

## Full Length Research Paper

# Isolation of a novel piperidide from *Achillea* 'Moonshine' using bioactivity guided fractionation for the treatment of acne

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Received 24 May, 2016; Accepted 19 July, 2016

*Achillea millefolium* is a flowering plant in the family, Asteraceae and is known to be used in traditional medicine as astringent, stimulant, tonic, antiphlogistic and for the treatment of cold and influenza. In an earlier study in the authors' laboratory, four different extracts of a variety of yarrow called *Achillea* 'Moonshine' were tested for anti-acne effect. The petroleum ether extract of *Achillea* 'Moonshine' was demonstrated earlier in the authors' laboratory to have phytochemicals that could scavenge free radicals, inhibit growth of *Propionibacterium acnes* and reduce tyrosinase activity which are needed for acne treatment. Using column chromatography, the petroleum ether extract was fractionated and each fraction was tested against *P. acnes* using TLC bioautography. The active compound from TLC bioautography was isolated by HPLC and identified using Nuclear Magnetic Resonance and Mass Spectrometry studies. This led to characterization of a novel piperidide, N-(21-hydroxy-21-(piperidin-1-yl) heneicosane-17, 19-diyl-1-yl) acetamide, from the petroleum ether extract of *Achillea* 'Moonshine' that contributed to the anti-acne activity. This study led to identification of a novel natural anti-acne compound that is anti-oxidant, anti-inflammatory and anti-microbial against acne causing organism.

**Key words:** *Achillea* 'Moonshine', petroleum ether extract, anti-acne activity, piperidides

## INTRODUCTION

Acne vulgaris is one of the most common skin disorders. It is the disorder of the pilosebaceous unit characterized by increased sebum production, colonization of *Propionibacterium acnes*, inflammation, redness and development of papules and pustules (Alexopoulos et al.,

2016). Acne affects about 85% of the teenage population, irrespective of their sex, and may continue to adulthood. It occurs mainly due to increased androgen production. Other factors like diet, medication, genetics, stress, etc. also play an important role in the development of acne

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(Nasri et al., 2015).

One of the major causes of the pathogenesis of acne vulgaris is the colonization of *P. acnes* near the sebaceous follicles. *P. acnes* is a Gram positive, anaerobic bacterium that is commensal to the human skin. When the skin follicles get blocked, the sebaceous glands become clogged with sebum and under these conditions, *P. acnes* start to flourish and lead to inflammation, causing inflammatory lesions in the dermis around the comedones and ultimately leading to acne (Knor, 2005; Tanghetti, 2013). This bacterium also influences the colonization of another bacterium, *Staphylococcus epidermidis*, which promotes pustules and nodule formation (Knor, 2005; Tanghetti, 2013).

However, there has been no significant change in the treatment and management of acne vulgaris since the last twenty five years. Topical retinoids have been the drug of choice for acne treatment, but there have been problems of skin irritation leading to decreased patient compliance. They are keratolytic, anti-comedogenic and mildly anti-inflammatory (Farrah and Tan 2016). Oral contraceptives reduce androgen production and have been accepted by women as an alternative medication for acne treatment. However, these contraceptives also have their own set of side effects (Lortscher et al., 2016) Antibiotics have long since played an integral role in acne treatment and management. Once widely used, tetracycline has fallen due to increasing cases of antibiotic resistance by *P. acnes*. Salicylic acid can also treat acne by preventing the abnormal cell shedding (Strauss et al., 2007). However, it does not kill the bacteria but just help to unclog the pores. Also, prolong use of benzoyl peroxides have some serious side effects including dryness of skin, flaking, painful itchiness, making the skin more sensitive to sunlight and many others (Harper, 2004).

Plant derived remedies are gaining increasing popularity for acne treatment. Both topical and oral formulations consisting of plants and herbs are investigated for acne treatment. Many patients turn to these alternative therapies for acne management in times when the conventional treatments fail to cure acne. These are also sometimes used as adjuvant therapies together with the traditional treatments (Fisk et al., 2014). Hence, there is a constant search for new and novel compounds that can treat acne. In this research, the authors have isolated and identified a novel compound from *Achillea* 'Moonshine' that can treat acne.

In North America, both native and introduced genotypes, and both diploid and polyploid plants of genus *Achillea* are found (Weakley, 2008). The species used in the traditional gardens has generally been superseded by cultivars with specific 'improved' qualities (Kindersley, 2008). One of such hybrid plant from the *Achillea* group, widely grown in the American gardens, is *Achillea* 'Moonshine'. It is a hybrid plant between *Achillea clypeolata* and *Achillea* 'Taygetea'. This is also the main

reason for using this variety in the study as it is readily available.

In the authors' previous study, the anti-acne activity of petroleum ether extract of *A. 'Moonshine'* was determined (Shah et al., 2015). *Achillea* has been traditionally used for healing wound, treat inflammation, as an anti-microbial and treat gastric and respiratory infections (Li et al., 2011; Moradi et al., 2013; Benedek and Kopp, 2007). This report was novel as this was the first time yarrow was determined to have acne treating abilities such as reducing inflammation, treating infections caused by *P. acnes* and *S. epidermidis* and removing dark pigmentation (Shah et al., 2015).

The next steps in this study were to use bioactivity guided fractionation, high performance liquid chromatography (HPLC), mass spectrometry and nuclear magnetic resonance (NMR) to identify the compounds contributing to the anti-acne effects in the petroleum ether extract. A novel compound from *Achillea* 'Moonshine' petroleum ether extract responsible for the anti-acne activity was isolated and characterized.

## MATERIALS AND METHODS

### Collection and extraction

Full bloomed *Achillea* 'Moonshine' were collected from Holly Days Nursery Inc. near Philadelphia (United States) in June 2013. The leaves and stems were washed three times with distilled water and grounded into fine powder using a simple grinder. 500 g of plant material (leaves and stem) was extracted with petroleum ether (3 × 1 L). The solvent was evaporated and the extracts were stored at 4°C till further use.

### Bacterial strains and media

The test organism, *P. acnes*, was obtained from American Type Cell Collection (ATCC). *P. acnes* was incubated in brain-heart infusion media for 48 h at 37°C under anaerobic conditions in an anaerobic jar, and the density was adjusted to approximately 0.5 McFarland Standard.

### Fractionation of the petroleum ether extract

#### Column chromatography

The petroleum ether extract was subjected to column chromatography to separate the extract into its component fractions. Silica (#200) was used as the stationary phase while petroleum ether: diethyl ether (1:1) was used as the mobile phase. In the setting up of the column (length 45c m, diameter 3 cm), the lower part of the glass column was stocked with glass wool with the aid of a glass rod. The slurry was prepared by mixing 45 g of activated silica mixed with 10% AgNO<sub>3</sub> and 200 ml of hexane. This slurry was poured down carefully into the column. The tap of the glass column was left open to allow free flow of solvent into a conical flask below. The column was allowed to stabilize, after which, the clear solvent on top of the silica gel was allowed to drain down until the level of the silica gel. The sample was prepared in a glass mortar by adsorbing 300 mg of the petroleum ether extract with 500 mg of

**Table 1.** TLC bioautography results of the fractions against *P. acnes*.

Fraction no.	R <sub>f</sub> values
5	0.19, 0.30
6	0.20, 0.29
7	0.19

silica gel. The dry powder was gently layered on top of the column. The mobile phase was added and the flow rate was adjusted to about 40 drops/min. The eluted fractions were collected in test-tubes. 40 fractions were collected at the end of the run. Fractions 1-12 were clear in appearance. Fractions 13 to 35 were yellowish green in color while the later fractions were blue to brown in color.

#### Thin layer chromatography and pooling of the fractions

The content of each test tube was spotted on a TLC plate to separate the different fractions based on their relative mobility in solvent systems and the TLC profile was observed under the UV light. These fractions were eluted with toluene: methanol (9.6:0.4) as the mobile phase. The developed plates were dried under stream of fast moving air. The plates were then observed under long and short UV and were sprayed with 1% sulphuric acid in methanol. The fractions which showed similar patterns were pooled together. These fractions were then stored in 4°C till further use.

#### TLC bio-autography of the fractions for antimicrobial activity

From each of the pooled fraction as described in the above procedure, 10 µl of sample was loaded onto TLC plates in a narrow band and eluted with toluene : methanol (9.6:0.4). The developed plates were dried under stream of fast moving air to remove the traces of solvent. The prepared chromatograms were dipped in the bacterial suspension of *P. acnes* (ATCC, approximately 10<sup>6</sup> CFU/ml) for 5-10 s. The plates were then incubated for 48 h at 37°C anaerobically in an anaerobic jar. Following incubation, the plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT). These plates were further incubated anaerobically for 8 h. White bands on the developed plates indicate that the reduction of INT to the colored formazan did not take place due to the presence of compounds that inhibited the growth of tested organisms (Raman et al., 1995).

#### Isolation of compounds from active fractions by preparative HPLC and identification of active peak

The fraction from the column chromatography that inhibited the growth of *P. acnes* was analyzed with HPLC. Preparative HPLC was performed to isolate individual compounds from the active fractions. The HPLC was performed on Agilent 1100 series apparatus with an auto sampler, quaternary gradient pump and a UV/Vis diode array detector. Zorbax 300SB-C18 column (4.6 mm x 25 cm) was used. Mobile phase A consisted of HPLC water with 0.1% formic acid while mobile phase B was acetonitrile with 0.1% formic acid. The gradient eluting phase was A : B (10:100 v/v) for 50 min. The mobile phase was pumped at 0.4 ml/min with the column temperature of 30°C. The injection volume was 75 µl. All the major peaks were isolated and the solvent was evaporated. The residue was re-dissolved in methanol and TLC bio-autography was

performed to identify the active isolated peak.

#### Identification of the active compounds separated by HPLC

The active compound was analyzed by IR, mass spectrometry and NMR.

#### Mass spectrometry

The mass spectrometry data was obtained by liquid chromatography-mass spectrometry (LC-MS) using Thermo Scientific™ Orbitrap™. The mobile phase was A : B (10:100 v/v) for 50 min, where A was water with 0.1% formic acid and B was acetonitrile with 0.1% formic acid.

#### Nuclear magnetic resonance

All NMR experiments were conducted on a Bruker Advance III 400 MHz NMR Spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 400.13 MHz. Spectra were recorded in CDCl<sub>3</sub> at 25°C using standard Bruker 1D and 2D sequences. Experiments for structure elucidation include <sup>1</sup>H, <sup>13</sup>C, 2D-COSY, HMBC and HMQC. The COSY (Correlation Spectroscopy) experiment indicates the hydrogen-hydrogen connectivity. The HMQC (Heteronuclear Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Coherence) experiments indicate the carbon and hydrogen connectivity.

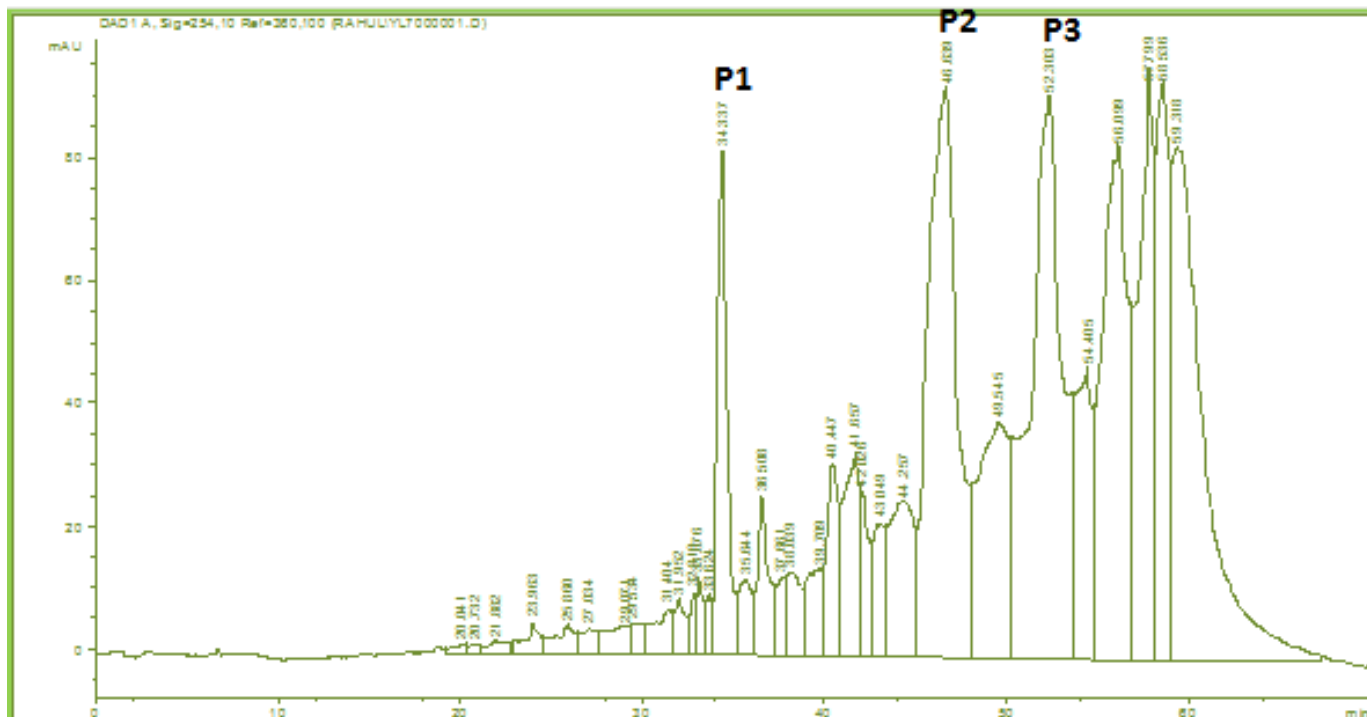
## RESULTS

#### Fractionation of petroleum ether extract of *Achillea* 'Moonshine' by column chromatography and pooling of the fractions

From the column chromatography, separation of the petroleum ether extract yielded 40 fractions which were collected at the end of the run. Initial fractions 1-12 were clear and were discarded because it contained only the mobile phase. Fractions 13 to 35 were yellowish green in color and gave positive tests for polyacetylenes (data not shown) (Wagner et al., 1986). The later fractions were blue to brownish in color and were discarded. These fractions tested positive for the presence of azulenes. However, due to insufficient sample quantity, further analysis was not possible. The fractions containing polyacetylenes (fractions 13-35), with similar profile were pooled together into 10 fractions (YL1- YL10).

#### TLC bioautography of the fractions

All the 10 fractions (YL1-YL10) were subjected to TLC bioautography assay for their antimicrobial activity against *P. acnes* after drying them in Savant™ SpeedVac concentrator and re-dissolving in hexane. Out of the 10 fractions, 3 fractions (YL 5, 6, 7) showed zone of inhibition against *P. acnes* (Table 1). The remaining fractions showed no zone of inhibition for *P. acnes* and



**Figure 1.** HPLC chromatogram of the YL-7 fraction. Peaks 1, 2 and 3 were collected for further analysis by TLC bioautography.

**Table 2.** TLC bioautography results of the collected peaks from the separated YL-7 fraction using HPLC.

Peak no.	R <sub>f</sub>
1	-
2	-
3	0.19

so were not tested further. All the active fractions (YL 5, 6, 7) had one common zone of inhibition at R<sub>f</sub> 0.19. Additional, TLCs were run and the compound at R<sub>f</sub> 0.19 was scrapped and collected. Due to insufficient amounts of fractions 5 and 6, only YL 7 was selected for further analysis.

#### Isolation of compounds from active fractions by preparative HPLC and identification of active peak

Fraction YL-7 was further fractionated by normal phase HPLC to isolate individual peaks and identify the active peaks. Figure 1 shows the HPLC chromatogram of YL-7. Three peaks for YL-7 (P1, P2 and P3) were isolated and each peak was tested again for its antimicrobial activity against *P. acnes* by TLC bioautography (Figure 2). Out of the three peaks, peak 3 was found to have anti-microbial

assay (Table 2).

#### Compound identification

##### YL-7 peak-3

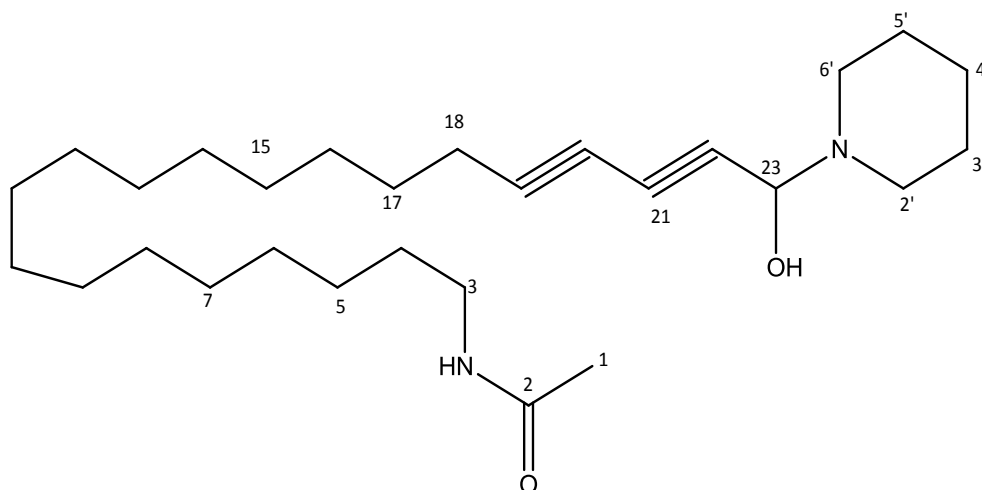
YL-7 peak-3 was identified as [N-(21-hydroxy-21-(piperidin-1-yl) henicososa-17, 19-diyl-1-yl) acetamide]. White Powder: UV λ<sub>max</sub> 253 nm, FT-IR (hexane) V<sub>max</sub> 3428.53cm<sup>-1</sup> (-OH, -NH), 2088.85 cm<sup>-1</sup> (-C≡C), 1643.92 cm<sup>-1</sup> (-C=O), 2922.53 cm<sup>-1</sup> (-CH<sub>3</sub> stretch), 2852.49 cm<sup>-1</sup> (-CH<sub>2</sub> stretch), 1461.87 cm<sup>-1</sup> (-CH<sub>2</sub> bend). HRESI m/z 445.12 (calculated for C<sub>28</sub>H<sub>47</sub>NO<sub>3</sub> 445.356).

The presence of 11 CH<sub>2</sub> groups was corroborated by the presence of their high field signals at δ<sub>H</sub> = 1.28 in <sup>1</sup>H-NMR and <sup>1</sup>H-<sup>1</sup>H COSY. This was also confirmed by <sup>13</sup>C-NMR (δ<sub>C</sub> = 29.7) (Table 3). The HMQC helped to determine the chemical shift of the carbons on the molecule through direct correlation with their hydrogens. Diagnostic HMQC correlations and <sup>1</sup>H-<sup>1</sup>H COSY also confirmed the presence of the piperidine ring (Figure 3).

The IR spectra indicated the presence of -OH and -CONH groups. The <sup>13</sup>C-NMR and <sup>1</sup>H-NMR spectral data (Table 3) allowed the assignments of most of the hydrogens and carbons signals of the proposed structure. The presence of 11 CH<sub>2</sub> groups was corroborated by the presence of their high field signals at δ<sub>H</sub> = 1.28 in <sup>1</sup>H-NMR (Figure 4) and <sup>1</sup>H-<sup>1</sup>H COSY (Figure 5). This was also



**Figure 2.** TLC bioautography of the compounds in Peak 3 separated from YL-7 fraction against *P. acnes*.



**Figure 3:** Structure of the isolated and identified compound, N-(21-hydroxy-21-(piperidin-1-yl)henicosa-17, 19-diy-1-yl) acetamide

confirmed by  $^{13}\text{C}$ -NMR ( $\delta_{\text{C}}=29.7$ ). The HMQC helped to determine the chemical shift of the carbons on the molecule through direct correlation with their hydrogens (Figure 6).

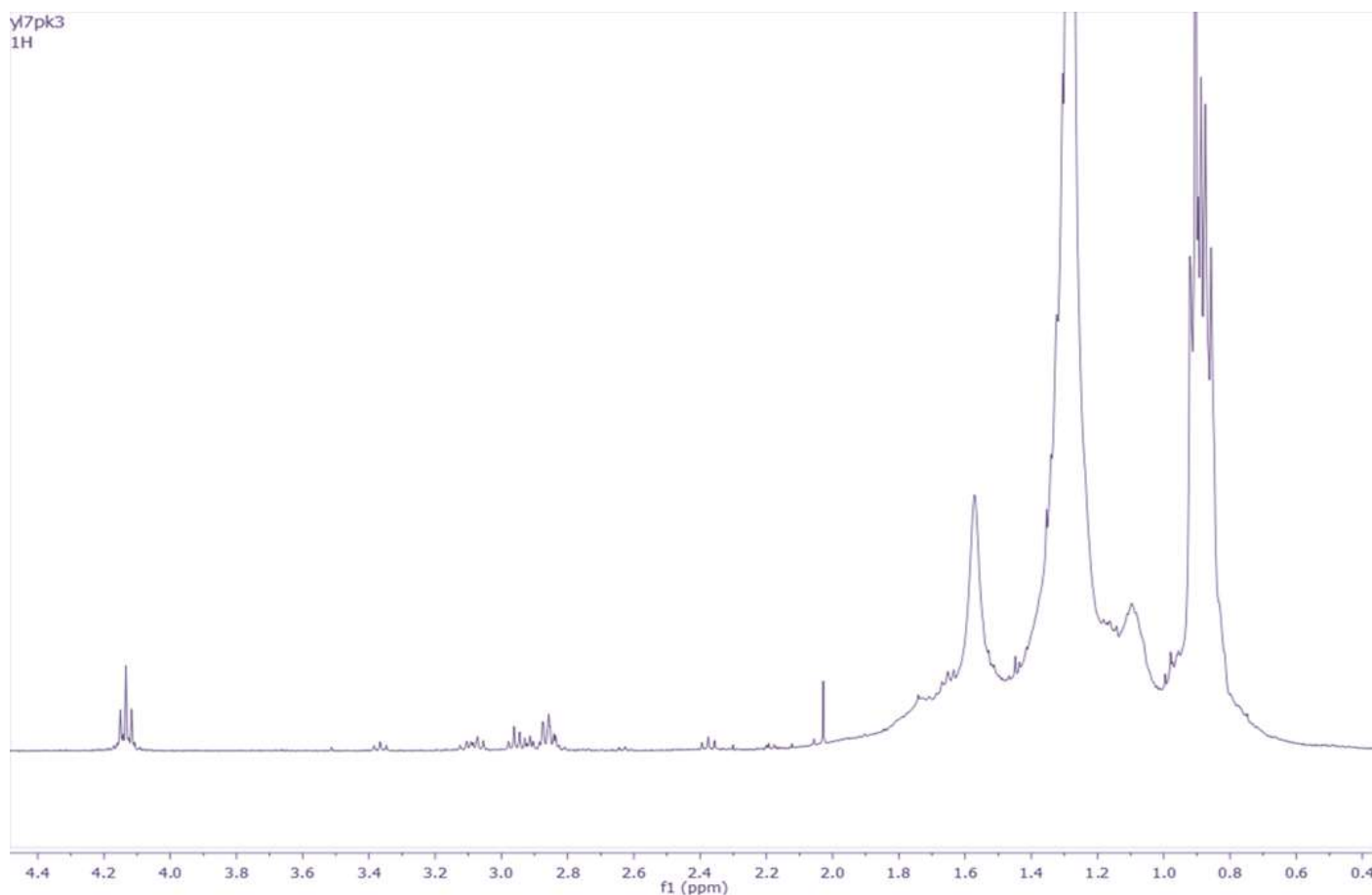
## DISCUSSION

Alkamides have mainly been reported in Piperaceae,

Asteraceae, Rutaceae and Aristolochiaceae families (Rios, 2012). However, polyacetylenic alkamides have only been found in Asteraceae where they frequently occur in Anthemideae and Heliantheae tribes. Within Anthemideae, presence of olefinic and acetylenic alkamides with upto three triple bonds is a typical trend of the *Achillea* genus. However, the biogenetic capacity replaces these polyacetylenes which are otherwise

**Table 3.**  $^{13}\text{C}$ -NMR and  $^1\text{H}$ -NMR values of the isolated compound

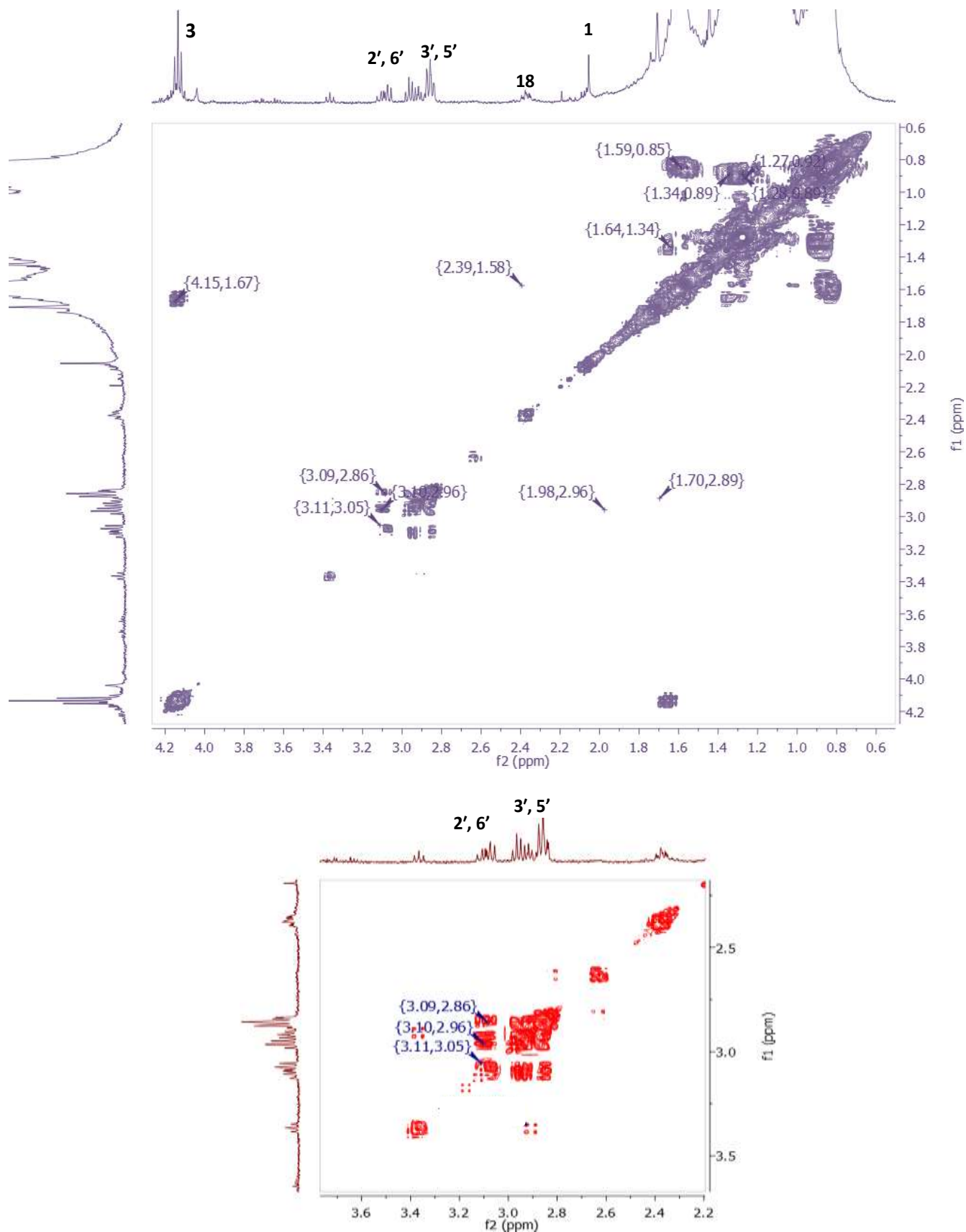
Carbon no.	$^{13}\text{C}$ (ppm)	$^1\text{H}$ (ppm)
23, 3	65.6	4.15
2', 6'	45.0	3.11, 3.05
3', 5'	27.3	2.96, 2.86
18	27.4	2.39
1	21.7	2.07
4'	27.4	1.98, 1.70
4	22.0	1.67
17	22.8	1.58
5	25.1	1.34
6-16	29.7	1.28

**Figure 4.**  $^1\text{H}$ -NMR spectra of the identified compound.

characteristic to Anthemideae. Alkamides are found generally in roots and they act as plant growth regulators either by promoting or inhibiting the growth and formation of roots in a dose-dependent manner (Campos-Cuevas et al., 2008). However, there are alkamides present in

leaves and stems of *Achillea* including roots.

The genus *Achillea* is rich in olefinic and acetylenic pyrrolidides, piperidides, and the corresponding dehydro derivatives (piperidides, pyrrolideides) which are alkamides with five or six membered cyclic or aromatic



**Figure 5.** Top and bottom:  $^1\text{H}$ - $^1\text{H}$  COSY of the identified compound. This analysis identifies the H-H bonds in the structure.

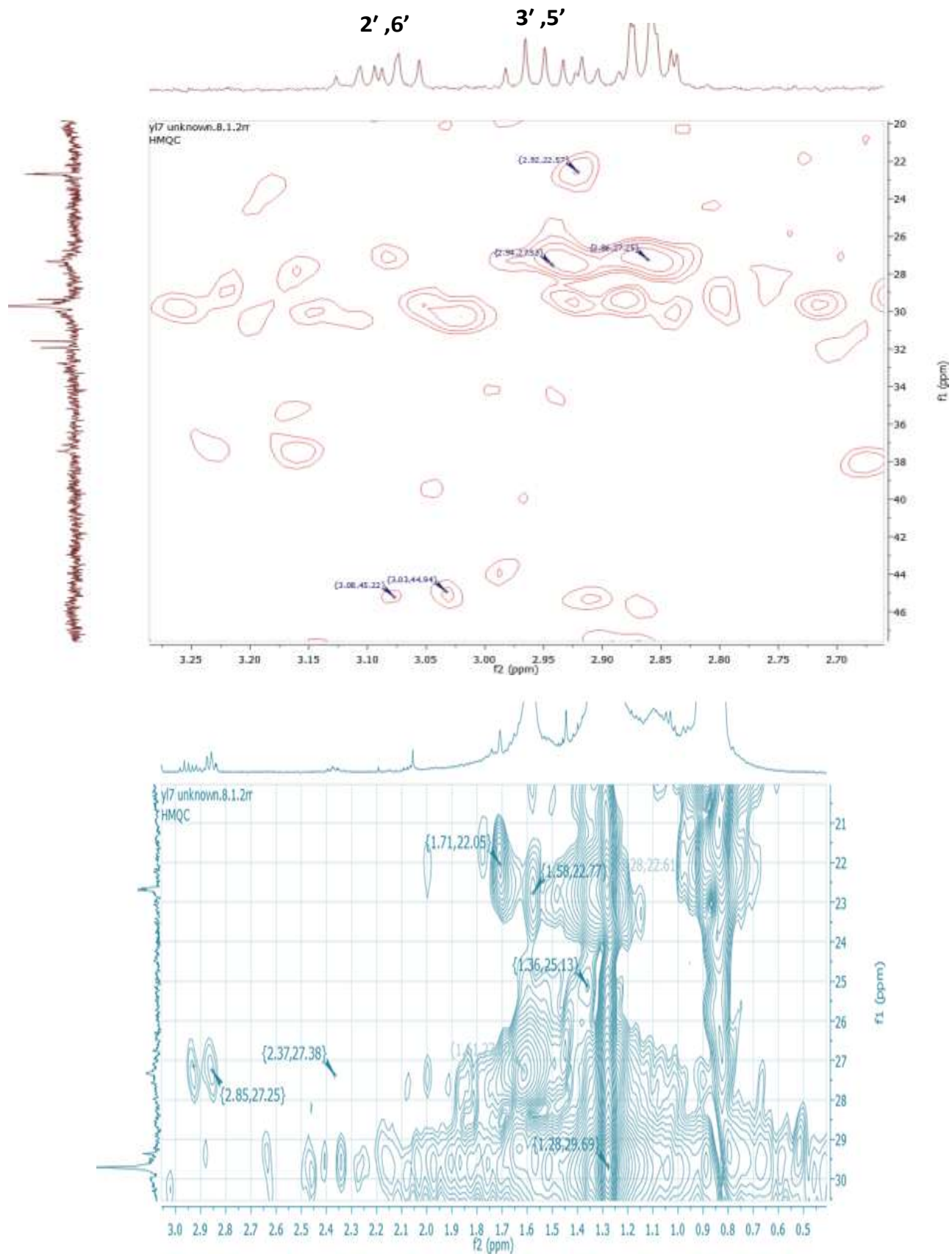


Figure 6. Top and bottom: HMQC spectra of the isolated compound.



rings with nitrogen. The distribution of cyclic amides is restricted to tribe Anthemidae. Most polyacetylenic cyclic amides found in this tribe consist of pyrrolidides and piperidides whereas polyacetylenic piperidides are rare (Bohlman, 1988; Bohlmann et al., 1973; Christensen, 1992; Greger, 1984, 1988). In this study, the novel anti-acne compound identified belongs to the piperidide family, which are known to be antimicrobial, antiviral, larvicidal, insecticidal, diuretic, pungent, analgesic, cannabimimetic and antioxidant (Rios, 2012). Some of the plant sources that are known to have anti-acne effect are *Aloe vera* (Grace et al., 2008), *Azadirachta indica*, *Curcuma longa*, *Hemidesmus indicus*, *Gossypium barbadense* (Kanlayavattanukul and Lourith 2011), essential oils of *Eucalyptus radiata* and *Melaleuca alternifolia* (Stevensen, 1998). But none of these studies have successfully identified a compound that could treat the array of acne causes such as free radicals, reducing inflammation, killing acne causing bacteria and also reduce pigmentation after treatment. This study is important as the novel alkamide is isolated from an extract that has already been demonstrated to have antimicrobial, free radical scavenging, anti-tyrosinase, anti-inflammatory activity and cytotoxicity assays necessary to characterize its anti-acne activity (Shah et al., 2015).

## Conclusion

From interpretation of all the spectral data, a novel piperidide with molecular formula  $C_{28}H_{47}NO_3$  was isolated and identified from *Achillea* 'Moonshine' petroleum ether extract. This piperidide can be used as a novel drug for acne management and treatment. The compound can be further tested on animal models to validate their activity *in vivo*. Efforts can be made to synthesize the compound on a laboratory scale and optimize its yield. Further clinical trials can be conducted to evaluate the compound's safety and efficacy.

## ACKNOWLEDGEMENTS

This research was funded by the Startup grant given to Dr. Bela Peethambaran by University of the Sciences, Philadelphia.

## Conflict of interests

The authors have not declared any conflict of interests.

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