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Isolation and Characterization of Larvicidal N-alkylamides from Acmella ciliata HBK

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Abstract

The transmission of pathogens by mosquito vectors predisposes several diseases leading to severe human mortality worldwide. The phenomenon of acquired resistance to insecticides or larvicides intensifies a thrust for discovery of novel alternatives for control of the diseases spread by these vectors. Present study demonstrates isolation of a couple of major N-alkylamides from *A. ciliata* and reveal larvicidal activity (LA) on larvae of *Aedes aegypti* and *Culex quinquefasciatus* that spread pathogens of dengue and filariasis respectively. The two compounds were identified to be 2*E*,4*E*-N-isobutyl undeca-2,4-diene-8,10-diynamide (NUD) and 2*E*,4*E*,8*Z*,10*E*-N-isobutyl dodeca-2,4,8,10-tetraenamide (NDT). The LC_{50} of NUD for late III – early IV instar larvae of *Ae. aegypti* was 44.19 ppm and 30.89 ppm respectively. The LC_{50} of NDT for the same were 18.28 ppm and 11.75 ppm respectively. NDT was found more toxic than NUD with both having a higher potency than methoprene (MET), the commercial larvicide. Quantitative structure-activity relationship (QSAR) study revealed that the chain length, degree of unsaturation and terminal methylation in the molecules influence the extent of LA. The compounds isolated in this study were further demonstrated for use as standards in analytical biochemistry. NUD and NDT content varied, in different parts of the plant, with both accumulating maximally at 19.01 mg/g and 68.55 mg/g, respectively in flowers. The LOD and LOQ for estimation of NUD by HPLC was 0.80 µg/ml and 2.51 µg/ml respectively, whereas, these were 5.50 µg/ml and 16.70 µg/ml, respectively, for NDT. To the best of our knowledge, this is the first report on the purification of larvicidal N-alkylamides from *A. ciliata* and demonstration of their use as biochemical standard.

1. Introduction

The mosquito vectors, *Aedes aegypti* L. and *Culex quinquefasciatus* Say., transmit pathogenic arboviruses that cause fatal diseases such as dengue, malaria, zika, chikungunya, Japanese encephalitis and lymphatic filariasis (Rozendaal, 1997; Pandey et al. 2011). According to the World Health Organization, nearly 230 million people in the globe are at risk of malaria with over 4,00,000 annual death (WHO, 2019). As on date the mosuitoes are controlled by application of organophosphates (chlorpyrifos and temephos), insect growth regulators (diflubenzuron and methoprene) and bacterial larvicides such as *Bacillus thuringiensis* and *B. sphaericus* (Beckage et al. 2004; Benelli et al. 2013). Despite their effectiveness, these agents are beset with health issues, environmental hazards and development of resistence in mosquitoes to the control agents (Nkya et al. 2013). Elimination of mosquito at larval stage, often at their aquatic breeding sites, is considered the most effective way of controlling incidences of mosquito-borne diseases (Rawani et al. 2017). In this context, plants belonging to angiosperm families of Asteraceae, Cladophoraceae, Labiatae, Meliaceae, Piperaceae and Rutaceae were explored and shown that they produce mosquito controlling agents (Park et al. 2002; Amer and Mehlhorn, 2006). Alternately, the plants were also explored for the production of bioactive drug molecules such as antimalarial artemisinin for the treatment of diseases (Tu, 2016).

One of the much studied natural product has been N-alkylamides obtained from the plants of *Echinacea*, *Heliopsis* and *Spilanthes* (syn: *Acmella*). Species belonging to *Acmella* are commonly called as toothache plants. They form ingredients in several herbal products. *Acmella* species have served as as raw material in the manufacture of the natural herbal product *Malarial-5* (an anti-malarial mixture of extracts from *Cassia occidentalis*, *Lippia chevelieri* and *Spilanthes oleracea*). Apart from this, *Acmella* is also used in the preparation of *Spolera®*, a German tincture used in the treatment of skin abrasions (Gasquet et al. 1993; Kasper et al. 2010; Silveira et al. 2016). The strong taste in *Acmella* preparations has been attributed to the presence of pungent N-alkylamides in the plant (Nakatani and Nagashima, 1992; Uthpala and Navaratne, 2020). N-alkylamides are reported from over 30 plant families and are formed by condensation of a fatty acyl moiety (of varied chain length and degree of unsaturation) with an amine group derived from an amino acid (Cortez-Espinosa et al. 2011; Rizhsky et al. 2016). The patents filed or granted for products containing N-alkylamides, particularly spilanthol, have also been increased since the last two decades (Silveira et al. 2018).

Though the extract from *Acmella* has been shown to exhibit acaricidal, anti-plasmodial, insecticidal, larvicidal, ovicidal, pesticidal and pupicidal properties, the potency of individual molecules present in the extract has not been demonstrated so far (Pandey et al. 2011; Sharma et al. 2012; Simas et al. 2013; De Oliveira et al. 2016; Silveira et al. 2016; Araujo et al., 2018). *Acmella ciliata* HBK Cass. (basionym: *Spilanthes ciliata* HBK., family Asteraceae) is an important medicinal plant distributed in South America, Celebes, Sumatra, India, and Thailand (Jansen, 1985; Kasper et al. 2010). Barring a study on the isolation of a novel anti-plasmodial endoperoxide N-alkylamide, (2*E*,7*Z*)-6,9-endoperoxy-N-isobutyl-2,7-decadienamide, from the aerial parts of *A. ciliata* (Silveira et al. 2016), the plant largely remains unexplored for larvicidal potency. Here we present our study on isolation, purification and characterization of two major N-alkylamides from *A. ciliata* and demonstrate their larvicidal activity (LA) on the larvae of *Ae. aegypti* and *Cx. quinquefasciatus*. Their physicochemical properties, under Lipinski's rule of five, were predicted using in silico tools, Swiss-ADME and

Molinspiration. QSAR study was carried out to understand the relationship between the physicochemical properties and the LA of test compounds to ascertain the key structural components that influence the potency of larvicidal N-alkylamides.The isolated N-alkylamides were further demonstrated for use as analytical standards. To the best of our knowledge, this is the first report on purification of NUD and NDT, the assessment of their LA against mosquito larvae and their utility as a standard for quantification of N-alkylamides in different parts of the plant.

2. Experimental

2.1 Plant Material

The flower, leaf, root, and stem of the plant were collected from the crop of *A. ciliata* maintained in the experimental garden of our department. The identity of the material was confirmed by Dr. S.N. Sharma, Taxonomist, Indian Institute of Integrative Medicine (IIIM), Jammu (India). A voucher specimen of the same was deposited (with accession number 22164) in the Institutional herbarium. The harvested plant parts were dried at 40 ± 2 ^{o}C in a hot air oven (Matri, India), pulverized in a mixer grinder (CHEFPRO^{+}, India) and stored in pet jars at room temperature until use.

2.2 Chemicals and Glasswares

The glassware such as boiling tubes, glass columns, TLC chamber, conical flasks, and beakers used in the study was procured from Borosil, India. TLC plates pre-coated with silica gel 60 F_{254} and the solvents (AR and HPLC grade) hexane, ethyl acetate, methanol, acetonitrile, deuterated chloroform ($CDCl_3$), and water were purchased from Merck, India. Silica gel (200–400 mesh size) for column chromatography was purchased from Avra Scientific, India. The standard larvicide methoprene (MET), Whatman No. 1 filters and potassium bromide (KBr) were procured from Sigma-Aldrich, USA. The autoclavable syringe filter and 0.45 μm nylon filter membranes were purchased from HiMedia, India.

2.3 Isolation, purification, and structural characterization of N-alkylamides from A. ciliata

2.3.1 Preparation of crude methanol extract and fractionation of N-alkylamides

About 200 g of the dry flower powder was treated with 2L of methanol in a conical flask and left for incubation on orbital shaker set at 100 rpm and 25 ^{o}C for 72 h. The content was then filtered through Whatman No. 1 filter paper and the filtrate was collected. The solvent from the filtrate was rotary evaporated (Buchi, India) to obtain the crude methanol extract. Normal phase column chromatography (Phrutivorapongkul et al. 2008) was followed for the fractionation of compounds from the extract. 200–400 mesh size silica gel was packed in a glass column served as the stationary phase and a series of a mixture of varying proportions of hexane and ethyl acetate (in the range of 10:0 to 1:1) was used as mobile phase. A total of 30 fractions of 50 ml each were collected and monitored by TLC. Fractions of a similar TLC profile were pooled together. This setup yielded two major pools, P1 (fractions D_5-E_5) and P2 (fractions C_5-D_4). Upon re-chromatography using hexane and ethyl acetate in 7:3 ratio as isocratic mobile phase, each pool yielded 30 sub-fractions of 15 ml each. The fractions containing solitary spots on TLC were pooled, rotary evaporated, weighed and stored at -20ºC until further use. The fraction obtained from P1 and P2 was labelled as compound 1 (C1) and compound 2 (C2) respectively.

2.3.2 Qualitative analysis of the putative N-alkylamides

2.3.2.1 High Performance Liquid Chromatography (HPLC)

A 5 mg/ml stock solution of the two compounds, C1 and C2, obtained as above was prepared in pure methanol. The stock solution was filtered through a nylon membrane (0.45 μm) and 10 μl of the stock was loaded on to the Shimadzu-HPLC system (LC: ATVP) fitted with a C_{18} reverse-phase column (250 × 4.6 mm), a guard column and a UV detector set at 254 nm (SPD-10AVP). The elution was done isocratically using a mobile phase comprising of 7:3 of acetonitrile (A) and milli-Q water (B) set at a flow rate of 1ml/min and a run time of 20 minutes.

2.3.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

The pure compounds C1 and C2 were subjected to FTIR spectroscopy to determine the functional groups present in them. 1 mg of the sample was mixed with KBr (in 1:100 proportion) and pulverized to make a fine powder. Pellet die of the FTIR was filled with the powdered material and scanned in the mid-IR range (400–4000 cm^{-1}) with a resolution of 4 waves cm^{-1} using a Shimadzu-FTIR (Model: Nicolet 6700) spectrophotometer. Plain KBr was used as blank.

2.3.2.3 High Resolution Mass Spectrometry (HRMS)

5 µl of 0.1 mg/ml solution of C1 and C2 prepared in methanol was injected, individually, into the Agilent Q-TOF HRMS (Model- 6530-B) operating at the positive mode electro-spray ionization (ESI). The nitrogen gas was used as the carrier gas and was operated at a flow rate of 8 L/min. The nebulizer was set at 350 ^oC with the capillary and fragmentation voltages set at 3500 V and 140 V, respectively. Data acquisition and analysis were done using Agilent's software (v. 12).

2.3.2.4 Nuclear Magnetic Resonance (NMR)

A Bruker-make 400MHz NMR (Model: Avance II) operating with a cryomagnet of field strength 9.4 T and radio waves of 400 MHz and 100 MHz was used respectively for recording ^{1}H-NMR and ^{13}C-NMR spectra. 25 mg of each compound was dissolved in 1 ml of deuterated $CDCl_3$ and transferred to an NMR tube. NMR spectra acquisition and analysis were carried out in Topspin software (v 2.0). The chemical structure and identity of the molecules were ascertained based on NMR data using ChemDraw software (v. 12).

2.4 Assessment of larvicidal activity of isolated N-alkylamides

2.4.1 Preparation of stock solutions

The stock solutions of 10000 ppm of the isolated NUD, NDT and the standard MET were prepared in pure acetone and diluted using dechlorinated water to obtain test samples having working concentrations in the range of 10 ppm to 100 ppm.

2.4.2 Larvicidal Assay

The larvicidal potency of the isolated compounds was tested following the guidelines of the World Health Organization (WHO, 2005) on larvae of *Ae. aegypti* and *Cx. quinquefasciatus* at the Indian Institute of Chemical Technology (IICT), Hyderabad. The mosquitoes were maintained at 26 ± 2 $^{\circ}C$, 70% relative humidity, 14 h light: 10 h dark cycle. A laboratory colony of late III - early IV instar larvae (n = 10) obtained from the reared mosquito species was placed in paper cups containing 100 ml of test solution supplemented with varying amounts of the test compound. The vehicle control and negative control consisted of acetone (0.5%) and water respectively. The percentage of mortality was recorded after 24 h of treatment. The lethal concentrations LC_{50} and LC_{90} were determined following Finney's probit method (Finney, 1971) using the formula:

$$\text{Mortality (\%)} = \frac{X - Y}{X} \times 100, \text{ where,}$$

'X' is the % survival in the untreated control and 'Y' is the % survival in the treated sample

2.5 Analysis of Quantitative Structure Activity Relationship (QSAR)

The reasoning for the difference in larvicidal potency exhibited by the test compounds was determined by QSAR analysis. It involved a test of correlation of the LA and the log P value. The log P is a physiochemical property that determines the drug-likeness of a compound under Lipinski's rule of five (Lipinski et al. 2001). The physicochemical properties were predicted using the bioinformatic tools Swiss-ADME and Molinspiration available at www.swissadme.ch and www.molinspiration.com, respectively (Cruz et al. 2014; Daina et al. 2017). The predicted log P values of the test compounds were plotted against the log $1/LC_{50}$ values (where LC_{50} represents the concentration of N-alkylamides at which 50% of the initial population of mosquito larvae gets killed) and the dataset was subjected to partial least-square analysis (GraphPad PRISM®, v. 6.0, USA, www.graphpad.com).

2.6 HPLC-assisted quantitation of N-alkylamides in different organs of A. ciliata

2.6.1 Preparation of stocks and calibration curves

The purified N-alkylamides obtained in section 2.3.1 were used as external standards. 1 mg/ml stock solution of NUD and NDT purified in this study was prepared in HPLC grade methanol and diluted to obtain them in the range of 20 µg/ml to 100 µg/ml and 100 µg/ml to 600 µg/ml respectively. The calibration curves were prepared by plotting the peak area (on the y-axis) versus the concentration of the isolated compounds (on the x-axis) using GraphPad PRISM®.

2.6.2 Estimation of N-alkylamides and Method Validation

For estimation of N-alkylamides, 25 g dry powder of test samples (flower, leaf, root and stem of *A. ciliata*) was extracted in methanol as described before. The crude extract was dissolved in HPLC grade methanol to obtain a 5 mg/ml working solution. The methanol extract of the test samples was analyzed in triplicates at intra-day and inter-day intervals, following the HPLC conditions described in section 2.3.2.1. The validity of the method was confirmed following International Conference on Harmonization (ICH, 1997). The limit of detection (LOD), limit of quantification (LOQ) and precision (expressed as percent relative standard deviation, RSD%) of the HPLC method were calculated as described earlier (Sharma et al. 2021).

2.7 Data Analysis

The significance ($P < 0.05$) of results was analyzed by the Duncan Multiple Range Test (DMRT) in IBM-SPSS software (v. 25).

3. Results

3.1 Isolation and purification of N-alkylamides from A. ciliata

Methanol extraction of *A. ciliata* flowers yielded *ca.* 30 g (15% by dry weight) of a brownish-yellow crude extract. HPLC of the crude showed the presence of 10 distinct peaks indicating as many compounds in the extract (**Fig 1A)**. Retention time (RT) of the peaks ranged from 2.36 min to 17.24 min, of which the peak with an RT of 8.8 min was the most prominent one **(Fig 1A)**. TLC-assisted purification of the crude by column chromatography yielded two compounds having RT of 5.1 min and RT 8.8 min, with utmost purity ~97% and a yield of 200 mg and 275 mg, respectively **(Fig 1B** and **1C)**. They were annotated as compound 1 (C1) and compound 2 (C2) respectively.

3.2 Structure determination of the isolated compounds

The results of FTIR, HRMS, ^{1}H-NMR, and ^{13}C-NMR of the purified compounds C1 and C2 are presented below.

Compound 1 (C1): Physically it was dark brown sticky compound. FTIR showed presence of functional groups with characteristic absorbance peaks at 3368 (NH), 2925, 2854 (C-H stretch), 1717, 1663 (C=O), 1456, 1370, 1316, 1268, 1077 (unsaturation) **(Fig 2A)**. HRMS indicated a *m/z* of 230.1543 $(M+H)^+$ for the compound. An introspection of the FTIR and HRMS data indicated that the molecule could have an empirical formula of $C_{15}H_{19}NO$ with a molar mass of 229.1467 g/mol **(Fig 3A)**. ^{1}H-NMR ($CDCl_3$, 400 MHz) data: δ 7.18 (1H, dd, H_3), 6.19 (1H, dd, H_5), 6.02-6.08 (1H, m, H_4), 5.82 (1H, d, H_2), 5.66 (1H, br s, NH), 3.16 (2H, t, $H_{1'}$), 2.38 (4H, d, C_6 and C_7), 1.98 (1H, s, H_{11}), 1.74-1.83 (1H, sept, $H_{2'}$), 0.92 (6H, d, $H_{3'}$ and $H_{4'}$) **(Fig 4A, Table 1)**. ^{13}C-NMR ($CDCl_3$, 100 MHz) data: δ 166.35 (C_1), 140.65 (C_2), 139.27 (C_3), 129.91 (C_4), 123.22 (C_5), 76.84 (C_8), 68.37 (C_9), 65.61 (C_{10}), 65.19 (C_{11}), 47.12 ($C_{1'}$), 31.39 ($C_{2'}$), 28.73 (C_7), 20.25 ($C_{3'}$), 20.25 ($C_{4'}$), and 18.95 (C_6) **(Fig 4B, Table 1)**.

Compound 2 (C2): Physically it was a pale-yellow sticky compound. FTIR showed presence of functional groups with characteristic absorbance peaks at 3414 (NH), 2958, 2925 (stretch of C-H), 1657, 1627 (C=O), 1555, 1465, 1316, 1264, 998, 878, 781, 725 (unsaturation), 668 **(Fig 2B)**. HRMS indicated a *m/z* of 248.2022 $(M+H)^+$. An introspection of the FTIR and HRMS data indicated that the molecule could have an empirical formula of $C_{16}H_{25}NO$ with a molar mass of 247.1936 **(Fig 3B)**. ^{1}H-NMR ($CDCl_3$, 400 MHz) data: δ 7.17 (1H, dd, H_3), 6.30 (1H, ddt, H_{10}), 6.15 (1H, dd, H_4), 6.07 (1H, dt, H_5), 5.96 (1H, br t, H_9), 5.77 (1H, d, H_2), 5.71 (1H, dq, C_{11}), 5.65 (1H, br s, NH), 5.25 (1H, dt, H_8), 3.15 (2H, t, $H_{1'}$), 2.23-2.27 (4H, m, H_6 and H_7), 1.78 (3H, dd, H_{12}), 1.78 (1H, sept, $H_{2'}$), and 0.91 (6H, d, $H_{3'}$ and $H_{4'}$) **(Fig 4C, Table 1)**. ^{13}C-NMR ($CDCl_3$, 100 MHz) data: δ 166.54 (C_1), 142.17 (C_2), 141.28 (C_3), 129.97 (C_4), 129.45 (C_5),

128.79 (C_8), 128.04 (C_9), 126.86 (C_{10}), 122.18 (C_{11}), 47.08 ($C_{1'}$), 33.13 ($C_{2'}$), 28.73 (C_6), 27.03 (C_7), 20.25 ($C_{3'}$), 20.25 ($C_{4'}$), and 18.45 (C_{12}) **(Fig 4D, Table 1)**.

Analysis of the data from the three spectroscopic methods above and a comparison with the published reports confirmed the identity of C1 as 2*E*,4*E*-N-isobutyl undeca-2,4-diene-8,10-diynamide and and C2 as 2*E*,4*E*,8*Z*,10*E* -N-isobutyl dodeca-2,4,8,10-tetraenamide. For convenience, the C1 and C2 were abbreviated as NUD and NDT respectively.

3.3 Assessment of larvicidal activity of purified N-alkylamides

The larvicidal potency of NUD and NDT were tested on larvae of *Ae. aegypti* and *Cx. quinquefasciatus*. The larvae of both mosquitoes were found susceptible to the test compounds. The compounds tested and the methoprene (MET) standard induced mortality on the larvae in a dose-dependent manner **(Supplementary Table 1 and 2)**. The LC_{50} and LC_{90} of compounds are presented in **Supplementary Fig 1** and **Table 2.** NDT, a C_{12} alkene N-alkylamide, showed higher toxicity than the NUD, a C_{11} alkyne N-alkylamide **(Supplementary Fig 1, Table 2)**. NUD and NDT showed a LC_{50} of 44.19 ppm and 18.28 ppm respectively for *Ae. aegypti*, while they were 30.89 ppm and 11.75 ppm, respectively, for *Cx. quinquefasciatus* larvae **(Supplementary Fig 1, Table 2)**. LC_{90} for NUD and NDT were 90.17 ppm and 56.86 ppm respectively against *Ae. aegypti*, while they were was 86.46 ppm and 21.76 ppm, respectively, for *Cx. quinquefasciatus* **(Supplementary Fig 1, Table 2)**. Both the compounds tested in this study in addition showed a potency exceeding that of MET standard with NDT being the most potent. NDT was 2 to 3 times more potent than NUD and 4 to 5 times that of MET **(Table 2).** Variation in the larvicidal potency could be attributed to the structural diversity in N-alkylamides resulting from the differences in chain length, type and degree of unsaturation **(Figure 4B** and **4D)**.

3.4 Analysis of Quantitative Structure Activity Relationship (QSAR)

The key physicochemical properties predicted using Swiss-ADME and Molinspiration tools indicated that both NUD and NDT did possess drug-like properties **(Table 3)**. The difference in the larvicidal potency observed with test compounds was validated by QSAR analysis. Lipophilicity, expressed as log P value, is one of the physicochemical features that determine the solubility and membrane permeability of molecules and therefore used as a descriptor to evaluate drug-likeness under Lipinski's rule of five. A correlation of the lipophilicity (log P) and LA (log $1/LC_{50}$) indicated that the log P value of MET fell outside the limit of Lipinski's rule of five. For N-alkylamides, on the other hand, it was found within the specified limits **(Fig 5, Table 3)**. It was intriguing to observe that MET had some deviation for drug-likeness under Lipinski's rule of five. Swiss-ADME-mediated prediction of the biological targets for N-alkylamides revealed that both N-alkylamides, NUD and NDT, had some common binding partners such as family A G-protein coupled receptors (GPCRs), nuclear receptors and voltage-gated ion channels **(Fig 6)**. A lower total polar surface area (TPSA), a determinant of the transport property related to intestinal absorption and blood-brain barrier, of the isolated N-alkylamides NUD and NDT in comparison to MET **(Table 3)** is indicative of high cellular permeability.

3.5 Determination of N-alkylamides content in different organs of A. ciliata

The calibration curves drawn for NUD and NDT isolated in the present study were linear with a high R^2 value **(Supplementary Fig 2**, **Table 5)**. HPLC of the test samples showed that the NUD and NDT content varied in the range of 2.06 mg/g to 68.55 mg/g by dry weight of the crude extract in different parts of *A. ciliata*. The compounds accumulated at its maximum in the flowers (19.01 and 68.55 mg/g, respectively) and turned out to be lowest in stem and leaf **(Table 4)**. The LOD and LOQ of the HPLC method for NUD were 0.80 µg/ml and 2.51 µg/ml, respectively, while, these were 5.50 µg/ml and 16.70 µg/ml, respectively, for NDT **(Table 5)**. The intra-day and inter-day precision of the method, expressed as percent relative standard deviation (RSD %), for estimation of NUD was 2.38 % and 2.71 %, respectively, whereas, the values of the same for NDT were 2.75 % and 3.37 % respectively **(Table 5)**.

4. Discussion

N-alkylamides differ in chain length, degree, type of unsaturation and for the distinct amino acid-derived amines such as isobutylamine, methylbutylamine and phenylethylamine present in the molecule (Cortez-Espinosa et al. 2011; Rizhsky et al. 2016). There have been reports on acaricidal, anti-plasmodial, insecticidal, larvicidal, ovicidal, pesticidal and pupicidal properties exhibited by

solvent extracts of *Acmella* (syn: *Spilanthes*) (Pandey et al. 2007, 2011; Silveira et al. 2016; Sharma et al. 2012; De Oliveira et al. 2016). The studies on LA of pure N-alkylamides, however, have been very limited (Jondiko, 1986; Saraf and Dixit, 2002; Simas et al. 2013). For example, it was shown that an extract of *S. acmella* was more toxic than the ones prepared from *S. calva* and *S. paniculata* against larvae of *Anopheles stephensi*, *An. culicifacies*, and *Cx. quinquefasciatus* (Pandey et al. 2007). Larvicidal assay-guided fractionation of hexane extract (of *S. acmella* flowers) later led to the identification of three N-alkylamides *viz.*, 2*E*,6*Z*,8*E*-N-isobutyl deca-2,6,8-trienamide (spilanthol), 2*E*,7*Z*,9*E*-N-isobutyl undeca-2,7,9-trienamide and 2*E*-N-(2-methylbutyl) undec-2-ene-8,10-diynamide (Pandey et al. 2011). Similarly, a mixture of phenylethylamide type N-alkylamides, namely nona-(2Z)-en-6,8-diynoic acid 2-phenylethylamide and deca-(2Z)-en-6,8-diynoic acid 2-phenylethylamide (in unknown proportion), and the hexane fraction of *A. oleracea,* from which these were isolated, showed LA with an LC_{50} of 7.6 ppm and 145 ppm, respectively, for *Ae. aegypti* larvae (Simas et al., 2013).

N-alkylamides isolated from *A. ciliata* differed from these studies by exhibiting a greater potency against the *Culex* larvae as compared to the *Aedes*. Our results are in contrast with Araujo et al. (2018) who reported higher toxicity of 70% ethanolic extract of *A. oleracea* on *Aedes* larvae as compared to *Culex*. Earlier, an isomer of NDT (configuration: 2*E*,4*E*,8*E*,10*Z*) and the chloroform extract of a related species *S. mauritiana* were shown to exhibit a LC_{50} at 0.01 ppm and 0.1 ppm respectively against *Ae. aegypti* (Jondiko, 1986). Whereas, LC_{50} of 10 ppm was recorded against the same for a mixture containing NDT (2*E*,4*E*,8*Z*,10*E*) and its isomer with the bond configuration 2*E*,4*E*,8*Z*,10*Z*, isolated from *Echinacea purpurea* (Clifford et al. 2002). The difference in the larvicidal potency of *Acmella* (syn: *Spilanthes*) extract could be attributed to the chemical structure of the molecules, chemical ecology of the plant extract, genotype, modulation in *de novo* biosynthesis of N-alkylamides and the environmental factors (Pandey et al. 2007, 2011; Simas et al. 2013; Araujo et al. 2018). From these observations it is inferred that distinct N-alkylamides interact differently at allosteric sites of the biomolecular targets, such as juvenile hormones, nuclear receptors or voltage-gated ion channels, resulting in varied toxicity (Du et al. 2013; Devillers et al. 2015). Also, it appears that a combination of N-alkylamides would be more effective than the use of solitary N-alkylamide in the control of mosquito larvae. The reason could be that the insects also would have evolved several receptors or allosteric receptors that requires a variety of N-alkylamides to exert the activity (Du et al. 2013).

The QSAR study between the biological activity and the chemical structure of isolated N-alkylamides was carried out to understand the influence of key structural properties of the molecules such as chain length, degree of unsaturation, and terminal methylation on the LA of N-alkylamides. For instance, NDT, a 12-carbon (*dodeca-*) fatty acyl chain molecule was more potent than the 11-carbon (*undeca-*) NUD. The higher LA of NDT as compared to NUD could further be attributed to the presence of terminal methylation, which otherwise has been shown to exert negative effects on antifungal activity of N-alkylamides (Cruz et al. 2014). Both the isolated N-alkylamides, NUD and NDT in their structure, possess a *trans*-double bond at C_2 which may be attributed for the toxicity of N-alkylamides on bacteria, fungi, and insects as reported earlier (Jacobson, 1954; Molina-Torres et al. 2004; Cruz et al. 2014). Our results of swiss-ADME-based prediction for the biological targets revealed that the N-alkylamides have affinity towards G-protein coupled receptors (GPCRs), nuclear receptors and voltage-gated ion channels as demonstrated previously for NAAs and pyrethroids (Zlotkin, 1999; Raduner et al. 2006; Du et al. 2013).

HPLC of the floral extract showed that NDT, an alkene N-alkylamide, was the most prominent molecule (>3.5-fold, in flower) and it elutes later than NUD, an alkyne N-alkylamide, in reverse-phase HPLC. The differences in their RTs indicate their differential affinity towards the reverse-phase C_{18} stationary phase column (Boonen et al. 2010; Rajendran et al. 2017). NUD and NDT content in *A. ciliata* flowers were comparably higher than those reported from *S. paniculata* and *A. oppositifolia* (Molina-Torres et al. 1996; Rajendran et al. 2017). The difference in N-alkylamide content, within *A. ciliata*, indicates the differential expression of genes involved in the N-alkylamide biosynthesis. Besides, the plant part used, their developmental stage, genotype and environmental factors influence the content of N-alkylamides in the plant (Boonen et al. 2010; Singh and Chaturvedi, 2012; Sharma et al. 2021).

Conclusion

The present study showed that *A. ciliata* harbours diverse forms of N-alkylamides. Specific N-alkylamides, namely NUD and NDT, isolated from flower-heads of the plant exerted remarkable toxicity on *Ae. aegypti* and *Cx. quinquefasciatus* larvae with their effective concentrations surpassing even the standard MET. To the best of our knowledge, this is the first report on the assessment of LA of pure larvicidal N-alkylamides from *A. ciliata.* The compounds could be alternative to synthetic larvicides therefore can be used for the elimination and control of mosquitoes in an eco-friendly manner.

Declarations

Acknowledgments

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Author's Contribution

Conceptualization: NA; Experimentation: RS; Drafting and Editing: RS and NA

Conflict of Interest

RS and NA declare no conflict of interest.

Ethics Approval

Not applicable

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Tables

Table 1

NMR Data on chemical shifts (**δ**) for proton and carbon of the compounds C1 and C2.

Carbon Position	^{13}C (δ, in ppm)		Proton Position	^{1}H (δ, in ppm)	
	C1	C2		C1	C2
C_1	166.35	166.54	NH	5.66 (br s, 1H)	5.65 (br s, 1H)
C_2	140.65	142.17	H_2	5.82 (d, 1H)	5.77 (d, 1H)
C_3	139.27	141.28	H_3	7.18 (dd, 1H)	7.17 (dd, 1H)
C_4	129.91	129.97	H_4	6.02-6.08 (m, 1H)	6.15 (dd, 1H)
C_5	123.22	129.45	H_5	6.19 (dd, 1H)	6.07 (dt, 1H)
C_6	18.95	28.73	H_6	2.38 (d, 4H)	2.23-2.27 (m, 4H)
C_7	28.73	27.03	H_7		
C_8	76.84	128.79	H_8	-	5.25 (dt, 1H)
C_9	68.37	128.04	H_9	-	5.96 (br t, 1H)
C_{10}	65.61	126.86	H_{10}	-	6.30 (ddt, 1H)
C_{11}	65.19	122.18	H_{11}	1.98 (s, 1H)	5.71 (dq, 1H)
C_{12}	-	18.45	H_{12}	-	1.78 (dd, 3H)
$C_{1'}$	47.12	47.08	$H_{1'}$	3.16 (t, 2H))	3.15 (t, 2H)
$C_{2'}$	31.39	33.13	$H_{2'}$	1.74-1.83 (sept, 1H)	1.78 (sept, 1H)
$C_{3'}$	20.25	20.25	$H_{3'}$	0.92 (d, 3H)	0.91 (d, 3H)
$C_{4'}$	20.25	20.25	$H_{4'}$	0.92 (d, 3H)	0.91 (d, 3H)

The data predicts compound 1 as NUD and compound 2 as NDT.

Table 2

Data on larvicidal potency and fiducial limits of N-alkylamides (NUD and NDT) isolated from *A. ciliata* on *Ae. aegypti* and *Cx. quinquefasciatus* larvae.

Compound Name	Mosquito species	Larval stage	LC_{50} (ppm)	FL (LC_{50}) (LCL-UCL)	LC_{90} (ppm)	FL (LC_{90}) (LCL-UCL)
NUD	*Aedes aegypti*	late III - early IV instar	44.19 ± 06.54 b	31.36 – 57.03	90.17 ± 29.77 b	31.80 – 148.53
NDT			18.28 ± 04.04 a	10.35 – 26.22	56.86 ± 26.95 a	04.03 – 109.70
MET			54.49 ± 04.03 c	46.58 – 62.40	75.02 ± 07.68 c	59.95 – 90.09
NUD	*Culex quinquefasciatus*		30.89 ± 04.82 b	21.44 – 40.35	86.46 ± 32.87 b	22.03 – 150.89
NDT			11.75 ± 01.86 a	08.09 – 15.41	21.76 ± 03.42 a	15.05 – 28.48
MET			59.71 ± 04.63 c	50.62 – 68.80	101.49 ± 14.40 b	1. – 129.73

[a] Mean ± SE; MET: standard Methoprene; FL: Fiducial Limit; LFL: Lower Fiducial Limit; UFL: Upper Fiducial Limit;

The significance ($P < 0.05$) of the results was assessed by Duncan's Multiple Range Test (DMRT)

Table 3

Details of physicochemical properties of the test compounds predicted by Swiss-ADME and Molinspiration tools.

Compound Name	Chemical Formula	MW (g/mol)	nRBs	nHBDs	nHBAs	TPSA (Å²)	Molar Refractivity	Log P		Violations
								Swiss-ADME	Molinspiration	
NUD	$C_{15}H_{19}NO$	229.32	7	1	1	29.10	72.60	3.11	3.51	None
NDT	$C_{16}H_{25}NO$	247.38	9	1	1	29.10	80.13	3.89	4.01	None
MET	$C_{19}H_{34}O_3$	310.47	11	0	3	35.53	94.91	4.77	5.36	1

MW: Molecular Weight; nRBs: Number of Rotatable Bonds; nHBDs: Number of Hydrogen Bond Donors;

nHBAs: Number of Hydrogen Bond Acceptors; TPSA: Total Polar Surface Area

Table 4

Data on NUD and NDT content in different parts of *A. ciliata*.

Part of the plant	Amount (mg/g) * Mean ± SD	
	NUD	NDT
Flower	19.01 ± 1.37 [a]	68.55 ± 3.49 [b]
Leaf	15.02 ± 8.60 [ab]	10.84 ± 3.40 [a]
Root	03.13 ± 1.85 [ab]	18.99 ± 3.41 [a]
Stem	02.06 ± 1.24 [b]	11.14 ± 3.20 [a]

* Significance ($P < 0.05$) of the results was assessed by Duncan's Multiple Range Test (DMRT).

Table 5

Data on the ICH parameters for estimation of N-alkylamides

Compound Name	Goodness of Fit (R^2)	Standard Error (%)	LOD (µg/ml)	LOQ (µg/ml)	RSD (%)	
					Intra-day	Inter-day
NUD	0.9953	2.23	0.8	2.51	2.38	2.71
NDT	0.9936	4.02	5.5	16.7	2.75	3.37

Figures

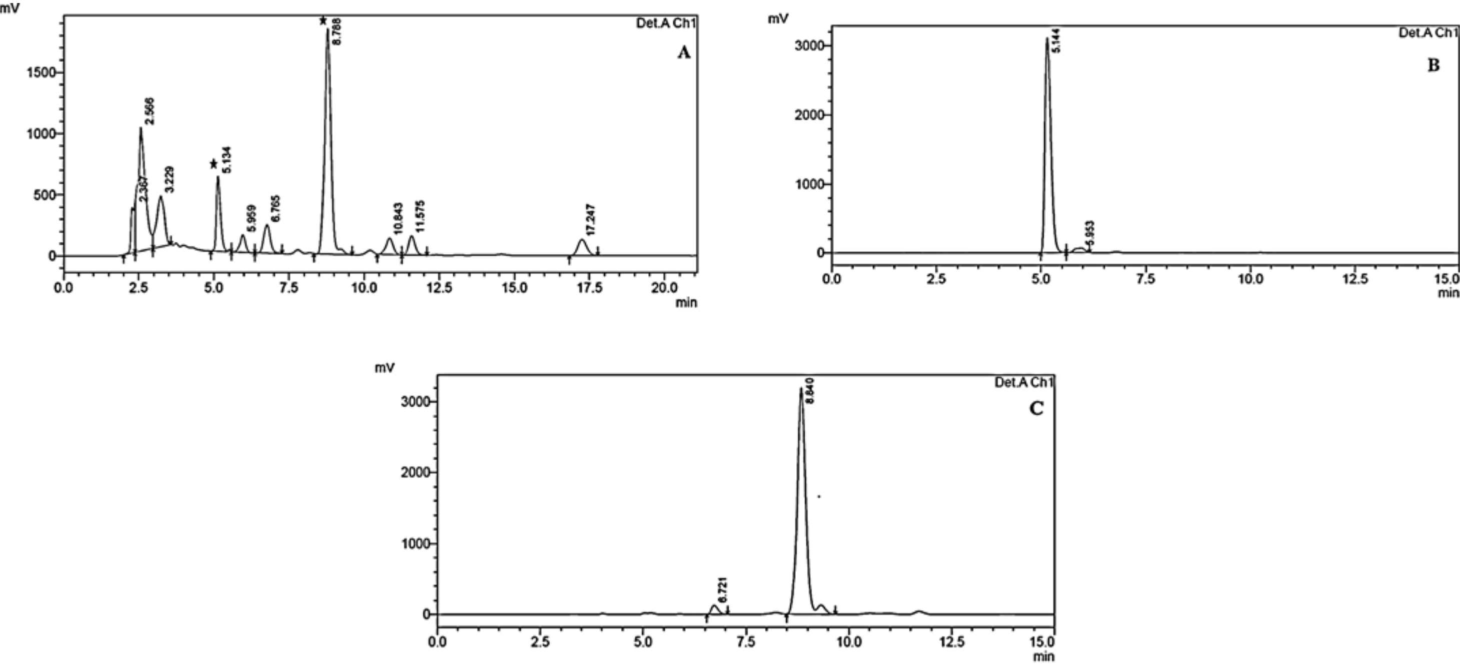

Figure 1

HPLC of crude methanol extract of (A) *A. ciliata* flower, (B) Compound 1 (C1) and (C) Compound 2 (C2). The compounds of interest are marked by an asterisk in (A).

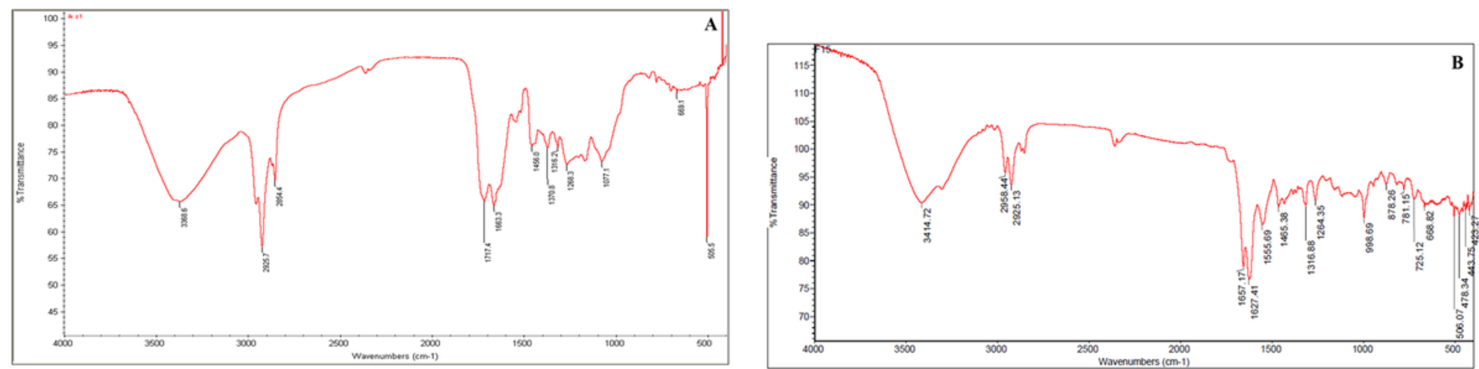

Figure 2

FTIR spectrum of (A) C1 and (B) C2.

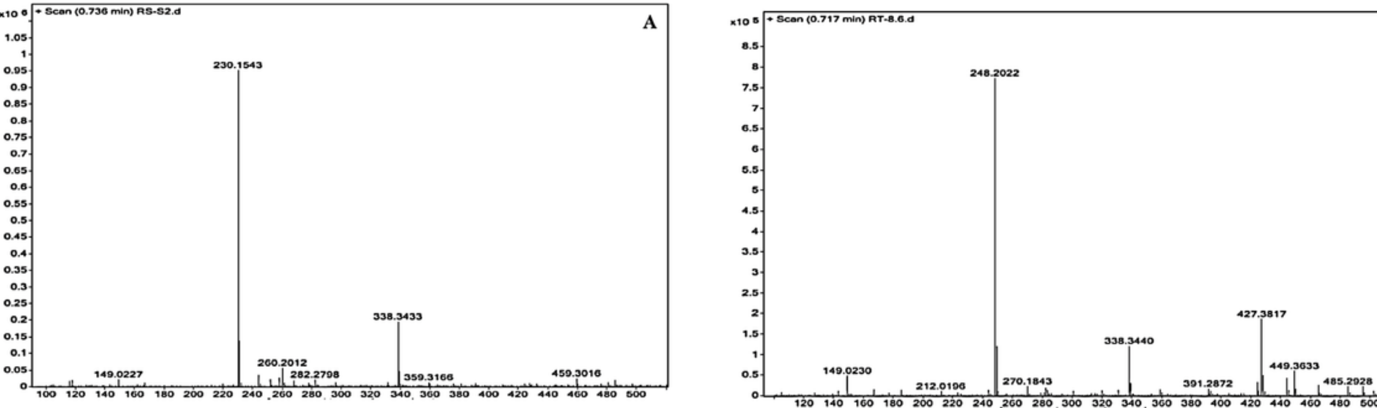

Figure 3

HRMS spectrum of (A) C1 and (B) C2 showing a *m/z* of 230.1543 and 248.2022 respectively.

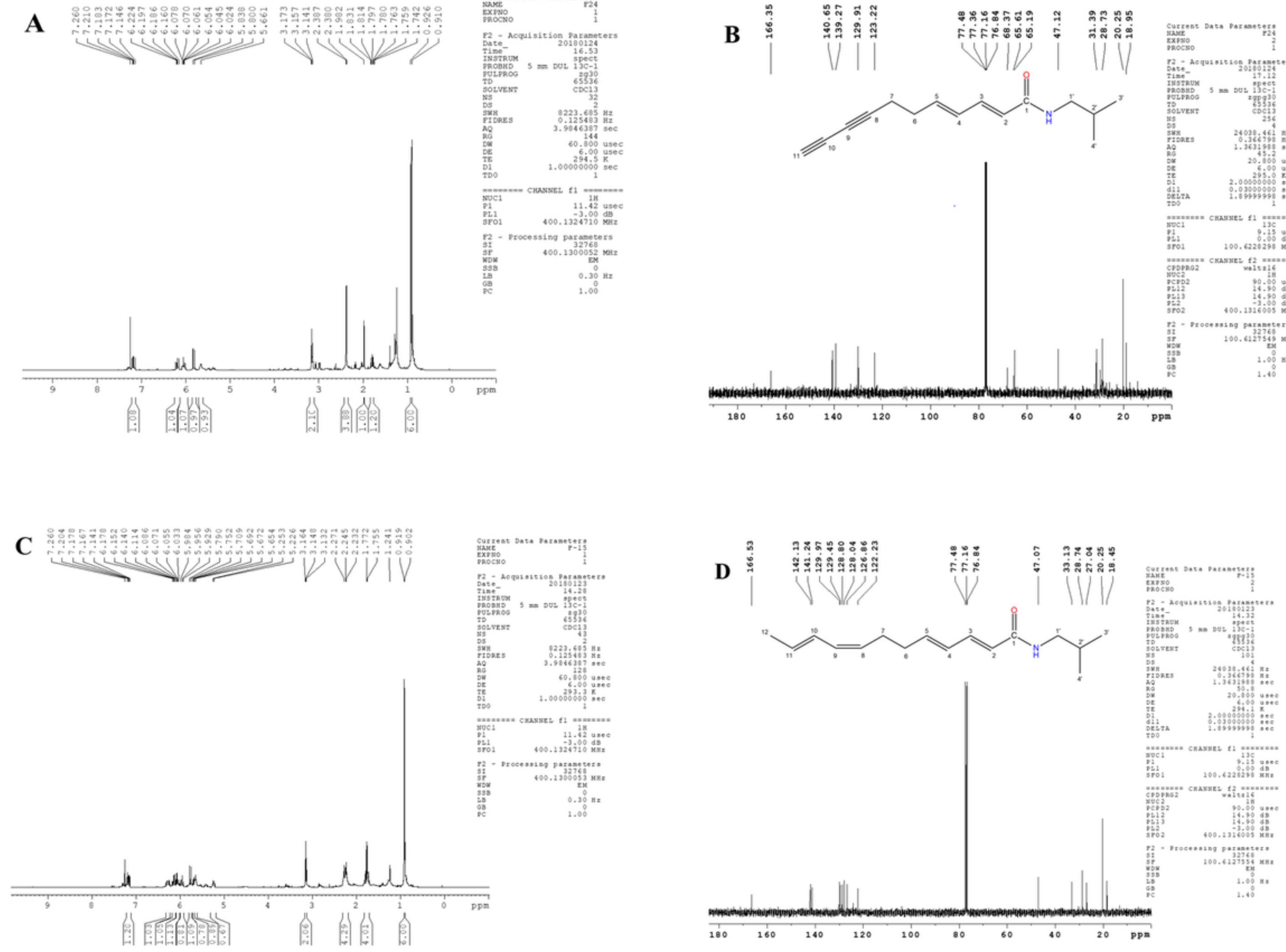

Figure 4

NMR spectrum and chemical structure of the isolated compounds. (A) ^{1}H-NMR of C1, (B) ^{13}C-NMR of C1, (C) ^{1}H-NMR of C2 and (D) ^{13}C-NMR of C2.

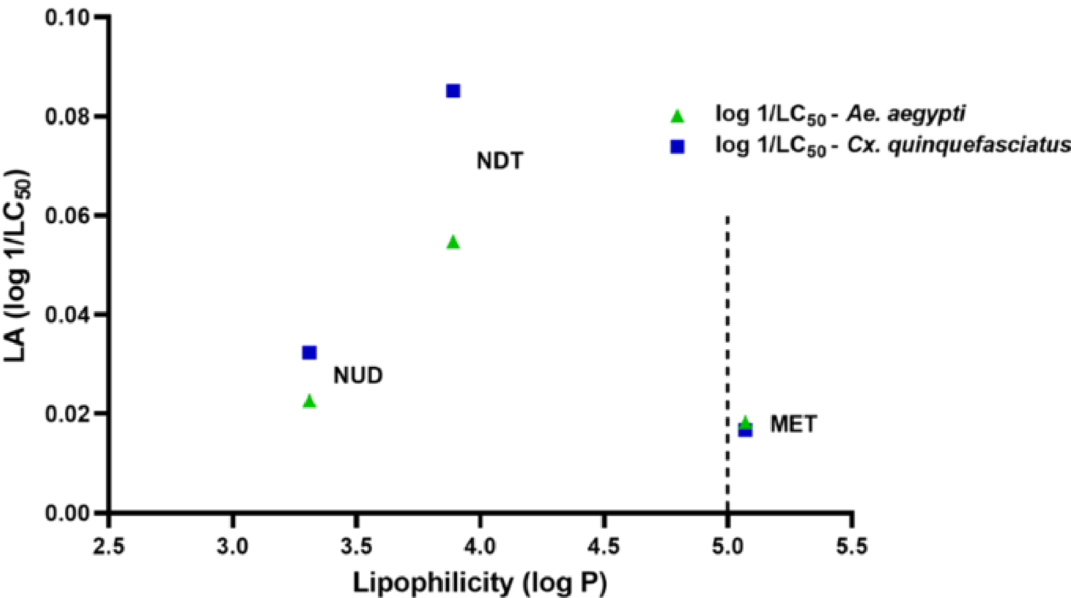

Figure 5

QSAR graph of LA versus log P for NUD, NDT, and MET. The dashed line at 5.0 denotes the threshold log P value as per Lipinski's rule of five for drug formulations.

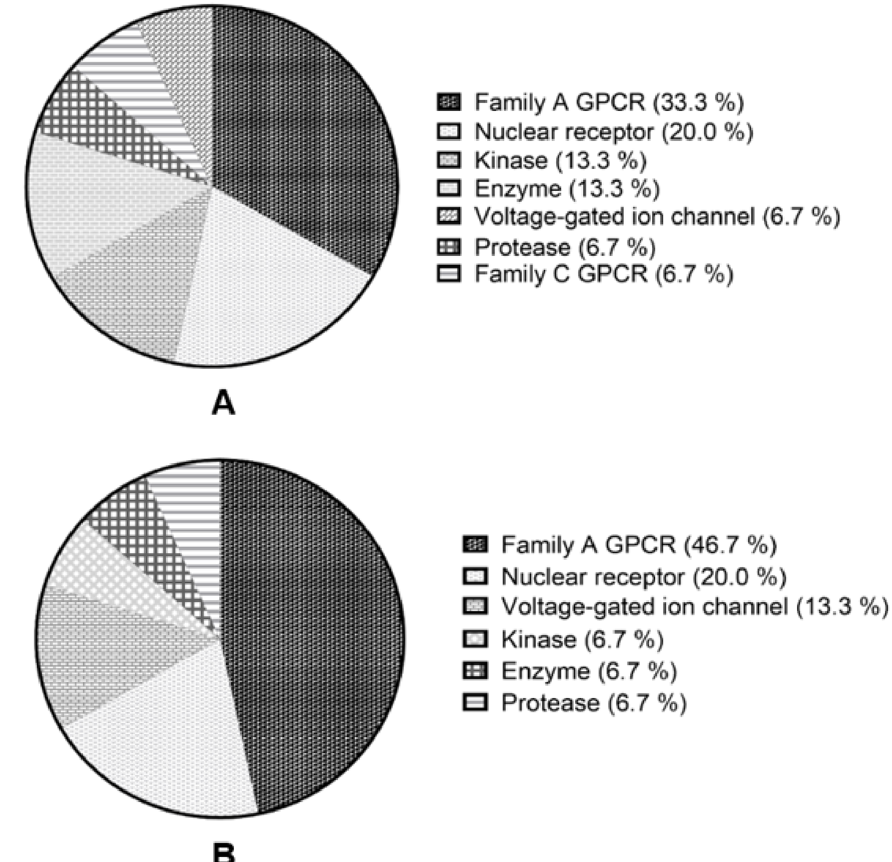

Figure 6

Prediction of molecular targets for the isolated N-alkylamides (A) NUD and (B) NDT by Swiss-ADME. The percentage likelihood for the same is given in parentheses.

Supplementary Files

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