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Characterization and GC-MS Profiling of Volatile Compounds in an Anti-Ophidic Formulation from Haryana

Madhu Sindhu* and Umesh Thakur
Department of Botany, Panjab University Chandigarh, India.

(Corresponding author: Madhu Sindhu)*
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ABSTRACT: Snakebites are a major public health concern around the globe with a high mortality rate. Modern and traditional approaches to healthcare systems signify abroad spectrum of healing practices for snake bites. This study reported a formulation said to be anti-ophidic in emergency cases of snake bites. Despite the dominance of conventional medicines in the treatment of snakebites, traditional medicines can offer significant benefits. In this quest, we explored its organoleptic and physico-chemical parameters and identified volatile compounds present in it through GC-MS analysis. From the ingredients. We identified the formulation as herbo-mineral preparation and all the physico-chemical parameters were found within the limits. The methanolic extract was prepared for GC-MS analysis. We identified a total of 15 chemical compounds. Out of them 2-Furancarboxaldehyde, 5-(2-furanylmethyl)- (20.16%), n-Hexadecenoic acid (9.33%), and 1-Heptatriacotanol (9.05%) were found to be highly abundant. Erythrocentaurin (4.19%), 3-O-Methyl-d-glucose (6.21%), 9-Octadecenamide, (Z)-, and Lupeol (7.18%) were present in moderate amounts. Some minor compounds such as 2-Methoxy-4-vinylphenol, Hexadecenoic acid, methyl ester (1.50%), and Ethyl iso-allocholate (1.10%) were also reported through the analysis. To our knowledge, this is the first documentation on standardization and analysis through GC-MS analysis of this formulation. Anti-inflammatory compounds such as 2-Methoxy-4-vinyl phenol, Erythrocentaurin, and n-hexadecenoic acid can help develop treatments for inflammation due to snakebites. PLA2 is prevalent in snake venom and the prevalence of n-Hexadecanoic acid in this formulation can be explored for its anti-ophidic properties. The GC-MS profiling revealed that this formulation is rich in compounds having anti-inflammatory, anti-necrotic, anti-ophidic, and vasodilation properties relevant to developing new treatments for the treatments of snakebites.

Keywords: Traditional formulations, Anti-ophidic, GC-MS, Physico-chemical.

INTRODUCTION

Plant products and minerals are the essential components of many traditional medicinal systems around the world, particularly in Unani and Ayurvedic traditions. Herbs are known to be effective and used around the world for different ailments. Integrating the mineral components into herbal preparations is a regular practice and is known to increase the potency and efficacy of the formulations (Aziz *et al.,* 2002). However, only limited scientific literature is available on these preparations due to multiple reasons such as lack of communication with traditional healers or practitioners, lack of proper research to find the active ingredients responsible for the healing, and limited scientific validation of therapeutic potential. This research aims to bridge the gap between the traditional and hidden practices and to find out the chemical profile of one such formulation.

In this context, during a field survey, we encountered a formulation used against snake bites and scorpion bites. The problem of snake bites is a global health concern claiming 25,000 to 125,000 deaths annually. Considering the global impact of snakebites, the World Health Organization (WHO) classified snakebites as Neglected Tropical Disease (NTD). Snakebites cause severe complications such as local tissue damage, necrosis, coagulation disorders, cardiovascular problems, renal complications, chronic inflammation, and death in many cases (Felix *et al.,* 2017). The primary treatment available for the treatment of snake bites is antivenom serum, which contains neutralizing

antivenom bodies. But due to high cost of production, high risk of immunological reaction, low stability at room temperature, and high specificity are related to antivenom therapy (Gutiérrez *et al.,* 2011).

Given these challenges associated with antivenom therapies, the exploration of complementary therapies becomes more important. Medicinal plants or formulations traditionally used for treating snake bites should be scientifically evaluated for anti-ophidic properties. These alternative treatments can offer potential advantages over present-day antivenom therapies due to low-cost production and stability at room temperature (Santhosh *et al.,* 2013). Standardization of herbo-mineral formulations is also required to ensure safety, efficiency, and efficacy. For standardization, several methods are used such as organoleptic evaluation, physico-chemical evaluation, analysis for the identification of chemical constituents ensuring the presence of correct herbal and mineral components, biological assays, microbial tests, and heavy metal testing (Shelar *et al.,* 2021).

In our study, we selected a formulation said to be effective against snake bites and scorpion bites. It is a less common and marketed formulation, documented from Haryana, India, and its ingredients were tracked down to study the scientific basis of this formulation. We classified it as a herbo-mineral formulation or 'Rasaushadhi.' These formulations are prepared by pharmaceutically processing the ingredients to make them therapeutically available (Chaudhary *et al.,* 2010). For standardization purposes, we did an organoleptic evaluation, physico-chemical evaluation, and GC-MS analysis for the identification and documentation of the chemical profile of the formulation, as open-source literature is not available for this formulation. Identification and documentation of the chemical profile of such formulations is essential for confirming the presence and concentration of ingredients. It is essential to find out the scientific basis of the utility of such formulations through the identification of chemical compounds present in them.

MATERIALS AND METHODS

Documentation and collection of study material. The documentation of the formulation was done in Haryana in October 2022 during a fieldwork survey. The formulation was identified by information through word of mouth. This formulation was used traditionally as first aid in the case of snake bites. The formulation was procured from the market and the source was tracked based on recommendations from local practitioners and community members. The ingredients were identified in the formulation.

Organoleptic evaluation. The formulation was taken to the laboratory and evaluated for organoleptic parameters such as physical form, colour, odour, taste, and texture using sensory methods.

Physico-chemical evaluation. The physico-chemical parameters of the formulation were evaluated as per the Standard Operating Procedures outlined in the Ayurvedic Pharmacopoeia of India (API), 2001. The physico-chemical properties included pH, electrical conductivity, loss on drying (moisture content at 105°C), water-soluble extractives, alcohol-soluble extractives, total ash, acid-insoluble ash, and water-soluble ash. Physico-chemical evaluation is essential for ensuring the quality, safety, and efficacy of the formulation (Sumbul *et al.,* 2012).

Preparation of methanolic extract. The formulation was brought to the laboratory and air-dried at room temperature and ground into a fine powder using an electric blender. A 20gm of the formulation was taken in an Erlenmeyer flask and macerated in 100 mL of methanol and left for 24 hours. Later, the solution was kept for 48 hours in an orbital shaker set at 150 rpm. The resulting mixture was filtered using Whatman filter paper No. 1. The filtered solution was taken in a petriplate and evaporated to dryness. The dried extract was stored at 4°C in an airtight container for further use.

Analysis of the methanolic extract of the formulation for identification of volatile compounds by Gas chromatography and mass spectrometry. For the analysis of a methanolic extract of the formulation, we used gas chromatography-mass spectrometry (GC-MS). It is an advanced hybrid analytical technique that combines chromatography and spectrometry, where compound separation capabilities associated with gas chromatography (GC) and detection capabilities associated with mass spectrometry (MS). The analysis was done using a Thermo-Trace 1300 GC coupled with a Thermo TSQ 8000 Triple Quadrupole Mass Spectrometer at Panjab University, Chandigarh. We used pure helium (99.999%) as a carrier gas at a constant flow of 1 mL/min. The injector volume and temperature were set at 10 µL and 250°C respectively, with a runtime of 20.09 minutes. For the separation of compounds, we TG 5MS column (30 m × 0.25 mm, 0.25 µm), which was composed of 5% diphenyl and 95% dimethyl polysiloxane. Results were interpreted by a chromatogram. We identified compounds by comparing their retention times and mass spectra with the NIST library database (National Institute of Standards and Technology, Gaithersburg, MD, USA). We interpreted the results and analyzed their retention indices, mass spectra, molecular formula, and molecular weights. Additionally, we evaluated and recorded the medicinal and therapeutic properties of these compounds. After the identification of compounds present in the formulation, Swiss ADME analysis was performed to identify potential drug candidates.

RESULTS AND DISCUSSION

The traditional marketed formulation was used as a first aid in the emergency cases of snakebites and scorpion

bites. The key ingredients found to be *Enicostemma axillare*, muktasukti, prawalchandraputi, and shudhhingul. We figured that it is aherbo-mineral composition (Table1). The aerial part of the *E. axillare* was used in the formulation, and well reported for its anti-inflammatory and anti-venom properties (Thondaiman and Saha 2017). Mukta sukti or the pearl oyster shell has been traditionally used in many Ayurvedic medicines (Sreejith, 2012). Prawalchandraputi is a famous Ayurvedic composition produced from coral by purification and calcination. It is primarily composed of calcium carbonate and magnesium, iron, and other minerals in trace amounts (Dubewar *et al.,* 2021). Shuddhhingul is also a mineral compound chemical known as mercury sulphide (HgS) has been traditionally used in ayurvedic formulations including eye conditions, liver disorders, diabetes, and skin disorders (Patel *et al.,* 2019). The combination of these ingredients suggests the approach for managing snakebites and scorpion bites.

The organoleptic analysis of the formulation reveals that the formulation is dark brown in colour and medium brown coarse powder, with a characteristic smell and taste (Table 2). The sensory evaluation of formulation is important to set standard adherence to traditional preparation methods. The results of the physico-chemical evaluation are presented in Table 3. The formulation depicts a pH of 5.77 which is slightly acidic and electrical conductivity values of 4.34 mS/m suggest a moderate level of electrolytes. The electrical conductivity can be attributed to mineral compounds such as muktasukti, and prawalchandraputi. Moisture content was found to be 8.2% and water and alcohol-soluble extractives were 12.75 ± 1.23% and 6.35 ± 5.4%, respectively. The total ash, acid-insoluble ash, and water-soluble ash were found to be 18.55 ± 0.1%, 7.36 ± 0.17% and 15.95 ± 0.58% respectively. The high value of ash in the sample can be attributed to the inclusion of mineral-rich ingredients like muktasukti and prawalchandraputi. Acid-insoluble ash suggests the presence of non-digestible minerals such as silica, which are typical with mineral compounds, whereas water-soluble ash reflects the compounds soluble in water, supporting the presence of bioavailable minerals from ingredients like coral and pearl oyster shells. All the observed physico-chemical parameters align with well-set standards of API and with known characteristics of the ingredients and support their inclusion.

The GC-MS chromatogram of the methanolic extract of the formulation is presented in Fig. 1. Several key compounds were identified by the GC-MS analysis of the methanolic extract of the formulation. The identified compounds were tabulated in Table 4 along with their molecular formula, molecular weight, retention time, and peak area percentage. The analysis reveals that the most abundant compound was 2-Furancarboxaldehyde, 5-(2-furanylmethyl)- (20.16%). Other compounds included n-Hexadecanoic acid (9.33%), 1-Heptatriacotanol (5.17% and 9.05%), Lupeol (7.18%), 9-Octadecenamide, (Z)- (6.32%), and 3-O-Methyl-d-glucose (6.21%) were present in moderate quantity Additionally, compounds like 10,10-Dimethyl-3,6,9,11,14,17-hexaoxa-10-silanonadecane (5.54%), Erythrocentaurin (4.19%), Benzoic acid, 4-ethylamino-, ethyl ester (3.81%), and Hexadecanoic acid, methyl ester (1.50%) were also identified. Minor compounds such as 2-Methoxy-4-vinylphenol (1.20%), 2,5-Cyclohexadien-1-one, 4-ethyl-3,4-dimethyl- (1.86%), 2-Butyl-5-methyl-3-(2-methylprop-2-enyl) cyclohexanone (1.14%), 4-Oxo-α-isodamascol (1.83%), and Ethyl iso-allocholate (1.10%) were also present and documented.

2-Methoxy-4-vinyl phenol (2M4VP) has shown significant anticancer effects on human pancreatic cancer cell lines. It reduced cell viability, inhibited PCNA protein expression, suppressed cell migratory activity, and decreased phosphorylation of FAK and AKT (Kim *et al.,* 2019). 2M4VP also exerts anti-inflammatory effects by inhibiting the production of NO and PGE2. It inhibits NOS and COX-2 expression and suppresses NF-κB and MAPK activation (Jeong *et al.,* 2011). The metalloproteinases in snake venom increase the expression of PGE2 and COX-2 levelsand lead to an increase in pain sensitivity. It can be a potential compound to treat the inflammation in the cases of snake bites as it can reduce the increased PGE2 and COX-2 levels due to snake venom. Chronic inflammation due to increase in the expression of proinflammatory cytokines is a common feature of snakebites and rheumatoid arthritis. Many treatments to reduce chronic inflammation include the targeting of NF-κB to reduce the expression of proinflammatory cytokines (Fernandes *et al.,* 2007; Jue *et al.,* 1999). As 2M4VP can reduce NF-κB levels, it can be a potential candidate in the treatment of snakebite and rheumatoid arthritis-induced inflammation (Moreira *et al.,* 2013).

Erythrocentaurin has been isolated from another species of *Enicostemma*, the *Enicostemma hyssopifolium*. This can be the marker compound to identify that *Enicostemma* sp. as it is also reported from this formulation contains *Enicostemma axillare* (Ghosal *et al.,* 1974). In another study, Erythrocentaurin has shown significant dose-dependent anti-inflammatory properties by inhibiting TNF-α secretion. This indicates its potential as a potent anti-inflammatory agent, contributing to its therapeutic value, as tissue necrosis is a common feature of snake bites. Harris and Cullen 1989). In another case, when some derivatives of erythrocentaurin were synthesized and tested for anti-Hepatitis B Virus (HBV) activity. Out of them two derivatives demonstrated the most potent inhibition of HBV DNA replication (El Menyiy *et al.,* 2021).

3-O-Methyl-D-glucose, also called methyl glucose has been frequently used in studies of blood-brain barrier transport and the distribution of hexoses in the brain

due to its presumed metabolic stability. The distribution spaces were 0.52 in the brain and heart, and 0.75 in the liver, indicating its limited metabolic transformation in these tissues. This can be a very important molecule to study in case of snake bites, where transportation of medicine to different tissues is required so that the drugs remain intact until they reach their target sites (Jay *et al.,* 1990).

Hexadecanoic acid, also called palmitic acid is a well-known bioactive compound and exhibits significant anti-microbial properties. It can be helpful in the development of antimicrobial products (Shaaban *et al.,* 2021). The methanolic extract of *Leucas aspera* that contained n-hexadecenoic acid in ample amounts along with other constituents had neutralized venom protease and hyaluronidase activities and venom hemolytic activity (Gopi *et al.,* 2014). A study suggests that n-Hexadecanoic acid as has demonstrated the inhibition of phospholipase A2 (PLA2) can control inflammation. The enzyme kinetics studies have demonstrated that n-hexadecanoic acid inhibits PLA2 as the acid positioned in PLA2's active site. These findings support the use of n-hexadecanoic acid-rich medicated oils in Ayurveda for treating rheumatic symptoms due to their anti-inflammatory properties (Aparna *et al.,* 2012). As PLA2 is prevalent in snake venom and inhibition of PLA2 by n-hexadecanoic acid can be an important compound to explore as an antiophidic compound (Hiu and Yap 2020; Xiao *et al.,* 2017). Its ADMET profile also shows that n-hexadecanoic acid is efficiently absorbed in the gastrointestinal tract and widely distributed across body tissues (Krishnaveni *et al.,* 2022).

A steroidal compound ethyl iso-allocholate has been observed in our GC-MS profile. In one study ethyl iso-allocate was isolated from *Trigonella foenum graecum* L. seeds, and has been shown to possess potent anticancer properties against A549 lung cancer cells. Some experiments using zebrafish models also demonstrated that ethyl iso-allocholate inhibited tumor growth by 80% but it exhibited no toxicity to normal tissues. (Thakur and Ahirwar 2018). In a study of an extract majorly containing ethyl iso-allocholate exhibits significant anti-inflammatory properties and antiviral activity. It particularly showed its effectiveness against SARS-CoV.

(Arsana *et al.,* 2022). These properties of iso-allocholate highlight its potential pharmaceutical utility in the cases of snakebites because of its anti-inflammatory properties and non-toxic to normal tissues.

9-Octadecenamide, commonly referred to as Oleamide and cis-9, 10-octadecenamide is detected in our GC-MS profileas a fatty acid primary amide with notable signaling actions in the cardiovascular system. Studies show that Oleamide influences voltage-gated Na+ channels and induces vasodilation in rat small mesenteric arteries facilitated by the release of endothelium-derived nitric oxide (Hiley and Hoi 2007). This compound is worth exploring as another study on scorpion alpha-toxins demonstrated the modulation of voltage-gated Na+ channels in a highly specific manner and subsequently altered sodium currents, affecting cellular excitability with potentially life-or-death consequences (Bosmans *et al.,* 2007). A triterpenoid lupeol has been identified on our GC-MS profile. It exhibits numerous pharmacological activities, including anticancer, antioxidant, anti-inflammatory, and antimicrobial effects. It is rapidly absorbed by animals and its derivatives have shown enhanced bioactivities, such as increased antiprotozoal and anticancer properties, and inflammatory properties (Liu *et al.,* 2021).

When the evaluation of identified compounds for drug-likeness was done using Swiss ADME, most of the identified compounds were found to be potential drug candidates. 2-Methoxy-4-vinylphenol, 2,5-Cyclohexadien-1-one, 4-ethyl-3,4-dimethyl-, 2-Butyl-5-methyl-3-(2-methylprop-2-enyl) cyclohexanone, 2-Furancarboxaldehyde, 5-(2-furanylmethyl)-, and Erythrocentaurin exhibited high drug-likeness due to their favourable physicochemical properties and moderate molecular weights. Whereas, 3-O-Methyl-d-glucose, 10,10-Dimethyl-3,6,9,11,14,17-hexaoxa-10-silanonadecane, Hexadecanoic acid, methyl ester, n-Hexadecanoic acid, Benzoic acid, 4-ethylamino-, ethyl ester, and 9-Octadecenamide (Z)- show moderate drug-likeness, but also potential issues in solubility or permeability. We found Ethyl iso-allocholate possessed good drug-likeness despite its larger size due to a good balance of properties. Lastly, Lupeol and 1-Heptatriacotanol were not found to be good candidates for drug development (Daina *et al.,* 2017).

Table 1: Reported ingredients in a traditional marketed formulation for first aid against snake and scorpion bites.

Sr. No.	Ingredients	Common Name	Part Used
1.	*Enicostemma axillare*	Kadu naay	Aerial parts
2.	Mukta sukti	—	Pearl oyster shell
3.	Prawalchandraputi	—	Coral compound
4.	Shuddhhingul	—	Purified cinnabar

Table 2: Observations of organoleptic analysis of the formulation.

Sr. No.	Properties	Observations
1.	Description	Powder
2.	Color	Dark brown powder
3.	Odor	Characteristic
4.	Taste	Characteristic
5.	Texture	Medium coarse

Table 3: Observations of physico-chemical analysis of the formulation

Sr. No.	Properties	Observations
1.	pH	5.77
2.	Electrical conductivity	4.34 mS/m
3.	Loss on drying	8.2 %
4.	Water- soluble extractives	12.75 ± 1.23 %
5.	Alcohol- soluble extractives	6.35 ± 5.4 %
6.	Total ash value	18.55 ± 0.1 %
7.	Acid- insoluble ash	7.36 ± 0.17 %
8.	Water- soluble ash	15.95 ± 0.58 %

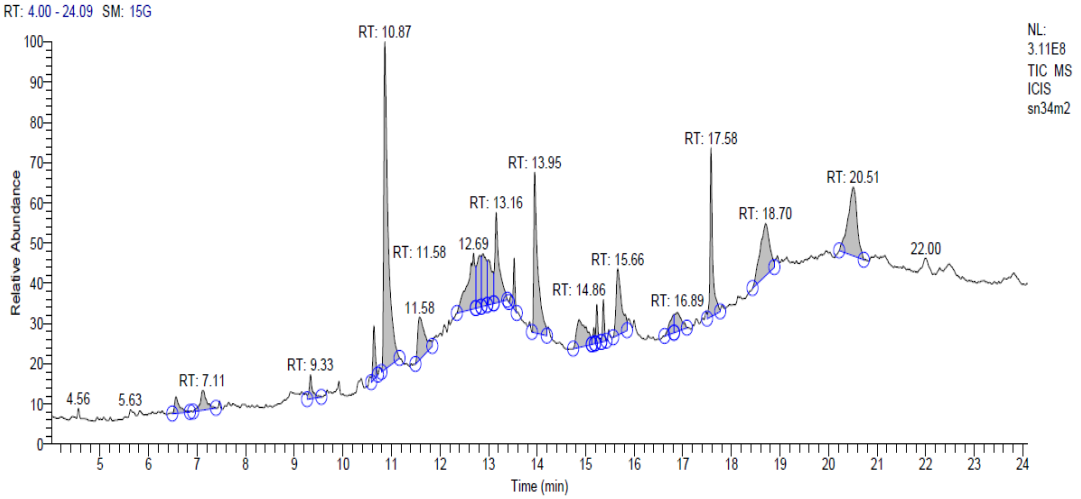

Fig. 1. Chromatogram showing bioactive phytoconstituents of methanolic extract of the formulation by the method of GC-MS.

Table 4: Phytochemicals identified in the methanolic extract of the formulation by GC-MS.

Compound Name	Molecular Formula	Molecular Weight (g/mol)	Retention Time	Peak Area Percentage
2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.17	6.56	1.20
2,5-Cyclohexadien-1-one, 4-ethyl-3,4-dimethyl-	$C_{10}H_{14}O$	150.22	7.11	1.86
2-Butyl-5-methyl-3-(2-methylprop-2-enyl) cyclohexanone	$C_{15}H_{26}O$	222.37	9.33	1.14
4-Oxo-α-isodamascol	$C_{13}H_{20}O_2$	208.30	10.64	1.83
2-Furancarboxaldehyde, 5-(2-furanylmethyl)-	$C_{10}H_8O_3$	176.16	10.87	20.16
Erythrocentaurin	$C_{10}H_8O_3$	176.17	11.58	4.19
3-O-Methyl-d-glucose	$C_7H_{14}O_6$	194.18	12.69	6.21
10,10-Dimethyl-3,6,9,11,14,17-hexaoxa-10-silanonadecane	$C_{14}H_{32}O_6Si$	324.48	13.16	5.54
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	13.53	1.50
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	13.95	9.33
Benzoic acid, 4-ethylamino-, ethyl ester	$C_{11}H_{15}NO_2$	193.24	14.86	3.81
Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436.6	15.23	1.10
9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281.47	17.58	6.32
Lupeol	$C_{30}H_{50}O$	426.7	18.70	7.18
1-Heptatriacotanol	$C_{37}H_{76}O$	537.0	30.51	9.05

CONCLUSIONS

Studies to discover treatments for snakebites are the requirement of time to achieve the goal of WHO to reduce the deaths due to snakebites to half by 2030. Due to the hefty limitations of present-day antivenom treatments exploration of alternative treatments is important to identify their constituents and subsequently the mechanism of action. We reported a formulation used majorly against snake bites and upon standardization physico-chemical parameters were found within the limits determined by API. We identified the 15 volatile compounds present in the formulation. The resulting compounds displayed a wide range of biological activities such as anti-inflammatory, anti-ophidic, anti-necrotic, antiprotozoal, anticancer, and anti-arthritic properties. Out of them anti-inflammatory, anti-ophidic, and anti-necrotic are of importance as chronic inflammation, and necrosis are the most common and deadly consequences of snake bites.

FUTURE SCOPE

As limited literature is available for this formulation, further research and isolation of compounds arerequired with different solvents. 2-Methoxy-4-vinyl phenol, Erythrocentaurin, 3-O-Methyl-D-glucose, Hexadecenoic acid, ethyl iso-allocholate, and 9-Octadecenamide were reported in the present formulation and previously reported in many studies for various biological characteristics. Advanced studies are required to explore the activity and effectiveness of these compounds in the cases of snakebites and scorpion bites.

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Conflict of Interest. None.

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