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Withania somnifera (L.) Dunal: A comparative study of antioxidant activities and GCMS analysis of aqueous and ethanol root extracts

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Abstract
Withania somnifera also known as winter cherry belongs to the family Solanaceae. It is an effective immunomodulatory agent and inhibited the myelosuppression induced by diverse immunotoxins. It increases longevity and useful for promoting physical and mental health and rejuvenates the body in debilitated conditions. Ashwagandha is also useful in epilepsy, stress, and neurodegenerative diseases. The objective of the study was to evaluate the antioxidant properties and GC-MS analysis of both aqueous and ethanol root extracts of *Withania somnifera*. Antioxidant activities such as DPPH radical, Fe^{3+} reducing power, and phosphomolybdenum reduction assays were carried out for aqueous and ethanol root extracts. The maximum DPPH radical scavenging activity of aqueous root extract was 92.82% and ethanol root extract was 40.49% at 120 µg/mL concentrations. The IC_{50} of DPPH˙ radical scavenging activity of aqueous and ethanol root extracts was 38.35 µg/mL and 148.14 µg/mL concentration respectively. The maximum Fe^{3+} reduction of aqueous root extract was 82.12% and ethanol root extract was 63.86% 120 µg/mL concentration, and their RC_{50} values were 23.56 µg/mL and 80.36 µg/mL concentration respectively. The maximum phosphomolybdenum reduction of aqueous toot extract was 94.50% and ethanol root extract was 86.13% at 120 µg/mL concentration, and their RC_{50} values were 13.70 µg/mL and 38.43 µg/mL concentration respectively. The GCMS analysis of aqueous root extract of *W. somnifera* showed the highest RT peak at 16.55 and the ethanol root extract of *W. somnifera* showed the highest RT peak at 17.45, which are of the same compound, n-hexadecanoic acid.

Keywords: *Withania somnifera*; DPPH; antioxidant; GC-MS; antibacterial.

Introduction
Withania somnifera, also known as ashwagandha, Indian ginseng, and winter cherry, has been an important herb in the Ayurvedic and indigenous medical systems for over 3000 years [1]. It is a perennial shrub from the Solanaceae or Nightshade family [2] The roots of the plant are categorized as immune boosters, which are reputed to promote health and longevity by augmenting defense against disease, arresting the aging process, revitalizing the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and by creating a sense of mental [3]. *W. somnifera* grows abundantly in India (especially Madhya Pradesh), Pakistan, Bangladesh, Sri Lanka, and parts of northern Africa [4]. *W. somnifera* is a small, woody shrub, that grows about two feet in height. An erect, evergreen, tomentose shrub, 30-150 cm high, found throughout the drier parts of India in waste places and on bunds. Roots are stout fleshy, whitish brown; leaves simple ovate, glabrous, those in the floral region smaller and opposite; flowers inconspicuous, greenish or lurid-yellow, in axillary, umbellate cymes; berries small, globose, orange-red when mature, enclosed in the persistent calyx; seeds yellow, reniform. The roots are the main portions of the plant used therapeutically. The bright red fruit is harvested in the late fall and seeds are dried for planting in the following spring [5].

Taxonomy
Kingdom: Plantae, Plants;
Subkingdom: Tracheobionta, Vascular plants;
Super division: Spermatophyta, Seeds plants;
Division: Angiosperma
Class: Dicotyledons
Order: Tubiflorae
Family: Solanaceae

Genus: *Withania*
Species: *Withania somnifera*

Fig 1: Roots of *Withania somnifera*

Materials and Methods

Collection of Plant material

The roots of *W. somnifera* were collected from the Siddha market at Chennai, Tamilnadu, India. The roots were washed, shade dried, and powdered by a mechanical blender and stored in an airtight container, in the refrigerator.

Extract preparation

About 10 g of root powder was boiled with distilled water in cookware and the supernatant was filtered and condensed in a hot plate at 50 °C, which yields pale brown gummy extract. Then, another 10 g of root powder was soaked in ethanol for 72 h and the supernatant was filtered and condensed in a water bath at 60 °C, which yields pale yellow gummy extract.

Determination of total phenols

Folin-Ciocalteau reagent method was used to determine the total phenolic compounds [6] with slight modifications. One hundred µL of aqueous and ethanol root extracts of *W. somnifera* was mixed with 900 µL of methanol in each test tube. Then, 1 mL of Folin Ciocalteau reagent (1:10 diluted with distilled water) and 1 mL of Na_2CO_3 (20% w/v) solution were added and shaken well.

Then, the mixture was allowed to stand for 30 min incubation in dark at room temperature. The absorbance of the supernatant was measured at 765 nm. The total phenolic content was expressed as (µg/mg of extract) gallic acid equivalent.

Determination of total flavonoids

The total flavonoid content of aqueous and ethanol root extracts of *W. somnifera* was determined by aluminium chloride reagent method with slight modification [7]. Five hundred µL of each extract (1mg/mL) was mixed with 0.5 mL of methanol, 0.5 mL of 5% (w/v) sodium nitrite solution and 0.5 mL of 10% (w/v) aluminium chloride solution. Then, 50 µL of 1 M NaOH solution was added and incubated for 30 min. The absorbance was measured at 510 nm. The result was expressed in terms of (µg/mg of extract) quercetin equivalent.

Antioxidant activity

DPPH˙ radical scavenging activity

The radical scavenging activity of aqueous and ethanol root extracts of *W. somnifera* was evaluated by using stable 1, 1-diphenyl 2-picrylhydrazyl (DPPH) free radical [8]. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations (50-300 µg/mL) of extracts. The mixture was then allowed to stand for 30 min incubation in dark. Ascorbic acid was used as the reference standard. One mL methanol mixed with 1 mL of DPPH solution was used as the control. The decrease in absorbance was measured by UV-Vis spectrophotometer at 517 nm. The percentage of DPPH˙ radical inhibition was calculated as:

$$\%\ \text{of DPPH}^{\cdot}\ \text{radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

Ferric (Fe^{3+}) reducing power activity

The reducing power of aqueous and ethanol root extracts of *W. somnifera* was determined by the potassium ferricyanide assay method [9]. Aqueous and ethanol extracts with different concentrations (20 – 120) µg/mL) were mixed with 1 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v) solution and 1 mL of phosphate buffer (0.2 M, pH 6.6) solution.

The mixture was then incubated at 50°C in a water bath for 20 min. Then, 500 µL of trichloroacetic acid (10%, w/v) and 100 µL of freshly prepared $FeCl_3$ (0.1%, w/v) solution were added and shaken well. The absorbance of the supernatant was measured at 700 nm. Ascorbic acid was used as the standard reference. The percentage of the Fe^{3+} reduction was calculated as:

$$\%\ \text{of}\ Fe^{3+}\ \text{reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \times 100$$

Phosphomolybdenum reduction activity

The reduction capacity of the aqueous and ethanol root extracts of *W. somnifera* was assessed by the phosphomolybdenum reduction method [10]. Aqueous and ethanol extracts with different concentrations (20 to 120 µg/mL) were mixed with 1 mL of reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM), and sulphuric acid (600 mM). The reaction mixture was incubated in a water bath at 95°C for 90 min. The absorbance of the green or blue colour complex was measured at 695 nm. Ascorbic acid was used as the standard reference. The percentage of Mo^{6+} reduction was calculated as:

$$\%\ \text{of phosphomolybdenum reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \times 100$$

Gas chromatography–Mass Spectrometry (GC–MS)

The aqueous and ethanol root extracts of *W. Somnifera* were injected into an HP-5 column (30 m X 0. 25 mm i.e with 0.25 µm film thickness), of Agilent technologies 6890N of JEOL GC Mate II GC-MS model. The chromatographic conditions were used as helium as carrier gas which has a flow rate of 1 mL/min, the injector was operated at 200 °C and column oven temperature was programmed at 50 °C-250 °C at a rate of 10 °C/min of injection mode.

The mass conditions were used as ionization voltage of 70 eV; ion source temperature of 250°C; interface temperature of 250 °C and the mass range of 50-600 mass units [11]. The database of the National Institute of Standards and Technology (NIST) was used for the interpretation of the mass spectrum of eluted compounds compared with the spectrum of compounds stored in the NIST library.

Results and Discussion

Total phenols and flavonoids

Herbal medicine and its derived products have been the mainstream of traditional medicines and the phytochemicals present in the plant and their food products are generally nontoxic and can prevent chronic diseases. Generally, the plant products have a high concentration of flavonoids and phenolic compounds, which play a vital role in protection against human diseases like lipid peroxidation involved in atherogenesis, thrombosis, carcinogenesis, hepatotoxicity, and a variety of disease conditions [12]. phenols and flavonoids, which are reported to have anti-asthmatic, anti-inflammatory, analgesic, and antioxidant activities. Most of the herbal plants contain flavonoids, which have been shown to have antispasmodic, diuretic, laxative, antihypertensive, and anti-inflammatory actions. Flavonoids and saponins are well known for their anti-inflammatory ability due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation. Phenols and flavonoids have good antioxidant properties and they were quantified in the aqueous and ethanol root extracts of *W. Somnifera*. The total phenolic content of aqueous and ethanol root extracts were 406.50 and 440.75 µg/mg of GAE and the total flavonoid content of aqueous and ethanol root extracts were 13.41 and 73.07 µg/mg of QE.

Table 1: Quantitative estimations of aqueous and ethanol root extracts of *W. somnifera*

Phytochemicals	Amount (µg/mg)	
	Aqueous extract	Ethanol extract
Phenols	406.50	391.75
Flavonoids	61.41	53.07

DPPH˙ radical scavenging assay

Most of the plant extracts can reduce the purple colour 1.1-diphenyl-2-picrylhydrazyl (DPPH) radical to the yellow colour 1.1-diphenyl-2-picrylhydrazine. The result showed that the aqueous and ethanol root extracts contain active constituents, which are capable of donating hydrogen atoms to DPPH free radicals to remove odd electrons which are responsible for radical reactivity. DPPH radical scavenging method is one of the best methods because its results are not affected by plant extracts mostly [13]. The maximum DPPH˙ radical scavenging activity of aqueous and ethanol root extracts were 92.82% and 40.49% at 120 µg/mL concentration. The aqueous root extract of *W. somnifera* has higher radical scavenging activity when compared to ethanol root extract. The IC_{50} values of aqueous and ethanol root extracts were 38.35 µg/mL and 148.14 µg/mL concentration and they were compared with standard (IC_{50} = 2.88 µg/mL concentration) ascorbic acid. Both, aqueous and ethanol root extracts of *W. somnifera* have significant capacity to reduce DPPH radical, and the reducing capacity increased with increasing concentration of the extract.

Table 2: DPPH radical scavenging activity of aqueous and ethanol root extracts of *W. somnifera*

Concentration (µg/mL)	% of inhibition	
	Aqueous extract	Ethanol extract
20	30.62±0.94	4.75±0.80
40	52.15±1.12	10.64±0.76
60	70.81±0.58	13.11±1.35
80	80.86±1.08	21.86±0.61
100	88.03±0.77	29.08±1.75
120	92.82±0.42	40.49±1.16

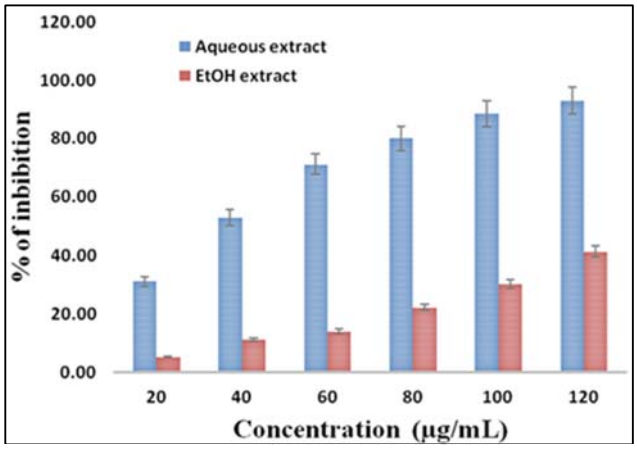


Fig 2: DPPH radical scavenging activity of aqueous and ethanol root extracts of *W. somnifera*

Ferric (Fe^{3+}) reducing power activity

The most important transition metals related to human disease are iron and copper. These elements play a pivotal role in the production of hydroxyl radicals *in vivo*. Hydrogen peroxide reacts with iron II (or copper I) to generate the hydroxyl radicals, causes tissue damage. Also, if transition metal ions are present, they involve redox reactions to contribute hydroxyl radical production and such conditions are found in areas of active inflammation, as well as various pathologic situations such as stroke, septic shock, ischaemia-reperfusion injury. Iron is released from ferritin by reducing agents including ascorbate and superoxide itself, and hydrogen peroxide can release iron from a range of haem proteins. Therefore, although the iron-binding proteins effectively chelate iron and prevent redox effects under normal physiological conditions, this protection can break down in disease states [14]. The reducing power assay was carried out by the reduction of Fe^{3+} to Fe^{2+} by the aqueous and ethanol root extracts of *W. somnifera* and the subsequent formation of the Ferro-ferric complex. The reduction ability increases with an increase in the concentration of the extract. The aqueous extract has good reducing power than ethanol extract. The maximum Fe^{3+} reduction in aqueous and ethanol root extracts were 82.12% and 63.86% 120 µg/mL concentration. The RC_{50} values of aqueous and ethanol root extracts were 23.56 µg/mL and 80.36 µg/mL concentration and they were compared with standard (RC_{50} = 29.11 µg/mL concentration) ascorbic acid.

Table 3: Ferric (Fe^{3+}) reducing power activity of aqueous and ethanol root extracts of *W. somnifera*

Concentration (µg/mL)	% of reduction	
	Aqueous extract	Ethanol extract
20	42.85±0.57	27.62±0.44
40	61.62±1.06	35.92±0.62
60	70.95±0.98	42.21±0.77
80	80.32±1.37	50.51±1.03
100	76.65±1.01	57.06±0.48
120	82.59±0.66	64.30±0.61

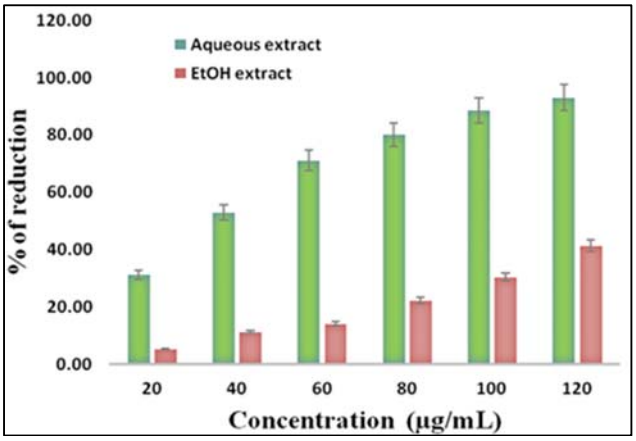


Fig 3: Ferric (Fe^{3+}) reducing power activity of aqueous and ethanol root extracts of *W. somnifera*

Phosphomolybdenum reduction activity

The total antioxidant activity of aqueous and ethanol root extracts of *W. somnifera* was measured by the phophomolybdenum reduction assay method which is based on the reduction of Mo (VI) to Mo(V) by the formation of green phosphate/Mo (V) complex at acidic pH.

The assay involves an electron transfer (ET) mechanism and many natural products, including phenolic compounds and flavonoids, can cause transition metal reduction [15]. The maximum phosphomolybdenum reduction of aqueous and ethanol root extracts were 94.50% and 86.13% at 120 µg/mL concentration.

The RC_{50} values of aqueous and ethanol root extracts were 13.70 µg/mL and 38.43 µg/mL concentration and it was compared with standard (RC_{50} = 5.97 µg/mL concentration) ascorbic acid.

Table 4: Phosphomolybdenum reduction activity aqueous and ethanol root extracts of *W. somnifera*

Concentration (µg/mL)	% of reduction	
	Aqueous extract	Ethanol extract
20	72.30±0.91	41.08±0.44
40	87.56±1.86	52.84±1.14
60	91.39±1.08	63.95±0.52
80	92.85±0.53	77.49±1.88
100	94.61±0.72	82.97±1.07
120	95.41±1.28	86.67±0.76

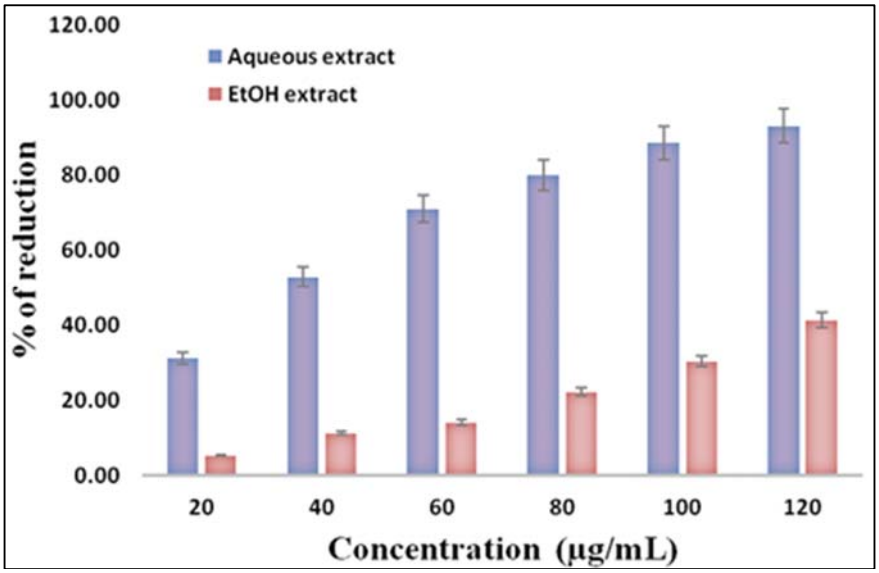


Fig 4: Phosphomolybdenum reduction activity of aqueous and ethanol root extracts of *W. somnifera*

GC-MS analysis

GC-MS analysis was carried out for the aqueous and ethanol root extracts of *W. somnifera* and the eluted compounds were shown in Tables 5a and 5b. n-Hexadecanoic acid has the highest RT in aqueous (RT=16.55) and ethanol (RT=17.45) root extracts of *W. Somnifera,* which has antioxidant anti-inflammatory hypocholesterolemic, nematicide, pesticide, hemolytic, 5-alpha reductase inhibitor, potent mosquito larvicide properties [16].

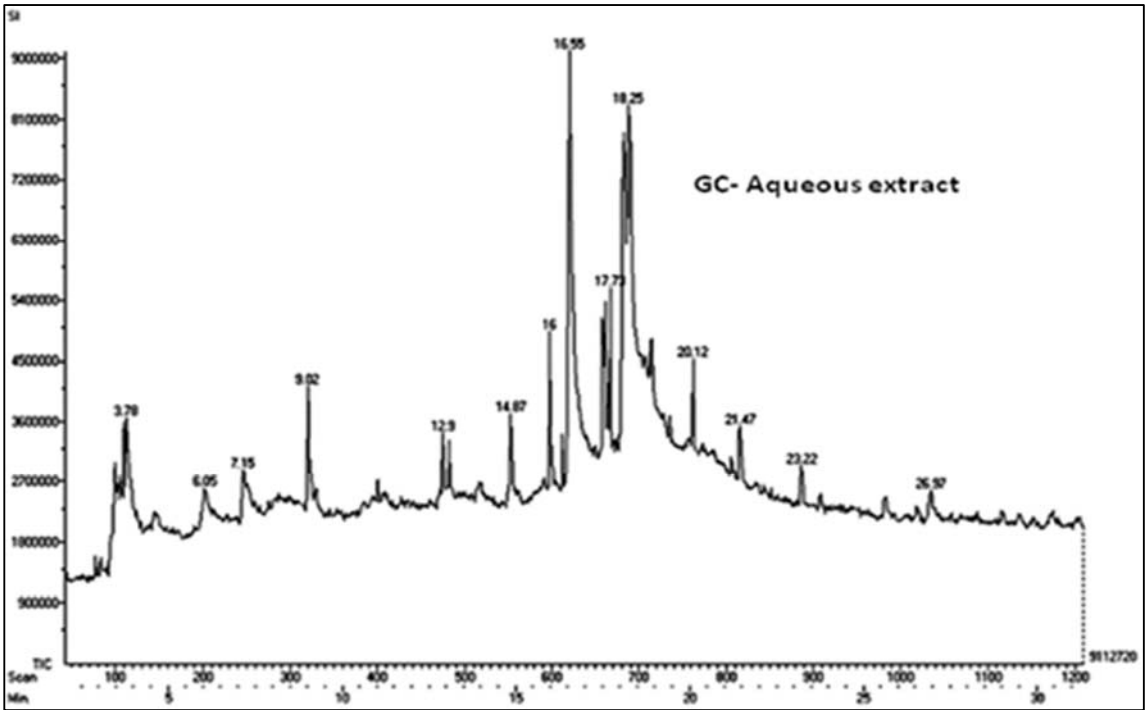
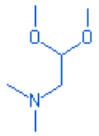
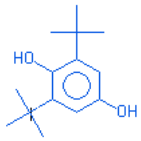
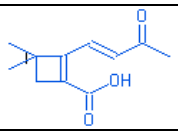
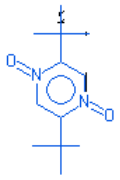
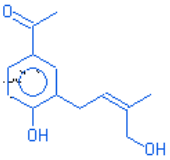
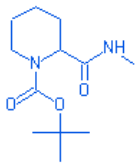
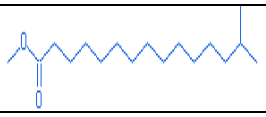
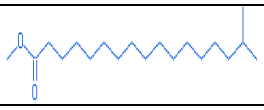
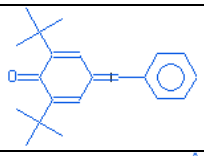
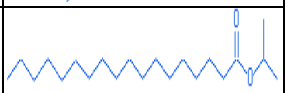
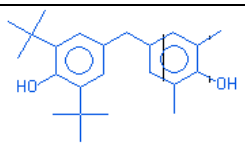
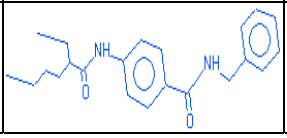


Fig 2: Gas chromatogram of aqueous root extract of *W. Somnifera*

Table 5a: GCMS analysis of aqueous root extract of *W. Somnifera*

S. No	RT	Name	Structure	Mol. Wt g/mol	Mol. Formula
1.	3.78	Dimethylaminoacetaldehyde dimethyl acetal		133.00	$C_6H_{15}NO_2$
2.	6.05	1,4 Benzenediol, 2,6-bis(1,1-dimethylethyl)		222.32	$C_{14}H_{22}O_2$
3.	7.15	1-Buten-3-one,1-(2- carboxy-4,4-dimethylcyclobutenyl)		194.23	$C_{11}H_{14}O_3$
4.	9.02	Pyrazine, 2-5-bis(1,1- dimethylethyl)- 1,4,dioxide		224.30	$C_{12}H_{20}N_2O_2$
5.	12.9	3-[4-Hydroxyisopent-2[Z]-enyl]-4-hydroxyacetophenone		220.24	$C_{13}H_{16}O_3$
6.	14.87	Azacyclohexan-2-carboxylic acid amide, 1-boc-N-methyl		242.31	$C_{12}H_{22}N_2O_3$

7.	16	Pentadecanoic acid, 14-methyl-, methyl ester		270.45	$C_{17}H_{34}O_2$
8.	16.55	n-Hexadecanoic acid		256.42	$C_{16}H_{32}O_2$
9.	17.73	Heptadecanoic acid, 16-methyl, methyl ester		298.51	$C_{19}H_{38}O_2$
10.	18.25	Oleic Acid		282.56	$C_{18}H_{34}O_2$
11.	20.12	2,6-Bis-(1,1-dimethylethyl)-4-phenylmethylenecyclohexa-2,5-dien-1-one		294.43	$C_{21}H_{26}O$
12.	21.47	Isopropyl Stearate		326.56	$C_{21}H_{42}O_2$
13.	23.22	Phenol, 2,6-bis[1,1-dimethylethyl]-4[[4-hydroxy-3,5-dimethylphenyl] methyl]		340.50	$C_{23}H_{32}O_2$
14.	26.97	Hexanamide, 2-ethyl-N-[4-benzylaminocarbonyl] phenyl-		352.47	$C_{22}H_{28}N_2O_2$

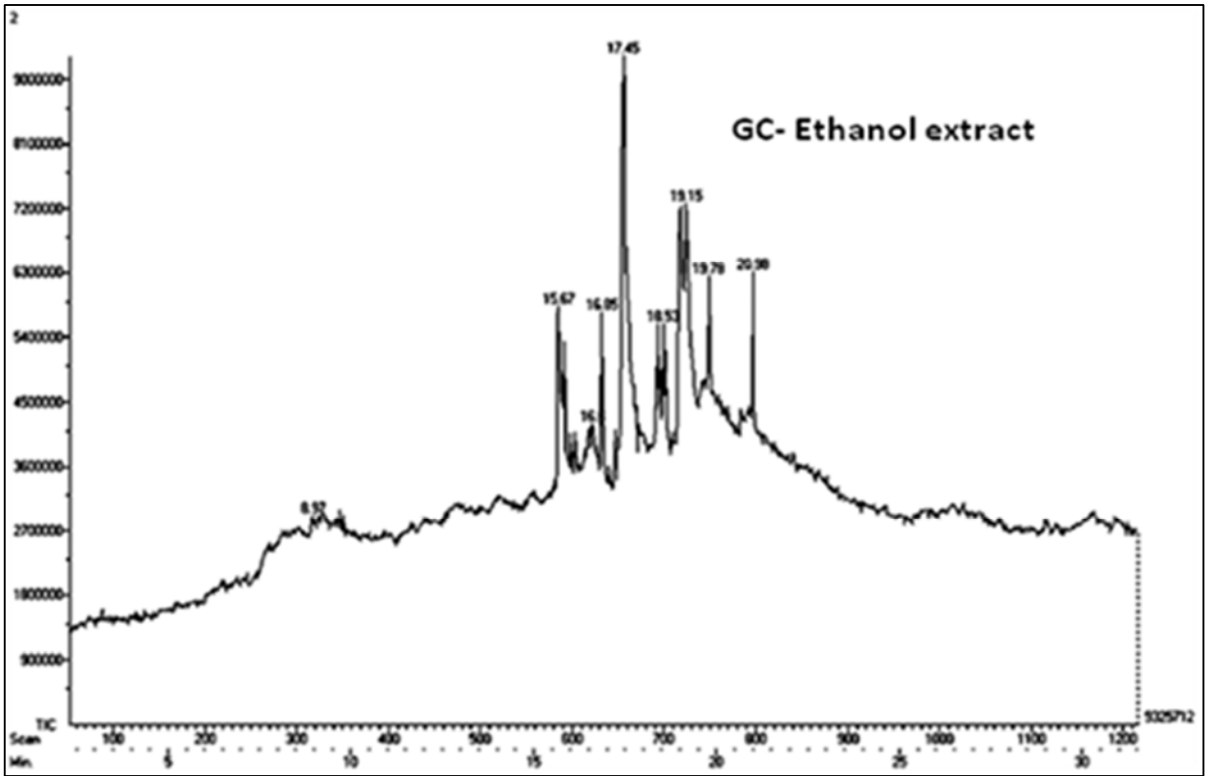
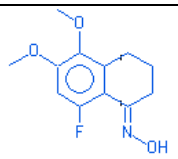
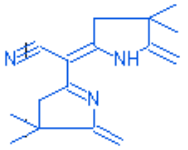
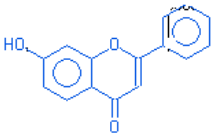
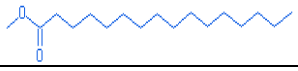
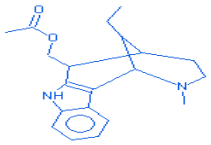
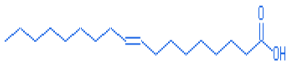
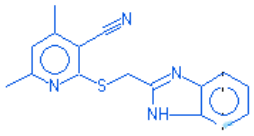
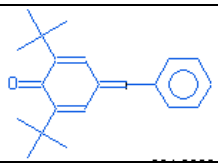


Fig 3: Gas chromatogram of ethanol root extract of *W. Somnifera*

Table 5b: GCMS analysis of ethanol root extract of *W. Somnifera*

S. No	RT	Name	Structure	Mol. Wt g/mol	Mol. Formula
1.	8.97	À-Tetraloxime, 8-fluoro-5,6-dimethoxy		239.56	$C_{12}H_{14}FNO_3$
2.	15.67	[4,4-Dimethyl-5-methylene-4,5-dihydro-3H-pyrrol-2-yl]-[4,4-dimethyl-5-methylene-pyrrolidin-2-ylidene]-acetonitrile		255.36	$C_{16}H_{21}N_3$
3.	16.6	4H-1-Benzopyran-4-one, 7-hydroxy-2-phenyl		238.24	$C_{15}H_{10}O_3$
4.	16.85	Hexadecanoic acid, methyl ester		270.45	$C_{17}H_{34}O_2$
5.	17.45	n-Hexadecanoic acid		256.43	$C_{16}H_{32}O_2$
6.	18.53	Dasycarpidan-1-methanol, acetate (ester)		362.44	$C_{20}H_{26}N_2O_2$
7.	19.15	Octadec-9-enoic acid		282.46	$C_{18}H_{34}O_2$
8.	19.78	2-[1H- Benzoimidazol-2-ylmethylsulfanyl]-4,6-dimethyl-nicotinonitrile		294.37	$C_{16}H_{14}N_4S$
9.	20.98	2,6-Bis[1,1-dimethylethyl]-4-phenylmethylenecyclohexa-2,5-dien-1-one		298.49	$C_{21}H_{26}O$

Conclusion

Free radicals are highly dangerous to cells and are generated continuously in the human body due to metabolism and diseases. Free radicals cause extensive damage to tissues and proteins, leading to various degenerative diseases and extensive lysis. Oxidative damage of cells can be protected by many synthetic drugs are available now, but they are associated with adverse side effects. An alternate solution to the above side effects is to consume natural antioxidants from food supplements, herbal folklore, and traditional medicine. To protect cells from free radicals, the organism has endowed with endogenous (catalase, superoxide dismutase, glutathione peroxidase/reductase) and exogenous (Vitamin C and E, carotene, uric acid) antioxidant defense systems, but these systems will not work in some critical conditions such as UV exposure, microbial infections, radioactive contamination and oxidative stress in which the free radicals coming out. So, there is an increasing demand for indigenous medicine in the protective biochemical functions of natural antioxidants contained in spices, herbs, and medicinal plants. Hence the present study indicates that aqueous and ethanol extracts of *W. somnifera* have significant antioxidant activities and quench the free radicals effectively and the aqueous extract showed slightly higher antioxidant activities than the ethanol extract. Further molecular studies are required to find out the health benefits of the extracts for the preparation of folklore to boost up the immunity and to retaliate against diseases.

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