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Research Article

PRELIMINARY PHYTOCHEMICAL ANALYSIS AND GAS CHROMATOGRAPHY MASS SPECTROMETRY SPECTRA OF CHLOROFORM EXTRACT FROM *SPERMACOSE HISPIDA* L. SEED

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ABSTRACT

Objectives: Globally, scientific evaluation of traditional uses of herbal medicine, isolation, and characterization of bioactive constituents from herbs are some of the leading research areas. *Spermacoce hispida* (SH) is well known for its hypolipidemic and anti-obesity activity. The aim of this study is to qualitatively analyze the presence of primary and secondary metabolites in various extracts of SH seeds and to examine the presence of bioactive principles of chloroform extract from SH seeds.

Methods: Physicochemical analysis such as ash content, acid-soluble ash, water-soluble ash, moisture content, fiber content, ethanol soluble extractive value, and water-soluble extractive value for seeds of SH was determined as per WHO guidelines. Cold percolative extracts of seeds of SH with different solvents were carried out. Preliminary phytochemical analysis for the presence of various primary and secondary metabolites in extracts was determined. Gas chromatography mass spectrometry (GC-MS) analysis of chloroform extract was carried out.

Results: Physicochemical analysis values were found to be present in permissible level (<5%). Yield of ethyl acetate (4.9/100 g), ethanolic (4.2/100 g), and hydroalcoholic extract (4.0/100 g) of seeds of SH was found to be higher than that of extract obtained by soaking with different low polar solvents. Secondary metabolites such as phenol, flavonoid, and tannin are present in ethyl acetate, ethanolic, hydroalcoholic extract. Fat and alkaloid are present in chloroform extract. GC-MS spectra show the presence of 30 different bioactive constituents. Among them, n-hexadecanoic acid was found to constitute (5.83%) highest peak area than the remaining compounds.

Conclusion: Seeds of SH is a rich source of primary and secondary metabolites and various bioactive phytoconstituents.

Keywords: Physicochemical analysis, Primary metabolites, Secondary metabolites, Yield, Gas chromatography mass spectrometry spectra.

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INTRODUCTION

Indigenous folk medicine is often coexists with Western medicine, Ayurvedic, Chinese, or Unani medicine but it is distinguishable from those medicines [1].

Mankind has been using herbal medicine for thw for healing various illness, starting from the human civilization. Radical healing effect of modern medicine makes people to looked down upon herbal medicine. Although herbal medicine is known for numerous pharmacological activities, significant adverse effects are reported [2].

Standardization of herbal medicine and isolation and characterization of active compound from herbal extract constitute an important position in Indian system of medicinal research. Primary and secondary metabolites such as phenols, flavonoids, tannins, saponins, and cardiac glycosides are isolated from herbal extract and used by pharmaceutical industries. The active compounds isolated from extracts exhibits numerous pharmacological activities such as analgesic, anti-inflammatory, antidiabetic, antitumor, and antiarthritic.

Spermacoce belongs to the family Rubiaceae contains 275 species. *Spermacoce hispida* (SH) a herb belongs to this family, is traditionally used for antiobesity and hypolipidemic activity. Phytochemical screening of petroleum ether extract and methanolic extract of SH confirms the presence of saponins, tannins, phenolics, steroids, essential oils, flavonoids, and terpenoids. Development of petroleum ether, methanolic and water extract in thin-layer chromatography plate with benzene:chloroform (4:1) as mobile phase shows the presence of 3,3 3 spots, water extract of SH shows the presence of tannins, phenolics, steroids, flavonoids, and terpenoids [3].

Gas chromatography mass spectrometry (GC-MS) analysis of methanolic extract of SH shows the presence of 25 compounds. Some of them are 9, 12-Octadecadienoic acid, ethyl ester (35.58%) are present with high % peak area. Methanolic extract of seed also shows the presence of α-tocopherol [4]. Characterization of seed oil by performing different analysis include specific gravity, refractive index, acid value, saponification value, and iodine value shows that the values were within the American Society for Testing and Materials standard specification [3].

Petroleum ether, methanol, water extracts of SH exhibits antimicrobial activity against *Bacillus subtilis, Escherichia coli, Candida albicans,* and *Aspergillus niger* [3]. Flavonoid-rich fraction of SH shows antioxidant, and free radical scavenging activity against 2,2-azinobis (3-ethylbenzthiazoline)-6-sulfonic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and exhibits inhibitory concentration (IC_{50}) value, which is less than the activity of ascorbic acid, atorvastatin [5]. Other reported pharmacological activities of SH are anticancer [3]. Anti-inflammatory [6], hypolipidemic activity [7], etc.

Recent research work on SH is very few. The aim of this study is to qualitatively analyze the presence of various metabolites in different extracts of SH seeds and bioactive constituents in chloroform extract should be studied by GC-MS.

METHODS

Extraction of plant material

SH seeds were purchased from Siddha medicine shop, Chennai. The seeds were washed in distilled water and dried at room temperature for 15 days. The seed was coarsely powdered. The powdered plant material

was stored at room temperature for further use. 100 g of plant material was soaked in 1 leach of different solvents such as hexane, chloroform, ethyl acetate, ethanol and hydroalcohol (ethanol: water - 70:30) for 72 hrs and filtered. The cold percolation of seeds was repeated for 3 times. The filtrate was mixed and concentrated under reduced pressure in rotary vaccum evaporator. The concentrated extract was stored at refrigerator and yield was calculated.

Physicochemical analysis

Determination of ash value

About 5.0 g of SH seeds was weighed in clean, dried and weighed crucible, weight of crucible with 5.0 g of SH seed was note down. Crucible with SH seed was kept in muffle furnace at 600°C for 6 hrs. Crucible with ash was weighed and the ash value of SH seed was calculated for 100 g.

Determination of water soluble ash

About 5.0 g of SH seed was weighed in clean, dried and weighed crucible, weight of crucible with 5.0 g of SH seed was noted down. Crucible with SH seed was kept in muffle furnace at 600°C for 6 hrs. Crucible with ash was removed from furnace, and the ash was dissolved in 100 ml of water and filtered using Whatmann No: 1 filter paper. Water-insoluble ash present in filter paper was kept in weighed, clean crucible and again maintained in muffle furnace at 100°C for 1 hr. The water-soluble ash value was calculated from the value of water-insoluble ash.

Determination of acid-soluble ash

About 5.0 g of SH seed was weighed in clean, dried and weighed crucible, weight of crucible with 5.0 g of SH seed was noted down. Crucible with SH seed was kept in muffle furnace at 600°C for 6 hrs. Crucible with ash was removed from furnace and the ash was dissolved in 100 ml of diluted hydrochloric acid and filtered using Whatmann No: 1 filter paper. Acid-insoluble ash present in filter paper was kept in weighed, clean crucible and again maintained in muffle furnace at 100°C for 1 hr. The acid soluble ash value was calculated from the value of acid-insoluble ash.

Determination of loss on drying

About 5.0 g of SH seed was taken in a dried Petri plate. The Petri plate was maintained at 50°C for 2 hrs. The final weight of plant material was weighed. The difference between initial and final weight of SH seed was calculated as loss on drying.

Determination of crude fiber content

About 2.0 g of SH seed was weighed accurately and transferred to a porcelain dish. 50 ml of 10.0% nitric acid was added and boiled for 10 minutes with constant stirring and filtered through fine mesh cotton cloth. The residue was washed with 5.0 ml of boiled water. The material from the cloth was collected in a porcelain dish and boiled with 50.0 ml of 2.5% caustic soda. Then, the liquid was filtered using a fine mesh cotton cloth. The residue was washed with 100.0 ml of boiling water. Then, the fiber was collected in a dried and weighed crucible. The crucible was then placed at 105°C for 2 hrs. That was then kept in a desiccator and cooled. The cooled crucibles were weighed. The measured weight of the residue from the crude fiber content was calculated.

Qualitative analysis of primary and secondary metabolites in various extracts of SH seeds

Test for alkaloids

A small portion of the extract was stirred with a few drops of dilute hydrochloric acid and filtered. The filtrate was used for the following tests:

- Dragendroff's reagent: An orange precipitate obtained exhibits the presence of alkaloids.
- Hager's reagent: A yellow precipitate formed shows the presence of alkaloids.
- Wagner's reagent: A brown precipitate obtained shows the presence of alkaloids.

Test for carbohydrates

A small quantity of extracts were dissolved separately in 5.0 ml of distilled water and filtered. The filtrate was subjected to Molisch's test to detect the presence of carbohydrates. Molisch's test: Filtrate was treated with 2-3 drops of 1% alcoholic alpha-naphthol solution and 2.0 ml of concentrated sulfuric acid. The formation of purple color showed the presence of carbohydrates.

Test for glycosides

Another portion of the extract was hydrolyzed with hydrochloric acid for few hours in a water bath, and the hydrolysate was subjected to Legal's and Borntrager's tests to detect the presence of different glycosides.

- Legal's test: To the hydrolysate, 1.0 ml pyridine and few drops of sodium nitroprusside solution were added and then it was made alkaline with sodium hydroxide solution. The formation of purple color indicated the presence of glycosides.
- Borntrager's test: Hydrolysate was treated with chloroform, and the chloroform layer was separated. To that equal quantity of dilute ammonia solution was added. The formation of yellow color showed the presence of glycosides.

Test for sterols

Liebermann-Burchard test: 1.0 g of the extract was dissolved in few drops of dry acetic acid. 3.0 ml of acetic anhydride was added followed by few drops of concentrated sulfuric acid by the sides of the test tube. The formation of green color showed the presence of sterols.

Test for proteins and aminoacids

Small quantities of various extracts were dissolved in a few ml of water and treated with.

- Ninhydrin reagent: A purple coloration obtained suggested the presence of amino acids.
- Biuret reagent: A violet coloration obtained placed the presence of proteins.
- Millon's reagent: A yellow precipitate formed displayed the presence of aminoacids.

Test for flavonoids

The extracts were tested for the presence of flavonoids using aqueous sodium hydroxide solution. An increase in the intensity of yellow color portrayed the presence of flavonoids.

GC-MS analysis of chloroform extract of SH seeds

About 10.0 mg of chloroform extract was dissolved in chloroform and analyzed in GC Clarus 500 Perkin Elmer using following experimental conditions: Column type - Elite -5 (5% diphenyl 95% dimethyl polysiloxane), Column dimension 30 mm × 0.32 mm), carrier gas - Helium 1 ml/minute, column temperature from 50°C to 285°C at the rate of 10°C/minutes and, 5 minutes hold at 285°C, injector and detector temperature - 290°C, injection mode - split, volume injected - 0.5 µl of solution prepared from 2 mg/100 ml in methanol. Total run time was 30 minutes. Mass spectrum was taken using mass detector - Turbo mass gold - Perkin Elmer. Transfer line temperature - 230°C, source temperature - 230°C, scan range is from 40 to 450 amu, ionization technique - electron ionization technique.

Statistical analysis

The values given are mean±standard error of triplicate.

RESULTS

Results of physicochemical analysis of raw SH seed are furnished in Table 1. Obtained and calculated ash value, acid-soluble ash, water-soluble ash, loss on drying, fiber content of SH seed was found to be <5.0/100.0 g.

Yield of extract obtained by soaking SH seed in different solvents with increasing polarity such as petroleum ether, chloroform, ethyl acetate, ethanol and hydroalcohol are mentioned in Table 2.

The presence/absence of primary and secondary metabolites such as carbohydrate, protein, amino acids, fat, alkaloid, tannin, phenol, flavonoid in different extracts of SH seeds obtained by soaking in solvents with different polarity are mentioned in Table 3.

Various compounds present in chloroform extract of SH seeds analyzed by GC-MS are mentioned in Table 4, Figs. 1-4. Nearly, 30 different compounds are found to be present in chloroform extract. 11 compounds are observed to be present with % peak area >2.0. 9.12 octadecadienoic acid (Fig. 2), n-hexadecanoic acid (Fig. 3), 9 - octadecanoic acid (Fig. 4), octadecanoic acid, were found to constitute 8.35%, 5.83%, 5.02%, 2.18% (Table 4). 1, Iodo-2-methylundecane was found to constitute nearly 3.02%.

DISCUSSION

Physicochemical analysis of raw SH seed was carried out to check the purity before initiating all experiments. All the values are found to be present within the permissible level of WHO guidelines. The results of Table 1 reveal that the collected plant material was pure in quality.

Extractive values constitute an important segment in selecting a herb in nutraceutical and pharmaceutical industry. Yield of extract from SH seeds (Table 2) reveals that ethanolic and hydroalcoholic extracts are obtained in high quantity rather than extracts obtained from low polar solvents.

Table 1: Physicochemical analysis of SH seeds

Parameters	g/100 g of sample
Ash value	4.4±0.4
Water soluble ash	2.2±0.4
Acid soluble ash	2.4±0.4
Loss on drying	1.2±0.03
Crude fiber content	4.5±0.5

Values are mean±SD of triplicate, SH: *Spermacose hispida*, SD: Standard deviation

Table 2: Yield of extract from SH seeds

Solvent used	Yield (g/100 g of plant)
Hexane	0.95±0.2
Chloroform	0.963±0.3
Ethyl acetate	4.017±0.34
Ethanol	4.223±0.25
Hydroalcohol (ethanol:water 70:30)	4.025±0.32
Water	4.362±0.33

Values are mean±SD of triplicate, SH: *Spermacose hispida,* SD: Standard deviation

Table 3: Qualitative analysis of various extracts from SH seed

Metabolites	Present/absent				
	Hexane	Chloroform	Ethyl acetate	Ethanol	HAE
Carbohydrate	Present	Present	Present	Present	Present
Protein	Present	Present	Present	Present	Present
Amino acids	Absent	Absent	Absent	Absent	Absent
Oil	Present	Present	Present	Present	Present
Phenol	Absent	Absent	Present	Present	Present
Flavonoid	Absent	Absent	Present	Present	Present
Tannin	Absent	Absent	Present	Present	Present
Alkaloid	Present	Present	Present	Present	+

SH: *Spermacose hispida*

Qualitative analysis of various primary and secondary metabolites was performed (Table 3). The primary metabolites such as carbohydrate, proteins, and oil were found to be present in all extracts of SH seeds including chloroform, hexane, ethyl acetate, ethanol and hydroalcohol. Moreover, amino acids were found to be absent in all extracts. The presence of oil in chloroform extract was observed to give more positive result rather than other extracts. The presence of saturated and other PUFA in oil should be observed by carrying out analysis with sophisticated instruments. Hence, GC-MS analysis for chloroform extract alone was continued. Quantitative analysis of oil in chloroform extract should be carried out.

Secondary metabolites such as phenol, tannin, and flavonoid were found to be present in extracts with high polarity starting from ethyl acetae. Alkaloid was found to be present in all extracts including extracts with low polar solvents. The presence of secondary metabolites favors SH seeds to exhibit numerous pharmacological activity. The antidiabetic activity [8], hypolipidemic and antioxidant activity [9], anti-inflammatory [10] of dietary flavonoids are reported earlier.

Atrial arrhythmia and heart failure can be treated by inhibiting Na/K ATPase using cardiac glycosides [11]. Tannins such as geraniin, and other oligomeric hydrolysable tannin, complex tannin isolated from various medicinal plants were reported to exhibit antitumor, inhibition of carcinogenesis, inhibition of lipid peroxidation inhibition of lipoxygenase xanthine oxidase, etc. [12]. Antioxidant and antiproliferative effect of tannins isolated from *Clerodendrum infortunatum* on HCT-15 cells were reported earlier [13].

Saponins are large molecules, contain a hydrophobic part, composed of a triterpenoid and a hydrophobic part consisting of several saccharide

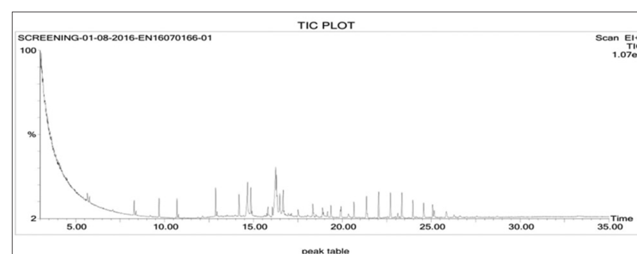

Fig. 1: Gas chromatography chromatogram of chloroform extract from *Spermacose hispida* seeds

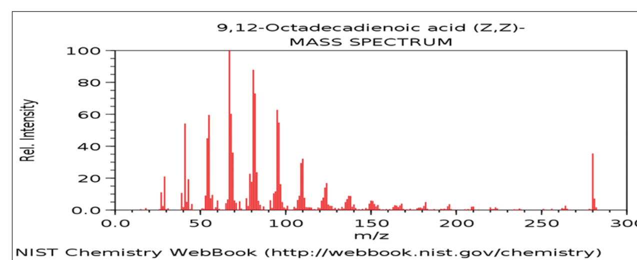

Fig. 2: Mass spectrometry spectra of 9, 12, octadecadienoic acid

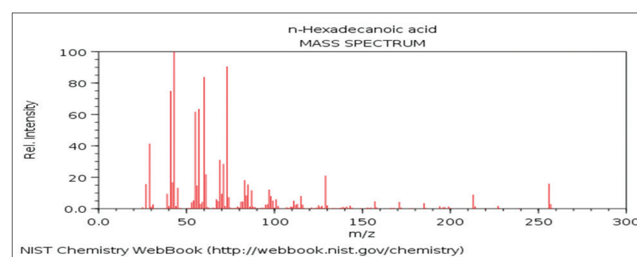

Fig. 3: Mass spectrometry spectra of n-hexadecanoic acid

Table 4: List of compounds present in chloroform extract of SH seeds observed by mass spectrometry

RT	Area	Area %	Name
3.149	1778408	0.79	Decane, 2-methyl
5.664	1687910	0.75	1-Nonene
8.290	3081564	1.36	3-Tetradecene, (Z)
9.691	3638538	1.61	Phenol, 2,4-bis (1,1-dimethylethyl)
10.700	3608353	1.60	1-Dodecene
12.871	5251670	2.33	3-Octadecene, (E)
14.190	4562686	2.02	Hexadecanoic acid, methyl ester
14.680	13153167	5.83	n-hexadecanoic acid
14.855	5320530	2.36	1-Nonadecene
15.818	2486356	1.10	9-Dodecenoic acid, methyl ester, (E)
16.063	1837099	0.81	Methyl stearate
16.250	18852652	8.35	9,12-Octadecadienoic acid, methyl ester, (E, E)
16.296	11352155	5.03	9-Octadecenoic acid, (E)
16.483	4914940	2.18	Octadecanoic acid
16.670	5240251	2.32	10-Heneicosene (c, t)
17.510	2025695	0.90	Undecanoyl chloride
18.339	2726082	1.21	1-Nonadecene
18.887	1823021	0.81	13-Tetradece-11-yn-1-ol
19.354	2869336	1.27	Diisooctyl phthalate
19.920	2493595	1.10	Dodecane, 2,6,11-trimethyl
20.334	1708918	0.76	1-Hexadecyne
20.649	3405545	1.51	1-Iodo-2-methylundecane
21.355	6659169	2.95	Nonadecane
22.038	6060631	2.68	Sulfurous acid, pentadecylpentyl ester
22.703	6824229	3.02	1-Iodo-2-methylundecane
23.345	6220642	2.76	Heptacosane
23.964	4629372	2.05	Sulfurous acid, hexyl pentadecyl ester
24.571	4365498	1.94	Sulfurous acid, hexyl pentadecyl ester
25.078	2880250	1.28	Silane, dimethyl (2-naphthoxy) hexadecyloxy
25.849	2690052	1.19	2-Piperidinone, N-[4-bromo-n-butyl]

SH: *Spermacose hispida*

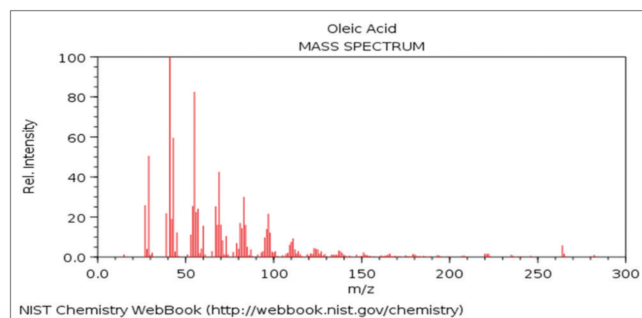

Fig. 4: Mass spectrometry spectra of 9, octadecanoic acid

residues linked to the hydrophobic scaffold through glycosidic bonds. They have many medicinal uses including microbial, antitumor, antiinsect hepatoprotective, hemolytic, and anti-inflammatory activities [14].

In general, 9,12 octadecadienoic acid, exists in different forms like linoleic acid, linolelaidic acid, rumenic acid. Cytotoxic and antitumor activity of octadecanoic acid extract suspected to be a rich source of oleic and linoleic acid was evaluated in human tumor cell lines [15]. N-hexadecanoic acid was found to inhibit phospholipase A2 and thus exhibits anti-inflammatory activity [16].

1-Nonene constitute 0.75% area. 1-Nonene was also found to be reported earlier in the essential oil isolated from *Farfugium japonicum* flowers and % area was calculated as 19.83. This essential oil was reported to exhibit anti-cancer activity, inhibition of nitric oxide synthase, cyclooxygenase mRNA gene expression and thus exhibits anti-inflammatory activity [17].

1-Dodecene constitute 1.6% in total % peak area. 1-dodecene was also reported to be present in essential oil of *Etlingera elatior* (24, 0.31%). This essential oil was reported to exhibits DPPH radical scavenging activity with IC_{50} value 995 µg/ml. The authors suggested the free radical scavenging activity of this essential oil might be due to the presence of 1-dodecene [18].

GC-MS analysis of ethanolic extract of *Solanum torvum* sw. the presence of 1-nonadecene (20.4%). The extract was found to exhibits antitumor activity, though the active compound of the extract is not elicited; the concentration of 1-nonadecene may responsible for antitumor activity [19].

In conclusion, SH yield higher extractive value when extracted with ethanol and hydroalcohol solvents. SH seeds are a rich source of various primary and secondary metabolites. Chloroform extract of SH seed is a rich source of saturated and unsaturated fatty acids with potent bioactive constituents such as 9, 12 octadecadienoic acid, n-hexadecanoic acid which is reported for their cytotoxic, anti-inflammatory activity, etc., SH seeds can be used as a good drug in pharmaceutical and nutraceutical industry.

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