus-maze

Anxiolytic activity was evaluated using elevated plus-maze (EPM) model.[13-16] The plus-maze apparatus consisting of two open arms (16 × 5 cm) and two closed arms (16 × 5 × 12 cm) having an open roof, with the plus-maze elevated (25 cm) from the floor was used to observe anxiolytic behavior in animals. The animals were fasted 18 h prior to the experiment. Test substances were administered orally using a tuberculin syringe fitted with an oral canula. The dose administration schedule was so adjusted that each mouse was having its turn on the elevated plus-maze apparatus 45 min after the administration of the dose. Each mouse was placed at the center of the elevated plus-maze with its head facing the open arms. During this 5 min experiment, the behavior of the mouse was recorded as (a) the number of entries into the open or closed arms, (b) average time spent by the mouse in each of the arms. During the entire experiment, the animals were allowed to socialize. Every precaution was taken to ensure that no external stimuli could invoke anxiety in the animals. The apparatus was cleaned using 70 % ethanol after each animal. Similar observations were recorded for the standard group (Diazepam 2 mg/kg, *p.o.*) as well as the control group (vehicle).

2.6. Light/dark test

The apparatus consisted a rectangular box (45 × 27 × 27 cm), partitioned into two compartments connected by a 7.5 × 7.5 cm opening in the wall between compartments[17] An animal was placed in the center of the light compartment and was observed for 5 min for the time spent in open (white/light) compartment and latency of the first crossing from light to dark compartment.

2.7. Mirrored chamber test

The apparatus consisted of a mirrored cube (30 cm on a side) open on one side that was placed inside a square wooden box (40 × 40 × 30.5 cm).[18-19] The mirrored cube was constructed of five pieces of mirrored glass. The mirrors used were mirrored on one surface only (back surface being painted dark brown). The three mirrored side panes, a top pane, and the floor pane faced the interior of the cube. The mirrored cube was placed in the centre of the wooden container to form a 5 cm corridor that completely surrounded the mirror chamber. During a 5 min test period, the following parameters were noted: (a) latency to enter the chamber, i.e., the time spent in seconds for the first entry into the chamber of mirrors and (b) the time spent with each entry which was calculated by dividing the total time spent with number of entries.

2.8. Open-field test

The open-field apparatus consisted of a rectangular plexiglass arena measuring 60 × 60 cm with 35 cm high walls.[20-21] The floor was marked with lines that divided it into 36 squares (10 × 10 cm). Behavior in the open-field box was observed and the following parameters were recorded: (a) the total number of squares traversed, (b) time spent in the perimeter (32 outer squares close to the outer walls), (c) time spent in the center (4 innermost squares) and (d) the number of entries into the center of the arena, over the 5 min period.

2.9. Confirmation of antianxiety activity using mCPP-induced hypolocomotion model

In this model, mCPP, a metabolite of the antidepressant drug trazodone was used.[22-25] Mice were divided into four groups — I to IV. Group I and III animals were administered vehicle whereas group II and IV animals were administered test sample (*p.o.*). Forty minutes after the above treatment, groups I and II were administered the vehicle (*i.p.*) while group III and IV animals received mCPP (7 mg/kg, *i.p.*). After 20 min of the treatment with the vehicle/mCPP, locomotor activity of the animals of all groups was observed in an actophotometer (Popular India) for 10 min.

2.10. Characterization of bioactive isolates

Bioactive isolates were subjected to IR, ^{1}H NMR, ^{13}C NMR and Mass spectroscopy. The following instruments at National institute of Pharmaceutical Education and Research, Mohali were used: IR spectrophotometer (Multi spoke FT-IR synthesis monitoring system, Perkin-Elmer, Germany); mass spectrometer (Finnigan, MAT, LCQ, USA) equipped with a pneumatically-assisted atmospheric-pressure chemical ionization (APCI). ^{1}H NMR and ^{13}C

NMR spectra were obtained on a 400 MHz NMR spectrometer, (Bruker 400, Ultra Shield, ZH079807, Avane, Germany) using $CDCl_3$ as solvent. Chemical shifts were expressed in parts per million (ppm) relative to tetramethyl silane (TMS) as an internal standard.

2.11. Statistical analysis

The results of biological studies have been expressed as mean ± SEM. Further the results of biological evaluations were analyzed by one way ANOVA. The observations obtained from the test groups were compared with standard/control by Tukey's Multiple Range Test. Differences were considered significant at $P < 0.05$.

3. RESULTS

3.1. Preparative TLC of EAF5.3.4

The TLC profile of EAF5.3.4 had shown the presence of two components at R_f 0.73 and 0.78 using the mobile phase toluene:chloroform in the proportion of 7:3. These two components were separated using preparative TLC. From EAF5.3.4, two compounds were obtained namely A1 (75 mg) and A2 (50 mg). The anxiolytic activity of the two compounds, A1 and A2 was evaluated using different models.

3.2. Confirmation of anxiolytic activity of A1 and A2 using different models

The anxiolytic activity of A1 and A2 was confirmed using elevated plus-maze- (Figs. 1 and 2), light/dark- (Figs. 3 and 4), mirrored chamber- (Figs. 5 and 6), open-field (Figs. 7,8,9 and 10) tests and mCPP-induced hypolocomotion model (Figs. 11 and 12).

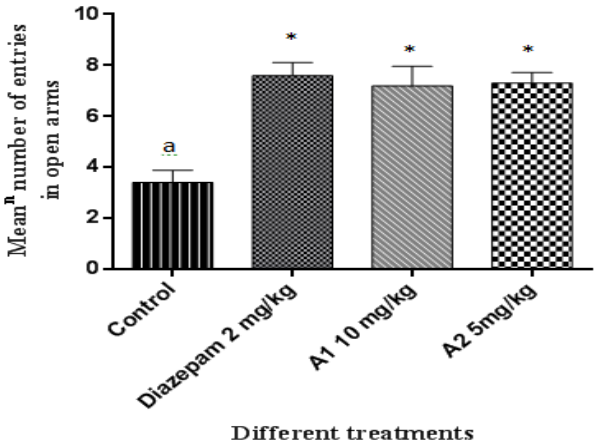


Fig. 1. Effect of different treatments on mean number of entries by mice in open arms of EPM. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. *P < 0.05 vs. control (vehicle); [a]P < 0.05 vs. diazepam (standard drug).

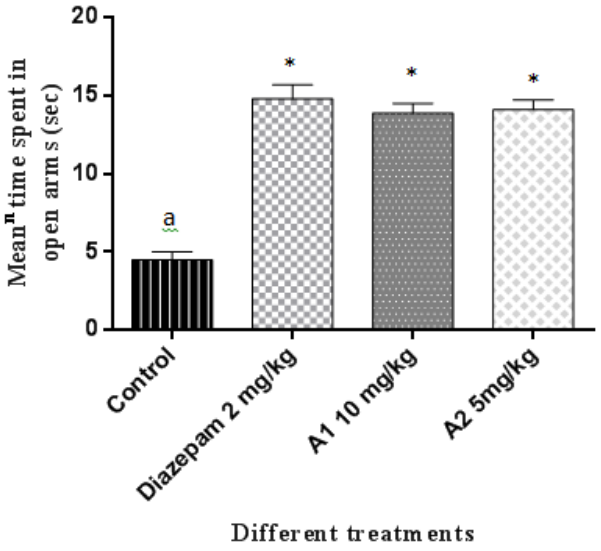


Fig. 2: Effect of different treatments on mean time spent by mice in open arms of EPM. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. *P < 0.05 vs. control (vehicle); ^{a}P < 0.05 vs. diazepam (standard drug).

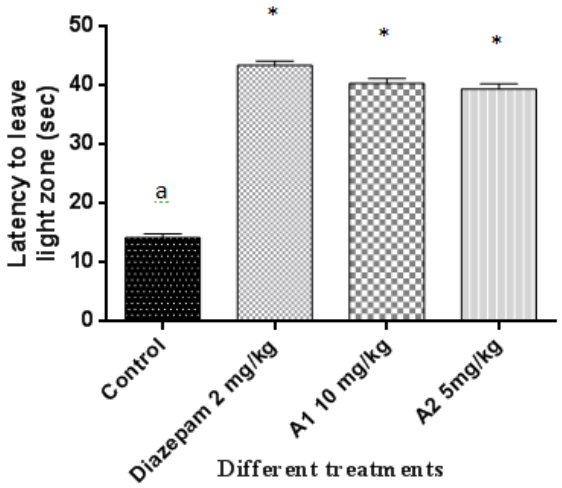


Fig. 3: Effect of different treatments on latency to leave light zone by mice in light/dark test. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. *P < 0.05 vs. control (vehicle); ^{a}P < 0.05 vs. diazepam (standard drug).

3.3. Characterization of A1 and A2

The authors carried out the characterization and interpretation of structures of both compounds (A1 and A2) as 6-methylheptyl-3′-hydroxy-2′-methylpropanoate and 2,2,4-trimethyloctan-3-one by spectral studies.[26]

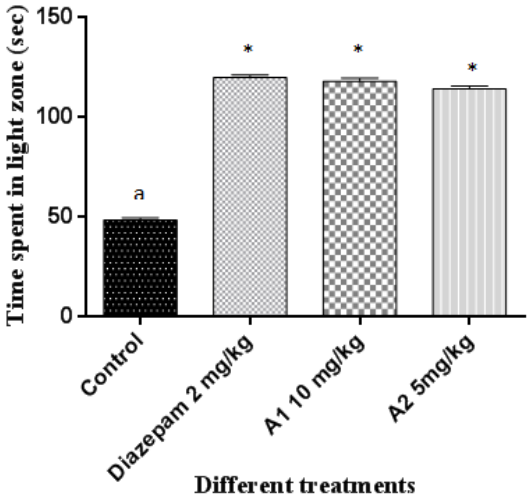


Fig. 4: Effect of different treatments on time spent in light zone by mice in light/dark test. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. *P < 0.05 vs. control (vehicle); [a]P < 0.05 vs. diazepam (standard drug).

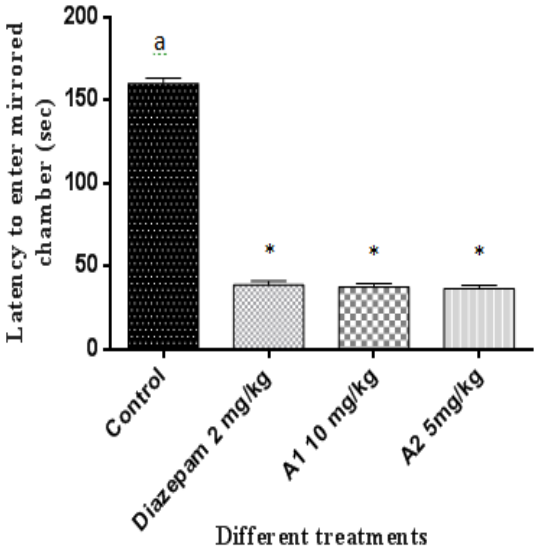


Fig. 5: Effect of different treatments on latency to enter mirrored chamber by mice in mirrored chamber test. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. *P < 0.05 vs. control (vehicle); [a]P < 0.05 vs. diazepam (standard drug).

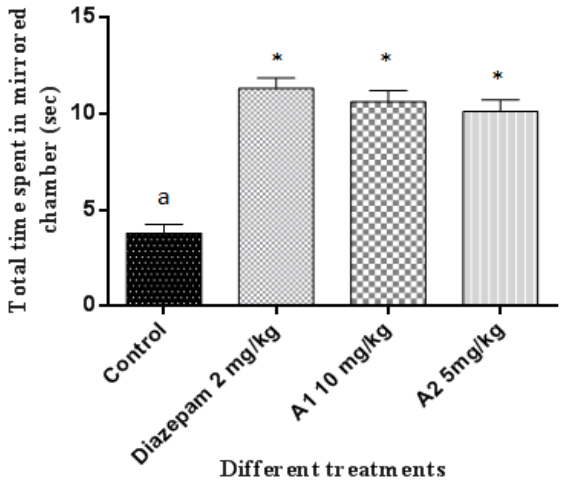


Fig. 6: Effect of different treatments on total time spent in mirrored chamber by mice in mirrored chamber test. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. *P < 0.05 vs. control (vehicle); [a]P < 0.05 vs. diazepam (standard drug).

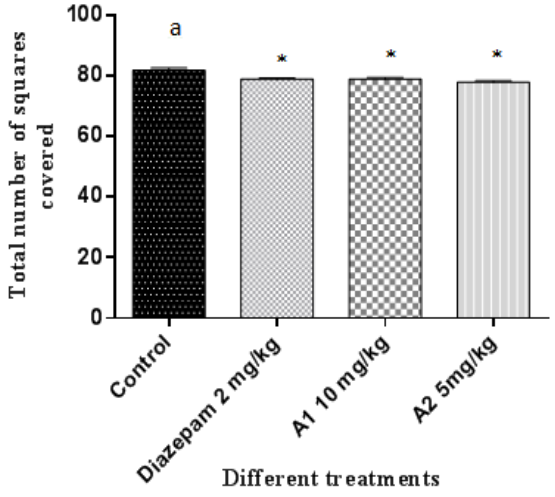


Fig. 7: Effect of different treatments on total number of squares covered by mice in open-field test. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. *P < 0.05 vs. control (vehicle); [a]P < 0.05 vs. diazepam (standard drug).

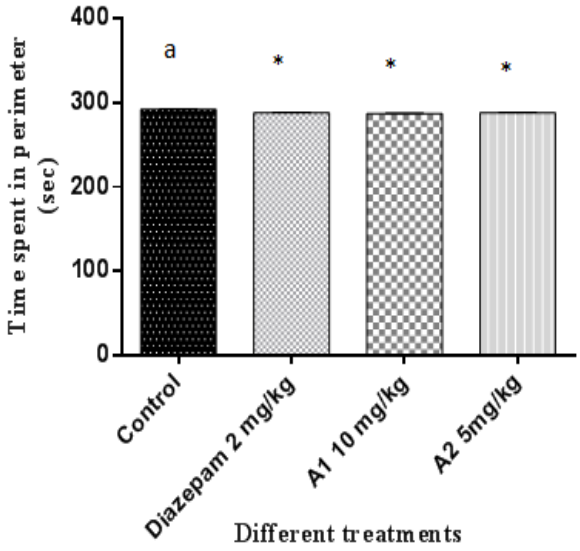


Fig. 8: Effect of different treatments on time spent in perimeter by mice in open-field test. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. *P < 0.05 vs. control (vehicle); [a]P < 0.05 vs. diazepam (standard drug).

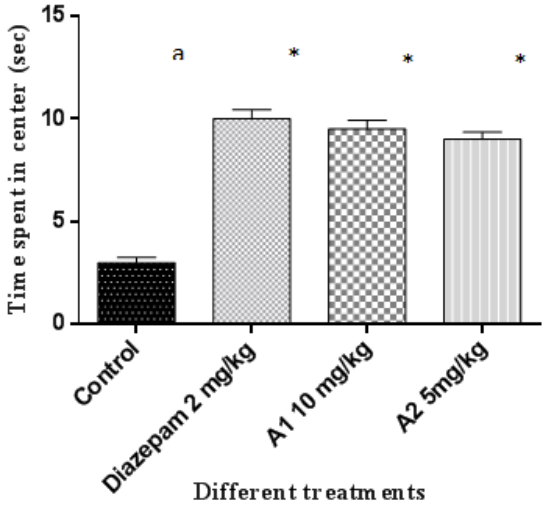


Fig. 9: Effect of different treatments on time spent in center by mice in open-field test. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. *P < 0.05 vs. control (vehicle); [a]P < 0.05 vs. diazepam (standard drug).

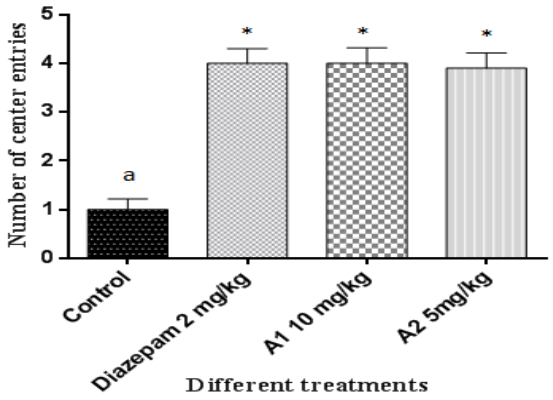


Fig. 10: Effect of different treatments on number of center entries by mice in open-field test. Values are expressed as mean ± S.E. (n = 6). The data was analyzed by one way ANOVA and post hoc Tukey's multiple range test. a = p < 0.05 vs. control (vehicle); b = p < 0.05 vs. diazepam (standard drug).

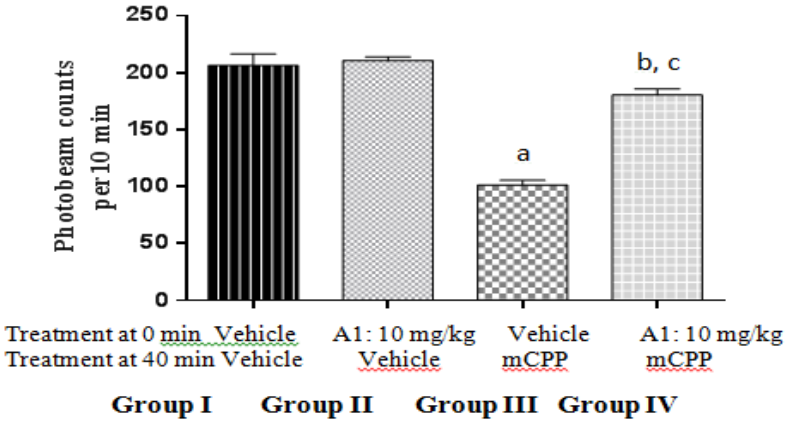


Fig. 11: Effect of A1 on mCPP-induced hypolocomotion. Values are expressed as mean ± S.E. (n = 6). ANOVA followed by Tukey's multiple range test. p < 0.05. a = significant with respect to Group I, b = significant with respect to Group II, c = significant with respect to Group III.

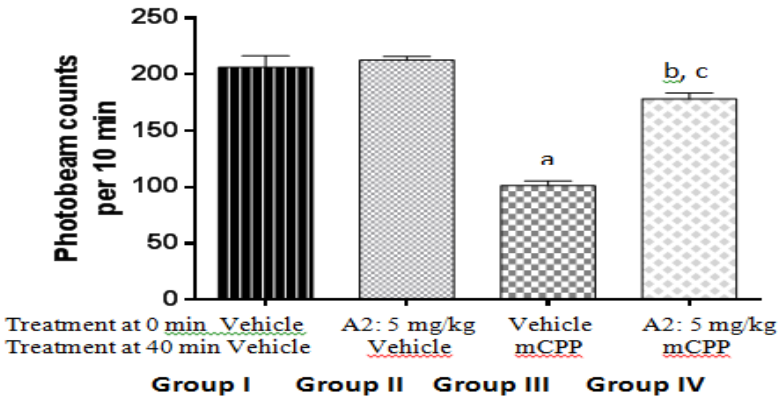


Fig. 12: Effect of A2 on mCPP-induced hypolocomotion. Values are expressed as mean ± S.E. (n = 6). ANOVA followed by Tukey's multiple range test. p < 0.05.

a = significant with respect to Group I,
b = significant with respect to Group II,
c = significant with respect to Group III.

4. DISCUSSION

Bioactivity guided fractionation of methanol extract of *S. media* ultimately led to the isolation of two compounds A1 and A2, which were found to be responsible for the antianxiety activity of *S. media*. The compounds A1 and A2 were characterized by IR, ^{1}H NMR, ^{13}C NMR and Mass spectroscopy. The chemical structure of A1 and A2 were interpreted as 6-methylheptyl-3′-hydroxy-2′-methylpropanoate and 2,2,4-trimethyloctan-3-one. These compounds have been reported for the first time from *S. media*.

Fats and lipids are common components of food and may perform essential roles. Fatty acids play an important role in brain cell structure as 36-60 % of nervous tissue in brain are lipids which include glycerophospholipids rich in docosahexaenoic acid (DHA) and arachidonic acid (AA), sphingolipids, cholesterol and its esters.[27] Dialkylamino alkyl esters of pivagabine are used as medicaments for the treatment of central nervous system disorders like acute stress disorder, bipolar disorder, anxiety disorders, obesity, Parkinson's disease, Alzheimer's disease, etc.[28] 3-hydroxybutyrate methyl ester enhances learning and memory, possibly through a signalling pathway requiring PUMA-G (protein up-regulated in macrophages by IFN-γ) that increases protein synthesis and gap junction intercellular communication.[29] Systemic administration of N^{G}-nitro-L-arginine methyl ester (L-NAME),[30-31] L-N^{G}-nitro arginine (L-NOARG)[32] and 7-nitro indazole (7-NI),[33-35] NOS inhibitors, showed an anxiolytic-like effect in the elevated plus-maze test that is a rodent model of anxiety.[36] *α*-Asarone exhibited anxiolytic-like effect in mice.[37] Thujone and carvone, components of the hydro-alcohol extract of *Aloysia polystachya* may have sedative, anxiolytic and antidepressant like properties.[38] Chronic administration of bupropione (2 and 5 mg/kg, *i.p.*) exhibited a significant protection against triazolam withdrawal-induced anxiety and hyper-locomotor activity in mice.[39]

To conclude the present investigation bioactivity guided fractionation of methanol extract of *S. media* ultimately led to the isolation of two compounds — 6-methylheptyl-3′-hydroxy-2′-methylpropanoate and 2,2,4-trimethyloctan-3-one, which were found to be responsible for the antianxiety activity of *S. media*.

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