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Extraction, Phytochemical Screening and Tlc Studies of Some Immunomodulator Preparation

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Abstract
Immunomodulation deals with modifying immunity response using either natural or synthetic substances. In the present investigation two marketed preparation viz., Trikatu churna, Giloy churna and one homemade churna were studied. In the study the herbs used were extracted our and was screened for active phytochemicals. Also, TLC was performed to ensure the active phyto-constituents present in individual herbs and immunomodulator preparations.

Key-words: Immunomodulator, Herbs, TLC

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Introduction

Immunomodulators have the ability to mount an immune response or defend against pathogens or tumors and can safely be used to alleviate hyper- or hypo- immune responses or against various diseases that accompany immune suppression. [1] A simple chromatographic technique such as TLC may provide valuable additional information to ascertain the identity of the plant material. This is particularly important for those species that contain different active constituents. Qualitative and quantitative information can be gathered regarding the presence or absence of metabolites or breakdown products. TLC fingerprinting is important for herbal drugs made up of essential oils, resins and gums which are complex mixtures of constituents that no longer have any organic structure. It is a powerful and comparatively rapid solution to distinguish between chemical classes where macroscopy and microscopy will be failed. Based on the concept of phyto-equivalence, to address the issue of quality control the chromatographic fingerprints of herbal medicines can be used. Methods based on information theory, similarity estimation, chemical pattern recognition, spectral correlative chromatograms (SCC), multivariate resolution, the combination of chromatographic fingerprints and chemometric evaluation for evaluating fingerprints are all powerful tools used for quality control of herbal Products. [2-3]

Material and Methods

For the present investigation two Marketed Ayurvedic Powders and one homemade preparation were selected that helps to improve immunity. The selected ayurvedic powders were: Trikatu Churna and Giloy Churna. Homemade preparation: The dried plant material of *Piper longum* (Piplai-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia* (giloy-stem) were powdered sieved and was mixed in the ratio mentioned below to prepare the churna.
1 part *Piper longum* Fruits
1 part *Zingiber officinale* Rhizome
1 part *Tinospora cordifolia* Stem

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Collection of Plant material

The plant parts viz., *Piper longum* (Piplai-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia* (Giloy-stem) were collected from the local vendors of Bhopal region of Madhya Pradesh and were authenticated by Dr. S.N. Dwivedi, Retd. Prof. & Head, Department of Botany Janata PG College, APS University, Rewa, (M.P.), Voucher specimen No. J/Bot/PLF012, J/Bot/ZOR013 & J/Bot/TCR014 were assigned.

Selection & Procurement of marketed immunomodulator preparation

The marketed ayurvedic preparations i.e., Trikuta Churna and Giloy Churna was selected after literature review and the herbs viz., *Piper longum* (Piplai-fruits), *Piper nigrum* (Kali mirchi-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia* (Giloy-stem) used in that preparation was procured from the local vendors of Bhopal District of Madhya Pradesh and was authenticated by Dr. S.N. Dwivedi, Retd. Prof. and Head, Janata PG College, APS University, Rewa (M.P.)

Extraction of selected preparations

Powdered plant materials viz., *Piper longum* (Piplai-fruits), *Piper nigrum* (Kali mirchi-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia* (Giloy-stem) of each separately were soaked in distilled water separately for 48h in a conical flask. Also, the drugs were separately extracted with methanol in soxhlet apparatus for 8hr. Then the extracts were filtered, concentrated and the solvent was removed by rotary evaporator. The extract was dried over desiccators and then the extracts were stored at 4ºC for further studies. [4-5]

Phytochemical screening

Aqueous and Methanolic extracts of *Piper longum* (Piplai-fruits), *Piper nigrum* (Kali mirchi-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia* (Giloy-stem) was tested for presence or, absence of different phytoconstituents like glycosides, terpenes, alkaloids, tannins, flavonoids, sterols, phenols etc.

The various extract obtained after extraction were subjected for phytochemical screening to determine the presence of various phytochemical present in the extracts. The standard procedure was adopted to perform the study [5-6].

Identification of Phytopharmaceuticals with TLC [7-14]

The methanolic extracts of *Piper longum* (Piplai-fruits), *Piper nigrum* (Kali mirchi-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia* (Giloy-stem) obtained were identified by chromatographic techniques. For that TLC and HPTLC was done using standard procedure.

TLC Profile of selected drug

The powdered drug material (100 g) was extracted with 250 ml of methanol. The extract was concentrated under reduced pressure and used for TLC fingerprinting.

Methodology

The aqueous extract of crude drug powder was subjected to TLC to find out the number of compounds present in it. The different steps followed in the development of thin layer chromatographic profile of plant extract are as follows (Mukherjee, 2002):

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Preparation of test sample
The crude drug powder 10 g was extracted separately with methanol. The extract was filtered and concentrated.

Preparation of plates
The absorbent silica gel G, about 25 g was taken in a glass mortar and about 35 ml of distilled water was added to it. The mixture was stirred with a glass rod until it became homogenous. The mixture was then allowed to swell for about 15 min. then additional 15 ml distilled water was added to it with stirring. The suspension was then transferred to a 150 ml flask fitted with a plastic stopper and was shaken vigorously for about 2 min. The suspension was then spread immediately on TLC plate (5 × 10 cm & 10 × 10 cm) to form a thin layer of the slurry.

Drying and storage of plates
The freshly coated plates were then air dried until the transparence of the layer had disappeared. The plates were then stacked in a drying rack and were heated in an oven for 30 min at 110 °C. The activated plates were kept in desiccators till required for further use.

Application of sample
Glass capillaries were used for applying test sample on TLC plates by keeping a minimum distance of 1 cm between the two adjacent spots. The spots of the sample were marked on the top of the plates to know their identity.

Chromatographic chamber, condition of saturation and development of TLC plates
Chromatographic rectangular glass chamber (16.5 × 29.5 cm) was used to develop the plates. To avoid insufficient chamber saturation and the undesirable age effect, a smooth sheet of filter paper approximately of 15 × 40 cm size was placed in the chromatographic chamber in U-shape in developing solvent. After being moistened, the paper was pressed against the walls other chamber, so as to adhere it to the walls. The chamber was allowed to saturate for 1.5 h before use. The experiment was carried out at room temperature in diffused daylight.

Development of solvent system
A number of developing solvent systems were tried, for aqueous extract by changing their concentration and combination to achieve satisfactory resolution.

Development of chromatogram
The spotted plates were kept in chromatographic chamber containing the appropriate solvent system. The chamber was covered with greased plate. The solvent system was allowed to run up to ¾ of the height of the TLC plate. The plates were taken out and air-dried after marking the solvent front.

Detection of spots
The air-dried plates were examined in daylight and in UV light (254 nm and 366 nm) for location of spots. The observations were recorded in Table.

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TLC fingerprinting studies

Solvent systems were developed for establishing the TLC patterns for the aqueous extracts of the selected drugs. Various visualization techniques were used to come up with the best TLC fingerprint, like UV 254, UV 366, iodinisation and spray reagents like anisaldehyde, ninhydrin, aniline phthalate, Folin's reagent, vanillin and sulphuric acid were also tried. The developed plates were dried in air, visualized in UV at wavelengths 254 nm and 366 nm and photographed.

Results and Discussion

Extraction of selected preparations

The powdered plant materials viz., *Piper longum* (Piplai-fruits), *Piper nigrum* (Kali mirchi-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia (Giloy-stem)* were extracted with water and methanol. The results of extraction were mentioned in table 1. The results for % extract of AETC, AEGC and AEHC were also mentioned in table 11.

Phytochemical screening

Aqueous and Methanolic extracts of *Piper longum* (Piplai-fruits), *Piper nigrum* (Kali mirchi-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia (Giloy-stem)* was tested for presence or, absence of different phytoconstituents like glycosides, terpenes, alkaloids, tannins, flavonoids, sterols, phenols etc. The results were presented in table 2 and 3.

Table 1: Estimation of % Yield of Various Extract of selected plant material

S/No.	Extract	Parameters			
		Nature of Extract	Color	pH	% Yield
1.	AEPLF	Solid powder	Blackish brown	7.0	10.32
2.	AEPNF	Solid powder	Blackish brown	7.1	11.28
3.	AEZOR	Solid powder	Light yellow	7.0	14.82
4.	AETCS	Solid powder	Light Brown	7.0	10.11
5.	MEPLF	Solid powder	Blackish brown	6.9	9.46
6.	MEPNF	Solid powder	Blackish brown	7.1	8.20
7.	MEZOR	Solid powder	Light yellow	7.0	11.50
8.	METCS	Solid powder	Light Brown	7.0	8.65
7.	AETC	Solid powder	Brown	7.0	13.42
8.	AEGC	Solid powder	Brown	7.0	10.22
9.	AEHC	Solid powder	Brown	7.0	11.32

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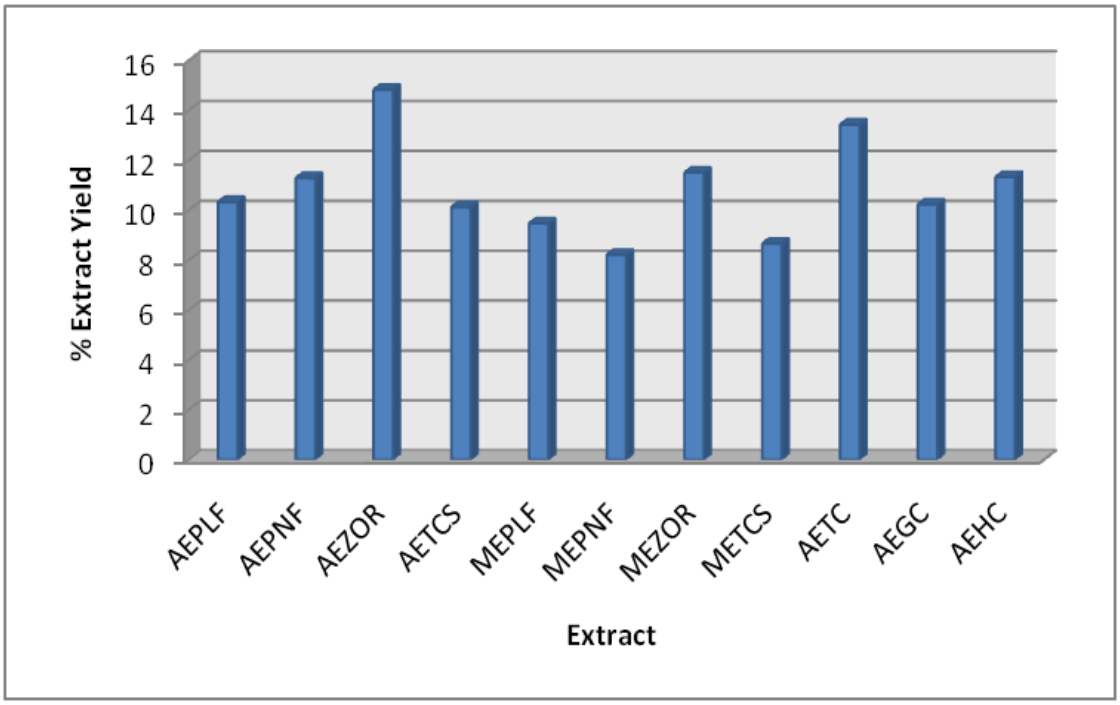
Abbr.: AEPLF: Aqueous extract of *Piper longum* (Piplai-fruits), AEPNF: Aqueous extract of *Piper nigrum* (Kali mirchi-fruits), AEZOR: Aqueous extract of *Zingiber officinale* (Adrak-rhizomes) and AETCS: Aqueous extract of *Tinospora cordifolia (Giloy-stem)*, MEPLF: Methanolic extract of *Piper longum* (Piplai-fruits), MEPNF: Methanolic extract of *Piper nigrum* (Kali mirchi-fruits), MEZOR: Methanolic extract of *Zingiber officinale* (Adrak-rhizomes) and METCS: Methanolic extract of *Tinospora cordifolia (Giloy-stem)*, AETC: Aqueous extract of Trikatu Churna, AEGC: Aqueous extract Giloy Churna, AEHC: Aqueous extract of Homemade Churna



Graph 1: % Extract Yield

Table 2: Phytochemical Screening of Various Aqueous Extract of selected plant material and preparation

S/No.	Constituents	Extract				Preparation		
		AEPLF	AEPNF	AEZOR	AETCS	AETC	AEGC	AEHC
1.	Carbohydrates	+	+	+	+	+	+	+
2.	Glycosides	-	-	+	+	-	+	+
3.	Alkaloids	+	+	+	+	+	+	+
4.	Protein & Amino acid	+	+	+	+	+	+	+
5.	Tannins & Phenolic compounds	-	-	-	-			
6.	Flavonoids	+	+	+	+	+	+	+
7.	Fixed oil and Fats	-	-	+	+	+	+	+
8.	Steriods &	+	-	+	+	+	+	+

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	Triterpenoids							
9.	Waxes	-	-	-	-	-	-	-
10.	Mucilage & Gums	-	-	+	-	-	-	-

+ = Present; - = Absent

Table 3: Phytochemical Screening of Various Methanolic Extract of selected plant material

S/No.	Constituents	Extract			
		MEPLF	MEPNF	MEZOR	METCS
1.	Carbohydrates	+	+	+	+
2.	Glycosides	-	-	+	+
3.	Alkaloids	+	+	+	+
4.	Protein & Amino acid	-	-	+	+
5.	Tannins & Phenolic compounds	-	-	-	-
6.	Flavonoids	+	+	-	-
7.	Fixed oil and Fats	-	-	-	-
8.	Steriods & Triterpenoids	-	+	+	+
9.	Waxes	-	-	-	-
10.	Mucilage & Gums	-	-	-	-

+ = Present; - = Absent

TLC Profile of *Piper longum*

Thin layer chromatography result confirmed the presence of different bioactive compounds. The result and observations were summarized in Table 5.4. TLC profiling of methanolic extract of *P. longum fruits* in Toluene: Ethyl acetate: Diethyl ether (10:3:1) and Benzene: Ethyl acetate: Diethyl ether (6:3:1) confirms the presence of diverse biomolecule in the plant.

TLC Profile of *Piper nigrum*

Thin layer chromatography result confirmed the presence of different bioactive compounds.The result and observations were summarized in Table 5.4. TLC profiling of methanolic extract of *P. longum fruits* in Toluene: Ethyl acetate: Diethyl ether (10:3:1) and Benzene: Ethyl acetate: Diethyl ether (6:3:1) confirms the presence of diverse biomolecule in the plant.

TLC Profile of *Zingiber officinale*

TLC is one of the important parameter equips with the qualitative and semi-quantitative information of the drug. If the drug is adulterated or exhausted which in turn may increase or decreases the number of spots and change in the *Rf* values. Thin layer chromatographic analysis of the methanolic extract of *Zingiber officinale* Rosc. was carried out via Toluene: Ethyl Acetate: Formic Acid (9:1:2). Spotted TLC plates were sprayed by Anisaldehyde- sulfuric acid and were also visualized in day light and UV short and long wavelength. The TLC profile along with images of TLC are illustrated in Table 4.

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TLC Profile of *Tinospora cordifolia*

Methanolic Extracts of *T. cordifolia* stems were obtained by preparative TLC (Thin layer chromatography). Samples were loaded on TLC plate in the form a streak with the help of micropipette and dried with dryer. The TLC plates were developed in solvent system i.e., Toluene: Ethyl acetate: Formic acid (5:4:1) at room temperature. The plates were removed, dried by evaporation of solvent then sprayed with anisaldehyde. Different bands were visualized at light UV 254 and 366 nm were observed and their Rf values were calculated.

TLC Profile of Trikatu Churna

Drug sample were soaked in aqueous alcohol (10%) for overnight, refluxed for 30 minutes on water bath and filtered. The filtrates were concentrated on water bath and made up to 10 ml in a standard flask separately. The TLC plates were developed in solvent system i.e., Toluene: Ethyl acetate (5:1.5) at room temperature. The plates were removed, dried by evaporation of solvent then sprayed with anisaldehyde. Different bands were visualized at light 366 nm were observed and their Rf values were calculated. After developing, the plates were dried and observed the colour spots at UV-254, UV-366 nm and vanillin-sulphuric acid spraying reagent and Dragendorff's reagent. Among the various solvent systems tested, the mixture containing toluene: ethyl acetate (5:1.5) gives the best resolution. In UV 254, 366 nm, visible light and Derivatization with Dragendorff's reagent *Piper longum*, *Piper nigrum*, Trikatu Churna and *Zingiber officinale* aqueous alcoholic extracts were shown Figure.

TLC Profile of Giloy Churna

Powdered materials were extracted with methanol and then the extracts were filtered through Whatman No. 1 filter paper concentrated under reduced pressure, and lyophilized. Known quantity of the extract was dissolved in methanol. Extract were applied on TLC silica gel plates. The plate was developed in chloroform: methanol: water (8 : 2 : 0.2) as mobile phase, in TLC chamber previously saturated with mobile phase vapour for 30 min at 24°C. After removal from the chamber, plates were completely dried in air at room temperature (24°C) and documented. The photographs were taken by illumination at UV 254 nm and 366 nm.

TLC Profile of Homemade Churna

Powdered materials were extracted with methanol and then the extracts were filtered through Whatman No. 1 filter paper concentrated under reduced pressure, and lyophilized. The TLC plates were developed in solvent system i.e., Benzene: Toluene: Ethyl Acetate: Formic Acid (4:3:1:2) at room temperature. The plates were removed, dried by evaporation of solvent then sprayed with anisaldehyde. Different bands were visualized at light 366 nm were observed and their Rf values were calculated. After developing, the plates were dried and observed the colour spots at UV-254 and UV-366 nm.

Table 5.4: TLC fingerprinting profile of methano*lic extract of selected plant material and preparation*

S/No.	Plants	Solvent system	No. of spots at UV 254 nm	No. of spots at UV 366 nm
1.	*Piper longum*	Toluene: Ethyl acetate: Diethyl ether (10:3:1)	7	11
2.	*Piper nigrum*	Benzene: Ethyl acetate:	12	15

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		Diethyl ether (6:3:1)		
3.	*Zingiber officinale*	Toluene: Ethyl Acetate: Formic Acid (9:1:2)	5	13
4.	*Tinospora cordifolia*	Toluene: Ethyl acetate: Formic acid (5:4:1)	8	17
5.	Trikatu Churna	Toluene: ethyl acetate (5:1.5)	12	10
6.	Giloy Churna	Chloroform: Methanol: Water (8 : 2 : 0.2)	9	13
7.	Homemade Churna	Benzene: Toluene: Ethyl Acetate: Formic Acid (4:3:1:2)	8	11

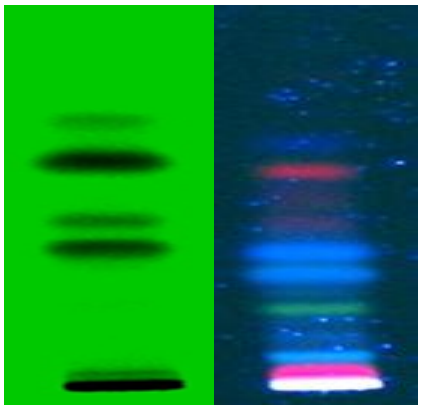

Fig. 1: Chromatogram of methanolic extract of *Piper longum* (fruits extract) in Toluene: Ethyl acetate: Diethyl ether (10:3:1), UV 254 nm and 366 nm

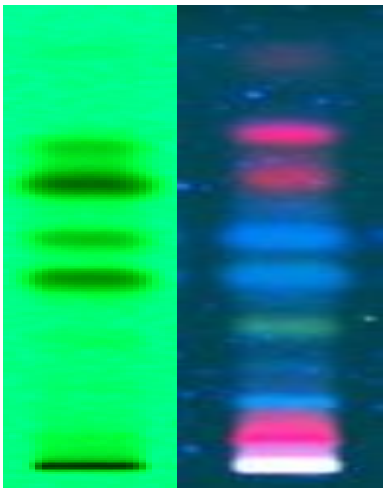

Fig. 2: Chromatogram of methanolic extract of *Piper longum* (fruits extract) in Benzene: Ethyl acetate: Diethyl ether (6:3:1), UV 254 nm and 366 nm.

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Fig. 3: Chromatogram of methanolic extract of *Zingiber officinale* (rhizome extract) in Toluene: Ethyl Acetate: Formic Acid (9:1:2), UV 254 nm and 366 nm.

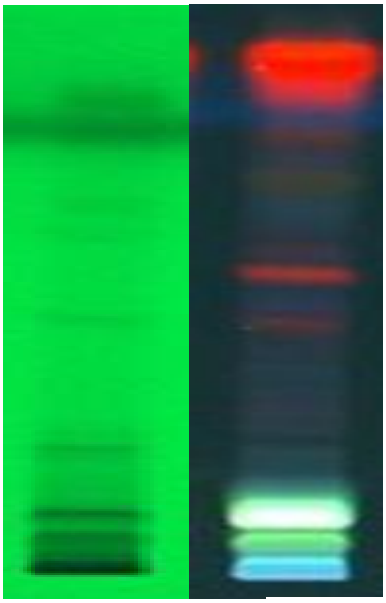

Fig. 4: Chromatogram of methanolic extract of *Tinospora cordifolia* (stem extract) in Toluene: Ethyl acetate: Formic acid (5:4:1), UV 254 nm and 366 nm.

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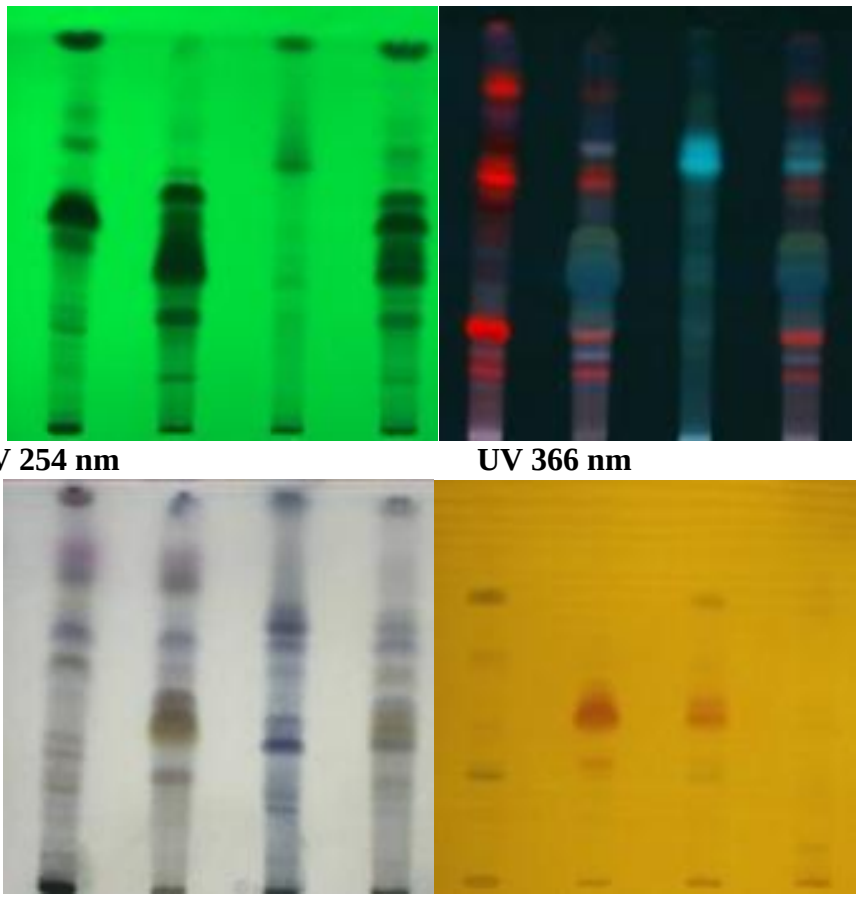

Fig. 5: Chromatogram of Trikatu Churna in Toluene: ethyl acetate (5:1.5), UV 254 nm and 366 nm. 1: *Piper longum* 2: *Piper nigrum* 3: *Trikatu churna* 4: *Zingiber officinale*

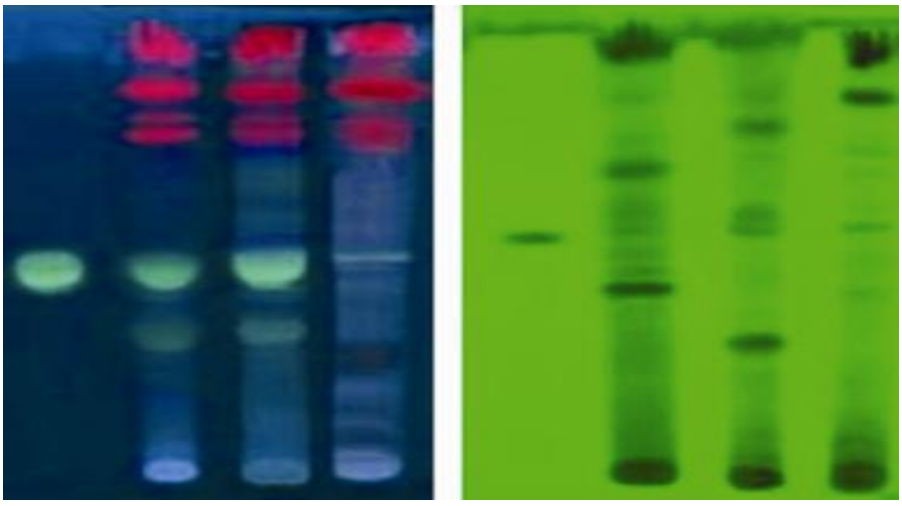

Fig. 6: Chromatogram of Giloy Churna in Chloroform: Methanol: Water (8: 2 : 0.2), UV 254 nm and 366 nm. 1: *Piper longum* 2: *Piper nigrum* 3: *Trikatu churna* 4: *Zingiber officinale*

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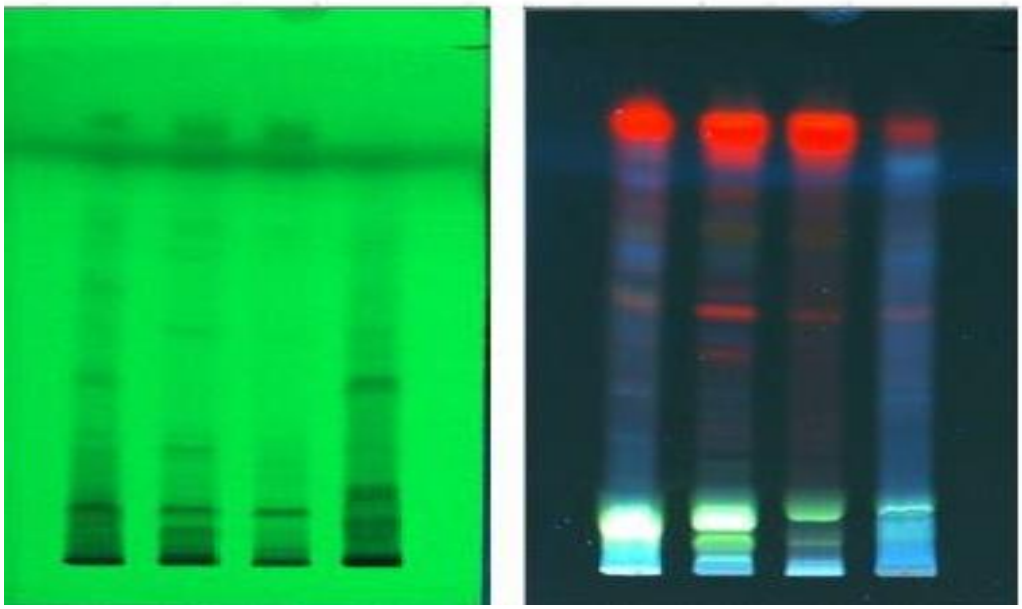

Fig. 7: Chromatogram of Homemade Churna in Benzene: Toluene: Ethyl Acetate: Formic Acid (4:3:1:2), UV 254 nm and 366 nm. 1: *Piper longum* 2: *Zingiber officinale* 3: *Tinospora cordifolia* 4: Homemade churna

Conclusion

In the present study Extraction, Phytochemical screening and Identification of phytochemicals by TLC of two marketed preparation viz., Trikatu Churna, Giloy Churna and one Homemade Churna were evaluated. The powdered plant materials viz., *Piper longum* (Piplai-fruits), *Piper nigrum* (Kali mirchi-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia (Giloy-stem)* were extracted with water and methanol. The results for % extract of AETC, AEGC and AEHC were also revealed. Phytochemical studies of Aqueous and Methanolic extracts of *Piper longum* (Piplai-fruits), *Piper nigrum* (Kali mirchi-fruits), *Zingiber officinale* (Adrak-rhizomes) and *Tinospora cordifolia (Giloy-stem)* was determined for the presence of active phytochemicals. The phytochemicals were identified by performing TLC studies of *Piper longum* (Piplai-fruits), *Piper nigrum* (Kali mirchi-fruits), *Zingiber officinale* (Adrak-rhizomes), *Tinospora cordifolia (Giloy-stem),* Trikatu Churna, Giloy Churna and Homemade Churna and investigation revealed that active constituents were present in all the three churna which were evaluated. The results showed the presence of piperine in *P. longum*, *P. nigrum*; zingeberene in Z. officinale and Tinosporaside in *T. cordifolia* in TC, GC and HC.

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