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CHROMATOGRAPHIC STUDY AND BEHAVIOURAL STUDY OF KSHIRBALA TAILA

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ABSTRACT

Kshirbala taila is a known Ayurvedic remedy for Rheumatoid Arthritis. Its basic physico-chemical evaluation profile is reported in Ayurvedic Pharamcopoiea of India. Detailed analysis was the objective of study. The formulation was prepared using *Sida cordifolia* roots, sesame oil and cow milk according to literature. The formulation was then subjected to various chemical investigations. Unsaponifiable matter was studied for preliminary phytochemical screening and chemoprofiling of expected phyto-constituents using chromatographic techniques. TLC confirmed presence of sesamin, b-sitosterol, lupeol and ephedrine presence. The pre and post treatment behavioral studies were also studied and reports revealed that pain induced stress symptoms are severe in untreated arthritic rats while treated rats show significantly less frequency of occurrence compared to negative control group.

Keywords: Kshirbala taila, Rheumatoid arthritis, Chromatographic, Behavioural study, *Sida cordifolia*.

INTRODUCTION

Kshirbala Taila is an Ayurvedic formulation meant for vata roga (inflammatory disorders) to be taken orally as well as can be applied on affected joints.[1-6] The formulation consists of *Sesamum indicum* seeds oil medicated with Sida cordifolia root powder and cow milk by Taila preparation method mentioned in Ayurvedic literature,[1-2] has developed HPTLC method for estimation of β-sitosterol in unsaponifiable matter of pumpkin seed oil. Rao V. et. Al. and Alam M. did standardization of Kshirbala taila. Rajendra also developed HPTLC method for estimation of lupeol and β-sitosterol. Bhatnagar and Sukumaran developed method for sesame lignans estimation by HPLC. Monica L., et. Al. (2000) and Anderson M. did provided optimized guidelines for behavioral study in Freund's Adjuvant induced arthritis model in Rats.

MATERIALS AND METHODS

Experimental

The selected anti-arthritic Ayurvedic formulations; Kshirbala taila were prepared according to Ayurvedic formulary of India.

For Preliminary phytochemical screening; The formulation was first saponified completely

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followed by collection of unsaponifiable matter. To the alcoholic/aqueous extract of unsaponifiable matter, tests were performedFor Sterol; Liberman Burchard's test: Concentrated chloroform extract was treated with equal quantity of acetic anhydride, mixed well and then drops of concentrated sulphuric acid was added to the mixture from the side of test tube gently. Blue or green colour may appear on the basis of type of steroid present.

Salkowski Test: Concentrated chloroform solution of the formulation when shaken with concentrated sulphuric acid and on standing yields red colour.

For Alkaloids; Mayer's Test: Concentrated alcoholic extra of the formulation is mixed with potassium bismuth Iodide solution and shaken well. White precipitates are due to alkaloids. Dragon dorff's Test: Concentrated alcoholic extract of the SNG formulation when mixed with dragon dorff's reagent - Potassium Mercuric Iodide solution, after gentle mixing showed slight orange precipitates. Wagner's test: Concentrated alcoholic extract (transparent) mixed with Wagner's reagent (Iodine + Potassium iodide in water) gives brown precipitates. Hager's test: Concentrated alcoholic extract of formulation (transparent) mixed with Hager's reagent (clear picric acid alcoholic solution) then yellow precipitates would come. Shinoda test (flavanoid test): Concentrated alcoholic extract of the formulation was kept in a test tube already having 1 cm magnesium ribbon. Then drops of concentrated hydrochloric acid were added. Evaluation of bubbles followed by red coloration was observed for flavonoid presence. For sugars; Molisch's test: After hydrolysis of the sample by drops of concentrated hydrochloric acid in warm condition, it was treated with α-naphthol followed by addition of concentrated sulphuric acid drop wise till ring forms. Fehling's test: After primary hydrolysis of the samples, they were mixed with fehling A solution ($CuSO_4$) and equal amount of Fehling B solution (10 % NaOH). On heating this mixture will yield yellow precipitates.

For protein; Nin hydrin's test: Unsaponifiable matter of the sample treated with 1% alcoholic ninhydrin reagent yield blue colour due to phenolic amino acids.

Millon's test: If there is soluble protein of extract when rated with millon's reagent (mercuric nitrate and nitrous acid) gives red colour.

Sakaguchi test: The extract (if contains protein) if treated with sakaguchi reagent (1-Naphthol and a drop of sodium hypobromite) and kept mixed for few minutes gives colored reaction. Xanthoprotic test: Extract (if contains aromatic amino acids) when treated with xanthoprotic reagent (concentrated nitric acid) gives yellow colored protiene nitrous complex.

HPLC method trials for ephedrine estimation

Shimadzu make HPLC instrument was used with connected PDA detector. Colum had a guard column. Both columns were prewashed with methanol:water (50:50). Mobile phase used was Acetonitrile, Deionized water (millipore). Formulation Extract was prepared by dissolving 30 mg of unsaponifiable matter was dissolved in 1 ml methanol and used for HPLC study for ephedrine presence.

Before developing HPTLC method for ephedrine estimation, HPLC method was under application for the same reason. Ephedrine was not getting detected in plain acetonitrile: H2O, acetonitrile: methanol and these systems with Glacial acetic acid, formic acid and slight nitric acid. According to assay procedure of ephedrine monograph [USP] Phosphoric acid is needed in the mobile phase, but addition of it increased column pressure drastically. So HPLC method was not continued for further development. Acetonitrile: H_2O (70:30), Methanol: H_2O (70:30), Acetonitrile: H_2O (55:45) and Acetonitrile: H_2O (20:80) at 1ml/min flow rate. Trials were performed till symmetrical peak, sharp retention time, no tailing and no extra contour was observed.

HPTLC method for Estimation of Ephedrine in Kshirbala taila

Stationary phase: Silica gel G (F_{254}, 0.2mm thickness). Spotting solution was Ephedrine

0.1mg in 1 ml methanol. From the stock solutions of ephedrine was spotted with 1 µl containing 1000 ng/spot. Instrument: Digital analytical balance (Shimadzu-AUX 220, Capacity-0.01mg to 200mg). HPTLC system Linomet V (Camag) (Semi-automatic application, band application by spray on technique (2-500 µL). Hamilton Syringe of maximum 100 µLvolume capacity. Camag Twin Trough chamber (10 × 10 cm and 20 × 10 cm), Camag win CATS software I.4.7. 2018, CAMAG automated dipping chamber (time controller), CAMAG derivatization Hot plate (automated and temperature controlled), Camag TLC Scanner IV (scanning speed upto 100 mm/s, spectral range 190- 800 nm)

Reagents: Solvent systems used for trials were Toluene: Chloroform : Ethanol (6:3:2), Ethyl acetate: Ethanol : Di-ethyl amine (7:3:0.2), Chloroform: Ethyl acetate : Methanol (1:2:0.75) and a drop of ammonia Solvent.

Optimized Mobile phase: Ethyacetate : Ethanol : Dietyl Amine (7.0:3.0:0.2)

Toluene: Chloroform: Ethanol (6.0:3.0: 2.0)

Derivatizing reagent: Ninhydrin reagent (1% alcoholic solution) + 10 ml glacial acetic acid at 110˚C for 3 min.

Validation of method[12]: Limit of detection was observed where signal to noise ratio was 1:3 and limit of quantification was measured at signal to noise ratio 1:10.Linearity of the method was determined between 50 to 1000 ng per spot concentration range for n=3. Regression equation was determined and ideal curve with minimum rsquare value were selected for optimum linear concentration range.For precision study, scanner repeatability and method reproducibility (n=7) were determined at 600 ng/spot concentration application. Interday and intraday precision study were performed according to guideline at three concentrations; 300, 500 and 600 ng/spot for n=3 times.Accuracy for the HPTLC method was studied by checking %recovery determination for combination of test extract (313 ng/spot) and standard ephedrine (150, 300 and 450 ng/spot) spiked.

Behavioral study

The formulations were given in polyarthritic rats induced by Freund's adjuvant at two doses (200 and 400 mg/Kg, p.o.) for 30 days. One group of rats were non arthritic (Normal), second group of rats were arthritic but without treatment (Negative control). Third and fourth group of rats were arthritic but with Kshirbala treatments at law and high dose (200 and 400 mg/Kg, p.o.). Fifth group of arthritic rats were given treatment of Indomethacin (10mg/Kg,p.o.). Arthritic pain associated pain induced behavioral signs like nibbling, scratching, restlessness, latency, paw dragging, biting and altered sleep pattern were measured before and after arthritis induction.

Requirements: Day light area, stop watch, Report sheet, labels etc.

Behavioral symptoms were observed and reported (n=3, morning, noon and evening – 3 times a day of single animal repeated for 6 animals per group). For pain signs; nibbling, scratching, paw dragging were measured for 1 minute. All the pain related behavioral symptoms of animals were noticed for 1 min per animal. Observations were documented on 0 and 30thdy of the dosing schedule. Other symptoms like rubbing, biting, shaking, poor grunting, and isolation were also observed but due to lack of significant difference, they were not reported. For activity study; total latency, total activity and total motility were measured. For sleep pattern study under stress; total sleep and reduced sleep were studied. All statistical analyses were performed with the Prism 7 software. Each group carried n=6 results represented as average ± standard error. For comparison between seven groups for 1 day results (30^{th} day) comparisons between independent groups performed, by one way ANOVA method. Resultant P value in a group compared to negative control group if less than 0.05 then it is said to be statistically significant and less than 0.005 – statistically very significant.

RESULT AND DISCUSSION

The formulations, raw materials in powdered form were subjected for saponification process followed by separation of pure unsaponifiable

matter. Unsaponifiable matter of all the formulations were treated with different reagents as per standard test procedures for preliminary phyto-chemical screenings and were found to have following results.

For formulations; unsaponifiable matter was treated with reagents for the study. Steroids: Green colour in liberman burchard's test was found positive for plant sterols. Phenolics: Decolorization of Potassium permanganate test was found positive which confirmed phenolics (some oxidizable compounds). Blueish black colour with Folin reagent confirmed phenolic presence. Light green colour with ferric chloride test also confirmed presence of phenolics. pH strip behaved acidic with unsaponifiable matter. Shinoda positive result confirmed presence of flavones type phenolics. Kreis tests and hold tests were also performed and reported. For ephedrine and related alkaloids general alkaloids tests were performed but all four (dragon dorff's, mayer's, hager's and wagner's tests) were found very slight or negative. Proteins were found absent by having negative results for Millon's test, Nin hydrin test, Sakaguchi test and Xanthoprotic test. Unsaponifiable matter contents of the formulations have shown presence of phytosterols, phenolics, lignans but absence of alkaloids etc.

Chemoprofiling of Phyto-constituents in Kshirbala taila by chromatographic studies

The formulations were checked for presence of ephedrine, β-sitosterol, Oleanolic acid, sesamin and lupeol using TLC technique. Out of them β-sitosterol, lupeol were found to be present in TLC of test extracts of formulations.

Development of TLC method for authentification of sesamin in Kshirbala taila

TLC system and trial results; Benzene: Chloroform (2:2) system gave band with broadening and large tail merged in to band at 0.14 R_f. While Benzene: Chloroform (2:3) gave tailed band at 0.18 R_f. Benzene: Chloroform: Methanol (1:3:1) showed thin band at solvent front at 0.98 R_f (near to solvent front). Benzene: Chloroform: Methanol (2:3:0.4) system gave band merged in to solvent front.Benzene: Chloroform (1.5:3) gave a tailed asymmetrical band at 0.25 R_f. Benzene: Chloroform: Methanol (1.5:3:0.25) system gave Thin band without tailing at 0.85 R_f. Hexane: Ethyl acetate (3.5:1.5) at 0.69 R_f gave a band (thin) without tailing.

Spot colour: pale to dark brown at R_f: 0.801

TLC result reflects sesamin can be identified by the developed system without interference of nearer sesamolin band and no tailing or band broadening. The bands are distinct with R_f between 0.2 to 0.9 thus easy to quantify with optimum accuracy.

The sesamin and sesamolin are having nearly same chemical properties [both are lignans and have same colour with anisaldehyde sulfuric acid reagent after derivatization] but can be separated by TLC at very law difference in R_f i.e. sesamin 0.801 and for sesamolin it is 0.75. Method was not selected for further estimation of sesamin in formulations as HPLC method is closed system and HPTLC is an open system thus factors responsible for deviation are much more in HPTLC than in HPLC.

HPLC method development for Ephedrine in Kshirbala Taila

HPLC on addition with phosphoric acid (according to USP method) could have shown detection of separate compound in HPLC but as it would have increased the column pressure very significantly so that method was not chosen. Trial methods and their mobile phases could not develop a method for detection and quantification of ephedrine.ACN: H_2O (70:30) system at 1 ml/minute flow rate and 257 nm wavelength gave small peak at 4.8 minute retention time. Methanol: water at 70:30 ratio, 1ml/min and 257 nm (scanned 254 to 260 nm) showed broad peak with very little contour at 2.4 minute time. Acetonitrile: water at 55:45 ratio, same flow rate and wave length were not able to gave a peak. Acetonitrile: water (20:80) system was also not able to gave a peak at 1 ml/min flow rate and 257 nm wave length. The result figure indicated that the ephedrine at above trial mobile phases and conditions do not show a sharp ideal peak. Thus

the trial method was not further studied for estimation of ephedrine in Kshirbala taila.

HPTLC method development for Ephedrine in KshirBala Taila

Trial results for ephedrine estimation in Kshirbala Taila by HPTLC

Toluene: Chloroform: Ethanol at (6:3:2) gave a band at 0.15 R_f where compound did not run completely from the spot zone.Ethyl acetate: Ethanol: Di-ethyl amine (7:3:0.2) gave a band at 0.6 R_f in which compound did resolved as a separate single band. System Chloroform: Ethyl acetate: Methanol and a drop of ammonia (1:2:0.75) gave single thin band with slight tailing and interference at 0.25 R_f.

R_f of ephedrine with this method is 0.78 with distinct light blue colour in pale purplish back ground. Densitometric chromatogram of ephedrine in test extract is super-imposable to that of ephedrine standard. The linearity regression curve of the ephedrine standard have shown 7.847 slope and 760.8 intercept. Method was found to be linear between 300 to 1000 ng/spot concentration range with R^2 value of 0.994. As ephedrine was found absent in all the formulations so, estimation was not done. As only Bala root have shown a band at too high spotting quantity; it was estimated from the regression curve.

Yield of ephedrine in KshirBala Taila: MF1, MF2, MF3, HF and LF all formulations; Ephedrine was found absent or it can be claimed that ephedrine was below detectable concentration range. Murchitatil taila also showed absence of ephedrine,which proves that ephedrine would have been degraded in formulations during process. So it was not required for further quantification. But Tila taila showed presence of it that too in very concentrated extract but below quantifiable limit. Spots of bala (*Sida cordifolia* roots) alcoholic extract has shown 0.0034 % w/w of ephedrine.

Method validation results: According to Figure, method was found to be linear between 300 to 900 ng/ml concentrations. Method has shown ephedrine detectable limit 110 ng/ml and minimum quantifiable limit 200 ng/ml. Scanner measurement repeatability for same band was found appropriate with 0.7201 co-efficient of variance. Method reproducibility for measurement of different band results of same concentration was found with 1.7336 co-efficient of variance. For Interday precision Method have shown co-efficient of variance between 1.3947 to 2.4075 for multiple measurements on various days at 500 to 700 ng/ml concentration. Intraday precision was observed with co-efficient of variance between 1.0181 to 1.239 for multiple measurements on various days at 500 to 700 ng/ml concentration. On addition of 50, 100 and 150 % of standard ephedrine in test extract followed by measurement of Area under curve have shown % recovery between 97.95 to 99.92 with Relative standard deviation between 0.8633 to 0.9263. Accuracy measured (between 450 to 780 ng/spot) by recovery study have specifically proved that ephedrine estimation is tough in extracts and unsaponifiable method with % recovery between 97.9514 to 99.8971. Limit of detection around 110 ng/spot and quantification starting from 200 ng per spot proved the sensitivity of method. Highest limit of linearity is assumed to be at 900 ng/spot after which no spotting were performed. No other peak is visible near to estimated compound thus the method is claimed as specific with great resolution.

Results of Table expresses that the method is repeatable, reproducible, precise, accurate, sensitive, economical and specific. This method is applicable for estimation of ephedrine (amino acid like compound) which is very tough to identify by Liquid chromatography in such a tough formulation like taila.

Behavioral study results; According to Table T. results; Group of untreated arthritic animals were showing excess scratching, biting, nibbling. At the other hand during walking animals affected paw dragging or slurred walk indicated pain or in-efficiency of leg to carry weight. Other treated groups have shown less pain signs at highest dose reduced nibbling to ground state and scratching was reduced by Kshirbala taila treatment.

Indomethacin treatment was found to reduce only paw dragging. For sleep pattern observation was done on total sleep time, which is for normal rat it was around 10.5 hours. Reduction in total time by 30 minutes indicates 1 point and thus total reduced sleep were recorded. Untreated rats have shown extreme change in sleep pattern, the highest reduction in sleep at 30th day was found around 2 hrs 22 minutes. Out of all other treatments Kshirbala taila at higher dose maintained natural sleep compared to negative control significantly. Motility was also affected by arthritis, swelling and pain in untreated group compared to non-arthritic group. Motility observed for 1 minute have shown that untreated arthritic group rats have shown reduced motility (may be due to pain, tenderness and paw swelling). Highest motility was observed in Kshirbala taila high dose and Indomethacin treated rats. High latency in untreated arthritic rats indicated pain and lack of comfort in walking in comparison with normal rats. On 0 day of dosing schedule, latency was minimum; animals were found equally active in all the groups. Positive control group showed minimum latency on 30th day of treatment.

CONCLUSION

Result from table reflects that taila preparation (heating and hydrolysis for prolong time) process must be responsible for degradation of ephedrine in Bala. Absence of ephedrine in all the formulations proves that, there is no need for quantification of this phyto-constituent in the formulation. Behavioral studies reflects that Kshir bala taila consumption reduces pain induced behavioral change and thus reduces morbidity of the Arthritic disease.

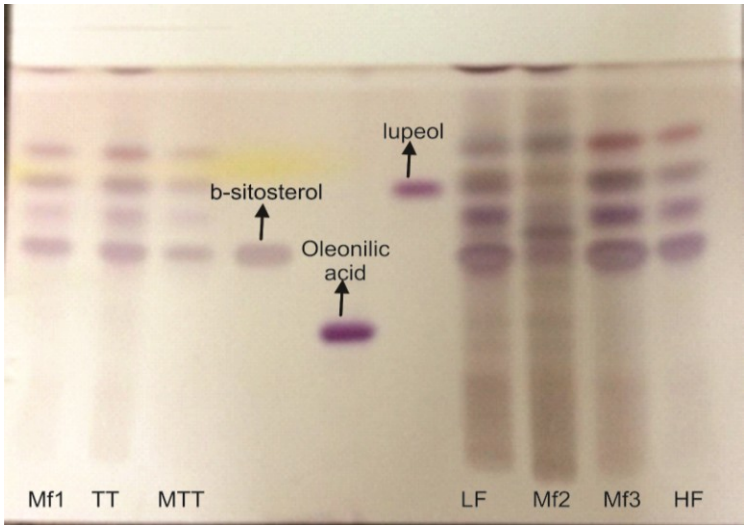

Figure 1: TLC of Kshirbala taila formulations with standards

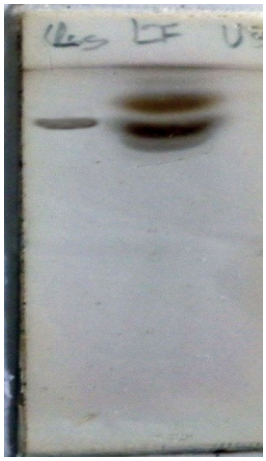

Figure 2: TLC of Sesamin (left) and KBT USM extract (right)

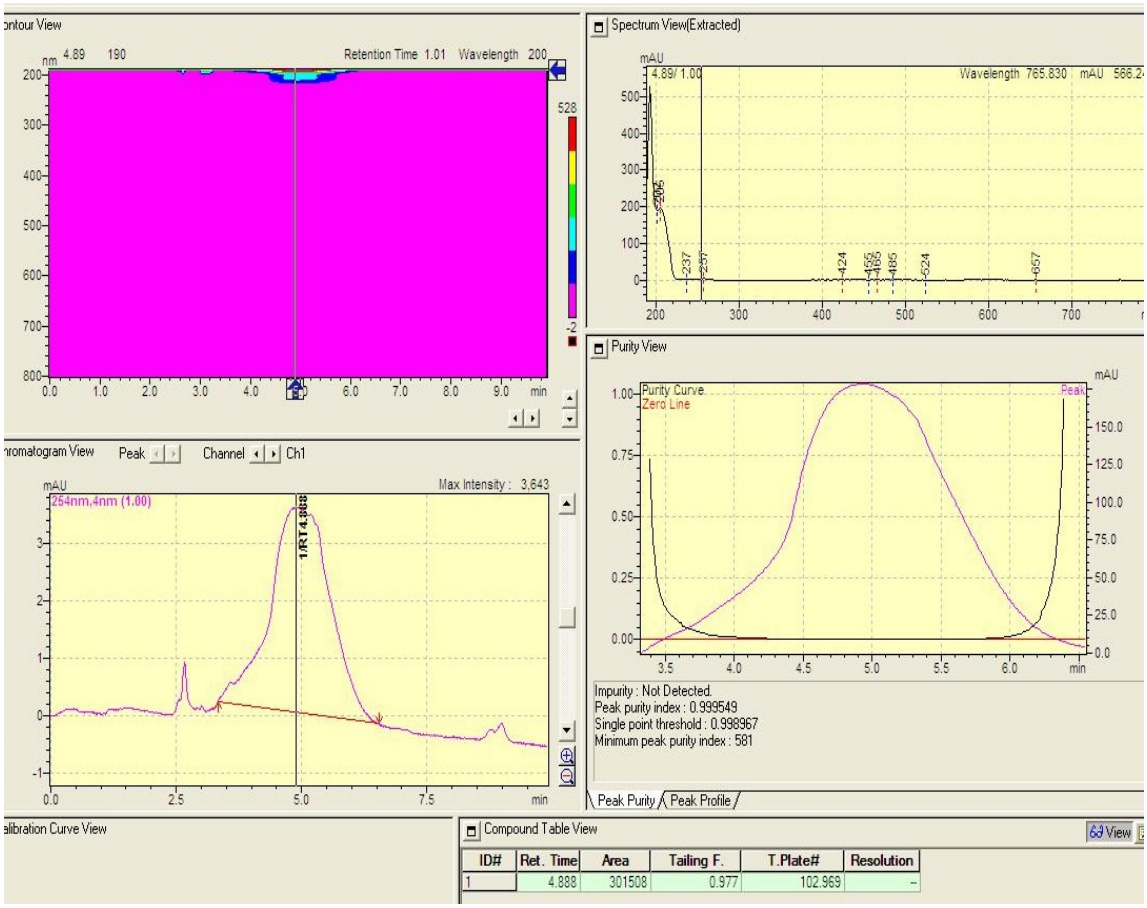

Figure 3: HPLC study results of ephedrine

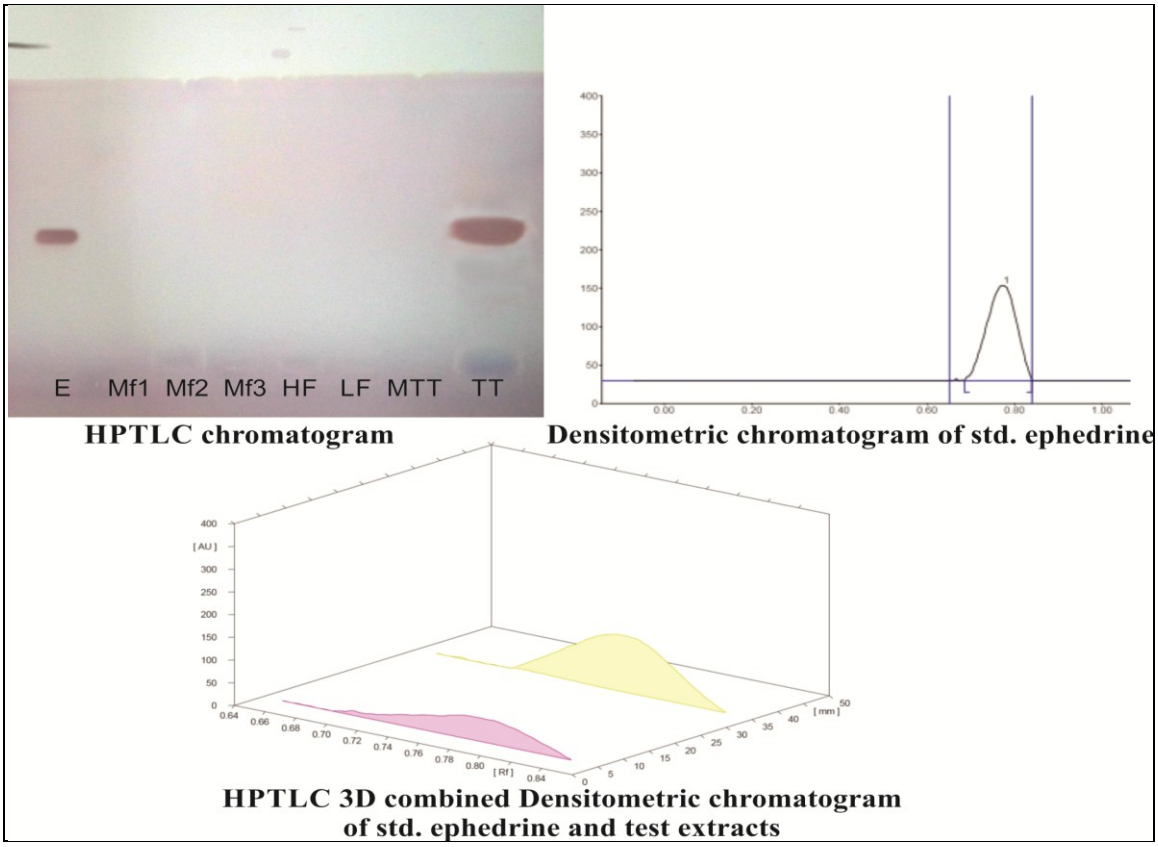

Figure 4: HPTLC study results for ephedrine in KBT

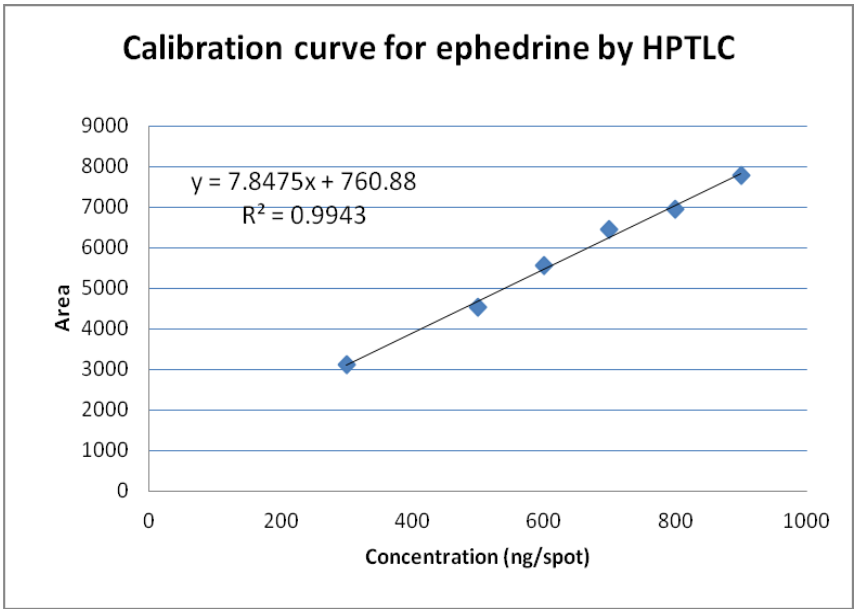

Figure 5: Calibration curve of ephedrine by HPTLC

Table 1: Repeatability and reproducibility for ephedrine by HPTLC

	Repeatability	**Reproducibility**
	5498.1	5498.1
	5509.2	5531.3
	5600.8	5650.3
	5552.1	5702.8
	5507.4	5583.1
	5486.2	5575.2
	5509.4	5411.6
Avg	5523.3142	5564.6285
Std. Dev.	39.7742	96.4697
Co-var.	0.7201	1.7336

Table 2: Interday and Intraday precision (ephedrine HPTLC)

	Conc. (ng/spot)	**1**	**2**	**3**	**Average**	**Std. Dev.**	**Co-var.**
Interday	**500**	4484.3	4583.2	4602.9	4556.8	63.5547	1.3947
	600	5583.1	5675.2	5411.6	5556.633	133.7781	2.4075
	700	6334.5	6481.6	6572.1	6462.733	119.9183	1.8555
Intraday	**500**	4488.9	4533.7	4600.7	4541.1	56.2661	1.239
	600	5498.1	5509.2	5600.8	5536.033	56.3634	1.0181
	700	6382.5	6467.5	6537.4	6462.466	77.5725	1.200

Table 3: Recovery results for ephedrine by HPTLC

Test (ng/spot)	Std (ng/spot)	Total Conc. (ng/spot)	Average (n=3)	std. dev.	Practical conc.	% recovery	% RSD
313	150	463	4319.53	37.2916	453.515	97.9514	0.8633
313	300	613	5567.5	49.3616	612.552	99.9270	0.8866
313	450	763	6741.9	62.4505	762.214	99.8971	0.9263

Table 4: Summary of HPTLC Validation Results for ephedrine in KBT

Parameters	For ephedrine
Linearity range	300-900 ng/spot
Correlation co-efficient	0.994
Repeatability Reproducibility Interday Intraday	0.7201 1.7336 1.3947 to 2.4075 1.0181 to 1.239
Accuracy (% recovery)	97.9514 to 99.927
Limit of detection	110 ng/spot
Limit of quantification	200 ng/spot
Limit of Linearity	900 ng/spot
Specificity	specific

Table 5: Yield of ephedrine in Kshirbala Taila

Evaluation parameter	M.F.1	M.F.2	M.F.3	H.F.	L.F.	Murchitatil taila	Bala root extract
Ephedrine Content(% w/w± SEM)	-nt	-nt	-nt	-nt	-nt	-nt	0.0034 ± 0.0001

(Average± standard deviation, n=3)

Table 6: Effect on pain related behavioral signs

	Nibbling		Scratching		Paw dragging		Biting	
	0 Day	30 Day	0 Day	30 Day	0 Day	30 Day	0 Day	30 Day
Normal	0.6	1	0.5	0.5	0	0	0	0
INDM	0.5	1.5	0.5	1.5	0	0.5	0	2
Negative control	0.5	1.67	0.5	2.4	0	2.33	0	2.1
KBT200	0.6	1.33	0	1	0	1	0	0.5
KBT400	0.6	0.67	0.5	0.83	0	0.67	0	1

Table 7: Effect on motility and sleep pattern

	Latency		Motility		Reduced sleep	
	0 Day	**30 Day**	**0 Day**	**30 Day**	**0 Day**	**30 Day**
Normal	0.5	0.5	4.0	3.5	0.0	0.1667
INDM	0.5	1.0	3.5	2.33	0.0	1.33
Negative control	0.5	2.833	4.0	1.667	1.0	2.33
KBT200	0.5	1.667	3.5	2.0	0.2	1.167
KBT400	0.5	1.83	3.5	2.167	0.1	1.33

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