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Biochemical and Antimicrobial Properties of Secondary Metabolites of Withania somnifera Dun

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University of Rajshahi

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Biochemical and Antimicrobial Properties of Secondary Metabolites of *Withania somnifera* Dun.

Thesis Submitted to the University of Rajshahi for the Degree

of

Doctor of Philosophy

Resubmitted

By

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March, 2014

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DEDICATED

TO

MY

BELOVED PARENTS

AND

HUSBAND

DECLARATION

I hereby declare that the work presented in the dissertation entitled **"Biochemical and Antimicrobial Properties of Secondary Metabolites of *Withania somnifera* Dun."** submitted for the degree of Doctor of Philosophy was carried out by me in the Department of Botany, University of Rajshahi, Bangladesh under the supervision of Professor M. Monzur Hossain, Department of Botany, University of Rajshahi.

I further declare that no part of the dissertation has been submitted for any other degree.

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CERTIFICATION

This is to certify that the work incorporated in the thesis entitled **"Biochemical and Antimicrobial Properties of Secondary Metabolites of W*ithania somnifera* Dun."** submitted by **Nadia Alam** was carried out under my supervision at the Department of Botany, University of Rajshahi, Bangladesh. It may be mentioned here that the candidate has already been published following three papers in reputed international journal.

1. Alam N, Hossain M, Khalil M I, Moniruzzaman M, Sulaiman S A and Gan S H, 2011. Recent advances in elucidating the biological properties of *Withania somnifera* and its potential role in health benefits. *Phytochemistry Reviews*, 11:97–112. **IF-4.33**
2. Alam N, Hossain M, Khalil M I, Moniruzzaman M, Sulaiman S A and Gan S H, 2011. High catechin concentrations detected in *Withania somnifera* (ashwagandha) by high performance liquid chromatography analysis. *BMC Complementary and Alternative Medicine*, 11:65. **IF-2.20**
3. Alam N, Hossain M, Khalil M I, Mottalib M A, Sulaiman S A and Gan S H, 2012. Methanolic extracts of *Withania somnifera* leaves, fruits and roots have strong antioxidant properties and antibacterial activities against gram-negative organisms (submitted). *BMC Complementary and Alternative Medicine,* 12:175. **IF-2.20.**

I wish her every success in her life.

Monzur Hossain
12/03/2014

(**Professor M. Monzur Hossain**)
Research Supervisor
Department of Botany

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The debt of my parents and thankful for their continual support. I am deeply grateful to my husband, Dr. Ibrahim Khalil, without whose guidance, patience and perseverance, the thesis would not have been completed. As a token of my appreciation I, herewith, dedicate this thesis to him.

The Author

LIST OF ABBREVIATIONS

2, 4-D	2, 4-dichlorophenoxy acetic acid
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
BA	6-benzyl adenine
CAT	Catalage
CEQ	Catechin equivalents
CH_3CO_2Na	Sodium acetate
DNSA	3,5-dinitrosalicylic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
$FeCl_3$	Ferric chloride
$FeSO_4$	Ferrous sulphate
FRAP	Ferric reducing antioxidant power
GABA	Gamma aminobutyric acid
GAEs	Gallic acid equivalents
GPx	Glutathioneperoxidase
GSH	Reduced glutathione
GST	Glutathione S-transferase
Ha	Hector
HCl	Hydrochloric acid
HP	Hydroperoxides
HPLC	High performance liquid chromatography
IC_{50}	Inhibition concentration 50 percent
IR	Infrared spectroscopy

K cal	Kilo calory
$K_3Fe(CN)_6$	Hexacyanoferrate
KOH	Potassium Hydroxide
Lbs/(inch)	Pound per square inch
LPO	Lipid peroxidation
MIC	Minimum Inhibitory Concentration
Min	Minute(s)
MS	Mass spectrometry
NA	Nutrient agar
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
NB	Nutrient Broth
NMR	Nuclear magnetic resonance
NO	Nitric oxide
RSA	Radical-scavenging activity
SD	Standard deviations
SOD	Superoxide dismutase
T3	Triiodothyronine
T4	Thyroxine
TBARS	Thiobarbituric acid reactive substances
TBHQ	Ttertiary butylhydroquinone
TCA	Trichloroacetic acid
TPTZ	2,4,6-tris (1-pyridyl)-1,3,5-triazine
WHO	World Health Organization
WSFEt	*W. somnifera* fruits
WSLEt	*W. somnifera* leaves
WSREt	*W. somnifera* roots

ABSTRACT

Withania somnifera is an important medicinal plant traditionally used in the treatment of many diseases. Plant derived natural products such as phenolic compounds, flavonoids, anthocyanin, ascorbic acids, terpenoids and steroids etc have received considerable attention in recent years due to their diverse pharmacological properties including antioxidative, antidiabetic, anticarcinogenic, antimicrobial, antiallergic, antimutagenic and anti-inflammatory activities. The present study was carried out to characterize the secondary metabolites, especially, phenolic acids, flavonoids and antioxidant as well as antimicrobial properties in methanolic extracts of *W. somnifera* fruits (WSFEt), roots (WSREt) and leaves (WSLEt).

WSFEt, WSREt and WSLEt were prepared by with 80% aqueous methanol and tested for the presence of phytochemicals using standard qualitative procedures. Total polyphenols, flavonoids, anthocyanin and ascorbic acids were determined spectrophotometrically. DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activities, ferric reducing antioxidant power (FRAP) assay, ferrous chelating activity, reducing power assay and Inhibition of β-carotene bleaching, were also determined by spectrophotometric methods, DNA damage protective activity was determined by agarose gel electrophoresis and finally phenolic acid profiles were determined by HPLC methods. The antibacterial activity of WSREt, WSFEt and WSLEt was determined by agar well diffusion method.

Preliminary phytochemical analysis of WSFEt, WSREt and WSLEt revealed the presence of glycosides, alkaloids, phytosterols, fixed oils, phenolic compounds and flavonoids.

High concentrations of both phenolics and flavonoids were detected in all parts of the plant with the former ranged between 17.80 ± 5.80 and 32.58 ± 3.16 mg/g (dry weight) and the latter ranged between 15.49 ± 1.02 and 31.58 ± 5.07 mg/g. The ascorbic acid content ranging between 62.60 mg/100g and 20.60 mg/100g. The total anthocyanin contents of WSLEt, WSFEt and WSREt were 12.5 ± 1.04 mg/100g, 5.66 ± 0.52 mg/100g and 2.86 ± 1.44 mg/100g respectively.

All of the three different plant parts showed strong DPPH radical scavenging activities (59.16 ± 1.20% to 91.84 ± 0.38%) and its IC_{50} values ranged between 101.73 µg/ml and 801.93 µg/ml. FRAP assay of WSLEt, WSFEt and WSREt showed

strong antioxidant properties and the highest value (1643.04 μM Fe/kg) was found in leaves while the lowest value (1130.18 μM Fe/kg) was found in roots. Ferrous chelating activity (IC_{50}) of the WSLEt, WSREt, WSFEt ranged between 0.22 ± 0.04 mg/ml and 0.65 ± 0.02 mg/ml. Reducing power assay among the three extracts, WSLEt showed higher absorbance compared to WSREt and WSFEt. Lipid peroxidation of WSLEt, WSREt and WSFEt exhibited 79.67%, 69.87%, and 72.11% peroxidation of linoleic acid emulsion at the concentration 60 μg/ml, respectively. DNA damage protective activity was also found at different concentration (2.5, 5, 10, 20 μg/ml) of WSLEt, WSREt and WSFEt.

Eight polyphenols (gallic, syringic, benzoic, p-coumaric and vanillic acids as well as catechin, kaempferol and naringenin) were identified by HPLC as well. Among all the polyphenols, catechin was detected in the highest concentration (13.01 ± 8.93 to 30.61 ± 11.41 mg/g).

Sensitivity test of WSREt, WSFEt and WSLEt showed strong antibacterial activity against five human pathogenic bacteria *E. coli*, *Salmonella*, *Citrobacter*, *Pseudomonas* and *Klebsiella.* WSLEt showed the highest zone of growth inhibition against *S. typhi.* The antifungal screening showed that methanolic extract of *W. somnifera* roots, fruits and leaves was active against *Fusarium oxysporum* and increasing concentration of the extract showed higher inhibitory effect of the fungus and *W. somnifera* roots extract have showed the highest activity compared to fruits and leaves.

The results indicate that *W. somnifera* is a plant with strong therapeutic properties thus further supporting its traditional claims. All major parts of *W. somnifera* such as the roots, fruits and leaves provide potential benefits for human health because of its high content of secondary metabolites especially phenolic compounds, flavonoids, ascorbic acids and anthocyanin as well as antioxidant and antimicrobial activity. *W. somnifera* leaves contain the highest amounts of polyphenols specially catechin which has promising medicinal and pharmacological value.

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CHAPTER I

INTRODUCTION

1. INTRODUCTION

Withania somnifera Dunal (*Solanaceae*), also known as Ashwagandha or winter cherry, is one of the most valuable plants in the traditional Indian systems of medicine. It is being used in more than 100 formulations in Ayurveda, Unani and Siddha and is therapeutically equivalent to ginseng (Sangwan *et al.* 2004). Phytochemically, this plant is unique because it possesses the largest and most structurally diverse set of *withanolides* (i.e., modified steroidal molecules based on an ergostane skeleton and named after the plant) with *glycowithanolides* being the major bioactive constituent of *W. somnifera*. Because the ethnopharmacological properties of the plant include adaptogenic, anti-sedative and anti-convulsion activities, it is no surprise that the plant is employed to treat various neurological disorders, geriatric debilities, arthritis, stress and behavior-related problems (Bhattacharya *et al.* 1987; Dhuley 2001; Kulkarni *et al.* 1998; Ray and Gupta 1994; Schliebs *et al.* 1973). Several modern molecular pharmacological studies have demonstrated a correlation between these therapeutic actions and one or more of the chemical constituents present in the herb (Bargagna-Mohan *et al.* 2006; Ichikawa *et al.* 2006; Kaileh *et al.* 2007; Kinghorn *et al.* 2004; Tohda *et al.* 2005). *W. somnifera* has long been used for all age groups of both sexes and even during pregnancy without any side effects (Sharma *et al.* 1985).

Figure 1.1: *W. somnifera* plant and root.

In Ayurveda, *W. somnifera* is considered a rasayana herb that works on a nonspecific basis to increase health and longevity. The roots and berries of the plant are used in herbal medicine. In Ayurveda, the fresh roots are sometimes boiled in milk, prior to drying, in order to leach out undesirable constituents. The berries are used as a substitute for rennet, to coagulate milk in cheese making.

1.1. HISTORY AND DISTRIBUTION

W. somnifera is used in India for about 4000 years. It is a very important herb in ayurveda, the traditional Indian medicine. It is used for tumors, inflammation (including arthritis) and a wide range of infectious diseases. The shoots and seeds are also used as food and to thicken milk in India.

Traditional uses of *W. somnifera* among tribal peoples in Africa include fevers and inflammatory conditions. *W. somnifera* is frequently a constituent of Ayurvedic formulas, including a relatively common one known as shilajit. It is distributed throughout the drier region of India, especially in wasteland ascending to an altitude of 2000m in the Himalaya.

1.2. MORPHOLOGY AND TAXONOMY OF *W. SOMNIFERA*

1.2.1. Morphology

W. somnifera is a small, woody evergreen shrub that grows up to the height of 0.5m to 1.5m. It can be found growing in Africa, the Mediterranean and India. As a result of this wide growing range, there are considerable morphological variations in terms of local species.

(i) Stem: They are solid, branched and usually erect. Stem and branches are covered with minute star shaped hairs.

(ii) Leaves: Simple, up to 10 cm long, ovate, petiolate and alternate.

(iii) Root: Roots are 20-30 cm long and 6-12 mm in diameter with few (2-3) lateral roots of slightly smaller size, straight, unbranched.

Outer surface is buff to grayish-yellow with longitudinal wrinkles and in the centre soft, solid mass with scattered pores.

(iv) Flower: The flowers of *W. somnifera* plants are hermaphrodite, small, about 1 cm long, greenish or lurid yellow in color; borne together in short axillary clusters. Each flower contains five stamens, two celled pistil, five sepals and five petals.

(v) Fruit: Ovoid berry-like, orange-red in color, 6mm diameter, smooth enclosed in an inflated and membranous calyx.

1.2.2. Taxonomic Position

W. somnifera is a perennial shrubby plant belongs to the family solanaceae and genus *Withania*. Solanaceae family has 90 genus. *Withania* contains about 26 species. There are over 20 other species of *Withania* that occur in the dry parts of India, North Africa, Middle East and the Mediterranean. These include *Withania coagulens* and *Withania simonii*, the roots of which are sometimes used interchangeably with those of *W. somnifera*.

1.3. BIOCHEMICAL CONSTITUENTS

The major biochemical constituents of the *W. somnifera* roots and leaves are steroidal alkaloids and steroidal lactones from a special class of constituents called withanolides (Elsakka *et al.* 1990). Laboratory analyses have revealed that over 35 chemical constituents are present in the *W. somnifera* roots (Rastogi and Mehrotra 1998), and withanolides are believed to account for the unique medicinal properties of *W. somnifera*. withanolides bear a resemblance, both in action and structure, to the active constituents of Asian ginseng (*Panax ginseng*), known as ginsenosides (Grandhi 1994).

The concentration of withanolides usually ranges from 0.001 to 0.5% dry weight (DW) basis in the roots and leaves (Anonymous, 2004). The withanolides are a group of naturally occurring C28-steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. The basic

structure (**Figure 1.2**) is designated as the withanolide skeleton (Alfonso *et al.* 1993; Glotter 1991).

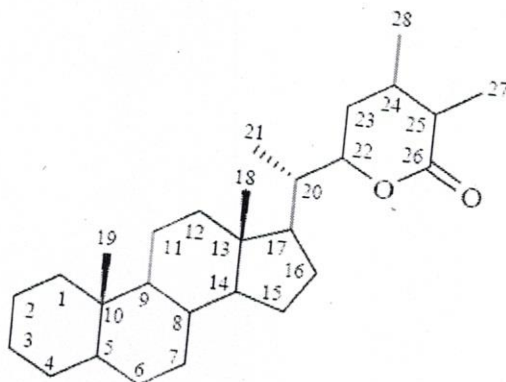


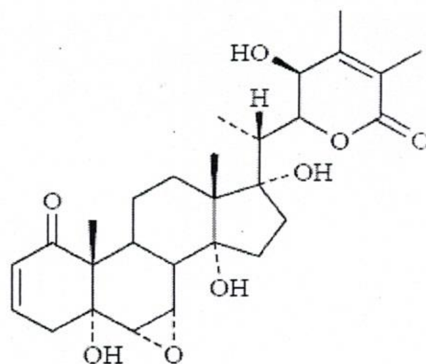
Figure 1.2: Withanolide skeleton.

The major chemical constituents of the *Withania sonifera*, the withanolides, are a group of naturally occurring C_{28}-steroidal lactone triterpenoids built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. The basic strucute of withanolide is a 22-hydroxyergostan-26-oic acid-26,22-lactone. There are many novel structural variants of withanolides with modifications either of the carbocyclic skeleton or the side chain and these have often been described as modified withanolides or ergostantype steroids related to withanolides. These compounds are generally polyoxygenated and it was found that plants elaborating them have an enzyme system capable of oxidizing all carbon atoms in a steroid nucleus. The characteristic feature of withanolides and ergosane-type steroids is one C_8 or C_9-side chain with a lactone or lactol ring but the lactone ring may be either six-membered or fivemembered and may be fused with the carbocyclic part of the molecule through a carbon-carbon bond or through an oxygen bridge. Appropriate oxygen substituents may lead to bond scission, formation of new bonds, aromatization of rings and many other kinds of rearrangements resulting in

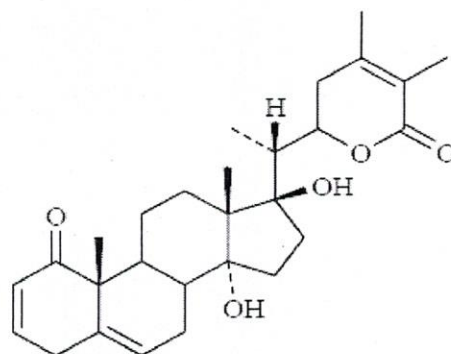
compounds with novel structures (Mirjalili *et al.* 2009). Chemical structure of some important bioactive compounds were shown in **Figure 1.3**.

Withanolide is the main alkaloid constituent of *W. somnifera*; the other alkaloid constituents include (Zhao *et al.*, 2002) somniferine, somnine, somniferinine, withananine, pseudo-withanine, tropine, pseudo-tropine, 3-a-gloyloxytropane, choline, cuscohygrine, isopelletierine and anaferine andanahydrine (Bone 1996; Elsakka *et al.* 1990).

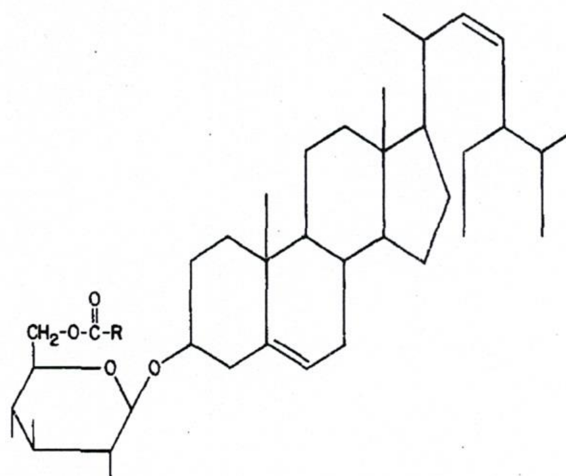
Zhao *et al.* (2002) were isolated five new withanolide derivatives from the roots of *Withania somnifera* on the basis of spectroscopic and physiochemical evidence. The new compounds were designed as (20*S*, 22*R*)-3a, 6a-epoxy 4b, 5b, 27-trihydroxy-1-oxowitha-24-enolide, 27-*O*-b-D-glucopyranosylpubesenolide 3-*O*-b-D-glucopyranosyl (1→6)-b-D-glucopyranoside (withanoside VIII, 9), 27-*O*-b -D-glucopyranosyl (1→6)-b -D-glucopyranosylpubesenolide 3-*O*-b-D-glucopyranosyl (1→6)-b–glucopyranoside (withanoside IX, 10), 27-*O*-b-D-glucopyranosyl-pubesenolide 3-*O*-b-D-glucopyranoside (withanoside X, 11), and (20*R*,22*R*)-1a, 3b, 20, 27-tetrahydroxywitha-5, 24-dienolide 3-*O*-b -D-glucopyranoside (withanoside XI, 12). Out of the five new comounds, withanolide A (2), (20*S*,22*R*)-4b ,5b ,6a,27-tetrahydroxy-1-oxowitha-2,24-dienolide (6), withanoside IV (14), withanoside VI (15) and coagulin Q (16) have showed significant neurite outgrowth activity at a concentration of 1mM on a human neuroblastoma SH-SY5Y cell line.



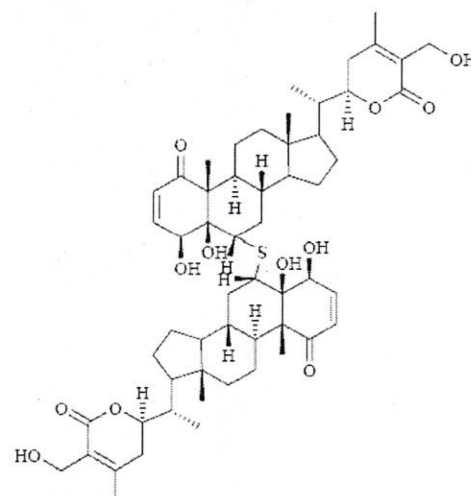
(7) Withanolide R



(8) Withanolide P



(9) Acyl steryl glucosides



(10) Ashwagandhanolide

Figure 1.3: Chemical structure of some important bioactive compounds in *W. somnifera*.

1.4. SOME OTHER COMPONENTS IN *W. SOMNIFERA*

Several reports demonstrated some other chemical compounds in *W. somnifera.* A new dimeric thiowithanolide, named ashwagandhanolide was detected in roots (Subaraju *et al.* 2006). Gupta *et al.* (1996) detected alkaloids in all the plant parts (roots, fruits, leaves), with the highest content found in leaves. The isolation of nicotine, somniferine, somniferinine, withanine, withananine, pseudowithanine, tropine, pseudotropine, 3α-tigloyloxytropane, choline, cuscohygrine, *dl*-isopelletierine and new alkaloids anaferine and anhygrine has been described detected in this medicinal plants (Gupta and Rana 2007). The reported total alkaloid content in the roots of Indian *W. somnifera* varies between 0.13 and 0.31%, though much higher yields (up to 4.3%) have been recorded in plants of other regions/countries. In addition to the alkaloids, the roots are reported to contain starch, reducing sugars, hentriacontane, glycosides, dulcitol, withanicil, an acid and a neutral compound. The leaves are reported to contain five unidentified alkaloids (yield 0.09%), chlorogenic acid, calystegines (nitrogen-containing polyhydroxylated heterocyclic compounds) withanone, condensed tannin and flavonoids were detected in the *W. somnifera* leaves. Four types of peroxidases have been purified and characterized from *W. somnifera* roots (Johri *et al.* 2005). Apart from these contents the plant also contain chemical constituents like withaniol, acylsteryl glucosides, starch, reducing sugar, hantreacotane, ducitol, a variety of amino acids including aspartic acid, proline, tyrosine, alanine, glycine, glutamic acid, cystine, tryptophan, and high amount of iron (Gupta and Rana 2007).

1.5. MECHANISMS OF ACTION OF WITHANOLIDES

The withanolides serve as important hormone precursors that can convert into human physiologic hormones as needed. *W. somnifera* is thought to be amphoteric; i.e., it can help regulate important physiologic processes. The theory is that when there is an excess of a certain hormone, the plant-based hormone precursor occupies cell membrane receptor sites so the actual hormone cannot attach and exert its effect. If the hormone level is low, the

plant-based hormone exerts a small effect. *W. somnifera* is also considered to be an adaptogen, facilitating the ability to withstand stressors, and has antioxidant properties as well.

1.6. BACKGROUND INFORMATION

W. somnifera is a well used popular medicinal plant in India subcontinent. Therefore, it attracts researchers attention for long time. A number of research groups have involved in conducting different types of experiments on *W. somnifera* time to time. The experimental finding on medicinal properties of *W. somnifera* are summerized in **Table 1.1**.

Table 1.1: Summary of the experimental findings on medicinal properties of *W. somnifera*.

Plant Parts	Experimental design	Results
Anti-stress effects		
whole herb	Chronic stress in rodents, received a mild electric shock and given the herb an hour before the foot shock.	Hyperglycemia, glucose intolerance, increase in plasma, corticosterone levels, gastric ulcerations, male sexual dysfunction, cognitive deficits, immunosuppression, mental depression and anti-stress adaptogenic effects (Bhattacharya *et al.* 2001; Bhattacharya *et al.* 1987; Bhattacharya and Muruganandam 2003).
whole herb	Anti-depressant and anti-anxiety effects are compared to the anti-depressant drug imipramine and the anti-anxiety drug lorazepam (Ativan®).	Anti-depressant and anti-anxiety effect (Archana and Namasivayam 1999).
Ethanolic extract of root	Rats subjected to multiple stress of cold, hypoxia and restraint (C-H-R) have been developed to evaluate adaptogenic properties with an oral dose of 100 mg/kg for 4-8 weeks.	Colonic temperature recovery, reduced stress, enhanced open field behavior, emotional stability with a significant enhancement in the functional sensitivity of 5-HT2 receptors in the brain and a reciprocal sub-sensitivity of the 5- HT1A receptors (Kaur *et al.* 2001; Tripathi *et al.* 1998).

Plant Parts	Experimental design	Results
Anti-ulcer activity		
Methanolic extract of whole herbs	*W. somnifera* was compared with ranitidine hydrochloride in a rat model.	*W. somnifera* reduced pylorus ligation-induced gastric ulcers, the ulcer index, the volume of gastric secretion, free and total acidity and protected against gastric mucosal damage possibly due to its antioxidant effects (Bhatnagar *et al.* 2005).
Antioxidant activity		
Methanolic extract of root and leaf	Root and leaf extracts were administered to diabetic rats using glibenclamide as control.	(i) Elevation in glucose and TBARS (ii) Significant reduction in glycogen, vitamins C and E, SOD, CAT, GPx, GST and GSH levels (Udayakumar *et al.* 2010)
Glycowithanolides	*W. Somnifera* was administered once daily for 21 days in chronic foot shock stress model.	(i) Increase in SOD and lipid peroxidation activities (Bhattacharya *et al.* 2001; Dhuley 1998) (ii) Decrease in CAT and GPx activities in some brain regions (Bhattacharya *et al.* 2001).

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Plant Parts	Experimental design	Results
Anti-tumor activity		
Methanolic extract of whole herbs	(i) Used in animal cell cultures (ii) Evaluated the antitumor effects in urethane-induced lung tumors in adult male mice	(i) Decreased levels of NF-□B, suppressed intercellular tumor necrosis factor and potentiated apoptotic signaling in cancerous cell lines (Ichikawa *et al.* 2006). (ii) Acts against cancer by reducing tumor size (Jayaprakasam *et al.* 2003).
Methanolic extract of root	Used against benzo(a)pyrene-induced lung cancer in male Swiss albino mice	Alters the levels of immunocompetent cells, immune complexes and immunoglobulins (Senthilnathan *et al.* 2006).
Anti-inflammatory activity		
Ethanolic extract of root	Powdered root orally administered to rats	Anti-inflammatory responses are comparable to that of hydrocortisone sodium succinate (Begum and Sadique 1988).
Ethanolic extract	Powdered root orally administered to rats	Reduction in inflammation (Anbalagan and Sadique 1981).

Plant Parts	Experimental design	Results
of root		
Ethanolic extract of root	A suspension orally administered to rats	Causes dose-dependent suppression of α2-macroglobulin (an indicator for anti-inflammatory drugs) (Anbalagan and Sadique 1984).
Ethanolic extract of root	Male Wistar rats orally administered 1000 mg/kg	(i) Decreases the GAGs content in the granuloma tissue (ii) Uncouples oxidative phosphorylation (Begum and Sadique 1987).
Ethanolic extract of root	A Freund's adjuvant-induced arthritis rat model orally administered 1000 mg/kg	(i) Causes a significant reduction in paw swelling (ii) observed degenerative changes by radiological examination (Begum and Sadique 1988).
Cardioprotective activity		
Hydro-alcohol extract of whole herbs	Isoprenaline-(isoproterenol)-induced myocardial necrosis in rats using Vitamin E as a control	(i) Decreases glutathione, SOD, CAT, creatinine phosphokinase and lactate dehydrogenase (ii) increases lipid peroxidation marker malonyldialdehyde levels

Plant Parts	Experimental design	Results
		(Gupta *et al.* 2004; Mohanty *et al.* 2004).
Ethanolic extract of whole herbs	Ischemic rats	Alleviates stress-induced changes and provides cardioprotection (Dhuley 1998, 2000).
Hypolipidemic and Anti-atherogenic Activity		
Ethanolic extract of whole herbs	CapsHT2, a medicine comprising of several plants including *W. somnifera* was investigated for its lipid-reducing and anti-atherogenic effects.	It acts against vascular intimal damage and atherogenesis, inhibits lipid peroxidation and enhances the release of lipoprotein lipase enzyme (Mary *et al.* 2003).
Hypoglycemic effect		
Ethanolic extract of whole herbs root and leaf	Alloxan-induced diabetic rats	Possess hypoglycemic and hypolipidemic activities (Udayakumar *et al.* 2009).

Plant Parts	Experimental design	Results
Ethanolic extract of whole herbs	Normal and streptozotocin-induced diabetic rat models	The maximum reduction was 47.1% and 42.7% in blood glucose levels of sub- and mild diabetic rats, respectively, with a dose of 750 mg/kg (Jaiswal *et al.* 2010).
Ethanolic extract of roots	NIDDM and hypercholesterolemic human subjects were treated	Decreases blood glucose and is comparable with an oral hypoglycemic drug (Andallu and Radhika 2000).
Hypothyroid activity		
Aqueous extract of dried Withania root	Administered to mice via gastric intubation	Observed significant increases in serum T4 (Panda and Kar 1998; Panda and Kar 1999).
Immunomodulatory activity		
W. somnifera root extract	Administered to Swiss albino mice before and during exposure to a skin cancer-causing agent	Observed a significant decrease in incidence and average number of skin lesions as compared with the control group (Prakash *et al.* 2002).

Plant Parts	Experimental design	Results
Antimicrobial activity		
Aqueous and alcohol extracts of the *W. somnifera* root and leaves	Using an agar well diffusion method	Possesses strong antibacterial activity against a range of bacteria (Owais *et al.* 2005).
W. somnifera root tubers	Isolated a monomeric glycoprotein with a molecular mass of 28 kDa in SDS-PAGE	Demonstrated potent antimicrobial activity against the tested phytopathogenic fungi and bacteria (Girish *et al.* 2006).
Hexane and diethyl ether extracts from both leaves and W. somnifera roots	Using an agar plate disc-diffusion assay against *Salmonella typhimurium* and *Escherichia coli*	From the six extracts tested, only methanol and hexane extracts of both leaves and roots were found to have potent antibacterial activity (Arora *et al.* 2004).
Methanol leaf extracts of *W. somnifera*	Antibacterial activity test	Showed significant antibacterial and antifungal activity when compared to root and bark extracts (Mahesh and Satish 2008).

Plant Parts	Experimental design	Results
W. somnifera extract in different organic solvents	Disc diffusion assay	Hexane and ethyl acetate extracts showed maximum inhibition against *A. niger*. Methanol extracts showed maximum activity against *F. oxysporium* (13 mm) and *A. flavus* (15 mm), while aqueous extract showed maximum activity against *F. moniliformis* (9.6 mm) (Singh *et al.* 2010).

1.7. AIM AND OBJECTIVE OF THE STUDY

Previous reports reveal that *W. somnifera* extract contains many active constituents that have potential activity against many diseases. A number of reports are available related to alkaloids and other medicinal properties of *W. somnifera*. However, report about secondary metabolites such as phenolic and flavonoid compounds, ascorbic acid and anthocyanin contents in different parts of *W. somnifera* as well as their antioxidant and antimicrobial activities are very limited.

Therefore, the present study was conducted with a view to achieve the following specific objectives.

1) To determine the total phenolic and flavonoid compounds, ascorbic acids and anthocyanin contents in *W. somnifera* by spectrophotometric analysis.
2) To determine the antioxidant properties of target plant by performing different types of antioxidant tests such as DPPH free radical scavenging activity, ferric reducing antioxidant power (FRAP) assay, Fe^{2+} chelation, reducing power assay and β-carotene bleaching.
3) To determine the DNA damage protective activities of target plant by agarose gel electrophoresis technique.
4) To determine the polyphenol profile of target plant by HPLC analysis.
5) To study the antimicrobial properties of target plant against selected pathogenic bacteria and fungi.

CHAPTER II

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Plant Materials

The *Withania somnifera* plant parts such as roots, fruits and leaves were collected from field grown plants after six months of cultivation in Botanical Garden, Rajshahi University, Bangladesh. The collected parts of medicinal plant were brought into the laboratory, cleaned and air-dried in shade and then grinded to a fine powder.

2.1.2. Chemicals and Reagents

A wide range of chemicals and reagents were used in the study. All chemicals used in the study were analytical grades and purchased from Sigma, USA; Fluka; BDH, UK and Merck, Germany.

2.1.3. Equipments

Following equipments were required in the study.

Grinder
Bath sonicator
Vacuum pump
Centrifuge
Rotary evaporator
Vortex
Lyophilizer
Micropipette
T 80 UV/VIS spectrophotometer, ChromoTek GmbH, Germany
Electrical oven
Analytical balance
HPLC system (Waters 2695, Milford, MA, USA)
HPLC column, Merck Purospher Star, RP-18e, (125 X 4 mm, 5 μm)

2.2. METHODS

Different methods and techniques used in the study are mentioned below under different subheads.

2.2.1. Preparation of Plant Extracts

W. somnifera roots (WSREt), fruits (WSFEt) and leaves (WSLEt) extract and preparation was performed according to a modified method described by Kahkonen *et al.* (1999).

- Each of the grinded dry plant materials (500 g) were weighed into a beaker followed by the addition of a total of 2 L of 80% aqueous methanol.
- The suspension was then stirred slightly.
- The beaker was sonicated for 5 min was kept in a shaker for 72 hours at room temperature.
- The mixture was filtered first through cloth and then through Whatman filters paper.
- The filtrates were evaporated by rotary evaporator.
- The concentrated extracts were further lyophilized and weighed.
- The final weight of the WSFEt, WSREt and WSLEt was 27.4, 42.7 and 54.2 g respectively. All the extracts were stored at -20 °C for further study.
- Stock solutions were prepared 1 mg/ml for each of the WSFEt, WSREt and WSLEt for further study.

2.2.2. Qualitative Phytochemical Screening of *W. somnifera*

The crude methanolic extract of leaves, roots and fruits of *W. somnifera* was tested for the presence of phytochemicals using standard qualitative procedures (Harborne 1973; Sofowora 1982; Trease and Evans 1989).

2.2.2.6. *Test for proteins*

Small quantity of the extract was dissolved in few ml of water and subjected to Xantho protein test. To 3 ml of the extract, 1 ml of concentrate nitric acid was added. A white precipitate was obtained. The solution was heated for 1minute and cooled under tap water. It was made alkaline by excess of 40% NaOH. Appearance of orange precipitate indicates the presence of protein.

2.2.2.7. *Test for phenolic compounds*

A small quantity of the extract was dissolved in few ml of water and subjected to $FeCl_3$ test. The dilute extract was treated with dilute $FeCl_3$ solution (5%) and appearance of violet colour shows the presence of phenolic compound and tannins.

2.2.2.8. *Test for flavonoids*

The extract was treated with concentrated Sulphuric acid. Appearance of yellowish orange show the presence of anthocyanins, yellow to orange color show the presence of flavones, and orange to crimson show the presence of flavonones

2.2.3. Spectrophotometric Analysis of Polyphenols and Antioxidants Properties

2.2.3.1. *Total polyphenols*

Phenolic compounds in *W. somnifera* were estimated by a spectrophotometric determination using a modified Folin-Ciocalteu method Singleton *et al.* (1999). Briefly, 100 µl of sample extracts (1 mg/ml) was mixed with 1 ml of Folin and Ciocalteu's phenol reagent (2 N). After 3 minutes, 1 ml of 10% Na_2CO_3 solution was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (T 80 UV/VIS spectrophotometer, ChromoTek GmbH, Germany). Gallic acid was used to calculate the standard curve (20, 40, 60, 80 and 100 µg/ml, r^2 = 0.993). Estimation of the phenolic compounds was carried out in triplicates. The results were expressed as mean ± standard deviations in milligrams of gallic acid equivalents (GAEs) per g of *W. somnifera* tissue dry weight (DW).

2.2.3.2. *Determination of total flavonoids*

The total flavonoid contents of the *W. somnifera* extracts were determined according to the colorimetric assay method developed by Zhishen *et al.* (1999). Briefly, 1 ml of properly diluted (1 mg/ml) WSREt, WSFEt and WSLEt were mixed with 4 ml of distilled water. At baseline, 0.3 ml of (5% w/v) $NaNO_2$ was added. After five minutes, 0.3 ml of (10% w/v) $AlCl_3$ was added followed by the addition of 2 ml of NaOH solution (1 M) six minutes later. After that, the volume was immediately made up to 10 ml, with the addition of 2.4 ml of distilled water. The mixture was shaken vigorously and the absorbance of the mixture was read at 510 nm. A calibration curve was prepared using a standard solution of catechin (20, 40, 60, 80 and 100 µg/ml, r^2 = 0.996). The results were expressed as mg catechin equivalents (CEQ) per g of *W. somnifera* (DW).

2.2.3.3. *DPPH free radical-scavenging activity*

The antioxidant capacity of the *W. somnifera* extracts was also studied through the evaluation of their free radical-scavenging effects on the DPPH radical. The determination was based on the method proposed by Ferreira *et al.* (2009b). Briefly, 1 ml (1 mg/ml) of WSREt, WSFEt or WSLEt was mixed with 2.7 ml of methanolic solution containing DPPH radicals (0.024 mg/ml). The mixture was vigorously shaken and left to stand for 60 min in the dark (until their absorbance remained unchanged). The reduction of the DPPH radical was determined by measuring the absorbance at 517 nm (Hatano, *et al.* 1988). The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the following equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The IC_{50} was determined as the concentration of the tested extract samples causing 50% reduction of the initial DPPH concentration, measured from the linear regression concentration curve of the test extract (mg/ml) against the percentage of the radical scavenging inhibition.

2.2.3.4. *Ferric reducing/antioxidant power assay (FRAP assay)*

The FRAP assay was performed according to a modified method described by Benzie and Strain (1999). Briefly, 200 µL solution of WSREt, WSFEt or WSLEt (1 mg/ml) was mixed with 1.5 ml of FRAP reagent. Then, the reaction mixture was incubated at 37°C for 4 min. After that, the absorbance was read at 593 nm against a blank that was prepared using distilled water. FRAP reagent was pre-warmed at 37°C and must be freshly prepared. This was done by mixing 10 volumes of 300 mM/L acetate buffer (pH 3.6) with 1 volume of 10 mmol 2,4,6-tris(1-pyridyl)-1,3,5-triazine (TPTZ) solution in 40 mM/L HCl with 1 volume of 20 mM ferric chloride ($FeCl_3.6H_2O$). A calibration curve was prepared, using aqueous solutions of ferrous sulphate ($FeSO_4.7H_2O$) at 100, 200, 400, 600 and 1000 µM/L. FRAP values were

expressed as micromoles of ferrous equivalent [μM Fe (II)] per kilogram of WSREt, WSFEt and WSLEt.

2.2.3.5. *Reducing power assay*

The Fe^{3+} reducing power of the extracts was determined by the method described by Oyaizu (1986) with slight modifications. The extracts (0.75 ml) at various concentrations were mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) [First, Phosphate Buffer A was prepared by diluting 31.2 grams $NaH_2PO_4.2H_20$ to 1000ml. Second, Phosphate Buffer B were prepared by diluting 53.61 grams $Na_2HPO_4.7H_20$ to 1000ml. Then, 62.5% of Buffer A was mixed with 37.5% of Buffer B. the pH was adjusted by using NaOH and H_2PO_4] and 0.75 ml of potassium hexacyanoferrate [K3Fe(CN)6] (w/v 1%), followed by incubating at 50 °C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 rpm for 10 min. 1.5 milliliter of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power.

2.2.3.6. *Fe^{2+} chelation*

The ferrous ion chelating activity was evaluated by a standard method (Haro-Vicente *et al.* 2006) with minor modifications. The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Briefly, various concentrations (0–120 μg/ml) of plant extract were added to 12.5 μM ferrous sulfate solution and the reaction was initiated by the addition of ferrozine (75 μM) [Ferrozine: 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine] disodium salt (Sigma P-9762) was dissolved in water to reach a concentration of 5 mg/ml [5 mM ferrozine (0.1 ml) dissolved in methanol]. The mixture was shaken vigorously and incubated for 20 min at room temperature, and then the absorbance was measured at 562 nm (Gulcin 2006). All tests were performed three times and the mean values were reported. EDTA was used as a positive control. The

percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated with the formula given below:

$$\text{Bounded ferrous ions (\%)} = (1 - \frac{\lambda 562\text{-S}}{\lambda 562\text{-C}}) \times 100$$

where $\lambda_{562\text{-C}}$ is the absorbance of the control and $\lambda_{562\text{-S}}$ is the absorbance in the presence of LAEP or standards. The control contains only $FeCl_2$ and ferrozine (Benzie and Strain 1996; Gulcin *et al.* 2010).

2.2.3.7. *Determination of anthocyanins*

Total anthocyanin content was determined by the pH differential method (Barnes et al. 2005). An aliquot of methanolic extract of *W. somnifera* (1 ml) was diluted to 10 ml with a pH 1.0 solution (125 ml of 0.2 M KCl and 375 ml of 0.2 M HCl). A second aliquot (1 ml) was diluted to 10 ml with a pH 4.5 buffered solutions (400 ml of 1 M CH_3CO2Na, 240 ml of 1 M HCl, and 360 ml of H_2O). Absorbance of the solutions was measured at 510 nm and the concentration of anthocyanins was calculated by the equation as below:

$$\text{Cmg/100 g} = [(\text{AbspH1.0} - \text{AbspH4.5}) \times 484.82 \times 1000/24825] \times \text{DF}$$

Where the term in parentheses presents the difference between absorbance (Abs) at 510 nm of the pH 1.0 and the pH 4.5 solution, 484.82 is the molecular mass of cyanidin-3-glucoside chloride, 24825 is it molar absorptivity (ε) nm in the pH 1.0 solution, and DF is the dilution factor. Each experiment was run in duplicates.

2.2.3.8. *Determination of ascorbic acid content in W. somnifera*

Ascorbic acid content was determined by following the spectrophotometric method described by Ferreira *et al.* (2009a). The sample (100 mg) was extracted with 10 ml of 1% metaphosphoric acid for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1ml) was then mixed with 9 ml of 2,6-dichlorophenolindophenol and the

absorbance was measured within 30 min at 515 nm against a blank. The content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (50, 100, 200, 400 µg/ml; Y = 3.2453X -.0703; R^2 = 0.9963) and the results were expressed as mg of ascorbic acid/100g of *W. somnifera*.

2.2.3.9. *Reducing sugar assay*

The total reducing sugar was determined using 3,5-dinitrosalicylic acid (DNSA) (Patil 2011). In principle the reducing sugar reduces DNSA to 3-amino- 5-nitrosalicylic acid resulting in the development of reddish–orangeish colouration which is measured spectrophotometrically at 540 nm (Saxena *et al.* 2010). A 1 ml aliquot of the diluted solution (0.5 mg/µL) of *W. somnifera* extract was mixed with equal volume of the DNSA solution and incubated in a boiling water bath for 10 min. The mixture was allowed to cool to ambient temperature for 10 min, mixed with 7.5 ml of Milli Q water and the absorbance was measured at 540 nm using a spectrophotometer. Glucose solutions of known concentration (100, 200, 400 and 600 µg/ml) were used as standards.

2.2.3.10. *Inhibition of β-carotene bleaching*

The antioxidant activity of methanolic extracts of *W. somnifera* solutions was evaluated by the β-carotene linoleate model system (Cao et al. 2009). A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two milliliters of this solution were pipetted into a 100 ml round-bottom flask. After the chloroform was removed at 40 °C under vacuum, 45 µL of linoleic acid, 400 µL of Tween 80 emulsifier, and 100 ml of distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing 60 µg/ml of the *W. somnifera* extracts. The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube the time zero absorbance was measured at 470 nm using a spectrophotometer. Absorbance readings were then recorded at 20 minutes interval until the

control sample changed color. A blank, devoid of β-carotene, was prepared for background subtraction (Mi-Yae *et al.* 2003).

Lipid peroxidation (LPO) inhibition was calculated using the following equation:

% of LPO inhibition = (β-carotene content after 2 hours of assay/initial β-carotene content) × 100. Ascorbic acid and TBHQ were used as standard.

2.2.3.11. *Effect of W. somnifera extract on pBR322 plasmid DNA scission induced by hydroxyl radical*

DNA damage protective activity of *W. somnifera* extract was determined using pBR322 plasmid DNA as target. Plasmid DNA was oxidized with H_2O_2 + UV treatment in presence of *W. somnifera* extract and checked on 1% agarose according to Russo et al. (2000) with minor modifications. The experiments were performed in a volume of 10 μl in an eppendrof tube containing 100 ng of pBR322 plasmid DNA (25 ng/μl stock, Takara Biomedicals, Japan) in 1xTE buffer (10 mM Tris–Cl and 1 mM EDTA), pH 8.0, 30% 4 μl H_2O_2 was added with and without 1 μl of different concentration (2.5, 5, 10, 20 μg/ml) of *W. somnifera* roots, fruits and leaves extracts in different tubes.

The reactions were initiated by UV irradiation and continued for 5 min on the surface of a UV transilluminator at 312 nm at room temperature. After irradiation the reaction mixture (10 μl) with gel loading dye (6x) was placed on 1% agarose gel for electrophoresis.

Untreated pBR322 plasmid DNA was used as a control. In each run of gel electrophoresis along with partial treatment i.e., only UV treatment and only H_2O_2. Gel was stained with ethidium bromide and photographed in Gel Doc. Quercetin (100 μM) was used as positive control.

2.2.4. HPLC Analysis of methanolic extract of W. somnifera roots, fruits and leaves

The HPLC method used for this investigation was based on the method published by Kaskoniene *et al.* (2009). Analysis of WSREt, WSFEt and WSLEt were performed by employing an HPLC system (Waters 2695, Milford, MA, USA) equipped with a Photodiode Array Detector (Waters 2996, Milford, MA, USA).

- The HPLC column was a Merck Purospher Star, RP-18e, (125 X 4 mm, 5 μm) fitted with a guard cartridge packed with the same type of stationary phase (Merck, Darmstadt, Germany).
- The linear 76 gradient was used at a flow rate of 0.5 ml/min with total analytical time of approximately 35 min.
- The binary mobile phase was consisted of a solvent A (ultra pure water with 0.1% of phosphoric acid) and solvent B (pure methanol with 0.1% of phosphoric acid).
- Elution from the column was achieved with the following gradient:
  - 0 min to 10 min of solvent B, increased from 35% to 55%
  - 10–25 min of solvent B, increased to 62%
  - 25–30 min of solvent B, increased to 85% and the final composition was kept constant till 35 min.
- All solvents used were of HPLC grade quality.
- The detection wavelength was done between 200 and 450 nm with specific monitoring at 265 nm.
- The identification of phenolic compounds was performed by comparing the retention time of each peak of the analytes with the reference standards.

The following Phenolic acids and flavonoids were purchased from Sigma (St. Louis, MO, USA) and were used as reference standards:

Phenolic acids

gallic

syringic

caffeic

vanillic

p-coumaric

benzoic and

transcinnamic acids

flavonoids

catechin

naringenin

luteolin

hesperetin

kaempferol

apigenin and

naringin

2.2.5. Antibacterial Properties of *W. somnifera*

2.2.5.1. *Sterility proofing of the extracts*

The extracts were tested for sterility after filtration with Millipore membrane (0.45µm) by introducing 2ml of this supposed sterile extract into 10ml of sterile nutrient broth. The extracts were Incubated at 37 ^{0}C for 24 hours. A sterile extract was indicated by the absence of turbidity or the clarity of the broth after the incubation period (Ronald 1995).

2.2.5.2. *Bacterial strains*

In vitro antimicrobial activity was examined for aqueous extract of *W. somnifera*. Pathogenic bacteria had been collected from Bangladesh Institute for Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM). The microorganisms studied include-

1) ***Escherichia coli***: Pathogenic strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible for haemolytic-uremic syndrome, peritonitis, mastitis, septicaemia and Gram-negative pneumonia (Todar 2008a).

2) ***Salmonella typhi***: *Salmonella* is a Gram-negative facultative rod-shaped bacterium. In humans, *Salmonella* are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a food borne infection/intoxication (Todar 2008b).

3) ***Citrobacter freundii***: The Citrobacter species, including *Citrobacter freundii*, are aerobic gram-negative bacilli. Its habitat includes the environment (soil, water, sewage), food, and the intestinal tracts of animals and humans (Wang, *et al.*, 2000). As an opportunistic pathogen, *C. freundii* is responsible for a number of significant opportunistic infections. It is known to be the cause of a variety of nosocomial infections

of the respiratory tract, urinary tract, blood and several other normally sterile sites in patients. *C. freundii* represents approximately 29% of all opportunistic infections (Whalen *et al.* 2007).

4) ***Pseudomonas aeruginosa***: *Pseudomonas aeruginosa* is a common bacterium that can cause disease in animals, including humans. It is found in soil, water, skin flora, and most man-made environments throughout the world. It uses a wide range of organic material for food; in animals, the versatility enables the organism to infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonizations occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal (Itah and Essien 2005). Because it thrives on most surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics. It is implicated in hot-tub rash. It is also able to decompose hydrocarbons and has been used to break down tarballs and oil from oil spills (Biopharma 2007).

5) ***Klebsiella pneumoniae***: *Klebsiella pneumoniae* is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin, and intestines (Ryan and Ray 2004). *K. pneumoniae* can cause the disease Klebsiella pneumonia. They cause destructive changes to human lungs inflammation and hemorrhage with cell death (necrosis) that sometimes produces a thick, bloody, mucoid sputum (currant jelly sputum). The most common infection caused by *Klebsiella* bacteria outside the hospital is pneumonia, typically in the form of bronchopneumonia and also bronchitis. These patients have an increased tendency to develop lung abscess, cavitation, empyema, and pleural adhesions. It has a high death rate of about 50% even with antimicrobial therapy. The mortality rate can be nearly 100% for persons with alcoholism and bacteremia. In addition to

pneumonia, *Klebsiella* can also cause infections in the urinary tract, lower biliary tract, and surgical wound sites (Rashid and Ebringer 2006).

All the microorganisms were maintained at 4^0C on nutrient agar slants. The bacterial strains were re-identified on the basis of morphological, cultural and biochemical characteristics (Cheesbrough 2000).

2.2.5.3. *Standardization of the bacterial cell suspension*

Five colonies of each test organism were picked into sterile test tube containing sterile nutrient broth and incubated at 37^0C for 24 hours. The turbidity produced by this organism was adjusted and used to match the turbidity (opacity) standard prepared as described by Monica (Monica 1984).

2.2.5.4. *Determination of antibacterial activity of different plant parts*

The antibacterial activity of the extracts (from different parts of the plant) was determined using agar well diffusion method following a published procedure with slight modification (Perez *et al.* 1990). Briefly-

- NA (NB+1.8% Agar) was inoculated with the given microorganism by spreading the bacterial inoculum in the media.
- Wells (8mm diameter) were punched in the agar and filled with 200 µl of the plant extracts (5 mg/ml).
- Control wells containing neat solvents (negative control) or a standard antibiotic solution of tetracycline (100 µg/ml) (positive control) were run parallel in the same plate.
- The plates were incubated at 37^0C for 24 hours.
- The antibacterial activity was assessed by measuring the diameter of the zone of inhibition for the respective drugs.
- The relative antibacterial potency of the given preparation was calculated by comparing its zone of inhibition with that of the standard drugs tetracycline.

2.2.5.5. *Determination of the minimum inhibitory concentration (MIC) of the extract*

The initial concentration of the plant extract (100mg/ml) was diluted using two fold serial dilution by transferring 5ml of the sterile plant extract (stock solution) into 5ml of sterile Nutrient broth to obtain 50mg/ml concentration. This process was repeated several times to obtain other dilutions: 25mg/ml, 12.5mg/ml, 6.25mg/ml and finally 3.125mg/ml (Ibekwe *et al.* 2001).

- After obtaining the different concentrations of the extracts, each concentration was inoculated with 0.1ml of the standardized bacterial cell suspension and incubated at 37^0c for 24 hours.
- The lowest concentration of the extract that inhibited the growth of the test organism was taken as the Minimum Inhibitory Concentration (MIC).
    - Negative controls were set up as follows
    - Nutrient broth only
    - Nutrient broth and sterile plant extract
- Finally positive control containing nutrient broth, and a test organism.

2.2.6. *The antifungal properties of methanolic extracts of W. somnifera leaves, fruits and roots against pathogen of basal rot disease*

This experiment was carried out to assess the antifungal properties of methanolic extracts of various parts of *W. somnifera* for the management of *Fusarium oxysporum* f. sp. cepae, isolated from onion suffering from basal rot disease.

Preparation of stock solution

Methanolic extract (10 g) of each plant part was dissolved separately in 2 ml dimethyl sulphoxide (DMSO) and volume was raised to 18 ml by adding sterile distilled water to prepare stock solution.

Bioassays with methanolic extract

Seventy six ml malt extract was autoclaved in 250 ml flasks and cooled at room temperature. Eight concentrations viz. 0.5, 1, 1.5, ...4% were prepared by addition of 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 ml of stock solution to 3.5, 3, 2.5, 2, 1.5, 1, 0.5 and 0 ml of sterile distilled water respectively to raise the volume of the medium 80 ml in each flask. This amount of the medium was divided into four equal parts that were treated as replications. For control, 2 ml of DMSO was added to 16 ml of water, and 4 ml of this mixture was added to 76 ml malt extract. Mycelial discs of *F. oxysporum* f. sp. cepae were prepared from the tips of 7 days old fungal culture using a sterilized 2 mm diameter cork borer and transferred to each 100-ml volume flasks each containing 20 ml of the medium. Flasks were incubated at 27±1 °C for 7 days. After that, the fungal mycelium was filtered and dried in an electric oven at 60 °C (Iqbal and Javaid 2012).

2.2.7. Statistical Analysis

All analyses were carried out in triplicates and the data were expressed as the means ± standard deviations (SD). The data were analyzed using Statistical Packages for the Social Sciences 12.0 (SPSS Inc., USA) and MS Excel 2003. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test was used to compare the phenol contents, FRAP values and DPPH scavenging activities of WSREt, WSFEt and WSLEt. The differences between means at 95% ($p < 0.05$) confidence level were considered statistically significant. Correlations were obtained by Pearson's correlation coefficient (r) in bivariate linear correlations.

CHAPTER III

RESULTS

3. RESULTS

3.1. PRELIMINARY PHYTOCHEMICAL ANALYSIS

Preliminary phytochemical analysis of methanolic extracts of leaves and fruits of *W. somnifera* revealed the presence of glycosides, alkaloids, phytosterols, fixed oils, phenolic compounds and flavonoids in extracts (**Table 3.1** and **Table 3.3**). The phytochemical tests of roots showed the presence of carbohydrate, glycosides, alkaloids, phytosterols, phenolic compounds and flavonoids (**Table 3.2**).

Methanolic extracts of *W. somnifera* leaves, roots and fruits were analyzed for the presence of carbohydrate, glycosides, alkaloids, phytosterols, phenolic compounds and flavonoids. The results of these experiments are summarized and presented in **Table 3.1**, **3.2** and **3.3**.

Table 3.1: Phytochemical screenings of *W. somnifera* leaf extracts

Serial No.	Phytochemical tests	Observation	Results
1	Carbohydrates: Molisch's test	There is no violet colored ring at the junction of two liquid	+
2	Glycosides: Fehling's test	Appearance of yellow or red color precipitate indicates the presence of reducing sugars	+
3	Alkaloids: Dragandroff's test	Appearance of organic precipitate shows the presence of alkaloids	+
4	Phytosterols: Salkowski test	After shaking, formation of golden yellow color in the lower layer indicates the presence of phytosterols	+
5	Fixed oil: Spot test	Appearance of oil strain on the paper indicates the presence of fixed oil	+
6	Proteins: Xanthoprotein test	Appearance of orange precipitate indicates the presence of protein	+
7	Phenolic compounds: $FeCl_3$ test	appearance of violet colour shows the presence of phenolic compound and tannins	+
8	Flavanoids: Concentrated H_2SO_4 test	Appearance of yellowish orange show the presence of anthocyanins, yellow to orange color show the presence of flavones, and orange to crimson show the presence of flavonones	+

Table 3.2: Phytochemical screenings of *W. somnifera* root extracts

Serial No.	Phytochemical tests	Observation	Results
1	Carbohydrates: Molisch's test	There is no violet colored ring at the junction of two liquid	+
2	Glycosides: Fehling's test	Appearance of yellow or red color precipitate indicates the presence of reducing sugars	+
3	Alkaloids: Dragandroff's test	Appearance of organic precipitate shows the presence of alkaloids	+
4	Phytosterols: Salkowski test	After shaking, formation of golden yellow color in the lower layer indicates the presence of phytosterols	+
5	Fixed oil: Spot test	Appearance of oil strain on the paper indicates the presence of fixed oil	–
6	Proteins: Xanthoprotein test	Appearance of orange precipitate indicates the presence of protein	+
7	Phenolic compounds: $FeCl_3$ test	appearance of violet colour shows the presence of phenolic compound and tannins	+
8	Flavanoids: Concentrated H_2SO_4 test	Appearance of yellowish orange show the presence of anthocyanins, yellow to orange color show the presence of flavones, and orange to crimson show the presence of flavonones	+

Table 3.3: Phytochemical screenings of *W. somnifera* fruit extracts

Serial No.	Phytochemical tests	Observation	results
1	Carbohydrates: Molisch's test	There is no violet colored ring at the junction of two liquid	+
2	Glycosides: Fehling's test	Appearance of yellow or red color precipitate indicates the presence of reducing sugars	+
3	Alkaloids: Dragandroff's test	Appearance of organic precipitate shows the presence of alkaloids	+
4	Phytosterols: Salkowski test	After shaking, formation of golden yellow color in the lower layer indicates the presence of phytosterols	+
5	Fixed oil: Spot test	Appearance of oil strain on the paper indicates the presence of fixed oil	+
6	Proteins: Xanthoprotein test	Appearance of orange precipitate indicates the presence of protein	+
7	Phenolic compounds: $FeCl_3$ test	appearance of violet colour shows the presence of phenolic compound and tannins	+
8	Flavanoids: Concentrated H_2SO_4 test	Appearance of yellowish orange show the presence of anthocyanins, yellow to orange color show the presence of flavones, and orange to crimson show the presence of flavonones	+

3.2. POLYPHENOLS AND ANTIOXIDANT ANALYSIS

The contents of total polyphenols (mg GAE/g) of WSREt, WSFEt and WSLEt were investigated using the modified Folin-Ciocalteu assay which is sensitive to phenol and polyphenols entities and other electron donating antioxidants such as ascorbic acid and vitamin E. The results of total polyphenols content and antioxidant analysis are shown in **Table 3.4-3.5** and **Figure 3.1-3.7**.

3.2.1. Total Polyphenol Contents

The sources of the analysed WSREt, WSFEt as well as WSLEt were significantly different ($p<0.05$), as shown in **Table 3.4**. Among the three different *W. somnifera* extracts, the concentrations of polyphenols was found to be the lowest in WSREt (17.80 ± 5.80 mg/g) and the highest in WSLEt (32.58 ± 3.16 mg/g). The values of total polyphenols content of the three extracts were significantly different which were shown in different letters (superscript) in each column in **Table 3.4**. The total polyphenols content of *W. somnifera* are highly correlated with flavonoids and DPPH free radical scavenging activities. Pearson's correlation matrix for total polyphenols have showed $r = 0.995$ ($p \leq 0.001$) with flavonoids and $r = 0.983$ ($p \leq 0.001$) DPPH. The correlation values of total polyphenols with flavonoids and DPPH free radical scavenging activities were shown in **Table 3.5**.

3.2.2. Flavonoid Contents

The total contents of flavonoids of the three different *W. somnifera* extracts were also determined. Flavonoids were detected in high concentrations and the lowest flavonoids content 15.49 ± 1.02 mg CEQ/g were found in WSREt and the highest content 31.58 ± 5.07 mg CEQ/g was found in WSLEt while 21.15 ± 5.32 mg CEQ/g was found in WSFEt and the value were shown in **Table 3.4**. The flavonoids content of the three *W. somnifera* plants parts were significantly different ($p < 0.5$) which were shown in **Table 3.4** (different superscript letters in each column indicating significant different). Flavonoids content of *W. somnifera* plant parts also have significantly positive correlation.

Flavonoids have showed Pearson's correlation value r = 0.995 ($p \leq 0.001$) with total polyphenols and r = 0.962 ($p \leq 0.001$) with DPPH. The value of Pearson's correlation were shown in **Table 3.5**.

Table 3.4: Spectrophotometric analysis of phenolics, flavonoids and antioxidant properties of methanolic extract of *W. somnifera* roots, fruits and leaves.

W. somnifera	Phenolics mg GAE/g	Flavonoids mg CEQ/g	% of DPPH inhibition
Roots	17.80±5.80^{c}	15.49±1.02^{c}	59.16±1.20^{c}
Fruits	22.29±1.99^{b}	21.15±5.32^{b}	70.38±0.84^{b}
Leaves	32.58±3.16^{a}	31.58±5.07^{a}	91.84±0.38^{a}

In each column, values with different letters (superscripts) indicate significant differences ($p < 0.05$).

Table 3.5: Correlations matrix among phenolics, flavonoids content and free radical scavenging activities.

	Phenolics	Flavonoids	DPPH
Phenolics	1	0.995(**)	0.983(**)
Flavonoids	0.995(**)	1	0.962(**)
DPPH	0.983(**)	0.962(**)	1

** Correlation is significant at the 0.01 level (2-tailed).

3.2.3. Ascorbic Acid Content of WSREt, WSFEt and WSLEt

Ascorbic acid content of WSREt, WSFEt and WSLEt was determined by spectrophotometric method and the highest content 62.60 mg/100g was found in WSLEt and moderate content was found in WSREt while the lowest content 20.60 mg/100g was found WSFEt. A great variation was observed in ascorbic acid content among WSREt, WSFEt and WSLEt which were shown **Figure 3.1**. Different letters in each column indicated significant difference.

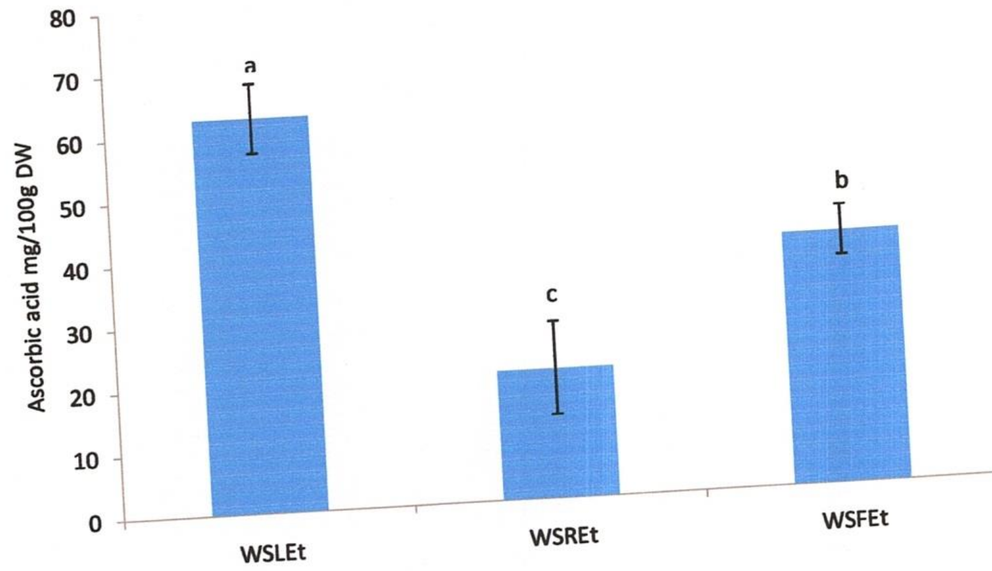


Figure 3.1: Ascorbic acid content of *W. somnifera* leaves, roots and fruits extracts. Different letters in each column indicate significant difference ($p<0.05$).

3.2.4. Total Anthocyanins of WSREt, WSFEt and WSLEt

The total anthocyanin content of WSLEt, WSREt and WSFEt was determined spectrophotometrically and the highest content of anthocyanin 12.5 ± 1.04 mg/100g was found in WSLEt and moderate content 5.66 ± 0.52 mg/100g was found in WSFEt while the lowest content 2.86 ± 1.44 mg/100g was found in WSREt (**Figure 3.2**). The values of total anthocyanin content of WSLEt, WSREt and WSFEt were significantly different ($p < 0.5$) and different letters in each column in **Figure 3.2** meant significant difference.

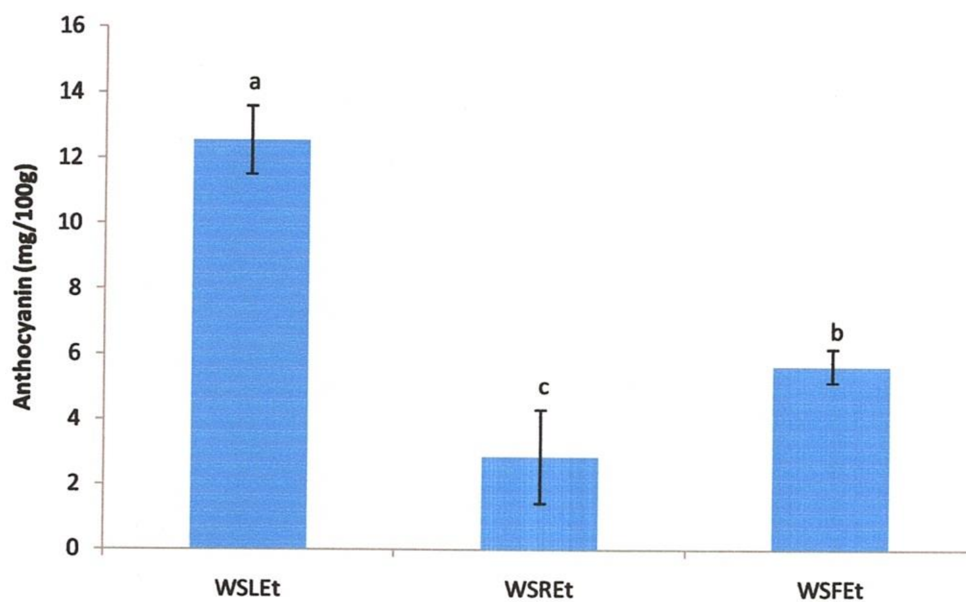


Figure 3.2: Anthocyanin content of *W. somnifera* leaves, roots and fruits extracts. Different letters in each column indicate significant difference ($p<0.05$).

3.2.5. DPPH Radical Scavenging Activity

There were significant differences in terms of their scavenging abilities present among the WSREt, WSFEt as well as WSLEt samples, expressed as percentage of inhibition on the DPPH radical (**Table 3.4**). Among the three extracts, the lowest scavenging activity was found in WSREt (59.16 ± 1.20%) while the highest activity was found in WSLEt (91.84 ± 0.38%). The DPPH radical scavenging test is one of the fastest tests available to investigate the overall hydrogen/electron donating activity of single antioxidants and health-promoting dietary antioxidant supplements. The reasons behind the markedly higher radical scavenging capacity exhibited by the different types of *W. somnifera* extracts probably lie in their diverse botanical origin. Antioxidant potential of *W. somnifera* extracts is directly related to its phenolic and flavonoids content.

3.2.6. DPPH Radical Scavenging Activity (IC_{50})

The IC_{50} values of DPPH radical scavenging activity for WSLEt, WSREt and WSFEt were 101.73 µg/ml, 801.93 µg/ml and 345.68 µg/ml respectively (**Figure 3.3**). Ascorbic acid and BHT were used as standard and the IC_{50} of these two standard were 1.08 µg/ml and 0.89 µg/ml. IC_{50} values of these three extracts were significantly different which were shown in **Figure 3.3**. Column of WSLEt, WSREt and WSFEt have different letters (a, b and c) indicated significant difference while the column of IC_{50} value of ascorbic acid and BHT have same letter (d) indicated non significant difference.

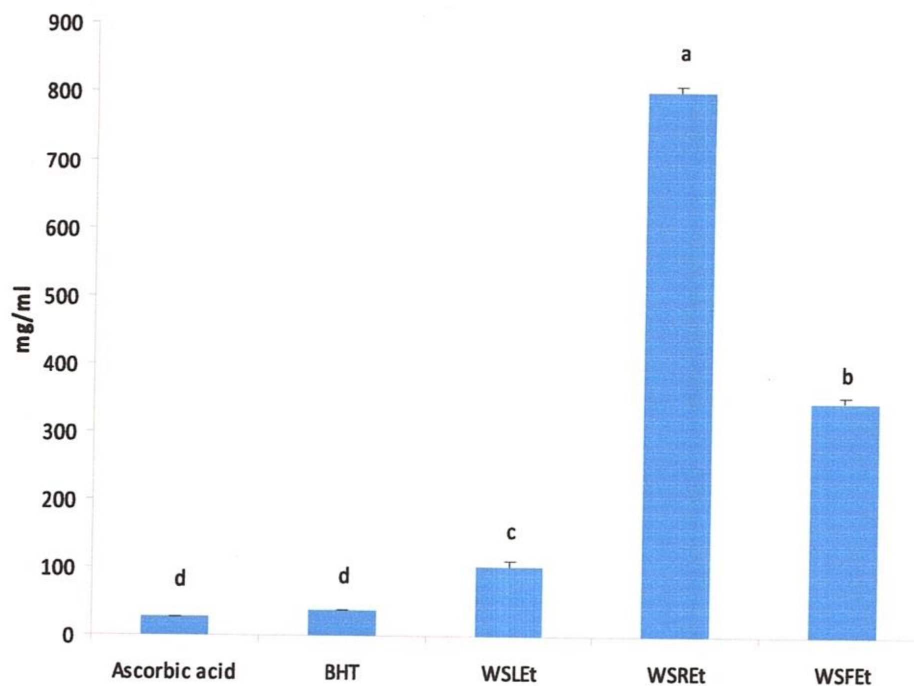


Figure 3.3: DPPH radical scavenging activity (IC_{50}) of *W. somnifera* leaves, roots and fruits extracts. Different letters in each column indicate significant difference ($p<0.05$).

3.2.7. Ferric Reducing Antioxidant Power Assay (FRAP)

FRAP assay directly gives an estimation of antioxidants or reductants present in a sample, and is based on the ability of the analyte to reduce the Fe^{3+}/Fe^{2+} couple. WSLEt contained the highest concentration of total antioxidant (1643.04 µM Fe/kg) while WSREt had the lowest (1130.18 µM Fe/kg) (**Figure 3.4**) and WSFEt contained 1564.50 µM Fe (Miyaka, *et al.*) /kg (**Figure 3.4**). The FRAP values of all the extracts were significantly different.

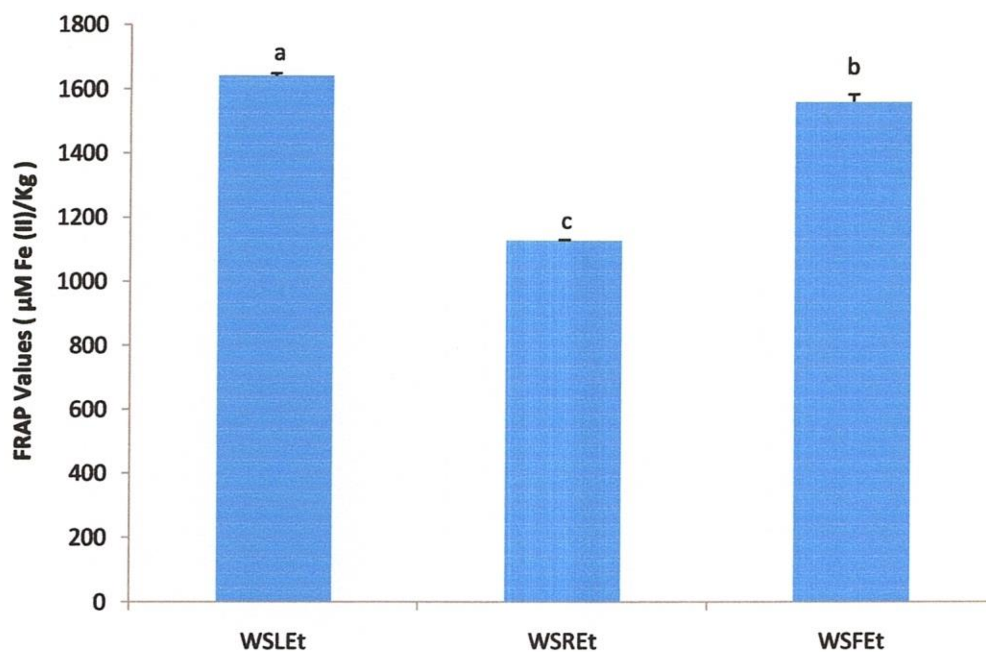


Figure 3.4: Ferrous reducing antioxidant assay (FRAP) of *W. somnifera* leaves, roots and fruits extracts. Different letters in each column indicate significant difference ($p<0.05$).

3.2.8. Ferrous Chelation Activity

Ferrozine produces a violet complex with Fe^{2+}. In the presence of a chelating agent, complex formation is interrupted and as a result the violet color of the complex is decreased. The results (**Figure 3.5**) demonstrated that formation of the ferrozine-Fe^{2+} complex is inhibited in the presence of the test and reference compounds. The IC_{50} values (**Table 3.1**) of the WSLEt, WSREt, WSFEt and EDTA were 0.22 ± 0.04 mg/ml, 0.37 ± 0.02 mg/ml, 0.65 ± 0.02 mg/ml and 0.13 ± 0.01 mg/ml respectively.

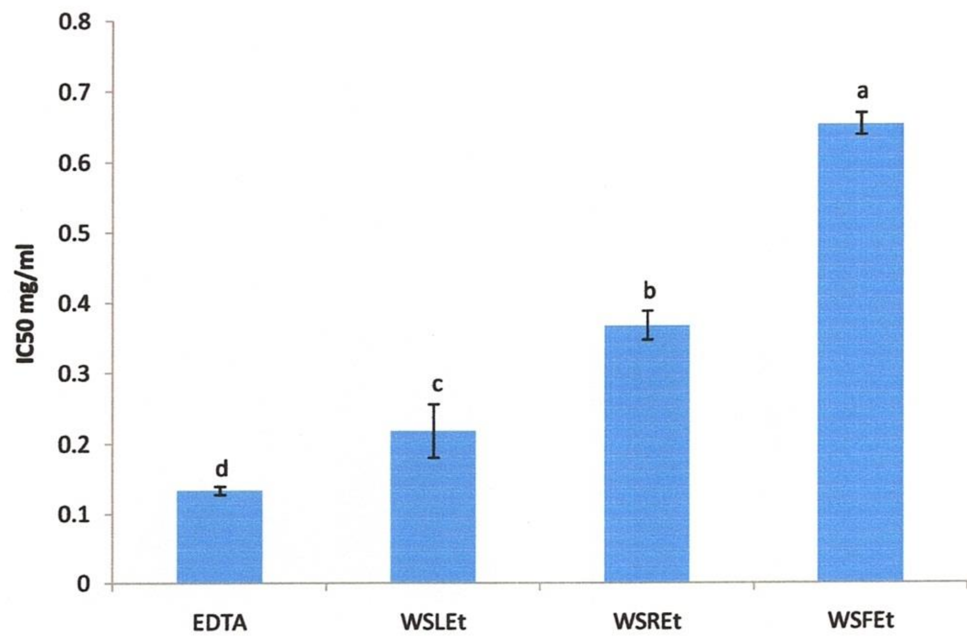


Figure 3.5: IC_{50} value of ferrous chelation activity of *W. somnifera* leaves, roots and fruits extracts. Different letters in each column indicate significant difference ($p<0.05$).

3.2.9. Reducing Power Assay

As illustrated in **Figure 3.6**, Fe^{3+} was transformed to Fe^{2+} in the presence of WSLEt, WSREt WSFEt and the reference compound BHT to measure the reductive capability. The absorbance was measured at different concentration (20, 40, 60 and 80 μg/ml) of the extracts and reference samples. Among the three extracts WSLEt showed higher absorbance compared to the WSREt and WSFEt while the highest absorbance was found for the reference compound BHT at different concentration (**Figure 3.6**).

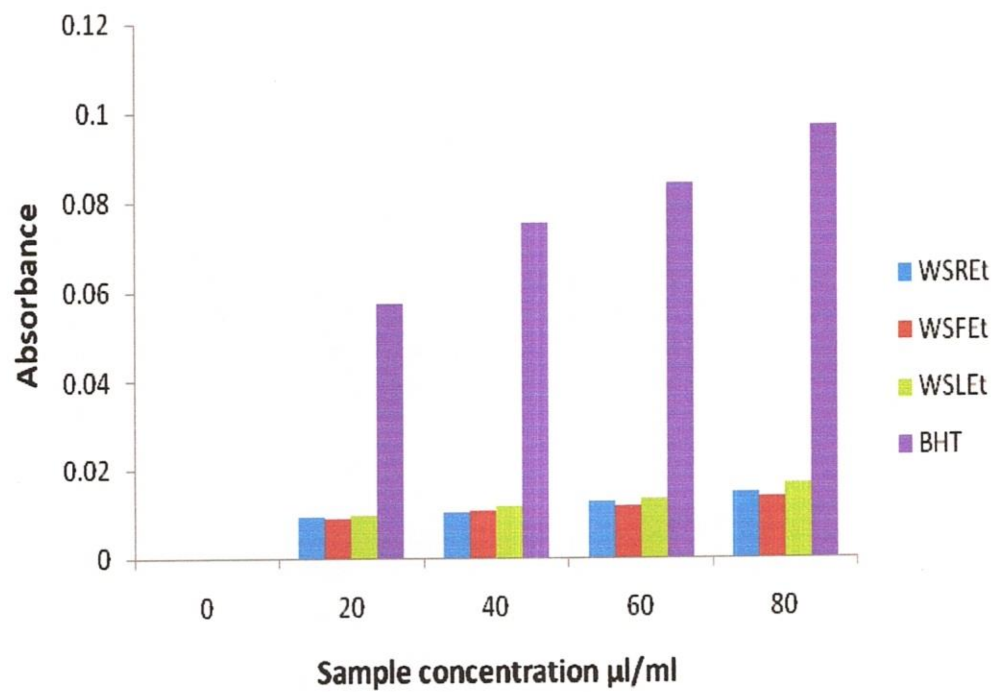


Figure 3.6: Reducing power assay of *W. somnifera* leaves, roots and fruits extracts.

3.2.10. Inhibition of β-Carotene Bleaching

The effect of 60 µg/ml WSLEt, WSREt WSFEt and the reference compound ascorbic acid and TBHQ on lipid peroxidation of a linoleic acid emulsion is shown in **Figure 3.7**. WSLEt, WSREt and WSFEt exhibited 79.67%, 69.87%, and 72.11% peroxidation of linoleic acid emulsion at the same concentration, respectively. On the other hand the reference compound ascorbic acid and TBHQ exhibited 100.28% and 104.52% peroxidation of linoleic acid emulsion. These results clearly indicate that WSLEt, WSREt and WSFEt showed remarkable antioxidant activity.

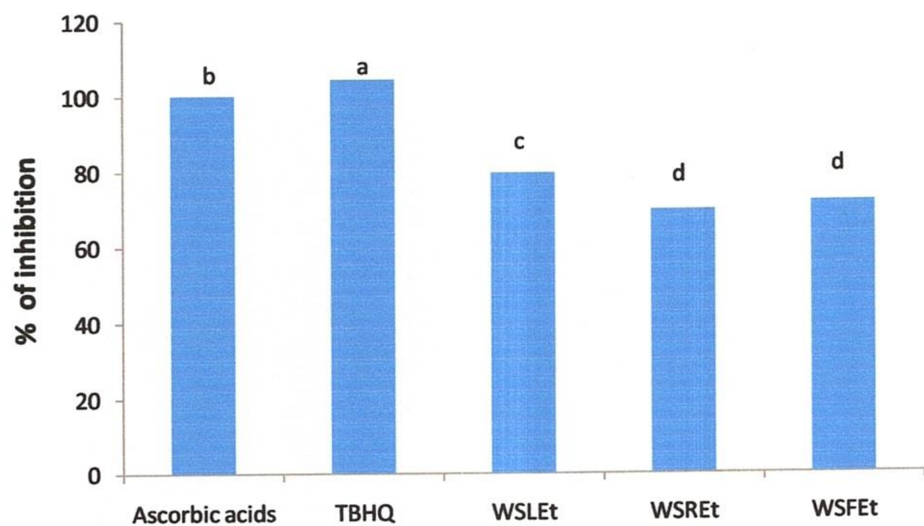


Figure 3.7: Percentage of lipid peroxidation (LPO) inhibition for *W. somnifera* leaves, roots and fruits extracts. Different letters in each column indicate significant difference ($p<0.05$).

3.2.11. Effect of *W. somnifera* extract on pBR322 plasmid DNA scission induced by hydroxyl radical

The protective effect of *W. somnifera* roots, fruits and leaves extract on H_2O_2 + UV-induced damage was studied on pBR322 plasmid DNA. **Figure 3.8** shows the electrophoretic pattern of DNA after UV-photolysis of 30% H_2O_2 in the presence or absence of different concentration (2.5, 5, 10, 20 µg/ml) of *W. somnifera* roots, fruits and leaves extract. DNA derived from pBR322 plasmid showed two bands on agarose gel electrophoresis (lane 1) the faster moving prominent band corresponded to the native supercoiled circular DNA (Sc DNA) and the slower moving faint band was the open circular form (Oc DNA).

It was noted that only UV treatment and only H_2O_2 treatment (lanes 2 and 3 respectively) could not induce damage as noted in combined treatment (lane 4). The UV irradiation of DNA in the presence of H_2O_2 (lane 4) resulting the cleavage of Sc DNA to give prominent Oc DNA and a faint linear (Lin) DNA indicating that $OH^{\cdot}$ generated from UV-photolysis of H_2O_2 produced DNA strand scission. DNA damage was reduced with the treatment of a standard antioxidant quercetin (lane 5). The addition of *W. somnifera* roots, fruits and leaves extract in different concentration (2.5, 5. 10, 20 µg/ml) to the reaction mixture of H_2O_2 induced the protection to the damage of native supercoiled circular DNA (lane 6-17).

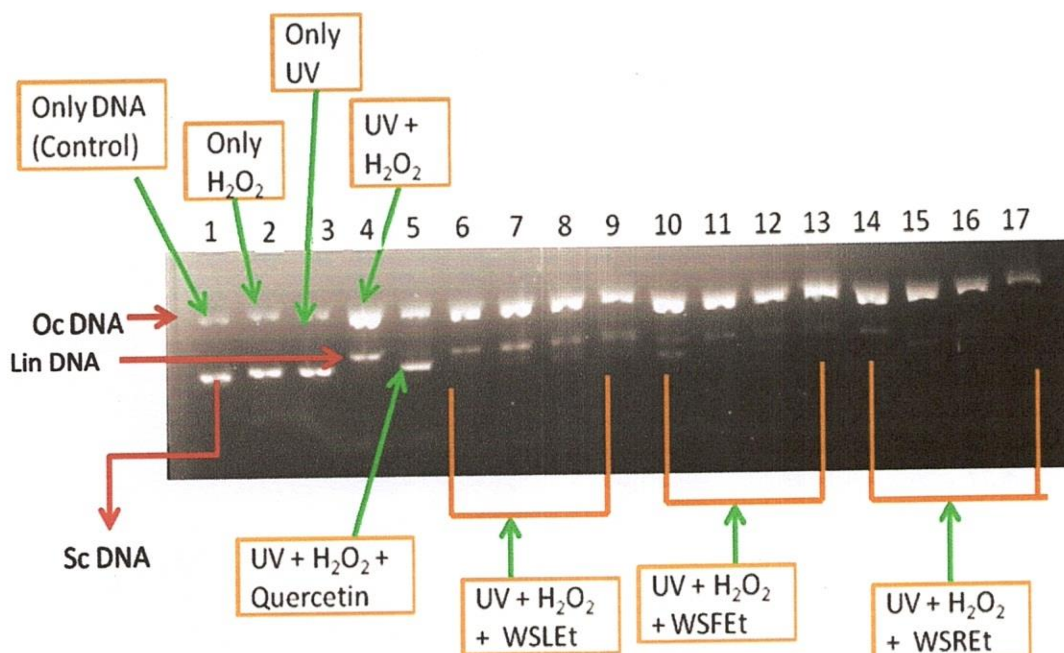


Figure 3.8: Electrophoretic pattern of pBR322 DNA after UV-photolysis of 30% H_2O_2 in the presence or absence of *W. somnifera* roots, fruits and leaves extract (2.5, 5. 10, 20 μg/ml). Lane 1: control, lane 2: only H_2O_2 treated, lane 3: only UV treated, lane 4: H_2O_2 + UV treated, lane 5: H_2O_2 + UV + quercetin (100 μM) and lane 6-9: WSLEt, WSFEt and WSREt (2.5, 5. 10, 20 μg/ml). Oc= Open circular, Lin= Linear, Sc= Supercoiled, WSREt, WSFEt and WSLEt: *W. somnifera* roots, fruits and leaves extract respectively.

3.3. HPLC ANALYSIS OF METHANOLIC EXTRACT OF *WITHANIA SOMNIFERA* ROOTS, FRUITS AND LEAVES

Individual chromatogram of fourteen phenolic and flavonoid standards were shown in **Figure 3.9(A-N)** and chromatograms of the fourteen mixture standards were shown in **Figure 10**.

Fourteen phenolic and flavonoid standards were compared with the chromatograms produced by the unknown *W. somnifera* extracts. The results on HPLC analysis of phenolic and flavonoids compounds present in WSREt, WSFEt as well as WSLEt are shown in **Table 3.6** and **Figures 3.11-3.13**. The HPLC analysis showed the only catechin is commonly found in all of the three extracts analyzed. Eight polyphenols (five phenolic acids and three types of flavonoids) have been identified and the phenolics patterns of all plant parts were confirmed to contain gallic, syringic, benzoic, p-coumaric and vanillic acids as well as the flavonoids catechin, kaempferol and naringenin (**Table 3.6**).

HPLC chromatograms obtained from WSREt, WSFEt and WSLEt are shown in **Figures 3.11-3.13**. Six phenolic compounds were detected in WSLEt whereas, three compounds were identified in WSFEt and only two compounds were identified in WSREt. The unknown compounds that may have had similar flavonoid and phenolic acid chromatographic behaviours (shown as extra peaks in the figures) were also detected. However, they could not be fully identified due to lack of standard compounds. **Figure 3.14** compared the total phenolic compounds of sample extracts obtained when using spectrophotometric and HPLC methods. Overall, spectrophotometric methods tend to report higher levels of phenolics when compared to HPLC method.

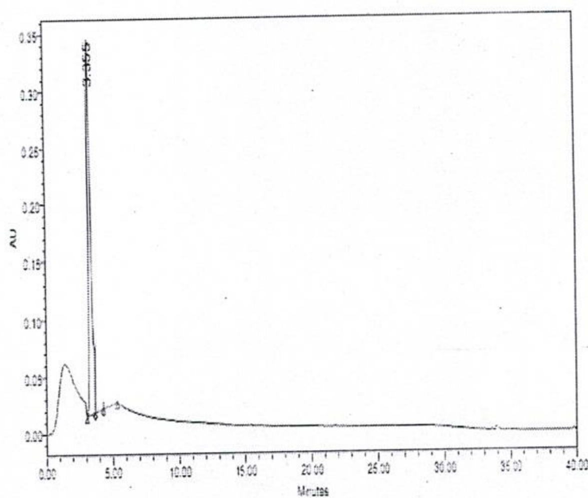
Table 3.6: Phenolic acids and flavonoids compounds detected in *Withania somnifera* roots, fruits and leaves using high performance liquid chromatography analysis.

SL No	Standard compounds	RT	λ_{max} (nm)	Quantity of the identified compounds (mg/g DW)		
				Roots	Fruits	Leaves
1	Catechin	3.36	278	12.82	19.48	28.38
2	Gallic acid	4.12	269, 216	ND	ND	0.18
3	Syringic acid	8.10	268, 216	ND	ND	0.30
4	Caffeic acid	8.31	287, 263	ND	ND	ND
5	Vanillic acid	8.61	224, 249, 269	ND	ND	0.15
6	p-coumaric acid	11.1	264, 286, 310	ND	ND	0.80
7	Benzoic acid	15.33	272, 241	0.19	ND	0.80
8	Naringenin	20.18	277, 292, 308	ND	0.50	ND
9	Transcinnamic acid	20.52	263, 280	ND	ND	ND
10	Luteolin	21.20	286, 249, 329	ND	ND	ND
11	Hesperetin	21.56	270, 295	ND	ND	ND
12	Kaempferol	25.59	361	ND	0.06	ND
13	Apigenin	26.79	264, 321, 325	ND	ND	ND
14	Naringin	28.56	283, 327	ND	ND	ND
	Total phenolic compounds			13.01 ±8.93	20.04 ±11.09	30.61 ±11.41

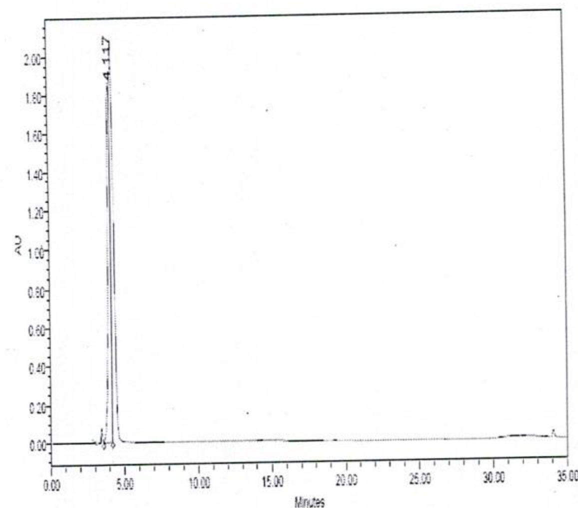
(ND = Not detected), DW=dry weight, RT, Retention time

In this investigation, the HPLC analysis for the phenolic compounds were performed according to the established method published by Kaskoniene et al. (2009). Phenolic compounds were identified in WSREt, WSFEt and WSLEt by comparing the retention times of each peak of the analytes with the reference standard. Out of fourteen standard, the lowest retention time 3.36 minutes was found for catechin and the hightest retention time was found for naringin. Individual chromatogram with retention time of the fourteen standard compounds were shown in the **Figure 3.9(A)** to **Fugure 3.9(N)** sequentially. The mixed peak of all the standard compounds were shown in **Figure 3.9** in a single chromatogram and the peaks were identified as peak 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 16 sequentially by comparing the retention time of the individual chromatogram of catechin, gallic acid, syringic acid, caffeic acid, vanillic acid, p-coumaric acid, benzoic acid, naringenin, transcinnamic acid, luteolin, hesperetin, kaempferol, apigenin and naringin respectively.

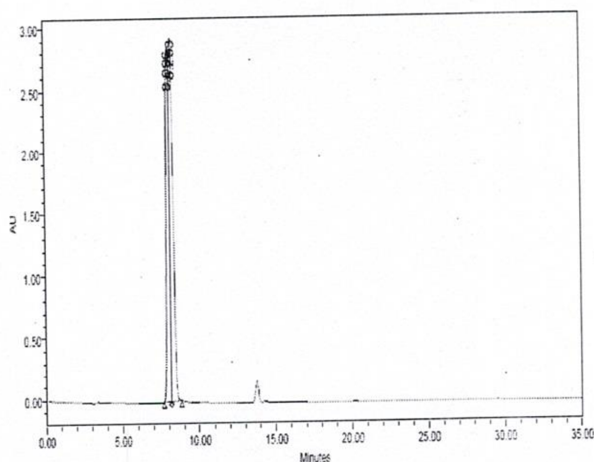
Catechin and benzoic acid were found by comparing the chromatogram of *W. somnifera* roots extract (**Figure 3.11**) with the chromatogram of **Figure 3.10** and catechin, naringenin and kaempferol were found in *W. somnifera* fruits extract (**Figure 3.12**) while six phenolic compounds catechin, gallic acid, syringic acid, vanillic acid, p-coumaric acid and benzoic acid were found in *W. somnifera* leaves extract (**Figure 3.13**).



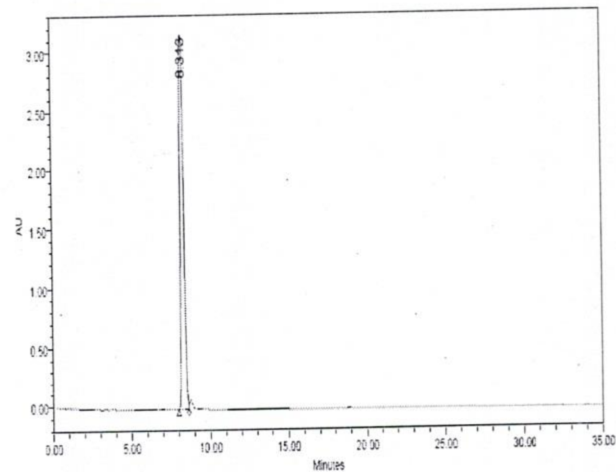
(A)



(B)

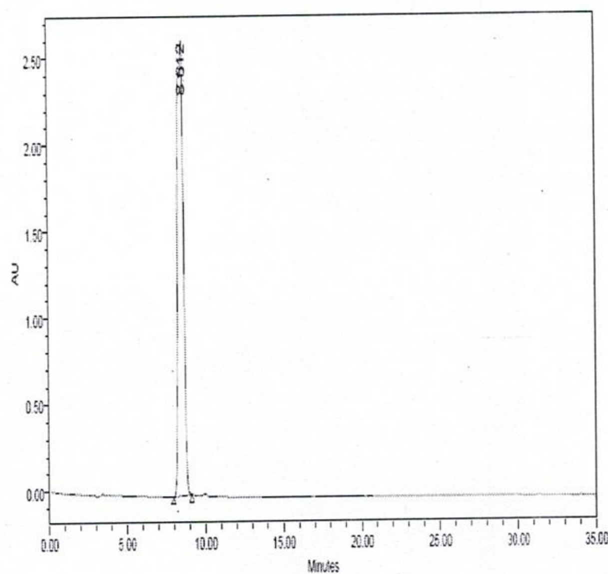


(C)

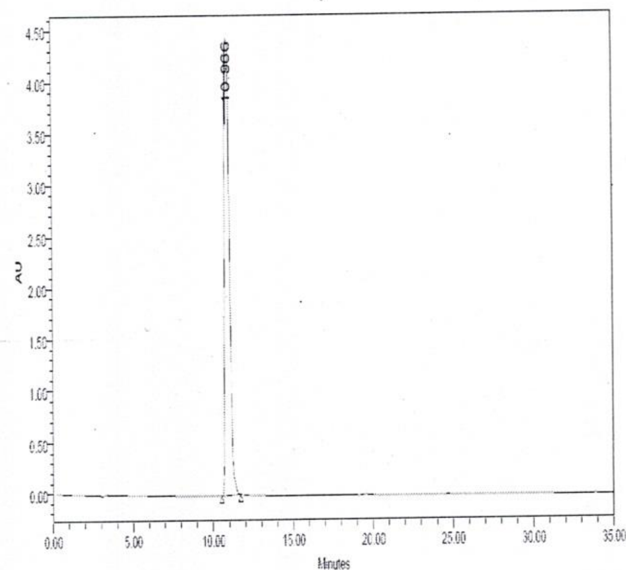


(D)

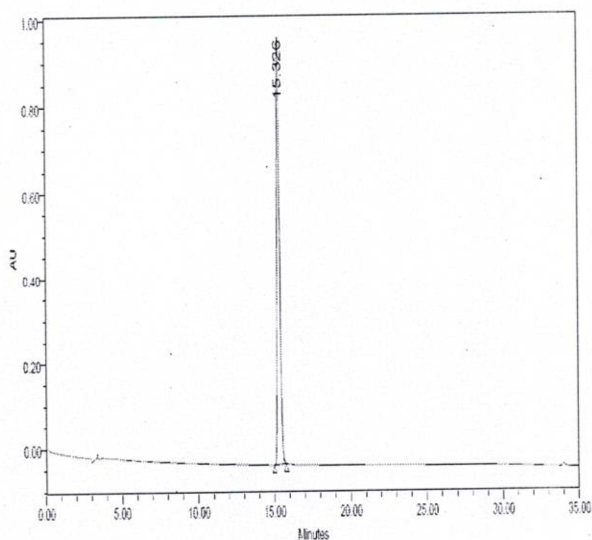
Figure 3.9 (A-D): HPLC chromatogram of (A) catechin (B) gallic acid (C) syringic acid (D) caffeic acid.



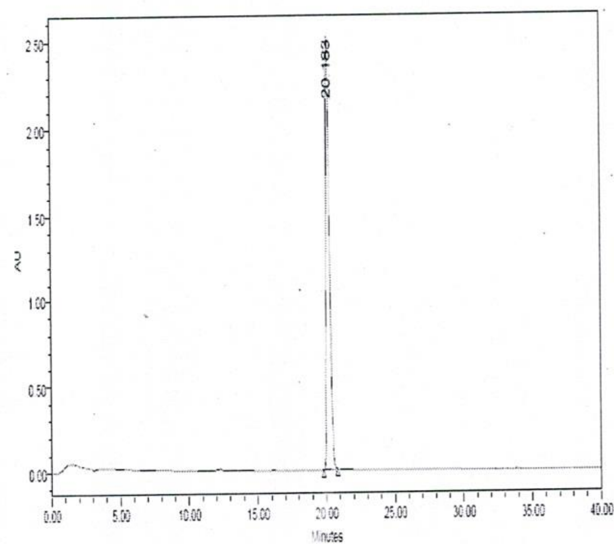
(E)



(F)

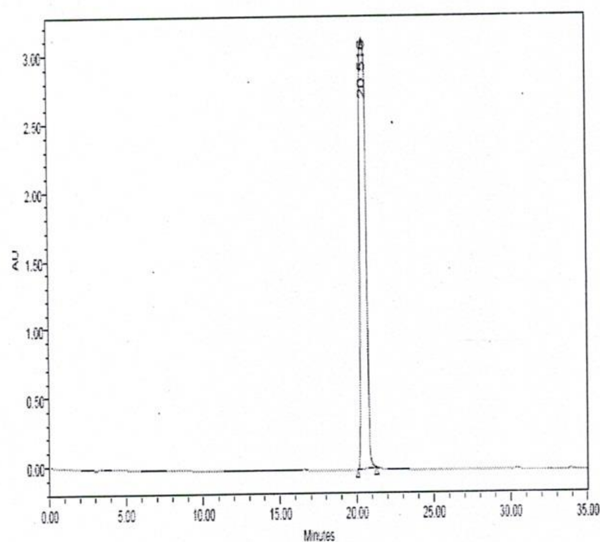


(G)

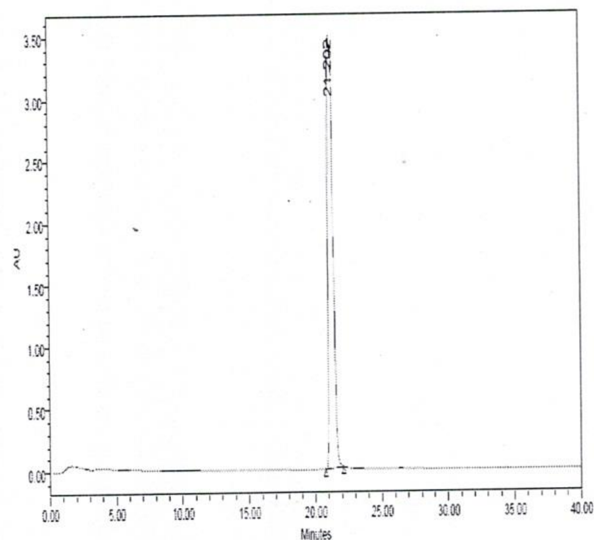


(H)

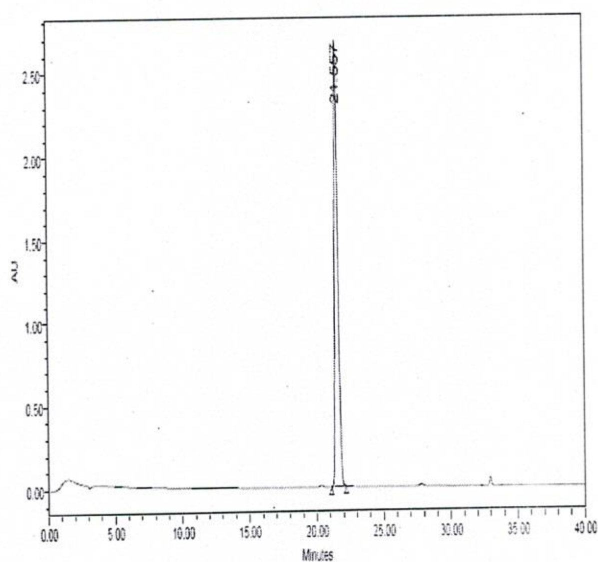
Figure 3.9(E-H): HPLC chromatogram of (E) vanillic acid, (E) p-coumaric acid (G) benzoic acid (H) naringenin



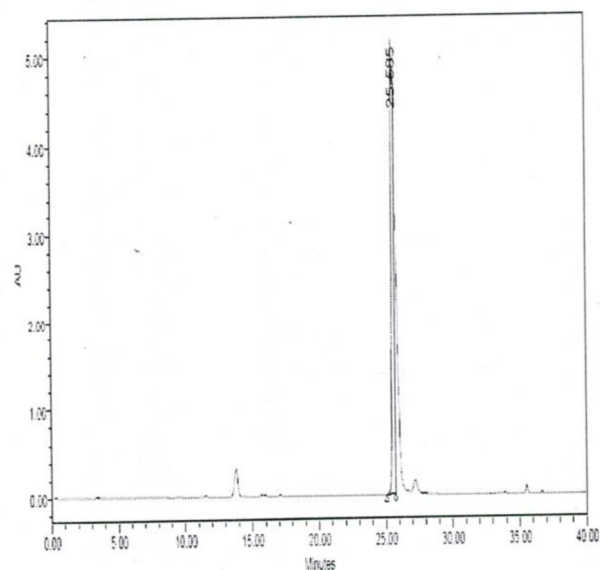
(I)



(J)

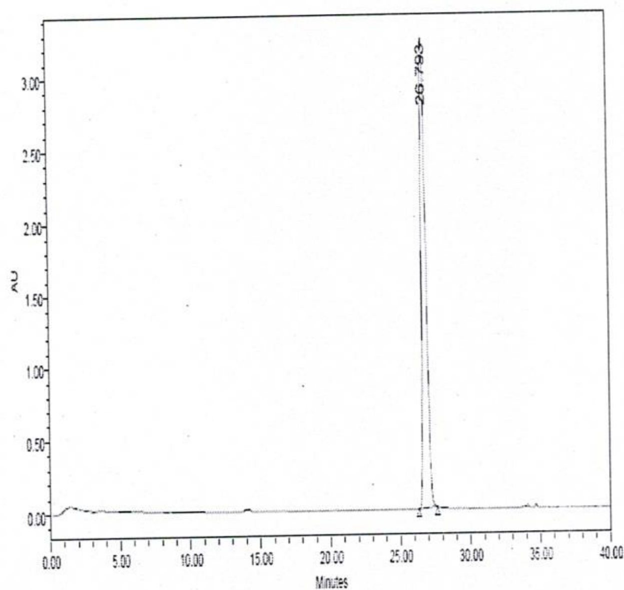


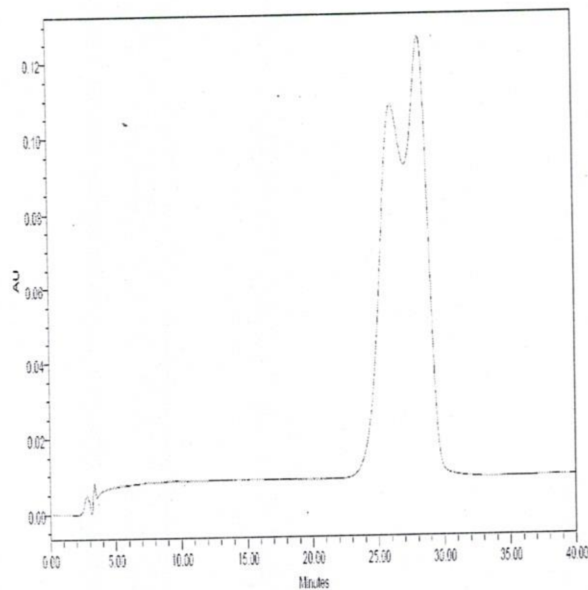
(K)



(L)

Figure 3.9(I-L): HPLC chromatogram of (I) transcinnamic acid (J) luteolin (K) hesperetin (L) kaemferol





(M) **(N)**

Figure 3.9(M-N): HPLC chromatogram of (M) apigenin (N) naringin.

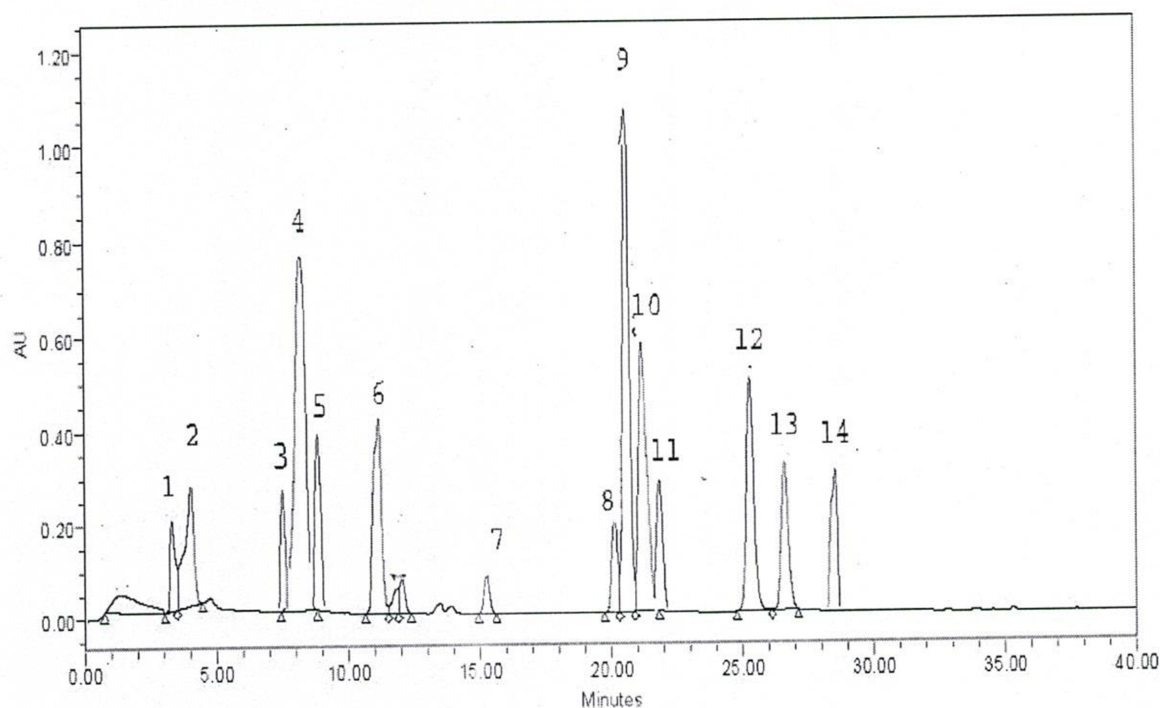


Figure 3.10: HPLC chromatogram of fourteen standard phenolic compounds: (1) Catechin (2) Gallic acid (3) Syringic acid(4) Caffeic acid (5) Vanillic acid (6) p-coumaric acid (7) Benzoic acid (8) Naringenin (9) Transcinnamic acid (10) Luteolin (11) Hesperetin (12) Kaempferol (13) Apigenin (14) Naringin

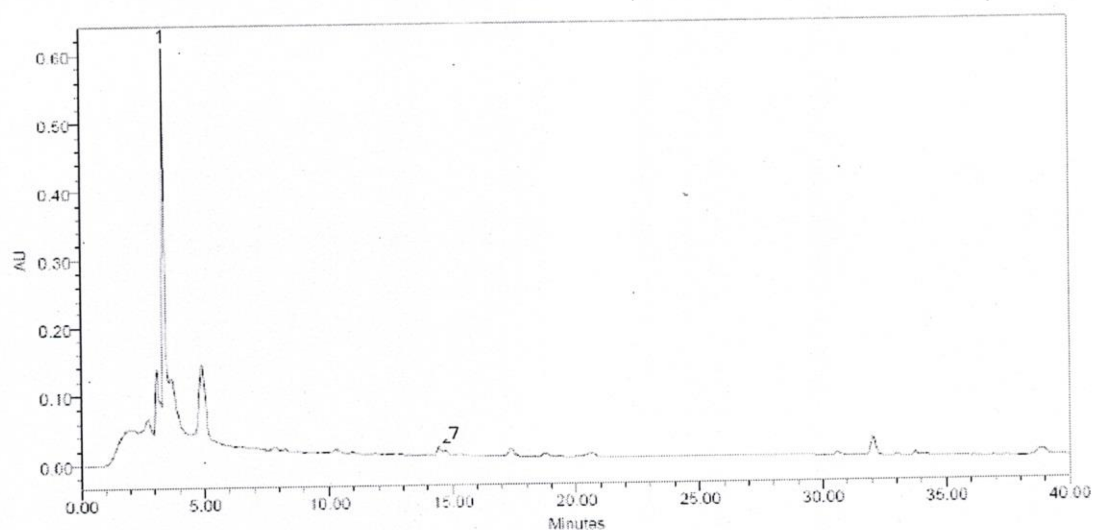


Figure 3.11: HPLC chromatogram of methanolic extracts of *W. somnifera* roots. (1) catechin and (7) benzoic acid.

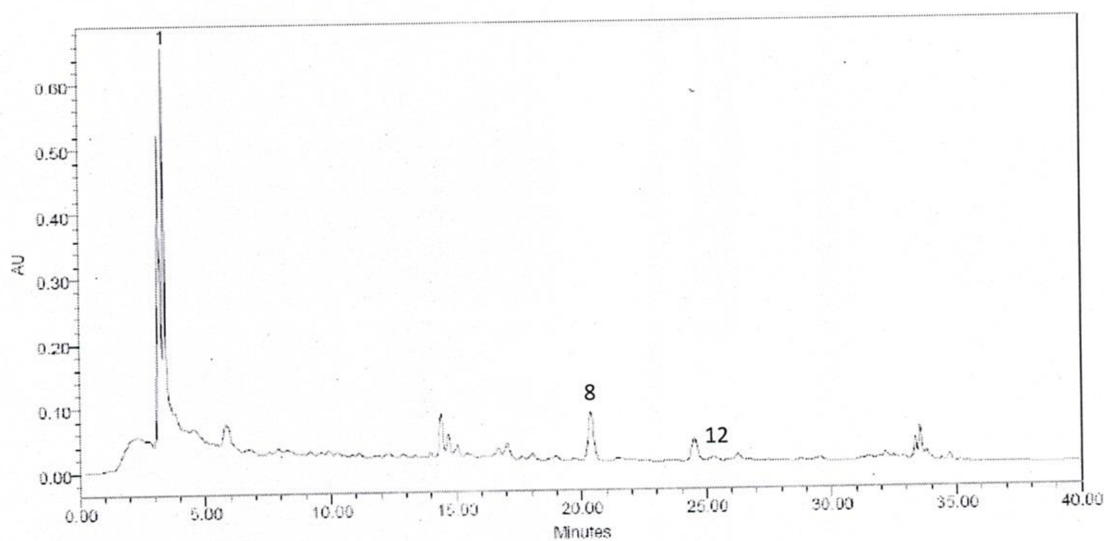


Figure 3.12: HPLC chromatogram of methanolic extracts of *W. somnifera* fruits. (1) catechin (8) naringenin and (12) kaempferol.

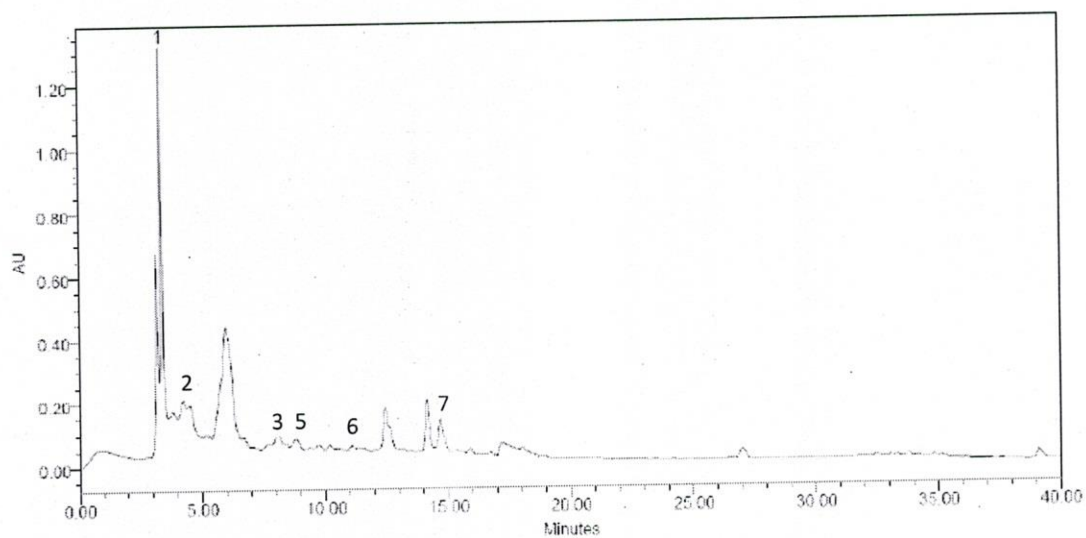


Figure 3.13: HPLC chromatogram of methanolic extracts of *W. somnifera* leaves. (1) catechin, (2) gallic acid, (3) syringic acid, (5) vanillic acid, (6) p-coumaric acid and (7) benzoic acid.

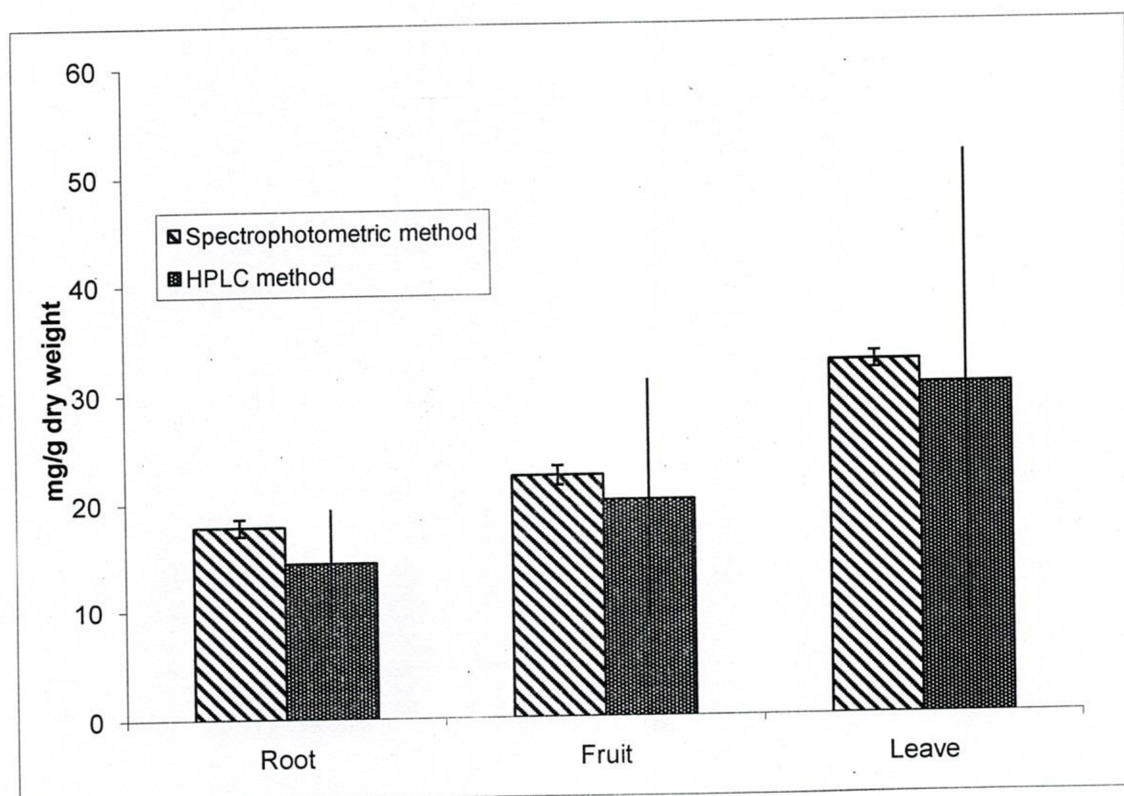


Figure 3.14: Comparison of total values of phenolic and flavonoid compounds (mg/g) of *W. somnifera* roots, fruits and leaves obtained by spectrophotometric and HPLC method ($p<0.05$).

3.4. ANTIMICROBIAL PROPERTIES OF *W. SOMNIFERA* ROOT, FRUITS AND LEAVES

3.4.1. Antibacterial activity

The antimicrobial activity of the WSREt, WSFEt and WSLEt and their potency was quantitatively assessed by the presence or absence of inhibition zone and zone diameter. The results on antimicrobial activities of different extracts are shown in **Table 3.7-3.8**. Eighty percent methanolic extract of the three plant parts showed antimicrobial potency against five pathogenic bacteria. WSLEt showed higher antibacterial activities against all the pathogenic bacteria tested compared to WSREt and WSFEt. The highest activity of WSLEt was found against *S. typhi* (32±0.75 mm zone of inhibition) while the lowest activity was found against *K. pneumoniae* (19±1.48 mm zone of inhibition). The highest zone of inhibition of WSFEt (14±0.79 mm) was found against *S. typhi* and the lowest zone of inhibition (08±1.61 mm) was found against *K. pneumonia*. In case of WSREt, the highest zone of inhibition (15±0.96 mm) was found against *E. coli* while the lowest zone of inhibition (09±1.32 mm) was found against *P. aeruginosa.*

Minimum inhibitory concentration (MIC) of the WSREt, WSFEt and WSLEt against the five pathogenic bacteria was shown in the **Table 3.8**. WSLEt showed the lowest MIC (6.25 mg/ml) against *S. typhi* while the highest value was 25 mg/ml against *C. freundii, P. aeruginosa* and *K. pneumonia*. The lowest MIC of WSFEt (25 mg/ml) was found for *E. coli* and *S. typhi* while the MIC 50 mg/ml was found for *C. freundii, P. aeruginosa* and *K. pneumonia*. For WSREt, The lowest MIC (25 mg/ml) was found against *E. coli* while the MIC 50 mg/ml was found against other four bacteria.

The antibacterial activity of WSREt, WSFEt and WSLEt against various bacterial strains was determined by agar diffusion method. The active

components of the root and leaves of *W. somnifera* were extracted with 80% aqueous methanol. The solid residues obtained after evaporation were re-suspended in sterile water at 5 mg/ml concentration. The well bored in bacterial culture plates were filled with 200 µl of suspension (1 mg crude extract/well). The negative control wells were exposed with the neat solvent (80% aqueous methanol). Each value represents mean±SD of three different observations.

Table 3.7: Diameters of zones of inhibition of the 80% aqueous methanolic extracts of WSREt, WSFEt and WSLEt on the test organisms.

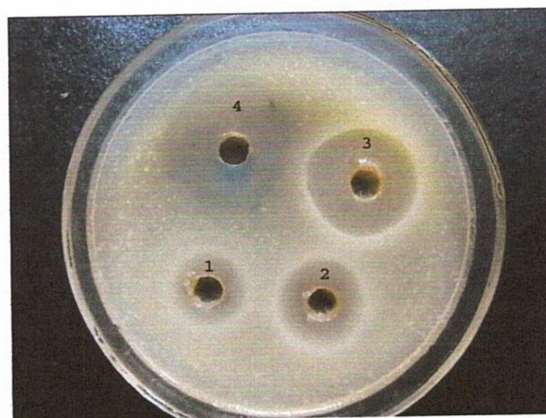
Test organisms	**Diameters of Zones of Inhibition (mm)**			
	WSREt	WSFEt	WSLEt	Tetracycline (100 µg/ml)
Escherichia coli	15±0.96	12±1.50	28±0.56	43±1.90
Salmonella typhi	16±0.43	13±0.79	32±0.75	47±1.42
Citrobacter freundii	11±0.86	09±1.21	23±1.27	38±1.22
Pseudomonas aeruginosa	10±1.32	8±1.43	26±1.08	32±0.59
Klebsiella pneumoniae	10±1.10	08±1.61	19±1.48	36±1.13

WSREt, WSFEt and WSLEt: *W. somnifera* roots, fruits and leaves extract respectively.

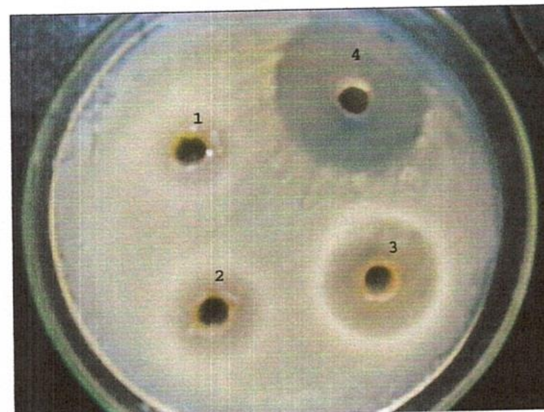
Table 3.8: Determination of minimum inhibitory concentration (MIC) of the methanolic extracts of WSREt, WSFEt and WSLEt on the test organisms.

Test organisms	**Concentrations (mg/ml)**			
	WSREt	WSFEt	WSLEt	Tetracycline
Escherichia coli	25.0	25.0	12.5	0.025
Salmonella typhi	50.0	25.0	6.25	0.0125
Citrobacter freundii	50.0	50.0	25.0	0.05
Pseudomonas aeruginosa	50.0	50.0	25.0	0.05
Klebsiella pneumoniae	50.0	50.0	25.0	0.025

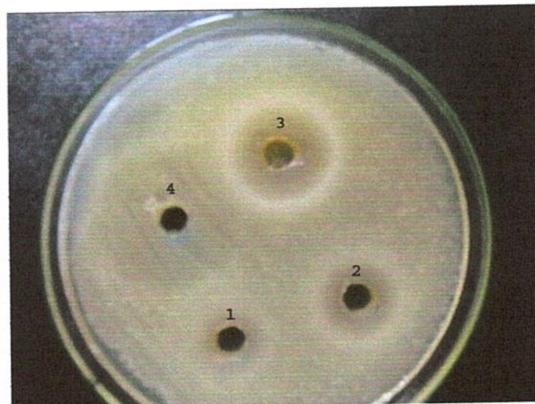
WSREt, WSFEt and WSLEt: *W. somnifera* roots, fruits and leaves extract respectively.



(A)

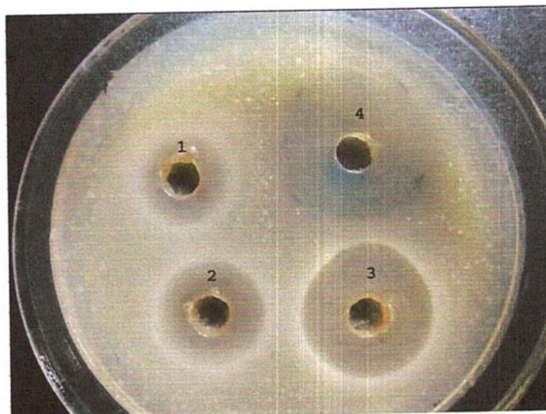


(B)



(C)

(D)



(E)

Figures 3.15(A-E) are the culture of (A) *C. freundii*, (B) *E. coli*, (C) *K. pneumoniae*, (D) *P. aeruginosa* and (E) *S. typhi* respectively, showing circular zone of growth inhibition. 1 = WSFEt (200 µl/well), 2 = WSREt (200 µl/well), 3 = WSLEt (200 µl/well) and 4 = Tetracycline (100 µg/well).

3.4.1. Antifungal activity of methanolic extracts of *W. somnifera* leaves, fruits and roots against pathogen of basal rot disease

The effect of 0.5–3.5% concentrations of the fruit extract was insignificant on fungal growth. The lower concentrations of 0.5–2.5% insignificantly reduced the fungal biomass by 6–15% while the higher concentrations stimulated the fungal growth. The highest concentration of 4% significantly enhanced fungal biomass by 50% over control. In general, there was a gradual increase in fungal biomass with the increase in concentration of the extract (**Figure 16**). The effect of lower concentrations of 0.5 to 2.0% of leaves extract was inhibitory and significant where a decrease of 38–50% in fungal biomass was recorded. In contrast, the effect of higher concentrations of 2.5 to 4.0% was stimulatory and insignificant where generally fungal biomass was increased gradually with the increase of extract concentration from 0.5% to 4% (**Fig. 16B**). Root extract played a very significant role in decreasing fungal growth as compared to control. The fungal biomass generally decreased as the concentration of extract increased from 0.5% to 4.0%. The effect of lower concentrations of 0.5 to 2.0% was insignificant where only 6 to 38% reduction in biomass of the fungus was noted. Conversely, the higher concentrations of 2.5 to 4.0% exhibited pronounced inhibitory effect on the fungal growth and significantly reduced the fungal biomass by 44 to 93% (**Fig. 16C**).

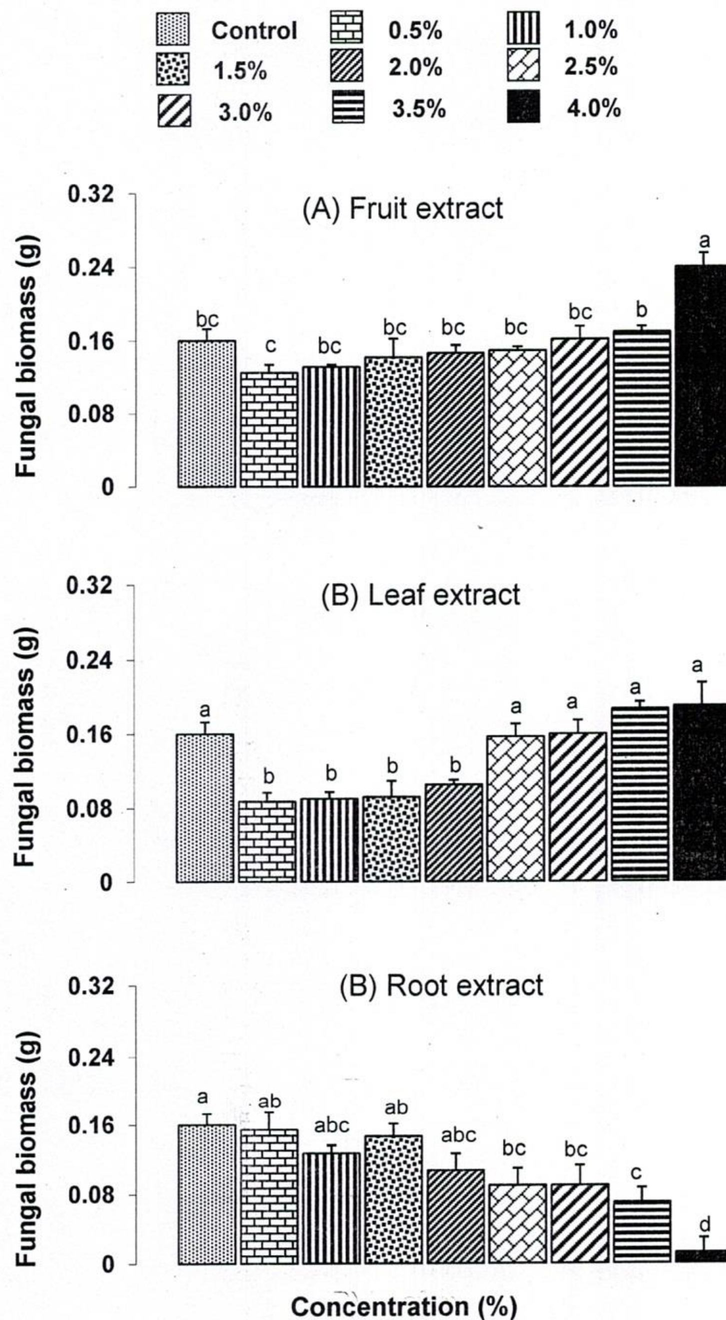


Figure 3.16: Effect of different concentrations of methanolic extract of *W. somnifera* roots, fruits and leaves on biomass of *Fusarium oxysporum* f.sp. cepae. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$).

CHAPTER IV

DISCUSSION

4. DISCUSSION

4.1. PHYTOCHEMICAL SCREENING OF *W. SOMNIFERA*

Preliminary phytochemical analysis of *W. somnifera* methanol extract showed the presence of carbohydrate, glycosides, alkaloids, phytosterols, fixed oils, phenolic compounds and flavonoids. The findings of the phytochemical screening of different plant parts of *W. somnifera* are in agreement with the studies of Santhi and Swaminathan (2011). Glycosides are known to work by inhibiting the Na^{+}/K^{+} pump. This causes an increase the level of sodium and calcium ion. This inhibition increases the amount of Ca^{2+} ions available for concentration of the heart muscle which improves cardiac output and reduces distention of heart; thus are used in the treatment of congestive heart failure and cardiac arrhythmia (Schneider and Wolfling 2004).

Flavonoids have been referred to as nature's biological response modifiers because of strong experimental evidence of their inherent ability to modify the body's reaction to allergen, virus and carcinogens. They show anti-allergic, anti-inflammatory, antimicrobial and anti-cancer activity [http://en.wikipedia.org/wiki/ flavonoids]. Flavonoids are phenolic compounds that are a major group of compounds act as primary antioxidants or free radical scavengers (Polterait 1997). Plant steroids are known to be important for their cardio tonic activities, possess insecticidal and antimicrobial properties. They are also used in nutrition, herbal medicine and cosmetics (Callow 1936).

Plant derived natural products such as phenolic compounds, flavonoids, terpenoids and steroids etc have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and antitumor activity (De Feudis *et al.* 2003; Takeoka and Dao 2003).

Phenolic phytochemicals have antioxidative, antidiabetic, anticarcinogenic, antimicrobial, antiallergic, antimutagenic and anti-inflammatory (Arts and Hollman 2005; Scalbert *et al.* 2005).

4.2. PHENOLIC COMPOUNDS, ASCORBIC ACIDS AND ANTHOCYANIN CONTENT OF *W. SOMNIFERA*

There are many reports on the analysis of alkaloids in *W. somnifera* e.g. withanolide, withaferin etc. but there is no available data on the analysis of phenolic compounds, ascorbic acids and anthocyanin content and antioxidant properties of *W. somnifera.* To our knowledge, this study is the first to identify phenolic compounds present in *W. somnifera*. The present study confirmed the presence of phenolic compounds, flavonoids and antioxidant activities in WSREt, WSFEt and WSLEt. High concentrations of phenolic compounds were found in the different parts of *W. somnifera* with significant variations in the amount. **Table 3.4** showed high content of phenolics and flavonoids in WSLEt (32.58 ± 3.16 and 31.58 ± 5.07 respectively) while that in WSREt was low (17.80 ± 5.80 and 15.49 ± 1.02 respectively). This indicates that the leaves of *W. somnifera* should be consumed for its antioxidant effects. Several reports have shown that different plant parts have variable polyphenol compositions, as shown by our findings (Udayakumar *et al.* 2010).

Udayakumar *et al.* (2010) reported that the presence of total phenolic compounds in WSREt was 28.26 mg/g while that of flavonoids was 17.32 mg/g. For WSLEt it was 5.4 mg/g total phenolic compounds and 5.1 mg/g flavonoids both of which were different from the present study perhaps due to the different source of *W. somnifera* and polyphenols of plant parts which may also be related to the colour, maturity and environment. However, the spectrophotometric method tend to overestimate the phenolics content with respect to the chromatographic method perhaps due to the fact that non-phenolic materials present in the investigated extracts interfered in the spectrophotometric analysis (Escarpa and González 2001).

"Polyphenols are a kind of chemical that may protect against some common health problems and possibly certain effects of aging," Dalia Akramiene, a physiologist at Kaunas University of Medicine in Lithuania, told Life Extension. "Polyphenols protect cells and body chemicals against damage caused by free radicals—reactive atoms that contribute to tissue damage in the body.

In a Finnish study of 1,380 middle-aged men, high intake of flavonoids, or polyphenols, was linked to healthier carotid arteries with less obstruction from atherosclerosis (Mursu et al. 2007). A large French study showed that people over 65 years of age who consumed a flavonoid-rich diet had less cognitive decline over a 10-year period (Letenneur et al. 2007). Polyphenols from different plants may work synergistically when consumed together, with benefits from the combination equaling more than the sum of the parts.

The 1st International Conference on Polyphenols and Health, held in 2005, reviewed impressive evidence strongly supporting a role for polyphenols in preventing degenerative diseases, especially cardiovascular disorders and cancer (Scalbert et al. 2005). Polyphenols are the most abundant antioxidants in the diet, and they promote health by a variety of mechanisms.

"Polyphenols can also block the action of enzymes that cancers need for growth and they can deactivate substances that promote the growth of cancers," Dr. Akramiene said "Increased consumption of polyphenols has been associated with a reduced risk of cardiovascular disease and possibly cancer and stroke."

The presence of Ascorbic acid in WSLEt, WSREt and WSFEt might be responsible for their therapeutic effects and uses in the traditional system of medicine (Trease and Evan 2005). As it is already discussed that the WSLEt, WSREt and WSFEt have ascorbic acid contents which support the conclusion of the works of Suner (1956) in which he concluded that the ascorbic acid is stable in solid form but oxidized in solution by dissolved oxygen.

The active parts of the *W. somnifera* used for the analysis of ascorbic acid contents showed variable amounts of the ascorbic acid, therefore the study carries important information beside other therapeutic effects of the analysed *W. somnifera*. This study also provides a scientific data base along with the reported constituents isolated from the *W. somnifera*.

Anthocyanins are one of the important phenolic substances in *W. somnifera* and belong to the flavonoids (Nielsen *et al.* 2003; Proteggente *et al.* 2002; Velioglu *et al.* 1998). The content of total anthocyanins in the WSLEt, WSREt and WSFEt (**Figure 3.2**) varies differently. This variation also observed in the phenolics, flavonoids and ascorbic acid contents of this study (**Table 3.4** and **Figure 3.1**). The content of total phenolics, flavonoids, ascorbic acids and anthocyanin were higher in WSLEt compared to WSREt and WSFEt which indicate higher antioxidant properties of *W. somnifera* leaves. The presence of all these parameters are the potential cause of antioxidant and antimicrobial properties of *W. somnifera*. The results also indicated that higher values of total phenolics, flavonoids, ascorbic acids and anthocyanin of WSLEt means the higher values of antioxidant properties.

In HPLC analysis, six compounds were identified in WSLEt while three were identified in WSFEt and two were identified in WSREt. Out of the eight phenolic compounds catechin was found in the highest concentration compared to others amounting 12.82 mg/g in WSREt, 19.48 in WSFEt and 28.38 mg/g in WSLEt (**Table 3.6**). This indicates that WSREt, WSFEt and WSLEt are rich sources of catechin. Catechin is one of the most important polyphenols that provide health benefits and is found in high quantities in green tea which is widely known for its strong antioxidant properties. There are many reports on catechin which described its therapeutic role in human health. Modern studies have found that catechin is responsible for antioxidant activity, anti-ageing properties and cardiac health maintenance (Lambert and Yang 2003). Catechins' beneficial effects are attributed to its ability to reduce

oxidative stress, lipid peroxidation, free radical generation and unhealthy low density lipoprotein (LDL) cholesterol-oxidation (Coimbra *et al.* 2006). There is also an evidence that suggests that catechins have a role in the protection against degenerative disorders (Cooper *et al.* 2005). Throughout the experiments, some catechins have also been shown to inhibit a key enzyme (squalene epoxidase) in the pathway of cholesterol biosynthesis (Abe *et al.* 2001). The potent antioxidant properties of catechin reduce free radical damage to cells and prevent the oxidation of LDL cholesterol (Cooper *et al.* 2005). Besides catechin, other phenolic compounds found in the WSREt, WSFEt and WSLEt may also contribute to its medicinal and antioxidant properties (Khalil *et al.* 2010). Further studies to isolate individual active principles and antioxidant activity of individual extracts of roots, fruits as well as leaves through radical scavenging assay and their pharmacological validation in terms of modern medicine will be of great pharmacological importance in future which is under our consideration.

4.3. ANTIOXIDANT PROPERTIES OF *W. SOMNIFERA*

In living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lysis (Halliwell and Gutteridge, 1998). Many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines (Yazdanparast and Ardestani, 2007; Yazdanparast *et al.* 2008). Recently, many natural antioxidants have been isolated from different plant materials (Jovanovic and Simic 2000; Packer and Ong 1997).

In the present study, we have demonstrated the antioxidant and radical scavenging mechanism of *W. somnifera* by using different *in vitro* bioanalytical methodologies. As reported in many studies, the activities of natural antioxidants in influencing diseases are closely related to their ability

to reduce DNA damage, mutagenesis, carcinogenesis and inhibition of pathogenic bacterial growth (Roginsky and Lissi 2005). Antioxidant capacity is widely used as a parameter for medicinal bioactive components. In this study, the antioxidant and radical scavenging activities of *W. somnifera* were compared to those of one or more references compounds like BHA, BHT, EDTA, TBHQ and ascorbic acid. These comparisons were made using a series *of in vitro* tests including DPPH free radical scavenging, FRAP, reducing power assay, metal chelating on ferrous ions (Fe^{3+}) activities and Inhibition of β-carotene bleaching.

Various antioxidant assay methods have been developed for food and biological samples. Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon that can lead to various pathological consequences (Hochstein and Atallah 1988). The resulting lipid hydroperoxides can affect membrane fluidity and the function of membrane proteins. In addition, lipid hydroperoxides can undergo iron-mediated, one-electron reduction and oxygenation to form epoxyallylic peroxyl radicals which trigger a chain reaction of free radical-mediated lipid peroxidation.

The end-products of lipid peroxidation are reactive aldehydes, such as 4-hydroxyl nonenal and malondialdehyde, many of which are highly toxic to cells (Yu and Yang 1996). In addition, reactive aldehydes generated by lipid peroxidation can attack other cellular targets, such as proteins and DNA; thereby propagate the initial damage in cellular membranes to other macromolecules. Because lipid hydroperoxides formed in membranes are important components of ROS generation *in vivo*. Their detoxification appears to be critical for the survival of an organism in oxidative stress (Dargel 1992). Therefore, antioxidants play a vital role in inhibition of lipid peroxidation or in protection against cellular damage by free radicals.

The scavenging ability of DPPH free radical is extensively used to screen the antioxidant potential of naturally-derived foods and plants. Methanol was

employed in this study to extract the low molecular weight and moderately polar substances because of its wide solubility properties. We found that WSREt, WSFEt and WSLEt exhibited free radical DPPH scavenging abilities (**Table 3.4** and **Figure 3.3**).

The correlations among the phenolic compounds, flavonoids and DPPH radical scavenging activities are shown in (**Table 3.5**). The correlation matrix showed that significant linear correlation exists between the results of all three analytical methods employed indicating that the three measurements are reliable indicators of antioxidant activities. The lowest linear correlation value at $r = 0.962$ ($p = 0.01$) and the highest correlation value at $r = 0.995$. Both phenolic compounds and DPPH radical scavenging activity are strongly correlated ($r = 0.995$ and 0.983 respectively) with DPPH radical scavenging activities. The significant correlations existing between phenolic compounds and DPPH radical scavenging activities indicate the strong antioxidant properties of the tested WSREt, WSFEt and WSLEt. Similar to our findings, some literature also reported strong correlation between the antioxidant capacity and total phenolic contents (Piljac-Žegarac *et al.* 2009) further suggesting that polyphenols are the major components responsible for the antioxidant effects of WSREt, WSFEt and WSLEt.

The values in FRAP assay varied from (1130.18 µM Fe/kg) to 1643.04 µM Fe/kg) (**Figure 3.2**). A relatively higher value of WSLEt indicated more reduction of ferric ions to ferrous ions. This high values of FRAP assay indicate the strong antioxidant properties of *W. somnifera*.

Iron can stimulate lipid peroxidation by the Fenton reaction ($H_2O_2 + Fe^{2+} = Fe^{3+} + OH^- + OH.$) and can also accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction (Halliwell 1991). Metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation (Duh *et al.* 1999).

According to the results of ferrous chelation, the WSREt, WSFEt and WSLEt are not as good as the standard EDTA (**Figure 3.5**); but the decrease in concentration dependent color formation in the presence of the extract indicates that it has iron chelating activity.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging (Yildirim, *et al.* 2000). On the other hand, as shown in **Figure 3.6**, the reducing power of the WSREt, WSFEt and WSLEt were compared with the standard BHT and found to be potential. Reducing power reflects the electron donating capacity of bioactive compounds and is associated with antioxidant activity. Antioxidants can be reductants and inactivate oxidants. The reducing capacity of a compound can be measured by the direct reduction of $Fe[(CN)_6]^3$ to $Fe[(CN)_6]^2$. Addition of free Fe^{3+} to the reduced product leads to the formation of the intense Perl's Prussian blue complex, $Fe_4[Fe(CN)_6]_3$, which has a strong absorbance at 700 nm. An increase in absorbance of the reaction mixture would indicate an increase in the reducing capacity due to an increase in the formation of the complex. There are a number of assays designed to measure overall antioxidant activity, or reducing potential, as an indication of a host's total capacity to with stand free radical stress. The ferric ion reducing antioxidant power assay takes advantage of an electron transfer reaction in which a ferric salt is used as an oxidant (Gulcin 2009). In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Gulcin 2010).

In this study, we attempted to isolate the active compounds responsible for antioxidant activities in WSREt, WSFEt and WSLEt. The antioxidant capacity

has been shown to be directly related with the total phenolic content (**Table 3.5**) which was in agreement with many previous reports (Azlim Almey *et al.* 2010; Liu *et al.* 2009; Shukla *et al.* 2009). In addition, the flavonoids contributed to almost all of the total phenolic content (**Table 3.4**), which indicated that the flavonoids in *W. somnifera* are important constituents responsible for the bioactivities. Phenolic acids are a major class of phenolic compounds, widely occurring in the plant. If there is an electron donation group, especially a hydroxyl group loaded on o- or p-positions of the phenolic compounds, it makes the compound polar and therefore antioxidant activities and metal chelating ability are increased (Duan *et al.* 1998; Weng 1993).

The results from these in vitro experiments, DPPH radical scavenging, FRAP, reducing power method, beta carotene bleaching, total polyphenol content, total flavonoid content, ascorbic acid content and anthocyanin content demonstrated that phytochemicals in *W. somnifera* plants might have significant effects on antioxidant activities. However, the quantity of polyphenols and flavonoids found in the *W. somnifera* roots, fruits and leaves extracts were not directly related to their antioxidant activities. The additive roles of phytochemicals might contribute significantly to the potent antioxidant activity. Hence, *W. somnifera* plants could be used as an easy accessible source of natural antioxidants in pharmaceutical and medical industries. For this reason, further work should be performed to isolate and identify the antioxidative components of *W. somnifera* medicinal plants.

Antioxidant of *W. somnifera* roots, fruits and leaves extract were investigated using a free radical-induced plasmid pBR322 DNA breaks system *in vitro*. With the attack of $^{\cdot}OH$ generated from the UV induced H_2O_2 supercoiled plasmid DNA was broken into three forms, including supercoiled (Sc), open circular (Oc) and linear form (Lin). Effects of *W. somnifera* roots, fruits and leaves extract on the hydroxyl radical-induced DNA damage was investigated as shown in **Figure 8**. All the treated lane (6-17) showed less dense Oc DNA

band compared to the Oc DNA band of UV induced H_2O_2 DNA damage (lane 4). There is no Sc DNA band in the UV induced H_2O_2 DNA damage (lane 4) while faint bands of Sc DNA was found in the *W. somnifera* roots, fruits and leaves extract treated lane (6-17) indicating DNA damage protective activities of used extracts. Although both $O_2^{\cdot -}$ and H_2O_2 are potentially cytotoxic, most of the oxidative damage in biological systems is caused by the $^{\cdot}OH$, which is generated by the reaction between $O_2^{\cdot -}$ and H_2O_2 in the presence of metal ions (Guitteridge, 1984). This damage can be reduced in the presence of standard flavonoid antioxidant, quercetin (100 μM) (lane 5) (Russo et al., 2000). DNA damage protective activity of *W. somnifera* roots, fruits and leaves extract is corresponding to its antioxidant potential.

Catechin isolated from *W. somnifera* showed high chelating activity for ferrous iron (Ruch *et al.* 1989). Our HPLC data (**Table 3.6**) confirmed that the good antioxidant and free radical scavenging activity of *W. somnifera* could be related to its major phenolic and flavonoid compounds such as gallic, syringic, benzoic, p-coumaric, vanillic acids, flavonoids catechin, kaempferol and naringenin. Regarding the high levels of phenolics and flavonoids in *W. somnifera* extract, it is possible that the antioxidant potential as well as modulatory effect on defences factors is due to its polyphenolic content. Our phytochemical investigations using HPLC may be confirm the mechanism of *W. somnifera* against oxidative cell injury.

4.4. ANTIMICROBIAL PROPERTIES

Since multidrug resistance of microorganisms is a major medical concern, screening of natural products in a search for new antimicrobial agents that would be active against these microorganisms is the need of the hour (Zgoda and Porter 2001). Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay (Singh and Kumar 2011; Tona *et al.* 1998). Many reports are available on the antiviral, antibacterial,

antifungal, anthelmintic, antimolluscal and anti-inflammatory properties of plants (Behera and Misra 2005; Bylka *et al.* 2004; Govindarajan *et al.* 2006; Kumaraswamy *et al.* 2002; Palombo and Semple 2001; Samy and Ignacimuthu 2000; Stepanovic *et al.* 2003). Some of these observations have helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings. In the present study, the 80% methanolic extract of roots, fruits and leaves of *W. somnifera* showed the activity against five pathogenic bacteria viz., *E. coli*, *Salmonella, Citrobacter, Pseudomonas* and *Klebsiella* at different extent.

Results of the antibacterial activities of leaves of *W. somnifera* showed more efficacy (**Table 3.7** and **Table 3.8**) compare to roots and fruits. In our biochemical analysis, the leaves have higher values of total phenol, flavonoids contents and antioxidant properties (**Table 3.4**) compare to roots and fruits. This result indicated that there is a correlation polyphenols content and antibacterial activity. Apart from antimicrobial activities, this plant extracts are also exploited for therapeutic purpose to cure several disorders (Afolayan *et al.* 2002).

The most susceptible organism detected was the *S. typhi.* All other tested bacteria also showed remarkable sensitivity against the WSREt, WSFEt and WSLEt. This result demonstrating that methanolic extracts contain the expected compounds for antibacterial activities.

There are some reports of methanolic extract of *W. somnifera* against different pathogenic bacteria and they have found significant antibacterial properties (Babayi *et al.* 2004; Farah 2010; Jain and Varshney 2011). The encouraging results indicate that the methanolic WSREt, WSFEt and WSLEt might be exploited as a natural drug for the treatment of several infectious diseases caused by these organisms and could be useful in understanding the relations between traditional cures and current medications.

The antifungal screening showed that methanolic extract of *W. somnifera* roots, fruits and leaves was active against *Fusarium oxysporum* and increasing concentration of the extract showed higher inhibitory effect of the fungus and *W. somnifera* roots extract have showed the highest activity compared to fruits and leaves (**Figure 15**).

Earlier, Dhuley (1998) reported the antifungal activity of *W. somnifera* root against *Aspergillus fumigates*. Recently Javaid and Munir (2012) reported that methanolic stem and fruit extracts of *W. somnifera* significantly declined growth of *Ascochyta rabiei* while methanolic root extract was not effective against this chickpea blight pathogen. It indicates that methanolic extracts of different parts of *W. somnifera* have specificity in their antifungal activity against different phytopathogens. Thus, there is a possibility of developing this plant a source of antifungal agent and further investigations are necessary to identify the bioactive principles.

4.5. CONCLUSION

On the basis of the results obtained in the present study, it is concluded that 80% methanolic extract of *W. somnifera* root, fruit, and leaf provide potential benefits for human health because of its high content of secondary metabolites especially phenolic compounds, flavonoids, ascorbic acids and anthocyanain. This study identified five phenolics (gallic, syringic, benzoic, p-coumaric and vanillic acids) and three flavonoids (catechin, kaempferol, and naringenin) in WSREt, WSFEt and WSLEt and catechin was found in high concentration compared to others which has promising medicinal and pharmacological value.

WSREt, WSFEt and WSLEt, which contains large amounts of flavonoids and phenolic compounds, exhibits high antioxidant and free radical scavenging activities. It also chelates iron and has reducing power. Methanol extracts of WSREt, WSFEt and WSLEt showed also high DPPH scavenging activity. Among the three plants parts of *W. somnifera* leaves showed the lowest IC_{50} of DPPH radical scavenging activities which indicate the highest radical scavenging activities of leaves. As known, there is a significant linear correlation between phenolic concentration and free radical scavenging activity, especially on DPPH radical.

Ferrous reducing antioxidant power (FRAP) assay estimated high values of total antioxidant content in WSREt, WSFEt and WSLEt while the highest concentration was found in leaves extract which is also promising antioxidant potential of *W. somnifera*.

Similarly, ferrous chelation, reducing power assay, Inhibition of β-carotene bleaching, DNA damage protective activities of WSREt, WSFEt and WSLEt have showed strong antioxidant properties.

The addition of *W. somnifera* extract to the reaction mixture of H_2O_2 induced the protection to the damage of native supercoiled circular DNA. This DNA damage protective activity of *W. somnifera* extract is corresponding to its antioxidant potential.

These *in vitro* assays indicate that *W. somnifera* extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

Susceptibility test of WSREt, WSFEt and WSLEt showed promising antibacterial activity against five pathogenic bacteria *E. coli*, *Salmonella, Citrobacter, Pseudomonas* and *Klebsiella* and WSLEt showed the highest growth inhibition activity *S. typhi.* WSREt, WSFEt and WSLEt also showed antifungal activities against *Fusarium oxysporum* at different extent. This finding indicates that methanolic extracts of WSREt, WSFEt and WSLEt contain the targeted compounds for antibacterial as well as antifungal activities. Thus our antimicrobial screening results also justify the traditional uses of *W. somnifera* in various ailments including infectious diseases.

CHAPTER V

REFERENCES

5. REFERENCES

Abe I, Kashiwagi Y, Noguchi H, Tanaka T, Ikeshiro Y, Kashiwada Y (2001) Ellagitannins and hexahydroxydiphenoyl esters as inhibitor of vertebrate squalene epoxidase. Journal of Natural Product 64: 1010-1014.

Afolayan A J, Grierson D S, Kambizi L, Madamombe I, Masika P J (2002) *In vitro* antifungal activity of some South African mVVedicinal plants. South African Journal of Botany 68: 72-76.

Alfonso D, Bernardinelli G, Kapetanidis I (1993) Withanolides from *lochroma coccineum*. Phytochemistry 34: 517-521.

Anbalagan K, Sadique J (1981) Influence of an Indian medicine (Ashwagandha) on acutephase reactants in inflammation. Indian Journal of Experimental Biology 19: 245-249.

Anbalagan K, Sadique J (1984) Role of prostaglandins in acute phase proteins in inflammation. Biochemical Medicine 31: 236-245.

Andallu B, Radhika B (2000) Hypoglycemic, diuretic and hypocholesterolemic effect of winter cherry. (*Withania somnifera*) root. Indian Journal of Experimental Biology 38: 607-609.

Anonymous (2004) Monograph: *Withania somnifera*. Alternative Medicine Reviews 9: 211-214.

Archana R, Namasivayam A (1999) Antistressor effect of *Withania somnifera*. Journal of Ethnopharmacology 64(1): 91-93.

Arora S, Dhillon S, Rani G, Nagpal A (2004) The in vitro antibacterial/synergistic activities of *Withania somnifera* extracts. Fitoterapia 75(3-4): 385-388.

Arseculeratne S N, Gunatilaka A A, Panabokke R G (1985) Studies of medicinal plants of Sri Lanka. Part 14: Toxicity of some traditional medicinal herbs. Journal of Ethnopharmacology 13: 323-335.

Arts I C, Hollman P C (2005) Polyhenols are disease risk in epidemiological studies. American Journal of Clinical Nutrition 81: 317-325.

Azlim Almey A A, Ahmed Jalal Khan C, Syed Zahir I, Mustapha Suleiman K, Aisyah M R, Kamarul Rahim K (2010) Total phenolic content and primary antioxidant activity of methanolic and ethanolic extracts of aromatic plants' leafs. International Food Research Journal 17: 1077-1084.

Babayi H, Kolo I, Okogun J I, Ijah U J J (2004) Antimicrobial Activity of Eucalyptus major and Eucalyptus baileyana Methanolic Extracts. Biokemistri 16: 106-111.

Bargagna-Mohan P, Ravindranath P P, Mohan R (2006) Small molecule anti-angiogenic probes of the ubuquitin proteasome pathway: potential applications to choroidal neovascularization. Investigative Ophthalmology and Visual Science 47: 4138-4145.

Begum V H, Sadique J (1987) Effect of *Withania somnifera* on glycosaminoglycan synthesis in carrageenin-induced air pouch granuloma. Biochemical Medicine and Metabolic Biology 38: 272-277.

Begum V H, Sadique J (1988) Long-term effect of herbal drug *Withania somnifera* on adjuvant-induced arthritis in rats. Indian Journal of Experimental Biology 26: 877-882.

Behera S K, Misra M K (2005) Indigenous phytotherapy for genito-urinary diseases used by the Kandha tribe of Orissa, India. Journal of Ethnopharmacology 102: 319-325.

Benzie I F F, Strain J J (1996) The ferric reducing ability of plasma as a measure of antioxidant power: the FRAP assay. Analytical Biochemistry 239: 70-76.

Benzie I F F, Strain J J (1999) Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods in Enzymology 299: 15–27.

Bhatnagar M, Jain C P, Sisodia S S (2005) Anti-ulcer activity of *withania somnifera* in stress plus pyloric ligation induced gastric ulcer in rats. Journal of Cell and Tissue Research 5(1): 287-292.

Bhattacharya A, Ghosal S, Bhattacharya S K (2001) Anti-oxidant effect of *Withania somnifera* glycowithanolides in chronic footshock stress-induced perturbations of oxidative free radical scavenging enzymes and lipid peroxidation in rat frontal cortex and striatum. Journal of Ethnopharmacology 74: 1-6.

Bhattacharya S, Goel R, Kaur R, Ghosal S (1987) Anti-stress Activity of Sitoindosides VII and VIII, New Acylsterylglucosides from *Withania Somnifera*. Phytotherapy Research 1: 32-39.

Bhattacharya S K, Bhattacharya A, Sairam K, . Ghosal S (2000) Anxiolytic-antidepressant activity of *Withania somnifera* glycowithanolides: an experimental study. Phytomedicine 7(6): 463-469.

Bhattacharya S K, Muruganandam A V (2003) Adaptogenic activity of *Withania somnifera*: an experimental study using a rat model of chronic stress. Pharmacol Biochem Behav. 75(3): 547-555.

Biopharma A V I (2007) Antisense antibacterial method and compound. World Intellectual Property Organization Retrieved 12 January 2012.

Bogdan C (2001) Nitric oxide and the immune response. Nature Immunology 2: 907-916.

Bone K (1996) Clinical Applications of Ayuvedic and Chinese Herbs. Phytotherapy Press: 137-141.

Bonjar GH (2004) Inhibition of Clotrimazole-resistant Candida albicans by plants used in Iranian folkloric medicine. Fitoterapia 75: 74-76.

Bylka W, Szaufer-Hajdrych M, Matalawskan I, Goslinka O (2004) Antimicrobial activity of isocytisoside and extracts of *Aquilegia vulgaris* L. Letters in Applied Microbiology 39: 93-97.

Callow R K (1936) Steroids. Proc. Royal, Soc London Series 157: 194.

Cao L, Si JY, Liu Y, Sun H, Jin W, Li Z, Zhao XH, Pan RL (2009) Essential oil composition, antimicrobial and antioxidant properties of Mosla chinensis Maxim. Food Chem 115: 801-805.

Cheesbrough M (2000) District laboratory practice in tropical countries. E.C.B.S. Cambridge University Press edition 2: 256-267.

Coimbra S, Castro E, Rocha-Pereira P, Rebelo I, Rocha S, Santos-Silva A (2006) The effect of green tea in oxidative stress. Clinical Nutrition 25: 790-796.

Cooper R, Morre D J, Morre D M (2005) Medicinal benefits of green tea: part I. Review of noncancer health benefits. Journal of Alternative and Complementery Medicine 11: 521-528.

Cooper R, Morre D J, Morre D M (2005) Medicinal benefits of green tea: part II. Review of anticancer properties. Journal of Alternative and Complementery Medicine 11: 639-652.

Dargel R (1992) Lipid peroxidation - a common pathogenetic mechanism? Experimental and Toxicologic Pathology 44: 169-181.

De Feudis F V, Padadopoulos V, Drieu K (2003) Gingo biloba extracts and cancer: a research area in its infancy. Funda Clin.Pharmacol 17: 405-417.

Dhuley J N (1998) Effect of Ashwagandha on Lipid Peroxidation in Stress-Induced Animals. Journal of Ethnopharmacology 60(2): 173-178.

Dhuley J N (2000) Adaptogenic and cardioprotective action of ashwagandha in rats and frogs. Journal of Ethnopharmacology 70(1): 57–63.

Dhuley J N (2001) Nootropic-like effect of Ashwagandha (*Withania somnifera*). Phytotherapy Research 15: 311-318.

Duan, Weng X C, Dong X W, Liu Y P, Li H P, Jin J R (1998) Antioxidant properties of butylated hydroxytoluene refluxed in ferric chloride solution. Food Chemistry 61: 101-105.

Duh P D, Tu Y Y, Yen G C (1999) Antioxidant activity of water extract of Harng Jyur (*Chrysenthemum morifolium* Ramat). Lebnes wiss Technol 32: 269-277.

Elsakka M, Grigorescu E, Stanescu U (1990) New data referring to chemistry of *Withania somnifera* species. Revista Medico-Chirurgicala a Societatii de Medici Si Naturalisti Din Iasi 94: 385-387.

Escarpa A, González M C (2001) Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods Analytica Chimica Acta 427(1): 119-127.

Farah Z J (2010) In vitro antimicrobial and phytochemical activity of *Operculina turpethum* L. Ethnopharmocology 67: 27-35.

Ferreira I C F R, Aires E, Barreira J C M, Estevinho L M (2009a) Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. Food Chemistry 114: 1438-1443.

Ferreira I C F R, Aires E, Barreira J C M, Estevinho L M (2009b) Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. Food Chemistry 114: 1438-1443.

Freiz B (1994) Antioxidant and antimicrobial analysis of some medicinal plants. American Journal of Medicine. 97: 58.

Girish K S, Ushanandini S, Harish Kumar K, Nagaraju S, Govindappa M, Vedavathi M, Kemparaju K (2006) Antimicrobial properties of a non-toxic glycoprotein (WSG) from *Withania somnifera* (Ashwagandha). Journal of Basic Microbiology 46(5): 365-374.

Glotter E (1991) Withanolides and related ergostane-type steroids. Natural Product Reports 8: 415-440.

Govindarajan R, Vijayakumar M, Singh M, Rao C H V, Shirwaikar A, Rawat A K S, Pushpangadan P (2006) Antiulcer and antimicrobial activity of *Anogeissus latifolia*. Journal of Ethnopharmacology 106: 57-61.

Grandhi A (1994) Comparative pharmacological investigation of ashwagandha and ginseng. Journal of Ethnopharmacology (Ireland) 3: 131-135.

Gulcin I (2006) Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology 217: 213-220.

Gulcin I (2009) Antioxidant activity of L-Adrenaline: An activity-structure insight. Chemical and Biological Interaction 179: 71-80.

Gulcin I (2010) Antioxidant properties of resveratrol: a structure-activity insight. Innovative Food Science & Emerging Technologies 11: 210-218.

Gulcin I, Elias R, Gepdiremen A, Chea A, Topal F (2010) Antioxidant activity of bisbenzylisoquinoline alkaloids from Stephania rotunda: cepharanthine and fangchinoline. Journal of Enzyme Inhibition and Medicinal Chemistry 25: 44-53.

Gupta A P, Verma R K, Misra H O, Gupta M M (1996) Quantitative determination of withaferin A in different plant parts of *Withania somnifera* by TLC densitometry. Journal of Medicinal and Aromatic Plant Sciences 18: 788-790.

Gupta G L, Rana A C (2007) *Withania somnifera* (Ashwagandha): A Review. Pharmacognosy Review 1: 129-136.

Gupta S K, Dua A, Vohra B P (2003) *W. somnifera* (Ashwagandha) attenuates antioxidant defense in aged spinal cord and inhibits copper induced lipid peroxidation and protein oxidative modifications. Drug Metabol Drug Interaction 19(3): 211-222.

Gupta S K, Mohanty I, Talwar K K, Dinda A, Joshi S, Bansal P, Saxena A K, Arya D S (2004) Cardioprotection from ischemia and reperfusion injury by *Withania somnifera*: a hemodynamic, biochemical and histopathological assessment. Molecular and Cellular Biochemistry 260: 39-47.

Halliwell B (1991) Reactive oxygen species in living systems: source, biochemistry, and role in human disease. American Journal of Medicine 91(3C): 14S-22S.

Halliwell B, Gutteridge J M (1998) Free radicals in biology and medicine. Oxford: Oxford University Press.

Harborne J B (1973) Phytochemical methods. A Guide to Modern Techniques of plant analysis, Chapman and Hall, London.

Harikrishnan B, Subramanian P, Subash S (2008) Effect of *Withania Somnifera* Root Powder on the Levels of Circulatory Lipid Peroxidation and Liver Marker Enzymes in Chronic Hyperammonemia. E-Journal of Chemistry 5: 872-877.

Haro-Vicente J F, Martinez-Gracia C, Ros G (2006) Optimization of in vitro measurement of available iron from different fortificants in citric fruit juices. Food Chemistry 98: 639-648.

Hatano T, Kagawa H, Yashura T, Okuda T (1988) Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects. Chemical & Pharmaceutical Bulletin 36: 2090-2097.

Hemalatha S, Wahi A K, Singh P N, Chansouria J P (2006) Hypolipidemic activity of aqueous extract of *Withania coagulans* Dunal in albino rats. Phytotherapy Research 20: 614-617.

Hochstein P, Atallah A S (1988) The nature of oxidants and antioxidant systems in the inhibition of mutation and cancer. Mutation Research 202: 363-375.

Ibekwe V I, Nnanyere N F, Akujobi C O (2001) Studies on Antibacterial activity and phytochemical qualities of various plant extracts. Int. J. Environ., Health and Human Development 2(1): 41-46.

Ichikawa H, Takada Y, Shishodia S, Jayaprakasam B, Nair M G, Aggarwal B B (2006) Withanolides potentiate apoptosis, inhibit invasion, and abolish osteoclastogenesis through suppression of nuclear factor-kappaB (NF-kappaB) activation and NF-kappaB-regulated gene expression. Molecular Cancer Therapy 5(6): 1434-1445.

Itah A Y, Essien J P (2005) Growth Profile and Hydrocarbonoclastic Potential of Microorganisms Isolated from Tarballs in the Bight of Bonny, Nigeria. World Journal of Microbiology and Biotechnology 21(6-7): 1317-1322.

Iuvone T, Esposito G, Capasso F, Izzo A (2003) Induction of nitric oxide synthase expression by *Withania somnifera* in macrophages. Life Science 72: 1617-1625.

Iwalokun BA, Ogunledun A, Ogbolu O, Bamiro BS, Omojola J (2004) In Vitro antimicrobial properties of aqueous garlic extract against multidrug-resistant bacteria and Candida species from Nigeria. J. Med. Food 7: 327-333.

Jain P, Varshney R (2011) Antimicrobial activity of aqueous and methanolic extracts of *Withania somnifera* (Ashwagandha). Journal of Chemical and Pharmaceutical Research 3(3): 260-263.

Jaiswal D, Rai P K, Watal G (2010) Hypoglycemic and antidiabetic effects of *withania coagulans* fruit ethanolic extract in normal and streptozotocin-induced diabetic rats. Journal of Food Biochemistry 34(4): 764-778.

Jaleel C A, Lakshmanan G M A, Gomathinayagam M, Panneerselvam R (2008) Triadimefon induced salt stress tolerance in *W. somnifera* and its relationship to antioxidant defense system. South African Journal of Botany 74(1): 126-132.

Javaid A, Munir R (2012) Bioassay guided fractionation of Withania somnifera for the management of chickpea blight pathogen Ascochyta rabiei. Int J Agric Biol 14: 797–800.

Jayaprakasam B, Zhang Y, Seeram N, Nair M (2003) Growth inhibition of tumor cell lines by withanolides from *Withania somnifera* leaves. Life Science 74: 125-132.

Johri S, Jamwal U, Rasool S, Kumar A, Verma V, Qazi G N (2005) Purification and characterization of peroxidases from *Withania somnifera* (AGB 002) and their ability to oxidize IAA. Plant Science 169: 1014-1021.

Jovanovic S V, Simic M G (2000) Antioxidants in nutrition. Ann NY AcadSci 899: 326-334.

Kahkonen M P, Hopia A I, Vuorela H J, P R J, Pihlaja K, Kujala T S, Heinonen M (1999) Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. Journal of Agricultural and Food Chemistry 47: 3954-3962.

Kaileh M, Vanden Berghe W, Heyerick A, H orion J, Piette J, Libert C, De Keukeleire D, Essawi T, Haegeman G (2007) Withaferin A strongly elicits IkappaB kinase beta hyper- phosphorylation concomitant with potent inhibition of its kinase activity. Journal of Biological Chemistry 282: 4253-4264.

Kaskoniene V, Maruska A, Kornysova O (2009) Quantitative and qualitative determination of phenolic compounds in honey. Cheminė Technologija 52(3): 74-80.

Kaur P, Mathur S, Sharma M, Tiwari M, Sdvastava K K, Chandra R (2001) A biologically active constituent of *W. somnifera* (ashwagandha) with antistress activity. Indian Journal of Clinical Biochemist 16(2): 195-198.

Kellner R, Mermett J M, Otto M, Widmer H M (1998) Analytical Chemistry: The approved text to the FECS curriculum: Analytical chemistry, Weinheim; Chichester: Wiley-VCH.

Khalil M I, Sulaiman S A, Boukraa L (2010) Antioxidant Properties of Honey and Its Role in Preventing Health Disorder. The Open Nutraceuticals Journal 3: 6-16.

Kinghorn A D, Su B-N, Jang D S, Chang LC, Lee D, Gu J-Q, Carcache-Blanco EJ, Pawlus AD, Lee SK, Park EJ, Cuendet M, Gills J J, Bhat K, Park H S, Mata-Greenwod E, Song L L, Jang M, Pezzuto J M (2004) Natural inhibitors of carcinogenesis. Planta Medica 70(8): 691-705.

Kulberg BJ (1997) Trends in immunotherapy of fungal infections. Eur. J. Clin. Microbiol. Infect. Dis. 16: 51-55.

Kulkarni S K, George B, Mathur R (1998) Protective effect of *Withania somnifera* root extract on electrographic activity in a lithiumpilocarpine model of status epilepticus. Phytotherapy Research 12: 451-453.

Kumaraswamy Y, Cox P J, Jaspars M, Nahar L, Sarker S D (2002) Screening seeds of Scottish plants for antibacterial activity. Microbiological Research 158: 353-357.

Kuttan G (1996) Use of *Withania somnifera* Dunal as an adjuvant during radiation therapy. Indian Journal of Experimental Biology 34: 854-856.

Lambert J D, Yang C S (2003) Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. Mutation Research 523-524: 201-208.

Letenneur L, Proust-Lima C, Le GA, Dartigues JF, Barberger-Gateau P (2007) Flavonoid intake and cognitive decline over a 10-year period. Am J Epidemiol. 165(12):1364-71.

Liu S C, Lin J T, Wang C K, Chen H Y, Yang D J (2009) Antioxidant properties of various solvent extracts from lychee (*Litchi chinenesis* Sonn.) flowers. Food Chemistry 114: 577–581.

Mahesh B, Satish S (2008) Antimicrobial activity of some important medicinal plant against plant and human pathogens. World Journal of Agricultural Sciences 4(S): 839-843.

Malhotra C L, Mehta V L, Prasad K, Das P K (1965) Studies on Withania ashwagandha, Kaul. IV. The effect of total alkaloids on the smooth muscles. Indian Journal of Physiology and Pharmacology 9: 9-15.

Mary N K, Babu B H, Padikkala J (2003) Antiatherogenic effect of Caps HT2,an herbal Ayurvedic medicine formulation. Phytomedicine 10: 474-482.

Mehta A K, Binkley P, Gandhi S S, Ticku M K (1991) Pharmacological effects of *Withania somnifera* root extract on GABAA receptor complex. Indian J Med Res. 94: 312-315.

Mi-Yae, Tae-Hun K, Nak-Ju S (2003) Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus* of *Hymenochaetaceae*) extracts. Food Chemistry 82: 593-597.

Mirjalili M H, Moyano E, Bonfill M, Cusido R M, Palazón J (2009) Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. Molecules and Cells 14: 2373-2393.

Miyaka Y, Yamamoto K, Tsujiihara N, Osawa T (1998) Protective effects of lemon flavonoids on oxidative stress in diabetes rats. Lipids 33: 689-697.

Mohanty I, Arya D S, Dinda A, Talwar K K, Joshi S, Gupta S K (2004) Mechanisms of cardioprotective effect of *Withania somnifera* in experimentally induced myocardial infarction. Basic Clin Pharmacol Toxicol. 94(4): 184-190.

Mohanty I R, Arya D S, Gupta S K (2008) *Withania somnifera* provides cardioprotection and attenuates ischemia-reperfusion induced apoptosis. Clinical Nutrition 27: 635-642.

Monica C (1984) Medical Laboratory Manual for Tropical Countries, Vol. 2. Microbiology. Butterworth and Co (Publishers) Ltd. Borough Green, Sevenoaks, Kent TN 158 PH. pp479.

Mursu J, Nurmi T, Tuomainen TP (2007) The intake of flavonoids and carotid atherosclerosis: the Kuopio Ischaemic Heart Disease Risk Factor Study. Br J Nutr. 98(4):814-8.

Nielsen I L F, Haren G R, Magnussen E L, Dragsted L O, Rasmussen S E (2003) Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography. Investigation of their pH stability and antioxidative potency. Journal of Agricultural and Food Chemistry 51: 5861-5866.

Owais M, Sharad K S, Shehbaz A, Saleemuddin M (2005) Antibacterial efficacy of *Withania somnifera* (ashwagandha) an indigenous medicinal plant against experimental murine salmonellosis. Phytomedicine 12(3): 229-235.

Oyaizu M (1986) Studies on product of browning reaction prepared from glucose amine. Japanese Journal of Nutrition 44: 307-315.

Packer L, Ong A S H (1997) Biological oxidants and antioxidants: Molecular mechanisms and health effects. Champaign, IL: AOCS Press.

Palombo E A, Semple S J (2001) Antibacterial activity of traditional. Australian medicinal plants. Journal of Ethnopharmacology 77: 151-157.

Panda S, Kar A (1998) Changes in thyroid hormone concentrations after administration of ashwagandha root extract to adult male mice. Journal of Pharmacy and Pharmacology 50: 1065-1068.

Panda S, Kar A (1999) *Withania somnifera* and Bauhinia pupurea in the regulation of circulating thyroid hormone concentrations in female mice. Journal of Ethnopharmacology 67: 233-239.

Perez C, Pauli M, Bazerque P (1990) An antibiotic assay by agar well diffusion method. Acta Biologica Medica Exp. 15: 113-115.

Perez C, Anesini C (1993) J. Ethnopharmacol. 44: 41-46.

Piljac-Zegarac J, Stipcevic T, Belscak A (2009) Antioxidant properties and phenolic content of different floral origin honeys. Journal of ApiProduct and ApiMedical Science 1(2): 43 - 50.

Prakash J, Gupta S K, Dinda A K (2002) *Withania somnifera* root extract prevents DMBA-induced quamous cell carcinoma of skin in Swiss albino mice. Nutrition and Cancer 42: 91-97.

Proteggente A R, Pannala A S, Paganga G, van Buren L, Wagner E, Wiseman S, van De Put F, Dacombe C, Rice-Evans C A (2002) The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. Free Radical Research 36: 217-233.

Rashid T, Ebringer A (2006) Ankylosing spondylitis is linked to *Klebsiella*-the evidence (Epub ahead of print). Clinical Rheumatology 26(6): 858-864.

Rastogi R P, Mehrotra B N (1998) Compendium of Indian Medicinal Plants. Central Drug Research Institute 6.

Ray A B, Gupta M (1994) Withasteroids, a growing group of naturally occurring steroidal lactones. Progress in the Chemistry of Organic Natural Products 63: 2-106.

Roginsky V, Lissi E A (2005) Review of methods to determine chain-breaking antioxidant activity in food. Food Chemistry 92: 235-254.

Ronald M A (1995) Micro-organisms in our World. Mosby Year Book, Inc. St. Louis. pp765.

Ruch R J, Cheng S J, Klaunji J E (1989) Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 10: 1003-1008.

Ryan K J, Ray C G (2004) Sherris Medical Microbiology (4th ed.). McGraw Hill. ISBN.

Samy R P, Ignacimuthu S (2000) Antibacterial activity of some folklore medicinal plants used by tribals in Western Ghats in India. Journal of Ethnopharmacology 69: 63-71.

Sangwan R S, Chaurasiya N D, Misra L N, Lal P, Uniyal G C, Sharma R, Sangwan N S, Suri K A, Qazi G N, Tuli R (2004) Phytochemical variability in commericial herbal products and preparations of *Withania somnifera* (ashwagandha). Current Science 86: 461-465.

Santhi M, Swaminathan C (2011) Evaluation of antibacterial activity and phytochemical analysis of leaves of *Withania somnifera* (L.) dunal. International Journal of Current Research 33(3): 010-012.

Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L (2005) Dietary polyphenols and the prevention of diseases. Critical Reviews in Food Science and Nutrition 45(4): 287-306.

Scalbert A, Johnson IT, Saltmarsh M (2005) Polyphenols: antioxidants and beyond. Am J Clin Nutr.:215S-7S.

Schliebs R, Liebmann S K, Kumar A, Ghosal S, Bigl V (1973) Systemic administration of defined extracts from *Withania somnifera* (Indian ginseng) and Shilajit differentially affects choilinergic but not glutamatergic and GAB Aergic marker in rat brain. Neurochemistry International 30: 181-190.

Schneider G, Wolfling J (2004) Synthetic Cardenolides and related compounds. Current Organic Chemistry 8: 14.

Senthilnathan P, Padmavathi R, Banu S M, Sakthisekaran D (2006) Enhancement of antitumor effect of paclitaxel in combination with immunomodulatory *Withania somnifera* on benzo(a)pyrene induced experimental lung cancer. Chemico-Biological Interactions 159(3): 180-185.

Sharada A C, Solomon F E, Uma Devi P (1993) Toxicity of *Withania somnifera* root extract in rats and mice. International Journal of Pharmacognosy 31(3): 205-212.

Sharma S, Dahanukar S, Karandikar S M (1985) Effects of long-term administration of the roots of ashwagandha and shatavari in rats. Indian Drugs 29: 133-139.

Shukla S, Mehtaa A, Johna J, Singha S, Mehtaa P, Vyasb S P (2009) Antioxidant activity and total phenolic content of ethanolic extract of *Caesalpinia bonducella* seeds. Food and Chemical Toxicology 47(8): 1848-1851.

Singh G, Kumar P (2011) Antibacterial potential of alkaloids of *Withania somnifera* L. & *Euphorbia hirta* L. International Journal of Pharmacy and Pharmaceutical Sciences 4(1): 78-81.

Singh G, Sharma P K, Dudhe R, Singh S (2010) Biological activities of *Withania somnifera*. Annals of Biological Research 1(3): 56-63.

Singleton V L, Orthofer R, Lamuela-Raventos R M (1999) Analysis of total phenols and other oxidation substrates and antioxidant by means of Folin-Ciocalteu reagent. Methods in Enzymology 299: 152-178.

Sofowora A (1982) Medicinal Plants and Traditional Medicine in Africa. John Wiley and Sons Ltd Nigeria: 33-34.

Stepanovic S N, Antic I, Dakic M, Svabic-vlahovic (2003) *In vitro* antimicrobial activity of propilis and antimicrobial drugs. Microbiological Research 158: 353-357.

Subaraju G V, Vanisree M, Rao C V, Sivaramakrishna C, Sridhar P, Jayaprakasam B, Nair M G (2006) Ashwagandhanolide, a bioactive dimeric thiowithanolide isolated from the roots of *Withania somnifera*. Journal of Natural Products 69: 1790-1792.

Sunar S P (1956) Bioquimica. Editorial Paz MontalvoMadrid 10(17).

Takeoka G R, Dao L T (2003) Antioxidant constituent of almond (*Prunus dulcis* (Mill) D.A.Webb) hulls. Journal of Agricultural and Food Chemistry 51: 496-501.

Todar K (2008a) "PathogenicE.coli" <http://www.textbookofbacteriology.net/e.coli.html>. *Online Textbook of Bacteriology*. University of Wisconsin-Madison Department of Bacteriology. Retrieved 12 January 2012.

Todar K (2008b) Todar's Online Textbook of Bacteriology. January 12, 2012. <http://www.textbookofbacteriology.net/salmonella.html>.

Tohda C, Kuboyama T, Komatsu K (2005) Search for natural products related to regeneration of the neuronal network. Neurosignals 44: 34-45.

Tona L, Kambu K, Ngimbi N, Cimanga K, Vlietinck A J (1998) Antiamoebic and phytochemical screening of some Congolese medicinal plants. Journal of Ethnopharmacology 61: 57-65.

Trease G E, Evan W G A (2005) Text book of Pharmacognosy. Baillere Tindell and Cassell, London.

Trease G E, Evans W C (1989) A text book of pharmacognosy Academic press, London. 22-40.

Tripathi A K, Dey S, Singh R H, Dey P K (1998) Alterations in the sensitivity of 5th receptor subtypes following chronic Asvagandha treatment in rats. Ancient Sciences of Life 17(3): 168-181.

Udayakumar R, Kasthurirengan S, Mariashibu T S, Rajesh M, Ramesh Anbazhagan V, Kim S C, Ganapathi A, Choi C W (2009) Hypoglycaemic and hypolipidaemic effects of *Withania somnifera* root and leaf extracts on alloxan-induced diabetic rats. International Journal of Molecular Sciences 10: 2367–2382.

Udayakumar R, Kasthurirengan S, Vasudevan A, Mariashibu T S, Rayan J J S, Choi C W, Ganapathi A, Kim S C (2010) Antioxidant Effect of Dietary Supplement *Withania somnifera* L. Reduce Blood Glucose Levels in Alloxan-Induced Diabetic Rats. Plant Foods for Human Nutrition 65: 91–98.

Vahidi H, Kamalinejad M, Sedaghati M (2002) Antimicrobial properties of Croccus sativus L. Iranian J. Pharm. Res. 1: 33-35.

Velioglu Y S, Mazza G, Gao L, Oomah B D (1998) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. Journal of Agricultural and Food Chemistry 46: 4113-4117.

Verma P, Srivastava S K, Nath A (2009) Stimulatory effect of *Withenia somnifera* on secretion and endocytotic pathways in the hepatic cell of *Clarias batrachus(Linn.)*. Journal of Ecophysiology and Occupational Health 9: 203-209

Visavadiya N P, Narasimhacharya A V (2007) Hypocholesteremic and antioxidant effects of *Withania somnifera* (Dunal) in hypercholesteremic rats. Phytomedicine 14: 136-142.

Wang J T, Chang S C, Chen Y C, Luh K T (2000) "Comparison of antimicrobial susceptibility of Citrobacter freundii isolates in two different time periods.". The Journal of Microbiology, Immunology and Infection 33(4): 258-262.

Weng X C (1993) Antioxidant and their antioxidation mechanism. J. Zhengzhou Grain College 3: 20-29.

Whalen J G, Mully T W, Enlgish J C (2007) Spontaneous Citrobacter freundii infection in an immunocompetent patient. Archives of Dermatology 143(1): 124-125.

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Yazdanparast R, Ardestani A (2007) *In vitro* antioxidant and free radical scavenging activity of *Cyperus rotundus*. Journal of Medicinal Food 10: 667-674.

Yazdanparast R, Bahramikias S, Ardestani A (2008) *Nasturtium oficinale* reduces oxidative stress and enhances antioxidant capacity in hypercholesterolaemic rats. Chemico-Biological Interactions 172: 176-184.

Yildirim A, Mavi A, Oktay M, Kara A A, Algur O F, Bilaloglu V (2000) Comparison of antioxidant and antimicrobial activities of Tilia (*Tilia argentea* Desf Ex DC), Sage (*Savia triloba* L.), and Black Tea (*Camellia sinensis*) extracts. Journal of Agricultural and Food Chemistry 48(10): 5030-5034.

Yu B P, Yang R (1996) Critical evaluation of the free radical theory of aging. A proposal for the oxidative stress hypothesis. Ann. NY Acad. Sci. 786: 1-11.

Zgoda J R, Porter J R (2001) A convenient microdilution method for screening natural products against bacteria and fungi. Pharmaceutical Biology 39: 221- 225.

Zhao J, Nakamura N, Hattori M, Kuboyama T, Tohda C, Komats K (2002) Withanolide derivatives from the roots of *Withania somnifera* and their neurite outgrowth activities. Chemical and Pharmaceutical Bulletin 50(6): 760-765.

Zhishen J, Mengcheng T, Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry 64: 555-559.

Ziauddin M, Phansalkar N, Patki P, Diwanay S, Patwardhan B (1996) Studies on the immunomodulatory effects of Ashwagandha. Journal of Ethnopharmacology 50: 69-76.

APPENDIX

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RESEARCH ARTICLE

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High catechin concentrations detected in *Withania somnifera* (ashwagandha) by high performance liquid chromatography analysis

Nadia Alam[1], Monzur Hossain[1], Md Ibrahim Khalil[2*], Mohammed Moniruzzaman[2], Siti Amrah Sulaiman[2] and Siew Hua Gan[3]

Abstract

Background: *Withania somnifera* is an important medicinal plant traditionally used in the treatment of many diseases. The present study was carried out to characterize the phenolic acids, flavonoids and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activities in methanolic extracts of *W. somnifera* fruits, roots and leaves (WSFEt, WSREt and WSLEt).

Methods: WSFEt, WSREt and WSLEt was prepared by using 80% aqueous methanol and total polyphenols, flavonoids as well as DPPH radical scavenging activities were determined by spectrophotometric methods and phenolic acid profiles were determined by HPLC methods.

Results: High concentrations of both phenolics and flavonoids were detected in all parts of the plant with the former ranging between 17.80 ± 5.80 and 32.58 ± 3.16 mg/g (dry weight) and the latter ranging between 15.49 ± 1.02 and 31.58 ± 5.07 mg/g. All of the three different plant parts showed strong DPPH radical scavenging activities (59.16 ± 1.20 to 91.84 ± 0.38%). Eight polyphenols (gallic, syringic, benzoic, p-coumaric and vanillic acids as well as catechin, kaempferol and naringenin) have been identified by HPLC in parts of the plant as well. Among all the polyphenols, catechin was detected in the highest concentration (13.01 ± 8.93 to 30.61 ± 11.41 mg/g).

Conclusion: The results indicating that *W. somnifera* is a plant with strong therapeutic properties thus further supporting its traditional claims. All major parts of *W. somnifera* such as the roots, fruits and leaves provide potential benefits for human health because of its high content of polyphenols and antioxidant activities with the leaves containing the highest amounts of polyphenols specially catechin with strong antioxidant properties.

Keywords: *Withania somnifera*, spectrophotometry, HPLC, polyphenols, antioxidant, free radical scavenging activity

Background

Ashwagandha [*Withania somnifera* L. Dunal] (Solanaceae) is an important medicinal plant, commonly-used as a domestic remedy for several diseases in India as well as other parts of the world [1]. It is described as an herbal tonic and health food in the famous book of Vedas and is considered as in 'Indian Ginseng' in traditional Indian system of healing [2]. Several recent reports have demonstrated immunomodulator and antitumor effect of *W. somnifera* as well [3]. Moreover, various parts of the plant have been reported to possess antiserotogenic, anticancer and anabolic properties and have shown beneficial effects in the treatment of arthritis, stress and geriatric problems [4]. *W. somnifera* is also made into dietary supplements with good nutritional properties and phytochemicals. Besides being used among the elderly to increase health vitality, a decoction of *W. somnifera* root is also used as nutrient and health restorative agent among postpartum ladies as it was purported to thicken and increase the nutritive value of the breastmilk when given to nursing mothers. The pharmacological effect of the roots of *W. somnifera* is attributed to its active ingredient, *withanolides* [5] which has a wide range of therapeutic applications [6].

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There is a great deal of evidence indicating that excessive free radical production and lipid peroxidations are actively-involved in the pathogenesis of a wide number of chronic diseases, including atherosclerosis [7], cardiac and cerebral ischemia [8], neurodegenerative disorders [9], carcinogenesis [10], diabetes [11] and rheumatic disorders [12] and contributes a major role in the ageing process [13]. Plant-derived antioxidants such as vitamin E, vitamin C, polyphenols including phenolic acids, phenolic diterpenes, flavonoids, catechins, procyanidins and anthocyanins are becoming increasingly important as dietary factors [14]. Supplementation with berry juice [15], flavones from skullcap, catechins from green tea, anthocyanins from chokeberry and condensed tannins from fava beans [16] are indices of oxidative stress protectant in rats. Furthermore, the growing interest in the substitution of synthetic food antioxidants by natural chemicals has fostered research on plant sources and the screening of raw materials for identifying new antioxidants. In this regard, polyphenols are being increasingly reported to exhibit antioxidant effects in foods [17]. Various plants have been analysed for the existence of phenolic acids by HPLC [18]. Plant acids are known to have anticarcinogenic activity [19]. and phenolic compounds are believed to be an important part of the general defence mechanism of many plants against infections [20]. Therefore, it is useful to measure the presence of phenolic compounds in natural substances.

Purification of phenolic acids is very difficult not only due to their isomeric similarities but also due to the influence of various effects such as acid-based treatment, temperature and light on their labile structures [21]. The determination of phenolic acids is important both for their characterization and to facilitate more efficient uses of important plant resources [22].

To date, very limited data exists on phenolic compounds reported in *W. somnifera* leaves, roots and fruits as well as their antioxidant effects to support their traditional claims. Therefore, we aimed to undertake this task in the present study as *W. somnifera* is widely-used. If the presence of phenolic and flavonoid compounds present in *W. somnifera* can be confirmed, the plant can be used as a good possible source of antioxidant.

Methods

Plant material

The *W. somnifera* plant parts such as roots, fruits and leaves were collected from field grown plants after six months of cultivation in Botanical Garden, Rajshahi University, Bangladesh. The collected parts of medicinal plant were brought into the laboratory, cleaned and air-dried in shade and then grinded to a fine powder.

Chemicals and reagents

Reagents such as 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and Folin-Ciocalteu's reactive were purchased from Sigma-Aldrich (St. Louis, USA). Sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), sodium nitrite ($NaNO_2$) and sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). All chemicals used were of analytical grades.

Preparation of Plant Extracts

W. somnifera roots, fruits and leaves extract (WSREt, WSFEt and WSLEt) preparation was performed according to a modified method described by Kahkonen et al., [23]. Grinded dry plant materials (500 mg) were weighed into a test tube followed by the addition of a total of 10 ml of 80% aqueous methanol. The suspension was then stirred slightly. The tubes were sonicated for 5 min and centrifugated for another 10 min at 1500 g and the resulting supernatants were collected. The extraction procedure was repeated and the supernatants were combined before being evaporated to a volume of approximately 1 ml. The concentrated extracts were further lyophilized and weighed.

Total Polyphenols

Phenolic compounds in *W. somnifera* were estimated by a spectrophotometric determination using a modified Folin-Ciocalteu method Singleton et al., [24]. Briefly, 100 μl of sample extracts (1 mg/ml) was mixed with 1 ml of Folin and Ciocalteu's phenol reagent (2 N). After 3 minutes, 1 ml of 10% Na_2CO_3 solution was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (T 80 UV/VIS spectrophotometer, ChromoTek GmbH, Germany). Gallic acid was used to calculate the standard curve (20, 40, 60, 80 and 100 μg/ml, $r^2 = 0.993$). Estimation of the phenolic compounds was carried out in triplicates. The results were mean values ± standard deviations and expressed as milligrams of gallic acid equivalents (GAEs) per g of *W. somnifera* dry weight (DW).

Determination of total flavonoids

The total flavonoid contents of the *W. somnifera* extracts were determined according to the colorimetric assay method developed by Zhishen et al., [25]. Briefly, 1 ml of properly diluted (1 mg/ml) WSREt, WSFEt and WSLEt were mixed with 4 ml of distilled water. At baseline, 0.3 ml of (5% w/v) $NaNO_2$ was added. After five minutes, 0.3 ml of (10% w/v) $AlCl_3$ was added followed by the addition of 2 ml of NaOH solution (1 M) six minutes later. After that, the volume was immediately made up to 10 ml, with the addition of 2.4 ml of distilled water. The mixture was shaken vigorously and the

absorbance of the mixture was read at 510 nm. A calibration curve was prepared using a standard solution of catechin (20, 40, 60, 80 and 100 μg/ml, r^2 = 0.996). The results were expressed as mg catechin equivalents (CEQ) per g of *W. somnifera* (DW).

DPPH free radical-scavenging activity

The antioxidant capacity of the *W. somnifera* was also studied through the evaluation of the free radical-scavenging effect on the DPPH radical. The determination was based on the method proposed by Ferreira et al., [26]. Briefly, 1 ml (1 mg/ml) of WSREt, WSFEt and WSLEt were mixed with 2.7 ml of methanolic solution containing DPPH radicals (0.024 mg/ml). The mixture was vigorously shaken and left to stand for 60 min in the dark (until their absorbance remained unchanged). The reduction of the DPPH radical was determined by measuring the absorbance at 517 nm [27]. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution.

High Performance Liquid Chromatography (HPLC)

The HPLC method was based on the method published by Kaškonienė et al., [28]. Analysis of WSREt, WSFEt and WSLEt were performed by employing an HPLC system (Waters 2695, Milford, MA, USA) equipped with a Photodiode Array Detector (Waters 2996, Milford, MA, USA). The HPLC column was a Merck Purospher Star, RP-18e, (125 × 4 mm, 5 μm) fitted with a guard cartridge packed with the same type of stationary phase (Merck, Darmstadt, Germany). The linear 76 gradient was used at a flow rate of 0.5 ml/min with total analytical time of approximately 35 min. The binary mobile phase consisted of a solvent A (ultra pure water with 0.1% of phosphoric acid) and solvent B (pure methanol with 0.1% of phosphoric acid). Elution from the column was achieved with the following gradient: 0 min to 10 min of solvent B, increased from 35% to 55%; 10-25 min of solvent B, increased to 62%; 25-30 min of solvent B, increased to 85% and the final composition was kept constant till 35 min. All solvents used were of HPLC grade quality. The detection wavelength was done between 200 and 450 nm with specific monitoring at 265 nm. The identification of phenolic compounds was performed by comparing the retention time and UV absorption (λ_{max}) of each peak of the analytes with the reference standards. Phenolic acids (gallic, syringic, caffeic, vanillic, p-coumaric, benzoic and transcinnamic acids) as well as flavonoids (catechin, naringenin, luteolin, hesperetin, kaempferol, apigenin, naringin) were purchased from Sigma (St. Louis, MO, USA) and were used as reference standards.

Statistical analysis

All analyses were carried out in triplicates and the data was expressed as means ± standard deviations (SD). The data was analyzed using (Statistical Packages for Social Science 12.0) (SPSS Inc., USA) and MS Excel 2003. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test was used to compare the phenol contents, FRAP values, DPPH scavenging activities and colour parameters of WSREt, WSFEt and WSLEt (Table 1). The differences between means at 95% ($p < 0.05$) confidence level were considered statistically significant. Correlations were obtained by Pearson's correlation coefficient (r) in bivariate linear correlations.

Results

Phenolic content

The contents of total polyphenols (mg GAE/g) of WSREt, WSFEt and WSLEt were investigated using the modified Folin-Ciocalteu assay which is sensitive to phenol and polyphenols entities and other electron donating antioxidants such as ascorbic acid and vitamin E. The sources of the analysed WSREt, WSFEt as well as WSLEt were significantly different ($p < 0.05$), as shown in (Table 1). Among the three different *W. somnifera* extracts, the concentrations of polyphenols was found to be lowest in WSREt (17.80 ± 5.80 mg/g) and highest in WSLEt (32.58 ± 3.16 mg/g).

Flavonoids content

The total contents of flavonoids of the three different *W. somnifera* extracts were also determined. Flavonoids were detected in high concentrations ranging from 15.49 ± 1.02 (WSREt) to 31.58 ± 5.07 (WSLEt) mg CEQ/g (Table 1).

DPPH radical scavenging activity

There were significant differences in terms of their scavenging abilities present among the WSREt, WSFEt as well as WSLEt samples, expressed as percentage of inhibition on the DPPH radical (Table 1). Among the three extracts, the lowest scavenging activity was found in WSREt (59.16 ± 1.20%) while the highest activity was found in WSLEt (91.84 ± 0.38%). The DPPH radical scavenging test is one of the fastest tests available to investigate the overall hydrogen/electron donating activity of single antioxidants and health-promoting dietary antioxidant supplements. The reasons behind the markedly higher radical scavenging capacity exhibited by the different types of *W. somnifera* extracts probably lie in their diverse botanical origin. Antioxidant potential of

Table 1 Spectrophotometric analysis of phenolics, flavonoids and antioxidant properties of *W. somnifera* roots, fruits and leaves.

W. somnifera	Phenolics mg GAE/g (DW)	Flavonoids mg CEQ/g (DW)	% of DPPH inhibition
Roots	17.80 ± 5.80[c]	15.49 ± 1.02[c]	59.16 ± 1.20[c]
Fruits	22.29 ± 1.99[b]	21.15 ± 5.32[b]	70.38 ± 0.84[b]
Leaves	32.58 ± 3.16[a]	31.58 ± 5.07[a]	91.84 ± 0.38[a]

In each column, values with different letters (superscripts) indicate significant differences ($p < 0.05$). DW = dry weight

W. somnifera extracts is directly related to its phenolic and flavonoids content.

Correlations

The correlations among the phenolic compounds, flavonoids and DPPH radical scavenging activities are shown in (Table 2). The correlation matrix showed that significant linear correlation exists between the results of all three analytical methods employed indicating that the three measurements are reliable indicators of antioxidant activities. The lowest linear correlation value at $r = 0.962$ ($p = 0.01$) and the highest correlation value at $r = 0.995$. Both phenolic compounds and DPPH radical scavenging activity are strongly correlated ($r = 0.995$ and 0.983 respectively) with DPPH radical scavenging activities. The significant correlations existing between phenolic compounds and DPPH radical scavenging activities indicate the strong antioxidant properties of the tested WSREt, WSFEt and WSLEt. Similar to our findings, some literature also reported strong correlation between the antioxidant capacity and total phenolic contents [29] further suggesting that polyphenols are the major components responsible for the antioxidant effects of WSREt, WSFEt and WSLEt.

HPLC analysis

Sixteen phenolic and flavonoid standards were compared with the chromatograms produced by the unknown *W. somnifera* extracts. HPLC analysis of phenolic and flavonoids compounds in WSREt, WSFEt as well as WSLEt showed that only catechin is commonly found in all of the three extracts analyzed. Eight polyphenols (five phenolic acids and three types of flavonoids) have been identified and the phenolics patterns of all plant parts were confirmed to contain gallic, syringic, benzoic, p-coumaric and vanillic acids as well as the flavonoids catechin, kaempferol and naringenin.

(Figures 1, 2, 3) show the HPLC chromatograms obtained from WSREt, WSFEt and WSLEt. Six phenolic compounds were detected in WSLEt whereas, three compounds were identified in WSFEt and only two compounds were identified in WSREt. The unknown compounds that may have had similar flavonoid and phenolic acid spectra and chromatographic behaviours (shown as extra peaks in the figures) were also detected. However, they could not be fully identified due to lack of standard compounds. (Figure 4) compared the total phenolic compounds of sample extracts obtained when using spectrophotometric and HPLC methods. Overall, spectrophotometric methods tend to report higher levels of phenolics when compared to HPLC method.

Table 2 Correlations matrix among phenolics, flavonoids content and free radical scavenging activities

Correlations

	phenolics	Flavonoids	DPPH
phenolics	1	0.995(**)	0.983(**)
Flavonoids	0.995(**)	1	0.962(**)
DPPH	0.983(**)	0.962(**)	1

** Correlation is significant at the 0.01 level (2-tailed).

Discussion

To our knowledge, our study is the first to identify phenolic compounds present in *W. somnifera*. The present study confirmed the presence of phenolic compounds, flavonoids and antioxidant activities in WSREt, WSFEt and WSLEt. High concentrations of phenolic compounds were found in the different parts of *W. somnifera* with significant variations in the amount. (Table 1) showed high content of phenolics and flavonoids in WSLEt (32.58 ± 3.16 and 31.58 ± 5.07 respectively) while that in WSREt was low (17.80 ± 5.80 and 15.49 ± 1.02 respectively). This indicates that the leaves of *W. somnifera* should be consumed for its antioxidant effects. Several reports have shown that different plant parts have variable polyphenols compositions, as shown by our findings [30].

There is very poor data on analysis of phenolic compounds in *W. somnifera*. Udayakumar et al., [30] from India reported that the presence of total phenolic compounds in WSREt was 28.26 mg/g while that of flavonoids was 17.32 mg/g. For WSLEt it was 5.4 mg/g total phenolic compounds and 5.1 mg/g flavonoids both of which were different from our study perhaps due to the different source of *W. somnifera* and polyphenols of plant parts which may also be related to the colour, maturity and environment. However, the spectrophotometric method tend to overestimate the phenolics content with respect to the chromatographic method

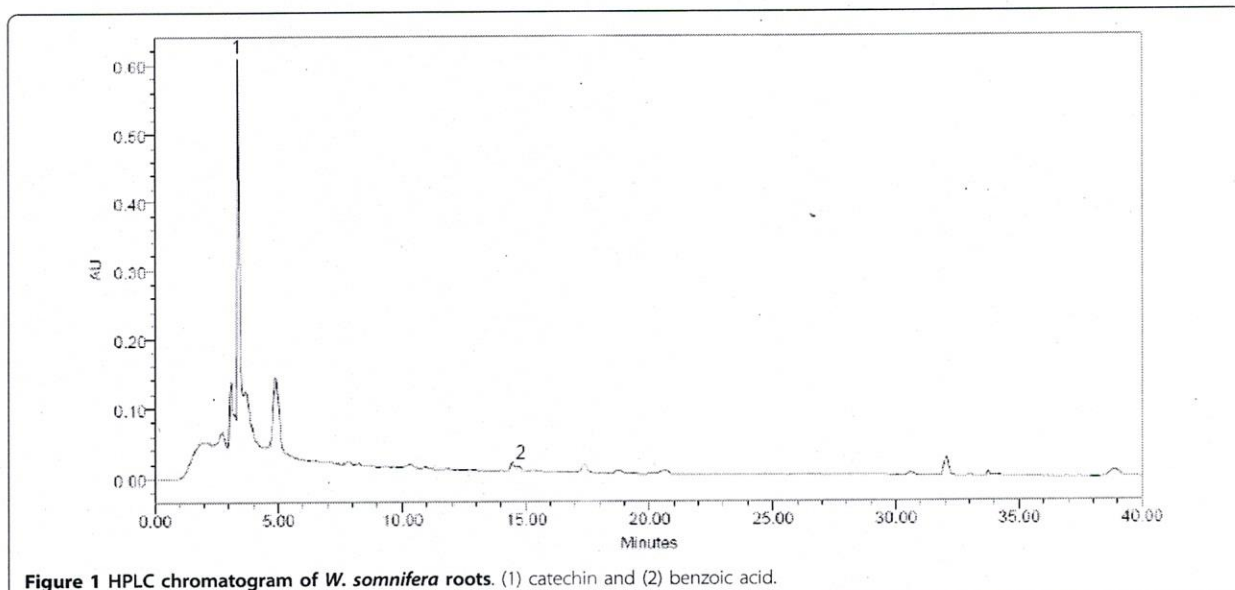

Figure 1 HPLC chromatogram of *W. somnifera* roots. (1) catechin and (2) benzoic acid.

perhaps due to the fact that non-phenolic materials present in the investigated extracts interfered in the spectrophotometric analysis [31].

The scavenging ability of DPPH free radical is extensively used to screen the antioxidant potential of naturally-derived foods and plants. Methanol was employed in this study to extract the low molecular weight and moderately polar substances because of its wide solubility properties. We found that WSREt, WSFEt and WSLEt exhibited free radical DPPH scavenging abilities (Table 1). In this study, we attempted to isolate the active compounds responsible for antioxidant activities in WSREt, WSFEt and WSLEt. The antioxidant capacity has been shown to be directly related with the total phenolic content (Table 2) which was in agreement with many previous reports [32-34]. In addition, the flavonoids contributed to almost all of the total phenolic content (Table 1), which indicated that the flavonoids in *W. somnifera* are important constituents responsible for the bioactivities.

In HPLC analysis, six compounds were identified in WSLEt while three were identified in WSFEt and two were identified in WSREt. Out of the eight phenolic compounds catechin was found in the highest

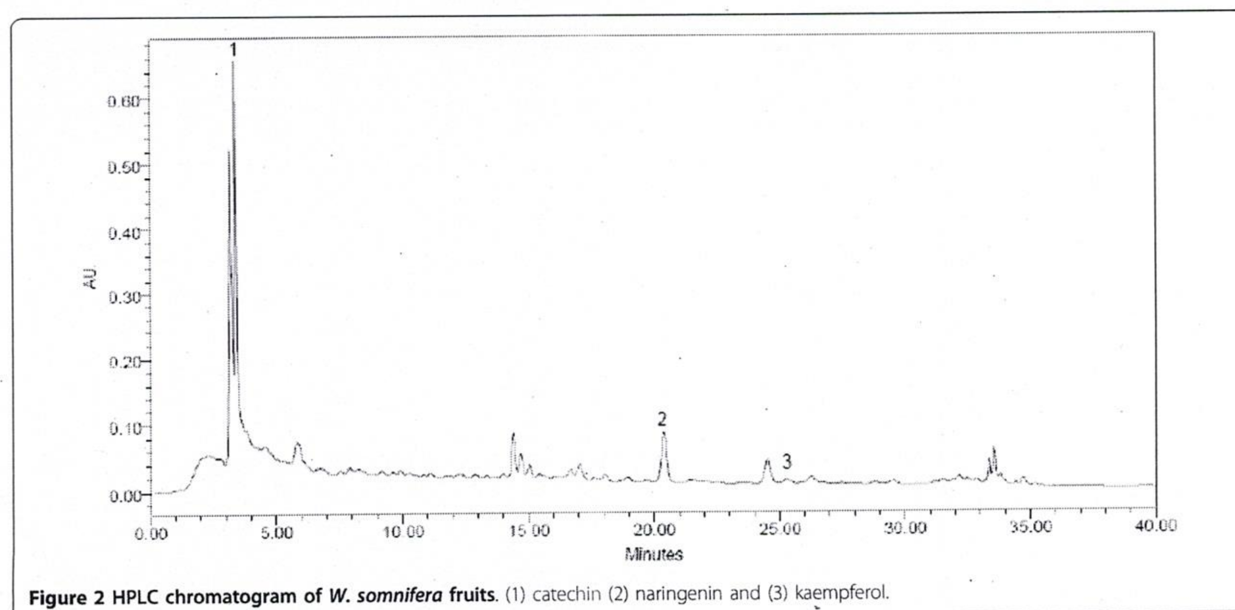

Figure 2 HPLC chromatogram of *W. somnifera* fruits. (1) catechin (2) naringenin and (3) kaempferol.

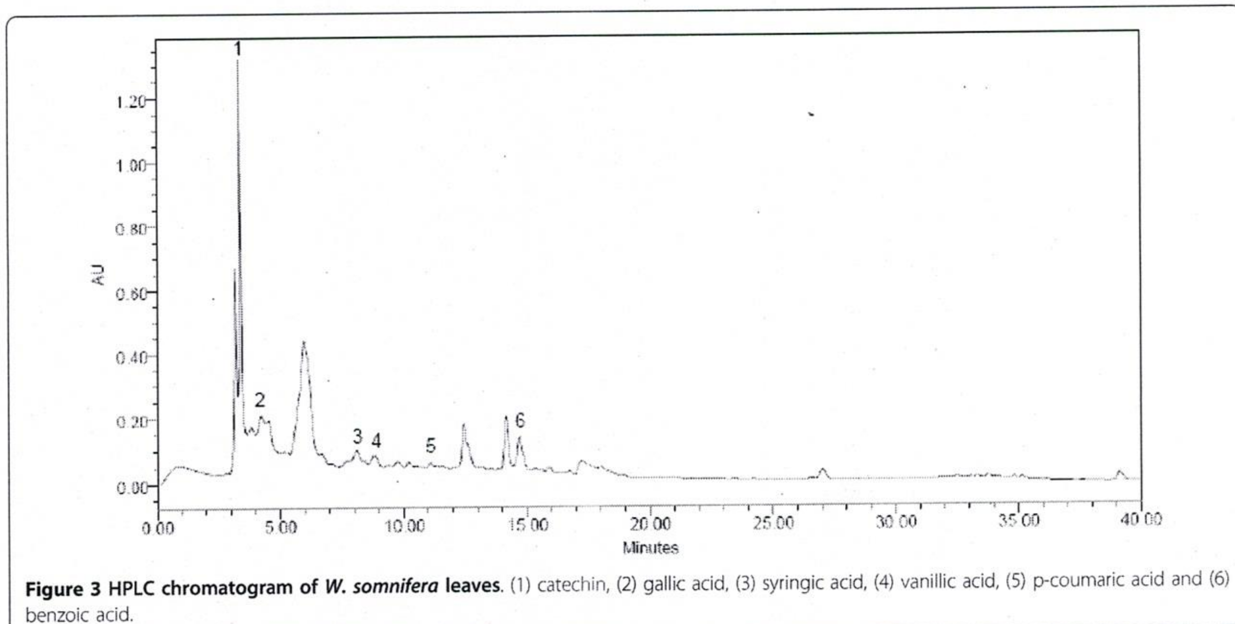

Figure 3 HPLC chromatogram of *W. somnifera* leaves. (1) catechin, (2) gallic acid, (3) syringic acid, (4) vanillic acid, (5) p-coumaric acid and (6) benzoic acid.

concentration compared to others amounting 12.82 mg/g in WSREt, 19.48 in WSFEt and 28.38 mg/g in WSLEt (Table 3). This indicates that WSREt, WSFEt and WSLEt are rich sources of catechin. Catechin is one of the most important polyphenols that provide health benefits and is found in high quantities in green tea which is widely known for its strong antioxidant properties. There are many reports on catechin which described its therapeutic role in human health. Modern studies have found that catechin is responsible for

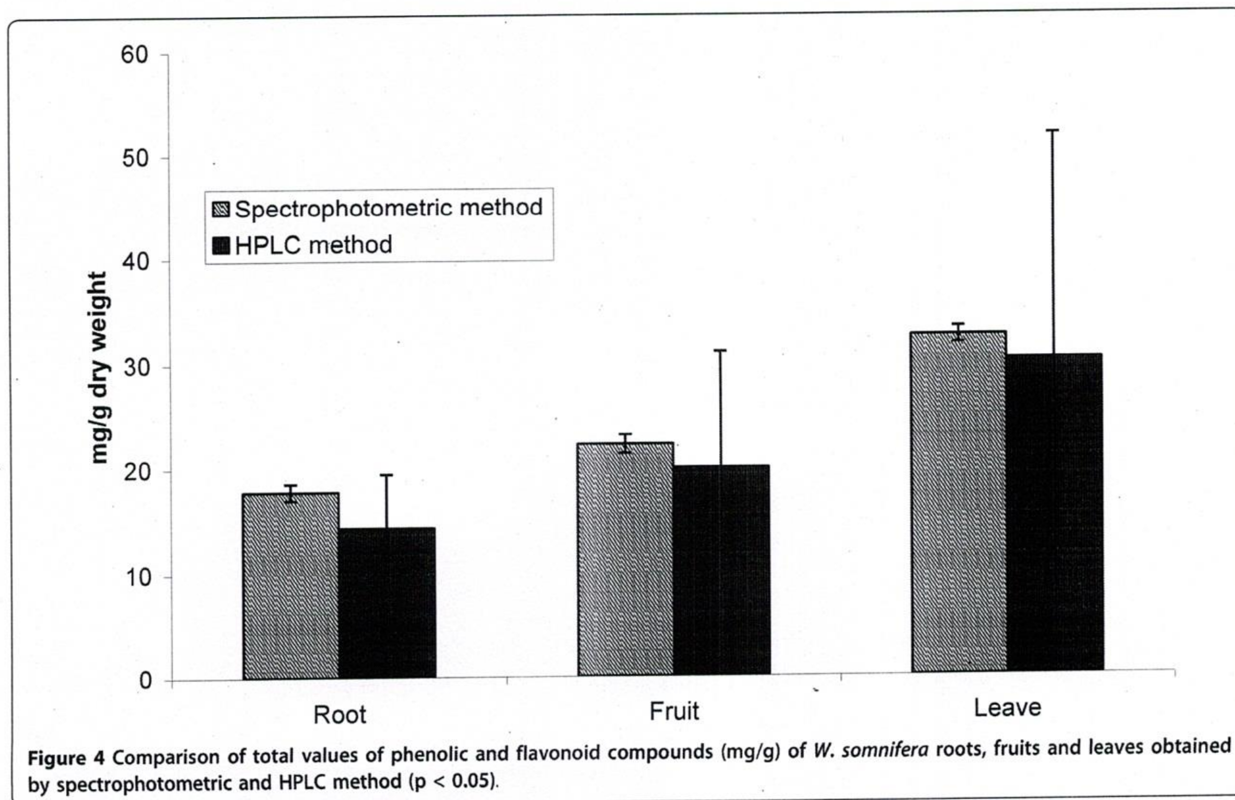

Figure 4 Comparison of total values of phenolic and flavonoid compounds (mg/g) of *W. somnifera* roots, fruits and leaves obtained by spectrophotometric and HPLC method ($p < 0.05$).

Table 3 Phenolic acids and flavonoids compounds detected in *Withania somnifera* roots, fruits and leaves using high performance liquid chromatography analysis.

SL No	Standard compounds	Retention time	λ_{max} (nm)	Quantity of the identified compounds (mg/g DW)		
				Roots	Fruits	Leaves
1	Catechin	3.36	278	12.82	19.48	28.38
2	Gallic acid	4.12	269, 216	ND	ND	0.18
3	Syringic acid	8.10	268, 216	ND	ND	0.30
4	Vanillic acid	8.61	224, 249, 269	ND	ND	0.15
5	p-coumaric acid	10.97	264, 286, 310	ND	ND	0.80
6	Benzoic acid	15.33	272, 241	0.19	ND	0.80
7	Naringenin	20.18	277, 292, 308	ND	0.50	ND
8	Kaempferol	25.59	361	ND	0.06	ND
	Total phenolic compounds			13.01 ± 8.93	20.04 ± 11.09	30.61 ± 11.41

(ND = Not detected), DW = dry weight

antioxidant activity, anti-ageing properties and cardiac health maintenance [35]. Catechins' beneficial effects are attributed to its ability to reduce oxidative stress, lipid peroxidation, free radical generation and unhealthy low density lipoprotein (LDL) cholesterol-oxidation [36]. There is also an evidence that suggests that catechins have a role in the protection against degenerative disorders [37]. Throughout the experiments, some catechins have also been shown to inhibit a key enzyme (squalene epoxidase) in the pathway of cholesterol biosynthesis [38]. The potent antioxidant properties of catechin reduce free radical damage to cells and prevent the oxidation of LDL cholesterol [39]. Besides catechin, other phenolic compounds found in the WSREt, WSFEt and WSLEt may also contribute to its medicinal and antioxidant properties [40]. Further studies to isolate individual active principles and antioxidant activity of individual extracts of roots, fruits as well as leaves through radical scavenging assay and their pharmacological validation in terms of modern medicine will be of great pharmacological importance in future which is under our consideration.

Conclusion

Five phenolics (gallic, syringic, benzoic, p-coumaric and vanillic acids) and three flavonoids (catechin, kaempferol, and naringenin) have been identified in WSREt, WSFEt and WSLEt and catechin was found in high concentrations especially in the leaves part, confirming the antioxidant potential and health benefits of *W. somnifera*.

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Authors' contributions
NA, MIK and MM have carried out the experimental parts of this investigation. MH, SAS and SHG supervised the work, evaluated the results and corrected the manuscript for publication. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References

1. Patwardhan B, Panse GT, Kulkarni PH: **Ashwagandha a review.** *Journal of the National Integrated Medicine Association* 1998, **30**:7-11.
2. Dhuley JN: **Adaptogenic and cardioprotective action of ashwagandha in rats and frogs.** *J Ethnopharmacol* 2000, **70(1)**:57-63.
3. Agarwal R, Diwanay S, Patki P, Patwardhan B: **Studies on immunomodulatory activity of *Withania somnifera* (ashwagandha) extracts in experimental immune inflammation.** *J Ethnopharmacol* 1999, **67(1)**:27-35.
4. Prakash J, Gupta SK, Kochupillai V, Gupta YK, Joshi S: **Chemopreventive activity of *Withania somnifera* in experimentally induced fibrosarcoma tumors in Swiss albino mice.** *Phytother Res* 2001, **15(3)**:240-244.
5. Budhiraja RD, Sudhir S: **Review of biological activity of withanolides.** *J Sci Ind Res* 1987, **46**:488-491.
6. Udayakumar R, Kasthurirengan S, Mariashibu TS, Rajesh M, Ramesh Anbazhagan V, Kim SC, Ganapathi A, Choi CW: **Hypoglycaemic and hypolipidaemic effects of *Withania somnifera* root and leaf extracts on alloxan-induced diabetic rats.** *Int J Mol Sci* 2009, **10**:2367-2382.
7. Parthasarathy S, Santanam N, Ange N: **Oxidised low-density lipoprotein, a two-faced Janus in coronary artery disease.** *Biochem Pharmacol* 1998, **56**:279-284.
8. Keller JN, Kindly MS, Holtberg FW, St Clair DK, Yen HC, Germeyer A, Steiner SM, Bruce-Keller AJ, Hutchins JB, Mattson MP: **Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite products, lipid peroxidation, and mitochondrial dysfunction.** *J Neurosci* 1998, **18**:687-697.
9. Perry G, Raine KA, Nunomura A, Watayc T, Sayre LM, Smith MA: **How important is oxidative damage? Lessons from Alzheimer's disease.** *Free Radic Biol Med* 2000, **28**:831-834.
10. Kamat JP, Devasagayam TPA: **Oxidative damage to mitochondria in normal and cancer tissues, and its modulation.** *Toxicology* 2000, **155**:73-82.

11. Gorogawa SI, Kajimoto Y, Umayahara Y, Kaneto H, Watada H, Kuroda A, Kawamori D, Yasuda T, Matsuhisa M, Yamasaki Y, *et al*: **Probucol preserves pancreatic-cell function through reduction of oxidative stress in type 2 diabetes.** *Diabetes Res Clin Pract* 2002, **57**:1-10.
12. Hänninen O, Kaartinen K, Rauma AL, Nenenen M, Törrönen R, Häkkinen S, Adlercreutz H, Laakso J: **Antioxidants in vegan diet and rheumatic disorders.** *Journal of Toxicology* 2000, **155**:45-53.
13. Khodr B, Khalil Z: **Modulation of inflammation by reactive oxygen species: implications for aging and tissue repair.** *Free Radic Biol Med* 2001, **30**:1-8.
14. Ferguson LR: **Role of plant polyphenols in genomic stability.** *Mutat Res* 2001, **475**:89-111.
15. Netzel M, Strass G, Kaul C, Bitsch I, Dietrich H, Bitsch R: **In vivo antioxidative capacity of composite berry juice.** *Food Res Int* 2002, **35**:213-216.
16. Zdunczyk Z, Frejnajel S, Wróblewska M, Juskiewicz J, Oszmianski J, Estrella I: **Biological activity of polyphenols extracts from different plant sources.** *Food Res Int* 2002, **35**:183-186.
17. Tang S, Kerry JP, Sheehan D, Buckley DJ, Morrissey PA: **Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation.** *Food Res Int* 2002, **35**:651-657.
18. Nuutila AM, Kammiovirta K, Caldentey KMO: **Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis.** *Food Chem* 2002, **76**:519-525.
19. Shahrzad S, Bitsch I: **Determination of some pharmacologically active phenolic acids in juices by high-performance liquid chromatography.** *J Chromatogr A* 1996, **741**:223-231.
20. Friend J: **Phenolic compounds are believed to be an important part of the general defence mechanism of many plants.** *Phytochemisty soc* 1985, **25**:367-372.
21. Keefer JF, Schuster SM: **Separation of citric acid cycle intermediates by high-performance liquid chromatography with ion pairing.** *J Chromatogr A* 1986, **383(2)**:297-305.
22. Scorska M, Matawska I, Gowniak K, Zgorka G: **Qualitative and quantitative analysis of phenolic acids in *Asclepias syriaca* L.** *Acta Pol Pharm* 2000, **57(1)**:69-72.
23. Kahkonen MP, Hopia AI, Vuorela HJPRJ, Pihlaja K, Kujala TS, Heinonen M: **Antioxidant Activity of Plant Extracts Containing Phenolic Compounds.** *J Agric Food Chem* 1999, **47**:3954-3962.
24. Singleton VL, Orthofer R, Lamuela-Raventos RM: **Analysis of total phenols and other oxidation substrates and antioxidant by means of Folin-Ciocalteu reagent.** *Meth Enzymol* 1999, **299**:152-178.
25. Zhishen J, Mengcheng T, Jianming W: **The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals.** *Food Chem* 1999, **64**:555-559.
26. Ferreira ICFR, Aires E, Barreira JCM, Estevinho LM: **Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract.** *Food Chem* 2009, **114**:1438-1443.
27. Hatano T, Kagawa H, Yashura T, Okuda T: **Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects.** *Chemical & Pharmaceutical Bulletin* 1988, **36**:2090-2097.
28. Kaškonienė V, Maruška A, Kornyšova O: **Quantitative and qualitative determination of phenolic compounds in honey.** *Cheminė Technologija* 2009, **52(3)**:74-80.
29. Piljac-Žegarac J, Stipčević T, Belščak A: **Antioxidant properties and phenolic content of different floral origin honeys.** *J ApiProd ApiMed Sci* 2009, **1(2)**:43-50.
30. Udayakumar R, Kasthurirengan S, Vasudevan A, Mariashibu TS, Rayan JJS, Choi CW, Ganapathi A, Kim SC: **Antioxidant Effect of Dietary Supplement *Withania somnifera* L. Reduce Blood Glucose Levels in Alloxan-Induced Diabetic Rats.** *Plant Foods Hum Nutr* 2010, **65**:91-98.
31. Escarpa A, González MC: **Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods.** *Anal Chim Acta* 2001, **427(1)**:119-127.
32. Azlim Almey AA, Ahmed Jalal Khan C, Syed Zahir I, Mustapha Suleiman K, Aisyah MR, Kamarul Rahim K: **Total phenolic content and primary antioxidant activity of methanolic and ethanolic extracts of aromatic plants' leafs.** *International Food Research Journal* 2010, **17**:1077-1084.
33. Liu SC, Lin JT, Wang CK, Chen HY, Yang DJ: **Antioxidant properties of various solvent extracts from lychee (*Litchi chinenesis* Sonn.) flowers.** *Food Chem* 2009, **114**:577-581.
34. Shukla S, Mehtaa A, Johna J, Singha S, Mehtaa P, Vyasb SP: **Antioxidant activity and total phenolic content of ethanolic extract of *Caesalpinia bonducella* seeds.** *Food Chem Toxicol* 2009, **47(8)**:1848-1851.
35. Lambert JD, Yang CS: **Cancer chemopreventive activity and bioavailability of tea and tea polyphenols.** *Mutat Res* 2003, **523-524**:201-208.
36. Coimbra S, Castro E, Rocha-Pereira P, Rebelo I, Rocha S, Santos-Silva A: **The effect of green tea in oxidative stress.** *Clin Nutr* 2006, **25**:790-796.
37. Cooper R, Morré DJ, Morré DM: **Medicinal benefits of green tea: part I. Review of noncancer health benefits.** *J Alternative Compl Med* 2005, **11**:521-528.
38. Abe I, Kashiwagi Y, Noguchi H, Tanaka T, Ikeshiro Y, Kashiwada Y: **Ellagitannins and hexahydroxydiphenoyl esters as inhibitor of vertebrate squalene epoxidase.** *J Nat Prod* 2001, **64**:1010-1014.
39. Cooper R, Morré DJ, Morré DM: **Medicinal benefits of green tea: part II. Review of anticancer properties.** *J Alternative Compl Med* 2005, **11**:639-652.
40. Khalil MI, Sulaiman SA, Boukraa L: **Antioxidant Properties of Honey and Its Role in Preventing Health Disorder.** *The Open Nutraceuticals Journal* 2010, **3**:6-16.

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RESEARCH ARTICLE **Open Access**

Methanolic extracts of *Withania somnifera* leaves, fruits and roots possess antioxidant properties and antibacterial activities

Nadia Alam[1], Monzur Hossain[1], Md Abdul Mottalib[2], Siti Amrah Sulaiman[3], Siew Hua Gan[4] and Md Ibrahim Khalil[3,5*]

Abstract

Background: *Withania somnifera,* also known as ashwagandha, is an important herb in ayurvedic and indigenous medical systems. The present study was designed to evaluate the antioxidant and antibacterial activities of an 80% aqueous methanolic extract of *W. somnifera* roots (WSREt), fruits (WSFEt) and leaves (WSLEt).

Methods: Several assays were performed to determine the antioxidant properties of this herb including 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity, ferric reducing antioxidant power (FRAP), ferrous chelation and inhibition of β-carotene bleaching.

Results: The values for DPPH, FRAP, ferrous chelation and inhibition of β carotene bleaching for the three types of extracts ranged from 101.73-801.93 μg/ml, 2.26-3.29 mM Fe/kg, 0.22-0.65 mg/ml and 69.87-79.67%, respectively, indicating that *W. somnifera*, particularly the leaves, possesses significant antioxidant properties. The mean ascorbic acid content was 20.60-62.60 mg/100 g, and the mean anthocyanin content was 2.86-12.50 mg/100 g. Antibacterial activities were measured using the agar well diffusion method and five pathogenic Gram-negative bacteria: *Escherichia coli, Salmonella typhi, Citrobacter freundii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The leaf extracts displayed the highest activity against *S. typhi* (32.00 ± 0.75 mm zone of inhibition), whereas the lowest activity was against *K. pneumoniae* (19.00 ± 1.48 mm zone of inhibition). The lowest minimum inhibitory concentration value was 6.25 mg/ml, which was against *S. typhi*, followed by 12.5 mg/ml against *E. coli*.

Conclusion: In addition to its antioxidant properties, *W. somnifera* exhibited significant antibacterial activities against Gram-negative bacteria, particularly *S. typhi*.

Keywords: Withania somnifera, Antioxidant, Antibacterial, Free radicals, FRAP

Background

Free radical reactions are important factors in the progression of chronic diseases such as cancers, hypertension, cardiac infarction, and atherosclerosis, as well as in rheumatism and cataracts [1]. Many synthetic drugs protect against oxidative damage, but these drugs have adverse side effects [2]. An alternative solution is to consume natural antioxidants from food supplements and traditional medicines [3]. Recently, many natural compounds with antioxidant and antimicrobial properties have been isolated from different plant materials [4].

W. somnifera Dunal (*Solanaceae*), also known as ashwagandha or winter cherry, is one of the most valuable plants in the traditional Indian systems of medicine. This plant is used in more than 100 formulations in Ayurveda, Unani and Siddha and is believed to be therapeutically equivalent to ginseng [5]. The ethnopharmacological properties of the plant include adaptogenic, anti-sedative and anti-convulsion activities, and the plant is used to treat various neurological disorders, geriatric debilities, arthritis, stress and behavior-related problems [6].

W. somnifera is also used as a dietary supplement because it contains a variety of nutrients and phytochemicals.

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A decoction of *W. somnifera* roots and leaves is used as a nutrient and health restorative by pregnant women and the elderly. *W. somnifera* thickens and increases the nutritive value of the milk when given to nursing mothers. Additionally, its fruits or seeds are used to curdle plant milk to make vegetarian cheeses [7]. It has been reported that all of the major parts of *W. somnifera* such as the roots, fruits and leaves provide potential benefits for human health because of their high content of polyphenols and antioxidant activities [8]. Previous reports on the activities of methanolic extracts of the whole *W. somnifera* plant against different pathogenic bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans* and *Candida albicans*, have also found significant antibacterial properties [9].

W. somnifera is traditionally used as a therapeutic agent for diarrhea, dyspepsia and gastrointestinal disorders [10]. It has been reported that the antioxidant activities in a plant are dependent on some phytoconstituents such as the phenolic compounds, the anthocyanin and ascorbic acids as well as many other important constituents [11]. In a previous research, the profiles of polyphenols composition in *W. somnifera* roots, fruits and leaves have been reported [8]. In this study, we report the anthocyanin and ascorbic acids contents as well as the antioxidant and antimicrobial properties of *W. somnifera* in different plant parts (the roots, fruits and leaves) to confirm the ethnomedical uses of this medicinal plant as well as to identify the plant part that gives the highest antioxidant activities.

Methods

Plant materials

In January 2009, WSR, WSF and WSL were collected from field-grown plants after six months of cultivation in the Botanical Garden of Rajshahi University (Rajshahi, Bangladesh). These plants were identified with the help of the available literature and were authenticated by a botanist, Professor Shah Alam from the Department of Botany, Rajshahi University (Rajshahi, Bangladesh). They were stored at the herbarium lab of the department and the voucher number of the collective plant was WS13. The collected parts of the medicinal plant were cleaned, air-dried in the shade and ground to a fine powder.

Chemicals and reagents

Reagents such as 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), Folin-Ciocalteu's reactive, 2,4,6-tris(1-pyridyl)-1,3,5-triazine (TPTZ) and ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] disodium salt were purchased from Sigma-Aldrich (St. Louis, USA). Sodium acetate trihydrate, glacial acetic acid, hydrochloric acid (HCl), potassium chloride (KCl), iron III chloride ferrous, iron II sulfate, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), ferrous chloride ($FeCl_2$), sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), ferric chloride ($FeCl_3.6H_2O$), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), potassium hexacyanoferrate [$K_3Fe(CN)_6$], trichloroacetic acid (TCA), HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, ethylenediaminetetraacetic acid (EDTA), 2,6-dichlorophenolindophenol, L-ascorbic acid, 3,5-dinitrosalicylic acid (DNSA), β-carotene, linoleic acid, Tween 80 emulsifier and tertiary butylhydroquinone (TBHQ) were purchased from Merck (Darmstadt, Germany). All chemicals used were of analytical grade.

Preparation of plant extracts

The WSR, WSF and WSL extracts were prepared according to a modified method described by Kahkonen et al. [12]. Ground dry plant materials (500 mg) were weighed in a test tube, followed by the addition of 10 ml of 80% aqueous methanol. The suspension was then gently stirred. The tubes were sonicated for 5 min (45°C) and centrifuged (25°C) for an additional 10 min at 1500 ×*g*. The resulting supernatants were collected. The extraction procedure was repeated three times, and the supernatants were combined before being evaporated using a rotary evaporator to a volume of approximately 1 ml. The concentrated extracts were then lyophilized and weighed. The extracts were resuspended in saline and used as a stock solution.

Determination of the ascorbic acid content of *W. somnifera*

The ascorbic acid content was determined based on the spectrophotometric method described by Ferreira et al. [13]. Briefly, the sample (100 mg) was extracted with 10 ml of 1% metaphosphoric acid for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 ml) was then mixed with 9 ml of 2,6-dichlorophenolindophenol, and the absorbance was measured within 30 min at 515 nm against a blank. The content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (50, 100, 200, 400 μg/ml; $Y = 3.2453X - 0.0703$; $R^2 = 0.9963$), and the results are expressed as mg of ascorbic acid/100 g of *W. somnifera* (dry weight of plant materials).

Anthocyanin determination

Total anthocyanin content was calculated using the pH differential method [14]. Briefly, aliquots of WSREt, WSFEt and WSLEt (1 ml) were diluted to 10 ml with a pH 1.0 solution (125 ml of 0.2 M KCl and 375 ml of 0.2 M HCl). A second aliquot (1 ml) was diluted to 10 ml with chloride-hydrochloride acid buffered solution (pH 4.5). The absorbance of the solutions was measured at

510 nm, and the concentration (C) of anthocyanins was calculated using the equation below:

$$C\ mg/100\ g = [(Abs\ pH\ 1.0 – Abs\ pH\ 4.5) \times 484.82 \times 1000/24825] \times DF$$

Where the term in parentheses represents the difference between the absorbance (Abs) at 510 nm of the pH 1.0 and pH 4.5 solutions, 484.82 is the molecular mass of cyanidin-3-glucoside chloride, 24,825 is the molar absorptivity (ε) nm in the pH 1.0 solution, and DF is the dilution factor. Each experiment was performed in duplicate. The results are expressed as mg of anthocyanin/100 g of *W. somnifera* (dry weight of plant materials).

DPPH free radical scavenging activity

The antioxidant capacities of the *W. somnifera* extracts were also studied by evaluating their free radical scavenging effects on the DPPH radical, which was based on the method proposed by Ferreira et al. [15]. Briefly, 1 ml of WSREt, WSFEt or WSLEt (1 mg/ml each) was mixed with 2.7 ml of a methanol solution containing DPPH radicals (0.024 mg/ml). The mixture was vigorously shaken and incubated at room temperature in the dark for 60 min, at which time their absorbances remained unchanged. Reduction of the DPPH radical was determined by measuring the absorbance at 517 nm [16]. Radical scavenging activity (RSA) was calculated as the percentage of DPPH discoloration using the following equation: $\%\ RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The 50% maximal inhibitory concentration (IC_{50}) was determined as the concentration of the tested WSREt, WSFEt or WSLEt sample resulting in a 50% reduction of the initial DPPH concentration, measured from the linear regression concentration curve of the test extract (μg/ml) against the percentage of the radical scavenging inhibition.

Ferric reducing/antioxidant power assay (FRAP assay)

The FRAP assay was performed according to a modified method described by Benzie and Strain [17]. Briefly, 200 μL of WSREt, WSFEt or WSLEt (1 mg/ml) was mixed with 1.5 ml of the FRAP reagent, and the reaction mixture was incubated at 37°C for 4 min. The absorbance was then read at 593 nm against a blank that was prepared using distilled water. The preparation of FRAP reagents was as follows: (i) Reagent A (300 mM/l acetate buffer (pH 3.6)); (ii) Reagent B, TPTZ solution, (0.031 g of TPTZ was added to 10 ml of 40 mM HCl and dissolved at 50°C); and (iii) Reagent C, ferric chloride solution, 0.54 g of ferric chloride was dissolved in 10 ml of distilled water. To make the FRAP reagent (30 ml), 2.5 ml of reagent B and 2.5 ml of reagent C were added to 25 ml of reagent A. The FRAP reagent was prewarmed to 37°C. Reagents B and C were freshly prepared immediately before the assay was performed. A calibration curve was generated using aqueous solutions of $FeSO_4$ at 100, 200, 400, 600 and 1000 μM. Ascorbic acid and BHT were used as positive controls. FRAP values were expressed as micromoles of ferrous equivalent [mmole Fe (II)] per kg of *W. somnifera* extract (DW).

Ferrous ion (Fe^{2+}) chelation

Ferrous ion chelating activity was evaluated using a standard method [18] with minor modifications. The reaction was performed in HEPES buffer (20 mM, pH 7.2). Briefly, different concentrations (0, 7.5, 15, 30, 60, or 120 μg/ml) of plant extract were added to 12.5 μM ferrous sulfate solution, and the reaction was initiated by the addition of ferrozine (75 μM). Ferrozine was dissolved in water to a concentration of 5 mg/ml. The mixture was shaken vigorously and incubated for 20 min at room temperature. The absorbance was then measured at 562 nm [19]. All tests were performed in triplicate, and mean values are reported. EDTA was used as a positive control. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated using the following formula:

$$\text{Bound } Fe^{2+}(\%) = [1 - (\lambda_{562} - S)]/(\lambda_{562} - C)\ X\ 100$$

Where λ_{562}-C is the absorbance of the control and λ_{562}-S is the absorbance in the presence of WSREt, WSFEt, WSLEt or the standards. The control contained only $FeCl_2$ and ferrozine [20]. The 50% maximal inhibitory concentration (IC_{50}) was determined as the concentration of the tested WSREt, WSFEt or WSLEt sample causing 50% inhibition of ferrozine–Fe^{2+} complex formation, measured from the linear regression concentration curve of the test extract (mg/ml) against the percentage of bound Fe^{2+}.

Inhibition of β-carotene bleaching

The antioxidant activities of the methanolic extracts of *W. somnifera* were evaluated using the β-carotene linoleate model system. A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two milliliters of this solution was pipetted into a 100 ml round-bottom flask. After the chloroform was removed at 40°C under vacuum, 45 μl of linoleic acid, 400 μl of Tween 80 emulsifier and 100 ml of distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing 0.2 ml of various concentrations of the *W. somnifera* solutions or phenolic extracts. The tubes were shaken and incubated at 50°C

in a water bath. Upon the addition of the emulsion to each tube, the time zero absorbance was measured at 470 nm using a spectrophotometer. The absorbance was then recorded at 20 min intervals until the control sample changed color. A blank, devoid of β-carotene, was prepared for background subtraction [21].

Lipid peroxidation (LPO) inhibition was calculated using the following equation:

% of LPO inhibition = (β – carotene content; after 2 hr of assay/initial β – carotene content) × 100. Ascorbic acid and TBHQ were used as positive controls.

Antibacterial properties of *W. somnifera*

Extract sterility

The extracts were filtered using Millipore nylon membranes (0.45 μm) and then tested for sterility by introducing 2 ml of the extract into 10 ml of sterile nutrient broth. The extracts were incubated at 37°C for 24 hr. A sterile extract was indicated by the absence of turbidity or the clarity of the broth after the incubation period [22].

Bacterial strains

The *in vitro* antimicrobial activities of WSREt, WSFEt and WSLEt were investigated. Five pathogenic bacteria, namely *Escherichia coli, Salmonella typhi, Citrobacter freundii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae,* were obtained from the Bangladesh Institute for Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM). All of the microorganisms were maintained at 4°C on nutrient agar slants. The bacterial strains were re-identified on the basis of their morphological, cultural and biochemical characteristics [23] as an additional confirmatory step.

Determination of antibacterial activity

The antibacterial activities of WSREt, WSFEt and WSLEt were determined using the agar well diffusion method following a published procedure with slight modifications [24]. Briefly, for the evaluation of antimicrobial activities, a fresh 24 hr culture of bacteria was suspended in sterile distilled water to obtain a turbidity of 0.5 McFarland units. The final inoculum size was adjusted to 5×10^5 CFU/ml. Nutrient agar (nutrient broth+ 1.8% agar) was inoculated with the given microorganism by spreading the bacterial inoculum on the media. Wells (8 mm diameter) were punched in the agar and filled with 200 μl of the plant extracts (5 mg/ml). Negative control wells containing neat solvent (80% aqueous methanol) or a standard antibiotic solution of tetracycline (100 μg/ml) (positive control) were run in parallel on the same plate. The plates were incubated at 37°C for 24 hr. Antibacterial activities were assessed by measuring the diameters of the zones of inhibition for the respective drugs. The relative antibacterial potency of a given preparation was calculated by comparing its zone of inhibition with that of the standard antibiotic tetracycline.

Determination of the minimum inhibitory concentration (MIC) of the extract

The initial plant extract (100 mg/ml) was serially diluted by transferring 5 ml of the sterile plant extract (stock solution) into 5 ml of sterile nutrient broth to obtain the following dilutions: : 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and finally 3.125 mg/ml [25]. After obtaining the different extract concentrations, each concentration was inoculated with 0.1 ml of a standardized bacterial cell suspension (approximately 10^6 CFU/ml) and incubated at 37°C for 24 hr. The lowest concentration of the extract that inhibited the growth of the test organism was taken as the MIC. The controls were prepared as follows: (i) nutrient broth only (positive control), (ii) nutrient broth and sterile plant extract, (iii) nutrient broth and a test organism (positive control), and (iv) the standard antibiotic tetracycline (positive control).

Statistical analyses

All analyses were performed in triplicate, and the data are expressed as the mean ± standard deviation (SD). The data were analyzed using the Statistical Package for the Social Sciences Version 12.0 (SPSS Inc., USA) and MS Excel 2003. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test was used to compare the data. Differences of $P < 0.05$ were considered significant.

Results

Ascorbic acid contents of WSREt, WSFEt and WSLEt

The highest concentration of ascorbic acid was found in WSLEt. WSREt had the lowest ascorbic acid concentration, and an intermediate concentration of ascorbic acid was found in WSFEt (Table 1).

Anthocyanin contents

The highest concentration of anthocyanins was also found in WSLEt (12.5 ± 1.04 mg/ 100 g), and WSREt had the lowest concentration (2.86 ± 1.44 mg/ 100 g) (Table 1).

Table 1 Ascorbic acid and anthocyanin contents (mg/ 100 g dry weight plant materials) in WSREt, WSFEt and WSLEt

Sample	Ascorbic acid	Anthocyanin
WSLEt	62.60±1.26^a	12.56±1.04^a
WSREt	20.60±1.06^c	2.86±1.44^c
WSFEt	40.00±0.40^b	5.66±0.52^b

Different letters in each column indicate a significant difference ($p<0.05$).

DPPH radical scavenging activity (IC_{50})

The DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extract [26]. The IC_{50} values for the DPPH radical scavenging activities of WSLEt, WSREt and WSFEt are shown in Table 2 in comparison with the ascorbic acid and BHT controls.

FRAP assay

The FRAP assay provides a direct estimation of the level of antioxidants or reductants present in a sample and is based on the ability of the analyte to reduce the Fe^{3+}/Fe^{2+} pair. WSLEt exhibited the highest FRAP value (3.29 mM Fe/kg), whereas WSREt had the lowest (2.26 μM Fe/kg). Ascorbic acid and BHT were used as positive controls, and the FRAP values of these controls were higher when compared to all three plant parts (Table 2). These results indicate that the leaves are a good potential source of antioxidants, followed by the fruits and roots.

Ferrous chelation activity

As an additional approach, the ferrous chelation assay was performed. Ferrozine produces a violet complex with Fe^{2+}, and in the presence of a chelating agent, complex formation is interrupted, resulting in a decrease of the intensity of the violet color. The results demonstrate that formation of the ferrozine-Fe^{2+} complex was inhibited in the presence of the test and reference compounds (Table 2).

Inhibition of β-carotene bleaching

The effect of 60 μg/ml of WSLEt, WSREt, WSFEt and the reference compounds ascorbic acid and TBHQ on the lipid peroxidation of a linoleic acid emulsion is shown in Table 2. Peroxidation of the linoleic acid emulsion was inhibited by 79.67, 69.87 and 72.11% when WSLEt, WSREt and WSFEt, respectively, were used, indicating that the leaves have the highest antioxidant properties. The reference compounds ascorbic acid and TBHQ exhibited 100.28% and 104.52% inhibition of peroxidation, respectively.

Antimicrobial properties

As shown in Table 3, the antimicrobial activities of WSREt, WSFEt and WSLEt and their potencies were quantitatively assessed by the presence or absence of a zone of inhibition and the zone diameter, respectively. The methanolic extracts (80%) of the three plant parts displayed antimicrobial activities against all five pathogenic bacteria. WSLEt displayed the highest antibacterial activity against all of the pathogenic bacteria tested compared with WSREt and WSFEt.

Overall, WSLEt exhibited the greatest zone of inhibition against all five microorganisms. For the specific plant parts, WSLEt exhibited the highest activity against *S. typhi*, whereas the lowest activity was against *K. pneumoniae*. The largest zone of inhibition for WSFEt was against *S. typhi*, and the smallest zone of inhibition was also against *K. pneumoniae*. For WSREt, the largest zone of inhibition was against *E. coli*, whereas the smallest zone of inhibition was against *P. aeruginosa*.

The MICs for WSREt, WSFEt and WSLEt against the five pathogenic bacteria are shown in Table 4. WSLEt had the lowest MIC against *S. typhi*, whereas its highest MIC values were against *C. freundii*, *P. aeruginosa* and *K. pneumonia*. The lowest MIC for WSFEt (25.00 mg/ml) was found for *E. coli* and *S. typhi*, whereas MICs of 50 mg/ml were found for *C. freundii*, *P. aeruginosa* and *K. pneumoniae*. For WSREt, the lowest MIC (25.00 mg/ml) was found for *E. coli*, whereas its MIC was similar for the four other bacterial species, that is, 50.00 mg/ml.

Discussion

This study is the first to report and compare the antioxidant and antibacterial properties of the different parts of *W. somnifera*, namely the leaves, roots and fruits. Our study is also the first to report on the ascorbic acid levels of WSFEt and WSLEt as well as the anthocyanin concentrations present in *W. somnifera*. We found that *W. somnifera* leaves have the highest antioxidant and

Table 2 Results of DPPH radical scavenging activity (IC_{50}), FRAP assay, Ferrous chelation activity and Inhibition of lipid peroxidation (LPO)

Sample	DPPH IC_{50} (μg/ml)	FRAP (mmole Fe/kg)	Fe^{2+} chelation IC_{50} (mg/ml)	% of LPO inhibition
WSLEt	101.73 ± 8.96^{c}	3.29 ± 8.03^{c}	0.22 ± 0.04^{c}	79.67 ± 3.85^{c}
WSREt	801.93 ± 7.92^{a}	2.26 ± 1.46^{e}	0.37 ± 0.02^{b}	69.87 ± 4.85^{d}
WSFEt	345.68 ± 8.98^{b}	3.13 ± 21.52^{d}	0.65 ± 0.02^{a}	72.11 ± 6.40^{d}
Ascorbic acid	25.95 ± 1.08^{d}	10.86 ± 9.52^{b}	NA	100.28 ± 1.07^{b}
BHT	38.14 ± 0.89^{d}	12.57 ± 13.23^{a}	NA	NA
EDTA	NA	NA	0.13 ± 0.01^{d}	NA
TBHQ	NA	NA	NA	104.516 ± 6.34^{a}

Different letters in each column indicate a significant difference ($p<0.05$). NA = Not applicable.

Table 3 Diameters of the zones of inhibition for the 80% aqueous methanolic extracts of WSREt, WSFEt and WSLEt on several species of Gram-negative bacteria

Test organisms	Diameters of zones of inhibition (mm)			
	WSREt	WSFEt	WSLEt	Tetracycline (100 μg/ml)
Escherichia coli	15.00 ± 0.96[c]	12.00 ± 1.50[d]	28.00 ± 0.56[b]	43.00 ± 1.90[a]
Salmonella typhi	13.00 ± 0.43[c]	14.00 ± 0.79[c]	32.00 ± 0.75[b]	47.00 ± 1.42 [a]
Citrobacter freundii	11.00 ± 0.86[c]	9.00 ± 1.21[c]	23.00 ± 1.27[b]	38.00 ± 1.22 [a]
Pseudomonas aeruginosa	9.00 ± 1.32[c]	10.00 ± 1.43[c]	26.00 ± 1.08[b]	40.00 ± 0.59 [a]
Klebsiella pneumoniae	10.00 ± 1.10[c]	8.00 ± 1.61[c]	19.00 ± 1.48[b]	36.00 ± 1.13 [a]

Each value represents the mean ± SD of three different observations. Different letters in each row indicate a significant difference ($p<0.05$).

antimicrobial activities as well as the highest ascorbic acid and anthocyanin contents. These observations may be related to the high phenolic and flavonoid contents reported in our previous study [8,27].

The highest concentration of ascorbic acid was found in WSLEt, whereas WSREt had the lowest ascorbic acid content. Hussain et al., [28] reported a higher vitamin C content (51.50 mg/ 100 g) in WSREt. In another study [29], the ascorbic acid contents of the leaves and roots of *W. somnifera* were compared, and the roots were found to possess a slightly higher ascorbic acid content. This variability may be due to differences in the source of the *W. somnifera* plant used. Anthocyanins are flavonoids [30] and are among the important phenolic substances in *W. somnifera.* Flavonoids are polyphenolic compounds with known properties that include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action [31]. The mechanisms of action of flavonoids are through scavenging or chelating processes [32]. It has been reported that the anthocyanins present in *W. somnifera* are chemically-similar to the ones present in black raspberry (*Rubus occidentalis*) [33]. As with ascorbic acid, the content of total anthocyanins in WSLEt, WSREt and WSFEt varies in different parts of the plant and is highest in the leaves. However, this level is lower than the highest concentration of anthocyanins found in tomato, which is 2.83 ± 0.46 mg/g [34].

Free radical DPPH scavenging ability is extensively used to indicate the antioxidant potential of naturally derived foods and plants. We found that *W. somnifera* exhibited good DPPH free radical scavenging abilities. WSLEt had the lowest IC_{50}. IC_{50} values between 206.77 and 224.96 μg/ml [35] have been reported in previous studies involving only *W. somnifera* leaves. These researchers indicated that the IC_{50} values for plant extracts harvested from polluted sites are higher when compared with those harvested from non-polluted sites.

A relatively higher FRAP value, as was determined for WSLEt, indicated a greater reduction of ferric ions to ferrous ions and higher antioxidant properties in the leaves when compared with the other plant parts (Table 2).

Metal chelating capacity is important because it reduces the concentration of transition metals that catalyze lipid peroxidation [36]. Ferrous chelation tests were performed, and the results showed that although WSREt, WSFEt and WSLEt did not display chelation activities as high as that of the EDTA standard (Table 2), the decrease in the concentration-dependent color formation in the presence of the extracts indicated that they did exhibit some amount of iron-chelating activity.

However, *W. somnifera* extracts exhibited high inhibitory effects to lipid peroxidation of linoleic acid. The activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging [37]. High inhibitory effect of lipid peroxidation of the extracts could be due to the abundant presence of antioxidant active compounds. Therefore, these extracts may be effective agents in retarding Fe^{2+} catalyzed lipid oxidation.

These results indicate that the antioxidant capacity of each extract may be related to the concentrations of ascorbic acid, anthocyanin and polyphenols. The antioxidant activities of these compounds depends on their molecular structures, that is, on the availability of phenolic hydrogens which result in the formation of phenoxyl radicals due to hydrogen donation [38].

For the antibacterial activity study, the 80% methanolic extract of all parts of *W. somnifera* displayed activity against five pathogenic Gram-negative bacteria, namely

Table 4 Determination of the minimum inhibitory concentration (MIC) of the methanolic extracts of the, WSFEt and WSLEt on the test organisms

Test organisms	Concentrations (mg/ml)			
	WSREt	WSFEt	WSLEt	Tetracycline
Escherichia coli	25.00	25.00	12.50	0.03
Salmonella typhi	50.00	25.00	6.25	0.01
Citrobacter freundii	50.00	50.00	25.00	0.05
Pseudomonas aeruginosa	50.00	50.00	25.00	0.05
Klebsiella pneumoniae	50.00	50.00	25.00	0.03

E. coli, S. typhi, C. freundii, P. aeruginosa and *K. pneumoniae,* to different magnitudes. *W. somnifera* leaves possessed the greatest antimicrobial effects. Phenolics, ascorbic acid and anthocyanins are associated with the antimicrobial efficiency of the plant because they cause hyperacidification at the plasma membrane interface of the pathogen, which potentially results in the disruption of the H^{+}-ATPase required for ATP synthesis [39].

The most susceptible organism was *S. typhi*, indicating that the *W. somnifera* extracts contain active compounds that can inhibit the proliferation and growth of *S. typhi.* which can cause diseases such as typhoid fever and foodborne illnesses. This finding may support the traditional uses of *W. somnifera* as a therapeutic agent for diarrhea, dyspepsia and gastrointestinal disorders [10]. The other tested bacteria also exhibited significant sensitivities against WSREt, WSFEt and WSLEt. These results demonstrate that the methanolic extracts contained the expected compounds for antibacterial activities against the five tested Gram-negative bacteria. *W. somnifera* may be exploited as a natural drug for the treatment of several infectious diseases initiated by these organisms. This finding is important in the quest for new antimicrobial agents because organisms with multidrug resistance are rapidly emerging.

Jain and Varshney [9] reported on the antibacterial activity of the methanolic extracts of the whole *W. somnifera* plant against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans* and *Candida albicans,* with zones of inhibition of 38, 36, 15, 38 and 32 mm, respectively. These results are similar to our reported zones of inhibition for WSLEt against two of the same organisms, *Escherichia coli* and *Pseudomonas aeruginosa,* at 28 ± 0.56 and 26 ± 1.08 mm, respectively. However, Jain and Varshney [9] reported that aqueous extracts of *W. somnifera* had higher antimicrobial activities (a zone of inhibition between 33 and 50 mm) when compared with methanolic extracts. In this study, methanol was used to extract low molecular weight and moderately polar substances because of its wide range of solubility. However, Jain and Varshney [9] did not report any MIC values for comparison with our own values.

Ascorbic acid, anthocyanin and polyphenols have been reported to exhibit antibacterial activities with distinguishing characteristics for their reactivities with proteins related to polyamide polymers [40]. The inhibition of microorganisms by these antioxidant compounds may be due to iron deprivation or hydrogen bonding with vital proteins such as microbial enzymes [41]. Antioxidant compounds such as ascorbic acid, anthocyanin and polyphenols are vulnerable to polymerization in air through oxidization reactions. Therefore, an important factor governing their toxicity is their polymerization size. It has been reported that the oxidized condensation of antioxidant compounds may result in the toxification of microorganisms [41]. These findings support the fact that ascorbic acid, anthocyanin and polyphenols may be responsible for the antimicrobial activities of WSREt, WSFEt and WSLEt.

The phytochemical constituents quantified in the present study such as ascorbic acid as well as anthocyanin and as also determined in our previous study i.e. the polyphenols and flavonoids [8] are of significant medicinal importance and may act as antioxidant, antimicrobial and immunomodulatory agents. In the future, the above phytoconstituents could be used as a major tool for obtaining a quality control profile for a drug.

Conclusion

Our results clearly indicate that *W. somnifera*, particularly the leaves, has remarkable antioxidant properties. Additionally, the leaves possess significant antibacterial properties against Gram-negative organisms, in particular, *S. typhi*. It will be beneficial to investigate the active compounds present in *W. somnifera* so that its leaves can be used to increase the armamentarium of antimicrobial agents and so that other possible therapeutic uses of the plant can be explored.

Competing interests
The authors declare no competing interests.

Authors' contributions
NA, MIK and MAM performed the experiments. MH, SAS and GSH supervised the work, evaluated the results and revised the manuscript for publication. All the authors read and approved the final manuscript.

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References

1. Ostrowska B, Rzemy KZ: **Antioxidant effects a basis of drug selection.** *Herba Pol* 1998, **44**(4):417.
2. Hazra B, Biswas S, Mandal N: **Antioxidant and free radical scavenging activity of *Spondias pinnata*.** *BMC Compl Alternative Med* 2008, **8**:63.
3. Yazdanparast R, Bahramikias S, Ardestani A: ***Nasturtium oficinale* reduces oxidative stress and enhances antioxidant capacity in hypercholesterolaemic rats.** *Chem Biol Interact* 2008, **172**:176–184.
4. Jovanovic SV, Simic MG: **Antioxidants in nutrition.** *Ann NY AcadSci* 2000, **899**:326–334.
5. Sangwan RS, Chaurasiya ND, Misra LN, Lal P, Uniyal GC, Sharma R, Sangwan NS, Suri KA, Qazi GN, Tuli R: **Phytochemical variability in commericial herbal**

products and preparations of *Withania somnifera* (ashwagandha). *Curr Sci* 2004, **86**:461–465.
6. Kulkarni SK, George B, Mathur R: **Protective effect of *Withania somnifera* root extract on electrographic activity in a lithiumpilocarpine model of status epilepticus.** *Phytother Res* 1998, **12**:451–453.
7. Facciola S: *Cornucopia - A Source Book of Edible Plants.* Vista: Kampong Publications; 1990.
8. Alam N, Hossain M, Khalil MI, Moniruzzaman M, Sulaiman SA, Gan SH: **High catechin concentrations detected in *Withania somnifera* (ashwagandha) by high performance liquid chromatography analysis.** *BMC Compl Alternative Med* 2011, **11**:65.
9. Jain P, Varshney R: **Antimicrobial activity of aqueous and methanolic extracts of *Withania somnifera* (Ashwagandha).** *J Chem Pharmaceut Res* 2011, **3**(3):260–263.
10. Acharyya S, Patra A, Bag PK: **Evaluation of the antimicrobial activity of some medicinal plants against enteric bacteria with particular reference to multi-drug resistant *Vibrio cholerae*.** *Trop J Pharmaceut Res* 2009, **8**(3):231–237.
11. Pietta P: *Flavonoides as antioxidant. J Nat Prod* 2000, **63**:1035–1042.
12. Kahkonen MP, Hopia AI, Vuorela HJ PRJ, Pihlaja K, Kujala TS, Heinonen M: **Antioxidant Activity of Plant Extracts Containing Phenolic Compounds.** *J Agric Food Chem* 1999, **47**:3954–3962.
13. Khalil MI, Moniruzzaman M, Boukraâ L, Benhanifia M, Islam MA, Islam MN, Sulaiman SA, Gan SH: **Physicochemical and Antioxidant Properties of Algerian Honey.** *Molecules* 2012, **17**:11199–11215.
14. Gulcin I: *Antioxidant properties of resveratrol: a structure-activity insight.* 2010, **11**:210–218.
15. Ferreira ICFR, Aires E, Barreira JCM, Estevinho LM: **Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract.** *Food Chem* 2009, **114**:1438–1443.
16. Hatano T, Kagawa H, Yashura T, Okuda T: **Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects.** *Chem Pharmaceut Bull* 1988, **36**:2090–2097.
17. Benzie IFF, Strain JJ: **Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified. version for simultaneous measurement of total antioxidant power and ascorbic acid concentration.** *Methods Enzymol* 1999, **299**:15–27.
18. Haro-Vicente JF, Martinez-Gracia C, Ros G: **Optimization of in vitro measurement of available iron from different fortificants in citric fruit juices.** *Food Chem* 2006, **98**:639–648.
19. Gulcin I: **Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid).** *Toxicology* 2006, **217**:213–220.
20. Gülçin I, Elias R, Gepdiremen A, Chea A, Topal F: **Antioxidant activity of bisbenzylisoquinoline alkaloids from Stephania rotunda: cepharanthine and fangchinoline.** *J Enzym Inhib Med Chem* 2010, **25**:44–53.
21. Mi Y, Tae Hun K, Nak Ju S: **Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus* of *Hymenochaetaceae*) extracts.** *Food Chem* 2003, **82**:593–597.
22. Atlas, Ronald M: *Micro-organisms in our World.* St. Louis, Missouri: Mosby-Year Book, Inc; 1995:765 blz. ISBN 0 8016 7804 8.
23. Cheesbrough M: **District laboratory practice in tropical countries.** *ECBS Cambridge University Press edition* 2000, **2**:256–267.
24. Perez C, Pauli M, Bazerque P: **An antibiotic assay by agar well diffusion method.** *Acta Biologica Medica Exp* 1990, **15**:113–115.
25. Ibekwe VI, Nnanyere NF, Akujobi CO: **Studies on Antibacterial activity and phytochemical qualities of various plant extracts.** *Int J Environ, Health and Human Development* 2001, **2**(1):41–46.
26. Koleva II, Van Beek TA, Linssen JPH, de Groot A, Evstatieva LN: **Screening of plant extracts for antioxidant activity: a comparative study on three testing methods.** *Phytochem Anal* 2002, **13**:8–17.
27. Kaileh M, Vanden Berghe W, Heyerick A, Horion J, Piette J, Libert C, De Keukeleire D, Essawi T, Haegeman G: **Withaferin A strongly elicits IkappaB kinase beta hyper- phosphorylation concomitant with potent inhibition of its kinase activity.** *J Biol Chem* 2007, **282**:4253–4264.
28. Hussain I, Khan L, Marwat GA: **Analysis of Vitamin C in Selected Medicinal Plants.** *J Chem Soc Pakistan* 2011, **33**(2):260–262.
29. Jaleel CA: **Antioxidant profile changes in leaf and root tissues of *Withania somnifera* Dunal *Plant*.** *Omics Journal* 2009, **2**(4):163–168.
30. Nielsen ILF, Haren GR, Magnussen EL, Dragsted LO, Rasmussen SE: **Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography. Investigation of their pH stability and antioxidative potency.** *J Agric Food Chem* 2003, **51**:5861–5866.
31. Frankel E: ***Nutritional benefits of flavonoids.*** *International conference on food factors: Chemistry and Cancer Prevention.* Hamamatsu: Japan Abstracts, C6- 2; 1995.
32. Kessler M, Ubeaud G, Jung L: **Anti- and pro-oxidant activity of rutin and quercetin derivatives.** *J Pharm Pharmacol* 2003, **55**:131–142.
33. Seeram, Navindra PS: **Berry Fruits: Compositional Elements, Biochemical Activities, and the Impact of Their Intake on Human Health, Performance, and Disease.** *J Agric Food Chem* 2008, **56**(3):627–629.
34. Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EGWM, Hall RD, Bovy AG, Luo J, *et al*: **Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors.** *Nat Biotechnol* 2008, **26**(11):1301–1308.
35. Sharma RK, Samant SS, Sharma P, Devi S: **Evaluation of antioxidant activities of *Withania somnifera* leaves growing in natural habitats of North-west Himalaya, India.** *Journal of Medicinal Plants Research* 2012, **6**(5):657–661.
36. Duh PD, Tu YY, Yen GC: **Antioxidant activity of water extract of Harng Jyur (*Chrysenthemum morifolium* Ramat).** *Lebnes wiss Technol* 1999, **32**:269–277.
37. Yildirim A, Mavi A, Oktay M, Kara AA, Algur OF, Bilaloglu V: **Comparison of antioxidant and antimicrobial activities of Tilia (*Tilia argentea* Desf Ex DC), Sage (*Savia triloba* L.), and Black Tea (*Camellia sinensis*) extracts.** *J Agric Food Chem* 2000, **48**(10):5030–5034.
38. Ramarathnam N, Ochi H, Takeuchi M: **Antioxidant defense system in vegetable extracts.** In *Natural Antioxidants; Chemistry, Health Effects and Application 1997.* Edited by Shahidi F. Champaign, IL: AOAC Press; 1997:76–87.
39. Vattem D, Lin YT, Labbe RG, Shetty K: **Antimicrobial activity against select food-borne pathogens by phenolic antioxidants enriched in cranberry pomace by solid-state bioprocessing using food grade fungus *Rhizopus oligosporus*.** *Process Biochem* 2004, **39**:1939–1946.
40. Haslam E: **Natural polyphenols (vegetable tannins) as drugs: possible modes of action.** *J Nat Prod* 1996, **59**:205–215.
41. Scalbert A: **Antimicrobial properties of tannins.** *Phytochemistry* 1991, **30**(12):3875–3883.

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Recent advances in elucidating the biological properties of *Withania somnifera* and its potential role in health benefits

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Abstract *Withania somnifera* (L.) Dunal (*Solanaceae*), also known as ashwagandha, is an important medicinal plant that is widely used as a home remedy for several diseases in the Indian subcontinent and other parts of the world. *W. somnifera* is a dietary supplement composed of various nutrients, polyphenols and alkaloids that have free radical scavenging capacity, as well as other chemical constituents that possess anti-inflammatory, antitumor, anti-stress, antioxidant, immunomodulatory, and rejuvenating properties. The mechanism of action for these properties are not fully understood. *W. somnifera* also appears to influence the endocrine, cardiopulmonary and central nervous systems. Toxicity studies reveal that *W. somnifera* can be used without side effects. The findings presented in this review are very encouraging and indicate that this herb should be studied more extensively to confirm these results and to reveal other potential therapeutic effects.

Keywords *W. somnifera* · Chemical constituents · Antioxidant · Therapeutic effects · Toxicity

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Abbreviations

ACP	Phosphatase
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
ATP	Adenosine triphosphate
CAT	Catalase
DM	Diabetes mellitus
ESR	Erythrocyte sedimentation
G6P	Glucose-6-phosphatase
GABA	Gamma aminobutyric acid
GAG	Glycosaminoglycan
GERL	Golgi-endoplasmic reticulum-lysosomes
GlcS	Glucosamine sulphate

GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione-S-transferase
HP	Hydroperoxide
HPA	Hypothalamic pituitary adrenal
LD_{50}	Lethal dose 50%
LPO	Lipid peroxidation
MDA	Malondialdihyde
MI	Myocardial infarction
MIR	Myocardial ischemia–reperfusion injury
NO	Nitric oxide
SOD	Superoxide dismutase
STDs	Sexually transmitted diseases
T_3	Triiodothyronine
T_4	Thyroxine
TBARS	Thiobarbituric acid reactive substances
WSG	*W. somnifera* glycowithanolides

Introduction

Withania somnifera Dunal (*Solanaceae*), also known as Ashwagandha or winter cherry, is one of the most valuable plants in the traditional Indian systems of medicine. It is used in more than 100 formulations in Ayurveda, Unani and Siddha and is therapeutically equivalent to ginseng (Sangwan et al. 2004). Phytochemically, this plant is unique because it possesses the largest and most structurally diverse set of *withanolides* (i.e., modified steroidal molecules based on an ergostane skeleton and named after the plant) with *glycowithanolides* being the major bioactive constituent of *W. somnifera*. Because the ethnopharmacological properties of the plant include adaptogenic, anti-sedative and anti-convulsion activities, it is no surprise that the plant is employed to treat various neurological disorders, geriatric debilities, arthritis, stress and behavior-related problems (Bhattacharya et al. 1987; Dhuley 2001; Kulkarni et al. 1998; Ray and Gupta 1994; Schliebs et al. 1973). Several modern molecular pharmacological studies have demonstrated a correlation between these therapeutic actions and one or more of the chemical constituents present in the herb (Bargagna-Mohan et al. 2006; Ichikawa et al. 2006; Kaileh et al. 2007; Kinghorn et al. 2004; Tohda et al. 2005). *W. somnifera* has long been used for all age groups of both sexes and even during pregnancy without any side effects (Sharma et al. 1985).

Fig. 1 *W. somnifera* plant and root

Because of the growing interest and worldwide therapeutic usage of *W. somnifera*, the objective of this paper is to review the chemical properties, therapeutic benefits and toxicity of this herb in the hopes of increasing awareness of its advantages and disadvantages so that the public can make informed decisions when using this herb (Fig. 1).

Biochemical constituents

The major biochemical constituents of the *W. somnifera* roots and leaves are steroidal alkaloids and steroidal lactones from a special class of constituents called withanolides (Elsakka et al. 1990). Laboratory analyses have revealed that over 35 chemical constituents are present in the *W. somnifera* roots (Rastogi and Mehrotra 1998), and withanolides are believed to account for the unique medicinal properties of *W. somnifera*. withanolides bear a resemblance, both in action and structure, to the active constituents of Asian ginseng (*Panax ginseng*), known as ginsenosides (Grandhi 1994).

The concentration of withanolides usually ranges from 0.001 to 0.5% dry weight (DW) basis in the roots and leaves (Anonymous 2004). The withanolides are a group of naturally occurring C28-steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. The basic structure (Fig. 2) is a 22-hydroxyergostan-26-oic acid-26,22-lactone and designated as the withanolide skeleton (Alfonso et al. 1993; Glotter 1991).

There are many novel structural variants of withanolides with modifications either of the carbocyclic skeleton or the side chain and these have often been described as modified withanolides or ergostan type steroids related to withanolides. These compounds are generally polyoxygenated and it was found that plants

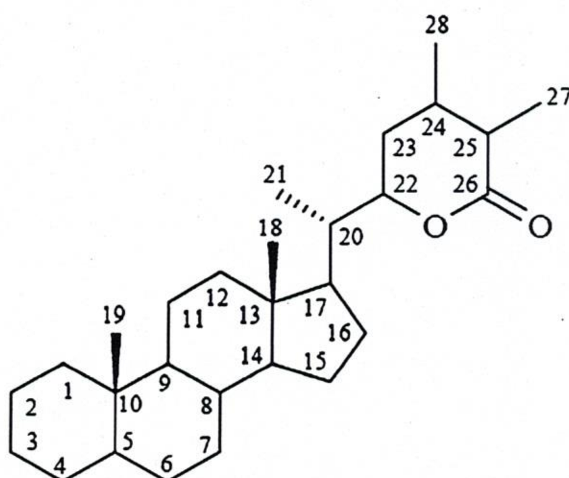

Fig. 2 Withanolide skeleton

elaborating them have an enzyme system capable of oxidizing all carbon atoms in a steroid nucleus. The characteristic feature of withanolides and ergosane-type steroids is one C_8 or C_9-side chain with a lactone or lactol ring but the lactone ring may be either six-membered or five membered and may be fused with the carbocyclic part of the molecule through a carbon–carbon bond or through an oxygen bridge. Appropriate oxygen substituents may lead to bond scission, formation of new bonds, aromatization of rings and many other kinds of rearrangements resulting in compounds with novel structures (Mirjalili et al. 2009). Chemical structure of some important bioactive compounds are shown in Fig. 3.

Withanolide is the main alkaloid constituent of *W. somnifera*; the other alkaloid constituents include (Zhao et al. 2002) somniferine, somnine, somniferinine, withananine, pseudo-withanine, tropine, pseudotropine, 3-a-gloyloxytropane, choline, cuscohygrine, isopelletierine and anaferine andanahydrine (Bone 1996; Elsakka et al. 1990).

Zhao et al. (2002) isolated five new withanolide derivatives from the roots of *W. somnifera* on the basis of spectroscopic and physiochemical evidence. The new compounds were designed as (20*S*,22*R*)-3a,6a-epoxy 4b,5b,27-trihydroxy-1-oxowitha-24-enolide, 27-*O*-b-D-glucopyranosylpubesenolide 3-*O*-b-D-glucopyranosyl (1 → 6)-b-D-glucopyranoside (withanoside VIII, 9), 27-*O*-b-D-glucopyranosyl (1 → 6)-b-D-glucopyranosylpubesenolide 3-*O*-b-D-glucopyranosyl (1 → 6)-b-D-glucopyranoside (withanoside IX, 10), 27-*O*-b-D-glucopyranosylpubesenolide 3-*O*-b-D-glucopyranoside (withanoside X, 11), and (20*R*,22*R*)-1a,3b,20,27-tetrahydroxywitha-5,24-dienolide 3-*O*-b-D-glucopyranoside (withanoside XI, 12). Out of the five new compounds, withanolide A (2), (20*S*,22*R*)-4b,5b,6a,27-tetrahydroxy-1-oxowitha-2,24-dienolide (6), withanoside IV (14), withanoside VI (15) and coagulin Q (16) have showed significant neurite outgrowth activity at a concentration of 1 mM on a human neuroblastoma SH-SY5Y cell line.

Some other components in *W. somnifera*

Several reports have demonstrated some other chemical compounds in *W. somnifera*. A new dimeric thiowithanolide, named ashwagandhanolide was detected in roots [Figure 3(10)] (Subaraju et al. 2006). Gupta et al. (1996) detected alkaloids in all the plant parts (roots, fruits, leaves), with the highest content found in leaves. Another study has detected nicotine, somniferine, somniferinine, withanine, withananine, pseudowithanine, tropine, pseudotropine, 3α-tigloyloxytropane, choline, cuscohygrine, *dl*-isopelletierine and new alkaloids anaferine and anhygrine in this medicinal plant (Gupta and Rana 2007). The reported total alkaloid content in the roots of Indian *W. somnifera* varies between 0.13 and 0.31%, though much higher yields (up to 4.3%) have been recorded in plants of other regions/countries (Johri et al. 2005). Apart from these contents the plant also contains chemical constituents like acylsteryl glucosides, starch, hantreacotane, ducitol, and a variety of amino acids including aspartic acid, proline, tyrosine, alanine, glycine, glutamic acid, cystine, tryptophan, and high amount of iron (Gupta and Rana 2007).

Therapeutics properties of *W. somnifera*

In Ayurveda, *W. somnifera* is used to prepare medicinal Ashwagandha. Although there are few scientific studies on the health benefits of Ashwagandha, it has been reported to possess anti-inflammatory, antitumor, antistress, antioxidant, immunomodulatory, hematopoietic and rejuvenating properties. Here we discuss experimental findings related to *W. somnifera* therapeutic usage with Table 1 summarising all of the findings.

Fig. 3 Chemical structure of some important bioactive compounds in *W. somnifera*. *1* Withaferin A *2* withanolide A *3* withanolide D *4* Withanone *5* withanolide E *6* withanolide Q *7* withanolide R *8* withanolide P *9* acyl steryl glucosides *10* ashwagandhanolide

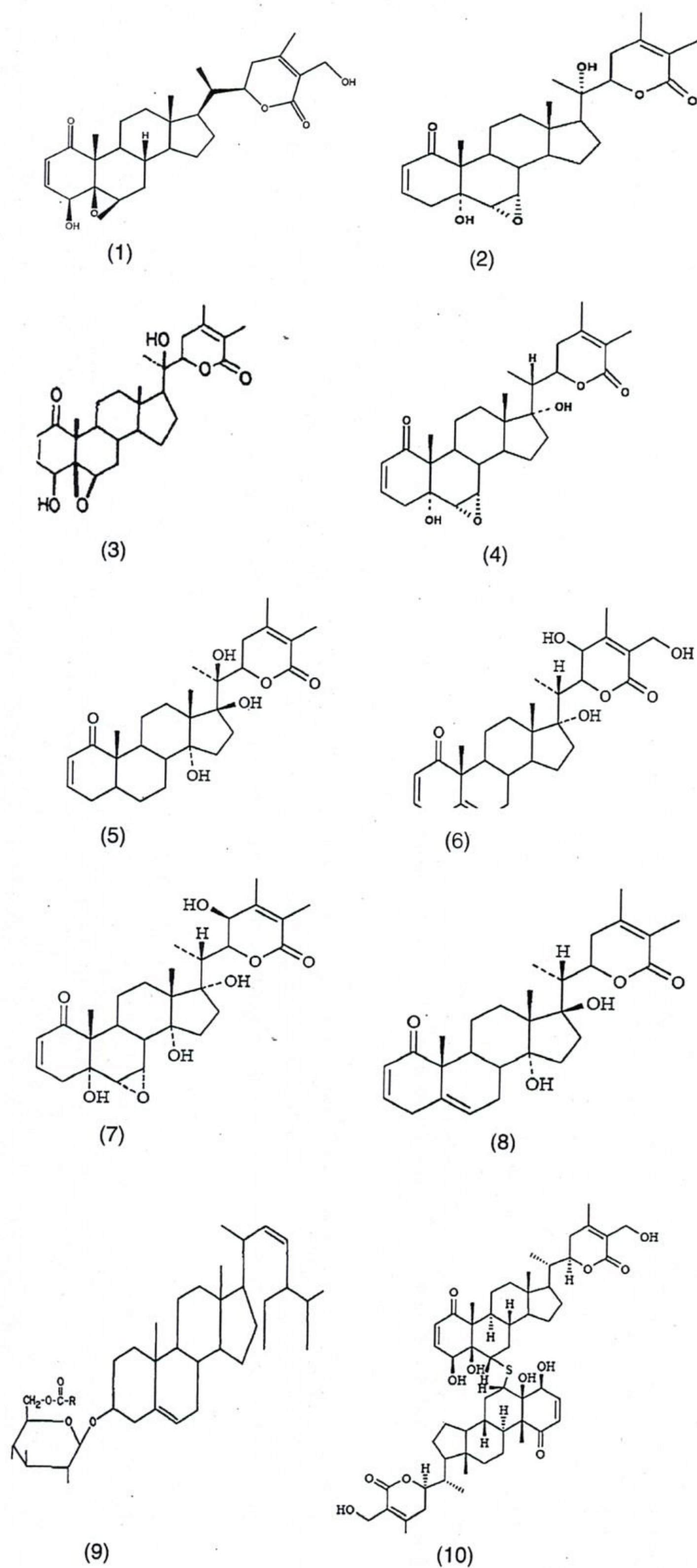

Table 1 Summary of the experimental findings on medicinal properties of *W. somnifera*

	Experimental design	Results
Anti-stress effects		
W. somnifera (whole herb)	Chronic stress in rodents, received a mild electric shock and given the herb an hour before the foot shock	Hyperglycemia, glucose intolerance, increase in plasma, corticosterone levels, gastric ulcerations, male sexual dysfunction, cognitive deficits, immunosuppression, mental depression and anti-stress adaptogenic effects (Bhattacharya et al. 2001; Bhattacharya et al. 1987; Bhattacharya and Muruganandam 2003)
W. somnifera	Anti-depressant and anti-anxiety effects are compared to the anti-depressant drug imipramine and the anti-anxiety drug lorazepam (Ativan®)	Anti-depressant and anti-anxiety effect (Archana and Namasivayam 1999)
W. somnifera root extracts	Rats subjected to multiple stress of cold, hypoxia and restraint (C–H–R) have been developed to evaluate adaptogenic properties with an oral dose of 100 mg/kg for 4–8 weeks	Colonic temperature recovery, reduced stress, enhanced open field behavior, emotional stability with a significant enhancement in the functional sensitivity of 5-HT2 receptors in the brain and a reciprocal sub-sensitivity of the 5-HT1A receptors (Kaur et al. 2001; Tripathi et al. 1998)
Anti-ulcer activity		
W. somnifera (methanolic extract)	*W. somnifera* was compared with ranitidine hydrochloride in a rat model	*W. somnifera* reduced pylorus ligation-induced gastric ulcers, the ulcer index, the volume of gastric secretion, free and total acidity and protected against gastric mucosal damage possibly due to its antioxidant effects (Bhatnagar et al. 2005)
Antioxidant activity		
W. somnifera root and leaf extract	Root and leaf extracts were administered to diabetic rats using glibenclamide as control	(i) Elevation in glucose and TBARS (ii) Significant reduction in glycogen, vitamins C and E, SOD, CAT, GPx, GST and GSH levels (Udayakumar et al. 2010)
W. somnifera glycowithanolides	*W. somnifera* was administered once daily for 21 days in chronic foot shock stress model	(i) Increase in SOD and lipid peroxidation activities (Bhattacharya et al. 2001; Dhuley 1998) (ii) Decrease in CAT and GPx activities in some brain regions (Bhattacharya et al. 2001)
Anti-tumor activity		
W. somnifera	(i) Used in animal cell cultures (ii) Evaluated the antitumor effects in urethane-induced lung tumors in adult male mice	(i) Decreased levels of NF-κB, suppressed intercellular tumor necrosis factor and potentiated apoptotic signaling in cancerous cell lines (Ichikawa et al. 2006) (ii) Acts against cancer by reducing tumor size (Jayaprakasam et al. 2003)
W. somnifera root powder	Used against benzo(a)pyrene-induced lung cancer in male Swiss albino mice	Alters the levels of immunocompetent cells, immune complexes and immunoglobulins (Senthilnathan et al. 2006)
Anti-inflammatory activity		
W. somnifera (powdered root)	Powdered root orally administered to rats	Anti-inflammatory responses are comparable to that of hydrocortisone sodium succinate (Begum and Sadique 1988)
W. somnifera (powdered root)	Powdered root orally administered to rats	Reduction in inflammation (Anbalagan and Sadique 1981)

Table 1 continued

	Experimental design	Results
W. somnifera root powder	A suspension orally administered to rats	Causes dose-dependent suppression of α2-macroglobulin (an indicator for anti-inflammatory drugs) (Anbalagan and Sadique 1984)
W. somnifera root powder	Male Wistar rats orally administered 1,000 mg/kg	(i) Decreases the GAGs content in the granuloma tissue (ii) Uncouples oxidative phosphorylation (Begum and Sadique 1987)
W. somnifera (root powder)	A Freund's adjuvant-induced arthritis rat model orally administered 1,000 mg/kg	(i) Causes a significant reduction in paw swelling (ii) observed degenerative changes by radiological examination (Begum and Sadique 1988)
Cardioprotective activity		
W. somnifera (hydro-alcohol extract)	Isoprenaline-(isoproterenol)-induced myocardial necrosis in rats using Vitamin E as a control	(i) Decreases glutathione, SOD, CAT, creatinine phosphokinase and lactate dehydrogenase (ii) increases lipid peroxidation marker malonyldialdehyde levels (Gupta et al. 2004; Mohanty et al. 2004)
W. somnifera	Ischemic rats	Alleviates stress-induced changes and provides cardioprotection (Dhuley 1998, 2000)
Hypolipidemic and anti-atherogenic activity		
W. somnifera	CapsHT2, a medicine comprising of several plants including *W. somnifera* was investigated for its lipid-reducing and anti-atherogenic effects	It acts against vascular intimal damage and atherogenesis, inhibits lipid peroxidation and enhances the release of lipoprotein lipase enzyme (Mary et al. 2003)
Hypoglycemic effect		
W. somnifera root and leaf extracts	Alloxan-induced diabetic rats	Possess hypoglycemic and hypolipidemic activities (Udayakumar et al. 2009)
Ethanolic extract of *W. somnifera*	Normal and streptozotocin-induced diabetic rat models	The maximum reduction was 47.1 and 42.7% in blood glucose levels of sub- and mild diabetic rats, respectively, with a dose of 750 mg/kg (Jaiswal et al. 2010)
W. somnifera roots powder	NIDDM and hypercholesterolemic human subjects were treated	Decreases blood glucose and is comparable with an oral hypoglycemic drug (Andallu and Radhika 2000)
Hypothyroid activity		
Aqueous extract of dried Withania root	Administered to mice via gastric intubation	Observed significant increases in serum T4 (Panda and Kar 1998; Panda and Kar 1999)
Immunomodulatory activity		
W. somnifera root extract	Administered to Swiss albino mice before and during exposure to a skin cancer-causing agent	Observed a significant decrease in incidence and average number of skin lesions as compared with the control group (Prakash et al. 2002)
Antimicrobial activity		
Aqueous and alcohol extracts of the *W. somnifera* root and leaves	Using an agar well diffusion method	Possesses strong antibacterial activity against a range of bacteria (Owais et al. 2005)
W. somnifera root tubers	Isolated a monomeric glycoprotein with a molecular mass of 28 kDa in SDS-PAGE	Demonstrated potent antimicrobial activity against the tested phytopathogenic fungi and bacteria (Girish et al. 2006)

Table 1 continued

	Experimental design	Results
Hexane and diethyl ether extracts from both leaves and *W. somnifera* roots	Using an agar plate disc-diffusion assay against *S. typhimurium* and *E. coli*	From the six extracts tested, only methanol and hexane extracts of both leaves and roots were found to have potent antibacterial activity (Arora et al. 2004)
Methanol leaf extracts of *W. somnifera*	Antibacterial activity test	Showed significant antibacterial and antifungal activity when compared to root and bark extracts (Mahesh and Satish 2008)
W. somnifera extract in different organic solvents	Disc diffusion assay	Hexane and ethyl acetate extracts showed maximum inhibition against *A. niger*. Methanol extracts showed maximum activity against *F. oxysporium* (13 mm) and *A. flavus* (15 mm), while aqueous extract showed maximum activity against *F. moniliformis* (9.6 mm) (Singh et al. 2010)

Anti-inflammatory properties of *W. somnifera*

Anbalagan and Sadique (1981) explored the ability of *W. somnifera* to ease arthritis symptoms and other inflammatory conditions and they found that the herb is an effective anti-inflammatory agent with higher activity as compared with hydrocortisone, a commonly-prescribed anti-inflammatory drug (Anbalagan and Sadique 1981). In another study, rats orally administered powdered *W. somnifera* roots an hour before injection with an inflammatory agent over a 3 days period showed that *W. somnifera* produces anti-inflammatory responses comparable to that of hydrocortisone sodium succinate (Begum and Sadique 1988).

Another study by Anbalagan and Sadique (1984) showed that *W. somnifera* caused a dose-dependent suppression of α_2-macroglobulin, an indicator for anti-inflammatory activity, in the serum of rats. In a study by Begum and Sadique (1987), air pouch granuloma was induced by subcutaneous injections of 4 ml of 2% (w/v) carrageenan in the dorsum of male Wistar rats and *W. somnifera* root powder (1,000 mg/kg) was orally administered. *W. somnifera* was found to decrease the glycosaminoglycan content in the granuloma tissue by 92% as compared with only 43.6% by hydrocortisone treatment, whereas no reduction was seen by phenylbutazone.

Begum and Sadique (1988) examined the effect of *W. somnifera* root powder orally administered daily to rats on paw swelling and bony degenerative changes in Freund's adjuvant-induced arthritis. *W. somnifera* was found to cause a significant reduction in both paw swelling and degenerative changes as observed by radiological examination, which was better than those produced by the reference drug hydrocortisone. Al-Hindawi et al. (1992) found that *W. somnifera* inhibited granuloma formation in cotton-pellet implantation in rats with comparable effects to hydrocortisone sodium succinate treatment.

Few studies have examined the mechanism of action of the anti-inflammatory properties of *W. somnifera*. In one study, rats injected with 3.5% formalin in their hind leg footpads showed a decrease in ^{14}C-glucose absorption in the jejunum (Somasundaram et al. 1983). Both *W. somnifera* and the cyclooxygenase inhibitor oxyphenbutazone maintained glucose absorption at normal levels and produced anti-inflammatory effects. Similar results were obtained in parallel experiments measuring ^{14}C-leucine absorption in the jejunum (Somasundaram et al. 1983). These studies suggest that cyclooxygenase inhibition may be involved in the *W. somnifera* mechanism of action.

Using a validated explant model of in vitro cartilage damage, Sumantran et al. (2008) evaluated the effects of *W. somnifera* root aqueous extracts and glucosamine sulphate (GlcS) on the levels of nitric oxide (NO) and GAGs secreted by the knee cartilage of chronic osteoarthritis patients. *W. somnifera* extracts significantly decreased NO release in the explants of a subset of patients (i.e., the anti-inflammatory response) and significantly increased the NO and GAG levels released in the explants of another subset (i.e., 'non-

responders'). Finally, it was suggested that the *W. somnifera* in vitro human cartilage damage model provides qualitative, statistically significant and quantitative preclinical data for the anti-inflammatory and chondroprotective activities of antiarthritic drugs.

Withaferin A is potent inhibitor of the pro-inflammatory transcription factors nuclear factor kappa B (NF-κB) and AP-1 and a promising agent for the treatment of the inflammatory cascade of cardiovascular diseases (Kaileh et al. 2007). In summary, *W. somnifera* has potential as an anti-inflammatory agent.

Antioxidant properties of *W. somnifera*

Many researchers have shown that *W. somnifera* has antioxidant effects of in vivo and in vitro (Bhattacharya et al. 1987, 2000; Gupta et al. 2003); these effects are related to the phenolic acids, flavonoids, alkaloids and other important phytochemicals present in various parts of the *W. somnifera* plant. Due to its varied therapeutic potential, *W. somnifera* is the subject of considerable scientific attention (Jaleel et al. 2008). high-performance liquid chromatography and MS/MS analysis of phenolic and flavonoids composition have showed that *W. somnifera* contain caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, catechin, tannic acid, kaempferol, quercetin and rutin (Prakash et al. 2002; Singh et al. 2010).

Researchers have discovered that some of the chemicals found in *W. somnifera* are powerful antioxidants. Studies conducted in rat brains showed that the herb produced an increased level of the three natural antioxidants SOD, CAT and GPx (Dhuley 1998). The antioxidant effect shown by the active components of *W. somnifera* roots may explain the reported anti-stress, cognition-facilitating, anti-inflammatory and anti-aging effects in experimental animals and in clinical situations (Bone 1996).

Another study showed that *W. somnifera* is a good source of nonenzymatic and enzymatic antioxidant components (Jaleel 2009). The main antioxidant constituents including nonenzymatic ascorbic acid, tocopherol and glutathione GSH and the enzymatic components SOD, ascorbate peroxidase, CAT, peroxidase and polyphenol oxidase levels were estimated from both leaves and roots. The analysis showed that the plant contains a significant quantity of nonenzymatic and enzymatic antioxidants in these organs.

Another study estimated the level of phenolic and flavonoid compounds in *W. somnifera* root and leaf extracts administered to diabetic rats and showed that the root extract has 28.26 mg/g total phenolic compounds and 17.32 mg/g flavonoids, while the leaf extract has less phenolic and flavonoid compounds (5.4 mg/g total and 5.1 mg/g, respectively). After treatment, the levels of urine sugar, blood glucose, liver glycogen and antioxidants such as vitamins C and E in plasma and SOD, CAT, GPX, GSH, thiobarbituric acid reactive substances (TBARS) and glutathione-S-transferase (GST) in the liver, kidney and heart were determined. Diabetic rats showed a significant ($P < 0.05$) elevation in glucose and TBARS and a significant ($P < 0.05$) reduction in glycogen, vitamins C and E, SOD, CAT, GPx, GST and GSH levels when compared to normal control rats receiving glibenclamide. These results suggest that the presence of phenolic compounds, including flavonoids, in *W. somnifera* root and leaf extracts and their antioxidant activity may play a vital role in blood glucose reduction in alloxan-induced diabetic rats (Udayakumar et al. 2010).

Bhattacharya et al. (2001) investigated the antioxidant activity of *glycowithanolides*, by using chronic foot-shock stress to induce changes in the rat brain frontal cortex and striatum. The stress procedure, performed once daily for 21 days, produced an increase in SOD and lipid peroxidation (LPO) activity with a concomitant decrease in CAT and GPx activities in both of the brain regions. *W. somnifera* glycowithanolides (WSG), orally administered 1 h prior to the 21-day stress procedure at doses of 10, 20 and 50 mg/kg, induced a dose-dependent reversal of the stress effects. WSG tended to normalize the augmented SOD and LPO activities and enhance the activities of CAT and GPx. These results indicate that at least part of chronic stress-induced pathology may be due to oxidative stress, which is mitigated by WSG, lending support to the clinical use of the plant as an anti-stress adaptogen.

Anti-aging effects of *W. somnifera*

In a double-blind clinical trial, a 3 g daily dose of *W. somnifera* was tested on a group of 101 healthy males, 50–59 years old, for 1 year. A significant improvement in hemoglobin (Hb), red blood cell

count, hair melanin and seated stature was observed, while serum cholesterol was decreased and nail calcium was preserved. ESR was also significantly decreased, with 71.4% of the subjects reporting an improvement in sexual performance (Bone 1996).

Anticancer, chemopreventive and immunomodulatory effects of *W. somnifera*

Research using animal cell culture has shown that *W. somnifera* decreases NF-kB levels, suppresses intercellular tumor necrosis factor and potentiates apoptotic signaling in cancerous cell lines (Ichikawa et al. 2006). To investigate its use in treating various forms of cancer, researchers have studied the antitumor effects of *W. somnifera*. One of the most exciting potential uses of *W. somnifera* in fighting cancers is due to its ability to reduce tumor size (Jayaprakasam et al. 2003; Prakash et al. 2002). In one study, the herb was evaluated for its effect on urethane-induced lung tumors in adult male mice. Following *W. somnifera* administration for a period of 7 months, the histological appearance of lungs treated with the herb was similar to those observed in control animal lungs (Jayaprakasam et al. 2003). *W. somnifera* has been reported to have anti-carcinogenic effects. Research (Winters 2006) has supported the polypharmaceutical use of *W. somnifera* and confirmed that the whole plant extract and several of its individual constituents possess antioxidant, anti-inflammatory, immunomodulating and anti-stress properties (Winters 2006).

A series of animal studies have demonstrated that *W. somnifera* has profound effects on the healthy production of white blood cells, which indicates that it is an effective immunoregulatory and chemoprotective agent (Kuttan 1996; Ziauddin et al. 1996). A recent study has suggested a possible mechanism for the increased cytotoxic effect of macrophages exposed to *W. somnifera* extracts (Iuvone et al. 2003). NO has been determined to have a significant effect on the macrophage cytotoxicity of microorganisms and tumor cells. Iuvone et al. (2003) demonstrated that *W. somnifera* increases NO production in mouse macrophages in a concentration-dependent manner. This effect was attributed to the increased production of inducible NO synthase, an enzyme generated in response to inflammatory mediators and known to inhibit the growth of many pathogens (Bogdan 2001).

Research has also shown that *W. somnifera* has stimulatory effects, both in vitro and in vivo, on cytotoxic T lymphocyte generation and demonstrated the potential to reduce tumor growth (Davis and Kuttan 2002). The chemopreventive effect was demonstrated in a study of *W. somnifera* root extract on induced skin cancer in Swiss albino mice given *W. somnifera* before and during exposure to the skin cancer-causing agent 7,12-dimethylbenz[a]anthracene (Prakash et al. 2002). A study of an alcohol extract of dried *W. somnifera* roots and the active component *withaferin A* isolated from the extract showed significant antitumor and radiosensitizing effects in in vivo experimental tumors and lacked any noticeable systemic toxicity. *Withaferin A* had a sensitizer enhancement ratio of 1.5 in the in vitro cell killing of V79 Chinese hamster cells at a nontoxic concentration of approximately 2 μM. This research indicates that *W. somnifera* could prove to be a good natural source of a potent and relatively safe radiosensitizer/chemotherapeutic agent (Devi 1996).

Central nervous system and adaptogenic effects of *W. somnifera*

In Ayurvedic medicine, *W. somnifera* is well known for its anti-stress activity. In a study that examined the effects of *W. somnifera* on chronic stress in rodents, the animals received a mild electric foot shock for a period of 21 days; the resulting animal stress produced hyperglycemia, glucose intolerance, increased plasma corticosterone levels, gastric ulcerations, male sexual dysfunction, cognitive deficits, immunosuppression and mental depression. The animals administered *W. somnifera* herb an hour before the foot shock experienced a significantly reduced stress level. This research confirms the theory that *W. somnifera* has significant anti-stress and adaptogenic effects (Bhattacharya et al. 2001; Bhattacharya and Muruganandam 2003).

Traditionally, *W. somnifera* has been used to stabilize mood in patients with behavioral disturbances. Studies of *W. somnifera* in rodents have revealed that the herb produces antidepressant and anti-anxiety activities that are comparable to the antidepressant drug imipramine and the anti-anxiety drug lorazepam (Archana and Namasivayam 1999). Studies of *W. somnifera* anti-anxiety effects conducted in the

Department of Pharmacology at the University of Texas Health Science Center indicated that *W. somnifera* extracts produce gamma aminobutyric acid (GABA)-like activity, which may account for the anti-anxiety effects of the herb (Mehta et al. 1991). In fact, *W. somnifera* is one of the most widely used tranquillizers in India, where it holds a similar position of importance as ginseng in China. It mainly acts on the reproductive and nervous systems, having a rejuvenating effect on the body and is used to improve vitality and aid recovery from chronic illness (Bhattacharya et al. 1987).

Chronic stress can cause cognitive deficit, immunosuppression, sexual dysfunction, gastric ulceration, irregular glucose homeostasis and a change in plasma corticosterone levels. In a chronic stress syndrome rat model, *W. somnifera* and *Panax ginseng* extracts were compared for their abilities to relieve some of the detrimental effects of chronic stress (Bhattacharya et al. 1987; Bhattacharya and Muruganandam 2003) and it was found that both of the extracts decrease the frequency and severity of stress-induced ulcers, reverse stress-induced inhibition of male sexual behavior and inhibit the effects of chronic stress on the retention of learned tasks. Although the activity of the *W. somnifera* extract was believed to be similar to the activity of the ginseng extract, *W. somnifera* has an advantage over *Panax ginseng* in that it does not appear to result in ginseng-abuse syndrome, a condition that is characterized by high blood pressure, water retention, muscle tension and insomnia (Bhattacharya et al. 1987).

Two acyl steryl glucosides, *sitoindoside VII* and *sitoindoside VIII*, which were isolated from *W. somnifera* roots, were screened for putative anti-stress activity using a diverse spectrum of stress induction models. Significant anti-stress activity was exhibited in all the test parameters used (Bhattacharya et al. 1987). Two new glycowithanolides, *sitoindoside IX* and *sitoindoside X*, which were also isolated from *W. somnifera*, were evaluated for their immunomodulatory and CNS effects (i.e., anti-stress, memory and learning) in doses of 100–400 mg/mouse and they produced the statistically significant mobilization and activation of peritoneal macrophages, phagocytosis and increased activity of the lysosomal enzymes secreted by activated macrophages. In oral doses of 50–200 mg/kg, both of these compounds produced significant anti-stress activity in albino mice and rats and augmented learning acquisition and memory retention in both young and old rats (Ghosal et al. 1989).

Anti-ulcer properties of *W. somnifera*

Anti-ulcer activity of methanolic *W. somnifera* extracts and its action against stress plus pylorus ligation-induced gastric ulcers in rats have been reported. Treatment with *W. somnifera* extract (100 mg/kg/day) for 15 days significantly reduced the ulcer index as compared with the control group. The extract also significantly reduced the gastric secretion volume and free and total acidities in rats. A significant increase in the antioxidant enzymes CAT and SOD and a decrease in malondialdihyde (MDA) was observed. *W. somnifera* extract was found to be an effective antiulcerogenic agent whose activity is comparable with the common antiulcer drug ranitidine hydrochloride. The study suggests that *W. somnifera* not only inhibits gastric hydrochloric acid secretion but also increases various defense factors including antioxidants to protect gastric mucosal damage (Bhatnagar et al. 2005).

Hypoglycemic effects of *W. somnifera*

Hyperglycemia is a major risk factor for cardiovascular disease. *W. somnifera* favorably alters blood and urine glucose levels, glycated Hb and liver enzymes in diabetic rats (Andallu and Radhika 2000; Udayakumar et al. 2009).

Udayakumar et al. (2009) investigated the hypoglycemic and hypolipidemic effects of *W. somnifera* root and leaf extracts on alloxan-induced diabetes in rats. In their study, both *W. somnifera* root and leaf extracts and the standard drug glibenclamide were orally administered to diabetic rats daily for 8 weeks. After treatment, the levels of the following were determined: urine sugar; blood glucose; Hb; glycosylated Hb (HbA1C); liver glycogen; serum and tissue lipids; serum and tissue proteins; liver glucose-6-phosphatase (G6P); and serum enzymes including aspartate transaminase (AST), alanine transaminase (ALT), acid phosphatase (ACP) and alkaline phosphatase (ALP). The levels of urine sugar, blood glucose, HbA1C, G6P, AST, ALT, ACP, ALP, serum

lipids, with the exception of HDL-c, and tissues such as liver, kidney and heart lipids were significantly ($P < 0.05$) increased, whereas Hb, total protein, albumin, albumin:globulin (A:G) ratio, tissue protein and glycogen were significantly ($P < 0.05$) decreased in alloxan-induced diabetic rats. Treatment of the diabetic rats with the extracts and glibenclamide restored the above parameters to their normal levels after 8 weeks of treatment, indicating that *W. somnifera* root and leaf extracts possess hypoglycemic and hypolipidemic activities in alloxan-induced diabetes mellitus (DM) rats.

Effects of *W. somnifera* on various cardiovascular diseases

Several medicinal plants have been reported to be beneficial for cardiac ailments in Ayurveda, including *W. somnifera* and *Terminalia Arjuna* (Arjuna), a medicinal plant traditionally used to promote "healthy hearts". *W. somnifera* is categorized as *Rasayanas* and is proposed to promote health and longevity, while Arjuna is primarily used for heart ailments such as coronary artery disease, heart failure, hypercholesterolemia, anginal pain and can be considered as a useful drug for coronary artery disease, hypertension and ischemic cardiomyopathy (Sandhu et al. 2010).

A study was conducted by Mohanty et al. (2008) to evaluate the cardioprotective mechanisms of *W. somnifera* in the setting of ischemia and reperfusion (IR) injury. *W. somnifera* pretreatment favorably restored the myocardial antioxidant balance and exerted marked anti-apoptotic effects and reduced myocardial damage, as confirmed by histopathologic evaluation. Therefore, the antioxidant and anti-apoptotic properties of *W. somnifera* may contribute to its cardioprotective effects. Dose-dependent and irreversible myocardial injury is a major side effect of doxorubicin, one of the most effective antineoplastic agents for the treatment of solid and hematopoietic malignancies. *W. somnifera* extract (Hamza et al. 2008) and a commercial polyherbal formulated product containing atenolol (CardiPro®) capsules (Mohan et al. 2006) have been shown to provide cardioprotection against doxorubicin-associated cardiotoxicity, as evidenced by reduced mortality, increased antioxidants and hypolipidemic action.

W. somnifera treatment has been found to increase heart rate, contractility and relaxation and decrease preload along with improved antioxidant enzymes and inhibit lipid-peroxidation comparable to vitamin E, a known cardioprotective antioxidant (Gupta et al. 2004; Mohanty et al. 2004). Thirunavukkarasu et al. (2006) stated that a formulation containing *W. somnifera* had energy-boosting properties in IR compromised hearts and recommended its use as a dietary supplement for cardioprotection. This formulation favorably altered the myocardial energy substrate, improved cardiac function and reduced the infarct size. In another study, *Marutham* (a polyherbal formulation containing *W. somnifera*), was found to be cardioprotective and to have antioxidant properties in isoproterenol-induced ischemic rats (Prince et al. 2008).

Stress is a major cardiovascular risk factor that leads to activation of the sympathoadrenal and hypothalamic pituitary adrenal (HPA) axis and causes oxidative stress. *W. somnifera* possesses a potent anti-stress effect and has been reported to alleviate stress-induced changes and provide cardioprotection in ischemic rats, which is similar to the properties ascribed to adaptogens like *Panax ginseng*. It also increases heart weight and glycogen in the myocardium and liver, indicating an increase in the anabolic process, in addition to enhancing the duration of contractility and the coagulation time (Dhuley 1998, 2000).

Hypolipidemic and anti-atherogenic properties of *W. somnifera*

W. somnifera has profound hypocholesteremic, hypolipidemic and antiatherogenic activities (Andallu and Radhika 2000; Hemalatha et al. 2006; Mary et al. 2003; Visavadiya and Narasimhacharya 2007). Mary et al. (2003) demonstrated the antiatherogenic activity of CapsHT2, a botanical medicine consisting of several plants including *W. somnifera* against vascular intimal damage and atherogenesis, which leads to various types of cardiovascular diseases. Its inhibition of platelet aggregation was comparable to heparin. This formulation altered the atherogenic index and reduced the body weight of rats and increased high-density lipoprotein cholesterol (HDL-c) levels in hyperlipidemic rats.

Hepatoprotective effects of *W. somnifera*

Mohanty et al. (2008) investigated the influence of *W. somnifera* root powder on the levels of circulating ammonia, urea, lipid peroxidation products such as TBARS, hydroperoxides (HP) and liver marker enzymes such as AST, ALT and ALP to determine its hepatoprotective effect in ammonium chloride-induced hyperammonemia. The results indicate that *W. somnifera* offers hepatoprotection by influencing the levels of lipid peroxidation products and liver markers in experimental hyperammonemia. The authors explained that these effects could be due to (i) the presence of alkaloids, *withanolides* and flavonoids, (ii) the normalization of the levels of urea and urea related compounds, (iii) its free radical scavenging property and (iv) its antioxidant property (Harikrishnan et al. 2008). The effect of *W. somnifera* root extract mixed-feed on the hepatic cell of *Clarias batrachus* was investigated by Verma et al. (2009). The study suggests that *W. somnifera* aqueous root extract contains different alkaloids such as flavonoids and the neurotransmitters GABA A and GABA B that activate the neuroendocrine system leading to hyperactivity of the endomembrane, smooth biosynthetic pathways and the exit of molecules through the surface via exocytosis.

W. somnifera effects against thyroid disorders

W. somnifera has also been investigated for its effect on thyroid activity in animal studies. An aqueous extract of dried *W. somnifera* root was given to mice via gastric intubation at a dose of 1.4 g/kg body weight daily for 20 days. Mouse serum was collected at the end of the 20-day period and analyzed for triiodothyronine (T3) and thyroxine (T4) concentrations and lipid peroxidation was measured in mouse liver homogenate by determining antioxidant enzyme activity. A significant increase in serum T4 was observed, indicating that the plant stimulates thyroid activity; however, no changes in T3 levels were observed. The researchers indicated that *W. somnifera* may also stimulate thyroid activity indirectly via its effect on cellular antioxidant systems. *W. somnifera* extract significantly decreased lipid peroxidation in liver homogenate and significantly increased CAT activity, which promotes free radical scavenging that can lead to cellular damage. These results indicate that *W. somnifera* is a botanical agent with potential for use in the treatment of hypothyroidism (Panda and Kar 1998, 1999).

Antimicrobial properties of *W. somnifera*

A study conducted by Owais et al. (2005) showed that both aqueous and alcohol extracts of the *W. somnifera* (root and leaf) possess strong antibacterial activity against a range of bacteria, as revealed by the in vitro agar well diffusion method.

Girish et al. (2006) isolated a monomeric glycoprotein, with a molecular mass of 28 kDa in SDS-PAGE, from *W. somnifera* root tubers. The protein, designated *W. somnifera* glycoprotein, demonstrated potent antimicrobial activity against the phytopathogenic fungi and bacteria tested, indicating that *W. somnifera* has potential as antifungal and antibacterial agents. Arora et al. (2004) evaluated the methanol, hexane and diethyl ether extracts from *W. somnifera* leaves and roots for antibacterial/synergistic activity by the agar plate disc-diffusion assay using the two bacterial species *Salmonella typhimurium* and *Escherichia coli*. From the six extracts tested, only the methanol and hexane extracts, from both leaves and roots, had potent antibacterial activities. In a study by Mahesh and Satish (2008), *W. somnifera* methanol leaf extracts showed significant antibacterial activity against *Bacillus subtilis*, *E. coli*, *Pseudomonas fluorescens*, *Staphylococcus aureus* and *Xanthomonas axonopodis pv. malvacearum* as well as antifungal activity against *Aspergillus flavus*, *Dreschlera turcica* and *Fusarium verticillioides* when compared to root and bark extracts, indicating that the leaf may be superior in terms of antibacterial properties.

Kambizia and Afolayan (2008) reported on the in vitro antimicrobial activities of water and methanol extracts of the two herbs *Aloe ferox* and *W. somnifera* on *Neisseria gonorrhoeae* and *Candida albicans*, which cause sexually transmitted diseases (STD). Extracts from both species, together with pure aloin from *A. ferox*, were evaluated for activity against six strains of *N. gonorrhoeae* and nine strains of *C. albicans*. The extracts showed activity against *N. gonorrhoeae* at concentrations ranging from 0.5 mg/ml (methanol extracts from both) to 10 mg/ml (water extract of *W. somnifera* only), whereas pure aloin was

found to inhibit the growth of both microorganisms. Only the *W. somnifera* methanol extract was effective against *C. albicans* at 20 mg/ml. Ghosh (Ghosh 2009) purified a 30 kDa monomeric acidic lectin-like protein from *W. somnifera* leaves by using a series of gel filtration and affinity chromatography methods before investigating the *W. somnifera* antibacterial activity. The protein antifungal activity was comparable with standard lectins such as concanavalin A, phytohemagglutinin and wheat germ agglutinin indicating that *W. somnifera* has good antifungal effects. Singh et al. (2010) studied the antifungal activity of *W. somnifera* extracts in different organic solvents using a disc diffusion assay. Hexane and ethyl acetate extracts showed a maximum inhibition zone against *Aspergillus niger* at 15 and 12.2 mm, respectively. Methanol extracts displayed maximum activity against *Fusarium oxysporium* (13.0 mm) and *A. flavus* (15 mm), while aqueous extracts showed maximum activity against *Nitella gracilis*, *F. moniliformis* (9.6 mm).

Another group of researchers also evaluated aqueous extracts of *Aloe ferox* and *W. Somnifera* for antiviral activity against herpes simplex virus type 1 (HSV-1) in vitro (Kambizia et al. 2007). The aqueous extracts showed detectable activity at a concentration of 1,000 μg/ml against the virus in monolayers of Vero African green monkey cell cultures. Their results indicate that the use of these two plant species for the treatment of STDs could have a scientific foundation.

Toxicity and adverse effects of *W. somnifera*

An important consideration when investigating the medicinal properties of an unknown compound is the diligent evaluation of its potential for harmful effects, which is usually evaluated through toxicity studies. For *W. somnifera*, no systematic studies have been reported that examined acute, subacute, subchronic or chronic toxicity of *W. somnifera* root powder, whole plant powder, or different extracts of the plant (i.e., water, alcohol, petroleum ether, purified alkaloids and glycosides). Although one preliminary toxicity study of *W. somnifera* was conducted, it was of insufficient quality to support its findings because too few animals were used, the body weight data was not collected and the survival data was not reported (Arseculeratne et al. 1985).

In one central nervous system study, a 2% suspension of *ashwagandholine* (total alkaloids from the *W. somnifera* roots) prepared in 10% propylene glycol using 2% gum acacia as a suspending agent was used to determine acute toxicity (Malhotra et al. 1965). The acute LD_{50} was 465 mg/kg (range: 332–651 mg/kg) in rats and 432 (range: 299–626 mg/kg) in mice. Another study showed that the LD_{50} of an alcohol *W. somnifera* root extract is 1,260 mg/kg in mice. No acute mortality was observed at a dose 1,100 mg/kg, but with a further dose increase of 100 mg/kg, there was a sharp increase in the death rate, indicating that *W. somnifera* is rather safe to be administered to mice (Sharada et al. 1993).

Conclusion and future perspectives

We conclude that *W. somnifera* extract contains many active constituents that have potential activity against many diseases. In summary, *W. somnifera* has therapeutic potential to be used as:

- an anti-inflammatory agent
- an antioxidant
- an anti-aging agent
- an anticancer agent, chemopreventive and immunomodulator
- an adaptogen
- an anti-anxiety and anti-depressant
- an anti-ulcer agent
- a cardioprotectant
- a hypolipidemic and anti-atherogenic agent
- an antihypertensive agent
- a hypoglycemic agent
- a hepatoprotectant
- a treatment for hypothyroidism
- an antimicrobial (antibacterial and antifungal) agent

or as an adjunct to the above treatments.

Several reports have shown that *W. somnifera* has antioxidant properties due to the presence of phenolic and flavonoid compounds in the plant but there is no available information about the specific phenolic and flavonoid compounds responsible for antioxidant properties. It is necessary to identify the specific phenolic and flavonoid compounds present in *W. somnifera* in future studies. On the other hand, it is not only necessary to find new pharmacological properties of

W. somnifera plant extracts, but it is even more important to know the compounds responsible for the actions in order to obtain plants with a higher production of the compounds of interest. Although many studies have examined the diverse therapeutic effects of *W. somnifera*, toxicity studies are lacking and need to be conducted to further confirm its safety. Further studies are needed to not only explore the clinical potential of this plant for therapeutic purposes, but perhaps to also reveal other potential therapeutic effects and its toxicity profile.

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References

Alfonso D, Bernardinelli G, Kapetanidis I (1993) Withanolides from *Iochroma coccineum*. Phytochemistry 34:517–521

Al-Hindawi MK, Al-Khafaji SH, Abdul-Nabi MH (1992) Anti-granuloma activity of Iraqi *Withania somnifera*. J Ethnopharmacol 37:113–116

Anbalagan K, Sadique J (1981) Influence of an Indian medicine (Ashwagandha) on acutephase reactants in inflammation. Indian J Exp Biol 19:245–249

Anbalagan K, Sadique J (1984) Role of prostaglandins in acute phase proteins in inflammation. Biochem Med 31:236–245

Andallu B, Radhika B (2000) Hypoglycemic, diuretic and hypocholesterolemic effect of winter cherry (*Withania somnifera*) root. Indian J Exp Biol 38:607–609

Anonymous (2004) Monograph: *Withania somnifera*. Altern Med Rev 9:211–214

Archana R, Namasivayam A (1999) Antistressor effect of *Withania somnifera*. J Ethnopharmacol 64:91–93

Arora S, Dhillon S, Rani G, Nagpal A (2004) The in vitro antibacterial/synergistic activities of *Withania somnifera* extracts. Fitoterapia 75:385–388

Arseculeratne SN, Gunatilaka AA, Panabokke RG (1985) Studies of medicinal plants of Sri Lanka. Part 14: toxicity of some traditional medicinal herbs. J Ethnopharmacol 13:323–335

Bargagna-Mohan P, Ravindranath PP, Mohan R (2006) Small molecule anti-angiogenic probes of the ubuquitin proteasome pathway: potential applications to choroidal neovascularization. Invest Ophthalmol Vis Sci 47:4138–4145

Begum VH, Sadique J (1987) Effect of *Withania somnifera* on glycosaminoglycan synthesis in carrageenin-induced air pouch granuloma. Biochem Med Metab Biol 38:272–277

Begum VH, Sadique J (1988) Long-term effect of herbal drug *Withania somnifera* on adjuvant-induced arthritis in rats. Indian J Exp Biol 26:877–882

Bhatnagar M, Jain CP, Sisodia SS (2005) Anti-ulcer activity of *Withania somnifera* in stress plus pyloric ligation induced gastric ulcer in rats. J Cell Tissue Res 5:287–292

Bhattacharya SK, Muruganandam AV (2003) Adaptogenic activity of *Withania somnifera*: an experimental study using a rat model of chronic stress. Pharmacol Biochem Behav 75:547–555

Bhattacharya S, Goel R, Kaur R, Ghosal S (1987) Anti-stress activity of Sitoindosides VII and VIII, new Acylsterylglucosides from *Withania Somnifera*. Phytother Res 1:32–39

Bhattacharya SK, Bhattacharya A, Sairam K, Ghosal S (2000) Anxiolytic-antidepressant activity of *Withania somnifera* glycowithanolides: an experimental study. Phytomedicine 7:463–469

Bhattacharya A, Ghosal S, Bhattacharya SK (2001) Anti-oxidant effect of *Withania somnifera* glycowithanolides in chronic footshock stress-induced perturbations of oxidative free radical scavenging enzymes and lipid peroxidation in rat frontal cortex and striatum. J Ethnopharmacol 74:1–6

Bogdan C (2001) Nitric oxide and the immune response. Nat Immunol 2:907–916

Bone K (1996) Clinical applications of Ayurvedic and Chinese herbs. Phytotherapy Press, Warwick, pp 137–141

Davis L, Kuttan G (2002) Effect of *Withania somnifera* on CTL activity. J Exp Clin Cancer Res 21:115–118

Devi PU (1996) *W. somnifera* Dunal (Ashwagandha): potential plant source of a promising drug for cancer chemotherapy and radiosensitization. Indian J Exp Biol 34:927–932

Dhuley JN (1998) Effect of ashwagandha on lipid peroxidation in stress-induced animals. J Ethnopharmacol 60:173–178

Dhuley JN (2000) Adaptogenic and cardioprotective action of ashwagandha in rats and frogs. J Ethnopharmacol 70: 57–63

Dhuley JN (2001) Nootropic-like effect of Ashwagandha (*Withania somnifera*). Phytother Res 15:311–318

Elsakka M, Grigorescu E, Stanescu U (1990) New data referring to chemistry of *Withania somnifera* species. Rev Med Chir Soc Med Nat Iasi 94:385–387

Ghosal S, Lal J, Srivastava RS, Bhattacharya SK, Upadhyay SN, Jaiswal AK, Chattopadhyay U (1989) Immunomodulatory and CNS effects of Sitoindosides IX and X, Two new Glycowithanolides from *Withania somnifera*. Phytother Res 3:201–206

Ghosh M (2009) Purification of a lectin-like antifungal protein from the medicinal herb, *Withania somnifera*. Fitoterapia 80:91–95

Girish KS, Ushanandini S, Harish Kumar K, Nagaraju S, Govindappa M, Vedavathi M, Kemparaju K (2006) Antimicrobial properties of a non-toxic glycoprotein (WSG) from *Withania somnifera* (Ashwagandha). J Basic Microbiol 46:365–374

Glotter E (1991) Withanolides and related ergostane-type steroids. Nat Prod Rep 8:415–440

Grandhi A (1994) Comparative pharmacological investigation of ashwagandha and ginseng. J Ethnopharmacol (Ireland) 3:131–135

Gupta GL, Rana AC (2007) *Withania somnifera* (Ashwagandha): a review. Pharmacogn Rev 1:129–136

Gupta AP, Verma RK, Misra HO, Gupta MM (1996) Quantitative determination of withaferin A in different plant parts

of *Withania somnifera* by TLC densitometry. J Med Aromat Plant Sci 18:788–790

Gupta SK, Dua A, Vohra BP (2003) *W. somnifera* (Ashwagandha) attenuates antioxidant defense in aged spinal cord and inhibits copper induced lipid peroxidation and protein oxidative modifications. Drug Metabol Drug Interact 19:211–222

Gupta SK, Mohanty I, Talwar KK, Dinda A, Joshi S, Bansal P, Saxena AK, Arya DS (2004) Cardioprotection from ischemia and reperfusion injury by *Withania somnifera*: a hemodynamic, biochemical and histopathological assessment. Mol Cell Biochem 260:39–47

Hamza A, Amin A, Daoud S (2008) The protective effect of a purified extract of *W. somnifera* against doxorubicin-induced cardiac toxicity in rats. Cell Biol Toxicol 24:63–73

Harikrishnan B, Subramanian P, Subash S (2008) Effect of *Withania Somnifera* root powder on the levels of circulatory lipid peroxidation and liver marker enzymes in chronic hyperammonemia. E-J Chem 5:872–877

Hemalatha S, Wahi AK, Singh PN, Chansouria JP (2006) Hypolipidemic activity of aqueous extract of *Withania coagulans* Dunal in albino rats. Phytother Res 20:614–617

Ichikawa H, Takada Y, Shishodia S, Jayaprakasam B, Nair MG, Aggarwal BB (2006) Withanolides potentiate apoptosis, inhibit invasion, and abolish osteoclastogenesis through suppression of nuclear factor-kappaB (NF-kappaB) activation and NF-kappaB-regulated gene expression. Mol Cancer Therapy 5:1434–1445

Iuvone T, Esposito G, Capasso F, Izzo A (2003) Induction of nitric oxide synthase expression by *Withania somnifera* in macrophages. Life Sci 72:1617–1625

Jaiswal D, Rai PK, Watal G (2010) Hypoglycemic and antidiabetic effects of *Withania coagulans* fruit ethanolic extract in normal and streptozotocin-induced diabetic rats. J Food Biochem 34:764–778

Jaleel CA (2009) Antioxidant profile changes in leaf and root tissues of *Withania somnifera* Dunal. Plant Omics J 2:163–168

Jaleel CA, Lakshmanan GMA, Gomathinayagam M, Panneerselvam R (2008) Triadimefon induced salt stress tolerance in *W. somnifera* and its relationship to antioxidant defense system. S Afr J Bot 74:126–132

Jayaprakasam B, Zhang Y, Seeram N, Nair M (2003) Growth inhibition of tumor cell lines by withanolides from *Withania somnifera* leaves. Life Sci 74:125–132

Johri S, Jamwal U, Rasool S, Kumar A, Verma V, Qazi GN (2005) Purification and characterization of peroxidases from *Withania somnifera* (AGB 002) and their ability to oxidize IAA. Plant Sci 169:1014–1021

Kaileh M, Vanden Berghe W, Heyerick A, Horion J, Piette J, Libert C, De Keukeleire D, Essawi T, Haegeman G (2007) Withaferin A strongly elicits IkappaB kinase beta hyperphosphorylation concomitant with potent inhibition of its kinase activity. J Biol Chem 282:4253–4264

Kambizia L, Afolayan AJ (2008) Extracts from *Aloe ferox* and *Withania somnifera* inhibit *Candida albicans* and *Neisseria gonorrhoea*. Afr J Biotechnol 7:12–15

Kambizia L, Goosenb BM, Taylorc MB, Afolayan AJ (2007) Anti-viral effects of aqueous extracts of *Aloe ferox* and *Withania somnifera* on herpes simplex virus type 1 in cell culture. S Afr J Sci 103:359–360

Kaur P, Mathur S, Sharma M, Tiwari M, Sdvastava KK, Chandra R (2001) A biologically active constituent of *W. somnifera* (ashwagandha) with antistress activity. Indian J Clin Biochem 16:195–198

Kinghorn AD, Su B-N, Jang DS, Chang LC, Lee D, Gu J-Q, Carcache-Blanco EJ, Pawlus AD, Lee SK, Park EJ, Cuendet M, Gills JJ, Bhat K, Park HS, Mata-Greenwod E, Song LL, Jang M, Pezzuto JM (2004) Natural inhibitors of carcinogenesis. Planta Med 70:691–705

Kulkarni SK, George B, Mathur R (1998) Protective effect of *Withania somnifera* root extract on electrographic activity in a lithiumpilocarpine model of status epilepticus. Phytother Res 12:451–453

Kuttan G (1996) Use of *Withania somnifera* Dunal as an adjuvant during radiation therapy. Indian J Exp Biol 34:854–856

Mahesh B, Satish S (2008) Antimicrobial activity of some important medicinal plant against plant and human pathogens. World J Agric Sci 4:839–843

Malhotra CL, Mehta VL, Prasad K, Das PK (1965) Studies on Withania ashwagandha, Kaul. IV. The effect of total alkaloids on the smooth muscles. Indian J Physiol Pharmacol 9:9–15

Mary NK, Babu BH, Padikkala J (2003) Antiatherogenic effect of caps HT2, an herbal Ayurvedic medicine formulation. Phytomedicine 10:474–482

Mehta AK, Binkley P, Gandhi SS, Ticku MK (1991) Pharmacological effects of *Withania somnifera* root extract on GABAA receptor complex. Indian J Med Res 94:312–315

Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazón J (2009) Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. Molecules 14:2373–2393

Mohan IK, Kumar KV, Naidu MU, Khan M, Sundaram C (2006) Protective effect of CardiPro against doxorubicin-induced cardiotoxicity in mice. Phytomedicine 13:222–229

Mohanty I, Arya DS, Dinda A, Talwar KK, Joshi S, Gupta SK (2004) Mechanisms of cardioprotective effect of *Withania somnifera* in experimentally induced myocardial infarction. Basic Clin Pharmacol Toxicol 94:184–190

Mohanty IR, Arya DS, Gupta SK (2008) *Withania somnifera* provides cardioprotection and attenuates ischemia-reperfusion induced apoptosis. Clin Nutr 27:635–642

Owais M, Sharad KS, Shehbaz A, Saleemuddin M (2005) Antibacterial efficacy of *Withania somnifera* (ashwagandha) an indigenous medicinal plant against experimental murine salmonellosis. Phytomedicine 12:229–235

Panda S, Kar A (1998) Changes in thyroid hormone concentrations after administration of ashwagandha root extract to adult male mice. J Pharm Pharmacol 50:1065–1068

Panda S, Kar A (1999) *Withania somnifera* and Bauhinia pupurea in the regulation of circulating thyroid hormone concentrations in female mice. J Ethnopharmacol 67: 233–239

Prakash J, Gupta SK, Dinda AK (2002) *Withania somnifera* root extract prevents DMBA-induced quamous cell carcinoma of skin in Swiss albino mice. Nutr Cancer 42:91–97

Prince PSM, Selvaraju S, Devika PT, Vaithianathan M (2008) Cardioprotective effect of Marutham' a polyherbal formulation on isoproterenol induced myocardial infarction in Wistar rats. Fitoterapia 79:433–438

Rastogi RP, Mehrotra BN (1998) Compendium of Indian medicinal plants. Central Drug Research Institute 6

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Ray AB, Gupta M (1994) Withasteroids, a growing group of naturally occurring steroidal lactones. Prog Chem Org Nat Prod 63:2–106

Sandhu JS, Shah B, Shenoy S, Chauhan S, Lavekar GS, Padhi MM (2010) Effects of *Withania somnifera* (Ashwagandha) and *Terminalia arjuna* (Arjuna) on physical performance and cardiorespiratory endurance in healthy young adults. Int J Ayurveda Res 1:144–149

Sangwan RS, Chaurasiya ND, Misra LN, Lal P, Uniyal GC, Sharma R, Sangwan NS, Suri KA, Qazi GN, Tuli R (2004) Phytochemical variability in commercial herbal products and preparations of *Withania somnifera* (ashwagandha). Curr Sci 86:461–465

Schliebs R, Liebmann SK, Kumar A, Ghosal S, Bigl V (1973) Systemic administration of defined extracts from *Withania somnifera* (Indian ginseng) and Shilajit differentially affects choilinergic but not glutamatergic and GAB Aergic marker in rat brain. Neurochem Int 30:181–190

Senthilnathan P, Padmavathi R, Banu SM, Sakthisekaran D (2006) Enhancement of antitumor effect of paclitaxel in combination with immunomodulatory *Withania somnifera* on benzo(a)pyrene induced experimental lung cancer. Chem Biol Interact 159:180–185

Sharada AC, Solomon FE, Uma Devi P (1993) Toxicity of *Withania somnifera* root extract in rats and mice. Int J Pharmacogn 31:205–212

Sharma S, Dahanukar S, Karandikar SM (1985) Effects of long-term administration of the roots of ashwagandha and shatavari in rats. Indian Drugs 29:133–139

Singh G, Sharma PK, Dudhe R, Singh S (2010) Biological activities of *Withania somnifera*. Ann Bioll Res 1:56–63

Somasundaram S, Sadique J, Subramoniam A (1983) Influence of extra-intestinal inflammation on the in vitro absorption of ^{14}C-glucose and the effects of anti-inflammatory drugs in the jejunum of rats. Clin Exp Pharmacol Physiol 10: 147–152

Subaraju GV, Vanisree M, Rao CV, Sivaramakrishna C, Sridhar P, Jayaprakasam B, Nair MG (2006) Ashwagandhanolide, a bioactive dimeric thiowithanolide isolated from the roots of *Withania somnifera*. J Nat Prod 69:1790–1792

Sumantran VN, Chandwaskar R, Boddul S, Patwardhan B, Chopra A, Wagh UV (2008) The relationship between chondroprotective and antiinflammatory effects of *Withania somnifera* root and glucosamine sulphate on human osteoarthritic cartilage in vitro. Phytother Res 22: 1342–1348

Thirunavukkarasu M, Penumathsa S, Juhasz B, Zhan L, Bagchi M, Yasmin T, Shara MA, Thatte HS, Bagchi D, Maulik N (2006) Enhanced cardiovascular function and energy level by a novel chromium (III)-supplement. Biofactors 27: 53–67

Tohda C, Kuboyama T, Komatsu K (2005) Search for natural products related to regeneration of the neuronal network. Neurosignals 44:34–45

Tripathi AK, Dey S, Singh RH, Dey PK (1998) Alterations in the sensitivity of 5th receptor subtypes following chronic Asvagandha treatment in rats. Anc Sci Life 17:168–181

Udayakumar R, Kasthurirengan S, Mariashibu TS, Rajesh M, Ramesh Anbazhagan V, Kim SC, Ganapathi A, Choi CW (2009) Hypoglycaemic and hypolipidaemic effects of *Withania somnifera* root and leaf extracts on alloxan-induced diabetic rats. Int J Mol Sci 10:2367–2382

Udayakumar R, Kasthurirengan S, Vasudevan A, Mariashibu TS, Rayan JJS, Choi CW, Ganapathi A, Kim SC (2010) Antioxidant effect of dietary supplement *Withania somnifera* L. Reduce blood glucose levels in alloxan-induced diabetic rats. Plant Foods Hum Nutr 65:91–98

Verma P, Srivastava SK, Nath A (2009) Stimulatory effect of *Withenia somnifera* on secretion and endocytotic pathways in the hepatic cell of *Clarias batrachus* (*Linn.*). J Ecophysiol Occupat Health 9:203–209

Visavadiya NP, Narasimhacharya AV (2007) Hypocholesteremic and antioxidant effects of *Withania somnifera* (Dunal) in hypercholesteremic rats. Phytomedicine 14:136–142

Winters M (2006) Ancient medicine, modern use of *Withania somnifera* and its potential role in integrative oncology. Altern Med Rev 11:269–277

Zhao J, Nakamura N, Hattori M, Kuboyama T, Tohda C, Komats K (2002) Withanolide derivatives from the roots of *Withania somnifera* and their neurite outgrowth activities. Chem Pharm Bull (Tokyo) 50:760–765

Ziauddin M, Phansalkar N, Patki P, Diwanay S, Patwardhan B (1996) Studies on the immunomodulatory effects of Ashwagandha. J Ethnopharmacol 50:69–76

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