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Quantification of Baicalein, Chrysin, Biochanin-A and Ellagic Acid in Root Bark of *Oroxylum indicum* by RP-HPLC with UV Detection

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Abstract

Oroxylum indicum (Syonakh), belonging to the family *Bignoniaceae,* has been used for the present study. Preliminary phytochemical screening revealed the presence of phytocostituents like flavonoids, alkaloids, tannins and anthraquinone in the root bark of *Oroxylum indicum.* The preliminary screening using TLC technique reflected the presence of four phytoconstituents such as baicalein, chrysin, biochanin-A and ellagic acid in both petroleum ether and hydrolysed *n*-butanol fractions. Modern phytochemical analysis in terms of quantification of different bioactive phytoconstituents in the root bark of O*roxylum indicum* was performed using reverse phase high performance liquid chromatography (RP-HPLC) fingerprint. To evaluate the quality of root bark of *Oroxylum indicum*, a simple rapid and accurate RP-HPLC method was developed for the assessment of four bioactive phytoconstituents viz. chrysin, baicalein, biochanin-A and ellagic acid. The components were quantified in different extracts viz. alcohol, petroleum ether, ethyl acetate and *n*-buatnol successively. Quantification of phytoconstituents was done by using RP-HPLC in petroleum ether and hydrolysed *n*-butanol fraction. Standard baicalein, chrysin biochanin-A and ellagic acid (Sigma-Aldrich) were employed for the development of the method. The RP-HPLC system used a base deactivated C18 column with water, methanol, acetonitrile and orthophosphoric acid as the mobile phase and detection was performed at 262 nm. The method was precise with relative standard deviation for these constituents that ranged between 0.5-1.0% (interday). The content of four phytoconstituents in the root bark of *Oroxylum indicum* was determined to establish the effectiveness of the method.

Keywords: *Oroxylum indicum*, chrysin, baicalein, biochanin-A, ellagic acid

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1. Introduction

Oroxylum indicum. (Syonakh) belonging to the family *Bignoniaceae,* was selected for the present study. This plant is used as an astringent, carminative, diuretic, stomachic, aphrodisiac and has high potential for stimulating digestion, curing fevers, coughs and preventing other respiratory disorders [1]. *Oroxylum indicum*, is used as one of the important ingredient in most commonly used Ayurvedic preparation, named as “Dasamula”. It is also used in other Ayurvedic formulation such as Amartarista, Dantyadyarista, Narayana Taila, Dhanawantara Ghrita, Brahma Rasayana, Chyavanaprasa Awalwha, etc. [2]. The plant is reported to possess anti-inflammatory, diuretic, anti-arthritic, antifungal and antibacterial activity [3]. The stem bark and leaves of this plant is reported to contain flavonoids namely, chrysin, oroxylin-A, scutellarin, baicalein, [4, 5]. Seeds of this plant are reported to contain ellagic acid [6]. Based on our earlier results of *TLC* study, we have suggested the presence of chrysin, baicalein, biochanin-A, and ellagic acid phytoconstituents in the root bark of *Oroxylum indicum.*

Baicalein is reported to possess an anti-inflammatory [7], anti-ulcer [8], antioxidant [9], hepatoprotective [10] and immunomodulatory activity [11], while chrysin and baicalein both are reported to have antibacterial, antifungal and antiviral activity [12, 13]. Furthermore, biochanin-A possesses anti-fungal action and tumor necrosis factor-α [14]. Ellagic acid is an important polyphenolic compound [15]. Most plant extracts are composed of complex phytoconstituents. Therefore, proper method is particularly desired for the quality control of the fraction of the title plant as well as that of pharmaceutical or nutraceutical products made therefrom. As no method is available to date for the co-quantification of chrysin, baicalein, biochanin A, and ellagic acid, an attempt has been made to develop *HPLC* method for quantification of these bioactive flavonoids in petroleum ether, and hydrolyzed n-butanol fraction of root bark of *Oroxylum indicum*.

2. Experimental

2.1. Procurement of plant material and extraction procedure

The fresh root bark of *Oroxylum indicum* was collected from Van-Aaushadhi Ektrikaran Udyan, Ahwa, Dang forest, Gujarat, India. The voucher specimen (#404) was deposited in the Department of Pharmacognosy and Phytochemistry at the L. M. College of Pharmacy, Ahmedabad. The root bark was dried and powdered to a 60 mesh size (≈250 μm). The powder of the root bark after defatting with petroleum ether (0.32% w/w) was dried, then moistened with ammonia (NH_3) solution, and extracted with chloroform (0.78% w/w), ethyl acetate (1.52% w/w) and *n*-butanol (1.68% w/w), successively. The dried fractions were then stored in airtight containers until usage.

2.2. Chemicals and Reagents

Pure samples of chrysin, baicalein, biochanin-A and ellagic acid were purchased from Sigma-Aldrich Chemical Co. Spectrochem HPLC grade methanol; acetonitrile, orthophosphoric acid and water were used. All different organic solvents and chemicals used for extraction under study were analytical grade (A.R. grade) obtained from S.D. Chem. Pvt. Ltd., Mumbai, India. Natural product polyethylene glycol (NP-PEG) was also obtained from S.D. Chem. Pvt. Ltd., Mumbai, India.

2.3. Phytochemical test

Phytochemical analysis of the crude powder drug was performed using standard methods. Tests for alkaloids, flavanoids, saponins, tannins, anthraquinone, and carbohydrates were carried out and were confirmed by quantitative analysis and also by thin-layer chromatography *(TLC).*

2.4. Preliminary *TLC* study

Hydrolysis of *n*-butanol: 5 g of *n*-butanol fraction was dissolved in a water: methanol (9:1) solution, hydrolysed using 2N HCl by refluxing the mixture for 2 h. After cooling, the ethyl acetate soluble fraction was separated, and used for the further studies (*TLC*, *HPLC*). *TLC* co-chromatography was performed on the petroleum ether fraction, the hydrolyzed *n*-butanol fraction, and standard baicalein, chrysin, biochanin-A and ellagic acid. 10 µl of each sample solution was spotted on the *TLC* plate along with a standard solution of baicalein, chrysin, biochanin-A and ellagic acid.

Stationary phase consisted of *TLC* Aluminum sheets pre-coated with silica gel 60 $F_{254,}$ thickness 0.2mm, (20×20 cm) (E Merck, Germany), mobile phase consisted of chloroform: ethyl acetate: acetic acid (5:4:1), detected at daylight showed yellow colored spot under (short-wave) UV light. Derivatization was done with natural product poly ethylene glycol (NP-PEG). On the basis of *TLC* study, an *HPLC* method was developed for the quantification of four phytoconstituents namely baicalein, chrysin, biochanin-A and ellagic acid in both the active fractions and development of fingerprints of the same.

Most plant extracts composed of complex phytoconstituents. Therefore, proper method is particularly desired for the quality control of the fraction of the title plant as well as that of pharmaceutical or nutraceutical products made therefrom. As no method is available to date for the co-quantification of chrysin, baicalein, biochanin-A, and ellagic acid, an attempt has been

made to develop *HPLC* method for quantification of these bioactive flavonoids in petroleum ether, and hydrolyzed *n*-butanol fraction of root bark of *Oroxylum indicum*.

2.5. Determination of phytoconstituents by RP-*HPLC*

Shimadzu 2010C integrated High performance liquid chromatographic system was used for this experiment. Shimadzu 2010C system equipped with 2010 quaternary gradient pump, 2010 UV-VIS detector, 2010 Column Oven, 2010 programmable auto sampler controlled by CLASS-VP software. The quantification of phytoconstituents in petroleum ether and hydrolyzed *n*-butanol extracts was performed by *HPLC* method on a base deactivated RP-phase [16] Complete separation of the phytoconstituents was achieved on 250 X 4.6 mm i.d. Hypersil BDS-RP-C18 5 µm column. The mobile phase consisted of Water: Methanol: Acetonitrile: Ortho phosphoric acid (60:30:38:1, v/v/v/v). Injection volume was10 µL used. The isocratic method was run for 35 min. The flow rate was 1ml/min at room temperature. The phytoconstituents were detected at 262 nm (UV-VIS detector). Oven temperature was ambient. The quantification of baicalein, chrysin, biochanin-A and ellagic acid was estimated by using calibrated Shimadzu LC-2010 quaternary RP-*HPLC* system. *HPLC* analysis of petroleum ether and hydrolyzed *n*-butanol fraction was carried out for developing finger printing and also to verify presence of chrysin, baicalein, biochanin-A, and ellagic acid in the root bark of *Oroxylum indicum.*

2.6. Sample preparation

1.0 mg mL^{-1} of test solution of petroleum ether and hydrolyzed *n*-butanol fraction was prepared in methanol and 4.0 µg/ml of chrysin, ellagic acid and baicalein and 40.0 µg mL^{-1} of biochanin-A. Final solutions were prepared in methanol.

2.7. Calibration curve

It was obtained using different concentration in the range of 2.92 - 20.4 µg mL^{-1} (chrysin), 5.30 - 37.1 µg mL-1 (baicalein), 2.98 - 20.8 µg mL^{-1} (ellagic acid) and 1.12 - 3.92 µg mL^{-1} (biochanin-A).

2.8. Validation of HPLC method

The method was validated by determining precision, accuracy, linearity and calibration curve, limit of quantification, limit of detection, ruggedness, robustness and solution stability.

3. Results

On preliminary phytochemical screening, the root bark of *Oroxylum indicum* showed presence of alkaloids, flavonoids, tannins, and anthraquinones. The *TLC* study was aimed at

checking the presence of similar kind of compound, if any, in active fractions but probably in different form. Baicalein, chrysin, scutellarine, oroxylin-A were reported to be present in stem bark and leaves of *Oroxylum indicum.* Our observations on *TLC* support the presence of baicalein, chrysin, biochanin-A and ellagic acid in the root bark of *Oroxylum indicum* (Figure 1). Based on results of *TLC* study, we have suggested the presence of four bioactive phytoconstituents viz. chrysin, baicalein, biochanin-A, and ellagic acid in the root bark of *Oroxylum indicum.*

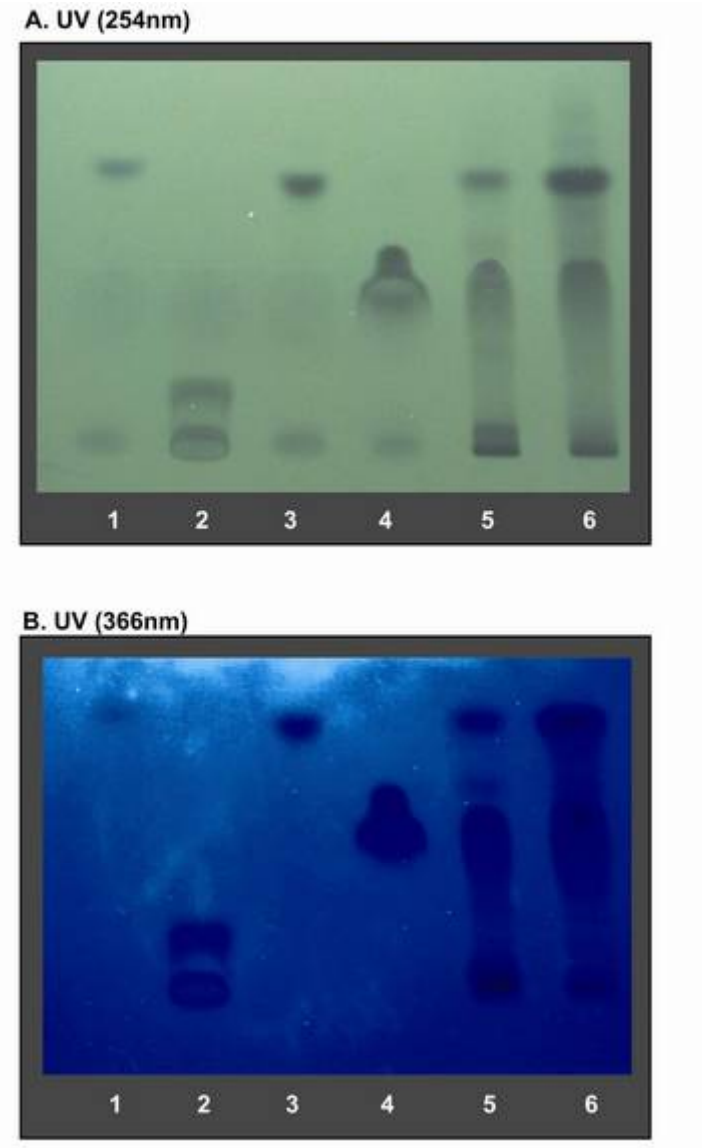

Fig.1: Co-chromatography of both the active fractions and standards (1: Biochanin A, 2: Ellagic acid, 3: Chrysin, 4: Baicalein, 5: Hydrolysed n butanol, 6: petroleum ether)

To evaluate the quality of root bark of *Oroxylum indicum*, a simple rapid and accurate RP-*HPLC* method was developed. Standard baicalein, chrysin biochanin-A and ellagic acid (Sigma-Aldrich) were employed for the development of the method. Phytochemical screening in

terms of quantification of different bioactive phytoconstituents in the root bark of *Oroxylum indicum* was performed using RP-*HPLC* fingerprint. Quantification of phytoconstituents was estimated by using RP-*HPLC* in both the active fractions viz. petroleum ether and hydrolyzed *n*-butanol fractions using standard chrysin, baicalein, biochanin-A and ellagic acid. These findings were supported by our earlier findings of *HPTLC* profile (data not presented here).

The RP-*HPLC* system used a base deactivated C18 column with water, methanol, acetonitrile and orthophosphoric acid as the mobile phase and detection was performed at 262 nm. The method was precise with relative standard deviation for these constituents that ranged between 0.5-1.0% (interday). The content of four phytoconstituents in the root bark of *Oroxylum indicum* was determined to establish the effectiveness of the method. *HPLC* method was validated by determining precision, accuracy, linearity and calibration curve, the limit of quantification, the limit of detection, ruggedness, robustness and solution stability.

Authentic standard baicalein, chrysin, biochanin-A and ellagic acid is being resolved at the retention time (R_t) 12.31, 24.42, 28.05 and 4.27 respectively and almost same R_t was observed with the use of petroleum ether and hydrolysed *n*-butanol fractions (Figure 2). A chromatogram was shown in Figure 2, which illustrates the separation of all the four phytoconstituents in this system. The detection wavelength was chosen at 262 nm because the chrysin, baicalein, biochanin-A and ellagic acid have better absorption and sensitivity at this wavelength. The quantification and validation of four phytoconstituents was done with help of calibration curve, which was prepared under same *HPLC* conditions (Figure 3). The accuracy of the method was determined by performing recovery studies. The follow- up extractions and *HPLC* analysis were accomplished in the same manner as detailed above. The recovery was determined as follows: Recovery (%) = (A-B)/C × 100% where, A is the amount of detections above, B is the amount of sample added without standards, C is the amount of the added standards. This was carried out at 50%, 100% and 150% level. The reproducibility of this method was satisfactory which ranged in 98.1%-101.3%. The relative standard deviations (R.S.D.) of recoveries of four components ranged between proving excellent accuracy and reproducibility of the method (Table 1).

For assay precision, replicates (n=5) of standard drug were analyzed to assess the interday variability in the assay. The precision data showing repeatability of the assay procedure is found satisfactory (Table 2 & 3). The co-efficient of variation of interday studies were both less than 5%. The system suitability parameters were calculated to confirm the specificity of the developed method and were showed in Table 2. The high percentage recovery and low percentage deviation was satisfactory and confirms the accuracy, precision and reliability of method.

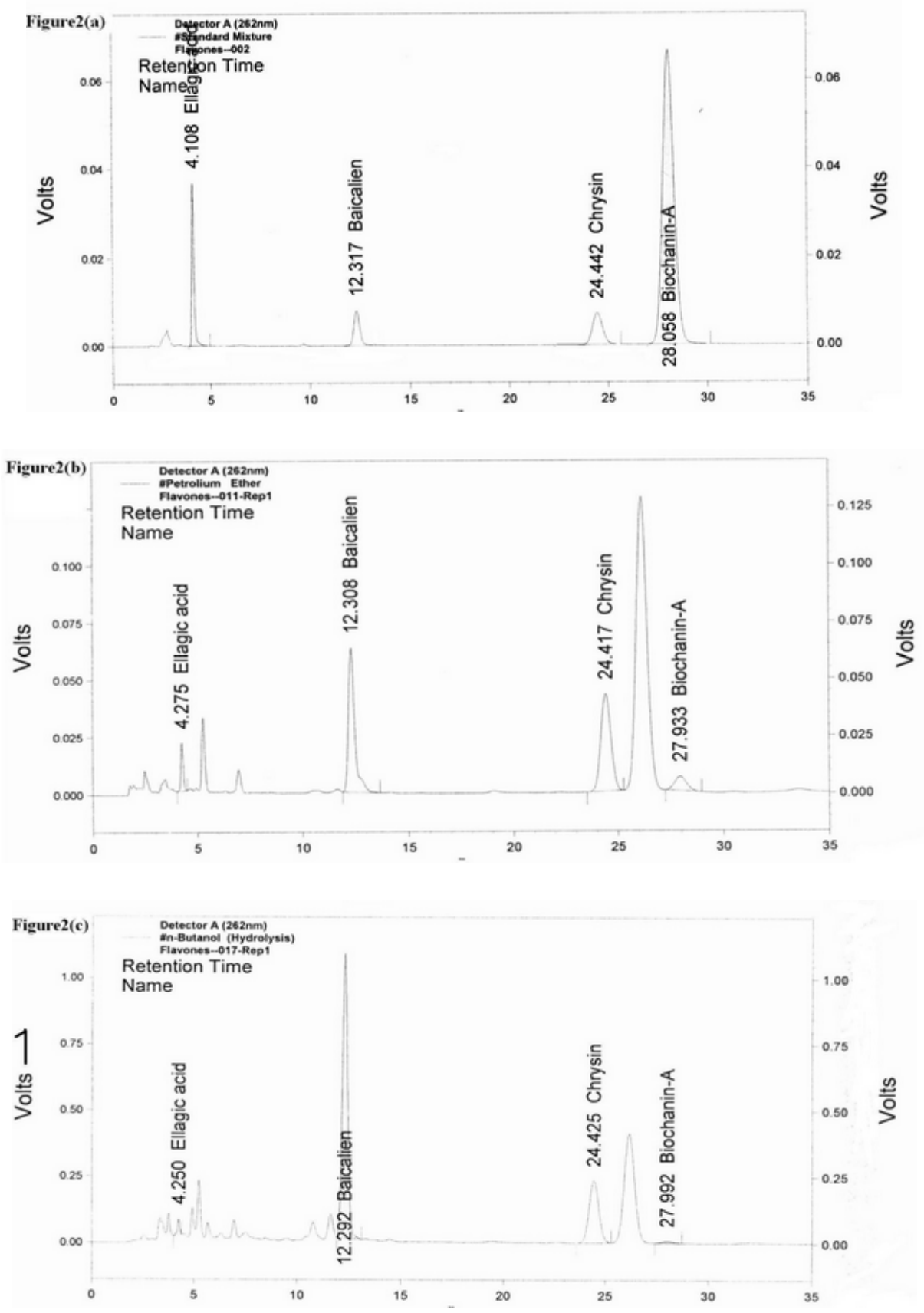

Fig.2: Chromatogram of (a) reference standard, (b) petroleum ether fraction, (c) hydrolyzed n-butanol fraction from the root bark of Oroxylum indicum

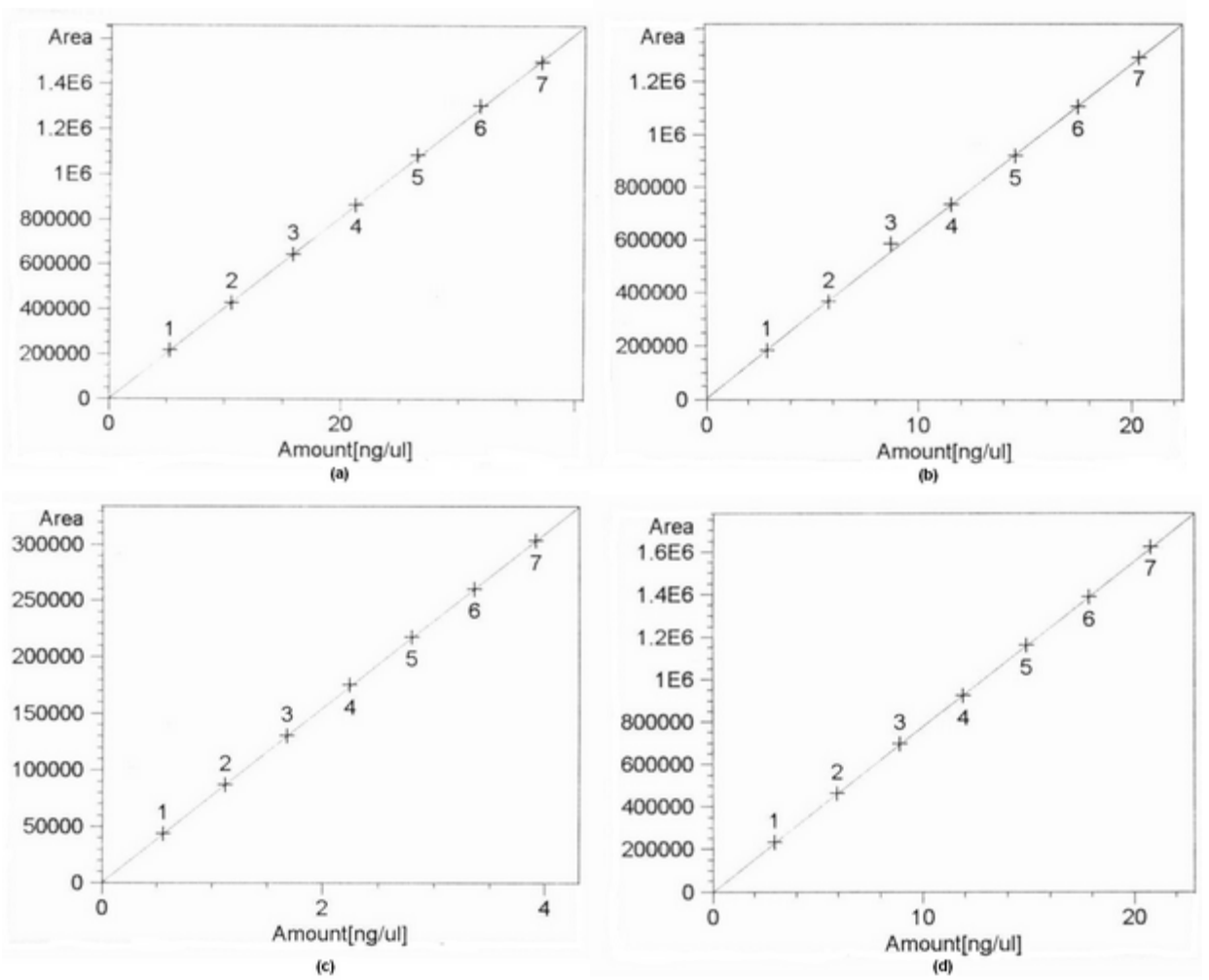

Fig.3: Calibration curve of standards (Linearity of detector response [(a) baicalein,, (b) chrysin, (c) biochanin-A and (d) ellagic acid]

Table 1: Recovery of baicalein, chrysin, biochanin-A and ellagic acid,
50% level

Standard	Amount added (mg)	Amount recovered (mg)	% Recovery (Mean) (n=5)	% RSD
Baicalein	1.4945	1.5002	100.3	0.7
Chrysin	0.6984	0.6957	99.6	0.5
Biochanin-A	0.1550	0.1521	98.1	1.0
Ellagic acid	0.0676	0.0682	100.8	1.4

100% level

Standard	Amount added (mg)	Amount recovered (mg)	% Recovery (Mean) (n=5)	% RSD
Baicalein	2.9890	3.0289	101.3	0.9
Chrysin	1.3968	1.3931	99.7	1.0
Biochanin-A	0.3100	0.3110	101.3	0.5
Ellagic acid	0.1392	0.1378	99.0	1.0

150% level

Standard	Amount added (mg)	Amount recovered (mg)	% Recovery (Mean) (n=5)	% RSD
Baicalein	5.978	6.012	100.5	0.8
Chrysin	2.7936	2.8141	100.7	1.0
Biochanin-A	0.6200	0.6189	99.8	1.1
Ellagic acid	0.274	0.271	98.9	1.3

Table 2: System suitability and system precision of baicalein, chrysin, biochanin-A and ellagic acid

Standard Compound	Concentration μg mL^{-1}	n	k'	R	Retention Time (min)	α	(Mean± SEM)
Baicalein	8.0	12.78 ± 0.003	9887.42	3.40	23.32	1.09	7.23
Chrysin	8.0	25.56 ±0.009	12031.24	7.76	17.54	1.05	16.51
Biochanin-A	26.0	29.34 ±0.007	12117.79	9.06	3.81	1.04	19.28
Ellagic acid	8.0	4.20 ±0.0000	6183.53	0.47	0.0	1.27	0.0

n: Theoretical plates
k': Capacity factor
R: Resolution
T: Asymetry factor
A: Selectivity factor

Table 3: Inter-day precision for determination of baicalein, chrysin, biochanin-A and ellagic acid

Standard Compound	Concentration (μg/ml)	Retention time (Min) (Mean± SEM)	% Assay of Drug (Mean± SEM)	% RSD of (n=5)
Baicalein	8.0	12.81 ± 0.007	2.60 ± 0.032	2.7
Chrysin	8.0	25.61 ± 0.015	1.46 ± 0.024	3.8
Biochanin-A	26.0	29.40 ± 0.017	0.20 ± 0.000	0.0
Ellagic acid	8.0	4.38 ± 0.003	0.15 ± 0.002	3.8

The proposed method was validated and system was calibrated for all the four phytoconstituents. The calibration graphs were constructed in the range of 2.92-20.4 µg/ml (chrysin), 5.30 - 37.1 µg mL^{-1} (baicalein), 2.98 - 20. µg mL^{-1} (ellagic acid) and 1.12 - 3.92 µg mL^{-1} (biochanin-A). These calibration ranges adequately covered the variations in amounts of phytoconstituents in the samples. The content of phytoconstituents in the petroleum ether and hydrolyzed *n*-butanol fraction of the root bark of Oroxylum indicum was calculated from the regression equation of the calibration curve (Y=mx+c, where m and c are slope and intercept respectively). The regression equations of the curves and their co-efficient of determination (R^2) are mentioned here; Chrysin: 63051.67294 x + 4268.30, 0.9997; baicalein: 40681.24663 x + 357.25, 0.9999; ellagic acid: 77825.25557 x – 1424.728, 0.9999; biochanin-A: 77426.10544 x + 238.833, 0.9999. Detection limit of these constituents were 0.656, 0.546, 3.13, 0.026 µg mL^{-1} for chrysin, baicalein, ellagic acid biochanin-A respectively. The limit of quantification was calculated experimentally i.e. 1.990, 1.650, 2.048, 0.079 for chrysin, baicalein, ellagic acid biochanin-A respectively. Ruggedness of the method was evaluated by carrying out the experiment on different instruments (Shimadzu), by different analysts and on different days. Ruggedness study signified the reproducibility of the method under different conditions (instruments, days and analysts). The robustness of method is the ability to remain unaffected by small changes in parameters such as flow rate, temperature and organic phase ratio. Experimental conditions were purposely altered and chromatographic characteristics were evaluated. To study the robustness, effect of flow rate was changed by (±10%), temperature (±5%), and organic phase ratio (±2%). The content of the constituents was not adversely affected by these changes as evident from the low value of relative standard deviation indicating that the method is robust. Standard and sample solution stability was evaluated at room temperature for 12 h. The deviation was found less than 2%. This indicated both standard and sample solution were stable up to 12 h at room temperature.

4. Discussion

Thin-layered chromatography is a technique in which, identification can be affected by observation of spots of identical R_f value and about equal magnitude obtained, respectively with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the same size and intensity of the spots usually serves for semi-quantitative estimation. In the present study, *TLC* observation showed spotting for four phytoconstituents namely biochanin-A, chrysin, baicalein, and ellagic acid. This study was performed in the petroleum ether and *n*-butanol fraction after hydrolysis of the respective glycosides from the root bark of *Oroxylum indicum*.

There is a strong need to adopt modern analytical method for quality control of plant material and herbal remedies. Application of fingerprinting technique using modern analytical techniques like *HPLC* can give high level of quality control of the plant. Chromatographic fingerprinting should be done with emphasis on identification and quantification of specific chemical marker compound representative of specific herb. But nevertheless in its, own limited sense the technique of chromatographic fingerprinting and specific marker compounds are very important tools available to modern analyst as an aid for the total quality control of a medicinal herb.

It should be done with emphasis on identification and quantification of specific chemical marker compound representative of specific herb.

The newly established RP-*HPLC* analysis was performed to develop complete chemo profile and described the quantification of chrysin, baicalein, ellagic acid biochanin-A in petroleum ether and *n*-butanol fraction after hydrolysis of the respective glycosides from the root bark of *Oroxylum indicum*. The advantage of this method lies in the simplicity of sample preparation and the low costs of reagents used. The proposed RP-*HPLC* conditions ensure sufficient resolution and the use of reference standard guarantees the precise quantification of the phytoconstituents. Results from statistical analysis of experiments are indicative of satisfactory precision and reproducibility. Therefore, this method was found accurate, precise, rapid, simple, and selective for quantitative monitoring of chrysin, baicalein, ellagic acid and biochanin-A in the root bark of *Oroxylum indicum*. Moreover, the hydrolysed *n*-butanol fraction showed 9.86 % of baicalein as compared to 3.24% in the petroleum ether fraction.

5. Conclusions

Preliminary phytochemical screening indicated that the root bark of *Oroxylum indicum* was rich in flavonoids. The *TLC* observations identified the presence of four phytoconstituents such as chrysin, baicalein, ellagic acid and biochanin-A in both petroleum ether and hydrolysed *n*-butanol fractions. The newly established RP-*HPLC* method described the quantification of chrysin, baicalein, ellagic acid biochanin-A in petroleum ether and n-butanol fraction after hydrolysis of the respective glycosides from the root bark of *Oroxylum indicum*. The advantages lie in the simplicity of sample preparation. The proposed RP-*HPLC* conditions ensure sufficient resolution and the use of reference standard guarantees the precise quantification of the phytoconstituents. Results from statistical analysis of experiments are indicative of satisfactory precision and reproducibility. Therefore, this method is accurate, precise, rapid, simple, and selective for quantitative monitoring of chrysin, baicalein, ellagic acid and biochanin-A in the root bark of *Oroxylum indicum.*

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