Revista Brasileira de Farmacognosia 27 (2017) 636–640

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Sedative and muscle relaxant activities of diterpenoids from *Phlomidoschema parviflorum*

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ARTICLE INFO

Article history:
Received 3 March 2017
Accepted 25 July 2017
Available online 17 August 2017

Keywords:
Rosane diterpenoids
Stachysrosane
Sedative
Skeleton muscle relaxant activity
Molecular docking

ABSTRACT

Phlomidoschema parviflorum (Benth.) Vved. (Basionym: *Stachys parviflora* Benth.) Lamiaceae, have significance medicinal importance as it is used in number of health disorders including diarrhea, fever, sore mouth and throat, internal bleeding, weaknesses of the liver and heart genital tumors, sclerosis of the spleen, inflammatory tumors and cancerous ulcers. The present contribution deals with the sedative and muscle relaxant like effects of diterpenoids trivially named stachysrosane and stachysrosane, isolated from the ethyl acetate soluble fraction of *P. parviflorum*. Both compounds (at 5, 10 and 15 mg/kg, *i.p*) were assessed for their *in vivo* sedative and muscle relaxant activity in open field and inclined plane test, respectively. The geometries of both compounds were optimized with density functional theory. The molecular docking of both compounds were performed with receptor gamma aminobutyric acid. Both compounds showed marked activity in a dose dependent manner. The docking studies showed that both compounds interact strongly with important residues in receptor gamma aminobutyric acid. The reported data demonstrate that both compounds exhibited significant sedative and muscle relaxant-like effects in animal models, which opens a door for novel therapeutic applications.

Introduction

Phlomidoschema parviflorum (Benth.) Vved. (Basionym: *Stachys parviflora* Benth.) belongs to the Lamiaceae (mint) family that distributed in the tropical and temperate regions of Pakistan (Ahmad et al., 2006). Plants of this genus have been extensively applied to treatment of wide panel of health disorders including diarrhea, fever, sore mouth and throat, internal bleeding, weaknesses of the liver and heart (Holiman et al., 1996), genital tumors, sclerosis of the spleen, inflammatory and cancerous ulcers (Skaltsa et al., 1999). The species of the genus *Stachys lavandulifolia* showed sedative and anxiolytic like effects in animal studies (Rabbani et al., 2003, 2005).

Phytochemical investigations of *Stachys* species have shown the occurrence of flavonoids, diterpenes, phenyl ethanoid glycosides, alkaloids, and saponins (Khanavi et al., 2005; Ahmad et al., 2006).

Various chemical constituents like stachyssaponin A, stachyssaponin B, parviflorosides A as well as parviflorosides B has been isolated from *P. parviflorum* (Ahmad et al., 2006, 2008). A new rosane-type diterpenoid from *P. parviflorum* has been isolated (Farooq et al., 2014).

Unfortunately, literature survey revealed that limited work has been done so far on *P. parviflorum*. Considering its medicinal importance, the present study is based on the sedative and muscle

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http://dx.doi.org/10.1016/j.bjp.2017.07.003

relaxant effects of compounds **1** and **2** isolated from *P. parviflorum* in two animal protocols. The computational studies of these compounds were carried out, in order to know the molecular mechanism at atomic level.

Materials and methods

General experimental procedure

Column chromatography was performed using silica gel (70–230 mesh, E-Merck) and silica gel (230–400 mesh, E-Merck). Analytical TLC was performed on precoated plates (silica gel 60 F_{254}). Optical rotations were recorded using a Jasco-DIP-360-digital polarimeter. UV and IR spectra were recorded with UV-240 and JASCO-320-A spectrophotometers, respectively. EI-MS and HR-EI-MS were recorded on a double-focusing varian MAT-312 spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-400 spectrometer, while 2D-NMR spectra were recorded on Bruker AMX-500 MHz NMR spectrometer. Chemical shifts in parts per million (δ) relative to tetramethylsilane as an internal standard, and scalar couplings (J) were reported in Hz.

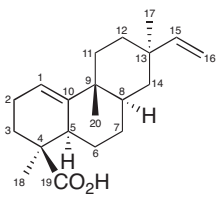
Plant material

The whole plant *Phlomidoschema parviflorum* (Benth.) Vved. (Basionym: *Stachys parviflora* Benth.), Lamiaceae, was collected from Abbottabad (Pakistan) in March 2012. The plant was identified by Dr. Manzoor Ahmad (Taxonomist, Department of Botany, Post-Graduate College, Abbottabad). A voucher specimen (No. 14230) has also been submitted in the herbarium of the same department.

Extraction and isolation

The air-dried whole plant (9 kg) was grinded and extracted with methanol at room temperature. The extract was evaporated on a rotary evaporator under reduced pressure to obtain a crude extract. The resulting methanol extract (2500 g) was suspended in water and successively partitioned to provide hexane (110 g), chloroform (80 g), ethyl acetate (35 g), *n*-butanol (95 g), and aqueous extract (150 g).

The ethyl acetate fraction was further subjected to column chromatography over a silica gel column using hexane with a gradient of ethyl acetate up to 100% followed by methanol. Eight sub-fractions were collected through column chromatography. Sub-fraction 7–8 was subjected to column chromatography and eluted with EtOAc/hexane 35:65 to purify compound **1** (20 mg). The combined less polar fractions 5–6 of this separation were re-subjected to column chromatography, which resulted compound **2** (17 mg) at EtOAc/hexane 30:70 polarity. The purity of compounds was checked on TLC and HPTLC plates. The structure of these compounds was recently reported by our group (Farooq et al., 2015).



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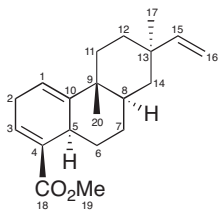

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Animals

NMRI mice (20–25 g) were used in various experiments. Animals were fed with standard laboratory food and water *ad libitum*. Animals were kept under standard condition of temperature and light. Before the start of experiment animals were acclimatized with laboratory conditions. All experimental protocols were approved from ethical committee for animal studies of University of Peshawar under reference number 14009 (PUP) keeping in mind the international guideline of animal studies.

Sedative properties in open field test

The apparatus used for this activity was consisted of an area of white wood (150-cm diameter) enclosed by stainless steel walls and divided in four squares by black lines. The open field was placed inside a light and sound-attenuated room. Animals were acclimatized under red light (40 W red bulb) 60 min before the start of experiment in laboratory with food and water available *ad libitum*. Animals were divided in various groups ($n = 6$) and were administered with normal saline (negative control, 10 ml/kg, *i.p.*), test compounds (5, 10 and 15 mg/kg, *i.p*) and bromazepam (0.5 mg/kg, *i.p.*). After 30 min, each animal was placed in the center of the box and the numbers of lines crossed were counted for each mouse (Rauf et al., 2017a,b). To assess process of adjustment to the novelty of the ground, mice were exposed to the apparatus for 5 min on two successive days.

Inclined plane test

The plane used in this procedure was consisting of two plywood boards. Both boards were connected with each other in such a way that one board form the base and other is fix with the base at 65°. Different groups ($n = 6$) were treated with bromazepam (1 mg/kg, *i.p.*), normal saline (10 ml/kg, *i.p.*) and test compounds (5, 10 and 15 mg/kg, *i.p*). After 30, 60 and 90 min of treatment the animals were placed on the upper part of the inclined plane for 30 s to hang or fall (Rauf et al., 2017a,b).

Statistical analysis

Results are expressed as mean ± S.E.M. One-way ANOVA was used for comparison test of significant differences among groups followed by Dunnet's multiple comparison post test. A level of significance ($p < 0.05$ or 0.01) was considered for each test.

Molecular modeling

Molecular modeling experiments have been performed on both compounds. Their geometries were fully optimized within the density functional theory (DFT) framework at the B3LYP/6-31G(d) level (Newman and Cragg, 2012; Malami et al., 2014). Vibrational calculations were subsequently performed to ascertain the nature of every localized structure: a stable structure corresponds to a minimum in the potential energy surface and consequently all frequencies are real. Partial atomic charges were next computed by using the Merz-Singh-Kollman ESP protocol (Newman and Cragg, 2012), to be used during docking simulations. Required quantum chemical calculations were performed with the Gaussian 09 suite of programs (Malami et al., 2014).

Docking calculations were performed using structural data from a crystal of $GABA_A$ receptor obtained from PDB database, with PDB code 4COF (Sussman et al., 1998). The simulations were carried out according to the procedure previously described by protocol (Newman and Cragg, 2012), by search of the global minimum of

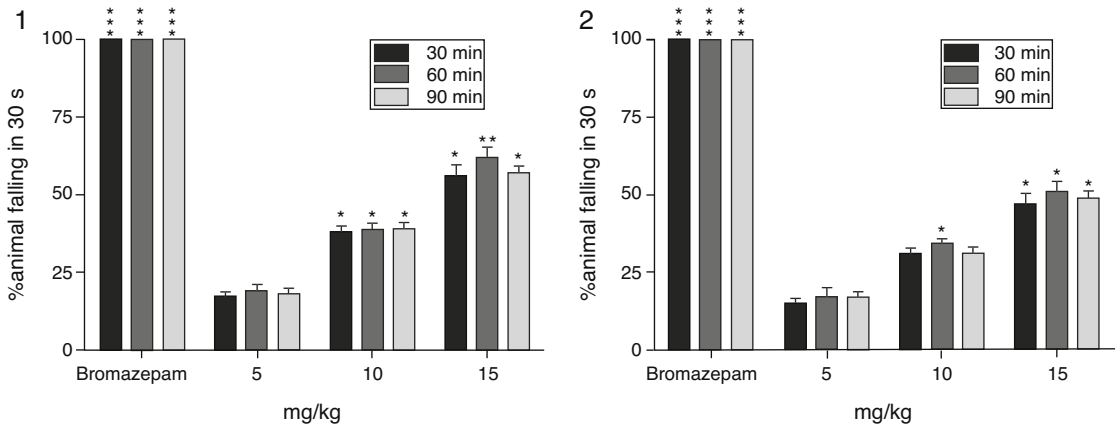

Fig. 1. Effect of isolated compounds **1** and **2** from *P. parviflorum* on muscle coordination in inclined plane, bar represents the percent time spent in seconds by which mice slide off the inclined plane, 30, 60 and 90 min after treatment with normal saline (10 ml/kg), compounds (5, 10 and 15 mg/kg) or bromazepam (1 mg/kg). $^{*}p < 0.05$, $^{***}p < 0.001$, all with respect to control.

the potential energy surface with the genetic algorithm implemented in the Lead Finder docking program. For the records, the full-atom model of $GABA_A$ used in docking calculations was prepared from the raw PDB structure 4COF. Water molecules were removed and the ionization states of the amino acids were assessed using Protonate3D function of the MOE software package (Chemical Computing Group, LLC). The same protocol was used for adding missing hydrogen atoms in the PDB files, where all charged side chains were considered in their default protonation states at neutral pH. Protein atomic partial charges were derived through the AMBER99 force field (Newman and Cragg, 2012), implemented in MOE. The size of the grid box for ligand docking was set to 120 Å in each direction from the geometric center of the Aquaporin model. The dG score produced by Lead Finder was taken as the predicted value of the ligand binding energy. Scoring function from Lead Finder considers Lennard–Jones term (LJ), metal interactions, solvation term, hydrogen bonds (H-bonds), and electrostatic interactions, internal energy of the ligand, contributions to entropy due to ligand torsions and a solvation penalty term. In this blind docking approach multiple docking runs started around geometric centers of all residues within the selected threshold.

Results

*Sedative effect of test compounds (**1** and **2**)*

The compounds caused dose-dependent effect on mobility at various test doses. Pretreatment of both compounds had significant reduction in mobility at 10 and 15 mg/kg. The overall effect of compound **1** and **2** was almost equally significant. The locomotion decreased by bromazepam was most significant than the test doses of isolated compounds (Table 1).

Muscle relaxant effect of test compounds

The results of muscle relaxant effect of test compounds (**1** and **2**) in inclined plane test are presented in Fig. 1. Both compounds elicited significant skeleton muscle relaxant like effect in a dose dependent manner. The maximum activity was noted at 15 mg/kg after 60 min of treatment. Compounds **1** and **2** produced almost similar relaxation of mice skeleton muscle but less pronounced then standard drug (bromazepam).

Table 1
Effect of isolated compounds **1** and **2** from *P. parviflorum* in open field test (locomotive activity).

Sample	Dose	No. of lines crossed
Control	5 ml/kg	130 ± 3.95
Bromazepam	0.5 mg/kg	9 ± 0.55[c]
	5 mg/kg	100 ± 5.03
Compound 1	10 mg/kg	82 ± 4.34[a]
	15 mg/kg	74 ± 3.60[b]
	5 mg/kg	101 ± 5.67
Compound 2	10 mg/kg	88 ± 4.67[a]
	15 mg/kg	79 ± 4.45[a]

Values represent the number of lines crossed by animal in box, 30 min after treatment with normal saline (10 ml/kg, control), compounds (5, 10 and 15 mg/kg) or bromazepam (0.5 mg/kg). Data presented as mean ± S.E.M. ($n = 6$).

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$, all compared with control.

Molecular modeling

The bromazepam and compounds **1** and **2** were first docked into the $GABA_A$ receptor, which acts as the host entity. The poses with the highest value for the protein–ligand interaction energy were next selected for a qualitative analysis. In Figs. 2–4 results obtained from blind docking for bromazepam, compound **1** and compound **2** are shown. These figures pointed out that main stabilizing interaction for bromazepan are due to pi–pi interactions between Phe200C, Tyr62B and aromatic scaffold of the ligand, hydrophobic interactions with same residue Tyr62B and hydrogen bond with Gln64B. Such large number of protein–ligand interactions explains why bromazepam interacts so strongly with $GABA_A$. Regarding docking results for compound **1** and **2**, we can see that in both cases they bind to the protein in the same area as bromazepam, through hydrophobic interactions with residues Phe200C, Asp43B and Tyr62B, and hydrogen bonds with Asn41B and Thr176B (compound **1**, Fig. 3) and Tyr205C (compound **2**, Fig. 4). There are not so many interactions for these compounds as with bromazepam but there is high shape complementarity of the two compounds with the binding site. Consequently, the hydrogen bonds, hydrophobicity and shape complementarity allow us to delineate the experimentally observed affinity of $GABA_A$ to these compounds.

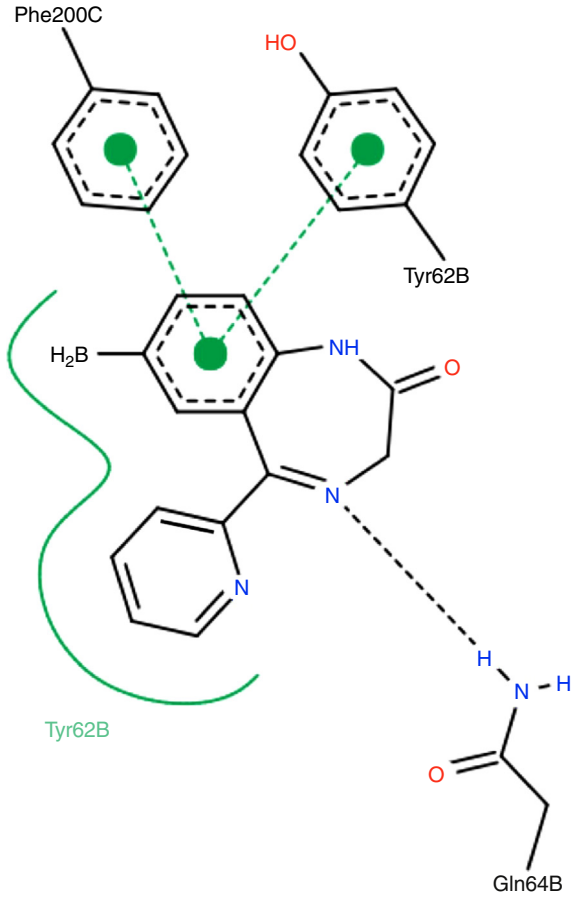

Fig. 2. 2D representation of main protein–ligand interactions established between bromazepam and $GABA_A$. Green line represents hydrophobic interactions, black dashed line shows hydrogen bonds and green dashed lines correspond to pi–pi interactions.

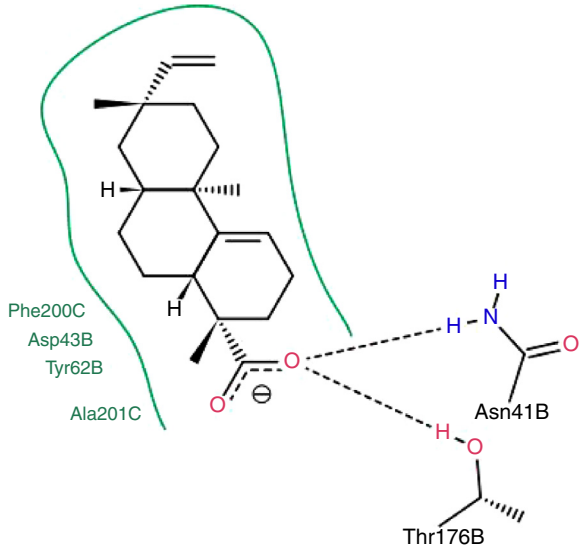

Fig. 3. 2D representation of main protein–ligand interactions established between compound **1** and $GABA_A$. Green line represents hydrophobic interactions, and black dashed lines show hydrogen bond interactions.

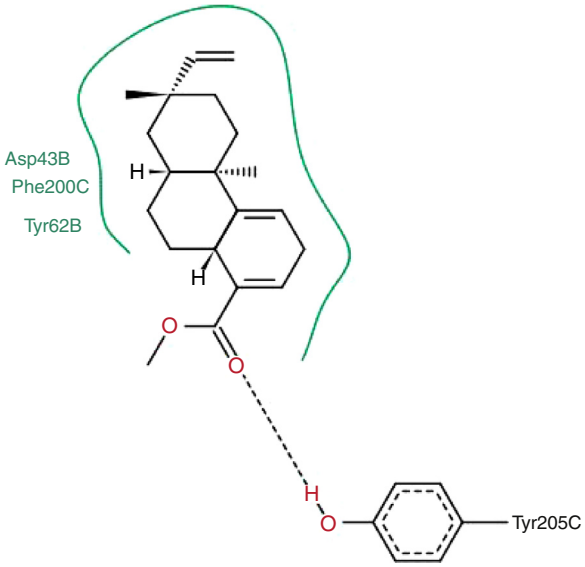

Fig. 4. 2D representation of main protein–ligand interactions established between compound **2** and $GABA_A$. Green line represents hydrophobic interactions, while black dashed line shows hydrogen bond interactions.

Discussion

The present study demonstrated a marked sedative and muscle relaxant effects of diterpenoids isolated from *P. parviflorum* in two animal models. The open field test is one of the simplest and commonly used screening tools for the assessment of sedative like potential (Uddin et al., 2014; Rauf et al., 2017a,b). Additionally, it can be used to evaluate exploration and anxiety behaviors. The number of central square entries and the duration of time spent in the central square are measures of exploratory behavior and anxiety. A high frequency/duration of these behaviors indicates high exploratory behavior and low anxiety levels and vice versa. The isolated compounds (**1** and **2**) lead to a significantly reduction of the movement of mice in the open field test apparatus. These evidences hint the sedative like nature of both compounds. Similarly, the muscle relaxant like property of compounds (**1** and **2**) also was tested in the inclined plane test that is commonly used assay (Elliot and White, 2001). It was initially designed to differentiating between neuroleptics from other centrally active drugs (Randall et al., 1961), but later on, the method was used for muscle relaxant like effect of test articles (Rivlin and Tator, 1977). The results showed marked skeleton muscle relaxant like effects of the test compounds.

The bromazepam, which demonstrated outstanding sedative and muscle relaxant effects, was used here as standard compound to provide a meaningful biological conclusions. Indeed, the benzodiazepines (BDZs) exhibit marked sedative and muscle relaxant effects mostly attributed to interfere with the action of gamma aminobutyric acid ($GABA_A$) (Dailly and Bourin, 2008), which makes bromazepam an excellent compound to provide a relative activity order. The significant sedative and muscle relaxant like effects of compounds in our study could be attributed to interfere with the action of $GABA_A$ like BDZ. However, further studies are required to ascertain its mechanism for sedative and muscle relaxant-like effects of these compounds for lead compounds in the therapeutic class with better efficacy and safety.

In this study we give also insights, through the use of molecular modeling techniques, about how the behavior results are correlated with the protein–ligand interactions established by the studied compounds. Our results suggest that main stabilizing interactions for bromazepam are due to pi–pi interactions between Phe200C,

Tyr62B and aromatic scaffold of the ligand, hydrophobic interactions with same residue Tyr62B and hydrogen bond with Gln64B. Such large number of protein–ligand interactions explains why bromazepam interacts so strongly with $GABA_A$. Regarding compounds **1** and **2**, our molecular modeling approach does not find so many interactions for these compounds as with bromazepam but there is high shape complementarity of the two compounds with the binding site. Therefore, the hydrogen bonds, hydrophobicity and shape complementarity allow us to delineate the experimentally observed affinity of $GABA_A$ to these compounds.

Conclusion

We can conclude that compounds **1** and **2** isolated from *P. parviflorum* possess significant sedative and skeleton muscle relaxant-like effects in animal models. Our study confirmed the folk uses of the plant as sedative by providing pharmacological rationale of isolated compounds from the plant. Results are fully consistent with the performed molecular modeling calculations, which demonstrate the molecular mechanism at atomic level. According to the computational simulations, both compounds **1** and **2** established similar interactions with $GABA_A$ than bromazepam though with less affinity given the lower number of protein–ligand interactions. The reported results not only help in rationalizing the observed selectivity of stachysrosane and stachysrosane toward $GABA_A$ but also could be used to guide the design of more selective compounds in further experimental and/or theoretical studies.

Author's contribution

AR, UF and AK supervised the whole project. SZ and AI involved in the isolation of compounds, JP, HDH, JP and HP performed computational study and HK performed biological screening. NJ, TBH, MFR, TA and SB make further editing and corrections in this MS and finalize for submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

We acknowledge Higher Education Commission (HEC) of Pakistan and COMSATS for financial support. This work was supported by the Fundación Séneca–Agencia de Ciencia y Tecnología de la Región de Murcia (Spain) under Project 19419/PI/14-1 and by the Centro de Alto Rendimiento de la Región de Murcia (Spain) under project CFE-CAR-23/15.

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