Plant Archives Vol. 24, No. 1, 2024 pp. 55-60 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org

DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2024.v24.no.1.008

DOCUMENTATION, STANDARDIZATION AND PHYTOCHEMICAL PROFILING OF METHANOLIC EXTRACT OF FORMULATION USED FOR SNAKE BITE MANAGEMENT IN JIND DISTRICT OF HARYANA, INDIA

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(Date of Receiving-28-10-2023; Date of Acceptance-05-01-2024)

ABSTRACT

This research is cantered on the formulation known as "Sarpdansh Amrita" developed by a Vaidya in the Jind district of Haryana, India. This formulation is employed by the local community as a first-aid remedy for snake bites and aphid stings. The study aims at physicochemical and phytochemical evaluation in methanolic extract of formulation. A total of 16 biochemical compounds were identified using GC-MS. The compounds identified in the formulation were compared with the mass spectra of the National Institute of Standards and Technology (NIST). The major identified phytochemicals include Lupeol;Lup-20(29)-en-3-one; Cis-13-Octadecenoic acid, methyl ester; Hexadecanoic acid, methyl ester; Bergamotol, Z-à-trans-; 9,12-Octadecadienoic acid (Z,Z)-, methyl ester; Undecanoic acid; Lanceol, cis, etc. The formulation holds promising pharmacological potential due to its abundant content of bioactive phytochemicals. The identified molecules can be further isolated, purified, and studied for pharmacological properties concerning their effect in case of snake bites.

Key words : Physico-chemical, Phytochemicals, GC-MS, Sarpdansh Amrita, Snake bites.

Introduction

Snake bite incidents pose a significant public health challenge globally, contributing to an annual death toll that ranges astonishingly from 25,000 to 125,000 fatalities and leaving countless individuals with enduring disabilities (Gutierrez *et al*., 2015). In acknowledgment of their impact, the World Health Organization added snake envenomation to the list of Neglected Tropical Diseases (NTDs) in 2009. Particularly affected are populations in rural tropics and subtropics, where limited access to healthcare is prime reason of mortality and morbidity (Harrison *et al*., 2009).

When snake bites, venom is injected into victims via fangs originating from venom glands of snake (Kardong *et al*., 1993). The snake venom contains pharmacologically active proteins and polypeptide complexes designed to immobilize prey. The consequences of snake envenomation can range from immediate effects to severe systemic outcomes, encompassing inflammation, oxidative stress, swelling, lipolysis, muscle necrosis, skin tissue damage, kidney and cardiovascular function disturbances (Kang *et al*., 2011).

The primary approach to treating snake venom involves the use of antivenom serum therapy. However, this method is encumbered by high costs, limited availability, and a significant risk of immunological reactions, whether immediate or delayed (Ryan *et al*., 2016). Given these challenges, the exploration of alternative snakebite treatments becomes imperative. Medicinal plants and traditional remedies hold promise for yielding compounds with antiophidic properties. Contemporary toxicologists are increasingly turning to traditional folk remedies due to their affordability, stability, and potential efficacy against venom toxins (Butt *et al*.,

Abbreviations - GC-MS: Gas chromatography and mass spectrometry, **SA:** Sarpdansh amrita, **GAE:** Gallic acid equivalents, **QE:** Quercetin equivalents, **LE:** Linalool equivalents, **TAE:** Tannic acid equivalents, **PGE2:** Petroselinic acid.

2015).

The assessment of local formulations plays a pivotal role in evaluating their effectiveness, potential toxicity, and quality control. To explore alternative medicines local practitioners known as "Vaids" were engaged as knowledgeable sources to collect relevant insights and a formulation was selected. The selection of a specific formulation was guided by its significance and feedback from individuals who had experienced positive outcomes from its use. In the Jind District of Haryana, a polyherbal preparation named "Sardansh Amrita" has gained attention among locals as an initial and preventive measure against snakebites, especially when immediate medical aid is lacking. This formulation also reported efficacy against aphid stings. The ongoing study encompasses physico-chemical and phytochemical assessments, coupled with GC-MS analysis, to scientifically evaluate the chemical composition of Sarpdansh Amrita (SA) as the formulation is not thoroughly accessed for the above properties. Transition from traditional to contemporary drugs can be accelerated through scientific examination of formulation.

Materials and Methods

Collection of study material

Ingredients of formulation are *Sapindus mukorossi* (fruit), family Sapindaceae, Safed Katha (Khadira), Neela Thotha (Copper sulphate) and Kalmi Shora (Potassium nitrate), cow ghee. *Sapindus mukorossi* (fruit) was purchased from a medical herb store and was authenticated from NISCAIR (National Institute for Science Communication and Policy Research), Pusa Gate, New Delhi with authentication no. – NIScPR/RJMD/Consult/2023/4485-86-7 12/6/2023. Safed Katha, Neela Thotha, and Kalmi shora were purchased from INDIAN JADIBOOTI registered under FSSAI license no.13319011000193.

Preparation of in-house formulation

An in-house formulation was prepared under the guidance of a local practitioner. Final product was prepared in the form of tablets. The sample was stored in airtight jar at room temperature for further analysis.

Preparation of extract

For analysis, a methanolic extract was prepared. The formulation was mixed with methanol in an Erlenmeyer flask, sealed with aluminium foil, and left undisturbed for 24 hours. Afterward, it was agitated in a shaker for 24 hours and then filtered using Whatman filter paper no. 1. The filtered solution was subsequently evaporated to yield dried extract, which was used to create a stock solution in methanol.

Organoleptic evaluation

Organoleptic parameters of formulation such as colour, odour, taste and texture were evaluated by sense organs.

Physicochemical analysis

The parameters like pH, loss on drying (moisture content at 105°C), water-soluble extractives, alcohol-soluble extractives, total ash, acid-insoluble ash and water-soluble ash of the formulation were evaluated. The procedure was followed as per Standard Operating Procedures [Ayurvedic Pharmacopoeia of India (API), 2001].

Qualitative phytochemicals screening

Preliminary phytochemical screening of methanolic extracts of the formulation was done used to identify phytoconstituents as per standard guidelines (API, 2001).

Quantitative phytochemical screening

Total phenolic content

The phenolic content of the extract was determined using the Folin-Ciocalteu reagent (FCR) method (Cicco *et al*., 2009). To plot the standard curve, gallic acid was taken at different concentrations of (25-250µg/ml). The absorbance was read at 750nm. Results were expressed in µg GAE/mg. Triplicates of the assay were done.

Total Flavanoid content

The total flavonoid content (TFC) was estimated using aluminium chloride colorimetric assay (Ebrahimzadeh *et al*., 2008). To plot the standard curve quercetin was taken at different concentrations (0.6-10 µg/ml). The absorbance was read at 415nm. Results were expressed as µg QE/mg of plant extract. Triplicates of the assay were done.

Total Terpenoid content

The total terpenoid content (TTC) was estimated using the method described by Ghorai *et al*. (2012). To plot the standard curve linalool was taken at different concentrations (78-2500 µg/ml). The absorbance was read at 538nm. Results were expressed as µg Linalool/mg of plant extract. Triplicates of the assay were done

Total Tannin content

Total tannin content was estimated using the method described by Oikeh *et al*. (2020). To plot the standard curve tannic acid was taken at different concentrations (0.1-10 µg/ml). The absorbance was read at 725nm. Results were expressed as µg Tannic acid /mg of plant extract. Triplicates of the assay were done.

Analysis of methanolic extract for secondary metabolites by Gas Chromatography-Mass Spectrometry

GC-MS analysis of methanolic extract of the formulation was done at SAIF-CIL, Panjab University, Chandigarh using the Thermo Scientific 1300 GC coupled with thermos TSQ8000 triple quadrupole MS was used. The capillary column (30*0.25µmID*0.25µmdf) with 5% diphenyl and 95% dimethyl polyxylane was used. The total run time of GC was 20.11 minutes and the injection volume was 1µl. Helium was used as carrier gas having a flow rate of 1ml/minute. The injector temperature, ion source temperature, oven temperature were maintained at 250°C, 250°C and 230°C, respectively. The mass spectrum was taken within the range of 50-600 at 70eV ionization voltage. Data interpretation was done using the National Institute Standard and Technology (NIST) and the identity of phytoconstituents was ascertained.

Results and Discussion

Vaidya's choice of ingredients stands validated, as a wealth of literature supports the selection of these components. These ingredients have found application across various ailments, substantiating their utility in disease management.

Sapindus mukorossi is a member of the Sapindaceae family and its pericarp of fruit is used in the formulation. It is traditionally used as an expectorant, natural detergent and abortefacient. Fruit is reported to have anti-inflammatory, hepatoprotective, antiplatelet aggregation activities (Ibrahim *et al*., 2008 and de Queiroz *et al*., 2017). Safed katha, also identified as Khadira, is sourced from the heartwood of *Acacia catechu*. In the treatment of snakebites, a formulation containing katha is orally administered to patients. It exhibits properties such as antioxidant, anti-inflammatory, antibacterial and antifungal. It helps in protecting the liver and wound healing and treating pain (Stohs *et al*., 2015). Neela thotha known as copper sulphate is a mineral origin drug. Despite its inherent toxicity, after purification it has utility within the framework of the Unani system of medicine. It is used in leprosy, bronchitis, chronic leucorrhoea, chronic gonorrhoea, epilepsy, tremor and piles. Once purified, it becomes a valuable component in the treatment of poisoning and induce emesis (Ahmad *et al*., 2021). Kalmi shora, or potassium nitrate, presents as a white crystalline salt. This compound showcases diuretic, anti-inflammatory, expectorant, vasodilator properties and pain relieving properties (Kalam *et al*., 2023).

The results of the organoleptic evaluation are depicted in Table 1. The organoleptic observations indicate that the prepared Sarpdansh Amrita is in the form of a dark brown tablet with a characteristic aroma and flavour. The mean value of pH, moisture content at 105°C, ash values (total ash, water-soluble ash and acid-insoluble ash), and extractive values (alcohol-soluble and water-soluble) are depicted in Table 2. Qualitative phytochemical screening for carbohydrates, phenols, flavonoids, saponins, tannins, steroids, glycosides, amino acids, terpenoids, and tannins was done and showed positive results for carbohydrates, phenols, flavonoids, saponins, glycosides, terpenoids and tannins. The results of quantitative phytochemical screening of the methanolic extract of the formulation are depicted in Table 3. In both qualitative and quantitative phytochemical analyses, the formulation demonstrates a scant presence of phenols (30.65 ± 5.42 µg GAE/mg of extract) and flavonoids (191.70 ± 5.03 µg QE acid/mg of extract), while showcasing a significant abundance of terpenoids (582.06 ± 83.36 µg LE /mg of extract), and tannins (390 ± 8 µg TAE /mg of extract).

Table 1 : Observations of organoleptic analysis of SA.

S. no.	Properties	Observations
1.	Description	Solid tablets
2.	Color	Dark brown
3.	Odor	Characteristic
4.	Taste	Characteristic
5.	Texture	Smooth

Table 2 : Observations of physicochemical analysis of SA.

S. no.	Properties	Observations
1.	pH	2.47
2.	Moisture content	5.07±0.60%
3.	Total Ash	34.12±2.13%
4.	Acid Insoluble Acid	14.55±1.34%
5.	Water soluble ash	27.92±1.13%
6.	Alcohol soluble extractive	19.46±0.94 %
7.	Water soluble extractive	24±1.6%

Table 3 : Quantitative phytochemical analysis of methanolic extract of SA.

S. no.	Phytochemical analysis	Results
1.	Phenolic content (µgGAE/mg of extract)	30.65±5.42
2.	Flavanoid content (µgQE/mg of extract)	191.70±5.03
3.	Terpenoid content (µgLE/mg of extract)	582.06±83.36
4.	Tannin content (µgTAE/mg of extract)	390±8

The GC-MS analysis of the methanolic extract of

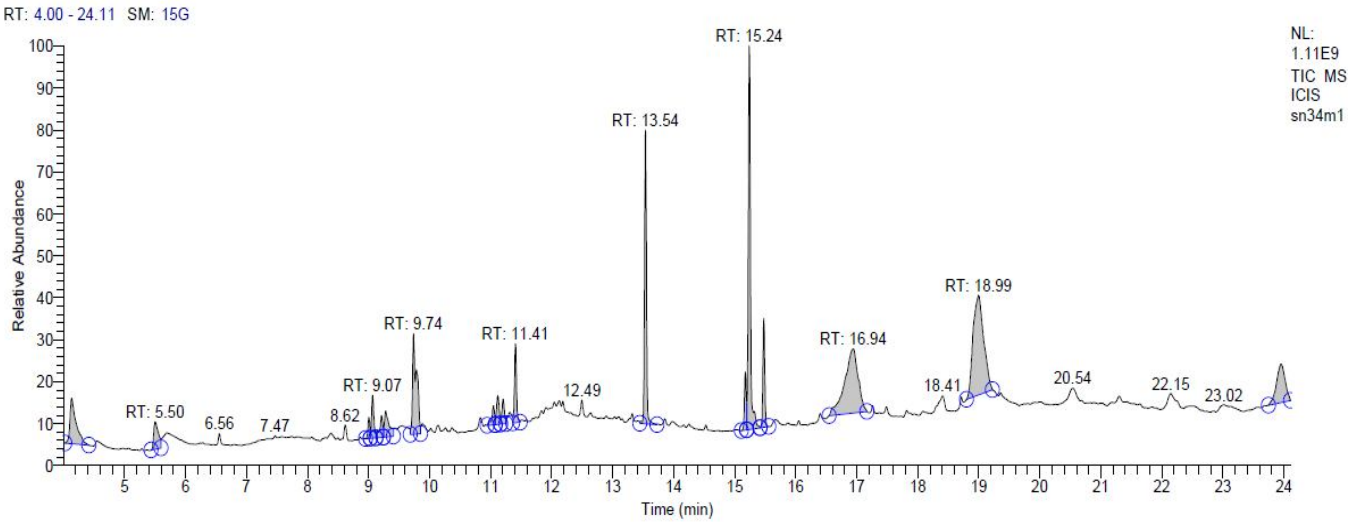

Fig. 1 : Chromatogram showing bioactive phytoconstituents of methanolic extract of SA by the method of Gas Chromatography-Mass Spectrometry.

the formulation shows the presence of 16 phytochemical compounds in the composition. The active principles with their retention (RT), molecular formula (MF), molecular weight (MW) and concentration (peak area percentage) are depicted in Table 4. The mass spectra of identified phytochemicals are depicted in Figure 1.A comprehensive list of 16 bioactive compounds was identified through GC-MS assessment of the methanolic extract *viz*. Lupeol (19.20%); Lup-20(29)-en-3-one(15.79%); Cis-13-Octadecenoic acid, methyl ester(15.45%); Hexadecanoic acid, methyl ester(10.46%); Bergamotol, Z-à-trans-(7.46%); Sitosterol (5.99%); 3-Acetoxy-3-hydroxypropionic acid, methyl ester(5.13%); Methyl stearate (3.82%); Methyl tetradecanoate (2.90%); 9,12-Octadecadienoic acid (Z,Z)-, methyl ester(1.96%); Undecanoic acid(1.81%); Lanceol,cis(1.42%); 1-Isopropenyl-3-propenylcyclopentane(1.16%);2,6,10-Dodecatrien-1-ol,12-acetoxy2,6,10-trimeth-,(E,E,E)-(1.26%); Diepicedrene-1-oxide(0.76%) and 1-Ethyl-4,4-dimethyl-cyclohex-2-en-1-ol(0.90%). *In silico* data of molecular docking properties to study absorption-distribution-metabolism-excretion-toxicity for these compounds was collected from the database of Indian Medicinal Plants, Phytochemistry and Therapeutics.

Diepicedrene-1-oxideis a sesquiterpenoid and an oxygenated component may function as an antioxidant (Assaeed *et al*., 2020). It has high blood brain permeation and gastrointestinal absorption ability. It is potentially as good pharmacokinetic drug as its successfully passes the Lipinski's rule of 5 and Ghose rule. 10-Methyl undecanoic acid is non-toxic to mammalian cells and possesses antifungal properties (Rossi *et al*., 2021). It is component of RS-1 analog of teicoplanin glycopeptide antibiotic clinically effective against gram positive bacterial infections (Campoli-Richards, 1990). Cis-Lanceol is a sesquiterpenoid demonstrates effectiveness against pneumolysin toxin, which modulates inflammatory responses (Hirst *et al*., 2004). It has good blood brain barrier permeation and gastrointestinal absorption abilities having bioavailability score of 0.55. It passes the Lipinski's rule of 5 and Ghose rule.n-Hexadecanoic acid commonly called as palmitic acid is saturated fatty acid. It is reported to have antioxidant, anti-inflammatory, hypocholestrolemic, antitumor, immunostimulant, haemolytic, 5-α-reductase inhibitor, lipoxygenase inhibitor properties (Ganesan *et al*., 2022). Structural and kinetic studies demonstrate that n-Hexadecanoic acid is an inhibitor of phospholipase A2. PLA2s are prevalent in reptile and arthropod venoms (Aparna *et al*., 2012). Thus, n-Hexadecanoic acid may serve as a superior candidate for anti-phospholipase A2 drugs showing promise as antidotes against a wide range of snake venoms and other animal toxins (Xiao *et al*., 2017). It successfully passes the Lipinski's rule of 5 and Ghose rule. It has high blood brain barrier permeation ability and good gastrointestinal absorption ability with high bioavailability score of 0.85.9,12-Octadecadienoic acid (Z, Z) commonly called as linoleic acid is omega-6-unsaturated acid is an anti-inflammatory activity (Pinto *et al*., 2017 and Mori *et al*., 2013). It is a component of cell membrane and an Arachidonic acid (ARA) precursor (Tallima *et al*., 2018). It showcases different properties such as hypocholestrolemic, antihistaminic, 5-α-reductase inhibitor, antiarthritic and antioxidant properties. It passes Lipinski's rule of 5 and Ghose rule having the bioavailability score of 0.85.Cis-13-octadecenoic acid, also known as petroselinic acid, is a mono-unsaturated fatty

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

No.	Name of the compound	RT	Molecular formula	MW	Peak area %
1.	3-Acetoxy-3-hydroxypropionic acid, methyl ester	4.13	$C_6H_{10}O_5$	162.14	5.13
2.	Diepicedrene-1-oxide	9.00	$C_{15}H_{24}O$	220.35	0.76
3.	Undecanoic acid, 10-methyl-, methyl ester	9.07	$C_{13}H_{26}O_2$	214.34	1.81
4.	2,6,10-Dodecatrien-1-ol, 12-acetoxy2,6,10-trimethyl-, (E,E,E)-	9.21	$C_{17}H_{28}O_3$	222.36	1.26
5.	Bergamotol, Z-à-trans-	9.74	$C_{15}H_{24}O$	220.35	7.46
6.	1-Ethyl-4,4-dimethylcyclohex-2-en-1-ol	11.05	$C_{10}H_{18}O$	154.25	0.90
7.	Lanceol, cis	11.12	$C_{15}H_{24}O$	220.35	1.42
8.	1-Isopropenyl-3-propenylcyclopentane	11.21	$C_{11}H_{18}$	150.26	1.16
9.	Methyl tetradecanoate	11.41	$C_{17}H_{34}O_2$	242.39	2.90
10.	Hexadecanoic acid, methyl ester	13.54	$C_{17}H_{34}O_2$	270.45	10.46
11.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	15.17	$C_{19}H_{34}O_2$	294.47	1.96
12.	Cis-13-Octadecenoic acid, methyl ester	15.24	$C_{19}H_{36}O_2$	296.5	15.45
13.	Methyl stearate	15.48	$C_{19}H_{38}O_2$	298.5	3.82
14.	Lup-20(29)-en-3-one	16.94	$C_{30}H_{48}O$	424.7	15.79
15.	Lupeol	18.99	$C_{30}H_{50}O$	426.7	19.20
16.	Sitosterol	23.95	$C_{29}H_{50}O$	414.70	5.99

RT = Retention Time, MW = Molecular Weight.

acid influences the lipolysis. It has anti-inflammatory, anticancer, hypocholestrolemic properties (Delbeke *et al*., 2008). Another contributor, lupeol is a pentacyclic triterpenoid, is non-toxic to cells and tissues. Its potent the reduction of prostaglandin E2 (PGE2) production leads to anti-inflammatory activity. It passes Lipinski's rule of 5, failed in the Ghose rule having bioavailability score 0.55. Studies show that it can inhibit reactive oxygen species production and restore anti-oxidant levels. Lupeol having the greatest peak area percentage is known to reduce the generation of proinflammatory cytokines and exhibit high wound-healing potential (Fernandez *et al*., 2001).

Phytochemical identified in this study shows that this formulation is a potential candidate for investigation as remedy of snake bite. Compounds identified are notable for having wound healing properties, anti-inflammatory activities, PLA2 inhibition capabilities and others. On basis of data made available by this study researchers can conceptualize the mechanisms and action of phytochemicals in the body. This can be effective in study of identified phytochemicals to get insights of interactions that are possible for effectiveness of traditional drugs.

Conclusion

The components utilized in this study are believed to play a significant role in addressing toxicity resulting from snake bites. The outcomes of this research could contribute to a more reasoned evaluation and assessment of the versatile therapeutic applications of the formulated mixture. This study has broadened the possibilities for developing innovative drugs by phytochemical drugs derived from this composition. These drugs have the potential to serve as enhanced therapeutic agents, promoting awareness of the importance of exploring locally utilized remedies for addressing envenomation cases.

Acknowledgements

The authors express their gratitude to traditional healers and individuals who generously shared their knowledge of medicinal practices. Special recognition is given to the late Vaidya Ramchander Ji for his invaluable guidance and wisdom. The authors also express thanks to the Department of Botany and SAIF-CIL, Panjab University, Chandigarh, India, for providing research facilities.

Funding

The authors would like to express their gratitude to the University Grants Commission (UGC) for providing financial assistance in the form of a fellowship.

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