N-alkylamides of Spilanthes (syn: Acmella): Structure, Purification, Characterization, Biological Activities and Applications – A review

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N-alkylamides of *Spilanthes* (syn: *Acmella*): Structure, purification, characterization, biological activities and applications – a review

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A B S T R A C T

Several species of *Spilanthes*, commonly known as the 'toothache' plants, are widely used by different communities as food ingredient in their traditional cuisines. Different parts of these plants are also used as medicine to treat diseases such as oral ulcers, stomatitis and toothache. The N-alkylamides (NAAs) present in *Spilanthes* species have been attributed to the biological properties such as anti-inflammatory, antimicrobial, aphrodisiac, antioxidant, insecticidal, larvicidal and astringent effects exhibited by this crop. Presence of NAA further allows usage of these plants as functional foods and cosmetics. Though interest in NAAs is enormous there is lack of studies with respect to the source material, mode of their action and user safety of NAA containing products. Therefore, a systematic survey of literature was done to present food and medicinal uses of *Spilanthes* plants, information on their NAAs, their distribution, biosynthesis, pharmacology, commercial potential and research lacunae. The review also attempts to caution the misnomer on the source material used for preparation of drugs from *Spilanthes* with a brief discussion on the plant's nomenclature and taxonomy.

Abbreviations

ATF-2	Activating Transcription Factor 2
cAMP	Cyclic Adenosine Monophosphate
CPC	Centrifugal Partition Chromatography
CREB-1	cAMP Responsive Element-binding Protein 1
FSH	Follicle Stimulating Hormone
IBA	Isobutylamide
JNK	Jun N-terminal Kinase
LH	Leutinizing Hormone
MAPK	Mitogen Activated Protein Kinase
MBA	Methylbutylamide
NAA	N-alkylamide
NF-κB	Nuclear Factor κB
PEA	Phenylethylamide
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
SFE	Supercritical Fluid Extraction.
STAT-4	Signal Transducer and Activator of transcription 4
TNF-α	Tumor Necrosis Factor α

1. Introduction

Plants produce a wide array of secondary metabolites which play key role in the adaptation and defense of plants in natural environment by conferring resistance against pathogenic microbes, pests and insects (Bourgaud et al., 2001; Pott et al., 2019). On the basis of their chemical structure, they are categorized broadly into alkaloids, flavonoids, phenolics, saponins and terpenes (Hussein and Al-Ensarry, 2018). Alkaloids are heterocyclic compounds invariably containing one or more N-atom in their cyclic ring structure. They taste bitter and exhibit base-like properties (Khan et al., 2013; Diaz et al., 2015). Most often an amino acid forms the precursor for alkaloids with the common amino acids being anthranilic acid, histidine, lysine, ornithine, phenylalanine and tryptophan (Hegnauer, 1963). On the basis of functional groups the alkaloids were grouped into 9 classes *viz.,* acridines, amides, amines, benzylisoquinolines, canthinones, imidazoles, indolquinazolines, furoquinolines and quinazolines (Price, 1963). A couple of recent studies report them as indoles, quinolines, isoquinolines, pyrrolidines, pyridines, pyrrolizidines, tropanes, terpenoids, steroids, peptide, cyclopeptide, true-, proto-, polyamine- and pseudo-alkaloids (Kurek, 2019; Memariani et al., 2020).

The N-alkylamides (NAAs) differ from alkaloids in that the nitrogen in most case occur in an open chain. They are basically fatty acid amides formed from a fatty acid chain and a decarboxylated amino acid, probably by a condensation reaction. Some recent literature consider NAAs under the umbrella of 'alkaloids', particularly those obtained from the ergot fungi due to their structural similarity with lysergic acid amide alkaloids such as ergine, ergometrine and lysergic acid diethylamide (LSD) (Schiff, 2006; Rios. 2012; Nascimento et al., 2012; Boonen et al., 2012). Sometimes they are described as proto-alkaloids or pseudo-alkaloids due to the lack of the N-atom in the ring structure as observed in certain clinically important alkaloids such as codeine, morphine and nicotine (Rios, 2012). The most common of NAAs is spilanthol reported from Asteraceae members such as *Spilanthes, Acmella* and *Heliopsis* (Wu et al., 2008; Deciga-Campos et al., 2012; Prachayasittikul et al., 2013; Castro-Ruiz et al., 2017). Traditionally, *Spilanthes* served as a remedy for alleviating toothache, throat infections and other oral ailments **(Table 1)**.

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Table 1
List of ethno-medicinal uses of extract from *Spilanthes* species.

S. No	Plant Name	Plant part used	Usage Location	Uses	Reference
1	*S. calva*	Fruit	Tamil Nadu, India	Toothache, throat infections, boils and wounds	Pushpagandan and Atal, 1986
2	*S. callimorpha*	Whole plant	China	Amenorrhea relief	Kong et al., 1986
3	*S. mauritiana*		Africa	Toothache and diarrhea	Jondiko, 1986
4	*S. oleracea*		Arunachal Pradesh, India	To facilitate capture of fishes	Tag et al., 2005
5	*S. africana*	Flowers and leaves	Western Uganda	Induce labor during childbirth	Mugisha and Origa, 2007
6	*S. paniculata*	Leaves	Tripura, India	To facilitate capture of fishes	Shil and Choudhary, 2009
7	*S. calva*		Tamil Nadu, India	Toothache and wound healing	Revathi and Parimelazhagan, 2010
8	*S. acmella*	Flowers			
		Roots	Madhya Pradesh, India	Throat infections	Vijender and Kumar, 2010
		Whole plant	India	Male aphrodisiac	Sharma et al., 2011
		Flowers, stem, and leaves	Meghalaya, India	Toothache	Sharma et al., 2014
		Flowers	Nagaland, India		Kichu et al., 2015
9	*A. uliginosa*	Leaves	Benin, West Indies	As a vegetable and in the de-worming sauces	Lagnika et al., 2016

Extracts of several of *Spilanthes* species were later shown to exhibit broad spectrum of biological activities such as antioxidant, gastroprotective, anti-proliferative, immuno-modulatory, diuretic, vasorelaxant, anti-inflammatory, enzyme inhibitory, antimicrobial, insecticidal and larvicidal properties which had led to attraction by several pharmaceutical industry to launch a variety of heath care products **(Table 2).** The objective of this review is to present an overview of current status of research on traditional medicinal herbs belonging to the genus *Spilanthes* (syn: *Acmella*) with special reference to their taxonomy, food use, medicinal properties, prominent bioactive NAAs and mode of action of NAAs. The electronic search engines namely, Web of Science, PubMed, Scopus, Science direct and Google scholar were searched for literature on keywords '*Spilanthes'*, '*Acmella'*, 'Toothache plant', 'N-alkylamides' and 'Fatty acid amides'. From about 270 hits, by excluding the duplicates and other plants, a total of 170 articles were selected for the study. The flora referred to were inspected at the central library of "The French Institute of Pondicherry" (UMIFRE 21), Pondicherry, India.

Spilanthol was first isolated in a crude form from the capitula of *Spilanthes oleracea* (Gerber, 1903; Jacobson, 1956; Alonso et al., 2018). Later on an insecticidal amide isolated from the roots of a Mexican plant *Erigeron affinis* DC, ascertained it to be N-isobutyl deca-2,6,8-trienamide and therefore was named as Affinin (Acree et al., 1945). However, in subsequent years the identity of the plant was confirmed to be *Heliopsis longipes* and it was proposed to discontinue the name 'Affinin' for any future considerations (Jacobson et al., 1947). It was further demonstrated that Affinin on interaction with UV or selenium became altered to all *trans*-spilanthol isomer loosing its toxicity against house flies (Jacobson, 1954). Both the generic names (Spilanthol and Affinin) are still used differentially in literature to refer to their origin in addition to referring them as synonyms (Molina-torres et al., 1996; Boonen et al., 2012; Castro-Ruiz et al., 2017). Apart from spilanthol, an array of pharmacologically active NAAs were isolated from *Spilanthes* and related plants. The importance of NAAs is exemplified by a growing number of patents granted to the products containing *Spilanthes* extracts and/or NAAs (Silveira et al., 2018). Considering the regulatory aspects for the benefit of all stakeholders recently NAA containing health products have been classified into five broad categories namely, medicinal products, foods, cosmetics, medical devices and biocidal products (Wynendaele et al., 2018). The products under foods were further subdivided into food supplements, functional foods, novel foods, PARNUTS foods, fortified foods and flavouring substances. It was emphasised that enumeration of dose-rate and side effects is critical for legal-regulatory classification of NAA containing products.

2. Food and ethnomedicinal uses of *Spilanthes*

'Let food be your medicine' is an old saying which is still in practice in indigenous global food systems (Payum, 2017). Recent studies indicate that there is an inverse relationship between the intake of antioxidant rich foods and the incidence of human diseases (Lagnika et al., 2016). Accordingly, several species of *Acmella* are found used as a food constituent in traditional cuisines **(Table 3)**. *Spilanthes* species, also known as *Jambu, Agrião bravo, Agrião do Pará, Paracress* and *Eye-Ball plant,* is variously used as condiment, appetite stimulant and as flavouring and seasoning agents in green salads **(Table 3).** In Brazilian, Japanese and West Indies cultures, *Spilanthes* species form part of the local cuisines as a leafy vegetable in the preparation of soups (tacacá), sauces (tacupi) and stews (Paulraj et al., 2013; Cruz et al., 2016; Lagnika et al., 2016; Rani et al., 2019; Uthpala and Navaratne, 2020). Tea prepared from the leaves is consumed as a beverage (Nascimento et al., 2013), whereas the decoction of the root is used as a purgative (Leng et al., 2011).

Prominent herbal companies manufacture preparations containing *Spilanthes* for use as nutrient supplements and as health care products. *Gattefossé SAS*, a French company, has a patented technology of preparing *A. oleracea* extract for use in cosmetic anti-wrinkle cream (Demarne and Passaro, 2008). The two products that came out of this are Gatuline® Expression AF and Gatuline® In-tense **(Table 3).** *A. Vogel*, a Swiss company, manufactures tinctures and toothpaste that contain aerial parts of *S. oleracea* as ingredients. *Spilanthes* derived products possessing analgesic, cleansing, detoxification and oral hygienic properties are also available in the market **(Table 3)**. *Takasago International Corp.*, a Japanese company, has developed a series of organoleptic products one of which contained Jambu oleoresin and spilanthol (IBA-10) as ingredients to give a tingling sensation. Use of these products in the manufacture of foods, pharmaceuticals and in personal care products is the focus of their current research (Nakatsu et al., 2001). Addition of spilanthol or a spilanthol containing plant extract has improved the tastes in potassium salt containing food or drink which otherwise had an unpleasant taste (Miyazawa et al., 2011).

3. Description of the genus *Spilanthes*

Spilanthes when first described consisted of just two species namely *Spilanthes insipida* and *Spilanthes urens* (Jacquin, 1760). Later, Richard (1807), described a related genus *Acmella* which differed from *Spilanthes* in having ray florets and absence of pappus (Jansen, 1981). Based on the nature of the floral heads alone, Cassini (1822) divided the genus *Spilanthes* into two sections, namely section - *Spilanthes* with discoid flower heads and section - *Acmella* with radiate flower heads, and the same was followed by Candolle (1836) and Moore (1907). Jansen ameliorated the generic status of *Spilanthes* and *Acmella* by providing convincingly reliable morphological, chromosomal and molecular evidences that were extremely suggestive of considering these genera as distinct, rather than Cassini's suggestion of considering *Acmella* as a section within *Spilanthes* solely on the basis of presence or absence of ray florets (Cassini, 1822; Candolle, 1836; Moore, 1907; Jansen, 1981,

Table 2
Details of Plant parts, Extraction methods, Bioassays and extent of Biological activities of the Extracts and NAAs from *Spilanthes* species.

S. No	Type of Bioactivity	Species (Country)	Plant Part	Extraction Solvent	Bioassay	Extent of Biological Activity (% Scavenging or IC_{50})*	Reference
1	Antioxidant Activity	*S. acmella* (Taiwan)	Dry flowers	Chloroform	DPPH (0.5 mM)	–	Wu et al., 2008
				Ethyl acetate		1.38 μM VCE/mg extract	
				Butanol		–	
		S. acmella (Thailand)	Dried aerial parts	Hexane	DPPH (200 μM)	–	Wongsawatkul et al., 2008
				Chloroform		381 μg/ml	
				Ethyl acetate		216 μg/ml	
				Methanol		223 μg/ml	
			Dried aerial parts	Chloroform	DPPH (100 μM)	1.90–73.23%	Prachayasittikul et al., 2009
				Ethyl acetate		15.15–39.59%	
				Methanol		84.65–92.05%	
		S. acmella (India)	Dry leaves (*in vivo*)	Methanol	DPPH (1.27 mM)	1085 μg/ml	Singh et al., 2014
			Dried callus			1343 μg/ml	
		A. uliginosa (West Indies)	Dry leaves	Dichloromethane	DPPH (0.50 mM)	–	Lagnika et al., 2016
				Methanol		500 μg/ml	
				Distilled water		500 μg/ml	
		S. acmella (India)	Whole plant	Methanol	DPPH (608.7 μM)	730 μg/ml	Swargiary et al., 2019
					ABTS	57 μg/ml	
		S. acmella (Taiwan)	Dry flowers	Chloroform		–	Wu et al., 2008
				Ethyl acetate		3.32 μM TE/mg extract	
				Butanol		–	
		S. acmella (Thailand)	Dried aerial parts	Hexane	NBT superoxide scavenging	00.41%	Wongsawatkul et al., 2008
				Chloroform		57.92%	
				Ethyl acetate		33.05%	
				Methanol		47.02%	
				Chloroform		15.38–20.69%	Prachayasittikul et al., 2009
				Ethyl acetate		27.59–52.41%	
				Methanol		%	
		A. oleracea (Sri Lanka)	Dry leaves	Methanol	FRAP	5.29 ± 0.85 mg TE/g plant material	Abeysiri et al., 2013
			Dry flowers			3.42 ± 0.59 mg TE/g plant material	
			Dry stems			1.42 ± 0.40 mg TE/g plant material	
			Dry leaves (field)	80% Methanol		09.23 ± 0.17 mg TE/g plant material	Abeysinghe et al., 2014
			Dry leaves (hydroponics)			10.27 ± 0.28 mg TE/g plant material	
			Dried callus			7.71 ± 0.61 mg TE/g plant material	
2	Gastro-protective	*A. oleracea* (Brazil)	Leaves (defatted and depigmented)	Chloroform: Methanol (2:1)	Ethanol induced gastric lesions	Rhamnogalacturonan: 1.5 mg/Kg BW of mice	Nascimento et al., 2013
3	Anti-proliferative	*S. acmella* (Taiwan)	Dry flowers	Hexane	MTT	25% inhibition	Wu et al., 2008
				Chloroform		19% inhibition	
				Ethyl acetate		09% inhibition	
				Butanol		07% inhibition	
		S. acmella (Thailand)	Dried aerial parts	Hexane		Above 10 μg/ml	Prachayasittikul et al., 2009
				Chloroform			
				Ethyl acetate			
				Methanol			
		S. acmella (Brazil)	Dry flowers	IBA-10		Above 100 μg/ml	Mbeunkui et al., 2011
				IBA-20			
				IBA-21			
				MBA-7			
		S. paniculata (India)	Dry flowers	Petroleum ether		43.16 μg/ml	Mishra et al., 2015
				Ethyl acetate		18.33 μg/ml	
				Ethanol		4.19 μg/ml	
		A. oleracea (Brazil)		Methanol		234 μg/ml	Gerbino et al., 2016
				Spilanthol (IBA-10)		260 μg/ml	

(*continued on next page*)

Table 2 (*continued*)

S. No	Type of Bioactivity	Species (Country)	Plant Part	Extraction Solvent	Bioassay	Extent of Biological Activity (% Scavenging or IC_{50})*	Reference
4	Immunomodulatory	*S. acmella* (India)	Dry leaves	95% Ethanol	Up - down or staircase method	500 mg/Kg BW of mice	Savadi et al., 2010
5	Diuretic	*S. acmella* (Sri Lanka)	Fresh flowers	Distilled water	Control Extract (0.5 g/Kg) Extract (1.0 g/Kg) Extract (1.5 g/Kg) Furosemide (13 mg/Kg)	10.2 ± 2.0 ml 08.6 ± 1.7 ml 06.7 ± 0.5 ml 53.4 ± 8.0 ml 08.1 ± 0.8 ml	Ratnasooriya et al., 2004
		S. paniculata (India)	–	–	Control Furosemide (0.01 g/Kg)	4.02 ± 0.35 ml 7.79 ± 0.27 ml	Ali et al., 2015
			Dry leaves	Distilled water	Extract (0.1 g/Kg) Extract (0.3 g/Kg) Extract (0.5 g/Kg)	6.27 ± 0.37 ml 6.39 ± 0.41 ml 6.95 ± 0.41 ml	
6	Vasorelaxant	*S. acmella* (Thailand)	Dried aerial parts	Hexane Chloroform Ethyl acetate Methanol	Isometric tension measurements	3.60×10^{-7} mg/ml 4.28×10^{-7} mg/ml 7.61×10^{-7} mg/ml 9.55×10^{-7} mg/ml	Wongsawatkul et al., 2008
7	Anti-inflammatory	*S. acmella* (Brazil)	Dry flowers, stem and leaves	Ethanol (85%)	Lipoxygenase inhibition	Flowers > Stem > Leaves	Dias et al., 2012
8	Enzyme Inhibition and Cytotoxicity	*A. oleracea* (Brazil)	Dry leaves, stem and inflorescence	Methanol	Tyrosinase inhibition	0.50 mM (CH_2Cl_2 fraction)	Barbosa et al., 2016
		S. acmella (Commercial)	Fresh plant	95% Ethanol	Cyt P_{450} mediated oxidation of p-nitrophenol	Significant increase at 25 µM	Raner et al., 2007
		S. acmella (Taiwan)	Dry flowers	Hexane fraction Chloroform fraction Ethyl acetate fraction Butanol fraction	RAW 267.4 macrophages	Below 80 µg/ml Below 80 µg/ml Above 80 µg/ml Above 80 µg/ml	Wu et al., 2008
		S. acmella (USA)	Dry flowers	IBA-10 IBA-20 IBA-21 MBA-7	Chinese hamster ovary cell line	Above 100 µg/ml	Mbuenkui et al., 2011
		S. calva (India)	Dried aerial parts	Isoprenylated flavonoid	Xanthine oxidase	16.56 µg/ml	Jayaraj et al., 2014b
		S. acmella (Brazil)	Dry flowers	96% Ethanol	HEp-2 L929 cell line	513 µg/ml	Soares et al., 2014
9	Anti-microbial	*S. acmella* (Thailand)	Dried aerial Parts	Hexane Chloroform Ethyl acetate Methanol	Agar dilution	0.256 mg/ml 0.064 – 0.256 mg/ml 0.064 mg/ml 0.128 – 0.256 mg/ml	Prachayasittikul et al., 2009
		A. uliginosa (West Indies)	Dry leaves	Dichloromethane Methanol Distilled water	Broth micro-dilution	0.625 – 1.25 mg/ml 1.25 mg/ml 5.0 mg/ml	Lagnika et al., 2016
		S. mauritiana (Africa)	Dried whole plant	Hexane Dichloromethane Acetone Methanol	Minimum Inhibitory Concentration (MIC)	2.50 mg/ml 1.96 mg/ml 1.96 mg/ml 1.72 mg/ml	Masoko, 2017

* VCE: Vitamin-C Equivalent, TE: Trolox Equivalent; '-': No data available.

Table 3
The list of foods and commercial products containing NAAs.

S. No	Product Name/ Produce	Manufacturer	Plant Source	Part	Format of Application	Function or Purpose	Reference/ Source of Information
1	Dr. SCHELLER	Dr. Scheller Cosmetics, Germany	*Spilanthes acmella*	Flowers	Cream	Anti-wrinkle	www.dr-scheller-cosmetics.com
2	SPILANTHES	Herb Pharm, USA		–	Liquid	Cleansing and Detoxification	www.herb-pharm.com
3	*Spilanthes* Paracress drops	A. Vogel, Switzerland	*Spilanthes oleracea*	Aerial parts		Prevention of infections	www.avogel.com
4	Dentaforce				Gel	Oral hygiene	
5	SPILANTHES [*ACMELLA OLERACEA*]	Herbal Terra, USA	*Acmella oleracea*		Liquid	Herbal Supplement	www.herbalterra.com
6	Buccaldol®	Alphamega, France	*Spilanthes* sp.	–	Gel	Analgesic and oral hygiene	www.medi-market.be
7	Gatuline® Expression AF	Gattefossé SAS, France	*Acmella oleracea*	Whole plants	Cream	Anti-wrinkle	Demarne and Passaro, 2008
8	Gatuline® In-Tense						
9	A composition of oral use	Takasago International Corp, Japan	*Spilanthes* sp.	Whole plants	Liquid	Organoleptic compositions	Nakatsu et al., 2001
10	Additive for Carbonated Beverage	Ogawa and Co. Ltd, Japan	*Spilanthes acmella*			Flavor improvement	Miyazawa et al., 2011
11	Leafy vegetable	Traditional	*Spilanthes* sp.	Aerial parts or roots	Infusions or decoctions	Wound healing	Paulraj et al., 2013
12			*Acmella oleracea*	Leaves	Sauces	As a vegetable, and to make de-worming sauce	Lagnika et al., 2016
13					Tea	As a beverage with analgesic property	Nascimento et al., 2013
14			*Acmella uliginosa*		Condiment	Flavoring and seasoning agents	Paulraj et al., 2013 Cruz et al., 2016
15	Flowers		*Spilanthes acmella*	Flowers	Raw spice	Appetizers	Nakatani and Nagashima, 1992
16	Whole plants		*Spilanthes americana*	Whole plants	Food	Columbian cuisine	Stashenko et al., 1996
17			*Spilanthes oppositifolia*		Spice	Mexican cuisine	Molina-Torres et al., 1996
18			*Spilanthes acmella*		Spice/ Food additive	Cuisines	Ley et al., 2006a
19			*Acmella oleracea*		Food	Brazilian typical recipes	Castro et al., 2014 Moreno et al., 2011
20	Aerial parts		*Acmella oleracea*	Aerial parts	Spice		Simas et al., 2013
21	Roots		*Spilanthes paniculata*	Root	Decoctions	Purgative	Mishra et al., 2015
22			*Spilanthes acmella*				Leng et al., 2011

'-': No data available.

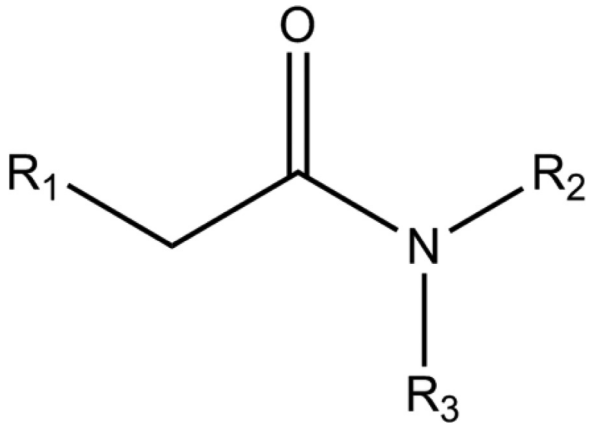

Fig. 1. Basic molecular skeleton of N-alkylamides (R_1 = fatty acyl chain; R_2 = residual amino acid moiety or its derivative; R_3 = $H/CH_3/OCH_3/OH$). Adopted from Boonen et al. (2012).

1985). While revising the status of *Spilanthes* in India, key details provided by Jansen appears to have been overlooked (Jansen 1981, 1985; Sivarajan and Remesan, 1987). According to Jansen, *Spilanthes* can be characterized by 16 chromosomes, while *Acmella* by 12 or 13 chromosomes (Jansen, 1981, 1985). Recently, the ploidy of *Acmella* was validated to be in multiples of 13, and suggested the possibility of producing the same by hybridization between the ancestral species with $n = 6$ and $n = 7$, followed by amphidiploidization (Rajalakshmi and Jose, 2011; Reshmi and Rajalakshmi, 2015; 2016a; 2016b). It was inferred that the polyploidy had greatly influenced these herbaceous genera and provided them with an advantage to grow in distinct habitual environments. Most of the rayed ones in the literature is still referred as *Spilanthes*, the same is followed in this review. The chronology of events clarifying the discrepancy in the taxonomy of genus *Spilanthes* or *Acmella* is presented in Supplementary Table 1. 'Flora of British India' by Hooker (1881) describes 4 species namely *S. acmella, S. calva, S. oleracea* and *S. paniculata* to occur in India. Species such as *S. radicans, S. uliginosa* and *S. ciliata* are suggested as immigrant to Indian subcontinent (Gamble, 1915; Hooker, 1981; Sivarajan and Philip, 1984). There continues an ambiguity in the past literatures over the generic specificity of plants that contains spilanthol. The misuse of botanical nomenclature within these genera often remain overlooked by research communities and folk herbalists (Saraf and Dixit 2002; Jirovetz et al., 2005; L. 2006; Nascimento et al., 2013; Freitas-Blanco et al., 2016, 2018a), whereas, internet sources have devastated the situation prevalently for plant researchers, herbal practitioners and traditional users by putting up plethora of botanical names. Until recently, research community has been lenient towards the use of *Spilanthes* in a broader synonymous sense (Saraf and Dixit 2002; Jirovetz et al., 2005; L. 2006; Moreno et al., 2011; Nascimento et al., 2013; Nomura et al., 2013; Freitas-Blanco et al., 2016, 2018a). The probable solution could be a global support and guidance for ascertaining, overcoming and subsequently fine-tuning the above raised quandaries with more and more evidence based studies on phytochemical, cytological as well as molecular characters which would surely ease the proper identification and usage of these valuable herbs in the near future (Rajalakshmi and Jose, 2011; Reshmi and Rajalakshmi, 2015; Barbosa et al., 2016).

4. General structure and biosynthesis of NAAs

The basic chemical skeleton of a NAA is presented in **Fig 1**. Almost all NAAs have one nitrogen atom bonded to three variable R groups. While R_1 invariably is a fatty acid chain, R_2 is the residual part of an amino acid and R_3 in most cases is a H-atom. Biosynthetic pathways are discovered by the use of isotope labeling experiments and by biochemical enzyme assays (Cortez-Espinosa et al., 2011; Rizhsky et al., 2016). Such studies indicate that NAAs are produced as a result of coupling of a fatty acid chain moiety (mostly unsaturated, rarely saturated) with an amine group by $O = C$–NH linkage (Boonen et al., 2012). This is an amide linkage that resembles the ubiquitously found in peptide bonds of proteins and has a partial double bond character owing to the presence of highly electronegative O and N atoms in near proximity with each other. There are nearly 200 fatty chains bonded to distinct amines found distributed in over 30 plant families including Asteraceae, Aristolochiaceae, Brassicaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Poaceae, Rutaceae, *etc.,* (Martin and Becker, 1984; Cortez-Espinosa et al., 2011; Rios, 2012; Boonen et al., 2012; Greger, 2015). The fatty acid chain length, degree of unsaturation, type of geometric orientation of the fatty chain and kinds of amine moieties afford enormous diversity to these molecules (Boonen et al., 2012; Rizhsky et al., 2016). A deduced biosynthetic pathway for NAAs in plants is presented in **Fig 3**. The fatty acid chain part of NAAs are derived from unsaturated fatty acid precursors such as oleic (18:1), linoleic (18:2) and linolenic (18:3) acids *via* chain shortenings at both carboxyl and methyl end (terminal) with successive dehydrogenation, dehydration and oxidative processes leading to the generation of characteristic alkene/alkyne products, modification of chain length and formation of epoxide structure respectively (Greger, 1984; Greger et al., 1985; Martin and Becker, 1985; Boonen et al., 2012; Silveira et al., 2016).

There are about 23 chemically distinct amine moieties found coupled to a variety of fatty acids that lead to occurance of diverse NAAs in plants **(Fig 2)**. Nine of these moieties are aliphatic (Fig 2A-I), 14 are aromatic (Fig 2J-W) structures and are derived from amino acids *via* decarboxylation reaction (Boonen et al., 2012; Greger, 2015). A polyacetylenic NAA was isolated from the basidiomycetes fungi *Poria sinuosa* is unique that its fatty acid part is coupled to an entire molecule of valine without any decarboxylation (Cambie et al., 1963). Valine serves as the precursor for isobutylamides (Fig 2A) and its derivatives such as hydroxy-isobutylamides (Fig 2B-C) and dehydro-isobutylamides (Fig 2D). The sole amine possessing N- methylation (Fig 2F) is said to have been derived from valine with an additional methylation. Isoleucine serve as precursor for methylbutylamides (Fig 2E), leucine serves for isopentylamine (Fig 2H), phenylalanine and tyrosine for the aromatic amines particularly for those having a phenylpropanoid skeleton such as phenylethylamine (Fig 2J), 4-methoxy phenylethylamine (Fig 2K), tyramine (Fig 2L), 4-hydroxy-5-methoxyphenylethylamine (Fig 2M), *E*-styrylamine (Fig 2N), *Z*-styrylamine (Fig 2O), benzylamines (Fig 2P) and its methoxy derivative 3-methoxy-benzylamine (Fig 2Q). The amines (Fig 2P and 2Q) reported from *Capsicum* species are exception to conventional NAAs with the prime member 'capsaicin' comprising of the fatty acid part derived from valine *via* deamination followed by chain elongation by a fatty acid synthase, and subsequently derives the amine group from aromatic amino acid phenylalanine (Stewart et al., 2007). On the other hand, lysine or cadaverine serves for piperidine (Fig 2R), piperideine (Fig 2S), and hydroxy-piperideine (Fig 2T). Ornithine/putrescine or proline serves for five-membered amines like pyrrolidine, pyrrolideine and pyrroline (Fig 2U-W) (Strunz, 2000; Boonen et al., 2012; Greger, 2015). The precursors for isohexylamine (Fig 2I), reported from *Piper nigrum,* remain unclear at the moment and cysteine is suggested as precursor for the only sulfur-containing amine (Fig 2G) reported from *Piper boehmeriifolium* (Tang et al., 2011; Greger, 2015).

Within Asteraceae, the occurance of MBAs in members of tribe Heliantheae (to which *Spilanthes* belongs), distinguishes themselves from the tribe Anthemideae (to which belong *Anacyclus* and *Piper*), with their members producing IBA (Fig 2A), piperidine (Fig 2R-T) and pyrrolidine (Fig 2U-W) type NAAs (Martin and Becker, 1984; Boonen et al., 2012). A set of IBAs also occurs in its hydroxylated (mono and di), de-hydroxylated forms (Fig 2B-D) and rarely with a methyl group substituting the R_3 hydrogen (Fig 2F). The PEA moiety is present in its methoxylated or hydroxylated forms or both (Fig 2K-M). Sometimes

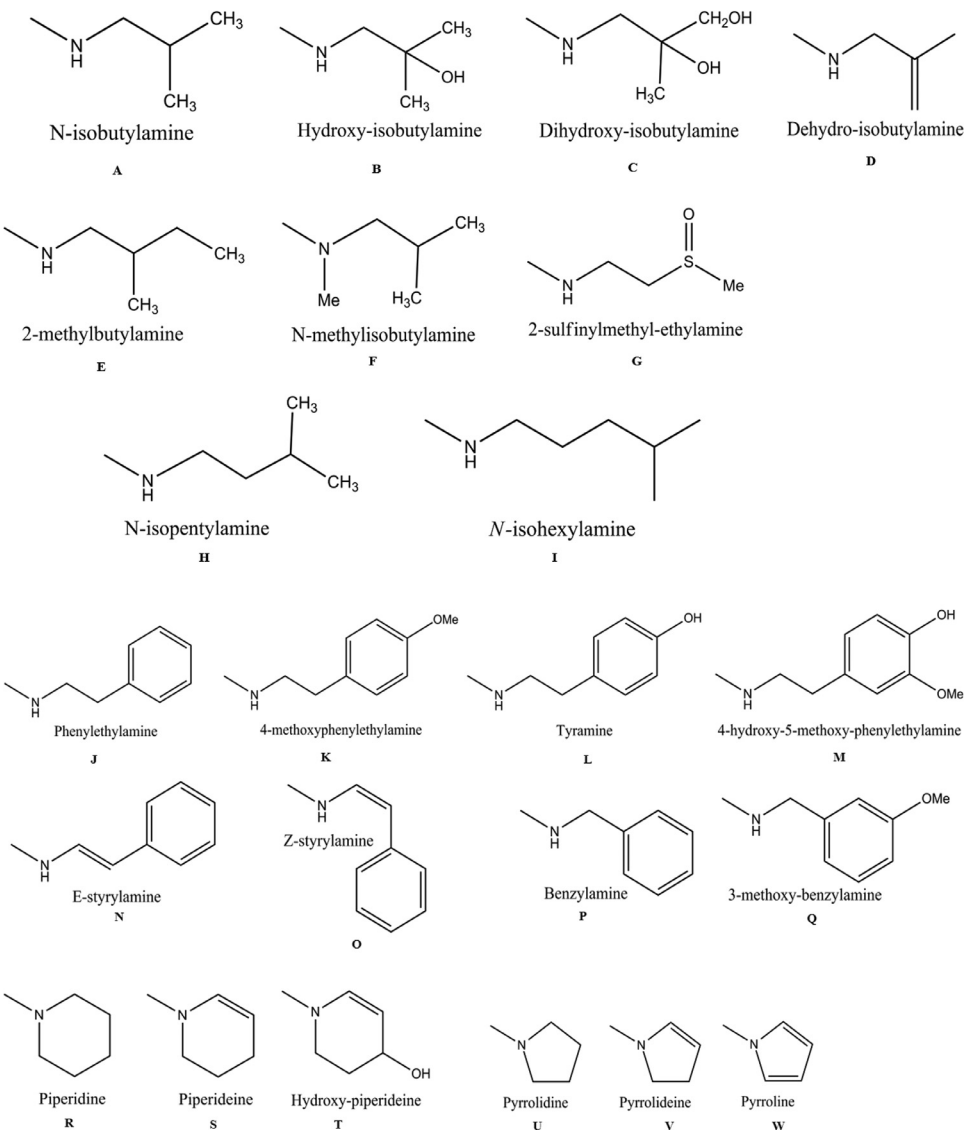

Fig. 2. Common amine moieties found in N-alkyamides of the plants (adopted from Greger, 2015).

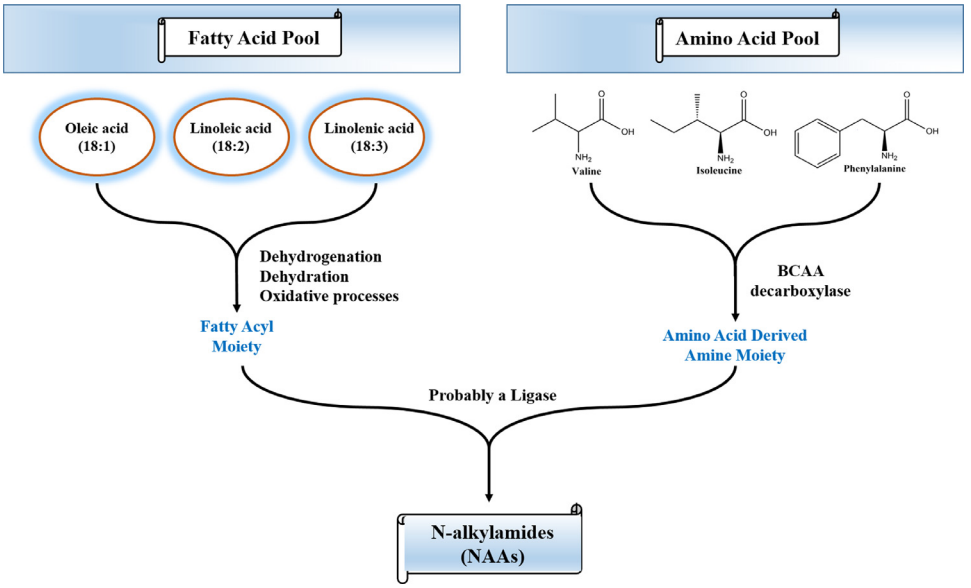

Fig. 3. A deduced biosynthetic pathway for N-alkylamides in plants reconstructed from Cortez-Espinosa et al., 2011; Boonen et al., 2012; Greger, 2015; Shephard Jr., 2013; Rizhsky et al., 2016.

they are found with an extra unsaturation at the carbon bonded to the amine (Fig 2N and 2O) (Greger, 2015). Occurance of NAAs carrying a PEA moiety and cinnamic acid chain was reported from leaves extracts of *S. ocymifolia* (Borges-Del-Castillo et al., 1984). It turns out that NAAs found in *Spilanthes* species fall under three major categories; (a) Isobutylamides (IBAs), (b) Methylbutylamides (MBAs) and (c) Phenylethylamides (PEAs) (Bae et al., 2010; Rios, 2012; Simas et al., 2013; Bhat et al., 2016). All together there were 49 NAAs isolated and characterized from different species of *Spilanthes* **(Table 4)**.

The final coupling step in biosynthesis of NAA has been elusive. It is however agreed that this is an enzyme mediated coupling step that results in the release of a molecule of water as a byproduct (Shephard Jr., 2013). In contrast, structurally related capsaicinoids present in the members of Solanaceae seems evolutionary different with both the acid and amine derived from amino acids *via* shikimic acid and polyketide pathways. It appears that in capcaisonoids biosynthesis there is a merging of the phenylpropanoid and the fatty acid synthesis pathways (Stewart et al., 2007; Mazourek et al., 2009). Recently a branched chain amino acid decarboxylase (BCAA decarboxylase) has been recombinantly expressed in *E. coli*, followed by the conversion of amino acids to amine with near equal specificity for both valine and isoleucine producing IBA and MBA respectively (Rizhsky et al., 2016). The expression of the BCAA decarboxylase was correlated with altered levels of NAAs in *Echinacea purpurea,* an Asteraceae member, treated with a pool of its endophytic bacteria suggesting a significant influence of plant-endophyte interactions in NAA biosynthesis (Maggini et al., 2017).

There are at least three different types of classifications followed for grouping of NAAs. Of the three, the one described by Boonen et al. (2012), defined simply as F_xM_y nomenclature, is the latest and more comprehensive for NAAs. According to this classification, an NAA should have at least one methyl group present at the amine side of the general formula of NAA, $ROCNH_2$. The NAAs were then grouped into 25 classes on the basis of saturation, unsaturation, heterocyclic-aromatic, heterocylic-non aromatic, carbocyclic-aromatic, carbocyclic-non aromatic fatty acid moiety in combination with an amino group which could either be substituted or non-substituted with a heteroatom (N,O,S), thus giving a total of 24 ($6 \times 2 \times 2$) classes. In addition, a cyclic amide (*e.g.* pyridine derivatives) is included as the 25th class. Based on this classification an online repository named "Alkamid®" has been put in place which currently has information on nearly 400 NAAs. There has also been an attempt to evolve a simple short-hand nomenclature scheme for naming NAAs. For example IBA-30 could be annotated as '$i4^N$-12:2$\Delta^{2E,4Z,8a,10a}$', where, '$i4^N$' represents that coupling of isobutyl chain with amine nitrogen, 12:2 shows the chain length and degree of unsaturation, the following numerals (2, 4) and letters (*E, Z*) in subscripts represent the position and orientation of double bonds (at 2nd and 4th position in *trans* (*E*) and *cis* (*Z*) configuration respectively). The following alphabet 'a' represents place of a triple bond if found in the fatty chain. If there is uncertainty in geometric configuration (*E* or *Z*) it can be indicated simply by numbers without assigning any symbol to denote the isomeric configuration. The same IBA-30 above for example in this situation will be represented as $i4^N$-12:2$\Delta^{2,4,8a,10a}$ for unknown 2nd and 4th position. The place of 'N' is substituted for molecules that have a S such as a thioester (Rizhsky et al., 2016).

5. Extraction, quantification and characterization of NAAs from *Spilanthes* species

The type of NAA and their content differed in different plants, plant parts and developmental stages with a further modification induced by the artificial and natural habitats in which they grow. NAAs have been isolated from flowers (Martin and Becker, 1984; Kadir et al., 1989; Nakatani and Nagashima, 1992; Ramsewak et al., 1999; Rios-Chavez et al., 2003; Wu et al., 2008; Pandey et al., 2011; Mbeunkui et al., 2011; Sharma et al., 2012), leaves (Singh and Chaturvedi, 2012; Simas et al., 2013), roots (Casado et al., 2009) and whole plants (Rios-Chavez et al., 2003; Li et al., 2007) of *Spilanthes* species **(Table 2)**. The variety of organic solvents used for extraction range from non-polar hexane to the most polar water (Ramsewak et al., 1999; Wu et al., 2008; Ratnasooriya et al., 2004; Ali et al., 2015). An overview of the methods employed for extraction, purification and characterization of NAAs is presented in **Fig 4**. The extraction methods adopted ranged from homogenization (Martin and Becker, 1984; Molina-torres et al., 1996; Rios-Chavez et al., 2003), cold percolation (Kadir et al., 1989), soaking (Jondiko, 1986; Leng et al., 2011; Singh and Chaturvedi, 2012), maceration (Phrutivorapongkul et al., 2008; Bae et al., 2010), soxhlet extraction (Casado et al., 2009; Sharma et al., 2011) to supercritical fluid extraction (Stashenko et al., 1996; Dias et al., 2012; Freitas-Blanco et al., 2018a). Among the conventional methods alcohol-based extraction often yielded better quantity of NAAs owing to a higher capability of alcohols to break the amide-amide interactions (Mbeunkui et al., 2011). Among various extraction methods, supercritical fluid extraction (SFE) was the most effective method for extraction of spilanthol (yielding upto 65% of dry weight) from flowers of *S. acmella* and *S. americana* (Dias et al., 2012; Stashenko et al., 1996). Centrifugal partition chromatography allowed a higher recovery of NAAs from the flowers of *S. acmella* as compared to traditionally used purification methods such as TLC and column chromatography (Mbeunkui et al., 2011). HPTLC-MS, a simple automated method used in separation sciences for identification of biological molecules as shown earlier for antimicrobials, is yet to be explored for separation and identification of NAAs from *Spilanthes* (Kasote et al., 2015).

Among the methods used for identification and quantification of NAAs in crude extracts, reverse phase HPLC turns out to be the most preferred **(Table 5)**. A range of HPLC chromatographic parameters such as composition of mobile phase and flow rates were tested and the most optimal combination was adopted **(Table 6).** Though a variety of NAA were identified by HPLC, the quatification of the individual NAA content was mostly done for IBA-10, IBA-26 and IBA-34 (**Tables 5** and **Table 6**). The NAA content was generally expressed in mg/g by fresh or dry weight basis of the plant material or of the crude extract. Acetonitrile and water when used as mobile phase in varying ratios resulted in varied retention time ranging from 4.97 to 64.2 min for prime NAA spilanthol (Bae et al., 2010; Sharma et al., 2011). The alkyne (or acetylenic) NAAs eluted (IBA-26) early in the reverse phase HPLC compared to the alkene (or olefinic) NAA (IBA-34) due to their differential affinity towards the stationary phase (Mudge et al., 2011; Rajendran et al., 2017). HPLC of ethanol extracts revealed spilanthol content (by dry weight) to be low (0.99 mg/g) in leaf-disk derived callus cultures of *S. acmella* as compared to leaves of *in vitro* derived (3.29 mg/g) and field grown (2.70 mg/g) plants (Singh and Chaturvedi, 2012). To date, the highest recorded spilanthol content is reported to be 84.52 mg/g in the ethanolic extract of flowers, followed by roots and aerial parts of *A. oleracea* (Cheng et al., 2015). Rajendran et al. (2017), however, observed a higher content of an acetylenic NAA (IBA-27, an isomer of IBA-26) in the floral heads as well as floral cultures of *S. paniculata* (on dry weight basis) as compared to spilanthol (IBA-10).

GCMS is the second most important analytical technique employed for identification and quantification of phyto-constituents of various *Spilanthes* species (**Table 7;** Baruah and Leclercq, 1993; Rios-Chavez et al., 2003; Jirovetz et al., 2005, L. 2006; Dias et al., 2012; Maimulyanti and Prahadi, 2016). This technique revealed a highest spilanthol content in stem, followed by flowers, leaves and roots of *in-vitro* grown plant, whereas, it was maximum in flowers of field grown plants (Rios-chavez et al., 2003; Dias et al., 2012). The choice of method for quantification of NAA could however be arrived at if the methods are employed simultaneously for estimation of compounds in the sample. The conglomerate of published reports and our own experience suggest that biosynthesis of spilanthol occurs in all part of the *Spilanthes* plant but the flower heads remain the major site of accumulation of NAAs. A range of techniques such as FTIR, HPLC, LCMS, HRMS and NMR were used to characterize individual NAAs purified from *Spilanthes* species

Table 4
List of major N-alkylamides reported from *Spilanthes* species.

Compound ID	Chemical Name*
(IBA) Isobutylamides	
IBA-1	(2E)-N-isobutyl octamonoenamide
IBA-2	(2E,4Z)-N-isobutyl octadienamide
IBA-3	(2E)-N-isobutyl-2-decamonoenamide
IBA-4	(2E,7Z)-N-isobutyl-2,7-decadienamide
IBA-5	(6Z,8E)-N-isobutyl-6,8-decadienamide
IBA-6	(4E,6E)-N-isobutyl-4,6-undecen-10-inamide
IBA-7	(2E,6Z)-N-isobutyl-8,9-dihydroxy-2,6-decadienamide
IBA-8	(2E,7E)-N-isobutyl-6,9-dihydroxy-2,7-decadienamide
IBA-9	(2E,7Z)-N-isobutyl-6,9-endoperoxy-2,7-decadienamide
IBA-10	(2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide (Spilanthol)
IBA-11	(2E,7Z,9E)-N-isobutyl-2,7,9-undecatrienamide
IBA-12	(2E,6Z)-N-isobutyl-2,6-dodecadienamide
IBA-13	**(2E,4E)-N-isobutyl-2,4-dodecadienamide**
IBA-14	**(2E,4E,8Z)-N-isobutyl-2,4,8-dodecatrienamide**
IBA-15	**(2Z,4E,8Z)-N-isobutyl-2,4,8-dodecatrienamide**
IBA-16	**(2E,4Z,10E)-N-isobutyl dodecatriene-8-ynamide**
IBA-17	**(2E,4E,10E)-N-isobutyl dodecatriene-8-ynamide**
IBA-18	**(2E,4Z,10Z)-N-isobutyl dodecatriene-8-ynamide**
IBA-19	(2Z)-N-isobutyl-2-decene-6,8-diynamide
IBA-20	(2Z)-N-isobutyl-2-nonene-6,8-diynamide
IBA-21	(2E)-N-isobutyl-2-undecene-8,10-diynamide
IBA-22	**(2Z)-N-isobutyl-2-undecene-8,10-diynamide**
IBA-23	**(2E)-N-isobutyl-2-dodecene-8,10-diynamide**
IBA-24	(7Z)-N-isobutyl-7-tridecene-10,12-diynamide
IBA-25	**(2E,7Z)-N-isobutyl-2,7-tridecadiene-8,10-diynamide**
IBA-26	(2E,4E)-N-isobutyl-2,4-undecadiene-8,10-diynamide (NUD)
IBA-27	(2E,4Z)-N-isobutyl-2,4-undecadiene-8,10-diynamide
IBA-28	**(2Z,4E)-N-isobutyl-2,4-undecadiene-8,10-diynamide**
IBA-29	(2E,4E)-N-isobutyl-2,4-dodecadiene-8,10-diynamide
IBA-30	**(2E,4Z)-N-isobutyl-2,4-dodecadiene-8,10-diynamide**
IBA-31	**(2Z,4E)-N-isobutyl-2,4-dodecadiene-8,10-diynamide**
IBA-32	(2E,5Z)-N-isobutyl-2,5-undecadiene-8,10-diynamide
IBA-33	(2E,7Z)-N-isobutyl-2,7-tridecadiene-10,12-diynamide
IBA-34	(2E,4E,8Z,10E)-N-isobutyl-2,4,8,10-dodecatetraenamide (NDT)
IBA-35	(2E,4E,8Z,10Z)-N-isobutyl-2,4,8,10-dodecatetraenamide
IBA-36	(2E,4Z,8Z,10E)-N-isobutyl-2,4,8,10-dodecatetraenamide
IBA-37	(2E,4E,8E,10Z)-N-isobutyl-2,4,8,10-dodecatetraenamide
IBA-38	**(2E,4E,8E,10E)-N-isobutyl-2,4,8,10-dodecatetraenamide**
IBA-39	(2E,4E,9E)-N-isobutyl-8,11-dihydroxy-2,4,9-dodecatrienamide
IBA-40	(2E,8E)-N-isobutyl-7-hydroxy-2,8-tridecadiene-10,12-diynamide
IBA-41	N-isobutyl decanamide
IBA-42	(2E)-N-isobutyl-2-decamonoenamide
IBA-43	**(2E,9Z) N-isobutyl hexadeca-2,9-diene-12,14-diyamide**
IBA-44	(2E,9Z) N-isobutyl pentadeca-2,9-diene-12,14-diynamide
IBA-45	(2E,4E) N-isobutyl nona-2,4-dien-8-ynamide
Methylbutylamides (MBA)	
MBA-1	(2E)-N-(2-methylbutyl)–2-undecene-8,10-diynamide
MBA-2	**(2E,4E)-N-(2-methylbutyl)–2,4-dodecadienamide**
MBA-3	**(2E,4E)-N-(2-methylbutyl)–2,4-undecadiene-8,10-diynamide**
MBA-4	**(2E,4Z)-N-(2-methylbutyl)–2,4-undecadiene-8,10-diynamide**
MBA-5	**(2Z,4E)-N-(2-methylbutyl)–2,4-undecadiene-8,10-diynamide**
MBA-6	**(2E,4Z)-N-(2-methylbutyl)–2,4-dodecadiene-8,10-diynamide**
MBA-7	(2E,6Z,8E)-N-(2-methylbutyl)–2,6,8-decatrienamide
MBA-8	(2E,4Z,8E,10E)-N-(2-methylbutyl)–2,4,8,10-dodecatetraenamide
MBA-9	(2E,4E,8Z,10E)-N-(2-methylbutyl)–2,4,8,10-dodecatetraenamide
MBA-10	**(2E)-N-(2-methylbutyl)-dodeca-2-ene-8,10-diynamide**
MBA-11	**(2E,9Z)-N-(2-methylbutyl)-pentadeca-2,9-diene-12,14-diynamide**
Phenylbutylamides (PEA)	
PEA-1	(2Z)-N-(2-phenylethyl)–2-nonene-6,8-diynamide
PEA-2	(2E)-N-(2-Phenylethyl)–2-nonene-6,8-diynamide
PEA-3	(2Z)-N-(2-Phenylethyl)–2-decen-6,8-diynamide
PEA-4	(2Z,4E)-N-(2-Phenylethyl)–2,4-undecadiene-8,10-diynamide
PEA-5	(2E,4Z)-N-(2-Phenylethyl)–2,4-octadienamide
PEA-6	(2Z,4E)-N-(2-Phenylethyl)–2,4-octadienamide
PEA-7	(3E,5E)-N-(2-Phenylethyl)–3,5-undecadiene-8,10-diynamide
PEA-8	(2E,4E)-N-hydroxy-(2-phenylethyl)–2,4-decadien-9-inamide
PEA-9	(2E,6Z,8E)-N-(2-Phenylethyl)–2,6,8-decatrienamide
PEA-10	(3E,6Z,8E)-N-(2-Phenylethyl)–3,6,8-decatrienamide
PEA-11	3-Phenyl-N-(2-Phenylethyl)–2-propenamide
PEA-12	N-(2-Phenylethyl)–2,3-epoxy-6,8-nonadiynamide
PEA-13	N-(2-Phenylethyl)–2,3-dihydroxy-6,8-nonadiynamide
PEA-14	(2E,4E)-N-(2-Phenylethyl) undeca-2,4-diene-8,10-diynamide

* Those in bold are restricted to *Echinacea*.

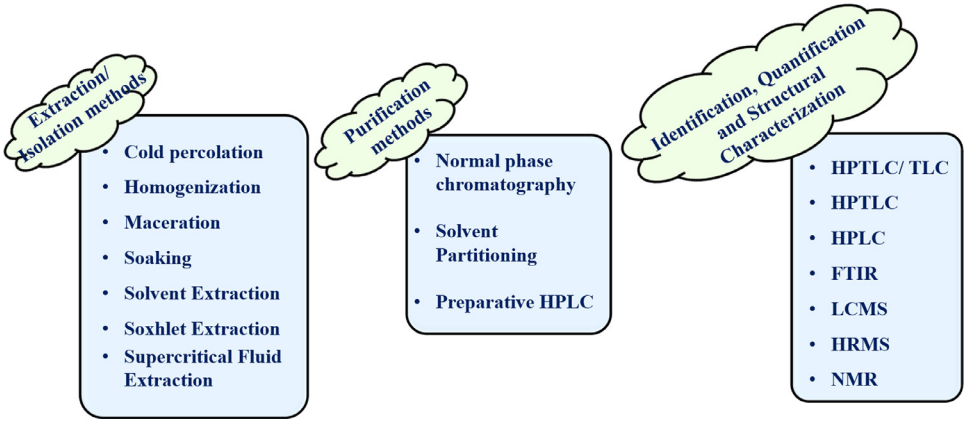

Fig. 4. A graphical presentation of the methods employed for extraction, isolation, purification and characterization of N-alkylamides.

Table 5
Quantitation of major NAAs of *Spilanthes* by HPLC.

S.No	Plant	Part of the plant	Extraction Method	Extraction Solvent	N-alkylamides (mg/g dry weight of plant material) IBA-10 (Spilanthol)	IBA-26 (NUD)	IBA-34 (NDT)	Reference
1	*Acmella oppositifolia*	Dried underground tissues	Maceration and Soaking (overnight)	Ethyl acetate	0.35	–	0.028	Molina-Torres et al., 1996
		Dried aerial tissue			0.17		0.0055	
2	*Spilanthes acmella*	Fresh whole plant	Maceration (7 days)	Water	01.40	–	–	Bae et al., 2010
				10% Ethanol	01.60			
				25% Ethanol	04.20			
				50% Ethanol	11.00			
				75% Ethanol	14.00			
				95% Ethanol	14.00			
3	*S. acmella*	Leaves (*in vitro* grown)	Soaking (12 h)	Methanol	3.29 ± 0.01	–	–	Singh and Chaturvedi, 2012
		Leaves (field grown)			2.70 ± 0.00			
		Callus (*in vitro* grown)			0.99 ± 0.01			
4	*Acmella oleracea*	Flower	Extraction	95% Ethanol	84.52 ± 0.81[A]	–	–	Cheng et al., 2015
		Aerial part			56.60 ± 3.14 [A]			
		Root			77.98 ± 0.13 [A]			
		Field grown (dried roots)			6.9			
5	*Spilanthes paniculata*	Flower	Soaking (12 h)	Methanol	0.83 ± 0.12	5.29 ± 0.05	–	Rajendran et al., 2017.
		Callus (flower)			2.23 ± 0.04	4.30 ± 0.22	–	
		Leaves (Field)			0.26	–	–	Rajendran and Chaturvedi, 2017
		Callus			1.75			

[A] – Values are in mg/g dry weight of the extract.

(Table 8). NMR turns out to be the method of choice for ascertaining the geometrical structure and configuration of NAAs (Martin and Becker, 1984; Ramsewak et al., 1999; Cech et al., 2006a; Matovic et al., 2007; N.J. 2011).

6. Chemical synthesis

Since initial isolation of spilanthol by Gerber in 1903, numerous chemical synthetic schemes have been put forward for synthesis of spilanthol with correct geometric configuration. Crombie and co-workers (1963) was first to demonstrate the chemical synthesis of spilanthol. Subsequently synthesis of the same *via* addition of allyltitanium over an aldehyde group as a key step yielded 88% pure spilanthol (Ikeda et al., 1984, 1987). A shortest route for spilanthol synthesis has been co-trimerization of one part of an unsaturated aldehyde (acrolein) with two parts of a acetylene (flammable gas) followed by palladium catalyzed coupling of MeZnCl with the vinylic bromide along with decarboxylated valine, an isobutyl amine residue, afforded spilanthol with 55% overall yield (Wang et al., 1998). Yet another method followed a controlled route for synthesis of the alkene geometry of the molecule (Alanso et al., 2018) or stereoselectively synthesized spilanthol from 4-bromobutanol in 6 steps with 47% overall yield (Nakamura et al., 2020). In all these methods the spilanthol yield ranged from 18 to 55%.

Apart from spilanthol, NAA such as *cis*-pellitorine [(2*E*,4*Z*)-*N*-isobutyl deca-2,4-dienamide] of *Artemisia dracunculus* was synthesized using lipase mediated conversion of a pearl ester (ethyl 2*E*,4*Z*-decadienoate) with ~80% productivity (Ley et al., 2004). An acetylenic NAA (IBA-28), present in *Echinacea,* was synthesized through organometallic coupling with the unsaturated amide moiety (Kraus et al., 2006). Ley et al. (2006b) demonstrated synthesis of a series of natural (IBA-10 MBA-7) and neoNAAs, and evaluated their sensory properties. One of the study shows stereo-selective synthesis of isomeric C_{12} NAAs (IBA-34, 35, 37 and 38) to be successful (Matovic et al., 2007). Synthesis of IBA-12 using two different routes proved that electrochemical synthesis (95%) was better as compared to chemical synthesis (57%) for yield (Palma et al., 2009). In addition, chemical synthesis of major *Echinacea* NAAs have been shown previously (N.J. Matovic et al., 2011). However, reports concerning scalable experiments for attaining

Table 6
Details of the HPLC parameters employed for identification and quantification of NAA in extracts from *Spilanthes* species.

S. No	Column specification and dimensions	Mobile Phase Used	Detector and Detection Lambda (λ)	Flow Rate (ml/min)	Retention Time for Spilanthol (min)	Reference
1	RP-8 (4 × 250 mm, 7 µm)	Methanol: Ethyl acetate (3:2 to 17:3)	ND	ND	ND	Martin and Becker, 1984
2	RP-18 (ND)	Acetonitrile: Water (40–60%)	PDA (ND)	ND	ND	Molina-Torres et al., 1996
3	Hichrom Excil 100–5 ODS (4.6 mm x 250 mm)	Methanol (10 to 100%)	PDA (228 and 280 nm)	1.0	ND	Wongsawatkul et al., 2008
4	RP-18 (4.6 × 250 mm, 5 µm)	A - 1% Acetic acid in water, B - Acetonitrile (A:*B* = 80:20)	UV (237 nm)	1.0	62.37	Boonen et al., 2010
5	C-18 (2.1 × 50 mm, 3 µm)	A - 1% Acetic acid in water B - Acetonitrile (A:*B* = 50:50)	PDA (254 nm)	0.2	4.97	Bae et al., 2010
6	Eclipse XDB-C18 (4.6 mm × 250 mm, 5 µm)	A - 0.1% Formic acid in water B - 0.1% formic acid in methanol (5–95–5% B over 15 min, 2 min hold and return to 5% over 1 min, final hold of 4 min at 5% B	PDA (254)	1.0	16.53	Mbuenkui et al., 2011
7	C-18 (4.6 × 250 mm, 5 µm)	A - 1% acetic acid in water, B - Acetonitrile (A:*B* = 50:50)	PDA (237 nm)	1.0	64.2	Sharma et al., 2011
8	Hypersil BDS RP-18 (4.6 × 250 mm)	Acetonitrile: water (93:7)	UV (237 nm)	0.5	7.34 ± 0.12	Singh and Chaturvedi, 2012
9	C-18 (4.6 × 250 mm)	Acetonitrile: water (45:55)	PDA (237 nm)	1.2	8.5	Cheng et al., 2015
10	Hypersil BDS RP-18 (4.6 × 250 mm)	Acetonitrile: water (60:40)	UV (237 nm)	0.5	13.45 ± 0.09	Rajendran et al., 2017
		Acetonitrile: water (93.7)			7.32	Rajendran and Chaturvedi, 2017

ND: Not Data available.

immense quantities of NAAs in desired pure form is still a major milestone to fulfill the global demand of scarcely available NAAs and evaluate their true clinical efficacy.

7. Biological activities

In this section varied biological properties attributed to the crude extracts and the purified NAAs obtained from different *Spilanthes* species is discussed. There are atleast nine biological properties that the extracts impart on higher organism (**Table 1** and **Table 2**). The remaining are the larvicidal, insecticidal, acaricidal and anthelmintic properties (**Table 9** and **Table 10**). In addition the metabolism of NAAs in human and their role in plant tissue cultures is also discussed.

7.1. Anti-oxidant

Antioxidant properties is the foremost biological property demonstrated for *Spilanthes* extracts. Antioxidant potential of *Spilanthes* extracts is generally tested by assays such as DPPH, ABTS and FRAP, with DPPH being the most preferred one (Wu et al., 2008; Wongsawatkul et al., 2008; Prachayasittikul et al., 2009; Abeysiri et al., 2013; Singh et al., 2014). Among several solvents extracts, the ethyl acetate and methanol extracts (of *S. acmella)* showed maximum DPPH radical scavenging activity with a IC_{50} of 216 and 223 µg/ml respectively (Wongsawatkul et al., 2008). For *S. acmella,* the DPPH radical scavenging was higher for *in-vivo* leaves compared to *in-vitro* callus demonstrating an organized structure to be a better source than unorganized callus (Singh et al., 2014). Among the plant parts, often flowers exhibited most of the antioxidant activity **(Table 1).** FRAP assay revealed a greater antioxidant potential for *A. oleracea* leaf extract as compared to the stem and flower extracts, thus indicating higher accumulation of phenolic compounds in these structures of the plant (Abeysiri et al., 2013). A hydroponically grown *A. oleracea* showed a greater antioxidant potential as compared the field grown and those raised from tissue cultures (Abeysinghe et al., 2014).

7.2. Anti-inflammatory and immunomodulatory

A pathogenic attack in an organism triggers production of chemotactic cytokines in macrophages which in turn leads direction of the circulatory neutrophils and monocytes to the site of inflammation or the attack (Matthias et al., 2007; Novarski et al., 2013). The inflammation and immunomodulation assays revealing the levels of mRNA or the inflammatory molecules such as nitric oxide (NO), prostaglandin E2 (PGE2), cytokines in macrophages aid in detection of the underlying cause and in making of treatment plans. Anti-inflammatory drugs are used to minimize the inflammation and pain. *S. acmella* extract acted as anti-inflammatory agent when it inhibited carrageenan-induced paw edema in albino wistar rats and protected them from acetic acid-induced writhing (Chakraborty et al., 2004). The treatment resulted in an increase in the macrophages count, leading to greater carbon clearance and immuno-prophylactic effects in the treated group as compared to the control (Savadi et al., 2010). Another study showed enhancement of neutrophil adhesion, haemagglutinating antibody titre and delayed hypersensitivity response in wistar male rats (Yadav et al., 2011).

In their pure form, NAAs inhibited the production of pro-inflammatory molecules in macrophages exposed to bacterial lipopolysaccharide (LPS) and influenza-A viruses (Lalone et al., 2007, 2009; Cech et al., 2010). Spilanthol, in particular, induced reduction in the levels of inducible NO synthase (iNOS), IL-8, TNF-α, mRNA and expression of COX enzymes. It also caused an increase in release of interleukins (IL-1β, IL-6) and tumor necrosis factor (TNF-α). It hindered the phosphorylation of cytoplasmic inhibitor-κB and DNA binding activity of NF-κB, indicating its inhibitory action on NF-κB (Matthias et al., 2007; Wu et al., 2008; Freitas- Blanco et al., 2018a). Inhibition of cytokine production, modulation of cAMP, activation of JNK, p38/MAPK kinases, ATF-2/CREB-1 are also suggested (Gertsch et al., 2004; J. 2006; Gertsch, 2008; Sharma et al., 2009). NAAs have been shown as potential ligands of cannabinoid receptors, thus forming a platform for identification of potential NAA candidates for treatment of various anti-inflammatory diseases. The structural

Table 7
Quantitation of major NAAs of *Spilanthes* by GCMS.

S.No	Plant	Part of the plant	Extraction Method	Extraction Solvent	N-alkylamides IBA-10 (Spilanthol)	IBA-26 (NUD)	IBA-34 (NDT)	Reference
1	*Spilanthes americana*	Fresh flowers	SFE	CO_2	17.1 ± 0.02 *	–	–	Stashenko et al., 1996
		Fresh leaves			21.4 ± 2.00 *			
		Fresh stem			10.1 ± 0.04 *			
		Fresh flowers	Steam distillation	CH_2Cl_2	1.50 ± 0.31 *			
		Fresh leaves			1.37 ± 0.91 *			
		Fresh stem			3.09 ± 0.31 *			
2	*Acmella radicans*	Fresh roots (*in vitro* grown)	Homogenization	Ethanol	0.05 ± 0.01 ^	–	–	Rios-Chavez et al., 2003
		Fresh stem (*in vitro* grown)			12.10 ± 2.30 ^			
		Fresh leaves (*in vitro* grown)			1.16 ± 0.11 ^			
		Fresh flowers (*in vitro* grown)			8.54 ± 0.07 ^			
		Fresh roots (field grown)			110.00 ± 10.74 ^	4.26 ± 0.36 ^	1.25 ± 0.07 ^	
		Fresh stem (field grown)			52.06 ± 10.03 ^	–	–	
		Fresh leaves (field grown)			4.94 ± 1.74 ^			
		Fresh flowers (field grown)			254.70 ± 6.50 ^			
3	*Spilanthes acmella*	Dry flower	Hydro-distillation	Water	02.8 ± 0.30 *	–	–	Dias et al., 2012
			Soxhlet extraction	Ethanol	25.7 ± 1.50 *			
			SFE	CO_2	65.4 ± 0.90 *			
		Dry leaves	Hydro-distillation	Water	09.7 ± 0.05 *			
			Soxhlet extraction	Ethanol	02.0 ± 0.01 *			
			SFE	CO_2	19.7 ± 0.20 *			
		Dry stem	Hydro-distillation	Water	18.8 ± 1.30 *			
			Soxhlet extraction	Ethanol	05.0 ± 1.10 *			
			SFE	CO_2	47.3 ± 4.50 *			

SFE: Supercritical Fluid Extraction.
* : Values are in percentage by dry weight of the tissue.
^ : Values are in μg/g fresh weight of the plant material.

transformation of the known NAAs may enhance both their receptor affinities and downstream pathways. Interaction of NAAs with the human (animal) cannabinoid receptors is a clear indication of an evolutionary convergence.

7.3. Anti-nociceptive

The flower extract from a couple of *Spilanthes* species have shown antinociceptive property. For example orally administered *S. acmella* flower extract attenuated persistent pain and hyperalgesia in albino rats *via* inhibition of prostaglandin synthesis, interruption of nociception transmission and exhibiting anti-histamine activity (Ratnasooriya and Pieris, 2005). Whole plant ethanolic extract of *S. ciliata* administered to normal rats was correlated with decreased levels of serum enzymes such as glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, bilirubin and alkaline phosphatase, increase in bile secretion (chloretic activity) and shortening of hexobarbitone-induced sleeping in swiss albino mice and elicitation of anti-lipid peroxidation properties (Suja et al., 2003). Such activities are indicative of effects due to radical scavenging and inhibition of liver enzymes involved in lipid peroxidation by the components of the *Spilanthes* extract. When administered intraperitoneally, spilanthol (30 mg/Kg) blocked the acetic acid-induced abdominal writhing and capsaicin-induced nociception in mice. The naltrexone (opioid receptor antagonist), p-chlorophenyl-alanine (inhibitor of serotonin synthesis) and flumazenil (antagonist of GABA receptor) were able to block the spilanthol-induced antinociception suggesting the involvement of opiodergic, serotoninergic and GABAergic systems (Deciga-Campos et al., 2010). However, the effect declined when mice were pretreated with a inhibitor of soluble guanylyl cyclase [1H-[1,2,4] oxadiazolo [1,2-a] quinoxalin-1-one] and a blocker of ATP-sensitive K^+ channels (glibenclamide), which supports the mechanism of action *via* nitric oxide-K^+ channels pathway. The orally administered *A. uliginosa* flowers extract (3–100 mg/Kg) produced significant anti-nociceptive responses in chemical and thermal induced nociception models suggesting the action regulated by both central and peripheral nervous system. Furthermore, the reversal of anti-nociception by naloxone (opioid receptor antagonist) suggests the participation of the opioid receptors (Ong et al., 2011). Spilanthol was able to modify anxiety behavior and prolong the time of sodium pentobarbital-induced hypnosis and decrease the time of pentylenetetrazole-mediated seizures (Deciga-Campos et al., 2012). Pretreatment with ethanolic extract (10, 30 and 100 mg/kg) of *A. oleracea* flowers reduced both neurogenic and inflammatory phases of the chemically-induced orofacial nociception. The extract (100 mg/kg) reversed the capsaicin-induced heat hyperalgesia (paw withdrawal latency) in hotplate test and the effects were partially reversed when treated with opioid antagonist naloxone (Nomura et al., 2013).

Table 8
Details of methods employed for determination of structure of NAAs from *Spilanthes* species.

S.No	Plant	Part of the plant	Method of Analysis	Compound ID*	Reference
1	*Acmella ciliata*	Fresh flowers	HRMS, NMR	IBA-10, MBA-7 and PEA-9	Martin and Becker, 1984
2	*Acmella caulirhiza*	Dry leaves	NMR	IBA-10 and IBA-45	Crouch et al., 2005
3	*Acmella decumbens*	Dry roots	NMR	IBA-6 and PEA-1, 8	Casado et al., 2009
4	*Acmella oppositifolia*	Dried aerial parts	GCMS, HPLC, NMR	IBA-10,34 and MBA-7	Molina-Torres et al., 1996
5	*Acmella oleracea*	Dried aerial parts	NMR	IBA-34	Phrutivoropongkul et al., 2008
6		Dry leaves	NMR	IBA-7,8 and PEA-1,3,13	Simas et al., 2013
8	*Acmella radicans*	Fresh flowers, leaves, roots and stem	GCMS and NMR	IBA-1,2,3,10,26,36,41,42 MBA-7,8 and PEA-2,5,6,9, 11,12	Rios-Chavez et al., 2003
9	*Spilanthes acmella*	Dry flowers	NMR	IBA-10,24,33 and MBA-1	Nakatani and Nagashima, 1992
10		Fresh flowers	NMR	IBA-10,11,21	Ramsewak et al., 1999
11		Dry flowers	NMR	IBA-10	Wu et al., 2008
12		Dried aerial parts	NMR	IBA-36	Phrutivorapongkul et al., 2008
13		Fresh whole plant	LCMS	IBA-4,10,20,21,27,33,35 MBA-1,7 and PEA-12	Boonen et al., 2010
14		Fresh whole plant	LCMS, NMR	IBA-10,20,21,25,27,34 and MBA-1,7	Bae et al., 2010
15		Dried whole plant	GCMS	IBA-10, 36	Leng et al., 2011
16		Dried aerial parts	FTIR, GCMS, NMR	IBA-10,21 and MBA-1	Moreno et al., 2011
17		Dry flowers	LCMS	IBA-4,10,25,27,35 MBA-7 and PEA-1	Sharma et al., 2011
18		Dry flowers	FTIR, NMR	IBA-10,11 and MBA-1	Pandey et al., 2011
19		Dry flowers	GCMS	IBA-5,10, MBA-7 and PEA-9	Dias et al., 2012
20		Dry leaves	LCMS	IBA-10	Singh and Chaturvedi, 2012
21		Dry flowers	NMR	IBA-10	Sharma et al., 2012
22		Dry flowers	GCMS	IBA-10	Costa et al., 2013
23	*Spilanthes americana*	Fresh flowers, leaves and stem	GCMS	IBA-5,10, MBA-7 and PEA-9	Stashenko et al., 1996
24	*Spilanthes callimorpha*	Dried whole plant	NMR	IBA-33,34,39,40 and PEA-1,4,14	Li et al., 2007
25	*Spilanthes paniculata*	Dry flowers Dried callus	HPLC and HRMS	IBA-10 and IBA-27	Rajendran et al., 2017

7.4. Anesthetic and antipyretic

The anesthetic effects of aqueous *S. acmella* extract was recognized when it was administered to pyrexia rats (Chakraborty et al., 2010). The efficacy of anesthesia however was lower (5.33 min) as compared to the standard drug Xylocaine (2.15 min). The anesthetic property of spilanthol (IBA-10) was exemplified when a group of Brazilian researchers developed anesthetic muco-adhesive films containing *A. oleracea* extract for topical use. Among the films developed, the one containing 10% crude and also treated with activated carbon showed higher flux and permeability coefficients for spilanthol as compared to the untreated ones (containing 10% and 20% crude alone) (Freitas-Blanco et al., 2016). Alonso et al. (2018) reported the efficiency of spilanthol (IBA-10) and its alkynic analogue [(2*E*,8*E*) N-isobutyl deca-2,8-dien-6-ynamide] in permeating pig ear skin *in-vitro* Franz-type diffusion cell model. Incidentally, the alkynic analogue presented a higher anesthetic effect *in-vivo* (tail flick model) as compared to spilanthol and the commercial standard EMLA® [Eutectic Mixture of Local Anesthetics, containing 2.5% of lidocaine and prilocaine].

7.5. Anti-microbial

There has been several reports on anti-microbial activity of the *Spilanthes* plant extracts (**Table 1**; Molina-Torres et al., 1996; 1999; 2004; Prachayasittikul et al., 2009; Cruz et al., 2014; Lagnika et al., 2016; Swargiary et al., 2019). Extract of *S. acmella* and *A. uliginosa* inhibited the growth of yeast cells, gram-positive and gram-negative bacteria (Prachayasittikul et al., 2009; Lagnika et al., 2016; Masoko, 2017). Among the solvent used for extraction, dichloromethane proved better as compared to the methanol or aqueous medium in inhibiting mycelial growth and sporulation in *Aspergillus* species (*A. flavus, A. parasiticus, A. ochraceus, A. nidulans, A. clavatus* and *A. fumigatus* (Lagnika et al., 2016). In pure form, alkyne type NAAs (IBA-21 and IBA-28) appears to have a better effect than alkene NAAs (IBA-13, IBA-14, IBA-34 and IBA-35) on inhibiton of *S. cerevisiae* growth and disruption of its cell wall (determined by sorbitol protection assay), while it was found inverse for disruption of cell membrane (as determined by membrane leakage assay) (Cruz et al., 2014). Direct use of spilanthol (IBA-10) suffered from lack of migration in well diffusion method (Molina-Torres et al., 1999). Observation of tyndall effect in a solution of NAAs indicated their limited solubility in aqueous medium (Raduner et al., 2006). Antimicrobial property and toxicity against pathogenic microbes by a *Spilanthes* extract, however, is yet to be validated.

7.6. Vasorelaxant and diuretic

An increase urine output, urinary Na^+/K^+ levels and decrease in urine osmolarity was observed in albino male rats administered with cold water extract of *S. acmella* (1.5 g/Kg). This suggested the role of NAAs as diuretic, probably through inhibition of anti-diuretic hormone (Ratnosooriya et al., 2004). Using phenylephrine-induced contraction of rat thoracic aorta, a maximum vasorelaxant efficacy by chloroform ex-

Table 9
Larvicidal activity of the solvent extract and some selected NAAs of *Spilanthes* species on Mosquito Larva.

Name of the Mosquito species	Larval stage	Name of the Plant	The Extract / the Test Molecule	LD_{50} (ppm)	LD_{90} (ppm)	LD_{100} (ppm)	Reference
Aedes	III instar	*Spilanthes*	IBA-	4.5	–	–	Saraf and
aegyptii	IV instar	*acmella*	10	5.0	–	–	Dixit, 2002
	III	*Acmella*	Ethanol extract	251.1	–	–	Simas et al.,
	or	*oleracea*	Hexane fraction	145.6	–	–	2013
	IV		Dichloromethane fraction	1193.0	–	–	
	III	*Spilanthes*	Methanol extract	04.07 *	–	50 *	Jondiko, 1986
	instar	*mauritiana*	Chloroform extract	07.38 *	–	0.1 *	
			IBA-37	> 50 *	–	0.01 *	
		Spilanthes	IBA-10	6.25	–	12.5 *	Ramsewak et al.,
		acmella	IBA-11	6.25	–	12.5 *	1999
			IBA-21	6.25 #	–	12.5 *	
	III or IV instar		Methanol extract	27.50 *	–	–	Swargiary et al., 2019
Anopheles	I and II instar	*Spilanthes*	IBA-	5.0	–	–	Saraf and
culicifacies	III and IV instar	*acmella*	10	5.09	–	–	Dixit, 2002
	III		Hexane	0.87	1.92	–	Pandey et al.,
	or	*Spilanthes calva*	ex-	0.92	1.99	–	2007
	IV	*Spilanthes paniculata*	tract	3.23	7.10	–	
Anopheles	III	*Spilanthes acmella*	Hexane	4.57	7.83	–	
stephensi	and	*Spilanthes calva*	extract	5.10	8.46	–	
	IV	*Spilanthes paniculata*		5.09	13.55	–	
	III	*Spilanthes*	Hexane extract (Root)	2.71	4.26	–	Pandey and
	or	*acmella*	Hexane extract (Flower)	4.57	7.83	–	Agrawal, 2009
	IV		Hexane extract (Leaf)	61.0	94.48	–	
	instar		Hexane extract (Stem)	86.92	110.14	–	
Culex	I and II instar		IBA-	4.0	–	–	Saraf and
quinquefasciatus	III and IV instar		10	4.5	–	–	Dixit, 2002
	IV instar		Ethanol extract	61.43	–	–	Pitasawat et al., 1998
	III		Hexane	3.11	8.89	–	Pandey et al.,
	or	*Spilanthes calva*	extract	3.54	9.92	–	2007
	IV	*Spilanthes paniculata*		3.36	6.33	–	
	instar	*Spilanthes*	Hexane extract (Root)	1.19	2.06	–	Pandey and
		acmella	Hexane extract (Flower)	3.11	8.89	–	Agrawal, 2009
			Hexane extract (Leaf)	14.54	27.66	–	
			Hexane extract (Stem)	26.33	47.83	–	
	III	*Acmella*	Essential	42.2 *	73.6 *		Benelli et al.,
Spodoptera littoralis	instar	*oleracea*	oil	68.1 @	132.1@	–	2018
Tuta	II		Hexane extract	1.83 @	–	–	Moreno et al.,
absoluta	instar		IBA-10	0.13 @			2011
			IBA-21	0.49 @			
			MBA-7	0.81 @			

- Values for LD_{30}.
* - Values are converted to ppm from µg/ml or mg/ml or mg/L for convenience of comparison, ND – Not Data Available,.
@ - Values are in µg/larva or µg/mg larva.

Table 10
Insecticidal and Acaricidal activity of the extracts and NAAs of *Spilanthes* species.

S. No	Plant species (Country)	Bioassay	The Extract / Test molecule	Insects/ Ticks	LD_{50} (μg/insect)	Reference
1	*Spilanthes acmella* (Malaysia)	Topical application	IBA-10	*Periplaneta americana*	2.46	Kadir et al., 1989
			Carbaryl		3.16	
			Bioresmethrin		6.27	
			Lindane		9.44	
2	*Acmella oleracea* (Brazil)		Hexane extract	*Solenopsis saevissima*	2.48	Moreno et al., 2011
				Tetragonisca. angustula	2.55	
			IBA-10	*Solenopsis saevissima*	0.18	
				Tetragonisca. angustula	0.35	
			IBA-21	*Solenopsis saevissima*	0.67	
				Tetragonisca. angustula	0.67	
			MBA-7	*Solenopsis saevissima*	1.33	
				Tetragonisca. angustula	1.10	
3	*Spilanthes acmella* (India)	Leaf dip method	Hexane extract	*Plutella xylostella*	5.14	Sharma et al., 2012
			IBA-10		1.49	
			Methanol extract		5.04	
4	*Acmella oleracea* (Brazil)	Larval packet test	Hexane extract	*Rhipicephalus microplus* [#]	0.8*	Castro et al., 2014
		Adult immersion test			79.7*	
5		Larval packet test	Methanol extract		3.1 [¥]	Cruz et al., 2016
			IBA-10		1.6 [¥]	
		Adult immersion test	Methanol extract	*Dermacentor nitens* [#]	12.5 [¥]	
			IBA-10		12.5 [¥]	
6		Adult immersion test	Ethanol extract	*Rhipicephalus sanguineus* [#]	24.88*	Oliviera et al., 2018
7		Topical application	Essential oil	*Musca domestica*	44.3*	Benelli et al., 2018

[#] : Ticks.
[¥] : Values for LD_{100}.
* : g/L.

tract of *S. acmella* was observed (Wongsawatkul et al., 2008). The ethyl acetate extract in contrast facilitated an immediate vasorelaxation attributed to the presence of phenolic and triterpenoids in the extract. Yet another study showed orally given extract (500 mg/kg) of *S. paniculata* exhibiting strong diuretic activity. Thus its role as loop diuretic (due to rise in urine volume, Na^+ and K^+ excretion without altering other renal functions) and the possibility of the impairment of basal secretion and/or action of anti-diuretic hormone was inferred (Ali et al., 2015). In contrast, the extract of *A. uliginosa* exhibited an opposite effect. Evaluation of the hematological parameters showed an increase in WBC levels in female wistar rats suggestive of the toxic effect of *A. uliginosa* leaf extract, while an increase in levels of creatinine in plasma indicated harmful effects on kidney filtration and a reduction in levels of alanine aminotransferase and aspartate transaminase directs harmful effects to liver (Lagnika et al., 2016).

7.7. Enzyme inhibition and cytotoxicity

Ethanolic extracts of *Echinacea purpurea, Spilanthes acmella* and *Hydrastis canadensis* were shown to inhibit the cytochrome P_{450}2E1 mediated oxidation of p-nitrophenol. The strong efficacy of *H. canadensis* in inhibition of the action of CYP_{450}2E1 was attributed to the presence of alkaloids such as berberine, hydrastine and canadine in the extract. The NAAs (IBA-10, 27, 28, 34 and 35) from *E. purpurea* and *S. acmella* inhibited the oxidation at low concentration (25 μM), whereas caffeic acid derivatives failed to exhibit the same (Raner et al., 2007). An isoprenylated flavonoid [6-(3-methylbut-1-enyl)−5,7-dimethoxy-4′-hydroxy flavone] isolated from *S. calva* inhibited xanthine oxidase (IC_{50}:16.56 μM), was suggested for the treatment of gout and other oxidative stress-related disorders (Jayaraj et al., 2014b). Spilanthol (~100%) present in the dichloromethane fraction of *A. oleracea* extract was shown to inhibit tyrosinase enzyme (IC_{50}: 0.5 mM), also known as polyphenol oxidase (Barbosa et al., 2016). Both hexane and chloroform extract (80 μg/ml) of *S. acmella* significantly reduced cell viability of RAW 264.7 macrophages, while ethyl acetate and butanol extracts did not (Wu et al., 2008). The hydro-ethanolic extract of *S. acmella* had no effect on HEp-2 and L929 cells at lower concentration (250 μg/ml), but reduced the cell number at higher (500 μg/mL) (Soares et al., 2014). Spilanthol (IBA-10) and few other NAAs (IBA-20, 21 and MBA-7) isolated from *S. acmella* exhibited toxicity against Chinese Hamster Ovary (CHO) cell lines with IC_{50} values >100 μg/ml, while, *A. oleracea* extract and spilanthol (IBA-10) had similar toxic effects on HEK293 cells with IC_{50} of 234 and 260 μg/ml respectively (Mbuenkui et al., 2011; Gerbino et al., 2016).

7.8. Metabolism in humans

Cytochrome P_{450} enzymes play key role in metabolism of lipids, cholesterol, vitamins and xenobiotic compounds (Beresford, 1993; Zanger and Schwab, 2013),. It was therefore hypothesized that even NAAs get metabolized by cytochromes P_{450} on reaching systemic circulation. Degradation of NAAs (IBA-13,21,23 and 35) was found to be time-dependent and requires NADPH in the liver microsomal fractions suggestive of NAA metabolism in human liver. The alkyne NAA (IBA-21) was observed inhibiting the metabolism of alkene NAA (IBA-35, abundant in *Echinacea*) suggesting that the metabolism of NAAs was dependent not only on chemical structure but also on co-occurance of other NAAs (Matthias et al., 2005). When *Echinacea* extract containing IBA-16,27–28,31,34–35, MBA-4,5,6 was incubated with the human liver microsomes, the carboxylic acid metabolite of NAAs (IBA-34–35) re-

mained as major products after 2 h incubation (Cech et al., 2006b). The NAAs were indicated to have suppressed the production of IL-2 in T-cells more potently as compared to their metabolized products (carboxylic acid and hydroxylated). This was further proved by interactions with the recombinant human xenobiotic-metabolizing P_{450s} such as CYP1A1, CYP1A2, CYP2A13 and CYP2D6 to result in the formation of epoxide, N-dealkylated and hydroxylated products from IBA-35. Whereas, in liver microsomes CYP2E1, CYP2C9 and CYP1A2 catalyzed the formation of hydroxylated, epoxides and dealkylation products respectively from IBA-35 (Toselli et al., 2010). Hence, it must be inferred that the structure of NAAs have crucial influence on metabolism of NAAs.

7.9. Sensorial properties

The NAAs are unique in exhibiting strong pungent taste accompanied by tingling and sialagogic effects (Nakatani and Nagashima, 1992; Dubey et al., 2013; Rajendran et al., 2017). These effects have been attributed to the fatty acid tail that possess a *trans*-unsaturation at C_2. Further unsaturation along the fatty acid chain appear to impact more vigorous effect with (*Z*)-*cis* type unsaturation favored over (*E*)-*trans* configuration (Ley et al., 2004; Sugai et al., 2005). Numbing effect is associated with IBAs and hydroxy-IBAs, with their geometric isomerism modifying these effects. If tested independently, at 0.31×10^{-5} g/ml, the tingling effect caused by IBAs prevail over hydroxy-IBAs (Sugai et al., 2005). Other sensory properties associated with NAAs include burning, pungency, scratching, numbing, warming and cooling effects. While spilanthol causes tingling and salivation, the trans-pellitorine [(2E,4E) N-isobutyl deca-2,4-dienamide], a common IBA from *Piper* and *Anacyclus* species, failed to cause any tingling sensation but induced only salivation (Ley et al., 2006b). A structurally similar ketol ester [(7*Z*,9*E*)−2-oxo-undeca-7,9-dienyl 3-methylbut-2-enoate] from *S. acmella* named acmellonate, lacks the amide group and elicits a weaker tingling response as compared to IBAs and MBAs (Ley et al., 2006a).

7.10. Larvicidal

There has been numerous reports on the tendency of *Spilanthes* extracts to act as mosquito larvicides **(Table 9)**. The effect has been found on all three common mosquitoes namely *Aedes, Anopheles* and *Culex* (Saraf and Dixit, 2002; Pandey et al., 2007; Pandey and Agarwal, 2009; Simas et al., 2013). Of the three *Spilanthes* species tested, the extract from *S. acmella* was found the most active followed by that from *S. calva* and *S. paniculata* (Pandey et al., 2007). Among extract (hexane) of different parts of micro-propagated *S. acmella,* leaf extract exhibited a greater larvicidal efficacy (low LD_{50}) against *Anopheles* and *Culex* species (Pandey and Agarwal, 2009). In addition, the potential of ethanol extract of *H. longipes* roots (2.48 mg/L), IBA-10 (4.24 mg/L) and its reduced amides [IBA-41 (18.33 mg/L) and IBA-42 (7.47 mg/L)] against larvae of *Anopheles albimanus* and *Aedes aegypti* is documented (Hernandez-Morales et al., 2015). The molecules specified are also present in *Spilanthes*, however, the efficacy of spilanthol alone was demonstrated as ovicidal, larvicidal and pupicidal agent at remarkably low concentrations resulting from inhibition of the nerve conduction (Saraf and Dixit, 2002). Recently Benelli et al. (2018), showed the larvicidal action of essential oil obtained from *A. oleracea* with an LD_{50} of 42.2 mg/L and 68.1 µg/larvae respectively. The compounds (*E*)-caryophyllene, its oxide, β-pinene, myrcene and ~4% spilanthol present in the oil have been attributed to the larvicidal activity against *Culex quinquefasciatus* and *Spodoptera littoralis* (Egyptian cotton worm). Thus, use of NAAs containing extracts can be a natural remedy for control of disease spreaded by mosquito vectors. Till date, the studies on larvicidal activities of *Spilanthes* NAAs have been confined to laboratory scale only and requires additional considerations to test their efficacy in field conditions as well.

7.11. Insecticidal and acaricidal

Both the crude as well as the NAAs purified from them have been demonstrated to show insecticidal activity **(Table 10)**. *Spilanthes* species could be a natural reservoir of highly potent insecticides. Spilanthol (IBA-10) was more potent against adult male American cockroaches (*Periplanata Americana)* with an LD_{50} of 2.46 µg/g of insect than the conventionally used insecticides carbaryl (carbamates), lindane (organochlorine), bioresmethrin (pyrethroid) (Kadir et al., 1989). Similar effects were reported for spilanthol (IBA-10), IBA-21 and MBA-1 against larvae of a lepidopterean pest *Tuta absoluta* (the tomato leafminer that attacks members of the plant family Solanaceae) and its adult predator *Solenopsis saevissima* and pollinator *Tetragonisca angustula* following 24 h of topical application. IBAs (IBA-10 and IBA-21) exhibited a stronger effects as compared to the MBA (MBA-1) (Moreno et al., 2011). Using leaf disk method, Sharma et al. (2012) found spilanthol (IBA-10) killed (LD_{50}: 1.49 g/L) diamond backmoth *Plutella xylostella* which affects cruciferous vegetables of family Brassicaceae and showed that it could cause upto 100% mortality at low concentration (2 g/L). Benelli et al. (2018) showed the insecticidal action of essential oil from *A. oleracea* against adult female housefly (*Musca domestica)* at a LD_{50} of 44.3 µg/adult. The insecticidal efficacy of the extracts containing NAAs, particularly of *Spilanthes* species, indicates that they could play a potential role as organic insecticide/pesticide in integrated pest management.

Interest of the search for novel acaricidal agents have been in literature since a couple of years **(Table 10)**. Hexane extract of *S. acmella* was found toxic on larvae and engorged females of an cattle tick *Rhipicephalus micropus* **(**Family: Ixodidae) with a LD_{50} of 0.8 g/L and 79.7 g/L respectively (Castro et al., 2014). Hexane extract of *A. oleracea* on treatment with the germ cells of semi-engorged females of *R. microplus* induced alterations in size and shape of oocytes, number of yolk granules, number, size and location of vacuoles in germ cells similar to those caused by commercial anti-tick products fipronil and permethrin (Oliveira et al., 2016). Similarly, methanol extract of *A. oleracea* as well as spilanthol on *R. microplus* and *Dermacentor nitens* induced 100% mortality at 3.1 g/L and 1.6 g/L respectively (Cruz et al., 2016). It was 12.5 g/L for both extract and spilanthol against horse tick *D. nitens*. The LT_{50} for the extract and spilanthol were 38 and 57 min for *R. microplus* and *D. nitens* respectively (at 12.5 g/L). After 24 h of treatment, a significant reduction in egg mass and hatching percentage was found if the females were treated with both the extract and spilanthol (IBA-10). *Amblyomma cajennense* ticks treated with *A. oleracea* showed morphological alterations in the glandular complex cells, higher cytoplasmic vacuolation in the secretory cells (of accessory glands) and reduced levels of polysaccharides, glycoprotein and lipoprotein in the secretion granules that are essential for functional maturation of spermatozoa (Anholeto et al., 2017). A dose-dependent effect was observed when semi-engorged females of dog tick *R. sanguineus* treated with the ethanolic extract of *A. oleracea* (Oliveira et al., 2018). Until recently, the reported literature suggests that the acaricidal effect of the plant extract is due to a synergistic effect of individual NAAs present in them. The acaricidal action of *Spilanthes* extract could thus be exploited further for developing a plan for the treatment of ticks in livestocks.

7.12. Anti-plasmodial

Variable anti-plasmodial activity of NAAs and the *Spilanthes* extracts have been documented by a few research groups. A LC_{50} of IBA-10 and IBA-30 to be 16.5 µg/ml and 41.4 µg/ml respectively against mildly chloroquine resistant *P. falciparum* PFB strain. But it was 5.8 µg/ml and 16.3 µg/ml respectively for Thai chloroquine-resistant *P. falciparum* K1 strain (Spelman et al., 2011). *S. acmella* ethanol extract as well as spilanthol (IBA-10) reduced parasitemia in mice infected with *P. yoelii* parasites (Spelman et al., 2011). IBA-10, 20, 21 and MBA-7 showed anti-plasmodial effect against chloroquine-sensitive *P. falciparum* D10 strain with LD_{50} of 26.43, 54.03, 29.34 and 33.73 µg/ml respectively

(Mbeunkui et al., 2011). The same study revealed that a semi-purified fractions of *S. acmella,* from which these NAAs were purified, exhibited higher anti-plasmodial ability suggestive of a synergistic effects of NAAs. Silveira et al. (2016) revealed the toxic effects of IBA-9, 10 and PEA-2 against *Trypanosoma brucei rhodesiense* and *P. falciparum strains* (NF54 and K1). Recently, Rajendran et al. (2017) showed the combinatorial effect of spilanthol (IBA-10) and IBA-27 on *P. falciparum* (3D7 strain) with an LC_{50} of 18.35 mg/L, when compounds were tested in equal proportions.

7.13. Anthelmintic

Orally administered ethanolic *S. calva* extract showed higher anthelmintic activity against both *Pheretima posthuma* (earthworm) and *Ascaridia galli* (roundworm) compared to the aqueous extract of the same (Jayaraj et al., 2014a), using *insilico* analysis the authors predicted the efficacy of the N-containing compound [3,4-dihydro-(1,4)-oxazino-(4,3-b)(1,2)-benzoxazol-1(10bH)-one] isolated from *S. calva* as a potent anthelmintic agent. It is said to have higher affinity for the β-tubulin through H-bonding, π-π and non-polar interactions within the inhibitor binding pocket. Singh et al. (2014) reported a dose-dependent ability of both aqueous and methanol extracts of dedifferntiated callus and field grown *S. acmella* against trematode parasites (fluke) of cattle, with the aqueous extract showing a stronger activity as compared to methanol extract at lower concentrations (5 and 10 mg/ml). A higher concentration produced paralysis in 45.7 and 87 min, and death in 83 and 126 min respectively. Recently, it has been shown that the hexane extract of dried aerial parts of *A.oleracea* induced lethality in the cestode *Taenia tetragona* and the nematode *Ascaridia perspicillum* with LC_{50} of 5128.61 ppm and 8921.50 ppm respectively. Shrinkage of the tegument, erosion of microtriches and distortion of the suckers occurred in cestode, whereas, it was collapse of the lips and shrunk cuticle in case of the nematode (Lalthanpuii and Lalchhandama, 2020). Anthelmintic potential of the chloroform extract of *S. acmella* was demonstrated with the intestinal cestode *Raillietina echinobothrida,* but was found weaker than the anti-worm medicine praziquantel, the extract induced shrinkage and folds on the main body with a severe damage on the suckers of the cestode as observed under SEM (Lalthanpuii et al., 2020).

7.14. Role of NAAs in plants

Spilanthol (IBA-10) and its semi-synthetic derivatives (IBA-41 and IBA-42) were tested to show a significant stimulatory effects on root development in *Arabidopsis*, IBA-10 enhanced the growth of primary root and root hair elongation, while, IBA-41 and IBA-42 were found more potent in stimulating elongation of root hair (Remirez-Chavez et al., 2004). IBA-41 has been shown to target cytokinin receptors in bringing about stimulatory effects on adventitious root formation in *Arabidopsis* (Lopez-Bucio et al., 2007; Campos-Cuevas et al., 2008). The effects caused by NAA in this manner was observed to be independent of auxin signaling resulting in the formation of lateral root primordia. Presence of NAA induces NO formation that possess a role in signal transduction. It appears NO is an intermediate in stimulating the expression of defense-related genes involved in the biosynthesis of jasmonic acid (JA) confering resistance to herbivores, insects and pathogen infections (Mendez-Bravo et al., 2010; A. 2011).

7.15. Miscellaneous

The trans-mucosal efficacy investigated in a Franz-type diffusion cell model demonstrated that spilanthol (IBA-10) could permeate buccal mucosa (Boonen et al., 2010). In yet another study, IBA-10 and pellitorine were shown to penetrate skin on topical application and were likely to pass endothelial gut as they could cross the Caco-2 cells (derived from human colorectal carcinoma) in the monolayer model. It was inferred that spilanthol, in particular, could cross oral-mucosa and the blood brain barrier (Veryser et al., 2014, 2016). A study by Sharma et al. (2011) showed that orally administered *S. acmella* (150 mg/kg/day) caused an increase in the frequency of mounting, intromission and ejaculation. It also enhanced the levels of hormones (FSH, LH and testosterone) in rat sera suggesting an aphrodisiac property for the extract. Spilanthol (50 µg) was suggested to be an anti-carcinogenic agent, when it was shown associated with the reduction of frame-shift mutations induced by 2-aminoanthracene and norfloxacin in *Salmonella typhimurium* strains (Arriaga-alba et al., 2013). Pretreatment with *S. acmella* extract (1 µg/ml) for 24 h prevented the toxicity of the pesticide (primicarb) in neuronal cells (SH-SY5Y) through an increase in their count, a decrease and an increase in the levels of calpain and calpastatin protein respectively. An alteration in calcium homeostasis is suggested for this effect (Suwanjang et al., 2017). Spilanthol (30 mg/Kg) reduced the severity of 5-fluorouracil induced intestinal mucositis by decreasing the histopathological changes, myeloperoxidase activity and increasing the villus height in the male mice (Freitas-Blanco et al., 2018b).

Future prospects and conclusion

N-alkylamides (NAAs), a unfathomed group of secondary metabolites, are widely distributed in the plants. Due to their diverse pharmacological properties, the plants synthesizing them are exploited at industrial scale. Genus *Spilanthes* is a rich source of such NAAs that awaits opening up of its potentials. To fulfill the emerging demand, alternatives for *Spilanthes* production and alleviation of NAA biosynthesis needs to be investigated basically to result in cost reduction and better availability of base material in the global market in the near future. Conclusive studies are further required to ascertain the mechanism of action of NAAs in their pharmacological activities. We hope that the data presented in this review would serve as a platform for future exploration and promote the usage of *Spilanthes* species as functional foods and in therapeutics.

Author's contribution

Design and concept by NA, literature survey and GNAL manuscript written by RS and NA.

Conflicts of interest

The authors declare no conflict of interests.
Sd/- Rahul Sharma, Neelakantan Arumugam.

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Supplementary materials

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