Indian Journal of Natural Products and Resources
Vol. 7(2), June 2016, pp. 116-124

Isolation and identification of withasomnine, withanolides and butein from industrial herbal marc of *Withania somnifera* (L.) Dunal

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Received 13 October 2015; Revised 27 January 2016

Herbal pharmaceutical industries consume thousands of tonnes of herbs annually, with a major share consumed for preparation of aqueous extracts as part of production process. Marc, the byproduct of aqueous extraction process produced in similar quantities is considered to be devoid of hydrophilic compounds and of low economic value. *Withania somnifera* (L.) Dunal is a herb having high therapeutic and frequency index and their increasing consumption in the recent decades have resulted in upsetting the demand-supply equations. The shortage of *W. somnifera* justifies their efficient and wholesome utilization to augment the natural sources. The present study was undertaken to evaluate phytochemical constituents present in the marc of *W. somnifera* roots collected from an Ayurvedic pharmaceutical industry. The HPTLC and GC-MS analyses is revealed presence of different compounds in marc such as withanolides, as well as oleic, linoleic and linolenic acids. Isolation and characterization by UV-Visible, FT-IR, ^{1}H-NMR, ^{13}C-NMR and Mass spectroscopy showed presence of withasomnine, an alkaloid and butein, a phenolic compound. The study explored potential for using marcs of *W. somnifera* produced in pharma industry as an alternate natural and economical source for pharmaceutically active compounds like polyphenols, flavonoids, alkaloids, etc.

Keywords: Aqueous extraction residues, Butein, Industrial herbal marc, *Withania somnifera* (L.) Dunal, Withanolides, Withasomnine.

IPC code; Int. cl. (2015.01)– A61K 36/00, C07G 5/00

Introduction

The traditional medicine systems in India and China have been centered on medicinal herbs for about 5000 years. Herbs based medicines have increasingly become popular owing to their natural origin, reduced side effects and high therapeutic efficacy. A majority of world population still rely on traditional medicines for their healthcare needs[1]. With more than 15,000 herbal pharmaceutical industries in India, the annual consumption for herbs is estimated to be of the order of several thousand tonnes of which about 80 % are collected from wild and rest from commercial cultivation[2]. The increased demands have resulted in over exploitation and fast depletion of medicinal plants like *Saraca asoka, Rauwolfia serpentina, Withania somnifera, Picrorhiza kurroa,* etc[3]. Among the different officinal parts used in pharma industry, consumption of roots account for more than about 30 % by weight and has speeded up destructive harvesting[4]. Human population dependent factors have also contributed to large scale herbal habitat destruction and deforestation in their natural geographies[5]. State laws, control over harvesting and other interventional practices have helped slow down their destruction, whereas commercial agriculture to some extent has augmented the herbal sources for meeting industrial production demands. Still, further studies identifying new methodologies and practices are necessary for the sustained availability of natural resources.

Withania somnifera (L.) Dunal (Family Solanaceae), also known as Indian Ginseng is a widely used therapeutically important plant[6,7] that serves as an ingredient in more than 200 formulations in traditional Indian medicine. *W. somnifera* is used for its properties such as immunomodulation, anti-tumor, anti-cancer, antioxidant, neuroprotective, anti-aging adaptogenic[8-14], etc. In ayurvedic pharma industry, it is reported that a major portion of supplied *W. somnifera* goes towards preparation of aqueous

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extract by methods described in texts of Ayurveda[15,16]. The aqueous extraction process generates extracts that are utilized in manufacturing of different formulations, while the residues from extraction process, called marcs, are treated as low economic value byproducts and are disposed of by burning as fuel or treated as agriculture manure.

More than 85 compounds from the roots of *W. somnifera* including steroidal lactones, saponins and phenolic compounds; the withanolides and alkaloids being principal compounds considered to be contributing to its pharmacological activities[17-19]. Among these phytoconstituents, alkaloids, steroids, terpenoids, etc. show low solubility in water and their yield from extraction is affected by factors such as solvent, temperature, solid/solvent ratio, method and time of extraction, nature and size of herbs, structure and stereochemistry of the compounds, etc[20]. Yield for phenolic compounds in ethanol and methanol extracts have been reported to be higher than those from their water extract[21], indicating incomplete extraction of phytochemicals when water alone is used as solvent, as in the case of traditional medicine manufacture. Therefore, there exists scope for evaluating the marcs obtained from water extraction process for the presence of active metabolites and further value addition.

With reference to phytochemistry and bioactive constituents, few studies have been reported on herbal marcs[22]. This paper discusses isolation and identification of bioactive compounds from herbal marcs of *W. somnifera* towards exploring feasibility of their use for extraction of medicinally and economically important compounds.

Materials and Methods

For the study, samples of *W. somnifera* and its marc (WSM) were collected from an ayurvedic medicine manufacturing industry in August 2012. The original root samples of *W. somnifera* and the marcs produced from them were identified by taxonomist Dr. P K Vasudevan Nair, Retired Professor, Government Victoria College, Palakkad, University of Calicut. Voucher specimens, AVP/HWS/20/2012 and AVP/HWSM/21/2012, respectively for herb and the marc samples were deposited at the R&D Department of The Arya Vaidya Pharmacy, Coimbatore.

Chemicals, reagents and instrumentation

Analytical grade chemicals were purchased from M/s. Merck Chemicals. Gallic acid, withanolide A and withaferin A were purchased from M/s. Sigma Aldrich. HPTLC and TLC were performed on silica gel 60F-254 aluminum plates, while column chromatography was performed with silica gel 60-120 mesh. The FT-IR analysis was performed for the range 650 to 4000 /cm using Perkin Elmer Spectrum One FT-IR instrument. For FT-IR, the sample was mixed with KBr and pressed into transparent discs of size 13 mm diam. × 0.3 mm thickness. Using ethanol as solvent, the wavelength for maximum absorption (λ_{max}) was determined on a Shimadzu UV-Vis Spectrophotometer 1800. The NMR spectra for the samples were recorded using Bruker Avance III 500 MHz NMR Spectrometer where methanol-D4 was used as solvent and chemical shifts were observed relative to standard tetramethylsilane. Further structural characterizations of isolated compounds were carried out using an Electron Ionization Mass Spectroscopy. GC-MS analysis was performed using Thermo Trace Ultra coupled with Thermo MS DSQII mass spectrometer. An MS capillary of 30 m × 0.25 mm / DB35 was used for chromatographic separation of compounds.

Preparation of extracts

The roots of *W. somnifera* and WSM obtained were separately dried and milled. About 100 g coarse powders were extracted successively using soxhlet extractor with solvents of increasing polarity such as n-hexane, ethyl acetate and ethanol in the solid/solvent ratio 0.33, respectively for 10 h each. The extracts were then concentrated in a rotary evaporator.

Phytochemical screening of extracts

Preliminary phytochemical screening was carried out on the different extracts for detecting the presence of phenols, flavonoids, tannins, steroids, terpenoids, phytosterols and saponins using alcoholic ferric chloride, Shinoda test, Breamer's reagent, Leiberman-Burchard reagent and foam test, respectively. Presence of alkaloids was analysed by Dragondorff's, Wagner's, Mayer's and Hager's reagents[23].

Total phenolic content

The total phenolic content was determined using Folin-Ciocalteu's reagent by UV-Visible spectroscopy method[24]. Observations were recorded at wavelength of 760 nm and the results were expressed as gallic acid equivalents.

Total flavonoid content

Total flavonoid content in the ethanol extract was evaluated by aluminum chloride colorimetric method[25]. Quercetin was used as standard and absorbance was measured at a wavelength of 415 nm using UV-Visible spectrophotometer.

Total alkaloids

A quantitative determination for total alkaloid content in the sample was carried out using standard methods[26].

Identification of phyto-constituents

Estimation of withanolides

The ethanol fraction was further fractionated twice with equal volume of chloroform. The chloroform fractions were pooled together, completely dried and estimated as the total withanolides content[27]. It was then dissolved in ethanol, filtered and subjected to HPTLC analysis. Pre-coated silica gel 60-F254 plates were used for loading 5 µL of withanolide extract. Solvent system of chloroform: ethyl acetate: ethanol: benzene (70 : 4 : 8 : 24 v/v) was used as mobile phase and visualization was made by heating at 110 ^{o}C for 15 min after spraying with anisaldehyde reagent (0.5 g anisaldehyde in 20 mL acetone, 80 mL water and 10 mL 60 % perchloric acid). Withanolide A and withaferin A were run as standard (5 µL of 1 mg/mL ethanolic solution)[28]. The quantitative determinations were carried out from the densitogram.

GC-MS analysis of hexane extract

Phytochemical composition of n-hexane extract was analysed using GC-MS. Hexane extract was heated with 5 mL of 5 % methanolic sulphuric acid. After cooling, it was diluted and extracted with 25 mL hexane, followed by vigorous shaking with 20 mL distilled water. Then, the hexane layer was separated and washed with 5 % sodium carbonate solution followed by 5 % sodium chloride solution. The separated hexane layer was dried over anhydrous sodium sulphate and then concentrated using rotary evaporator. This was dissolved further in GC-grade n-hexane and 1 µL of the solution was subjected to GC[29]. After 5 min solvent delay time at 70 ^{o}C, the temperature was raised to 250 ^{o}C at 6 ^{o}C/min isocratic and cooled down to 70 ^{o}C to obtain the mass spectrum.

Isolation of compounds

Bio-activity guided isolation of phenolic compound

Compounds present in the ethanol extract of *W. somnifera* were separated by column chromatography. Silica gel was used as stationary phase and the extract was eluted through the wet filled column using toluene : chloroform : ethanol (4 : 3 : 3) solvent system. Five mL of fractions were collected and pooled according to thin layer chromatography results. The four fractions which answered positive to phenolic test was further screened for *in vitro* antioxidant activity by DPPH (2'2-diphenyl-1-picryl hydrazine) radical scavenging assay[30], using Vitamin C as standard. The third fraction which exhibited the highest DPPH radical scavenging activity was further purified and characterized by different methods, viz. UV-Visible, IR, ^{1}H-NMR, ^{13}C-NMR and Mass spectroscopy.

Isolation of alkaloid

The alkaloid fraction of WSM was further separated by preparative TLC method and fractionated by column chromatography. Silica gel was used as stationary phase and the extract was eluted through a wet filled column using a chloroform : ethanol (9 : 1) solvent system. Five mL of fractions were collected and pooled according to thin layer chromatography results. The fourth fraction was separated, concentrated and characterized by different spectroscopy methods, UV-Visible, IR, ^{1}H-NMR, ^{13}C-NMR and Mass spectroscopy.

Results and Discussion

The quantitative determinations of phytochemicals extracted with different solvents by successive solvent extraction were observed as 0.39±0.07, 0.87±0.08 and 5.26±0.76 % for n-hexane, ethyl acetate and ethanol, respectively. The results were expressed as mean from five different determinations with standard deviations. Similar analysis on *W. somnifera* samples collected before their extraction with water, yielded percentage extractive values of 0.61±0.05, 1.32±0.13 and 16.88±0.68 % for n-hexane, ethyl acetate and ethanol, respectively. This indicates that WSM retained about 34.66 % of total extractable matter compared to the original *W. somnifera*.

Preliminary phytochemical studies carried out on different extracts showed presence of phytosterols in hexane extract and phenols, flavonoids and glycosides in ethyl acetate extract. Ethanol extract contained phenols, flavonoids, tannins, steroids, glycosides and alkaloids. These results were comparable with that of extracts prepared from *W. somnifera* before extraction. The results of biochemical assays on WSM showed phenolic compounds, flavonoids and alkaloids to the extent of 6.62±0.43, 1.01±0.10, and

1.48±0.11 %, respectively and those obtained for *W. somnifera*, were 16.80±0.84, 5.49±0.92 and 1.62±0.79 %, respectively. The results indicate that about 39.40, 18.40 and 91.36 % of phenolic compounds, flavonoids and alkaloids, respectively were retained in the WSM compared to *W. somnifera* roots. The total withanolide content in the marc was estimated as 0.01 % and that in *W. somnifera* before extraction was observed as 0.021 %. HPTLC fingerprinting verified presence of withanolide A and withaferin A at concentration of 1.22 µg/mg (R_f 0.89) and 0.76 µg/mg (R_f 0.66), respectively (Plate 1).

The GC-MS spectrum obtained was analysed using NIST mass spectral library and other literarture[29]. The GC-MS profiles, as shown in Plate 2 and 3, helped in detection of six nonpolar compounds: oleic acid, linoleic acid, linolenic acid, anethole, octodecanoic acid and pellitorine in the esterified (methyl ester) hexane extract of WSM. The details of identified compounds are given in Table 1. A peak observed at m/z 87 could be associated with loss of $-(CH_2)_2CO_2CH_3^+$ from the molecular ion and base peak at m/z 74 could be due to McLafferty rearrangement. Structures of the six identified compounds are shown in Fig. 1 and 2.

Isolation of compounds

Bio-activity guided isolation of phenolic compound

Four phenolic fractions obtained from column chromatography were assessed for their antioxidant activity (Fig. 3). The third fraction exhibited highest DPPH radical scavenging activity and hence this fraction was further purified and identified by spectroscopy methods.

The compound was obtained as yellow solid, 0.048 g with melting point 211 ºC, practically insoluble in water and freely soluble in alcohol and ethyl acetate. Two peaks were observed in the UV-Visible spectrum at 262 and 380 nm. In the FT-IR spectrum, a broad band was observed at 3393 /cm, indicating the presence of intramolecular hydrogen, bonded with >C=O, 2372, 1597, 767 /cm corresponding to phenolic hydroxyl groups and a band at 1640 /cm that is characteristic of chalcones >C=O group. The ^{1}H-NMR spectrum showed up fielded aromatic proton signals at δ 8.02, 7.96, 7.90, 7.89 ppm and hydroxyl proton signals at δ 4.2, δ 4.03 and δ 6.02 of meta coupled hydroxyl protons. For two trans coupling olefinic protons (Hα and Hβ), signals were observed at δ 8.42 and 7.52. The ^{13}C-NMR spectrum for the new compound indicated a peak at δ 190 corresponding to carbonyl carbon, peaks at 143.7, 143.6, 138.5 for oxygenated aromatic carbons and those at δ 129.9, 128.5, 128.2 for aromatic carbons. Two quaternary aromatic signals were observed at δ 129 and 128 ppm. Quadrat at δ 94.6 and 131.0 ppm represent olefinic carbons. Molecular ion peak in the Mass spectrum was observed at m/z 272 and other peaks at 255 [$M^{+\cdot}$ - H_2O], 163, 150, 137, 123, 110, 96. The base peak with 100 % intensity was observed at m/z 137 representing $[C_7H_5O_3]^+$ fragment ion, which confirmed the structure. From spectroscopy results and literature concordance[31,32], the compound was identified as 3, 4, 2' 4'-tetrahydroxy chalcone, a polyphenol compound with molecular formula $C_{15}H_{12}O_5$, commonly known as butein. The mass spectrum for the isolated compound and its structure is shown in Plate 4.

The presence of naringenin chalcone (4, 2', 4', 6'-tetrahydroxy chalcone) having the same molecular formula ($C_{15}H_{12}O_5$) as butein has been reported in *W. somnifera*[33]. The mass spectrum discussed above does indicate a molecular ion peak at m/z 272 that could correspond to both butein and naringenin chalcone. However, observing m/z values for fragment ions in

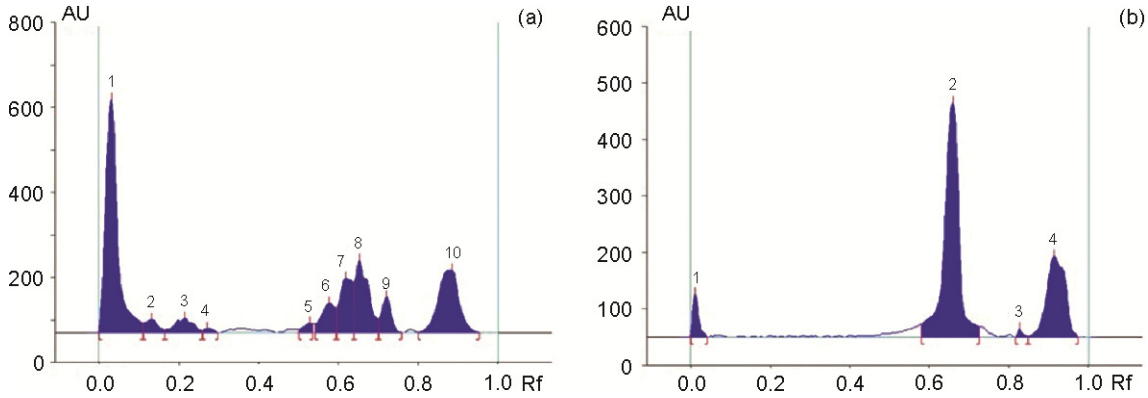

Plate 1—HPTLC densitogram of *Withania somnifera*

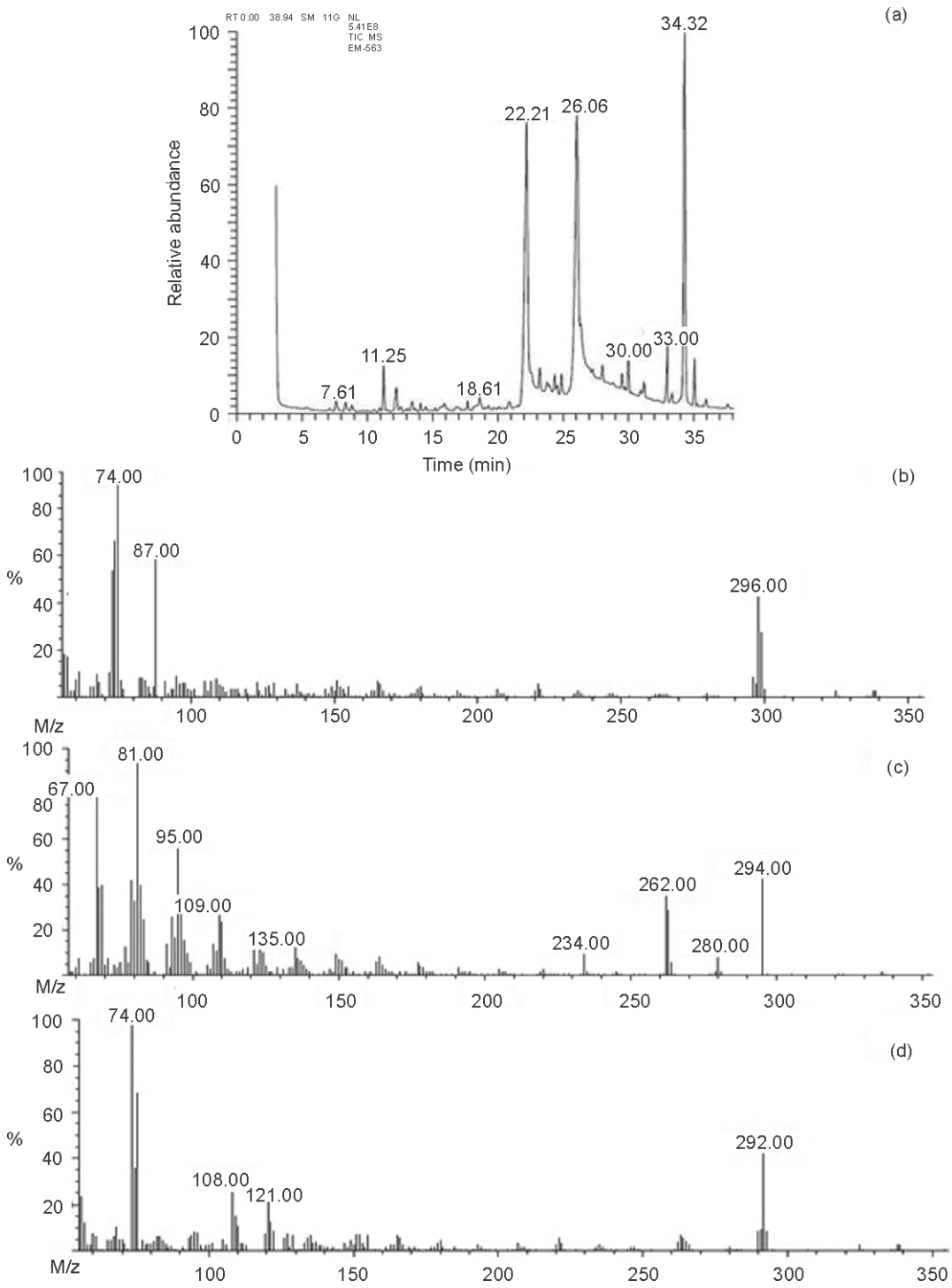

Plate 2—a) GC of hexane extract of *Withania somnifera*, with MS profiles indicating b) oleic acid, c) linoleic acid and d) linolenic acid.

mass spectrum obtained, a base peak was observed at m/z of 137 that would represent $[C_7H_5O_3]^+$ fragment ion. There was however no base peak at m/z 153 as to be expected for $[C_7H_5O_4]^+$ fragment ion of naringenin chalcone[34]. The melting point (211 ºC) also confirmed the isolated compound as butein.

In this study, isolation of bioactive compound from *W. somnifera* resulted in the recovery of a natural phenolic compound 3, 4, 2' 4'-tetrahydroxy chalcone (butein), belonging to flavonoid family. The presence of butein has earlier been reported in a few unrelated genera such as *Butea monosperma* and *Semecarpus*

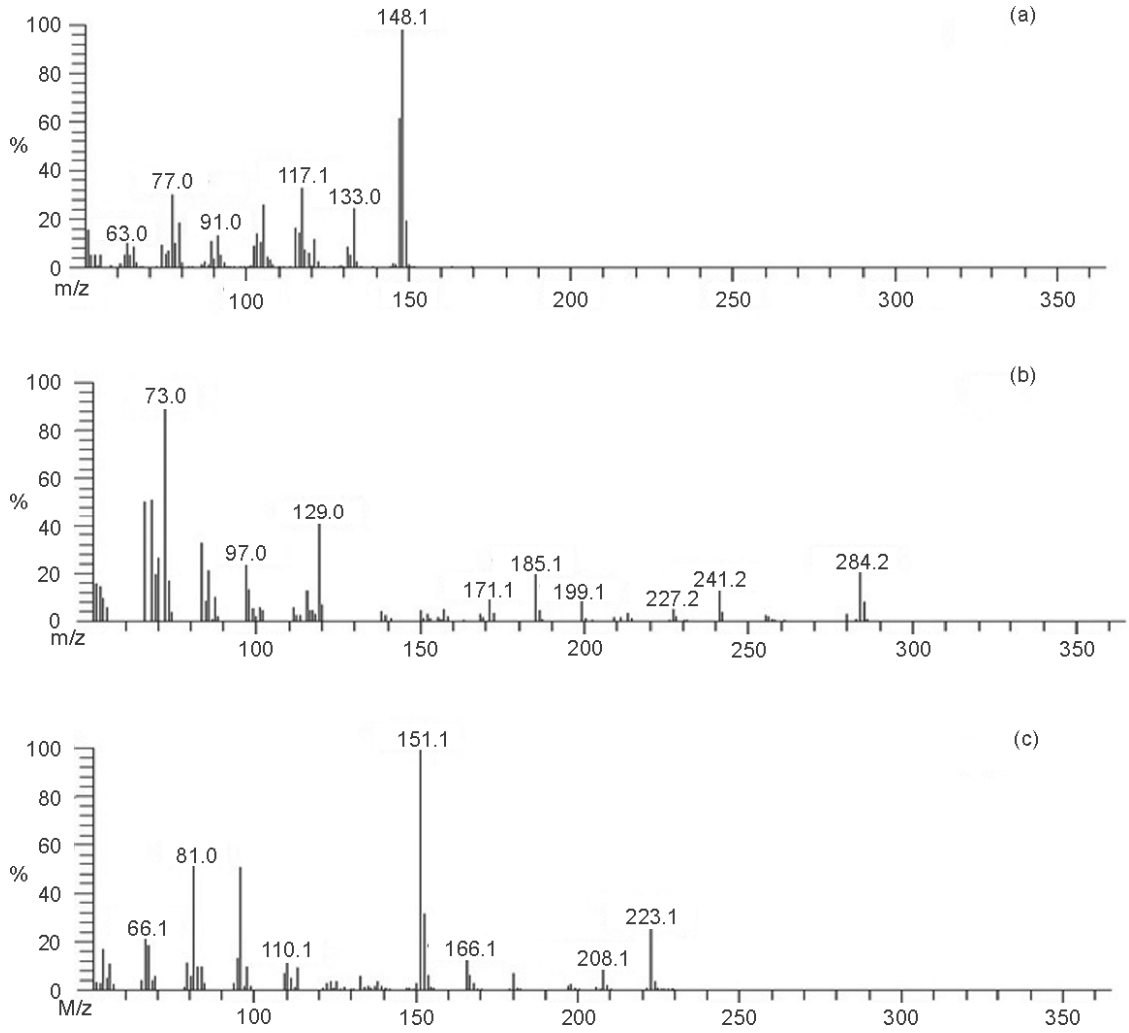

Plate 3—MS profiles showing a) anethole, b) octodecanoic acid and c) pellitorine.

Table 1—Compounds isolated from marc of *Withania somnifera* by GC-MS.

Phyto-compound	Retention time (Min)	MS data (m/z)	Quantity of identified compounds (dry extract weight, mg/g)
1-methoxy-4-(1-propenyl) benzene [Anethole]	11.25	148 [M^+], 133 (26 %), 117 (35 %), (30 %)	0.018±0.22
9-octadecanoic acid [Oleic acid]	22.21	296 [M^+], 87 (58 %), 74 (100 %), 55 (16 %)	0.83±0.30
(2-methylpropyl)-2,4-decadienamide [Pellitorine]	23.22	223 [M^+], 151 (100 %), 81 (50 %)	0.01±0.42
octadecanoic acid [Stearic acid]	26.06	284 [M^+], 185 (20 %), 129 (42 %), 73 (92 %)	0.86±0.28
9,12-octadecanoic acid [Linoleic acid]	34.32	294 [M^+], 109 (20 %), 95 (35 %), 81(100 %), 67 (70 %)	1.06±0.5
octadeca-9,12,15-trienoic acid [Linolenic acid]	35.14	292 [M^+], 121 (18 %), 108 (20 %), 74 (100 %), 55 (22 %)	0.19±0.32

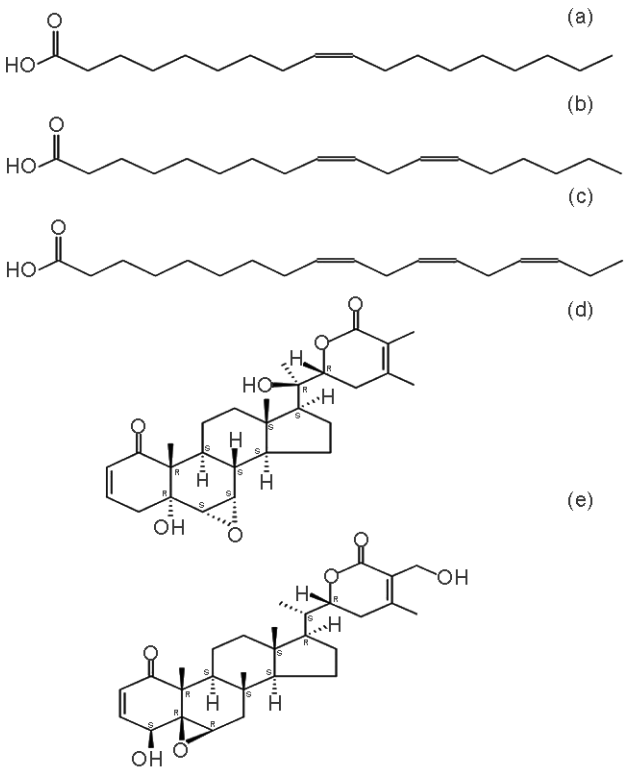

Fig. 1—Chemical structure of a) oleic acid, b) linoleic acid, c) linolenic acid, d) withanolide and e) withaferin A.

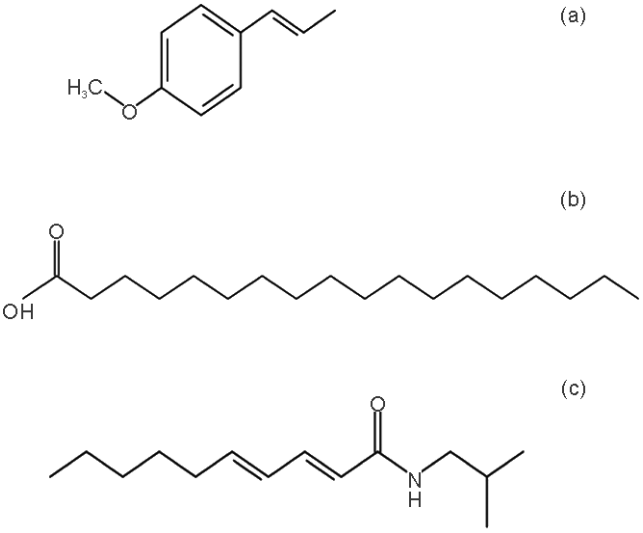

Fig. 2—Chemical structure of a) anethole, b) octodecanoic acid and c) pellitorine

anacardium L[35]. Analytical study in this work has helped report presence of butein in *W. somnifera*. The presence of butein was also detected in the fresh sample of *W. somnifera* roots, which is being reported for the first time. Butein exhibits less solubility in water and is therefore present in water extracted roots. The strong antioxidant properties reported for butein

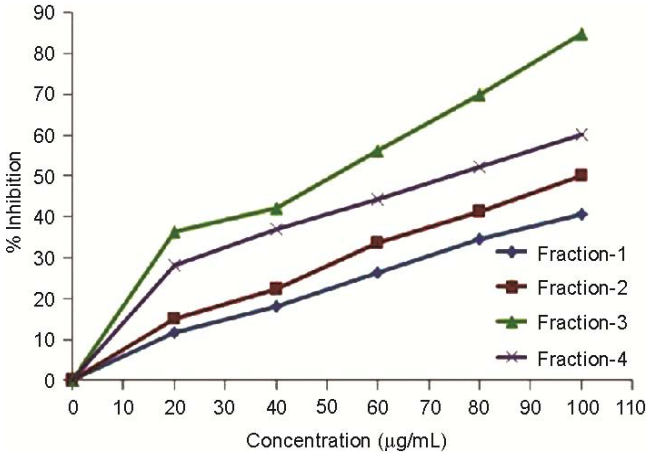

Fig. 3—DPPH activity of different samples of *Withania somnifera*

attributed to its catechol structure, phenolic hydroxyl groups and ability to donate electrons to stable free radicals, which are known to enhance the antioxidant activity of polyphenols[36-38]. Besides its use as anti-cancer compound, it finds use as food additive and as intermediate for synthesis of different pharmaceutical compounds[39]. Thus, the newly isolated compound explicates many of the biological activities of *W. somnifera* and the wide spectrum of applications in food and pharmaceutical sectors.

Isolation of alkaloid

The fourth fraction separated from the alkaloid extract of WSM was identified by spectroscopy as 3-phenyl-5, 6-dihydro-4H- pyrrolo [1,2,-b] pyrazole (withasomnine) $C_{12}H_{12}N_2$- 3.48 mg. In the FT-IR (KBr) spectrum n_{max} values were observed at 3045 /cm due to heterocyclic ring stretching vibrations, aromatic stretching vibrations at 2982 /cm, characteristic –C=N–vibrations at 1605 and 1260 /cm aromatic –C=C- ring stretching vibrations at 1420 /cm.

The result of ^{1}H-NMR analysis performed on the WSM showed signals at (CD_3OD)- δ 7.80 (s- N=CH), 7.42,7.46 (m- phenyl), 7.30-7.45 (m-phenyl), 7.12- 7.29 (m- phenyl), 4.15 (t- NH_2-CH_2). The ^{13}C-NMR at 75 MHz showed (CD_3OD)- δ 142.2 (N-C), 140.8 (N=CH), 133.5 (C), 128.8 (CH), 125.5(CH), 125.0(CH), 115.1(C-PH), 47.6(CH2), 26.4 (CH2) 24.2 (CH2).

Molecular ion peak (100 %) in the GC-MS was obtained at m/z 184 [$M^{+\cdot}$], 156, 148, 115, 102 and 77 ([C_6H_5]$^+$ fragment ion), which confirmed the presence of an aromatic ring in the compound. From spectroscopy results and the literature concordance[40],

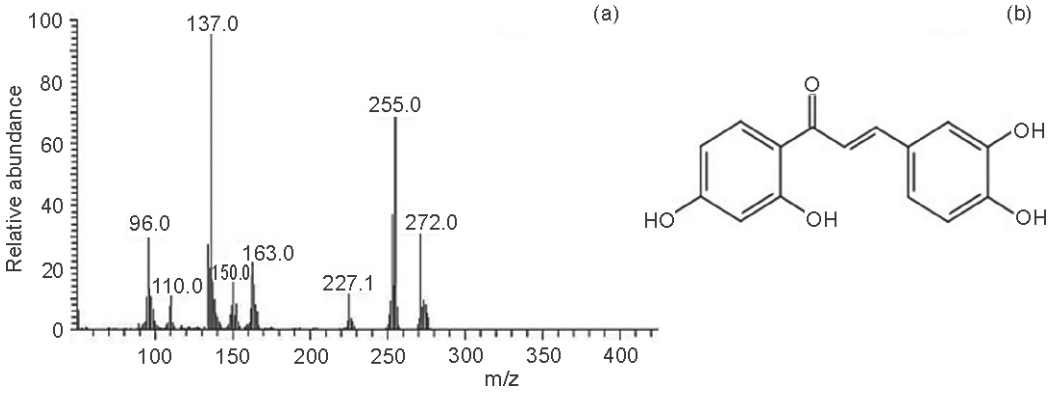

Plate 4—a) Mass spectrum and b) Chemical structure of butein (3, 4, 2' 4'-tetrahydroxy chalcone)

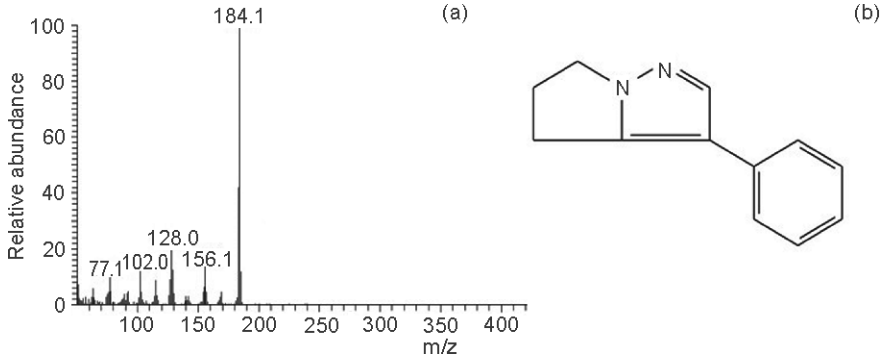

Plate 5—a) Mass spectrum and b) Chemical structure of withasomnine

the compound was identified as withasomnine, an alkaloid with molecular mass 184. The mass spectrum for the isolated compound and its structure are shown in Plate 5.

Conclusion

A phytochemical study was undertaken to evaluate marc of ***W. somnifera,*** collected from a reputed Indian traditional medicine manufacturing industry. Preliminary phytochemical analysis confirmed presence of phenols, flavonoids, steroids, phytosterols, alkaloids, etc. in the marc. Analysis carried out by GC-MS and HPTLC revealed presence of oleic acid, linoleic acid, linolenic acid, anethole, octodecanoic acid, pellitorine, withanolide A and withaferin A in the marc of ***W. somnifera***. Two compounds, withasomnine and butein were isolated from the marc by column chromatography and characterized by spectroscopy methods. The study demonstrated good potential for use of marcs for recovery of bio-actives. Since marc is generally considered as byproduct discard from pharmaceutical manufacture process, the study points to enormous potential for exploiting hitherto unused marcs of ***W. somnifera*** and other similar herbs for further production processes. Further studies are proposed to explore economic feasibility for recovery of phyto compounds from marcs of different plant parts thereby helping to reduce the herb's bulk consumption in pharma industry. This utilization of marc as an alternate source may slow down the rapid depletion of natural sources for medicinal plants such as ***W. somnifera.***

Acknowledgement

The study has been undertaken as part of research work under Manonmaniam Sundaranar University, Tirunelveli. The authors are grateful to Sophisticated Analytical Instrumentation Facility, IIT-M, Chennai; National Research Institute for Panchakarma, Thrissur; South Indian Textile Research Association, Coimbatore and CARe-Keralam, Thrissur for the analytical tests and technical discussions.

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