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Phytochemical screening and standardization of Nimba, Babbul and Manjistha Churna (A polyherbal mixture of *Azadirachta indica*, *Acacia nilotica* and *Rubia cordifolia*) through HPTLC

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Abstract
Nimba (*Azadirachta indica*), Babbul (*Acacia nilotica*) and Manjistha (*Rubia cordifolia*) are three widely used medicinal plants in Ayurveda- the traditional Indian system of medicine. The present study was aimed to screen the phytochemical constituents through preliminary phytochemical tests of Nimba, Babbul and Manjistha churna (equiproportional, powdered mixtutre of *Azadirachta indica*, *Acacia nilotica* and *Rubia cordifolia*) and to standardize this poly herbal mixture through High Performance Thin Layer Chromatography fingerprinting. The preliminary phytochemical screening of the extract revealed the presence of bioactive compounds like alkaloids, saponins, polyphenols, tannins, steroids carbohydrate and proteins. The HPTLC fingerprint profile, obtained from this study, of the same herbal formulation may be used for authenticity and quality.

Keywords: ayurveda, Nimba, Babbul, Manjistha, polyherbal formulation, phytochemical analyses, bioactive compounds, HPTLC, standardization

Introduction
Ayurveda is the traditional Indian system of medicine being practiced for thousands of years. In Ayurveda natural products like plants, animals and minerals are used for the treatment of various diseases, mostly the plants are used to derive therapeutic materials [1]. These medicinal plants are rich sources of beneficial constituents and it is believed in Ayurveda that complex diseases can be treated with combination of medicinal plants rather than single drug, used in western system of medicine [2]. Nimba *(Azadirachta indica)* [3], Babbul *(Acacia nilotica)* [4] and Manjistha *(Rubia cordifolia)* [5] are such medicinal plants having various medicinal properties and used to treat various diseases. In this study Extract of Nimba, Babbul and Manjistha Churna *i.e.* equiproportional, powdered mixture of useful parts (table no.1) of these three herbs is used for preliminary phytochemical screening and standardization through High Performance Thin Layer Chromatography (HPTLC). The present study was aimed to determine the phytochemical constituents of Nimba, Babbul and Manjistha churna as well as to standardize this particular formulation which will provide another useful resource for future.

Materials and Methods
Plant material
The ingredients (Table no. 1) were procured from the local market. The collected drugs were identified and authenticated at the teaching pharmacy of Department of Dravyaguna, Parul Institute of Ayurved, Limda, Waghodia, Vadodara.

Phytochemical analysis
Preliminary phytochemical screening and phytochemical studies through HPTLC were carried out at Vasu Research Centre, Makarpura, Vadodara-390010, Gujarat, India as per the standard procedures.

Table 1: Ingredients (Plant materials)

Sl. No.	Ingredients	Latin Name	Part Used	Quantity
1	*Nimba*	*Azadirachta indica*	Stem bark	1 part
2	*Babbul*	*Acacia nilotica*	Stem bark	1 part
3	*Manjistha*	*Rubia cordifolia*	Root	1 part

- Above ingredients are taken in equal proportions (1:1:1); made into fine powdered form and mixed well. This mixture of powders is stored in a closed vessel for future use.

Preliminary phytochemical tests [6, 7]
The preliminary phytochemical screening was performed according to the standard procedure. The procedures are as follows:

Test for alkaloids
Wagner's test: About 1ml of extract and 1ml of Wagner's reagent (dilute iodine solution) are added and mixed. Formation of reddish-brown precipitates indicates the presence of alkaloids.

Dragendroff's Test
To a few milligrams of extract dissolved in alcohol, a few drops of acetic acid and dragendroff's reagent were added and shaken well. An orange red precipitate formed indicates the presence of alkaloids.

Mayer's Test
To a few milligrams of extract dissolved in acetic acid, a few drops of mayer's reagent was added. A dull white precipitate formed indicates the presence of alkaloids.

Hager's Test
To a few milligrams of extract dissolved in acetic acid, 3 ml of hager's reagent was added, the formation of yellow precipitate indicates the presence of alkaloids.

Test for carbohydrates
Molisch's Test
To the extract, 1 ml of α-naphthol solution and conc. sulphuric acid were added along the sides of test tube. Violet colour formed at the junction of the two liquids indicates the presence of carbohydrates.

Fehling's Test
A few milligrams of extract were mixed with equal quantities of Fehling's solution A and B. The mixture was warmed on a water bath. The formation of a brick red precipitate indicates the presence of carbohydrates.

Benedict's Test
To 5 ml of Benedict's reagent, a few milligrams of extract was added, and boiled for two minutes and cooled. Formation of a red precipitate indicates the presence of carbohydrates

Test for steroids
Libermann Burchard Test
To the extract dissolved in choloro form, 1 ml of acetic acid and 1 ml of acetic anhydride were added, then heated on a water bath and cooled. Few drops of con.H_2SO_4 were added along the sides of the test tube. Appearance of bluish green color indicates the presence of steroids.

Salkowski Test
The extract was dissolved in chloroform and equal volume of con. H_2SO_4 was added. Formation of bluish red to cheery red colour in chloroform layer and green fluorescence in the acid layer indicates the presence of steroids.

Test for Saponins
To a few milligrams of extract, distilled water was added and shaken. Stable froth formation indicates the presence of saponin.

Test for Tannin
To the extract a few drops of dilute solution of ferric chloride was added, formation of dark blue colour shows the presence of tannins.

Test for Flavonoids
Shinoda's test: To the extract in alcohol, a few magnesium turnings and few drops of conc. H_2SO_4 were added and heated on a water bath. Formation of red red to pink colour indicates the presence of flavonoids.

Test for Phenol
To the extract in alcohol, added two drops of alcoholic ferric chloride. Formation of blue to blue black indicates the presence of phenol.

Test for Coumarins
To the extract in alcohol, a few drops of 2 N sodium hydroxide solution was added. Dark yellow colour formation indicates the presence of coumarins.

Test for Triterpenoids
The extract was warmed with tin bits and few drops of thionyl chloride. Formation of pink colour indicates the presence of triterpenoids.

Test for Carboxylic Acid
Extract dissolved in water is treated with sodium bicarbonate. Brisk effervescence indicates the presence of carboxylic acid.

Test for resin
Few milligrams of the sample was mixed with water and acetone. Turbidity indicates the presence of resin.

Test for quinine
A few milligrams of alcohol extract was treated with 0.5% of sodium hydroxide. Deep colouration like pink, purple or red indicates the presence of quinine.

High Performance Thin Layer Chromatography [8, 9]
Preparation of Test Solution
0.5 g of sample is weighed in an Iodine Flask and to it 10 mL of Methanol is added. It is refluxed for 30 Minutes. Then the sample is filtered through Whatman Filter Paper and the filterate of the sample is taken. The test solution thus obtained for HPTLC fingerprinting.

Preparation of Spray reagent [Anisaldehyde – sulphuric acid reagent]
0.5 mL Anisaldehyde is mixed with 10 mL Glacial acetic acid, followed by 85 mL Methanol and 5 mL Sulphuric acid (98 %). 8 µl of the above extract were applied on a pre-coated Silica gel 60 F254 on aluminum plates to a band width of 7 mm using Linomat 5 TLC applicator. The plate was developed in Toluene: Ethyl acetate: Formicacid (7.0: 2.0:1.0). The developed plates were visualized in short UV 254, 366, and then derivatised with Anisaldehyde Sulphuric acid reagent and scanned under UV 254nm, 366nm and 540nm. Rf and densitometric scan were recorded.

Table 2: Phytochemical constituents of Nimba, Babbul and Manjistha Churna

Sr. No.	Parameters	Results
1	Alkaloid	+
2	Anthraquinone	-
3	Flavonoids	-
4	Saponins	+
5	Total Polyphenols	++
6	Tannins	+
7	Steroids	++
8	Triterpenoids	-
9	Starch	-
10	Carbohydrates	++
11	Proteins	+
Key word: "+, ++, +++" indicates Present in increasing intensity and " - " indicates Absent.		

Table 3: R f value at 254nm

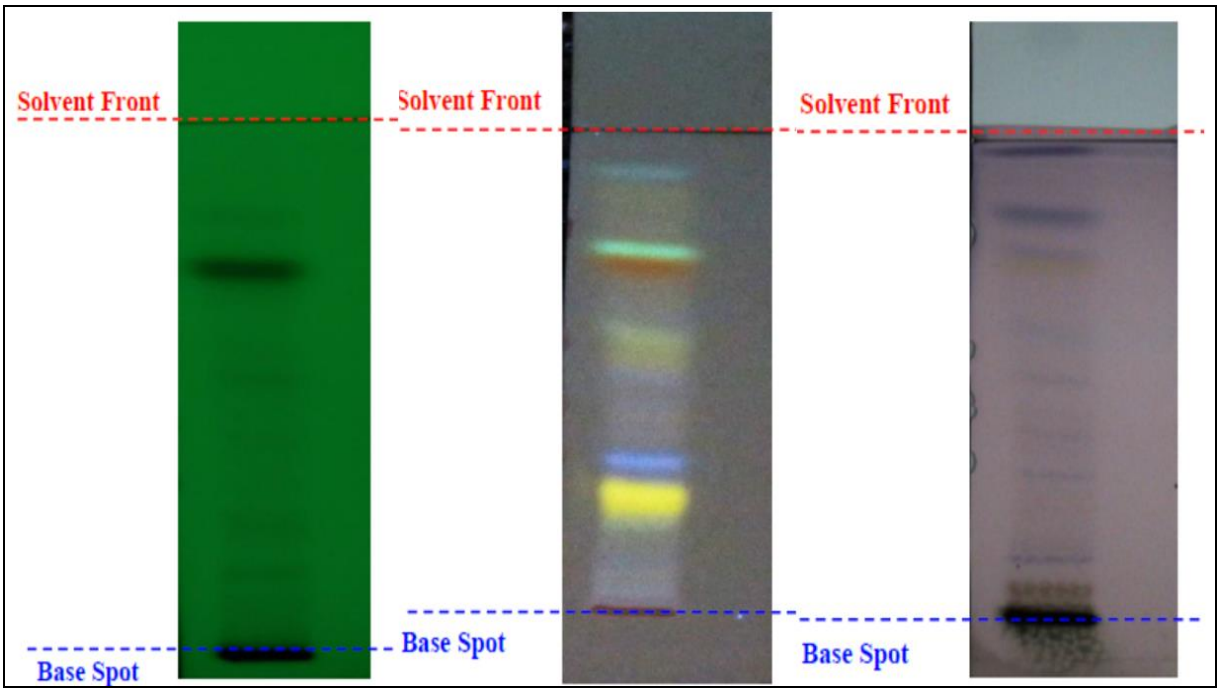
Spot No.	Track T
1	0.13
2	0.19
3	0.27
4	0.37
5	0.45
6	0.51
7	0.58
8	0.64
9	0.80
10	0.92

Table 4: R f value at 366nm

Spot No.	Track T
1	0.13
2	0.27
3	0.34
4	0.37
5	0.45
6	0.55
7	0.58
8	0.64
9	0.80
10	0.82
11	0.88
12	0.92

Table 5: R f value at 540nm

Spot No.	Track T
1	0.13
2	0.19
3	0.27
4	0.34
5	0.37
6	0.41
7	0.45
8	0.52
9	0.64
10	0.80
11	0.88
12	0.98



At 254nm at 366nm at 540nm (after Derivatization)

Track T- Nimba, Babbul and Manjistha Churna
Solvent system: Toluene: Ethyl acetate: Formic acid (7.0: 2.0:1.0)

Fig 1: HPTLC photo documentation of alcohol extract of Nimba, Babbul and Manjistha Churna

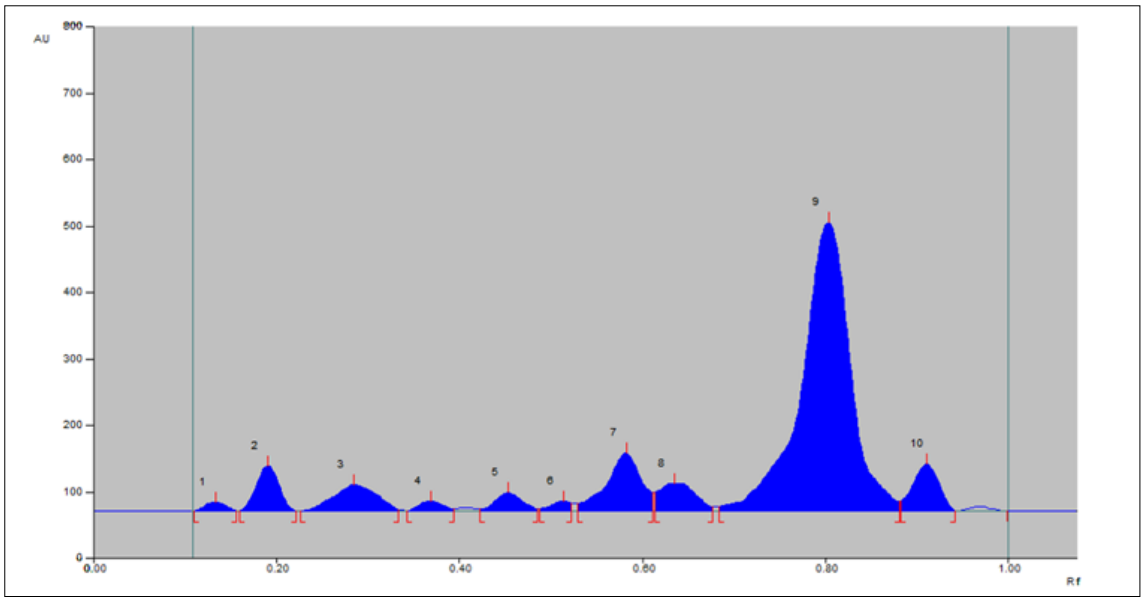


Fig 2: HPTLC 2D Chromatogram at 254 nm

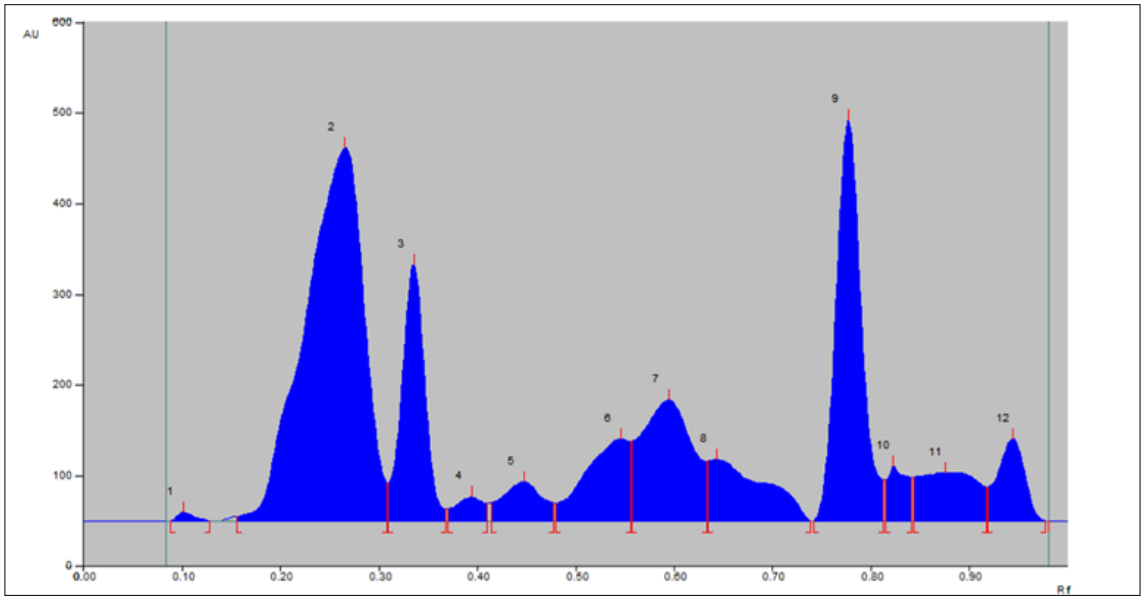


Fig 3: HPTLC 2D Chromatogram at 366 nm

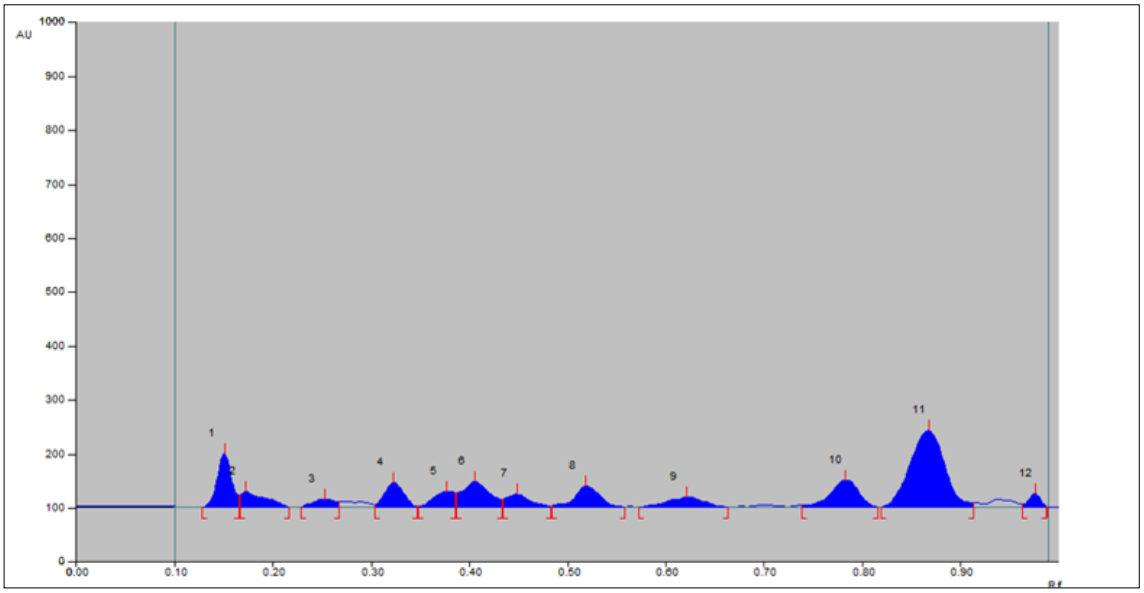


Fig 4: HPTLC 2D Chromatogram at 540 nm

Results and Discussion

Preliminary Phytochemical Tests

The phytochemical screening results showed the presence of alkaloids, carbohydrate, saponin, polyphenols, tannins, steroids and proteins in the extract of Nimba, Babbul and Manjistha Churna (Table 2). Most of the identified phytochemical compounds have been reported to have various biological activities *viz*. the alkaloids and saponins possess antimicrobial properties; Phenolics acid possess antiulcer, anti- inflammatory, antioxidant, antispasmodic, and antidepressant activities; tannin has got anti-inflammatory, antiseptic, antioxidant and haemostatic properties [10].

High Performance Thin Layer Chromatography

TLC photo documentation of Nimba, Babbul and Manjistha Churna showed 10, 12 and 12 spots under 254 nm, 366 nm and 540 nm after derivatization respectively. Spot with Rf 0.13, 0.27, 0.37, 0.45, 0.64 and 0.80 were commonly detected in all the three detection methods. All the three methods gave optimum separation of different bands and hence all of them may be used as TLC fingerprint pattern to identify the composition of the mixture (Table no. 3, 4,5). Densitometric scan at 254 nm revealed 3 high peaks corresponding to 3 different compounds in the ethanol extract, compounds with Rf 0.92, 0.58 and 0.80 were the peaks (Figure 2). At 366 nm there were three high peaks, with Rf 0.34, 0.27 and 0.80 being the major peaks detected (Figure 3). At 540 nm there were three high peaks, with Rf 0.80, 0.13 and 0.88 being the major peaks detected (Figure 4).

Conclusion

Preliminary phytochemical tests of the extract of Nimba, Babbul and Manjistha Churna showed the presence of alkaloids, carbohydrate, saponin, phenols, tannins which are reportedly bioactive in nature and may add up to the therapeutic effect of the polyherbal drug. HPTLC fingerprint profile of the same polyherbal formulation may be used for authentication and quality control.

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