Materials and Methods

3.0. MATERIALS AND METHODS

The present work entitled "**Extraction and purification of withanolides from dry roots of *Withania somnifera***" was conducted at Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Deemed University for Women, Coimbatore. The materials used for the study and experimental methods followed are presented in this chapter.

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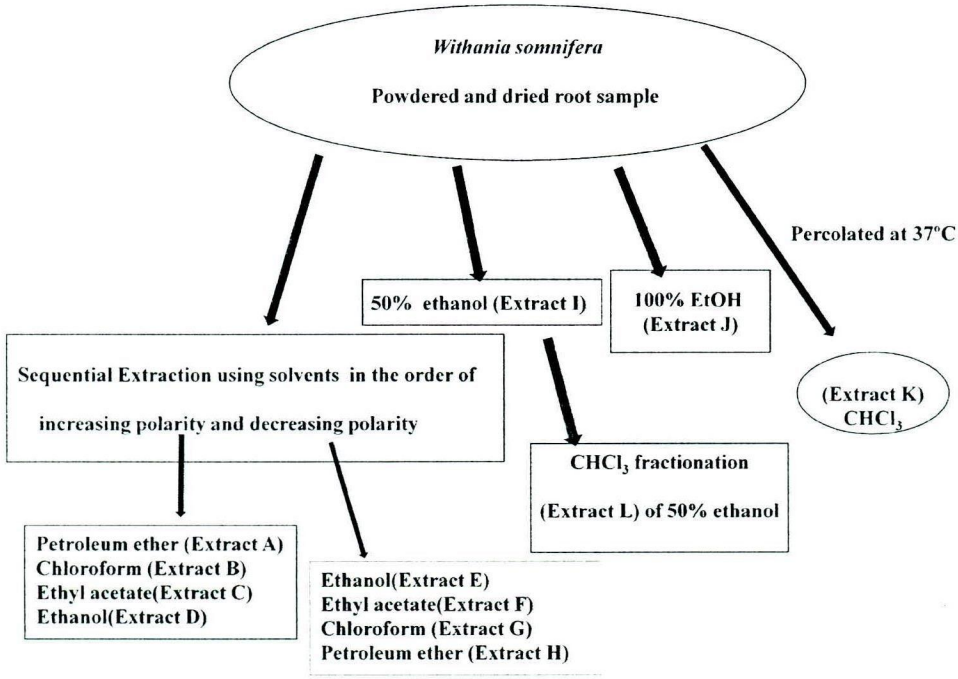
3.1. Collection of samples

Dried root samples of *Withania somnifera* collected from Nagpur during the month of January 2008 was used for the present study.

3.2 Extraction of Secondary metabolites from *Withania somnifera* roots

The extraction of *Withania somnifera* roots was performed using powdered sample with various solvents following the protocol of Cho *et al.*,(2008) that the extraction yield of total solid of pulverized red ginseng (10~40 mesh size) was high when compared with that of non-pulverized sample.

Figure 3.1
Secondary metabolite extraction from *Withania somnifera* roots using various solvents



Various types of extraction can be applied to raw material resulting in significant changes in quantities and proportions of active components (Schilter *et al.*, 2003). According to the conditions of the extraction namely type of solvent, duration of extraction, light conditions and temperature during extraction, the secondary metabolite content of the extract will change (Liu, 2000).

The extraction of finely powdered 2g of *Withania somnifera* roots using 25 ml of respective solvents with different polarity was carried by constant stirring at 85 rpm for 12 hours in an incubated shaker maintained at 37°C. The color and the total volume of the each extracts obtained were noted.

Thin layer chromatographic analysis and multi wavelength scanning of various extracts from the *Withania somnifera* roots was performed to analyze the compounds that were extracted with solvents of different polarity.

3.2.1. Sequential extraction of samples

2g of powdered *Withania somnifera* roots was extracted sequentially with 25 ml of solvents in the order of increasing as well as decreasing polarity. The extracts were named as Extract A, Extract B, Extract C, Extract D, Extract E, Extract F, Extract G and Extract H as shown in the figure 3.1.

Sequential extraction of powdered *Withania somnifera* root sample was performed in the present study using solvents namely petroleum ether, chloroform, ethyl acetate and ethanol in the order of increasing as well as in decreasing polarity. This was performed in order to analyze the best solvent for the extraction of withanolides from *Withania somnifera* roots. Before sequential addition of every solvent (25ml) for extraction, the residue was dried followed by subsequent extraction.

3.2.2. Ethanol extraction of samples

2g of powdered sample of *Withania somnifera* was subjected to extraction with 25ml of 50% ethanol and this process was repeated for four times. The extracts obtained were then filtered through Whatmann No.1 filter paper and named as Extract I. The volume and the color of Extract I were noted. Similarly for obtaining 100% ethanol extracts (Extract J) another 2g of *Withania somnifera* roots was used.

3.2.2.1 Chloroform fractionation of 50% ethanol extracts

50% ethanol (hydro alcoholic) extracts (Extract I) of *Withania somnifera* roots was incubated in a shaker maintained at 37°C using equal volume of chloroform by constant stirring at 85 rpm.

The chloroform layer was then separated from Extract I using separating funnel and this process of fractionation was repeated until the organic layer turned colorless. The alcoholic chloroform fraction named as Extract L was pooled and the hydro alcoholic extract after fractionation (fraction M) was concentrated at 50°C by simple distillation. The concentrated chloroform fraction (Extract L) and hydro alcoholic extract obtained after fractionation (fraction M) was analyzed by TLC and double beam spectrophotometer.

3.2.2.2 Test for glycosides

Chloroform fraction of 50% ethanol extract (Extract L) obtained from *Withania somnifera* root was dried and 4mg of the dried residue was used for the assay of the glycosides.

Dried residue was suspended in 10ml of water and mixed with 0.5 ml of strong lead acetate solution followed by vigorous shaking. The supernatant was removed by filtration and mixed thoroughly with 5ml of chloroform. Subsequently, the upper layer was aspirated off carefully followed by removal of solvent by simple evaporation. The residue obtained was suspended in 3ml of glacial acetic acid containing 2 drops of 5% ferric chloride solution. This was followed by careful and slow addition of 2ml concentrated H_2SO_4 along the wall of the test tube. A reddish brown color formed at the junction of two liquids and conversion of upper layer slowly into bluish green color was considered as an indication of positive reaction (Owais *et al.*, 2005).

3.2.3 Chloroform extraction of samples

Similarly chloroform extracts (Extract K) of *Withania somnifera* was obtained by extracting 2g of samples directly with 25 ml of chloroform. This process was repeated for

4 times and the extracts were filtered through Whatmann No.1 filter paper. The pooled chloroform extracts were concentrated in a water bath maintained at 50°C.

3.3 Analysis of sample extracts

All the sequential extracts obtained using the solvents in the order of increasing polarity and decreasing polarity, 50% ethanol (Extract I), 100% ethanol (Extract J), chloroform fraction of 50% ethanol extract (Extract L) and chloroform extracts (Extract K) obtained from 2g of finely powdered *Withania somnifera* roots was made up to a volume of 10 ml. And this was used for analysis by Systronics double beam spectrophotometer 2202 and thin layer chromatography.

3.3.1 Thin layer chromatography

In the present study the extracts prepared using various solvents were analyzed by thin layer chromatography following the method of Sharada *et al.*, (2007).Thin layer chromatography of extracts was performed on Merck Silica gel 60 F_{254} plates. A TLC plate of size 9cm x 4cm was taken and origin was marked at 1.2 cm from the base of plate. The solvent system chloroform: methanol in the ratio 18:2 was prepared. 10µl of methanol and ethanol extracts of samples were spotted on the TLC plate, air dried and placed in a chromatographic chamber saturated with solvent. The run was performed until the solvent reached the top of the plate. 10% of sulphuric acid was sprayed over the plate and air dried for 2-3 minutes. The spots developed by mild heat using a spirit lamp were recorded and analyzed.

3.3.2 Multi wavelength scanning

The presence of various compounds in 10ml of sequential extracts (Extract A to Extract H),Extract I, Extract J, Extract K and Extract L from 2g of *Withania somnifera* roots were analyzed by scanning between wave length 200 nm and 300nm using the instrument Systronics double beam spectrophotometer 2202.

3.4 Purification of withanolides by column chromatography

In the present study, the extract of sample showing fine resolution with maximum number of spots upon TLC analysis and maximum absorbance by dual beam spectrophotometer was subjected to column chromatography in order to purify the withanolide glycosides from *Withania somnifera* roots.

3.4.1 Column Preparation

Silica gel of 100-200 mesh analytical grade was prepared by washing in distilled water. After the silica gel is free of very fine particles that greatly impede solvent flow through the column, it is dried to constant weight at 100°C.The dried silica gel was then stirred thoroughly with petroleum ether (60-80°C) and this slurry was poured into the glass column of length 50 cm and 78 cm respectively.

A glass column of 0.8 cm diameter and 50 cm long with a 10ml solvent reservoir on one end and stop cock on the other was used for packing the adsorbent. A small plug of glass wool at the bottom of the column prevents the silica gel from entering the stopcock.

7.0 g of silica gel (100-200 mesh) suspended in 20 ml of petroleum ether (60-80°C) and this slurry prepared was poured carefully and slowly into a glass column of height 50 cm using a funnel. Stop pouring frequently to swirl the slurry so that the silica is evenly mixed. The slight force was applied along the length of column gently with a rubber stopper or with the end of a pencil, which will improve the packing of the silica gel.

Never let the solvent level drop below the top of the column while collect excess solvent that elutes the silica gel through the stopcock at the bottom and reuse for further packing of silica gel in glass column. A pipette with petroleum ether was used to rinse any silica stuck to the sides at the top of the column into the solvent layer resulted in 28.5 cm height silica gel column. Once the column is packed, add a protective layer of cotton plug to the top of the silica for protecting the column while adding solvent for elution.

Housley and Bentley (1956) suggested that employing a column of different length, or changing the degree of hydration of the silica gel may provide solutions to some problems obtained while purification using silica gel column chromatography.

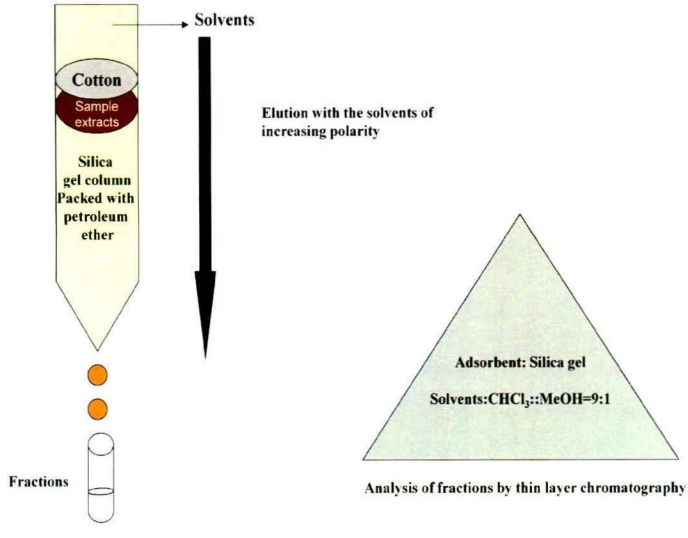
28g of silica gel suspended in 75 ml of petroleum ether (60-80°C) was poured into the glass column of 78cm long with 1cm diameter resulted in 40 cm silica gel column. Approximate bed volume of petroleum ether in 28.5 cm and 48 cm silica gel column was found to be 10ml and 30 ml respectively.

In the present study the concentrated extract obtained from 5g of *Withania somnifera* roots was subjected to silica gel column of 28.5 cm. Highly concentrated extract from 15-50g of sample was then subjected to 48 cm silica gel column by directly pipetting into the column. This can be done only when the extracting solvent does not undesirably alter the elution pattern of regular eluting solvents. After pipetting the sample extracts into the silica gel column, the cotton plug was inserted to avoid any disturbances in the column while pouring the mobile phase on it.

3.4.2 Elution of silica gel column

Silica gel in the column chromatography consists of polar hydroxyl groups, the withanolide glycosides and other compounds from the sample extracts being polar preferred to adsorb on it. Thus the non-polar components of the extract tend to elute before the more polar ones. And in the present study the silica gel column was further accompanied by elution using the solvents of increasing polarity.

Figure 3. 2 Purification of withanolides by silica gel column chromatography



The first eluting solvent namely petroleum ether was followed by solvents of increased polarity namely chloroform and ethyl acetate. For routine survey of work a suggested solvent schedule as shown in table 3.1 was followed in the present study.

After determining which of these solvents in specified volume are important for complete elution of desired compound, the others may be eliminated from the solvent schedule, thus shortening the chromatographic process. The solvent in different ratios of desired volume to elute the particular compound was made to pass through the column by gravity and the specified volume of eluates were collected as the solvent drips from the bottom of the column.

Table 3.1

Solvent schedule for the Purification of Withanolides on silica gel column

S.No	Petroleum ether	Chloroform	Ethyl acetate
1	100	0	0
2	75	25	0
3	50	50	0
4	25	75	0
5	0	100	0
6	0	75	25
7	0	50	50
8	0	25	75
9	0	0	100

3.4.3 Analysis of eluants

Several fractions of the eluant of desired volume are collected sequentially in labeled tubes. Each fraction was concentrated and the composition of it was analyzed by thin layer chromatography as mentioned above.

For longer duration (48cm silica gel) column with the highly concentrated extract, the fractions collected were combined after analysis by thin layer chromatography while the elution of column was performed until the desired compound appeared as spot upon TLC.