

Comparative Pharmacognostic Study of Different Parts of *Withania somnifera* and its Substitute *Ruellia tuberosa*

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Abstract *Withania somnifera* (L.) Dunal. (Solanaceae) is a therapeutically important medicinal plant widely used in Ayurveda and traditional systems of medicine in all over the world. Since this valuable plant is not commercially cultivated in Sri Lanka, traditional practitioners use *Ruellia tuberosa* L. (Acanthaceae) as a substitute for *Withania somnifera*. However, use of *R. tuberosa* as a substitute without scientifically proven data on important quality standards might adversely affect on the therapeutic properties of herbal drugs. Present study investigates the important pharmacognostic aspects of *W. somnifera* and *R. tuberosa*. Comparative quality parameters on morphological, anatomical, powder microscopical, phytochemical, physicochemical and brine shrimp toxicity of different parts of (leaf, bark and roots) *W. somnifera* and *R. tuberosa* by using established protocols. Results demonstrated that all major phytochemical groups tested were present in leaves, bark and roots of both plants. Physicochemical analysis exhibited the higher total ash, water soluble ash and acid insoluble ash in all parts of *R. tuberosa*. However, TLC profiles exhibited the higher number of spots in all 3 parts for *W. somnifera* over *R. tuberosa*. Potent of brine shrimp toxicity was increased as leaf>bark>roots for *R. tuberosa* and bark>root>leaf for *Withania somnifera*. Therefore, *W. somnifera* could be differentiated from *R. tuberosa* by comparing polymorphic macroscopic, microscopic, phytochemical, physicochemical characters either singularly or as a whole. The presence of certain similarities in major phytochemical groups, and in brine shrimp toxicity of *W. somnifera* and *R. tuberosa* partially justifies the use of *R. tuberosa* as a substitute for *W. somnifera* in traditional systems of medicine in Sri Lanka which needs to be confirmed after further clinical trials.

Keywords: *Withania somnifera*, *Ruellia tuberosa*, phytochemical parameters, physicochemical analysis, brine shrimp toxicity assay

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1. Introduction

Pharmacognosy is the study of the physical, chemical, biochemical and biological properties of drugs, drug substances or potential drugs or drug substances of natural origin. Study of pharmacognosy is a reliable tool, by which complete information of the crude drug could be obtained [1]. With respect to quality control, correct identification of the species concerned from commonly available adulterants or substitutes, in fresh, dried or powdered state is of prime importance [2]. The process of standardization could be achieved by pharmacognostic studies which help in identification and authentication of the plant materials [3,4]. Since adulterants or substitutes closely resemble the genuine material, macroscopic or microscopic evidences alone could not always provide evidence for complete identification. On the other hand misidentification of species and the subsequent substitution with adulterated materials reported a real danger in Chinese Traditional Medicine [5,6]. Therefore, implementation of rigorous standardization process with multi-techniques procedure is required for proper

standardization in order to avoid harmful adulteration, substitution, contamination and degradation [7].

Withania somnifera (L.) Dual (Solanaceae) is a therapeutically important, widely used medicinal plant in traditional and Ayurveda systems of medicine for the treatment of mental health, tumor, genotoxicity, arthritis, hypertension, tremors, diabetes, general debility, and angiogenesis activities and maintain the vigour and stamina [8-13]. Further, *W. somnifera* is well known for its other biological activities like adaptogenic/anti-stress [14,15], immunomodulatory [16,17], anti-ageing [14,15,18,19], anti-fatigue [10,14,15], antioxidant [18,20], anti-parkinsonism [21,22], antiulcerogenic [15-23], support healthy thyroid function [24]. Since there is no commercial cultivation of *W. somnifera* in Sri Lanka, traditional practitioners widely used *Ruellia tuberosa* L. (Acanthaceae), as a cheap substitute for *W. somnifera*, which is traditionally used for diuretic, anti-pyretic, analgesic, anti-hypertensive and anthelmintic properties [25]. However, use of *R. tuberosa* as a substitute without scientifically proven data on important quality standards might adversely affect the therapeutic properties of herbal drug which incorporate *R. tuberosa* as a substitute.

Moreover, even though, this substitution has been practiced since long times, available information on comparative morphological, powder microscopical, physicochemical, phytochemical and basic toxicological studies using brine shrimp assay of *W. somnifera* and *R. tuberosa* are scattered. Therefore, present study was undertaken to compare morphological, powder microscopical, physicochemical, phytochemical and bioactivity of different parts of *W. somnifera* and *R. tuberosa* in order to scientifically validate the traditional claims of use of *R. tuberosa* instead of *W. somnifera*.

2. Materials and Methods

2.1. Plant Material

Plant materials of *Withania somnifera* and *Ruellia tuberosa* were collected from the institutional research plots maintained under similar soil and climatic conditions. Herbarium specimens of both plants were prepared and deposited (HTSMP 19& HTSAP-20) in the institutional herbarium.

2.2. Preparation of Free Hand Sections

Free hand transverse sections of leaf of both plants were prepared using razor blades. Suitable sections were selected and taken through an alcohol series and subsequently strained with 1% safranin in 50% ethanol. Stained material was made into temporary mounts using glycerin.

2.3. Phytochemical Studies

2.3.1. Preparation of Extracts

Coarsely powdered material of each plant part (leaf, bark and root 10 g per each) of both species was separately extracted in 50 mL of methanol by using Soxhlet apparatus. The extract was concentrated at 45°C using rotavapour (BuchiRotavapour, Type-R-114A29 B-480, Switzerland).

2.4. Thin Layer Chromatography

The Thin Layer Chromatography (TLC) was performed as described by Stahl [27] with little modifications. TLC plates (Pre-coated silica gel 60 A, 20 X 20 cm; 0.2 mm thickness) and developed using Cyclohexane: Dichloromethane: Ethyl acetate: Methanol (5:1:4:0.4) as mobile phase. Developed TLC plates were visualized under UV 366 and subsequently after spraying with Vanillin sulphuric acid. Colour and the R_f values of each spot was recorded.

2.5. Qualitative Screening of Phytochemicals

Methanolic extracts of leaf, bark and root extracts of *W. somnifera* and *R. tuberosa* samples were screened for the presence of preliminary phytochemicals such as alkaloids, flavonoids, saponins, steroid glycosides and tannins according to the method described by reference [26].

2.6. Determination of Physico-Chemical Parameters

2.6.1. Quantification of Total Ash

Dried materials (2 g) were ignited at 500–600°C until the sample turn into white color. Then the total ash content of ignited sample was determined as methods described in WHO guidelines [5].

2.6.2. Quantification of Water-Soluble Ash

Ignited ash sample was mixed with 25 mL of distilled water and boiled, Then it was filtered using a Whatman ashless filter-paper. Insoluble matter was washed with hot water and ignited for 15 min at 450°C. The residue was allowed to cool in a desiccator for 30 minutes [5].

2.6.3. Quantification of Acid-Insoluble Ash

A crucible containing ash was gently boiled with 25 mL of HCl. Insoluble matter was collected to Whatman ashless filter-paper and washed with hot water until the filtrate become neutral. Then the acid insoluble matter was transferred to original crucible and ignited to a constant weight at 450°C. Then the residue was allowed to cool in a desiccator for 30 minutes [5].

2.6.4. Quantification of Total Extractable Matter

Hot extraction method - Four grams of coarsely powdered samples of leaf, bark and root were separately refluxed with 100 mL of methanol for 1 h. Then the mixture was filtered and total weight was re-adjusted by adding methanol and concentrated in a rotavapour (BuchiRotavapour, Type-R-114A29 B-480, Switzerland) at 45°C. The residue was dried at 105°C for 6 h and allowed to cool for 30 min. [5].

2.6.5. Brine Shrimp Toxicity Assay (BST)

Brine shrimp assay was performed as described by Michael [28] with slight modifications. *Artemia salinae* eggs were incubated in 500 mL of brine water (35 ppt, pH 7.5) under illumination at 28°C ±2°C for 24 h and larvae were transferred to 12 well plates containing 1 mL of aerated artificial brine water. The extracts of 3 different concentrations (1 ppm, 5 ppm, 20 ppm and 50 ppm) were added into the wells and left for 24 h. artificial brine water was used as the control. The numbers of death larvae were counted under light microscope.

2.6.6. Statistical Analysis

Results of physico-chemical parameters and antioxidant activity were analyzed by general linear model (GLM) ANOVA test followed by Duncan's Multiple Range Test (DMRT) and presented as means ± SE.

3. Results and Discussion

In the present study attempts were made to compare the important pharmacognostic parameters of *Withania somnifera* and its common substitute *Ruellia tuberosa* by means of morphological, anatomical, phytochemical, physicochemical and brine shrimp toxicity. Distinguished morphological characters of *Withania somnifera* and its common substitute *Ruellia tuberosa* are demonstrated in Figure 1 and distinguished vegetative and reproductive characters are given in Table 1. As shown in Table 1, growth habit, flower type, plant height, leaf phyllotaxy,

petiole length flower color, shape of corolla, type of inflorescence, fruit type and seed color morphological characters for differentiation of *W. somnifera* from *R. tuberosa* at the plant collection point and in the raw material stage.

characters for differentiation of *W. somnifera* from *R. tuberosa* at the plant collection point and in the raw material stage.

Table 1. Morphological characters of *Withania somnifera* and *Ruellia tuberosa*

Morphological characters	Morphological type of <i>Withania somnifera</i>	Morphological type of <i>Ruellia tuberosa</i>
Plant habit	Erect, much branched	Erect, much branched
Growth habit	Shrub	Perennial herb
Plant height	132-150 cm	45-60cm
Nature of the stem	Aerial	Aerial
Leaf	Simple	Simple
Leaf margin	Serrate, Slightly wavy	Undulate
Leaf shape	Elliptic-oblong	Elliptic-oblong-Obovate
Leaf apex	Acute	Obtuse to somewhat acute
Leaf base	Cuneate	Cuneate
Leaf Venation	Pinnately reticulate	Reticulate
Leaf Phyllotaxy	Alternate	Opposite
Leaf texture	Soft	Soft
Colour of the Dorsal surface	Green	Green
Colour of the ventral surface	Light Green	Green
Average Leaf length	7.0-8.3 cm	6-7 cm
Leaf width	5-6 cm	3-3.5 cm
Petiole	Petiolate	Petiolate
Length of petiole	2.48-3.1 cm	0.8-1.2 cm
Nature of the leaf surface	Pubescence on both sides	pubescence on both sides
Flower colour	Greenish yellow colour	Purple
Shape of corolla	Bell shape	Tubular shape
Number of petals	5	5
Type of inflorescence	Cymose often solitary cyme	Axillary cymes
Androecium	5 stamens	4 stamens
Calyx	Papery inflated calyx	Shortly tubular or funnel shape
Fruit type	Spherical berry	Club shaped
Colour	Red	Bluish purple
Seed colour	Pale yellow	Brown to dark brown
Number of seeds/ fruit	27-33	8-11
Root system	Stout and tap root type	Tuberous root system
Stem Colour	Light green	Green
Shape	Sub quadrangular	Sub quadrangular
Internodal space	2-3 cm	3-4.5 cm



Figure 1. Mature plant of (A) *Withania somnifera* and (B) *Ruellia tuberosa*

Distinguished morphological (vegetative and reproductive) characters are key parameters which have been widely used for field species recognition, specially to delimit taxonomic ambiguity among species or the populations in the same species [29].

As demonstrated in Figure 2, *W. somnifera* possess anomocytic stomata and much branched trichomes while *R. tuberosa* possess diacytic stomata and un-branched trichomes. Further, epidermal trichome frequency, veins, and shape of the stem, were key polymorphic features of leaf powder of *W. somnifera* and *R. tuberosa*. These distinguished characters either singularly or as a whole could be easily used for differentiation of raw materials of both plants at the powdered stage. Use of gross morphological, foliar anatomical features such as stomata and trichomes have been well reported for authentication of controversial medicinal plants such as *Plectranthus hadiensis* and *Plectranthus amboinicus* [30], *Munronia*

pinnata and *Andrographis paniculata* [31], *Senna* and *Munronia pinnata* morphotypes [32] & [31]. Therefore, the results of the present study are in agreement with previous studies.

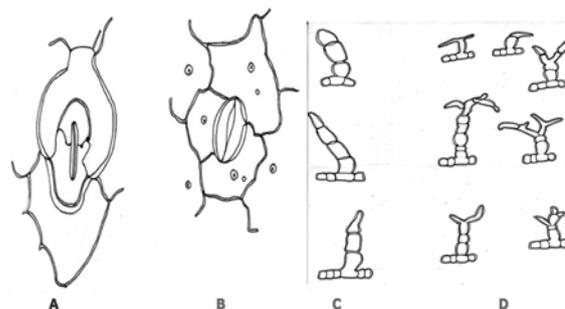


Figure 2. Line drawings of stomata and trichome types found in *Withania somnifera* and *Ruellia tuberosa*. [A= stomata of *Ruellia tuberosa*; B= stomata of *Withania somnifera*; C= unbranched trichome of *Ruellia tuberosa* and D= Much branched trichomes of *Withania somnifera*. [Mag: -10x3x40]

Table 2. Distinguished polymorphic anatomical and powder microscopic characters of *Withania somnifera* and *Ruellia tuberosa*

Character	<i>Withania somnifera</i>	<i>Ruellia tuberosa</i>
Stem	Circular	Rectangular
Stomata	Anomocytic	Diacytic
Trichome	Predominantly branched	Not branched
Trichomes frequency	Abundant	Comparatively low
Veins	Reticulate	Reticulate
Trichome types	Glandular/ non glandular	Glandular/ non glandular

Thin Layer chromatographic profiles of leaf, bark and root extracts of *W. Somnifera* and *R. tuberosa* compare and found that numbers of spots are greater in all three parts of *W. somnifera* over the *R. tuberosa*. Further, spots with similar Rf values were observed under UV 366 nm (Rf 0.08, 0.15, 0.37, 0.65 and 0.91) and after spraying with Vanillin sulphate (Rf 0.75, 0.85 and 0.87) for leaf extracts of *W. somnifera* and *R. tuberosa* 0.85 and 0.87) for leaf extracts of *W. somnifera* and *R. tuberosa*.

As shown in Figure 3, prominent, bright purple (Rf 0.90), dark green (Rf 0.78) and greenish colour spots distinguished for *Withaniasomnifera* while dark yellow spots (Rf 0.49 and 0.96) are characteristics to *Ruellia tuberosa* root extracts. On the other hand two black colour spots (Rf 0.38 &0.43) are common for root extracts of both plants.

As demonstrated in Table 3, it was revealed that total ash, water soluble ash, acid insoluble ash and hot extraction values were comparatively higher in *R. tuberosa* over the *W. somnifera* (Table 3). The order of increase of total ash and water soluble ash was varied as leaf>bark > roots for both plant species. Moreover, the higher extractable matter was reported from hot extraction method for both plant species. This may be due to enhancement of extraction in hot extraction procedure.

Observed higher extractable matter content of leaf are in agreement with previous studies [31], which reported the presence of comparatively higher extractable matter content in leaf extracts of *A. paniculata*

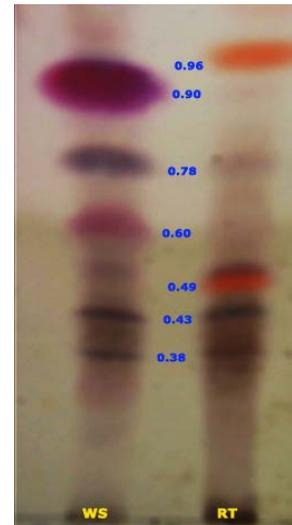


Figure 3. Thin layer chromatographic profile of root extract of *Withania somnifera* and *Ruellia tuberosa* [WS- *Withania somnifera* root extract; RT- *Ruellia tuberosa* root extract]

Table 3. Physicochemical characters of *Withania somnifera* and *Ruellia tuberosa*

Parameter	Plant species					
	<i>Withania somnifera</i>			<i>Ruellia tuberosa</i>		
	Leaf	Root	Bark	Leaf	Root	Bark
Moisture content	10.5±0.25	7.4±0.36	13.1±0.68	12.4±0.22	12.4±0.22	11.3±0.31
Total ash	11.2±0.06	4.5±0.12	10.0±0.99	13.3±0.34	14.4±0.22	17.2±0.11
Water soluble ash	7.3±0.15	1.9±0.10	3.7±0.19	6.9±0.09	2.1±0.11	7.1±0.16
Acid insoluble ash	0.53±0.01	0.82±0.07	0.57±0.04	0.1±0.02	1.6±0.33	0.9±0.10
Hot extractable matter	22.5±0.22	16.6±0.39	8.4±0.11	11.5±0.03	20.8±0.06	7.3±0.52
Cold extractable matter	17.6±0.48	13.5±0.11	5.6±0.29	8.3±0.22	19.0±0.36	7.2±0.13

Results are the means of 3 replicates ±SE

Table 4. Comparative phytochemical analyses of different parts of *Withania somnifera* and *Ruellia tuberosa*

Metabolite	Phytochemical Analysis					
	Plant species					
	<i>Withania somnifera</i>			<i>Ruellia tuberosa</i>		
	Root	Leaf	Bark	Root	Leaf	Bark
Alkaloids	+	+	+	+	+	+
Flavanoids	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Steroidal Glycosides	+	+	+	+	+	+
Tannins	+	+	+	+	+	+

+ = Presence

Table 5. Brine shrimp toxicity of different parts of *Withania somnifera* and *Ruellia tuberosa*

Part of the plant	LC ₅₀ (ppm)		LC ₅₀ (ppm)	
	<i>Withania somnifera</i>	95% Fiducial value	<i>Ruellia tuberosa</i>	95% Fiducial value
Root	23.54 ± 3.18 ^a	15.05-21.04	27.20 ± 5.08 ^b	20.35 - 47.53
Leaf	17.53±1.44 ^a	18.85 - 33.75	16.31 ± 1.48 ^b	13.76 - 19.96
Bark	16.64 ± 2.46 ^a	12.78 - 23.99	19.84 ± 1.86 ^a	16.82 - 24.84

The therapeutic value of drugs, which are mainly based on herbal materials, depends greatly on the bioactive constituents present in the raw materials. Therefore, use of authentic raw materials with proper bioactive molecules play an important role in efficacy, safety and quality control of drugs. As demonstrated in Table 4, root leaf and bark extracts of *W. somnifera* and *R. tuberosa* exhibited the presence of major chemical groups such as alkaloids, flavanoids, saponins, steroid glycosides and tannins in both *W. somnifera* and *R. tuberosa*.

Brine shrimp toxicity assay is considered as a simple, inexpensive, and a reliable tool for preliminary screening of cytotoxicity and it has consistent with the correlation with cytotoxicity in plant extracts [33,34]. Therefore, it is commonly used as a preliminary tool for screening cytotoxicity in plant crude extracts [35]. As demonstrated in Table 4, all parts of *W. somifera* and *R. tuberosa* exhibited mild cytotoxicity. Order of potency of *W. somnifera* was bark>leaf>root while it was leaf > bark > root for *R. tuberosa*. Presence of less cytotoxicity in root is an evidence of safety of root extracts of both plants.

This result is in agreement with Prabu *et al.* [36], who investigated acute and sub-acute oral toxicity of *W. somnifera* root extracts using rat models and found that there was no any toxic signs up to 2000 mg/kg body weight per day. Further, According to Meyer *et al.* [33], crude plant extract is toxic (active) if it has an LC50 value of less than 1000 µg/mL while non-toxic (inactive) if it is greater than 1000 µg/mL. On the other hand, presence of slightly higher cytotoxicity in bark and leaf extracts of *W. somnifera* and *R. tuberosa* might be due to presence biologically active constituents in leaf and bark extracts. Results of the present study are in agreement with Siriwardane *et al.*, [37], who investigated that the presence of higher content of anticancer potential withaferin A, in leaf and bark extracts of *W. somnifera*. Moreover, Sharmin *et al.* [38], which proved the presence of cytotoxicity in leaves of *A. oleraceae*.

4. Conclusions

Even though the plants belong to two genera and possess different morphological and powder microscopical characters, there are certain similarities in preliminary phytochemical screening, thin layer chromatographic profiles and brine shrimp toxicity of different parts of *W. somnifera* and *R. tuberosa*. Presence of certain phytochemical groups, both in preliminary phytochemical and physicochemical screening, thin layer chromatographic profiles, and brine shrimp toxicity partially justifies the traditional claim of use of *R. tuberosa* as a substitute for *W. somnifera* in traditional systems of medicine in Sri Lanka. However, further studies on separation and bioactivity guided isolation of active constituents, and clinical trials are needed to confirm the above results. Information gathered through the present study could be directly used for the standardization and quality control of *W. somnifera* and *R. tuberosa* as well as to upgrade the Sri Lankan pharmacopeia.

Conflict of Interest

Authors declare that there is no conflate of interests

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