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Research Article

HPTLC ANALYSIS OF RHIZOME OF PICRORRHIZA KURROA AND AAROGYAWARDHINI BATI

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Abstract

Aim: To evaluate chemical constituent of rhizome of *Picrorhiza kurroa*.and Aarogyawardhini bati.

Materials and Methods: *P. kurroa* forms a major ingredient of many Ayurvedic preparations prescribed in the treatment of asthma, Diabetes, Hypertension, fever and several ailments of the liver and spleen. Most of the preparations are used for the treatment of fever or diseases of the skin or diseases of the liver. One of the best –known preparations is Arogyavardhini bati which contains. 50% of extract *P. Kurroa* of and is used for treatment of fever, diabetes, obesity, skin diseases and liver diseases. This work presents an attempt to evaluate Aarogyawardhnin bati. Presence of *P. kurroa* in the Arogyawardhini bati was confirmed through HPTLC fingerprinting of the formulation. HPTLC fingerprinting and compared with the authentic ingredients as reference. It was observed that the chromatographic analyses complement each other in their findings and can be used effectively for the identification of the raw materials in the Aarogyawardhini bati. **Results:** Identification of tannin, steroid and bitter glycoside was confirmed through HPTLC fingerprinting. **Conclusion:** A rapid, simple, accurate and specific HPTLC method for estimation of tannin, kutkoside and steroid present in the Rhizome of *Picrrorhiza kurroa* and Aarogyawardhini bati has been developed.

Keywords: Ayurvedic formulation, Chromatographic fingerprinting studies Indian traditional medicine.

1. Introduction

Natural products have been an important resource for the maintenance of life for ages. It is evident that plants played an important role in daily life. One of the best known examples is Soma, a plant that was pressed to yield juice, which was used as a medicine[1-2].

Even today, natural products have become increasingly important as a source of Pharmacotherapeutics, either directly, for the treatment of chronic diseases, or as raw materials from which more or less complex chemical structures with particular biological activity are isolated. Medicinal plants are of great importance in providing healthcare to a large portion of the population in India. One of the species that emerged from such an inventory is *P. kurroa* Royle. *P. kurroa* forms a major ingredient of many Ayurvedic preparations prescribed in the treatment of asthma, Diabetes, Hypertension, fever and several ailments of the liver and spleen[3]. A number of plants are described in Ayurveda for use in the treatment of allergic disorders, namely psoriasis, eczema, bronchial asthma, etc. In one of the reports of review of various systems of system of medicine, about 444 classical preparations out of which 37 contain *Picrorhiza* rhizome powder. Most of the preparations are used for the treatment of fever or diseases of the skin or diseases of the liver. One of the best –known preparations is Arogyavardhini bati which contains. 50% of extract *P. Kurroa* of and is used for treatment of fever, diabetes, obesity, skin diseases and liver diseases. Dry rhizome contains Kutkin 3.4%w/w. Kutkin is the stable mixture of two C-9 iridoid glycosides of Picroside I and Kutkoside[4-5]. Picrrorhiza also contain steroid and phenolic compound. Literature survey reveals that their evaluation of the Aarogyawardhnin bati has not been done yet. However, the formulation also contains a number of pharmacologically active crude drugs which needs to be studied. Thus in the present study, evaluation of the formulation and HPTLC studies on the plant ingredients present in the marketed formulation.

2. Materials and Methods

2.1 Plant material

The rhizome of *P. kurroa* purchased from the local market in Mumbai, was authenticated through comparison of morphological characters with the specimen of the crude drug at the herbarium of Agharkar Research Institute; Pune.

The voucher specimen ((AHMA R 095)) is deposited in the institute for reference (The certificate from the Agharkar Research Institute is attached). Authentication is the first important step in investigation of any herbal drug.

2.2 Formulation

Aarogyawardhini Bati of one batch, Manufacturer by Baidyanath Company, Calcutta, was procured from a local store.

2.3 Extraction

The air dried crude drug was pulverized to obtain coarse powder. The total aqueous extract was prepared by decoction method with ratio of distilled water: Drug (1:5). The powdered drug was defatted by extracting with Pet-ether (60-80^{0}C), followed by extraction with methanol using Soxhlet extractor. The extract thus obtained was concentrated by recovering the solvent through rotary flash evaporator. The concentrated extract was then evaporated to dryness in vacuum oven at temperature not more than 50^{0}C. The dried extract was stored at 2-8^{0}C in refrigerator. The extract was further used for the detection of Phytoconstituents.

2.4 Phytochemical analysis

Most of the preparations are in use for the treatment of fever or diseases of the skin or diseases of the liver. One of the best –known preparations is Arogyavardhini Bati which contains 50% of *P. kurroa* and is used for treatment of fever, diabetes, obesity, skin diseases and liver diseases. The label claim of Arogyavardhini Bati is as follows,

Shudda parad 6mg, Shduddha Gandhak 6 mg, lauha Bhasma 6 mg, Abhrak Bhasma 6mg,Tamara Bhasma 6mg,Harre 12mg, Awala 12mg, Shuddha Shilajit 18mg, Chitrak Mool 24mg, Shuddha Guggul 24mg , Kutaki 132mg,etc. The preparation was standardized for the following parameters and it was also evaluated for the stated activities.

2.4.1 Total phenolic content estimation (TPC) [4]

Total phenolic content (TPC) was analyzed by Folin–ciocalteu colorimetric method using Gallic acid as a reference standard.

Preparation of Reagents

I Preparation of Standard Gallic acid Solutions

1. Stock I solution-Weighed accurately about 100mg of Gallic acid (Loba Chemie) and transferred quantitatively to a 100ml volumetric flask. Gallic acid was dissolved in about 50ml of distilled water; the final volume was made up to the mark with distilled water to get concentration of 1000ppm.

2. Stock II solution- Transferred 10 ml of Stock I solution in 100 ml volumetric flask and make up the volume was up to the mark with distilled water to get concentration of 100ppm.

II Preparation of Sodium carbonate solution (200g/L)

Weighed accurately 20gm Sodium carbonate (Loba Chemie) transferred to 100ml volumetric flask. The solids were dissolved in about 50ml of distilled water and volume was up to the mark with distilled water.

III Preparation of Samples

Methanolic Extract of *P. kurroa*

Weighed accurately about 100mg of the Methanolic extract transferred to 100ml volumetric flask. The solids were dissolved in about 50ml of distilled water and the volume was up to the mark with distilled water to get concentration of 1000ppm of the extract.

Arogyawardhini Bati

Weighed accurately about 200mg of powdered Arogyawardhini Bati tablet and transferred to 100ml volumetric flask. The powdered formulation dissolved in about 50ml of distilled water and the volume was upto the mark with distilled water to get concentration of 1000ppm of the extract.

2.4.2 Estimation of Total Steroid content[5]

I Preparation of the standard solution

The total sterols were estimated by using cholesterol (Himedia Lab Pvt. Ltd. Mumbai) as a reference standard. Weighed accurately about 200mg of cholesterol and was dissolved in minimum amount of glacial acetic acid and volume was made up to 100ml with glacial acetic acid.

II Preparation of sample solutions

Arogyawardhini Bati solution

Weighed accurately about 0.5gm of the powdered Arogyawardhini Bati tablet and was hydrolyzed with 10ml of conc. hydrochloric acid. The sterols were then extracted three times with 10ml of chloroform. Pooled all the chloroform extracts and evaporated to obtain the residue. The residue was dissolved in glacial acetic acid to obtain a solution.

Methanolic Extract of *P. kurroa*

Weighed accurately about 0.5gm of the Methanolic Extract and was hydrolyzed with 10ml of conc. hydrochloric acid. The sterols were then extracted three times with 10ml of chloroform. Pooled all the chloroform extracts and evaporated to obtain the residue. The residue was dissolved in 6ml of glacial acetic acid to obtain a solution.

Procedure

Transferred 0.2, 0.4, 0.6, 0.8 and 1 ml of the standard cholesterol solution to pre-calibrated (5ml) dry test tubes, and added acetic anhydride (1ml) and from the sides of the test tube conc. sulphuric acid (1-2 drops) were slowly added and the test tubes were allowed to stand in dark for 20 mins. The green color developed was read on UV visible spectrophotometer (Elico SL 159) at 540 nm against the reagent blank. The samples were treated in the similar way to develop colors and absorbance were read at 540 nm against reagent blank. The results were obtained by extrapolation using the calibration curve (Fig. 2).

2.5 HPTLC fingerprinting

Methanolic extract of *P. kurroa* and Arogyawardhini Bati was compared for the presence of Kutkoside, Gallic acid and Guggulosterone through HPTLC fingerprinting using parameters like R*f*, wavelength and peak area. The label of claim of Arogyawardhini Bati is as follows- Shudda parad 6mg, Shduddha Gandhak 6 mg, lauha Bhasma 6 mg, Abhrak Bhasma 6mg,Tamara Bhasma 6mg,Harre 12mg, Awala 12mg, Shuddha Shilajit 18mg, Chitrak Mool 24mg, Shuddha Guggul 24mg , Kutaki 132mg etc.

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2.5.1 HPTLC Fingerprinting of methanolic extracts of *P. kurroa* and Arogyawardhini Bati[6]

The appropriate quantity of dried methanolic extract of *P. kurroa* and Arogyawardhini Bati powder were dissolved in methanol to get the stock solution of 2mg/ml and 7.5mg/ml respectively. From the stock solutions, 20 µl and 50 µl of solution of *P. kurroa* and Arogyawardhini were applied in triplicate on HPTLC (Silica gel GF 254) plates using

Linomat 5 respectively. The chromatograms were developed using ascending mode with mobile phase composition of ethyl acetate: methanol (47:3) as mobile phase (Fig. 3 and Table 2). The plates were then dried and scanned with Camag scanner 3 and the data were processed with Win Cats 1.4.4 software at 254nm (Table 2, Fig. 3 and Fig. 4).

2.5.2 HPTLC Fingerprinting of Methanolic extracts of Guggul and Arogyawardhini Bati[7]

Suitable quantities of methanolic extract of Guggul and Arogyawardhini Bati powder were dissolved in methanol to get the stock solution of 1/ml and 10mg/ml respectively. From the stock solutions, 20 µl and 50 µl of solution of Guggul and Arogyawardhini respectively were applied on HPTLC (Silica gel GF 254) plates using Linomat 5. The chromatograms were developed using ascending mode with Petroleum ether: ethyl acetate (3:1) as mobile phase (Fig .7 and Table 3). The plates were then dried and scanned with Camag scanner 3 and the data were processed with Win Cats 1.4.4 software at 254nm. The results are presented- (Fig. 7, Fig. 8, Fig. 9, Fig. 10 and Fig. 11).

2.5.3 Determination of HPTLC Fingerprinting of Gallic acid in Methanolic extracts and Arogyawardhini Bati[8]

The appropriate quantities of *Gallic acid* and methanolic extract Arogyawardhini Bati were dissolved in methanol to get the stock solution of 1mg/ml and 10mg/ml respectively. From the stock solutions, 5 µl and 50 µl of solution of *Gallic acid* and Arogyawardhini Bati were applied on HPTLC (Silica gel GF 254) plates using Linomat (5) respectively. The chromatograms were developed using ascending mode with Toluene: ethyl acetate: glacial acetic acid: Formic acid (20:45:20:5) as mobile phase (Fig 12, Table4). The plates were then dried and scanned with Camag scanner 3 and the data was processed with Win Cat 1.4.4) software at 284nm

The results are presented**- (**Fig. 12, Fig. 13, Fig. 14 and Fig. 15).

3. Result

The rhizome of *P. kurroa* revealed presence of tannins, glycosides and steroids in the preliminary phytochemical analysis, hence, the efforts were made to quantify the methanolic extract for total polyphenolic and steroidal content. One of the formulations viz Arogyawardhini bati, selected for the study, contains Shudda parad (6mg), shuddha Gandhak (6mg), Lauha Bhasma (6mg), Abhrak Bhasma (6mg), Tamara Bhasma (6mg), Harre (12mg), Awala (12mg), Shuddha Shilajit (18 mg), Shuddha Guggul (24mg), Chitrak Mool (24mg), Kutaki (132mg) was also quantified for the total polyphenolic and steroidal content.

The total polyphenolic content was estimated using Gallic acid as a reference standard, while the total steroidal content was determined using cholesterol as a reference standard using spectrophotometric methods like, Folin-Ciocalteu and Liebermann Burchard methods respectively.

The total polyphenolic content in the methanolic extract of *P. kurroa* and Arogyawardhini bati were extrapolated from the standard curve of Gallic acid. (fig.1.). The total polyphenolic content in methanolic extract of *P. kurroa* and Arogyawardhini bati were found to be 0.004561 and 0.049% w/w respectively (Table 2). The higher content of total polyphenolic in the Arogyawardhini bati is due to the presence of tannins containing drug like, Awala.

The total steroid content in the methanolic extract of *P. kurroa* and Arogyawardhini bati was extrapolated from the standard curve of cholesterol (Fig. 2). The total steroid content methanolic extract of *P. kurroa* and Arogyawardhini bati were found to be 0.0086 and 0.024 % w/w respectively. Higher content of total steroid was observed in Arogyawardhini bati due to presence of Guggul, which contains steroidal compounds like Guggulosterone – E, and Guggulosterone – Z (Table 2).

4. HPTLC Analysis

HPTLC analysis of the methanolic extract of *P. kurroa* for the glycosides revealed presence of two major spots. These spots were visualized by spraying with Vanillin-Sulphuric acid, lead to formation of bluish-brown color. Based on the reaction given by the constituents with Vanillin-Sulphuric acid and R_f value, the spots were identified on the Preliminary basis to be picroside and kutkoside, by comparing with the reported data[6]. Further, the presence of kutkoside could be confirmed through carrying out wavelength scan of the spot corresponding to R_f value (0.24), without derivatizing with Vanillin-Sulphuric acid. The wavelength scan revealed that the constituent has λ_{max} at 295nm which correspond to kutkoside (295nm) as reported in literature. Analysis of kutkoside using the external reference standard of kutkoside could not be carried out as the reference standard was not affordable.

Presence of *P. kurroa* in the Arogyawardhini bati was confirmed through HPTLC fingerprinting of the formulation. HPTLC Chromatogram of Arogyawardhini bati indicated allied spots with Rf values and the reaction to Vanillin-Sulphuric acid with the constituents of *P.kurroa* (Fig. 3, Table 2). Further, the presence of kutkoside in Arogyawardhini bati was confirmed by spiking the methanolic extract of *P. kurroa* in the formulation and carrying

out HPTLC analysis of the same. The HPTLC analysis (Fig. 5) indicated rise in the peak area of the spot with R_f (0.24) which was corresponding to kutkoside. Presence of kutkoside in Arogyawardhini bati was also confirmed by carrying out wavelength scan an of the spot corresponding to R_f (0.24) and it was observed that the wavelength scans coincide with each other (Fig. 6).

The fingerprint analysis presented above thus confirmed the presence of kutkoside, which is one of the major glycosides of *P. kurroa* in Arogyawardhini bati., however, quantitative analysis of the same could not be carried out.

Presence of steroid viz, Guggulosterone – E, and Guggulosterone – Z in the Arogyawardhini bati was also confirmed through HPTLC fingerprinting technique. Standard Guggul sample was co-chromatogramed with Arogyawardhini bati and the spots corresponding to Guggulosterone were detected through reaction with Liebermann Burchard reagent (Fig.**7** and Table 3). The wavelength scan and spiking methods were utilized and the presence of Guggulosterone – E (R_f-0.22) with $\lambda_{max.}$ at 254 nm and Guggulosterone – Z (Rf 0.33) with $\lambda_{max.}$ at 254 nm could be detected in the Arogyawardhini bati (Fig. 8, Fig. 9, Fig.10 and Fig.11).

Presence of Gallic acid in Arogyawardhini bati was confirmed by spiking method and wavelength scan of spot with R_f 0.64. (Table 4, Fig. 13, Fig.14 and Fig. 11). and Table 4 indicated that spiking with standard Gallic acid leads to increase in the area of the peak corresponding Rf 0.64 (Fig. 14) and wavelength scan of the spot corresponding to Rf 0.64 in the Arogyawardhini bati has $\lambda_{max.}$ at 284 nm which corresponds to Gallic acid ($\lambda_{max.}$ 284nm).

5. Discussion

In the present study,Rhizome of Picrrorhiza kurroa and an ayurvedic formulation, *Aarogyawardhini bati*, was purchase local market. Preliminary phytochemical analysis indicated presence of tannins, Steroid and bitter glycosides.

Chromatographic technique like HPTLC was utilized for fingerprinting and quantitative determination of phytoconstitutes present in the rhizome and the formulation containing *P. kurroa.* The glycosides like picroside and kutkoside in the extract were detected and confirmed by comparing the R_f, λ_{max} with the reported data in the Indian Herbal Pharmacopoeia. The extract thus standardized by fingerprinting was utilized as the reference standard for detection and confirmation of kutkoside in the Arogyawardhini bati. The Presence of kutkoside in Arogyawardhini

bati was confirmed by spiking the extract of rhizome of *P. kurroa* in the formulation followed by coinciding the wavelength scan.

The presence of steroidal constituents like Guggulosterone-E and Guggulosterone-Z were detected and confirmed by comparing with fingerprinting of Guggul as stated above. The technique of spiking and wavelength scan was utilized for steroidal constituents in the formulation.

The total polyphenolic content of the formulation and the rhizome were determined spectrophotometrically, by Folin-Ciocalteu method using Gallic acid as reference standard. The content of total polyphenolics in the rhizome and Arogyawardhini bati were observed to be 0.00456 and 0.049 %w/w respectively.

The steroidal content of rhizome and formulation was estimated using Liebermann-Burchard method using cholesterol as a reference standard. Arogyawardhini Bati and the rhizome were found to contain 0.0086 and 0.024 %w/w of steroid.

6. Conclusion

A rapid, simple, accurate and specific HPTLC method for estimation of tannin, kutkoside and steroid present in the Rhizome of *Picrrorhiza kurroa* and Aarogyawardhini bati has been developed. The data could be used as a QC standard. The method used in this work resulted in good peak shape and enabled good resolution of constituents of the plant material.

Table-1: Content of total polyphenolics and steroid in rhizome of *P. kurroa* and Arogyawardhini bati.

Constituents	***P. kurroa* %w/w**	**Arogyavardhini bati %w/w**
	Mean ± S.E.M.	Mean ± S.E.M.
Polyphenolics	0.004561 ± 5.179	0.049 ± 0.00057
Steroid	0.0086 ± 0.00015	0.024 ± 0.00066

Table-2: Chromatographic data of HPTLC analysis of methanolic extracts of *P. kurroa* and Arogyawardhini Bati.

Track	Start position (R_f)	Start height (AU)	Max position R_f	Max height (AU)	Max %	End position R_f	End height (AU)	Area (AU)	Area %
1	0.23	77.8	0.31	212.7	9.12	0.37	17.6	12478.4	16.24
2	0.21	39.5	0.27	137.3	8.05	0.34	2.1	7085.7	15.19
3	0.19	67.4	0.30	431.6	23.08	0.34	17.6	24806.9	40.14
4	0.20	65.8	0.31	427.0	23.14	0.38	13.0	25760.5	40.63
5	0.21	111.4	0.32	418.6	18.82	0.38	29.1	26613.8	31.73
6	0.22	112.3	0.32	434.4	18.74	0.37	31.0	27271.9	30.40
7	0.22	151.6	0.32	425.7	17.25	0.40	25	29320.5	30.33

Table-3: Chromatographic data of HPTLC analysis of methanolic extracts of Guggul and Arogyawardhini Bati.

Track	Guggulosterone	Start position (R_f)	Start height (AU)	Max position R_f	Max height (AU)	Max %	End position R_f	End height (AU)	Area (AU)	Area %
A	E	0.21	40.7	0.26	125.6	5.66	0.30	8.4	4248.2	10.42
	Z	0.30	8.4	0.35	72.1	3.27	0.41	0.6	2285.2	5.60
G	E	0.22	94.1	0.25	229.9	16.22	0.29	37.4	8216.7	17.58
	Z	0.30	88.0	0.34	249.2	17.58	0.40	17.9	9397.5	20.10
A+G	E	0.21	261.5	0.24	281.0	7.23	0.26	59.2	8940.0	4.99
	Z	0.26	259.5	0.31	316.9	8.15	0.35	33.7	18455.5	10.30

Table 4: Chromatographic data of HPTLC analysis of Methanolic extracts of *Gallic acid and* Arogyawardhini Bati.

Track	Start position (R_f)	Start height (AU)	Max position R_f	Max height (AU)	Max %	End position R_f	End height (AU)	Area (AU)	Area %
A	0.53	168.3	0.64	322	29.21	0.75	2.6	28418.6	38.88
G	0.48	81.6	0.65	273.8	25.91	0.79	0.1	27782.2	38.73
A+G	0.42	19.8	0.60	514.9	61.53	0.73	0.2	46273.1	83.83

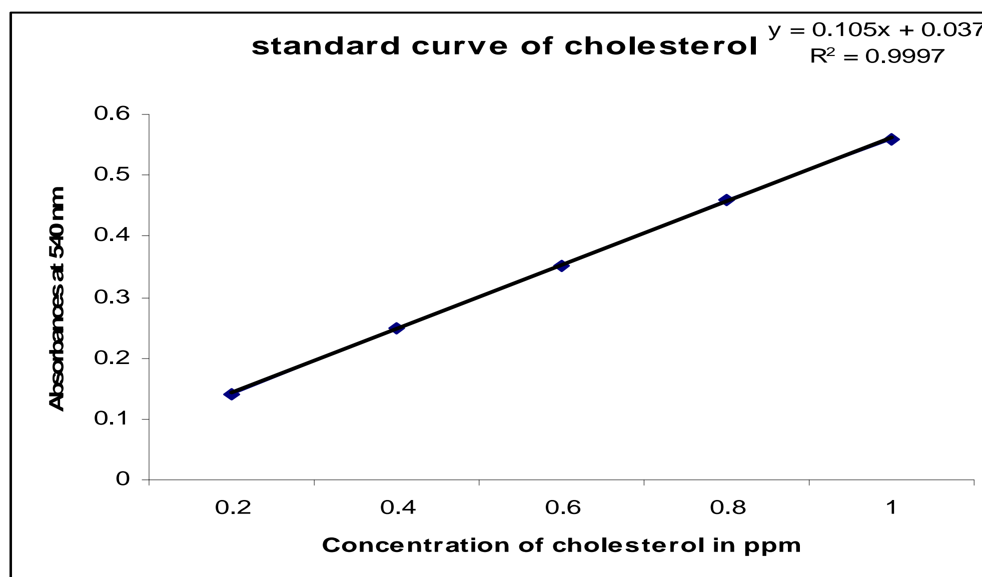


Fig.1: Standard curve for Determination of total polyphenolics By Folin- Ciocalteu method.

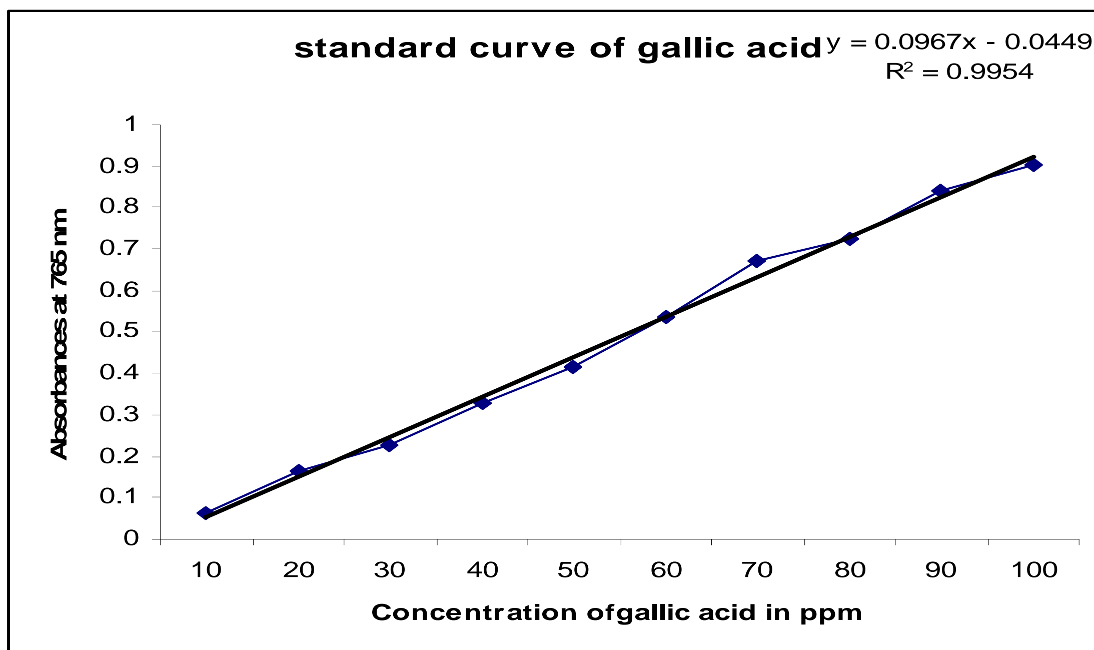


Fig. 2: Standard curve of Cholesterol by Liebermann Burchard method.

Fig. 3: HPTLC Plate image at visible light developed with Vanillin-Sulphuric acid Reagent. 1&2: Arogyawardhini Bati methanolic extract (50 µl), 3 & 4- methanolic extract of *P.kurroa* (20 µl), 5-7: methanolic extract of Arogyawardhini Bati and *Picrorhiza kurroa* (50 + 20 µl).

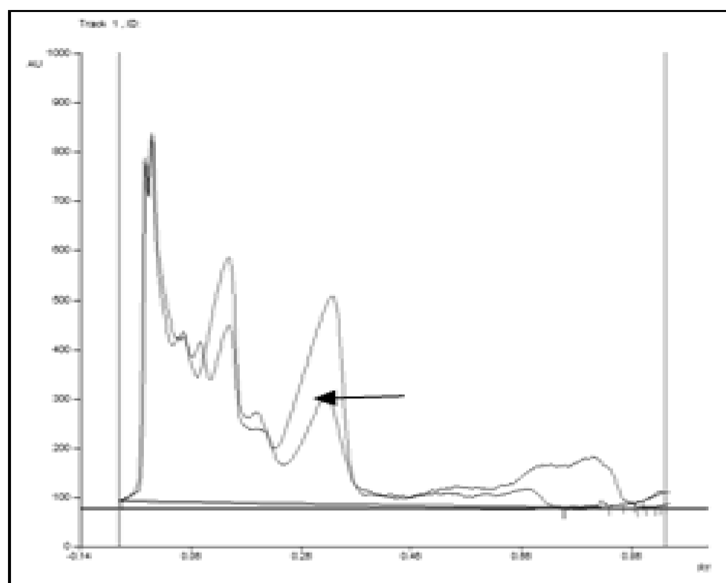


Fig. 4: Tracks 1&2: Arogyawardhini Bati (50 µl), Tracks 3 & 4- methanolic extract of *Picrorhiza kurroa* (20 µl), Tracks 5-7: Arogyawardhini Bati and methanolic extract of *P. kurroa*. (50 + 20 µl).

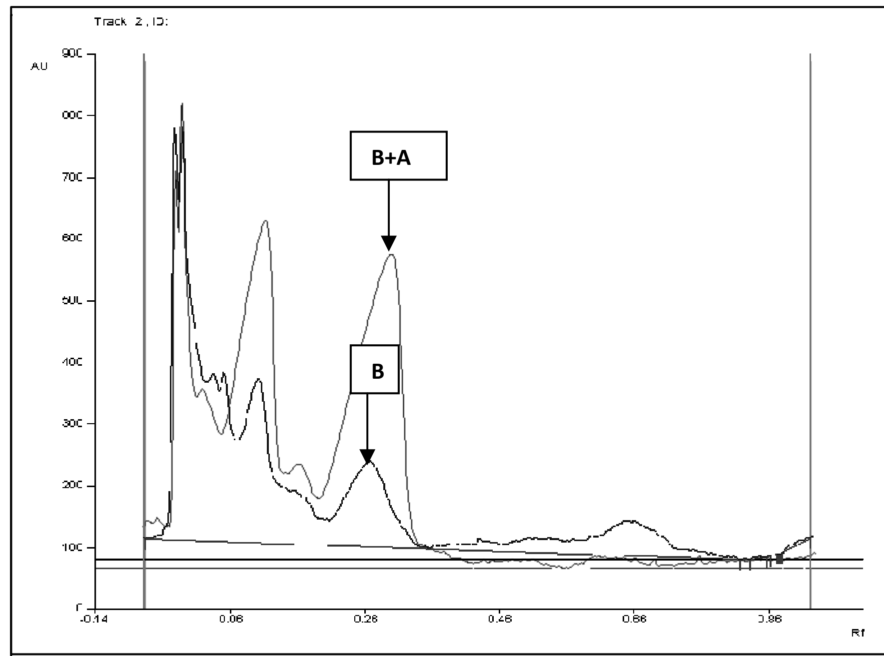


Fig.5: Effect of Spiking of methanolic extract of *P. kurroa* (A) in Arogyawardhini Bati (B) on the chromatogram of methanolic extract of *P. kurroa*.

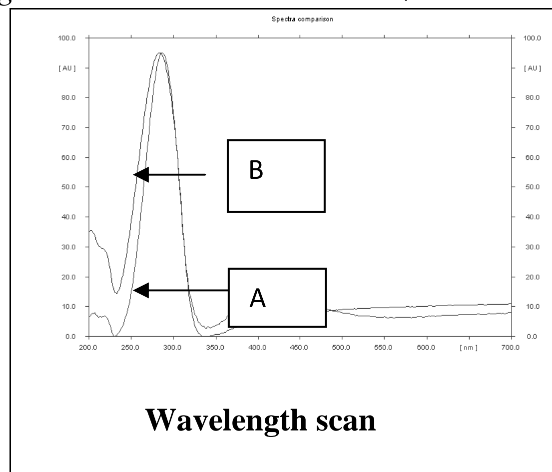


Fig.6: Wavelength scans of spot with Rf 0.24 of methanolic extract of *P. Kurroa* (A) and Arogyawardhini Bati (B).

Fig.7: HPTLC Plate image at visible light developed with Liebermann-Burchard Reagent. A - Arogyawardhini Bati (50 µl), G- methanolic extract of Guggul (20 µl), A + G - ethanolic extract of Arogyawardhini Bati and methanolic extract of Guggul (50 + 20 µl)

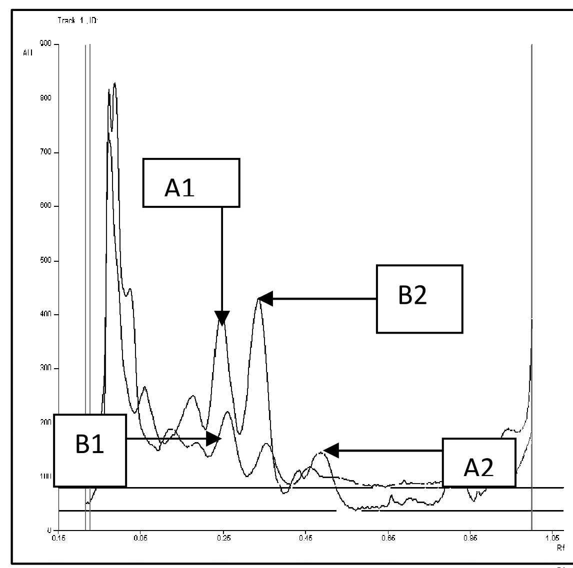
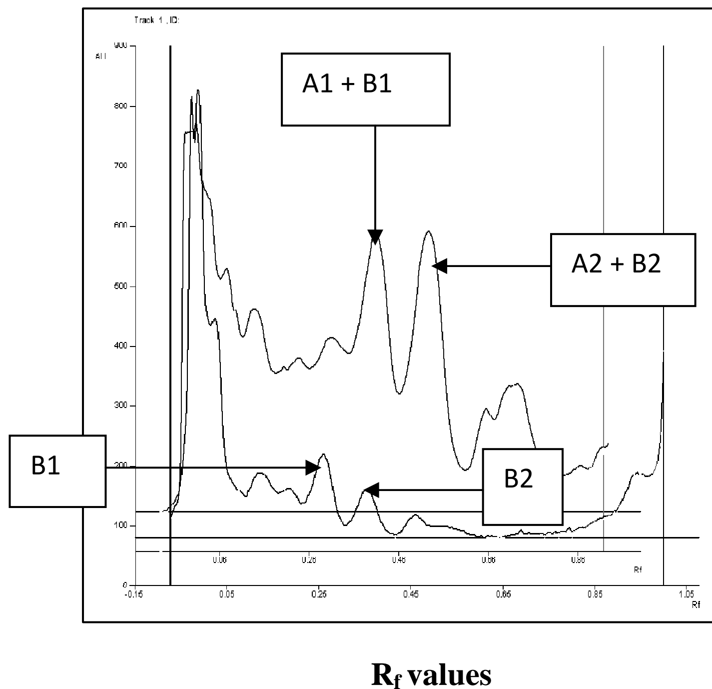
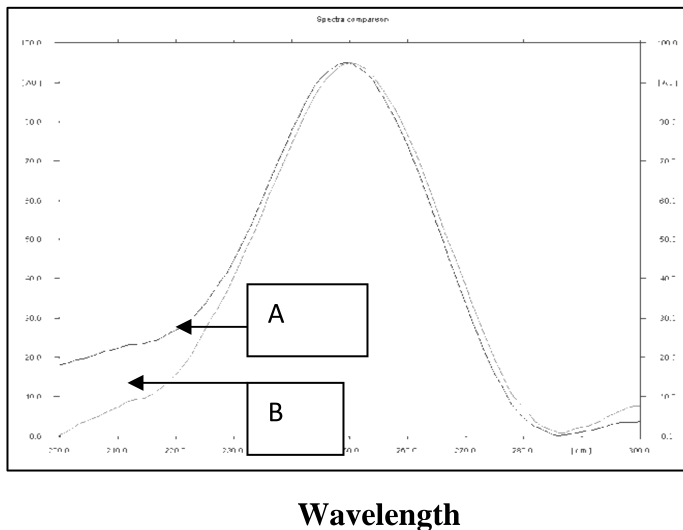


Fig.8: Fingerprint of Methanolic extracts of *Guggul* (A1, A2) and Arogyawardhini Bati. (B1, B2), A1- Guggulosterone –E, A2 - Guggulosterone –Z, B1- Guggulosterone –E, B2- Guggulosterone –Z.



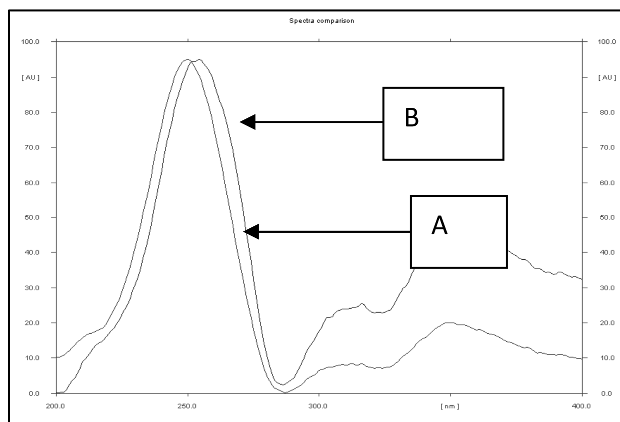
R_f values

Fig. 9: Spiking of Methanolic extracts of *Guggul* (**A1, A2)** and Arogyawardhini Bati. (**B1, B2), A1-** Guggulosterone –E, A2 - Guggulosterone – Z, **B1-** Guggulosterone –E, **B2-** Guggulosterone –Z.



Wavelength

Fig. 10: Wavelength scan spot with R_f 0.22 of Methanolic extracts of *Guggul* (**A**) in Methanolic extract of Arogyawardhini Bati (**B**), A- Guggulosterone – E in Guggul.B- Guggulosterone – E in Arogyawardhini Bati.

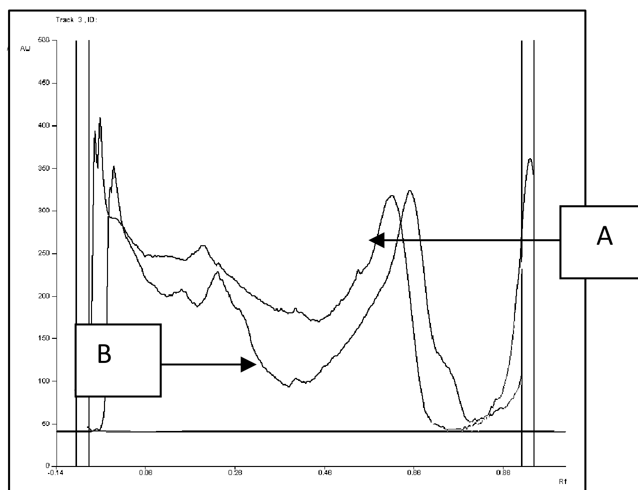


Wavelength scan

Fig. 11: Wavelength scan spot with Rf 0.33 of Methanolic extracts of *Guggul* (**A**) in Methanolic extract of Arogyawardhini Bati (**B**), A- Guggulosterone –Z in Guggul. Guggulosterone –Z in Arogyawardhini Bat.

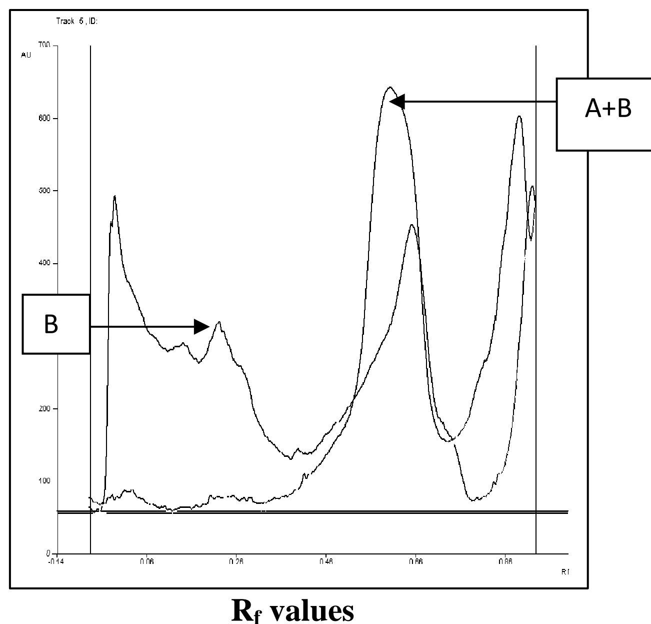
A G A+G

Fig. 12: HPTLC Plate image at visible light developed with 5 % Ferric Chloride Reagent. A - Arogyawardhini Bati (50 µl), G- methanolic extract of Gallic acid (5 µl), A + G - Arogyawardhini Bati and methanolic etract of Gallic acid (50 + 5 µl)



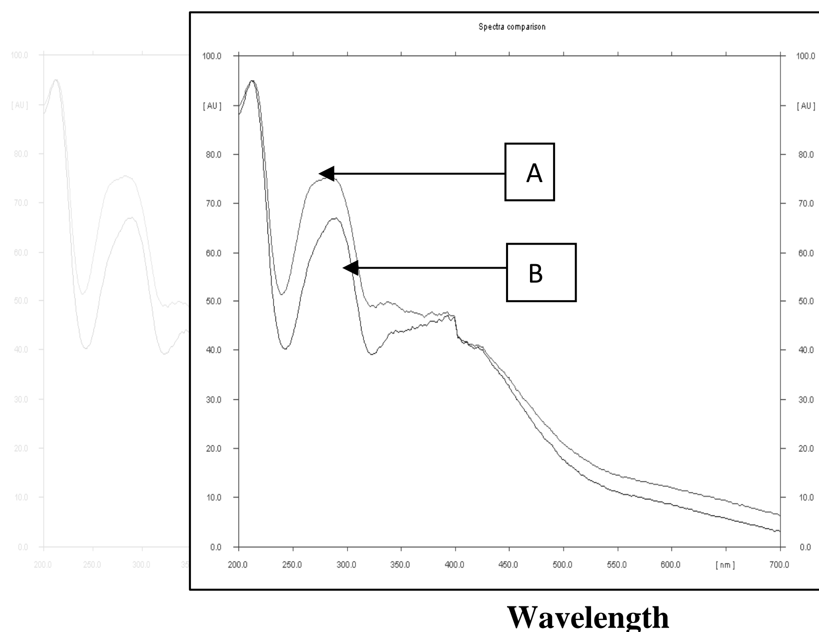
R_f values

Fig. 13: Fingerprint of *Gallic acid* (A) and Methanolic extract of Arogyawardhini Bati.(B).



R_f values

Fig. 14: Spiking of *Gallic acid* (A) in methanolic extract of Arogyawardhini Bati(B).



Wavelength

Fig. 15: Wavelength scan spot with R_f 0.64, extract of *Gallic acid* (A) in Methanolic Extract of Arogyavardhini Bati (B).

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