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ORIGINAL ARTICLE

Identification of bioactive metabolites against adenosine A1 receptor using NMR-based metabolomics

Kashif Ali · Muzamal Iqbal · Nancy Dewi Yuliana · Yeon-Ju Lee · Seungil Park · Saem Han · Jeong-Woo Lee · Hyi-Seung Lee · Robert Verpoorte · Young Hae Choi

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Abstract Marine sponges are relatively less explored for their chemical features but highly anticipated resource for bioactive compounds. In this paper we report the screening of marine sponges crude extracts for their potential to bind the adenosine A1 receptor. Many samples showed very promising activity and in order to identify the active components, a metabolomics-chemometrics approach is employed. Nuclear magnetic resonance spectroscopy is used for the metabolic profiling of the marine sponges and partial least squares (PLS) and orthogonal PLS (OPLS) algorithms are used to correlate the metabolomics with bioactivity data. Using several two dimensional-NMR techniques, the resonances responsible for the separation of high activity samples from the medium and low activity samples were identified as associated to metabolites like halisulfate 1, halisulfate 3–5, and suvanine (1–5), all belongs to sesterterpenes class. The reference compounds for these metabolites are also tested for the activity, which endorse the findings of the applied methodology.

Keywords Marine sponges · Halisulfates · Suvanine · Adenosine A1 receptor binding · NMR spectroscopy · Chemometrics

1 Introduction

Natural products, chemical compound or substance produced by a living organism, are known to have a key role as resource to supply the candidates of drugs. Terrestrial organisms are considered as the primary source of such products as a number of useful natural products are originated from plants (Harborne 2001). However, considering the fact that almost 75 % of the Earth's surface is covered with water, less significant attention has been paid towards marine natural products research. Natural products from marine sources are gaining much attention now and among them sponge metabolites acquired a unique place due to the great diversity in structures, biosynthetic pathways, and bioactivities. In the area of drug research, numerous new biologically active compounds have been identified in sponge; possess anti-inflammatory, anti-tumor, immunosuppressive, anti-viral, anti-malarial, and/or antibiotic activities (Sipkema et al. 2005).

High-throughput screening of compounds, in combination with high-throughput synthesis and combinatorial chemistry, for the development of a drug fails to meet the expectations as no dramatic increase in the number of lead compounds is observed in the recent past (Yuliana et al.

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K. Ali (✉) · M. Iqbal · N. D. Yuliana · R. Verpoorte · Y. H. Choi
Natural Products Laboratory, Institute of Biology, Leiden University, 2300 RA Leiden, The Netherlands
e-mail: kashifali.81@gmail.com

K. Ali
Department of Biosciences, Shaheed Zulfikar Ali Bhutto Institute of Science & Technology (SZABIST), Karachi 75600, Pakistan

N. D. Yuliana
Division of Food Chemistry, Department of Food Science and Technology, Bogor Agricultural University, IPB Dramaga Campus, Bogor 16002, Indonesia

Y.-J. Lee · S. Park · S. Han · J.-W. Lee · H.-S. Lee
Marine Natural Product Chemistry Laboratory, Korea Institute of Ocean Science and Technology, Ansan 426-744, Republic of Korea

2011). The unparallel structural and functional diversity of natural products (mainly secondary metabolites) provides a suitable alternative thus making their comprehensive, qualitative, and quantitative analysis, referred as 'Metabolomics', a hot topic for the current researchers.

Obesity is the condition develops due to the imbalance between energy intake and energy expenditure. For the development of anti-obesity drug, several strategies like appetite suppression, reduced or no nutrient absorption, and higher energy expenditure, can be applied (Chiesi et al. 2001). The adenosine A1 receptor, a G-protein coupled receptor, can be and have been used as a target in anti-obesity drug development by blocking the receptor by an antagonist or inverse agonist (Barakat et al. 2006; Johansson et al. 2008). This adenosine A1 receptor assay was used to identify the potential inhibitors among the metabolites isolated from marine sponges.

Many platforms available for the metabolome analysis (Verpoorte et al. 2008), but nuclear magnetic resonance (NMR) spectroscopy is the method of choice. The key features of NMR come from its reproducibility, non-selectivity, simple sample preparations, and less measuring time as compared to other methods, although criticized for its low sensitivity. The identification of known and specially unknown metabolites in a complex mixture is another great advantage of using NMR as it is primarily known well for the structural classification of the compounds. Many articles have been published using NMR spectroscopy as a tool to study complex mixtures of metabolites derived from the natural sources (Ali et al. 2011; Brescia et al. 2002; Charlton et al. 2002).

Multivariate data analyses methods are the integral part of any metabolomics study. Principal component analysis (PCA) is routinely used for the unbiased reduction of the multidimensional metabolomics data (Ali et al. 2009). The use of projections to latent structures (PLS)-based methods is successfully applied to correlate the metabolomics data with transcriptomics data (Tohge et al. 2005; Hirai et al. 2004) and also recently with the bioactivity data (Yuliana et al. 2011; Ali et al. 2012). In the later case, using PLS-based correlation methods is proved very effective as it provides insight about the active ingredients of the crude extract or identifies active fractions of the crude extract without any further chromatographic separation.

The present study is an attempt to correlate the metabolic profiling data and Adenosine A1 receptor binding activity data of the marine sponges using NMR spectroscopy and PLS-based chemometric methods. The active metabolites in the crude extract of sponge were identified and their activities were confirmed by using the reference compounds.

2 Materials and method

2.1 Sponge sampling and taxonomic identification

The sponges were collected by hand using SCUBA at a 15 m depth offshore of Weno Island, Chuuk state, Federated States of Micronesia, and identified by Dr. Chung J. Sim, Department of Biology, Han Nam University, Korea. Voucher specimens are currently deposited at the Sponge Collection of Korea Institute of Ocean Science and Technology. The details of the samples with their taxonomic classification are available as a supplementary material (SF1).

2.2 Extraction and isolation of reference compounds

Collected specimens (*Psammocinia* sp.) were immediately frozen and stored at −25 °C until investigated. Lyophylized specimens were macerated and extracted with MeOH (2L × 3) and CH_2Cl_2 (2vL × 3). The combined extracts were successively partitioned between H_2O and *n*-BuOH; the *n*-BuOH solution was concentrated and partitioned between 15 % aqueous MeOH and *n*-hexane. An aqueous MeOH layer was fractionated by C18 reversed-phase column chromatography using a sequential mixture of MeOH and H_2O as eluents resulting six fractions (50, 40, 30, 20, 10, 0 % aqueous MeOH). On the basis of the results of ^{1}H NMR analyses of obtained fractions, the fraction eluted with 20 % aqueous MeOH was found to contain suvanine (**5**), of which the minor impurities were removed by MPLC using C18 column chromatography. For halisulfates, extraction, partition and fractionation were carried out following the same protocol described above. Among obtained fractions, 20 % aqueous MeOH and 10 % aqueous MeOH fractions contain halisulfate derivatives judged by ^{1}H NMR data. Further purification of each fraction by C18 HPLC column chromatography resulted in the isolation of halisulfates 1 and 3–5 (**1**–**4**), respectively.

2.3 NMR sample preparation

n-BuOH extracts of sponge (10–30 mg) samples were dissolved in 1.0–3.0 mL of methanol-d_4 containing 0.03 % TMS (tetramethylsilane, w/v, Sigma–Aldrich, St. Louis, MI, USA). The final concentration was kept 10 mg/mL by addition of equivalent amount of methanol-d_4 containing 0.03 % TMS. The mixture was mixed at room temperature for 1 min, with ultrasonication for 20 min (Branson 5510E-MT, Branson Ultrasonics, Danbury, CT, USA), and followed by centrifugation at 17,000 *g* at room temperature for 5 min. The supernatant (800 μL) was transferred to a 5 mm NMR glass tube and analyzed.

2.4 NMR measurements

1D-^{1}H NMR spectra, 2D *J*-resolved spectra as well as ^{1}H–^{1}H homonuclear and inverse detected ^{1}H–^{13}C correlation experiments were recorded at 25 °C on a Bruker 600 MHz AVANCE II NMR spectrometer (600.13 MHz proton frequency) equipped with TCI cryoprobe and Z-gradient system. CD_3OD was used for internal lock purposes. The detailed parameters followed our previous work (Kim et al. 2010a, b).

2.5 Adenosine A1 receptor assay

The assay was performed similarly as explained by Chang et al. (2004) with a modification in the volume of the total mixture in the assay to 200 µL. Chinese hamster ovary (CHO) cells, stably expressing human adenosine receptors, were used to prepare the membranes as described by Dalpiaz et al. (1998). The same method was used to test the reference compounds for the receptor binding activity at the concentration of 25 µg/mL.

2.6 Data analysis and statistics

The ^{1}H NMR spectra were automatically reduced to ASCII files. Spectral intensities were scaled to TMS signal (δ 0.0) and reduced to integrated regions of equal width (δ 0.04) corresponding to the region of δ 0.0–10.0. The regions of δ 4.85–4.95 and δ 3.2–3.4 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Projections to latent structures (PLS) and orthogonal PLS (OPLS) with scaling based on Unit Variance were performed with the SIMCA-P + software (v. 12.0, Umetrics, Umeå, Sweden).

3 Results and discussion

3.1 Adenosine A1 activity profiling

The adenosine A1 receptor binding activity of all the sponge samples was evaluated and shown in Fig. 1. The percentage of activity is calculated considering the positive control as 100 % active, which means higher the percentage shown by the extract, higher will be the binding activity of the extract to the adenosine A1 receptor (Supplementary data SF2). For the convenience, the samples are designated as high, medium, or low activity samples. Samples having activity more than 80 % are designated as high activity samples, while the samples showed activity of less than 40 % are the low activity samples. The remaining samples (from 40 to 79 %) are labeled as medium activity samples. It can be easily observed that many sponge samples from *Callyspongia*, *Psammocinia*, and *Sarcotragus* genus showed some really promising bioactivity results which lead us to further investigate the extracts and to identify the active ingredients in those samples.

3.2 Multivariate data analysis

Principal component analysis (PCA) is considered as fundamental in any metabolomics study to underline the unbiased systemic variation in the data. The projections to latent structures (PLS), an extended form of PCA, normally use to establish the relationship between two data sets i.e. X (predictors) and Y (response). In our case the X-data is the metabolomics data and the Y-data is the adenosine A1 receptor binding activity data. The idea behind using this PLS modeling is to predict the chemical shifts from the metabolomics data responsible for the bioactivity of the sponge extract. The score plot of PLS with two components shows the samples grouped on the basis of activity. Samples with high activity are on the positive side of the component 1, while the low activity samples are having a negative component 1 score (Fig. 2a).

Supervised multivariate analyses methods like PLS needs to be validated and the most common method is the permutation test. The permutation test assesses the goodness of fit and the predictive power of the model, represented by R2 and Q2, respectively. To be a valid model, the R2 and Q2 values should be close to 1 and the R2Y and Q2Y intercepts should be less than 0.3–0.4 and 0.05, respectively (Errikson et al. 2006). The PLS model was validated using the permutation test through 100 permutations and as shown by Fig. 2b, the R2Y and Q2Y intercepts fulfills the criteria by having the values of 0.17 and −0.18, respectively. The lower R2 (0.56) and Q2 (0.43) values explain the highly diverse metabolic profiles the analyzed sponge samples.

Structured noise present in the X-data set can cause systemic variation and to remove this noise, an extension of PLS method known as orthogonal PLS (OPLS) can be used. This OPLS modeling extracts the relationship between the two data blocks and divides the systemic variation of X into two model parts: the predictive or parallel part which correlates the X and Y-data, and the orthogonal part which indicates the variation in X-data unrelated to Y-data set. By applying the OPLS method, the samples with high and low activities are nicely separated by the predictive component having positive and negative scores, respectively (Fig. 2c). The OPLS model was validated by cross validation-analysis of variance (CV-ANOVA) with a p value of 4.98×10^{-28}.

In order to identify the active ingredients in the sponge extracts, we use Y-related coefficient plot. As shown in Fig. 2d, many signals are positively correlated with the

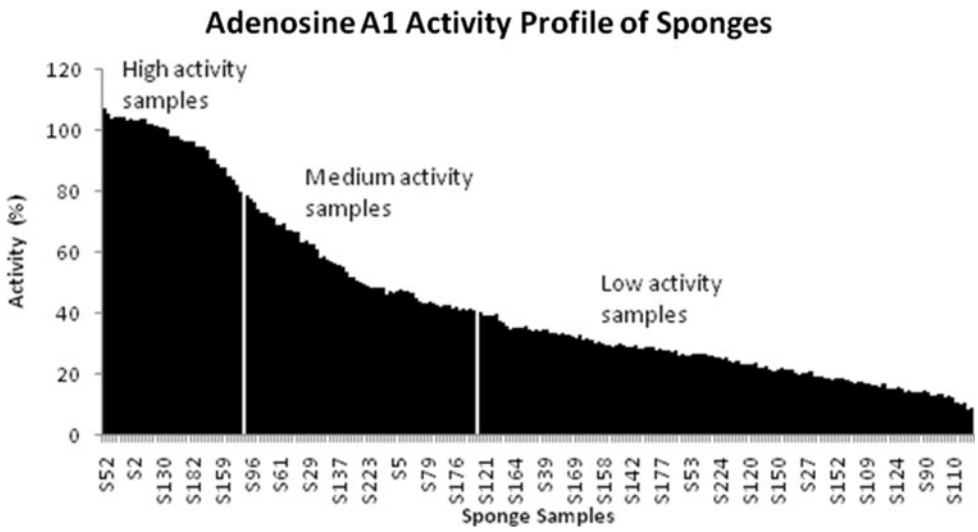

Fig. 1 The adenosine A1 receptor binding activity profiles of the sponge samples. The *bars* showed the % activity of all the samples and can be divided into low (8–39 %), medium (40–79 %), and high (80–107 %) activity classes

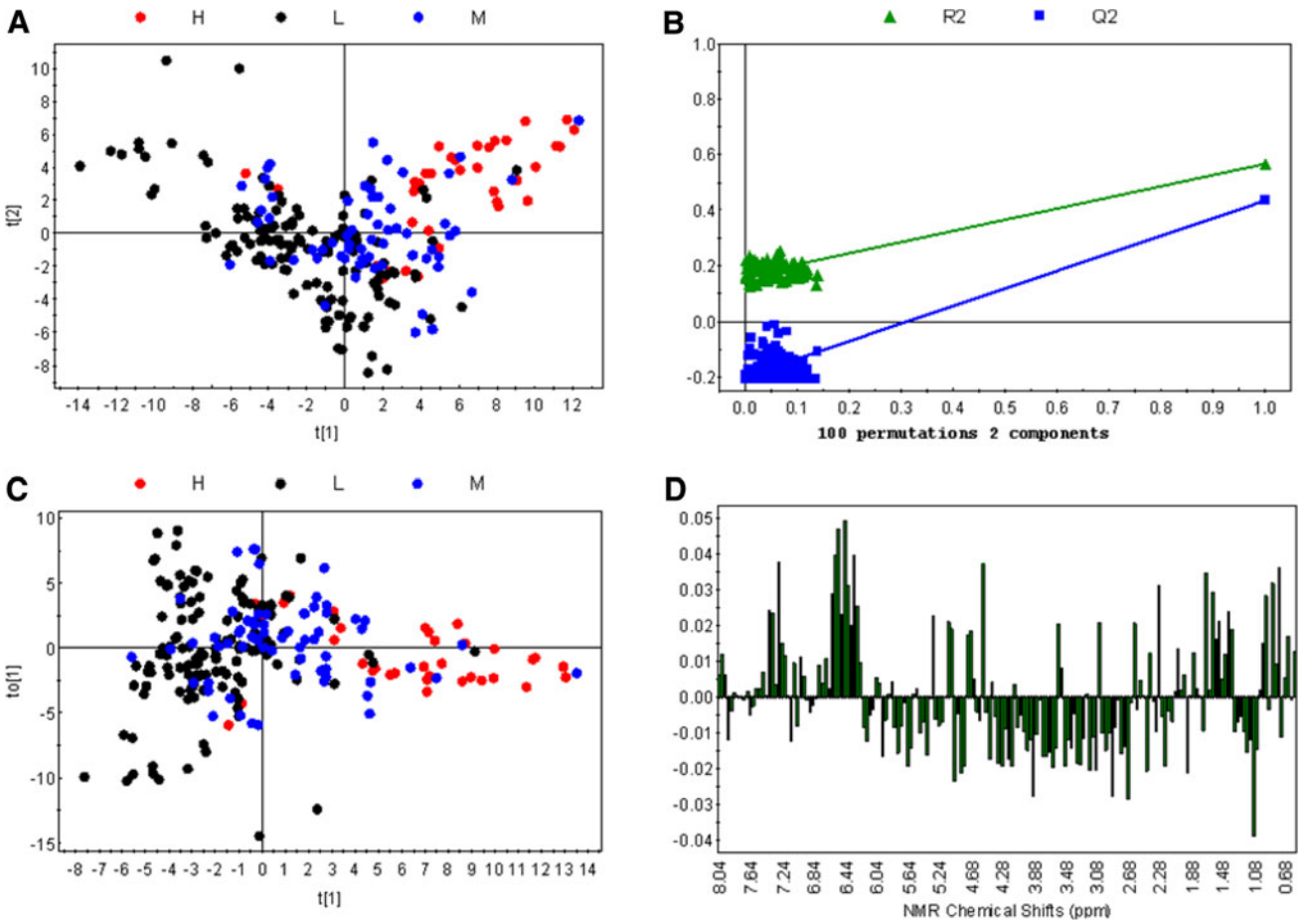

Fig. 2 Multivariate data analyses for sponge metabolic and activity profiling data correlation. The PLS score plot (**a**) shows grouping the samples based on their activity. The permutation test plot (**b**) for the PLS modeling. The OPLS score plot (**c**) shows the distinction among the samples based on the predictive component (x-axis). The loading coefficient plot (**d**) shows the positively (on the positive y-axis) and negatively (negative y-axis) correlated signals to the adenosine receptor binding activity

high activity of the sponge extracts. In order to shortlist the signals, we choose VIP (variable importance in the projections) scores of each positively correlated signal. The VIP is the weighted sum of squares of PLS weights and it is indicated that it is directly proportional with the influence of predictor on the separation on score plot means higher

the VIP value more the predictor is responsible for separation (Errikson et al. 2006). It has been reported that the predictors (values from the X-data set i.e. NMR chemical shifts) with VIP values higher than 0.7 are significant in separation. The predictors having more than 1.5 VIP values were selected and subjected to identification using 1D and 2D-NMR spectra of the reference compounds.

3.3 Identification of metabolites

Sponges are known to synthesize a diversity of terpenoids and other metabolites having polyprenyl groups, among them sesterterpenoids composed a minor group as very few carbon skeletons are reported for them (Faulkner 2002). Among the sulfate containing metabolites from marine sponges, steroidal sulfates are dominating (D'Auria et al. 1993; Fu et al. 1994) with few reports of sesterterpenes sulfates, e.g. halisulfates (Kernan and Faulkner 1988). These halisulfates are isolated from the sponge family Halichondriidae (*Coscinoderma* sp.) and have unique structure by possessing a furan or hydroquinone moiety along with a sulfated side chain (Müller and Faulkner 1997; Fu et al. 1999). Also the salts of sesterterpene sulfates like suvanine and sulfricin are reported from the genus Ircinia (Manes et al. 1985, 1988; Wright et al. 1989).

The NMR spectra of the crude extracts of sponge showed the characteristic signals of hydroquinone moiety at δ 6.48 (dd, $J = 8.0$, 3.0 Hz), δ 6.56 (d, $J = 3.0$ Hz), and δ 6.64 (d, $J = 8.0$ Hz), suggested the presence of halisulfate 1(**1**). The other NMR resonances associated to this compounds were observed at δ 5.37 (brs), δ 5.39 (t, $J = 7.0$ Hz), δ 4.49 (dd, $J = 5.5$, 1.1 Hz), δ 3.23 (d, $J = 7.0$ Hz), and δ 2.02 (t, $J = 8.0$ Hz). The methyl signals at δ 0.71 (s), δ 0.78 (s), δ 0.86 (s), and δ 0.90 (d, $J = 7.0$ Hz) were also observed in the spectra. The ^{1}H-^{1}H COSY spectrum showed the correlations between the protons at δ 6.53 and δ 6.69 of the hydroquinone group. Similar observation was made for the signals at δ 5.39 and δ 3.23 using the COSY spectrum.

While searching for the other metabolites, halisulfates with the furan moiety were also identified. The NMR spectra also showed the presence of three singlets around δ 7.42, δ 7.28, δ 6.32, and triplets around 2.39 ($J = 6.0$ Hz), which evident the presence of other halisulfates in the sponge extracts. By examining the spectra halisulfate 3 (**2**) and 4 (**3**) were identified with a characteristics signals at δ 0.96 (s), δ 0.94 (s), δ 0.84 (d, $J = 7.0$ Hz), and δ 0.81 (d, $J = 7.0$ Hz). For halisulfate 5 (**4**), resonances like δ 1.12 (s), δ 1.02 (s), δ 0.80 (s), and δ 0.78 (s), along with two doublets at δ 0.74 and δ 0.76 of δ 7.0 Hz were observed. The signals at δ 7.42 and δ 7.28 were also found correlated with the signal at δ 6.32 in the COSY spectrum.

Suvanine (**5**), a tricyclic sesterterpene salt, was identified due to the presence of characteristic signals at δ 6.35 (dd, $J = 1.7$, 0.9 Hz), δ 7.43 (t, $J = 1.8$ Hz), and a broad singlet δ 7.29. The singlets of CH3 groups were also observed at δ 0.75, δ 0.80, δ 0.82, and δ 0.92. The COSY spectrum confirms the identification by showing the correlation between the protons at δ 7.43 and δ 7.29 with the proton at δ 6.35. The assignments were made by using the previously published NMR data of these metabolites (Kernan and Faulkner 1988; Manes et al. 1988; Müller and Faulkner 1997), and also by comparing the NMR data of the reference compounds analyzed under same parameters. The structures of these metabolites are shown in Fig. 3, while the *J*-resolve and COSY spectra, having some of the above-mentioned resonances, are shown in Fig. 4.

The chemometrics methods explained above showed many of the above metabolites' resonances positively correlated to the adenosine receptor binding activity. For halisulfate 1, signals at δ 6.48, δ 6.56, and δ 1.69 showed high VIP values i.e. 2.71, 2.11, and 1.69, respectively. For others, i.e. halisulfate 3, 4, and 5, the corresponding VIP values for the resonances at δ 7.28, δ 6.32, δ 0.94, and δ 0.84, are 1.79, 1.93, 2.30, and 2.18. High VIP values like 2.03, 2.15, and 2.30, were observed for Suvanine associated signals at δ 6.35, δ 0.75, and δ 0.92, respectively. Based on above observations, it can be postulated that the halisulfates and Suvanine are the mainly responsible for the high adenosine receptor binding activity in sponge extracts.

3.4 Adenosine receptor binding activity of reference compounds

To test the hypothesis that halisulfates and suvanine are the mainly responsible for the high adenosine A1 receptor binding activity of sponge extract, the reference compounds of these metabolites were tested for the same activity. As shown in Fig. 5, all the compounds were found highly active (more than 86 %) in binding the receptor. Among the halisulfates, halisulfate 5 is the most active followed by halisulfate 3 and halisulfate 1, while halisulfate 4 is the least active among them. Suvanine was also found with less activity in comparison with other compounds showing 88.03 % binding to the receptor. This suggests that minor differences in the structures of the metabolites belong to the same class can lead to the differences in the activity. As Fig. 5 suggest, the halisulfate 3, 4, and 5, even having the same furan moiety, show different activities. The activity profiling of the reference compounds confirms the findings of the metabolomics and activity profiling correlation using chemometrics method and advocate the importance of the applied methodology in the natural product research.

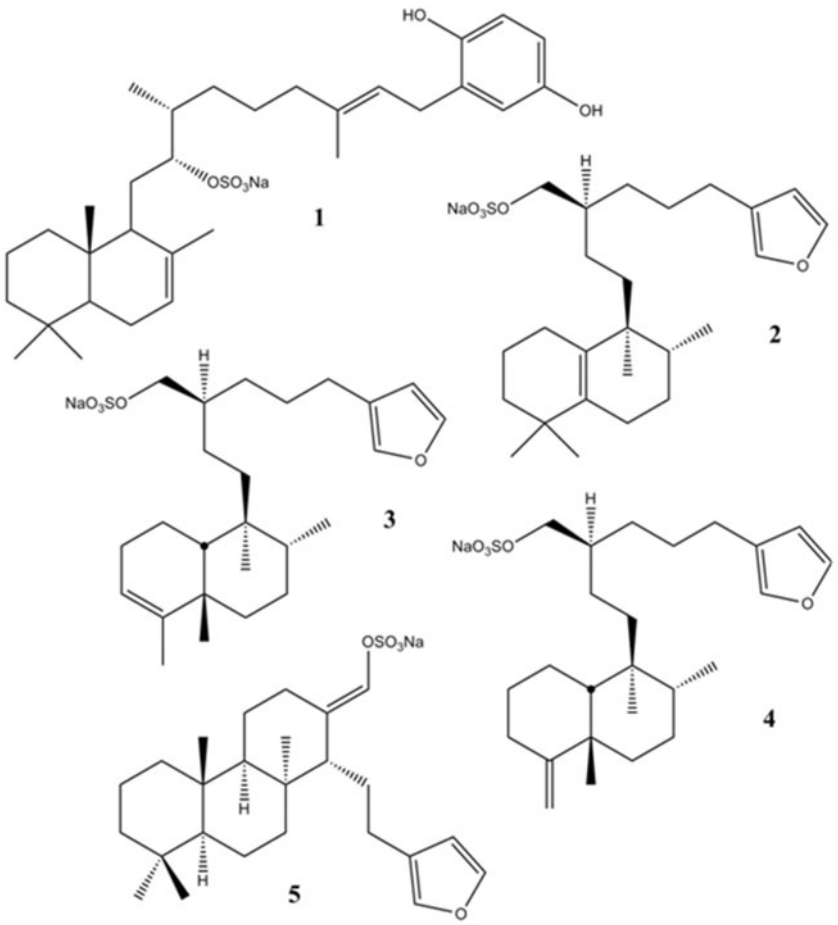

Fig. 3 Chemical structures of the identified halisulfates (**1**–**4**) and suvanine (**5**)

Although there is a lot the explore related to natural products from terrestrial plants, marine sponges have attracted many researchers providing the huge pool of chemically diverse and medicinally important natural products. Natural products from marine sponges have shown to be bioactive having anti-inflammatory, antitumor, immunosuppressive, antiviral, antimalarial, or antibiotic activities (Sipkema et al. 2005; Keyzers and Davies-Coleman 2005). One of the major obstacles in marine product research is the sample collection and correct taxonomic identification of the samples. Also researchers have to be sure that the product is from the studied sample or from any associated microorganism living symbiotically with the test sample. Very careful sample collection and proper pre-treatment of the samples is the pre-requisite for marine natural product research.

Being a relatively new area of natural product research, not so much has been published regarding the metabolic profiling or fingerprinting of marine sponge until recently Ivanišević et al. (2011) use this approach to identify the taxonomic markers in sponge. The use of chemometrics methods to identify the active ingredients in the crude extracts is also not an old concept and showed some successful attempts (Ali et al. 2012; Yuliana et al. 2011; Cardoso-Taketa et al. 2008).

This chemometrics-based active metabolite identification approach is successfully applied to marine sponge extract and metabolites like halisulfates and suvanine, previously known for their enzyme inhibition and antimicrobial activities (Kernan and Faulkner 1988), have been identified to be active in binding to the adenosine A1 receptor. These findings were endorsed by the activity evaluation of these pure compounds also showing the effectiveness of this methodology in underlining the active constituents of the crude extract of the unknown composition.

4 Conclusion

The crude extracts of the marine sponges resulted from methanol:water (1:1) extraction showed promising adenosine A1 receptor binding activity. The NMR spectroscopy leads to the identification of many characteristic metabolites and in combination with chemometrics methods like PLS and OPLS, the metabolites responsible for the high activity are underlined. Sesterterpenes like halisulfate 1,

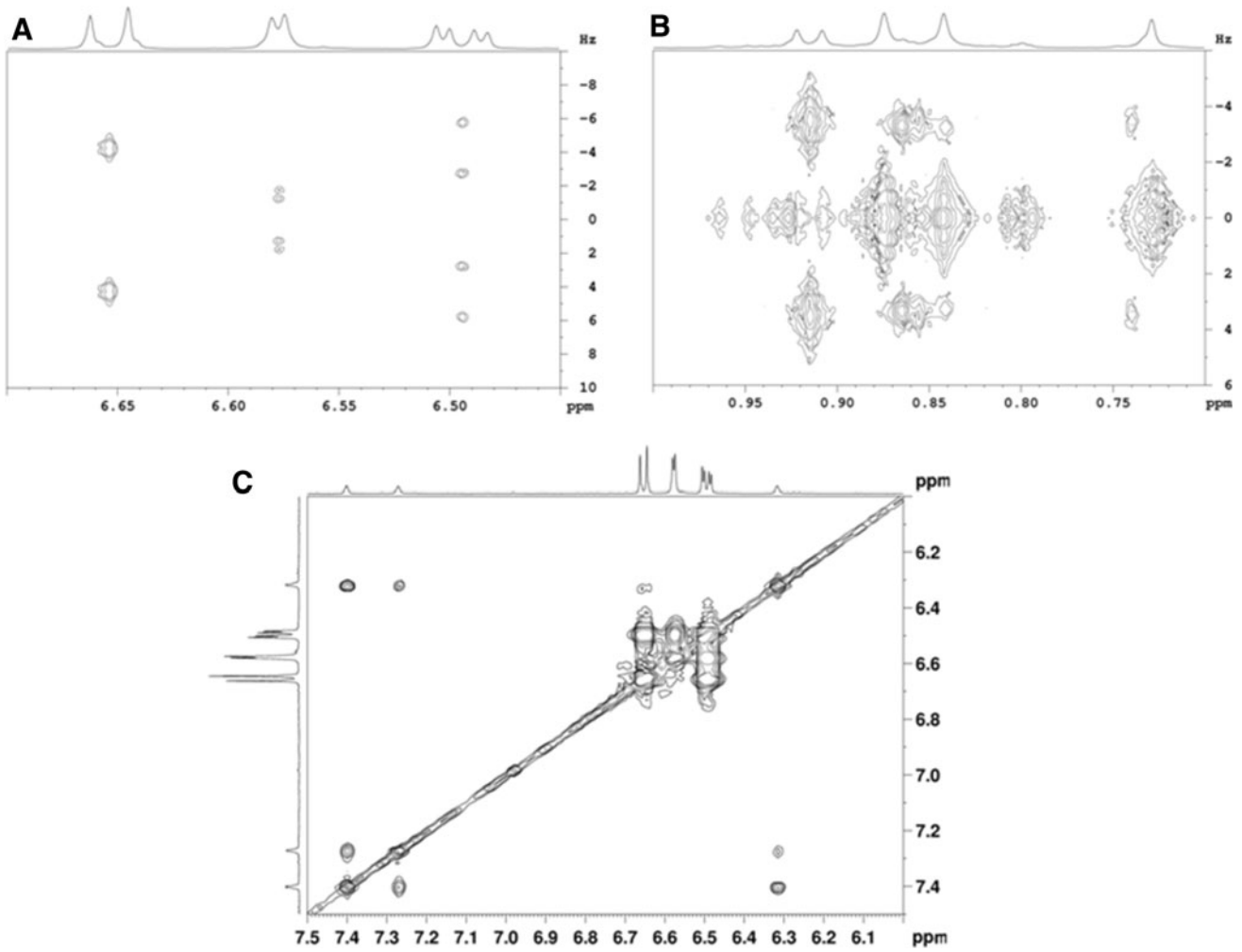

Fig. 4 The *J*-resolve (**a**, **b**) and COSY (**c**) spectra of crude sponge extract showing some characteristic proton resonances and their correlations, respectively

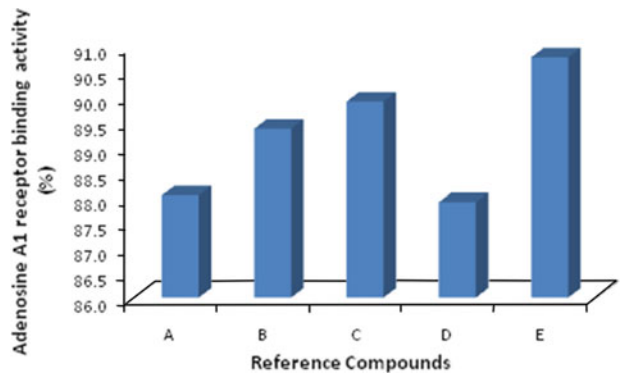

Fig. 5 The adenosine A1 receptor binding activity profiles of the sponge samples. The *bars* represent the % activity of all the reference compounds tested. *A* Suvanine (**5**), *B* halisulfate 1 (**1**), *C* halisulfate 3 (**2**), *D* halisulfate 4 (**3**), *E* halisulfate 5 (**4**)

3–5 (**1**–**4**) and suvanine (**5**) are identified as active components in the sponge extracts. By examining the reference compounds for these metabolites for the adenosine receptor binding activity, these findings were authenticated as they found highly active. Natural product isolation from sponge can provide some interesting leads in drug discovery and the presented metabolomics-chemometrics approach can proved to be very useful to search for the pharmacologically important leads in the large pool of extracts or fractions without any chromatographic separation.

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