Results and Discussion

4.0 RESULTS AND DISCUSSION

Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterized by their structure containing a triterpene or steroid aglycone and one or more sugar chains.

Over 85% of the herbs most commonly used in Traditional Chinese Medicine were observed to contain saponins (in addition to poly phenols) in significant detectable amounts, while the herbal products and most commonly used formulae were explicitly rich in these components (Liu and Henkel, 2002).

Withania somnifera belonging to the solanaceae family contains the saponins known as withanolide glycosides (steroidal lactone + sugar residues) as indicated by Ghosal *et al.*,(1988).Biological activity of saponins (such as anti-cancer and anti-cholesterol activity) has led to the emergence of these saponins as commercially significant compounds with expanding applications in food, cosmetics, and pharmaceutical sectors. The realization of their full commercial potential requires development of new processes/processing strategies to address the processing challenges posed by their complex nature.

The results of the present study "**Extraction and purification of Withanolides from dry roots of *Withania somnifera***" were discussed under the following headings.

4.1 Collection of sample

4.2 Extraction of secondary metabolites from *Withania somnifera* roots

4.2.1 Sequential extraction of samples

4.2.2 Physical characteristics of sequential extracts

4.2.3 TLC pattern of sequential extracts

4.2.4 Scanning of sequential extracts by double beam spectrophotometer

4.3 Hydro alcoholic, alcoholic and chloroform extracts of samples

4.3.1 Physical characteristics of hydro alcoholic, alcoholic and chloroform extracts

4.3.2 TLC pattern of hydro alcoholic, alcoholic and chloroform extracts

4.1 Collection of sample

In the present study *Withania somnifera* root was collected from a local market in Nagpur. The samples were dried, powdered and used for the study.

4.2 Extraction of secondary metabolites from *Withania somnifera* roots

Secondary metabolites from Dry roots of *Withania somnifera* was extracted using various solvents.

4.2.1 Sequential extraction of samples

As saponins (water soluble components) mostly obtained in the aqueous alcoholic extracts, in the present study the sequential extraction from *Withania somnifera* roots was performed as followed by Yoo *et al.*,(2009) using petroleum ether, chloroform, ethyl acetate and ethanol in the order of increasing and decreasing polarity to determine the extraction efficiency of saponins in various solvents.

4.2.2 Physical Characteristics of Sequential extracts

The physical characteristics of the sequentially extracted solvents in the order of increasing and decreasing polarity were determined by visual observation.

Withania somnifera roots were yellowish Brown or light brown. The outer surface of it is bugg to grey yellow with longitudinal wrinkles (Kokate *et al.*, 1996). Extracts of *Withania somnifera* roots obtained in the present study namely ethanol, chloroform and ethyl acetate extracts were found to be orangish yellow to pale yellow similar to the color of the sample.

Table 4.1

Physical Characteristics of Sequential Extracts

Sequential extracts	**Volume of the extract**	**Colour of the extract**
Increasing polarity		
Petroleum ether (Extract A)	10ml	Pale green
Ethyl acetate (Extract B)	10 ml	Orangish yellow
Chloroform (Extract C)	10ml	Pale yellow
Ethanol (Extract D)	12 ml	Orangish yellow
Decreasing polarity		
Ethanol (Extract E)	16ml	Orangish yellow
Ethyl acetate (Extract F)	13ml	Pale yellow
Chloroform (Extract G)	10ml	Pale yellow
Petroleum ether (Extract H)	10ml	Colourless

The extracts obtained in the order of both increasing and decreasing polarity was then further subjected to thin layer chromatographic analysis in order to analyze the constituents extracted in the solvents.

4.2.3 TLC pattern of sequential extracts

As Fayez and Saleh (1967) analyzed the glycosides from *Solanum wrightII* using chloroform–ethanol-ammonia solution (1%) in the ratio (2:2:1) as the solvent system, similarly in the present study 20µl of the sequential extracts obtained in the order of both increasing and decreasing polarity was analyzed by the solvent system chloroform and methanol in the ratio 18:2.

TLC analysis of sequential extracts revealed the presence of various compounds with different R_f values as indicated in table 4.2. Sharada *et al.*, (2007) extracted withanolides

using lyophilized plant parts or *in vitro* cultured tissues of *Withania somnifera* with ethanol: water (1:1) and the extract obtained was further fractionated using chloroform found to contain total withanolides. And this chloroform fraction was analyzed by TLC with solvent system chloroform: methanol (96:4) and the spots were visualized by spraying vanillin reagent [vanillin: boric acid: methanol: sulphuric acid in the ratio of 0.5g: 50g: 500 cm^3:10 cm^3 (50 %)].

Table 4.2

TLC pattern of sequential extracts

R_f values	Increasing polarity				Decreasing polarity			
	A	B	C	D	E	F	G	H
0.1		√	√	√	√	√		
0.175		√	√	√	√	√		
0.275		√	√	√	√	√		
0.35	√	√	√	√	√	√	√	
0.4	√	√	√	√	√	√	√	
0.5	√	√	√		√	√	√	
0.55	√	√	√		√	√		
0.67	√	√	√		√	√		
Total no.of spots	5	8	8	5	8	8	3	0

√: Presence of the spot

The sequential extraction performed in the order of increasing polarity revealed that extract A (obtained using petroleum ether, non polar solvent) was found to contain five compounds corresponding to the R_f values 0.67, 0.55, 0.5, 0.4 and 0.35. The intensity of the obtained spots was found to be more predominant as shown in **plate** 4.**1**.

The sequential extracts namely extract B and extract C obtained using solvents chloroform and ethyl acetate respectively in the order of increasing polarity was found to contain eight spots corresponding to R_f values 0.67, 0.55, 0.5, 0.4, 0.35, 0.275, 0.175 and 0.1. But the intensity of the spots corresponding to R_f values 0.67, 0.55, 0.5, 0.4 and 0.35 obtained in extract B and extract C was found to be successively decreased when compared

to extract A. The extract D obtained in the order of increasing polarity was found to contain only five compounds with R_f values 0.4, 0.35, 0.275, 0.175 and 0.1.

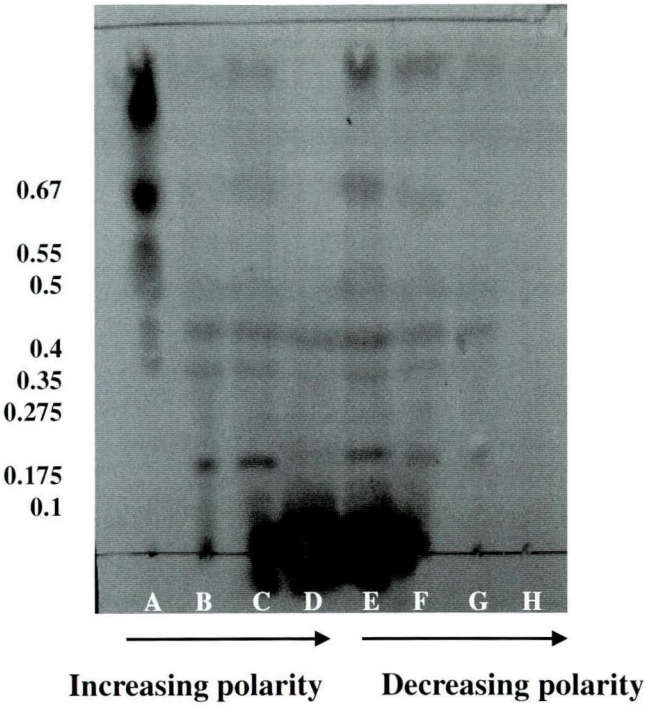
Preliminary phytochemical screening of petroleum ether and alcohol extract of *Symplocos racemosa* was carried out by Devmurari (2010) who found the presence of carbohydrate, glycoside, saponins, terpenoid and alkaloids in alcoholic extract whereas ether extract have shown presence of glycoside, phytosterol and steroids revealing that in the present study petroleum ether being non-polar solvent extracted the compounds with R_f values 0.67, 0.55, 0.5, 0.4 and 0.35 predominantly when compared to the other extracts containing the compounds with lower R_f values. It can be said that saponins were most predominantly extracted in alcohol when compared to the petroleum ether.

More over withanolide glycosides (steroidal aglycones+ sugar residues) obtained in the extract may be the spots with the lower R_f values compared to the withanolide aglycones when the chloroform and methanol in the ratio 9:1 was employed in the present study as solvent system for TLC analysis.

The sequential extracts namely extract E and extract F obtained in the order of decreasing polarity revealed the presence of eight spots as in the case of increasing polarity corresponding to the R_f values 0.67, 0.55, 0.5, 0.4, 0.35, 0.275, 0.175 and 0.1 but the extract G was found to contain only three spots corresponding to the R_f values 0.5, 0.4 and 0.35 with decreased intensity. No spots were obtained in extract H which indicates that the extraction of active compounds from *Withania somnifera* root was an exhaustive process (Ganzera *et al.,* 2003) which was clearly revealed in the present study by thin layer chromatographic analysis as shown in **plate 4.1**.

TLC pattern of sequential extracts performed in the order of decreasing polarity indicated that first solvent ethanol being more polar extracted eight compounds from *Withania somnifera* roots. The spots obtained for the ethyl acetate extract (Extract F) were found to be the same as that of the ethanol extract (Extract E) but with the decreased intensity. The chloroform extract (extract G) was found to contain the presence of three compounds corresponding to the R_f values 0.35, 0.4 and 0.5. It was found that no spots were obtained in petroleum ether extract revealing that most of the components were extracted in the ethanol, ethyl acetate and chloroform solvent as indicated in table 4.2.

Plate 4.1 Thin layer chromatographic pattern of Sequential extracts of *Withania somnifera* roots



Sequential extracts

Increasing polarity-A-petroleum ether extract , B-chloroform extract , C-ethyl acetate extract and D-ethanol extract

Decreasing polarity-E-Ethanol extract, F-ethyl acetate extract, G-chloroform extract and H-petroleum ether extract.

For the present study aimed at the purification of polar withanolide glycosides from *Withania somnifera* roots, extraction can be carried in the order of increasing polarity.TLC analysis of sequential extracts in the order of increasing polarity indicates that the extract A obtained with non polar solvent extracted four non polar components (**plate 4.1**). Chloroform being comparatively less non polar extracted most of the non polar and polar components in extract B which was followed by ethyl acetate solvent.

Pawar and Bhutani (2006) obtained polar saponins in both the ethyl acetate fractions of 70% methanolic extract and parent methanolic extract of *Bacopa monniera*. Bagavan *et al.*, (2008) found the highest larval mortality in the ethyl acetate extract of *Achyranthes aspera* led to the separation and identification of a potential saponin as a mosquito larvicidal compound indicating that the ethyl acetate may also have extracted the saponins from *Withania somnifera* roots.

The spotted area of ethanol extract (Extract A and Extract E) on TLC plate after developing with 10% sulphuric acid was found to be blackened in both increasing and decreasing polarity indicating that the ethanol being more polar extracted more sugars. Moreover TLC pattern of sequential extracts performed in the order of increasing polarity revealed that most of the non polar components were extracted in the petroleum ether, chloroform and ethyl acetate solvents and therefore the ethanol extract was found only with spots corresponding to the polar components.

Similarly Jubie *et al.*, (2008) performed the successive extraction of the powdered stem bark of *Alangium salviifolium* using petroleum ether, ethyl acetate, chloroform and methanol in the order of increasing polarity. The phytochemical analysis revealed the presence of steroids, saponins and flavonoids in petroleum ether extract whereas the chloroform had extracted the compounds namely alkaloids, steroids and saponins. The ethyl acetate extract contain alkaloids, steroids and flavonoids. The methanol extract was found to contain alkaloids, steroids, tannins and saponins.

Krasteva *et al.*, (2004) found the presence of flavonoids in the dry ethyl acetate extract from the above ground parts of *Astragalus corniculatus*. Yoo *et al.*, (2009) found that recovery yield and bioefficacy of crude saponin content of aqueous extract obtained after the sequential extraction with hexane was high when compared to sequential extraction

with aqueous solvents followed by hexane. Moreover the results obtained suggested that total crude saponin extraction was hardly affected by the hexane extraction process.

Therefore, TLC pattern of sequential extracts performed in the order of increasing and decreasing polarity revealed that the extraction of withanolides from *Withania somnifera* roots can be carried with petroleum ether for defatting and chloroform, ethyl acetate followed by ethanol in the order of increasing polarity can be performed for obtaining the purified withanolide glycosides.

4.2.4 Scanning of sequential extracts by double beam spectrophotometer

In the present study, the absorbance of the extracts at 254nm was detected using the Dual beam spectrophotometer 2202 to determine the maximum quantity of saponins in the sequential extracts obtained in the order of increasing polarity from *Withania somnifera* roots as Ciddi (2006) analyzed the presence of withaferin A in the methanolic extract at UV 254 nm.

Similar to the present study Matsuda *et al.*, (2001) analyzed the withanolide glycosides from *Withania somnifera* by the UV spectrum. Sequential extracts obtained from 2g of *Withania somnifera* roots in the order of increasing polarity using solvents namely petroleum ether, Chloroform, Ethyl acetate and Ethanol extracts of the sample was subjected to scanning for the presence of saponins at 254nm by double beam spectrophotometer 2202.The results revealed that absorbance at 254nm was maximum for extract D (2.503) followed by extract B (1.836), extract C (1.102) and extract A (1.004). Similarly Yamaguchi *et al.*, (1988) conducted the chromatographic detection of saponins at shorter UV wavelengths, e.g., 202 nm for detection of Panax ginseng roots.

The scanning of sequential extracts performed in the order of increasing polarity revealed that the maximum quantity of withanolides was found in ethanol extracts (Extract D) followed by chloroform (Extract C) when compared to ethyl acetate extracts (Extract B). Absorbance for withanolides at 254nm for petroleum ether extract (Extract A) was minimum suggesting that petroleum ether can be used for defatting the *Withania somnifera* roots before extraction with the ethanol or chloroform.

4.3 Hydro alcoholic, alcoholic and chloroform extracts of samples

In the present study exhaustive extraction of 2g of *Withania somnifera* roots was carried as suggested by Ganzera *et al.*,(2003) using solvents in various percentage such as 50% ethanol, 100% ethanol and 100% chloroform was performed. This study was similar to the one conducted by Ichikawa *et al.*, (2009) who evaluated the extraction efficiency of methanol concentrations on saponin extraction from *Codonopsis lanceolata* root powder in order to comparatively analyze the best solvent for the extraction of active components from *Withania somnifera* roots.

4.3.1 Physical characteristics of hydro alcoholic, alcoholic and chloroform extracts

The physical appearance of concentrated 50% ethanol (E), 100% ethanol (F) and the chloroform extracts (I) was determined by visual observation was found to be yellowish orange to dark red in color.

50% ethanol extracts (E) after exhaustive extraction with 2g of *Withania somnifera* roots for 4 times using 25ml of solvent was found to be 60ml whereas the volume of 100% ethanol (F) and chloroform extract (I) was found to be 55 ml and 25 ml respectively.

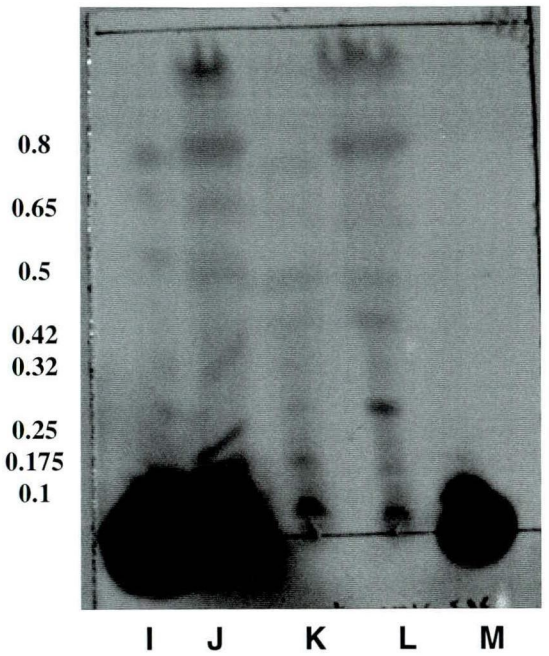
4.3.2 TLC pattern of hydro alcoholic, alcoholic and chloroform extracts

TLC analysis was performed using 10µl from 10 ml concentrated hydro alcoholic (Extract I), alcoholic (Extract J) and chloroform extracts (Extract K) of samples. The presence of similar spots corresponding to the R_f values 0.8, 0.65, 0.5, 0.42, 0.32, 0.25, 0.175 and 0.1 upon TLC analysis of all the extracts was found as shown in **plate 4.2**.

Moreover TLC pattern of 50% (Extract I) and 100% ethanol extracts (Extract J) revealed that the spotted area was more blackened when compared to that of 100% chloroform extracts (Extract K) indicating that more sugars were extracted in the polar ethanol solvent when compared to the non polar chloroform solvent.

The poor resolution of spots upon TLC analysis of 50% ethanol extract (Extract I) was found when compared to 100% ethanol (Extract J) and chloroform extracts (Extract K). Malinow *et al.*, (1979) revealed that the solvents namely acetone and 95% ethanol removed

Plate 4.2 TLC pattern of alcoholic, hydro alcoholic and chloroform extracts of sample



Extract I-50% ethanol extract Extract J -100% ethanol extract Extract K- chloroform extract

Extract L-chloroform fraction of 50% ethanol Fraction M- 50% ethanol after fractionation

not only saponins, but also fat, protein and carbohydrates while preparing the "saponin-free" alfalfa by sequential organic solvent extraction.

Kandil *et al.,* (1994) identified flavonoids and phenolic compounds from the aqueous ethanolic extract of *Withania somnifera* leaves which indicated that flavonoids are better extracted in ethanol. The results obtained indicated that 50% ethanol extract (Extract I) being highly polar may extracted more polar components such as oligosaccharides, proteins, phenolic compounds, flavonoids etc as suggested by Malinow *et al.*, (1979) and Kandil *et al.*, (1994) which may be a reason for the poor resolution of spots upon TLC.

4.3.3 Scanning of hydro alcoholic, alcoholic and chloroform extracts of samples

In the present study, 50% ethanol (Extract I), 100% ethanol (Extract J) and 100% chloroform extracts (Extract K) from 2g of *Withania somnifera* roots was scanned for the withanolides at 254 nm using double beam spectrophotometer.

The results obtained indicated that the absorbance of 50% ethanol extracts at 254nm was comparatively very high (2.740) followed by 100%, chloroform (2.380) and ethanol (2.375) extracts of sample.

The result obtained was supported by the studies of Gafner *et al.*, (2004) who indicated that the amount of total saponins were found to be higher quantity (61.7 ± 0.1 mg/g dry root) in 50% aqueous ethanol extract from *Panax quinquefolius* root than the other solvents such as 20% ethanol, 40% glycerin, and 40% water and 65% (v/v) aqueous glycerin.

Malinow *et al.,* (1979) found that *Codonopsis lanceolata* root extracted with 30, 50 and 70% methanol showed higher contents of all saponins than samples extracted with 0, 90 or 100% methanol. Moreover they found that more than 94% of extracted saponins were retained in sample solutions when extracted with 50% methanol after storage for 48 hour at room temperature and therefore, 50% methanol was selected as an optimum solvent for saponin extraction from *Codonopsis lanceolata* root powder.

Thus the present study aimed at the purification of withanolide glycosides which belong to saponins can be performed by extracting *Withania somnifera* roots using 50%

ethanol. Moreover the ethanol is relatively cheap for herbal medicine preparation compared to the other solvents.

4.4 Silica gel column chromatographic analysis of alcoholic extract

In the present study an attempt was made to remove the more polar components such as oligosaccharides, proteins, phenolic compounds, flavonoids etc that was extracted along with withanolides from *Withania somnifera* roots.

The concentrated 50% alcohol extracts (Extract I) of samples was subjected to silica gel column chromatography followed by elution with water and then with 100% alcohol (Extract J) was performed. The alcoholic fraction of silica gel column upon thin layer chromatographic analysis revealed that the blackening of the spotted area was less compared to the TLC pattern of the concentrated crude alcoholic extract as shown in **plate 4.3**.

More over the resolution of spots obtained for aqueous alcoholic extract (Extract J) was comparatively poor than the alcoholic fraction after column chromatography indicating that the highly concentrated ethanolic extracts of samples may have a mixture of compounds like flavonoids, alkaloids and phenolics along with sugars and withanolides that hindered the resolution spots upon TLC.

The result obtained indicates that the elution of the silica gel column containing the concentrated alcoholic extract with water removed the sugars to a certain quantity. But alcoholic extract (Extract J) when subjected to silica gel column chromatography followed by elution with water can have the possibility of eluting the withanolide glycosides and therefore less withanolide glycosides can be obtained in 100% alcohol fraction.

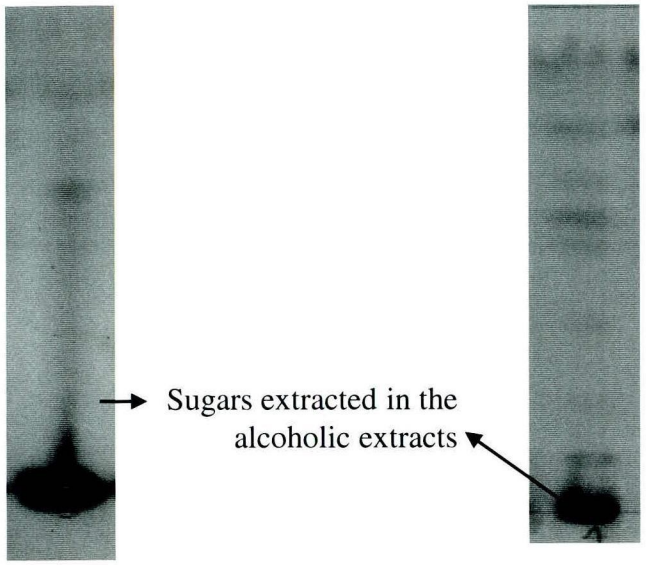
4.5 Fractionation of hydro alcoholic extracts of sample

In the present study an attempt was made to further fractionate 50% ethanol extract of *Withania somnifera* roots with the chloroform (less polar solvent) in order to separate the withanolides completely from the other soluble compounds.

4.5.1 Physical characteristics of chloroform fraction of 50% ethanol extract

The color of 50% ethanol extract (Extract I) obtained in the present study was found to be turbid and dark brownish to dark yellowish orange in color. The fractionation of hydro

Plate 4.3 TLC pattern of concentrated alcoholic extracts before and after silica gel column chromatography



Before silica gel column chromatography

After silica gel column chromatography

alcoholic extracts using equal volume of chloroform for 12 hours maintained at 82 rpm was performed and the chloroform fraction (Extract L) was found to be clear reddish yellow in color. And this process of fractionation was found to have similar results to that of the alcoholic fraction obtained after elution with the water from alcoholic extract containing silica gel column.

4.5.2 TLC pattern of chloroform fraction and 50% ethanol extract after fractionation

TLC pattern of chloroform fraction revealed the presence of all the spots (withanolide glycosides and withanolides) with fine resolution in chloroform fraction (Extract L) as shown in **plate** 4.2. 50% ethanol extract after exhaustive fractionation (Fraction M) using chloroform was further analyzed by thin layer chromatography which was found to contain no spots indicated the absence of withanolides in fraction M.

The results obtained indicates that as withanolides being steroidal lactones have more affinity towards the less polar solvent, chloroform during the process of fractionation under shaking at 82 rpm for 12 hours. Moreover the chloroform fraction from 50% ethanol extract (Extract L) of sample obtained after fractionation has shown the positive results for the presence of glycosides.

4.6 Standardized procedure for the extraction of withanolides from Root samples

For the present study aimed at the purification of withanolide glycosides, hydro alcoholic extraction of *Withania somnifera* roots was performed as it has been found to contain both the withanolides and withanolide glycosides apart from the other water soluble components.

The results obtained revealed that for the large scale extraction of saponins defatting of *Withania somnifera* root powder using petroleum ether was performed followed by extraction with 50% ethanol as the studies on the extraction process revealed that most saponins are soluble in aqueous solutions and diluted alcohols, but almost insoluble in non polar solvents like hexane and petroleum ether.

As suggested by Hostettmann and Marston (1995) that defatting the powdered sample using non-polar organic solvents (i.e. petroleum ether or hexane) can be performed either prior to the extraction step or on the extract itself in the present study defatting process was performed prior to the extraction step.

Similar process of defatting the dried and powdered fruit of *Withania somnifera* was performed with petroleum ether (60–80°C) at room temperature for 48 hours by Abou-Douh (2002) while isolating two new withanolides.

Thus the non-polar solvent namely the petroleum ether can be employed for the purification of withanolide glycosides (saponins) via the removal of non-polar compounds before stepping into the extraction process using 50% ethanol as solvent.

Kitagawa (1985) found a common method for the preliminary purification of saponins that involves the partitioning of saponins between aqueous extracts and a water immiscible solvent such as n-butanol after the extraction step.

Similarly in the present study the fractionation of 50% ethanol extract (Extract I) using chloroform was performed as a preliminary purification step to separate all the withanolides in Extract I from that of aqueous soluble components. Moreover the chloroform fractions of 50% ethanol (Extract L) have shown the presence of glycosides in it confirming the presence of withanolide glycosides in it.

Sangwan *et al.*, (2007) isolated withanolides from *Withania somnifera* roots using four volumes of 50% methanol followed by defatting and depigmentation three times with an equal volume of *n*-hexane and recovering withanolides from the defatted methanolic solution into chloroform by liquid–liquid partitioning.Chloroform phases were pooled and redissolved in known volume of methanol just prior to TLC and HPLC analysis.

Similarly Chaurasiya *et al.*, (2007) extracted the wet leaves of *Withania somnifera* with 20 ml of methanol:water (25:75, v:v) at room temperature and the extract obtained was defatted using n-hexane. The methanol-water fraction was further fractionated twice with an equal volume of chloroform and found that the chloroform fractions have shown the presence of withanolides.

Bhattacharya *et al.,* (2001) found that chloroform insoluble (water soluble) fraction obtained from aqueous alcoholic extract of *Withania somnifera* roots was found to contain sitoindosides VII–X and withaferin-A as the major bioactive entities which was controversial to the present study where TLC pattern of chloroform fraction (Extract L) obtained from 50% ethanol extract revealed similar spots to that of ethanol extract before fractionation (Extract I and Extract J).

In the present study, the chloroform fraction obtained from 50% ethanol extract (Extract L) was used for the purification of saponins from *Withania somnifera* roots as suggested by Kim and Park (2001) that solvent partitioning might be efficient purification of saikosaponins followed by the preparative liquid chromatography that have a meaningful importance in food and medical sectors.

4.7 Purification of withanolides by adsorption column chromatography

Chromatography, firstly introduced by the Russian botanist Micharl Iswett is a method for separating the components of a mixture by differential distribution between a stationary phase and a mobile (moving) phase. Initially used for the separation of colored substances from the plants (Greek, Chromos meaning colored) and is now the most extensive technique for the separation and purification of colored/colorless organic compounds. Tswett (1905) developed this technique of chromatography and demonstrated its use by separating plant pigments.

For the purification of withanolides from *Withania somnifera* roots adsorption column chromatography was used as suggested by Ray and Gupta (1994) and Singh *et al.,* (1998) who indicated that different separation techniques such as column chromatography (CC), preparative TLC and centrifugal adsorption chromatography can be used for the isolation of withanolides.

Silica gel (SiO_2) and alumina (Al_2O_3) are two adsorbents commonly used by the organic chemist for column chromatography. These adsorbents are in different mesh sizes and for the present study, the silica gel of 100-200-mesh size was used. The solvent or eluant is allowed to flow down the column by gravity, or percolation was frequently referred to as liquid-solid chromatography or gravity column chromatography.

Chloroform (a non polar solvent) and ethyl acetate (less non polar solvent) in various ratios were used for the separation of withanolides from *Withania somnifera* roots in the present study similar to the one conducted by Gupta *et al.*, (2008) who isolated 27-hydroxy-withanolide B after subjecting the $CHCl_3$ extract from *Withania somnifera* leaves to silica gel column chromatography with the mobile phase containing chloroform and methanol solvent in the order of increasing polarity ($CHCl_3$, 2% MeOH in $CHCl_3$, 5% MeOH in $CHCl_3$ and MeOH).

Fractions of desired volume were collected in various test tubes as the solvent drips from the bottom of the column as suggested by Unger, 1979 that the impurity concentration in the silica gel is forming a gradient along the column height with increasing purity towards the exit.

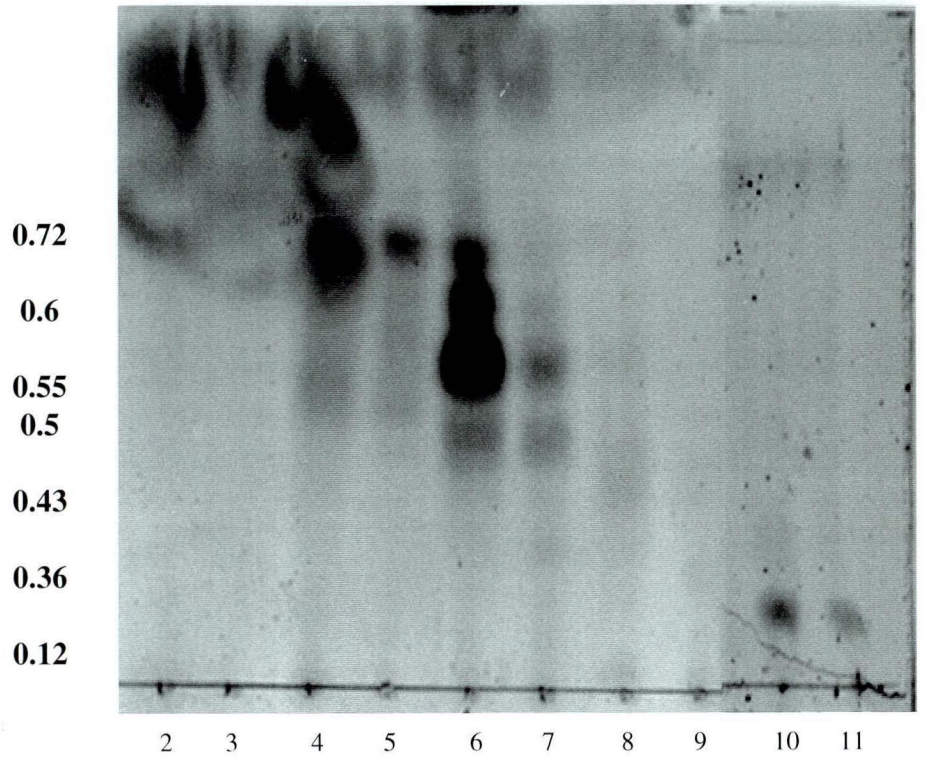
TLC analysis is generally used to determine the system for a column chromatographic separation as most of the fractions obtained from column chromatography in the present study are colorless. Thin layer chromatography employed in the present study was performed using solvent system chloroform and methanol in the ratio of 18:2.

4.7.1 Silica gel column chromatographic analysis of extract K

In the present study aimed for the purification of withanolides from *Withania somnifera* roots, the concentrated chloroform extract (extract K) obtained from 2.5g of root sample after defatting with 25ml of petroleum ether was subjected to silica gel column (height 48cm and diameter 1cm) in order to standardize the process of purification of withanolides as indicated in **plate 4.4**.

The mobile phase containing chloroform and ethyl acetate as solvent of increasing polarity was used for the silica gel column packed in petroleum ether (60-80°C). The column on elution with chloroform (100%) was found to be clear and pale yellow in color. But further elution with the increasing concentration of ethyl acetate in solvent mixture made the silica gel to appear turbid and colorless.

Plate 4.4 TLC pattern of fractions obtained from silica gel column chromatography containing extract K



Approximate bed volume of the silica gel column chromatography was found to be 20-25 ml of petroleum ether (60-80°C) and this was collected as fraction 1 and found to contain no spots upon TLC.

The elution of silica gel column chromatography using solvents in the order of increasing polarity was performed with 50 ml volume of different ratios of chloroform and ethyl acetate as indicated in table 4.3.

11 fractions of 25 ml was collected and analyzed by thin layer chromatography. TLC pattern of fraction 2 and 3 revealed the presence of spot corresponding to R_f value 0.72 on elution with 100% chloroform as solvent. The fraction 4 and 5 revealed the presence of spots corresponding to R_f value 0.72, 0.6, 0.55 and 0.5 when eluted with the solvent chloroform and ethyl acetate in the ratio 75:25.

Table 4.3 Solvents of different polarity used for the elution of silica gel column containing extract K.

S.No	Ratio of solvents used		Volume of solvents used (ml)	Fractions collected
	Chloroform	Ethyl acetate		
1	100	0	50	2 -25ml 3-25ml
2	75	25	50	4-25ml 5-25ml
3	50	50	50	6-25 ml 7-25ml
4	25	75	50	8-25ml 9-25ml
5	0	100	50	10-25ml 11- 25ml

On eluting the column using solvents chloroform: ethyl acetate in the ratio 50: 50 obtained the fractions 6 which was found to contain the spots corresponding to R_f value 0.72, 0.6, 0.55 and 0.5. The fraction 7 collected was then found to contain spots with R_f value 0.55

and 0.5 but spot corresponding to R_f value 0.72 and 0.6 was found comparatively at the decreased intensity as shown in **plate 4.4.**

The silica gel column containing the chloroform extract when eluted with the solvent chloroform and ethyl acetate in the ratio 25:75 resulted in the fraction 8 and 9 with spots corresponding to R_f value 0.43 whereas the fraction 9 was found to contain the spot with R_f value 0.36 in addition to the spot with R_f value 0.43. And on elution with 100% ethyl acetate, the fractions 10 and 11 was found with the spot with the R_f value 0.12.

In the present study fractions of 25 ml volume was collected, TLC pattern of collected fractions revealed that the fraction 10 and 11 contain only the lower most purified spot while elution with 100% ethyl acetate whereas all the other non polar compounds were separated in various ratios of non polar solvent. Fractions 6 and 7 obtained while elution with chloroform: ethyl acetate in the ratio 50:50 was found to contain predominant spots when compared to the other fractions.

Table 4.4

Solvents used for eluting silica gel column containing extract K from 15g of dry sample.

S.No	Ratio of solvents		Volume of solvents used (ml)	Fractions collected
	Chloroform	Ethyl acetate		
1	100	0	50	5-9
2	75	25	50	10-14
3	50	50	50	15-19
4	25	75	50	20-24
5	0	100	50	25-33

But the volume of the fraction containing lower most purified spot obtained from 2.5g of *Withania somnifera* roots was less and in order to obtain the compound with lower R_f value in considerable quantity, the large scale purification was performed by extracting higher quantity of powdered *Withania somnifera* roots.

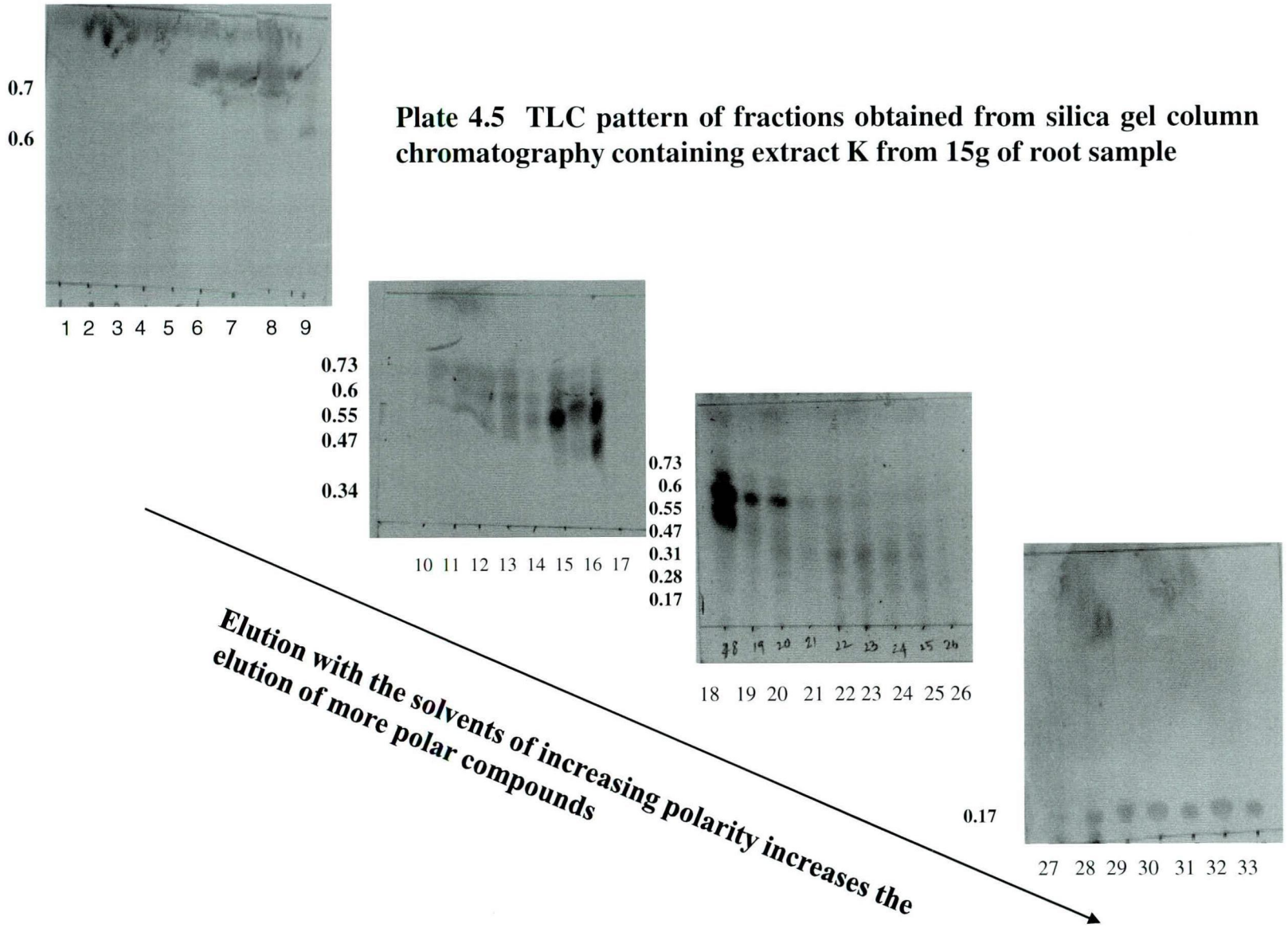


Plate 4.5 TLC pattern of fractions obtained from silica gel column chromatography containing extract K from 15g of root sample

15g of defatted *Withania somnifera* roots was extracted exhaustively using chloroform and this highly concentrated 3 ml of chloroform extract (extract K) was then subjected to silica gel column of height 48 cm and diameter 1cm with the bed volume of 25ml petroleum ether for obtaining the purified withanolide glycosides in consistent quantity. Further elution with 50 ml solvent of chloroform and ethyl acetate in different ratio as indicated in table 4.4 was performed followed by the fractions with 10ml volume was collected.

The collected fraction of 10ml volume was further concentrated to 2ml and then followed by thin layer chromatographic analysis. TLC pattern of collected fractions revealed that on increasing the polarity of the solvents increases the elution of more polar compounds as indicated in **plate 4.5.** The bed volume of 48 cm silica gel column was 25 ml and thus the fractions 1-4 revealed no spots on elution using 100% chloroform whereas the fraction 5, 6, 7 and 8 contained spots corresponding to R_f values 0.7. Fraction 9 was found to contain spot with R_f value 0.6 in addition to that of spot with R_f value as 0.7.

The spots with R_f value 0.73, 0.6 and 0.55 was obtained in the collected fractions 10-14 on elution with chloroform and ethyl acetate in the ratio 75:25.On increasing the polarity of eluting solvents by increasing the ratio of ethyl acetate from 25% to 50% revealed the presence of additional spot with R_f value 0.47 but the spot corresponding to R_f value 0.73 was found with less intensity upon TLC analysis of fractions 15-19.

The fractions 20-24 obtained by elution with the silica gel column using 25:75 ratio of chloroform and ethyl acetate was found to contain the spots with the R_f value 0.17,0.28 and 0.31. The collected fractions 25-26 from silica gel column on elution using 100% ethyl acetate revealed the presence of spot corresponding to R_f value 0.17,0.28 and 0.31 whereas the fractions 28-33 revealed the predominant presence of spot corresponding to R_f value 0.17.

Thin layer chromatographic pattern revealed that the fractions 15-19 on elution with the solvent chloroform and ethyl acetate in the ratio 50:50 was found to contain pre dominant spots compared to the spots observed in the other fractions. Moreover, when the polarity of the eluting solvent when increased increases the elution of more polar compounds.

Thus the purification of compound with lower R_f value 0.17 obtained by subjecting the chloroform extract (extract K) to silica gel column chromatography confirmed that the polar saponins of *Withania somnifera* roots was also obtained in considerable quantity when extraction was carried using the solvent chloroform.

4.7.2 Silica gel column chromatographic analysis of extract L

In the present study, the chloroform fraction obtained from 50% ethanol extract (extract L) using 5g of defatted *Withania somnifera* roots was subjected to silica gel column as suggested by Chapman *et al.*, (1997) who indicated that Saikosaponin purification has been generally done by sequential solvent extraction using petroleum ether, n-hexane, ethyl acetate, n-butanol and acetone followed by column chromatography.

Similarly in the present study, the chloroform fraction of 50% ethanol extract (extract L) obtained from defatted *Withania somnifera* roots containing the withanolides was subjected to silica gel column chromatography followed by elution with the solvents in the order of increasing polarity was performed.

An attempt was made to purify the withanolides from chloroform fraction of 50% ethanol extract (extract L) using various ratios of petroleum ether, chloroform and ethyl acetate and the results obtained were described under the method 1 and method 2 respectively.

Method 1

The chloroform fraction of 50% ethanol extract (extract L) from 5g of *Withania somnifera* roots was subjected to small silica gel column of 28.5 cm height and diameter 0.8 cm with the bed volume of 10 ml petroleum ether. And the elution of silica gel column containing extract L was followed with 20 ml of solvent containing different ratios of petroleum ether, chloroform and ethyl acetate .This was performed in order to identify the particular components eluted in specific ratio as indicated in table 4.5. The fractions of 5ml volume were collected and 10µl of concentrated fraction was analyzed by thin layer chromatography.

TLC pattern of fractions 1-7 revealed the presence of no predominant spots on eluting 28.5 cm silica gel column with 100% petroleum ether whereas the fractions 8-15 was

found to contain spots corresponding to R_f value 0.7 and 0.6 obtained by eluting with solvent containing petroleum ether and chloroform in the ratio 75:25 and 50:50.

The fractions 16-23 was found to contain spots corresponding to R_f values 0.7 and 0.6 obtained by elution with solvent petroleum ether and chloroform in the ratio 25:75 and 0:100 respectively. Elution with chloroform and ethyl acetate in the ratio 75:25 revealed the presence of spots corresponding to R_f values 0.7, 0.6 and 0.5 as indicated in **plate 4.6.**

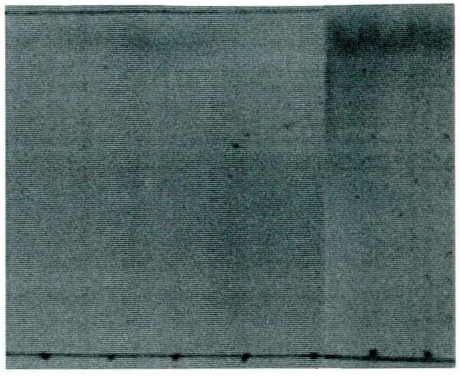
Table 4.5

Different ratios of petroleum ether, chloroform and ethyl acetate as mobile phase for silica gel column containing extract L.

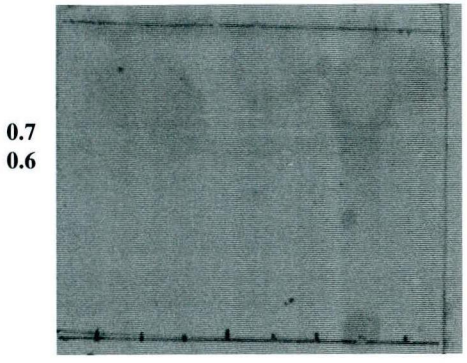
S.No	Ratio of solvents used			Volume of solvents used (ml)	Fractions collected
	Petroleum ether	Chloroform	Ethyl acetate		
1	100	0	0	20	3-7
2	75	25	0	20	8-11
3	50	50	0	20	12-15
4	25	75	0	20	16-19
5	0	100	0	20	20-23
6	0	75	25	20	24-27
7	0	50	50	20	28-31
8	0	25	75	20	32-35
9	0	0	100	20	36

The solvent chloroform and ethyl acetate in the ratio of 50:50 when used for eluting the silica gel column with extract L resulted in fractions 28-31 with compounds corresponding to R_f values of 0.7, 0.6, 0.5, 0.4 and 0.36 whereas the fraction 29, 30, 31 and 32 have showed the presence of spots corresponding to R_f value 0.5, 0.36 and 0.1 obtained by elution with the solvent chloroform and ethyl acetate in the ratio of 50:50. The results

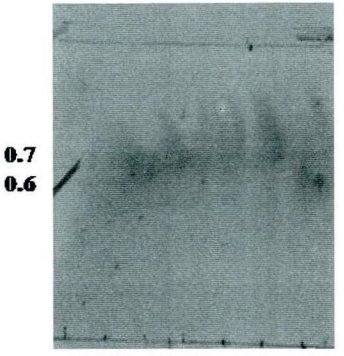
Plate 4.6 TLC pattern of fractions obtained from silica gel column chromatography containing extract L Method 1



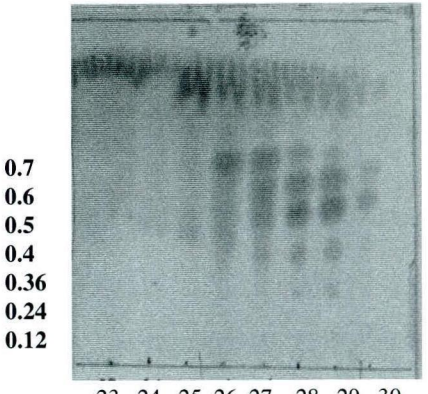
1 2 3 4 5 6 7



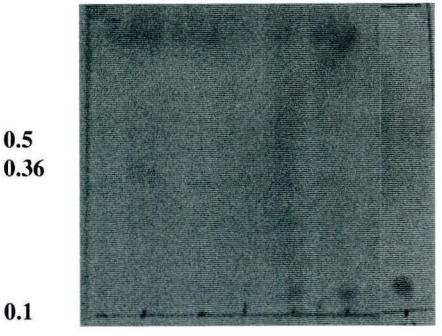
8 9 10 11 12 13 14 15



16 17 18 19 20 21 22



23 24 25 26 27 28 29 30



31 32 33 34 35 36

obtained in the present study indicated that the molecules with different polarity partitioned to different extents thereby move through the column at different rates.

Further eluting the column using solvents chloroform and ethyl acetate in the ratio of 25:75 followed by 100% ethyl acetate resulted in fractions 32-36 with spot 0.5 was contained in fractions 31 and 32 whereas the fraction 34 and 35 was found to contain spots corresponding to R_f value 0.36 as well as 0.1. Lastly, the fraction 36 was found to contain only spots with R_f value 0.1.

TLC pattern of fractions 3-24 obtained by the elution of silica gel column using the solvents chloroform in combination with petroleum ether revealed the presence of only two spots indicating that the various ratios of non polar solvents chloroform and petroleum ether can be suitable only for the separation of the most non polar compound corresponding to the R_f value 0.7 and 0.6.Therefore various ratios of chloroform and ethyl acetate can be suitable for obtaining purified withanolide glycosides.

Similarly Monroe (2003) while performing the separation of ferrocene and acetyl ferrocene on adsorption column chromatography using the hexane and tert-butyl methyl ether (TBME) indicated that polarity was the most important factor in separating the compounds.

Sangwan *et al.*, (2007) obtained withanolide A as pool of fractions eluted with ethyl acetate and n-hexane in the ratio 2:3 from silica gel column containing the methanol extract of *Withania somnifera* roots.

TLC pattern of fractions 25-29 obtained by elution using the mobile phase chloroform: ethyl acetate in the ratio 25:75, 50:50 and 75:25 revealed the fine separation of maximum spots indicating that the ratio of polar solvent ethyl acetate when increased increases the elution of the less non polar compounds. Finally the fractions obtained by eluting the column with 100% ethyl acetate as solvent revealed the presence of only one polar compound.

Method 2

In the present study, in order to critically analyze the compounds separated using chloroform and ethyl acetate as mobile phase, chloroform fraction of 50% ethanol extract

obtained from *Withania somnifera* roots was subjected to silica gel column (height 28.5cm,diameter 0.8cm and bed volume 10ml) followed by elution using the solvent containing various ratios of chloroform and ethyl acetate eliminating the petroleum ether as indicated in table 4.6.

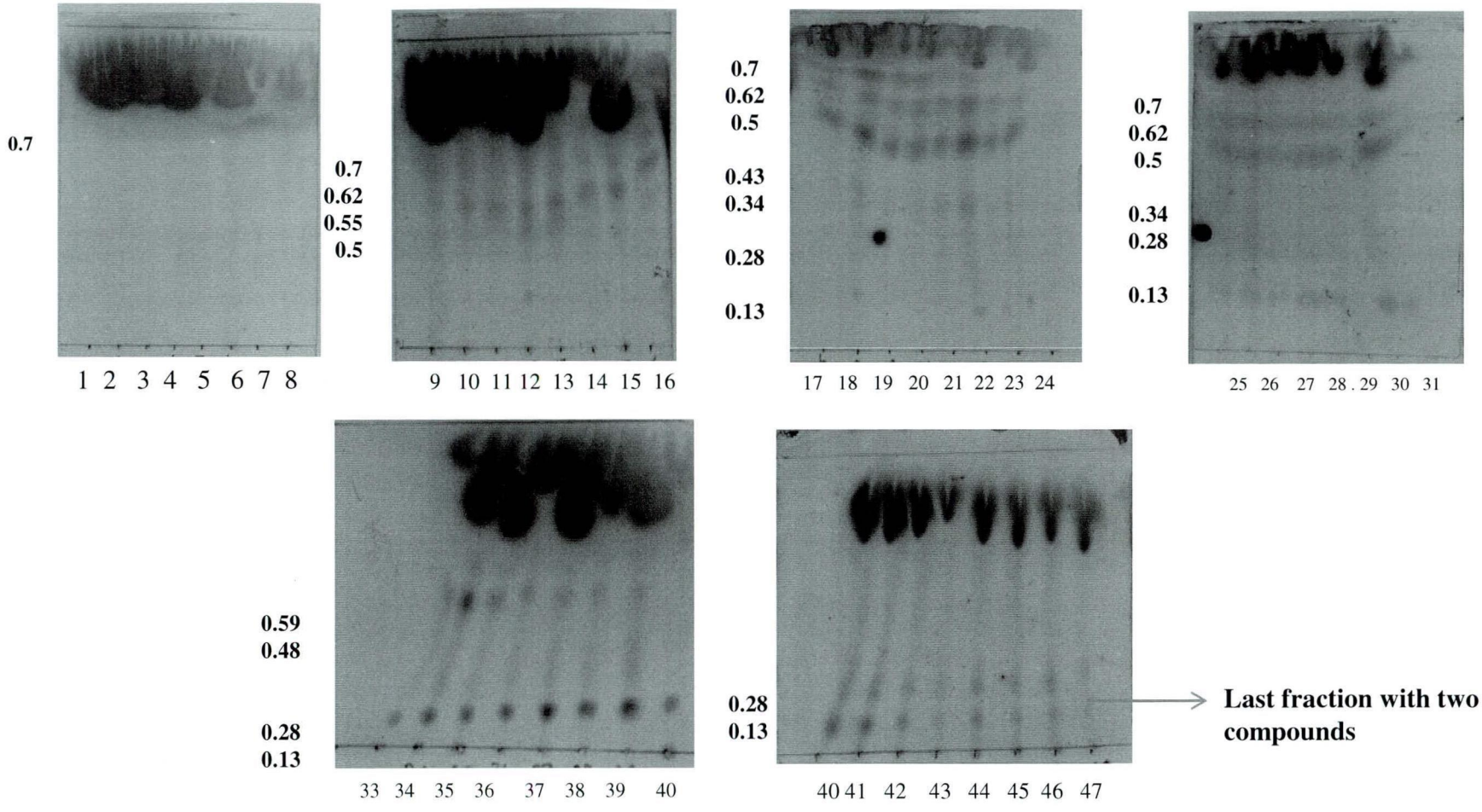
Table 4.6

Elution of silica gel column containing extract L with different ratios of chloroform and ethyl acetate

S. No	Ratio of solvents used		Volume of solvents used (ml)	Fractions collected
	Chloroform	Ethyl acetate		
1	100	0	20	3-5
2	90	10	20	6-8
3	80	20	20	9-11
4	70	30	20	12-14
5	60	40	20	15-17
6	50	50	20	18-20
7	40	60	20	21-24
8	30	70	20	24-27
9	20	80	20	27-29
10	10	90	20	30-32
11	0	100	20	33-48

Approximately 5ml fractions of silica gel column was collected and further concentrated to approximately 2 ml followed by 10µl of the fraction was used for TLC analysis. TLC pattern of fractions 3-5 and 6-8 obtained by elution with chloroform 100% and chloroform: ethyl acetate in the ratio 90:10 as eluting solvent revealed the presence of only one spot with R_f value 0.7.

Plate 4.7 TLC pattern of fractions obtained from silica gel column chromatography containing Extract L Method 2



Eluting the silica gel column containing the extract L using chloroform: ethyl acetate in the ratio 80:20 followed by chloroform : ethyl acetate in the ratio 70:30 and 60:40 revealed the presence of spots with R_f values 0.7, 0.62, 0.55 and 0.5 in the collected fractions 9 -11 and 12-17 respectively. The collected fractions 18-21 by elution with the solvent chloroform: ethyl acetate in the ratio 50:50 resulted in additional spot corresponding to R_f value 0.43 and 0.34 along with other spots 0.7, 0.62 and 0.5 respectively.

The fractions namely 22 to 32 were found to contain spots corresponding to R_f values 0.7, 0.62, 0.5, 0.43, 0.35 as well as 0.13 upon eluting the column with the solvent chloroform: ethyl acetate in the ratio 40:60, 30:70, 20:80 and 10:90 respectively. The elution carried with 100% ethyl acetate resulted in fractions from 33 to 38 was found to contain spots corresponding to R_f value 0.59, 0.48, 0.28 and 0.13 whereas the fractions 39 to 47 contains two spots corresponding to R_f value 0.28 and 0.13 respectively with no single purified spots.

The results obtained in the present study indicated that as 100% ethyl acetate being more polar effectively solvate the polar constituents consequently eluting highly polar molecules rapidly through the column. Thus from the present study it is understood that if a solvent is too polar, movement of compound becomes too rapid, and little or no separation of the components of a mixture will result. And thus the proper choice of an eluting solvent is thus crucial to the successful application of column chromatography as a separation technique.

The TLC pattern of fractions obtained in method 2 revealed that in order to elute the compounds with R_f values 0.7, 0.62, 0.55 and 0.5 from silica gel column containing the extracts of *Withania somnifera* roots, the elution can be carried using chloroform: ethyl acetate in the ratio 80:20. No purified lower most spot in the fractions obtained upon eluting with 100% ethyl acetate indicated that the polarity of the solvent gradient must be increased only after the complete elution of the less polar compounds.

On comparing the different ratio of chloroform: ethyl acetate, the ratio 80:20 was found to be comparatively less polar than 50:50 and thus involved in the elution of compound corresponding to the R_f value 0.7, 0.62, 0.55 and 0.5 upon TLC analysis whereas the ratio

50:50 of chloroform and ethyl acetate was involved in the elution of additional spots with R_f values 0.43 and 0.35 respectively as indicated in **plate 4.7**.

When the polarity of the eluting solvent was increased with chloroform and ethyl acetate 20: 80, the lower most spot corresponding to the R_f value 0.17 and its adjacent spot with R_f value 0.28 are eluted more quickly.

Comparison of R_f values of compounds eluted in methods 1 and 2.

The compounds obtained as fractions upon eluting the silica gel column chromatography with various ratios of three different solvents namely petroleum ether, chloroform and ethyl acetate was analyzed by thin layer chromatography. The calculated R_f values of spots obtained in TLC was compared with those obtained in two different methods as mentioned in table 4.7.

It was found that the compounds corresponding to R_f values 0.7 -0.73, 0.59-0.62 were obtained upon eluting the silica gel column containing the extract L with petroleum ether: chloroform (75:25,50:50,25:75,0:100) and chloroform: ethyl acetate (80:20,50:50,40:60).

In the method 2, the spots with R_f values 0.59-0.62 was obtained in chloroform: ethyl acetate 30:70, 20:80, 10:90 indicating that simultaneous analysis of fractions after the elution of single fraction is necessary for determining whether the same mobile phase can be continued or not until the compound corresponding to that specific R_f values was eluted which again might not interfere in the purification of other compounds.

The compound corresponding to R_f value 0.55 and 0.5 were obtained as fractions upon elution with increasing concentration of ethyl acetate from 20% to 50% in chloroform whereas the compound with R_f value 0.4 was obtained upon eluting the silica gel containing chloroform fraction (extract L) with ethyl acetate 25% to 50% in chloroform.

TLC pattern of fractions upon eluting the column with chloroform: ethyl acetate 50:50 and 40:60 in followed methods revealed the presence of spots with R_f value 0.36. The spots namely 0.1 - 0.13 were obtained in fractions when chloroform and ethyl acetate 40:60, 30:70, 20:80 and 0:100 were used as mobile phase.

Table 4.7

Comparison of R_f values of compounds eluted in methods 1 and 2

R_f values	Method 1	Method 2
0.73-0.7	Petroleum ether : Chloroform 75:25,50:50,25:75,0:100 Chloroform: Ethyl acetate 75:25,50:50	Chloroform: Ethyl acetate 100:0,90:10,80:20,60:40, 50:50,40:60
0.59-0.62	Petroleum ether : Chloroform 75:25,50:50,25:75,0:100 Chloroform: Ethyl acetate 75:25,50:50	Chloroform: Ethyl acetate 100:0,90:10,80:20,60:40 50:50,40:60, 30:70,20:80,10:90
0.55	-	Chloroform: Ethyl acetate 80:20,70:30
0.48-0.5	Chloroform: Ethyl acetate 75:25,50:50	Chloroform: Ethyl acetate 70:30,60:40,50:50, 40:60,30:70, 20:80,10:90
0.43-0.4	Chloroform: Ethyl acetate 75:25,50:50	Chloroform: Ethyl acetate 50:50
0.34-0.37	Chloroform: Ethyl acetate 50:50	Chloroform: Ethyl acetate 50 :50 40 :60
0.24-0.28	-	Chloroform: Ethyl acetate 40:60,30:70,20:80,0:100
0.1-0.13	Chloroform: Ethyl acetate 25:75,0:100	Chloroform: Ethyl acetate 40:60,30:70,20:80,0:100

The results obtained revealed that for purification of polar components (withanolides glycosides) namely with R_f value 0.28 and 0.1,elution of the silica gel column containing the sample extract with chloroform: ethyl acetate 40:60 followed by step wise gradient increase of the ethyl acetate in mobile phase was essential.