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IN-VITRO ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF *SALIX ALBA* L. ALONG WITH SIMULTANEOUS HPTLC ANALYSIS OF SALICIN AND FERULIC ACID

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Keywords:

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ABSTRACT: Now a day's interest towards natural and has been growing due to the unhealthy consequences of chemicals in the health industry; though, herbal substances possess several quality control and confirm pharmacological action issues. This present study was designed to determine the effects of *Salix alba* L. methanolic extract (MESAL) for its antioxidant and anti-inflammatory activity in rat models. Further to establish and validate a sensitive, fast and reproducible high performance thin layer chromatographic (HPTLC) method of two biomarker compounds Salicin and Ferulic acid from MESAL. The anti-inflammatory activity was studied by the carrageenan induced rat paw oedema method while DPPH free radical scavenging ability was utilized to determine the antioxidant activity. Additionally, the separation was performed by HPTLC with quantification of markers (Salicin and Ferulic acid). Among the different combinations of mobile phases used, best separation was achieved in Toluene: Ethyl acetate: Methanol: Formic Acid (5:3:1:1v/v/v/v). The MESAL exhibited antioxidant activity with a maximal inhibitory concentration (IC_{50}) value of 400 µg/ml and exerted anti-inflammatory activity, wherein 70% protection was shown at 400 mg/ml. Whereas the HPTLC method gave compact spots of Salicin and Ferulic acid at Rf 0.22 ± 0.02 and 0.68 ± 0.02, respectively. The MESAL displayed potent antioxidant and anti-inflammatory properties. Statistical analysis proves that the HPTLC method is repeatable and selective for the estimation of the said drugs, thus can be used for routine analysis and quality control of raw material of *Salix alba* L.

INTRODUCTION: Noteworthy work is essential to evaluate herbal drugs for their quality, safety and efficacy; there is required of a well-defined particular strategy for routine analysis of herbal raw materials and formulations with regard to constituents responsible for its efficacy [1, 2].

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S. alba L., universally recognized as White Willow (particularly, the bark) is the original source of salicin, a weaker precursor of aspirin [3].

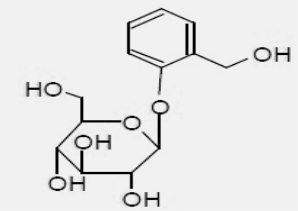
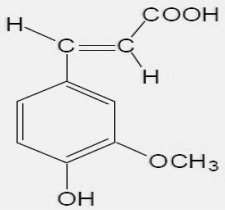
The chemical component like glycosides (1.5-11%) predominantly salicylates (salicin, salicortin, populin, fragilin, tremulacin); tannins (8-20%); aromatic aldehydes and acids distinctively salidroside, vanillin, syringin, salicylic acid, caffeic and ferulic acids; Salicyl alcohol (saligenin); Flavonoids have been isolated and identified from the plant [4-6]. *Salix alba* L. has been used as antioxidant, antiacetylcholinesterase, antimigraine, mouth wash agent, antiestrogenic and antigenotoxic

activity [7-8]. Ferulic acid, a phenolic acid has wide conveyance plant kingdom and is more bio-available than other dietary flavonoid and monophenolics studied **Table 1**. It has been detailed to be a powerful antioxidant, anti-inflammatory and is accounted to terminate free radical chain reaction and decrease the chance for coronary heart diseases [3, 9-14]. Salicin is the metabolic antecedent of salicylic acid; a ponder compound. Chemically talking is closely related to aspirin and includes an exceptionally comparative activity within the human body. Salicin is an alcoholic beta-glycoside that contains D-glucose **Table 1.** Having antipyretic and analgesic effects, salicin can be utilized for the treatment of fever and diseases, like arthritis [15].

Tragically, deficiently data is accessible concerning the dispersion of Salicin and Ferulic acid in the *S. alba* L. There is no synchronous strategy that has been detailed for quantitation of Salicin and Ferulic acid from the *S. alba* L as well as a pharmacological activity like antioxidant and anti-inflammatory of methanolic extract of *S. alba* L.

In the present study, an endeavor has been made to create a simple, rapid, and accurate HPTLC method for estimation of the two biomarker compounds Salicin and Ferulic acid from methanolic extract of *S. alba* L, and the extracts were further evaluated to investigate the *in-vitro* antioxidant and anti-inflammatory activity of methanolic extract of *S. alba* L.

TABLE 1: CHEMICAL PROPERTIES OF SALICIN AND FERULIC ACID

Particular	Details	
	Salicin	**Ferulic acid**
CAS number	138-52-3	1135-24-6
Chemical Formula	$C_{13}H_{18}O_7$	$C_{10}H_{10}O_4$
Chemical Name	2-(Hydroxymethyl)phenyl-β-D-glucopyranoside	(E)-3-(4-Hydroxy-3-methoxyphenyl)prop-2-enoic acid
Representation		
Molecular Weight	286.27	194.18
Description	NSAID. Salicyclic acid prodrug	Hydroxycinnamic acid; antioxidant properties.

MATERIALS AND METHOD:

Chemicals and Reagents: Standard Salicin and Ferulic acid was purchased from Natural Remedies India and used without further purification, owing to its high purity, at least 99% w/w. 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) were purchased from Hi-Media Lab. Pvt. Ltd., Mumbai, India. HPLC water (Millipore equipment, France) was used to prepare the stock solution. Analytical-reagent grade solvents like tween 80, ascorbic acid, Diclophenac Na, carrageenan, toluene, ethyl acetate, formic acid, and methanol were obtained from Merck Ltd (India). HPTLC aluminium plates precoated with silica gel 60F254 (20 × 20 cm, 0.2 mm thickness) were obtained from Merck, India.

Plant Material: The dried methanolic extract of *S. alba* L. was purchased from Phyto concentrate, India. Their identity and authentication were confirmed by the Department of Pharmacognosy Dr. D. Y. Patil institute of Pharmaceutical research and sciences, Pimpri, India by correlating their morphological and microscopical characters with those given in literatures.

Experimental Animals: Male Wister rats weighing 150-180 g were gotten from the National Institute of Biosciences, Pune, and utilized for this examination. The animals were separated into bunches and kept in plastic cages (47 × 34 × 18 cm) beneath 12 h light/12 h dark cycle at room temperature (22 °C) with standard diet and water were given *ad libitum*.

The animals were allowed to acclimatize to laboratory conditions earlier to experimentation. All experiments were conducted during the light period of 12 h of the day/night cycle. All the experiments were allowed and conducted as per the rules of the Institutional Animal Ethical Committee.

Acute Toxicity Study: Acute oral toxicity study was carried out in accordance with the guidelines of the OECD-423 (acute toxic class method). Eighteen rats (six for each group) were used for the research of acute toxicity. Overnight the animals were kept fasting, offering only water. Different extract doses (0.5, 1, and 2 g / kg) were suspended and administered orally in 0.5 percent aqueous Tween 80. For indications of toxicity *e.g.* autonomy, central nervous system and behavioral modifications, the animals were continually noted for 12 h and death was observed for 24 h [16-17].

DPPH Radical Scavenging: MESAL's antioxidant activity was assessed using photometric assay of DPPH. The test extract (2 ml) at different concentrations (100, 200, and 400 µg/ml) was mixed with 0.5 mM DPPH (in 1 ml of methanol) in a cuvette. The absorbance at 517 nm was taken after 30 min of incubation in the dark at room temperature. Ascorbic acid was utilized as a positive control. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using Equation [18-19].

$$\% \text{ RSA} = [(A_0 - A_S) / A_0] \times 100$$

Where A_0 and A_S are the absorbance of the control (containing all reagents, except the test compound) and test compound respectively.

Anti-inflammatory Activity: Acute inflammation was induced by carrageenan injection into the hind paws of rats. Rats were divided into five groups (6 rats/group). The control group received 0.5% Tween 80 (1 ml/kg, p.o.) served as the vehicle only, while other groups received (100, 200 & 400 mg/kg, p.o.) and standard drug diclophenac Na (75 mg/ kg, p.o.) respectively.

To develop the paw edema, 0.1 ml of 1% carrageen and was injected into the subplantar surface of the right hind paw of each rat 1 h after the administration of MESAL. The paw volume was measured initially at 1, 2, 3, 4, and 24 h after carrageenan injection using a plethysmometer [20-21]. The percent inhibition of edema volume was calculated using the formula as follows: %

Inhibition = [(Predrug reading - Postdrug reading) × 100]
Predrug reading

Statistical Analysis: Data is expressed as Mean ± SEM and was analyzed for the significance of variance by one-way ANOVA followed by Tukey multiple comparison tests using PRISM software.

Quantification of Salicin and Ferulic acid using HPTLC:

Preparation of Standard Solution of Salicin and Ferulic Acid: Standard solution was prepared by dissolving Salicin and Ferulic acid in 50 mL methanol (stock solution). 1 ml of stock solution was diluted to 10 mL and was used as a working standard for the analysis.

Preparation of Test Solution for Analysis: Methanolic extract of *S. alba* L was accurately weighed in 50 mL volumetric flask and added to 25 mL Methanol. It was allowed to sonicate for 15 minutes. Then make up the volume with methanol.

HPTLC-Photodensitometry: The samples were applied in the form of a band of width 6 mm with CAMAG 100 µL syringe on precoated silica gel $60F_{254}$ aluminium plate (20 cm × 10 cm with 0.2 mm thickness) using Linomat 5 applicator CAMAG (Switzerland) fitted with a CAMAG 100 µL syringe. The ascending development was carried out in the mobile phase Toluene: Ethyl acetate: Methanol: Formic Acid (5:3:1:1v/v/v/v) in a CAMAG twin trough chamber (20 x10 cm) previously saturated with mobile phase for 15 minutes. The volume applied on each track was 10 µL. The plate was allowed to run approximately 80 mm from the point of application. After development, plates were dried by dryer. The densitometric scanning was performed using CAMAG TLC scanner-3 operated by win CATS software V at 254 nm for Salicin and Ferulic acid. The slit dimension was 5×0.45 mm with the scanning speed of 20 mm s^{-1}. Evaluation was done *via* peak area with linear regression.

Calibration Graph of Standard Salicin and Ferulic acid: The stock solution of Salicin and Ferulic acid was diluted to different concentrations between of working concentration. These were applied in duplicate on the HPTLC plate for the preparation of the calibration graph. The calibration graph was plotted by using the concentrations versus the average peak area at 254 nm. The

linearity of the detector response for the standards was determined by means of linear regression.

Method Validations: Validation of the analytical method was done according to the International Conference on Harmonization guideline. The method was validated for specificity, linearity, recovery, robustness, and precision [22-27].

Specificity: Specificity was ascertained by analyzing blank, standard, and samples. The bands for Salicin and Ferulic acid from sample solutions were established by comparing the R_f and spectra of the bands to those of the standards. The peak purity of all the compounds was analyzed by comparing the spectra at three different levels, *i.e*., start, middle, and end positions of the peak.

Precision: Precision of the method was studied by performing System, Method, and intermediate precision studies. The sample application and measurement of peak area were determined by performing seven replicate measurements of the same band using a sample solution containing Salicin and Ferulic acid.

Solution Stability: The sample solution and standard solution were prepared as per the proposed method and subjected to stability study at room temperature for 24 h. The sample solution was analyzed at initial and at 6 different time intervals up to 24 h. The change in response of Salicin and Ferulic acid in sample solution with respect to time is calculated as absolute percent difference against initial response.

Robustness: Composition of the mobile phase, the volume of the mobile phase, time from spotting to development, and time from development to scanning was involved in this study. The composition and volume of the mobile phase were varied in the range of ± 10% of the used optimized conditions. Time variations were varied from the optimized times in the range of ± 20%. The effect of these changes on the R_f value was evaluated by calculating the relative standard deviations (RSD) for each parameter.

Accuracy (Recovery): The accuracy of the method was ascertained by spiking the pre analysed samples with a known amount of Salicin and Ferulic acid (80, 100, and 120%).

The average percentage recovery was estimated by applying values of peak area to the regression equations of the calibration graph.

RESULTS AND DISCUSSION:

DPPH Radical Scavenging Assay: DPPH is a stable free radical in aqueous or methanol and ethanol solution and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It is used as a substrate to evaluate the antioxidative activity of antioxidants. The reduction capability of DPPH radicals was determined by a decrease in its absorbance at 517 nm.

The decrease in absorbance of DPPH radical was caused by an antioxidant, and was due to the scavenging of hydrogen donation. In another study, antioxidant properties of hot ethanolic extract of *S. alba* L. bark which was assessed by 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) free radical scavenging. The extract showed significant antioxidant activity.

Table 2 shows the dose-response of DPPH radical scavenging activity of the MESAL of 100, 200, and 400 µg/ml, compared with Ascorbic acid. It was observed that the MESAL of 400 µg/ml had higher activity than that of the other extractives. At a concentration of 100 and 200 µg/ml of Mesal, the scavenging activity reached 239.56 and 170.96, respectively. While at 400 µg/mL of MESAL, the scavenging activity reached 120.47, that of the ascorbic acid was 80.92%. From the above scavenging activity, it was found that 400 µg/mL of MESAL having higher antioxidant activity as compared to ascorbic acid. The antioxidant activity of 400 µg/mL of MESAL may be due to presence of ferulic acid in their extract [16-18].

TABLE 2: IC_{50} FOR DPPH SCAVENGING OF MESAL

Sample Name	Concentration (µg/ml)	DPPH Scavenging Activity
Mesal	100	239.56 ± 5.1
	200	170.96 ± 4.5
	400	120.47 ± 2.5
Ascorbic acid	100	80.92 ± 3.1

Acute toxicity studies of Mesal: The acute toxicity results showed that MESAL was safe up to a dose of 2000 mg/kg body weight. Based on acute toxicity data, two different dosages 100, 200, and 400 mg/kg (p.o.) were selected for in vivo antiinflammatory studies [16-17].

Anti-inflammatory Activity: Carrageenan induced inflammation is useful in detecting orally active anti-inflammatory agents; therefore, it has significant predictive value for anti-inflammatory agents acting through mediators of acute inflammation. The development of edema induced by carrageenan injection causes an acute and local inflammatory response. In past studies, anti-inflammatory effects by plethysmometric measurement of formalin-induced paw edema on methanolic extracts of *S. alba* L. The result indicates that the extracts inhibited the paw edema size and shows inhibition of the inflammation. As seen in table 2, Mesal at 100, 200, and 400 µg/ml significantly decreased carrageenan-induced rat paw edema. The anti-inflammatory effects of 100 and 200 µg/ml dose of Mesal determined inflammation as 0.923 and 0.817 respectively at 24 h. For the same hour, a 400 µg/ml dose of Mesal produced inflammation of 0.783. In comparison, the anti-inflammatory effect of Diclofenac at 75 mg/kg was 0.767 at the same time. These results suggest that the Mesal at 100, 200, and 400 µg/ml exhibits the anti-inflammatory property in the acute phase of inflammation, but the anti-inflammatory activity is more significant in dose 400 µg/ml, and mechanism of action may be associated with inhibition of the some of the inflammatory mediators like histamine, serotonin, bradykinins, and prostaglandins [17-21].

TABLE 3: EFFECT OF MESAL ON CARRAGEENAN INDUCED RAT PAW EDEMA

Group	Average Inflammation (mm) ± SEM					
	0 hour	1 hour	2 hour	3 hour	4 hour	24 hour
Control	0.760±0.029	0.913±0.046	0.920±0.017	0.928±0.024	0.913± 0.007	0.908± 0.044
Mesal 100 mg/kg	0.761±0.049	0.943±0.043	0.967± 0.021	0.981±0.013	0.951±0.015	0.923±0.027
Mesal 200 mg/kg	0.756±0.010	0.872±0.030	0.890±0.019	0.871±0.008	0.849±0.023	0.817±0.036
Mesal 400 mg/kg	0.751±0.033	0.863±0.045	0.870±0.012	0.852± 0.031	0.815±0.019	0.783±0.023
Diclophenac 75 mg/kg	0.752± 0.019	0.871± 0.038	0.804± 0.041	0.792± 0.037	0.785±0.031	0.767±0.026

The values are presented in the form of means ± standard error. One-way ANOVA followed by Tukey multiple comparison tests using PRISM software.

Method Optimization for the HPTLC-Densitometric Measurements: The reported methods of Salicin and Ferulic acid estimation like HPLC requires derivatization or working at lower wavelength for sample detection due to lack of UV absorbing chromophore. HPTLC offers several advantages over reported methods [27]. It facilitates automatic application and scanning in situ. The composition of the mobile phase for development of chromatographic method was optimized by testing different solvent mixtures of varying polarity [26-27]. The solvent system consisting of Toluene: Ethyl acetate: Methanol: Formic Acid (5:3:1:1v/v/v/v) given dense, compact and well separated bands of the drug. This mobile phase showed good resolution of Salicin and Ferulic acid peak from the different extract of *Salix alba*. Densitometric scanning of all the extracts showed compounds with R_f value 0.22 ± 0.02 and 0.68 ± 0.02, identified as Salicin and Ferulic acid. The present method is quicker as the time needed for plate development is reduced considerably to less than half an hour for chamber saturation and development of plate compared to the previously reported method. The scanning wavelength selected was 254 nm for Salicin and Ferulic acid. At this wavelength, the Salicin and Ferulic acid showed optimum response **Fig. 1-2.**

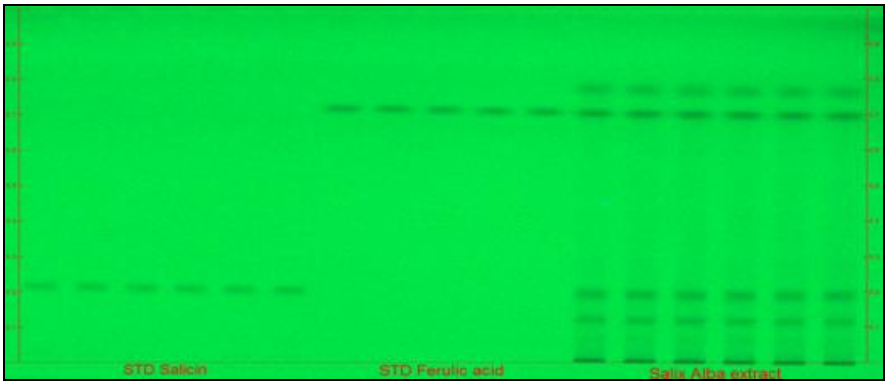


FIG. 1: HPTLC PROFILE OF *SALIX ALBA* L. BEFORE DERIVATIZATION UNDER UV 254 nm

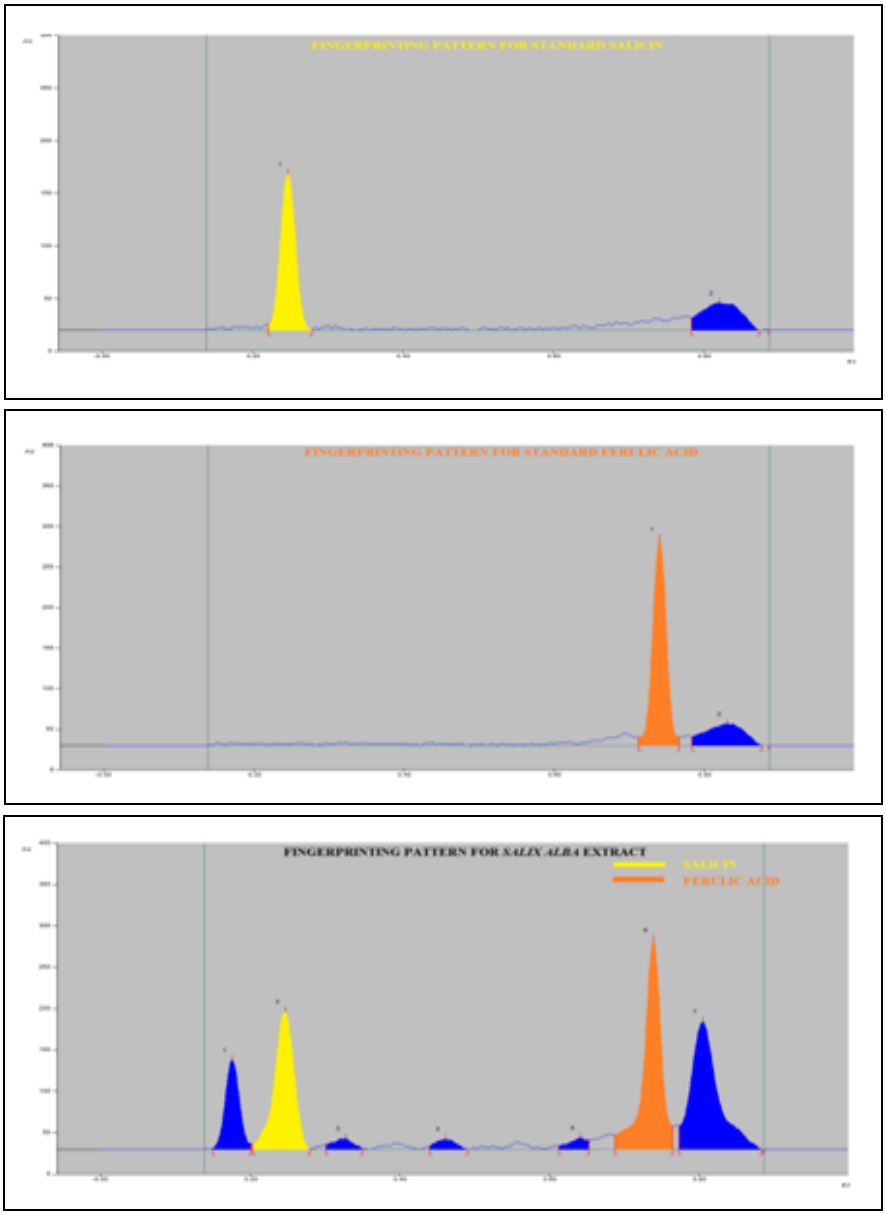


FIG. 2: COMPARATIVE CHROMATOGRAM OF STANDARD SALICIN AND FERULIC ACID WITH *SALIX ALBA* EXTRACT

Method Validation:

Linearity: Peak areas were found to have a good linear relationship with the concentration than the peak heights **Fig. 3**. For Salicin and Ferulic acid, the r^2 was found 0.9984 and 0.9991. The correlation coefficients, y-intercepts, and slopes of the regression lines of the compound were calculated and presented in **Table 4**.

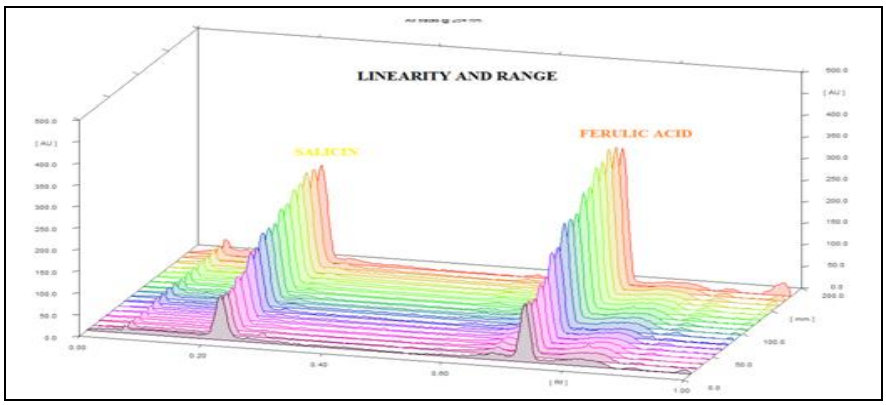


FIG. 3: 3-D CHROMATOGRAM OF LINEARITY OF SALICIN AND FERULIC ACID

TABLE 4: SUMMARY OF LINEAR REGRESSION AND VALIDATION DATA

Parameters	Salicin	Ferulic acid
Linearity range	5-50 ng/spot	10-100 ng/spot
Linear regression equation	y = 0.19694x + 0.25244	y =0.13162x + 0.16696
Slope± SD	0.19694	0.13162
Intercept±SD	0.25244	0.16696
Correlation coefficient ®	0.9994	0.9993
Determination coefficient (r2)	0.9984	0.9991

Specificity: The peak purity tests of Salicin and Ferulic acid spots were assessed by comparing their respective spectra at peak start, peak apex, and peak end positions of the spot[16]. The results of spectral comparison for Salicin and Ferulic acid were found to be specific at peak start–peak apex and at peak apex–peak end, respectively.

The closeness of peak purity values to 1 indicates that the spots were only attributed to a single compound. A good correlation (r= 0.992) was also obtained between standard and sample spectra of Salicin and Ferulic acid. The UV spectra comparison of the spots of the standards and all extracts were presented in **Fig. 4**.

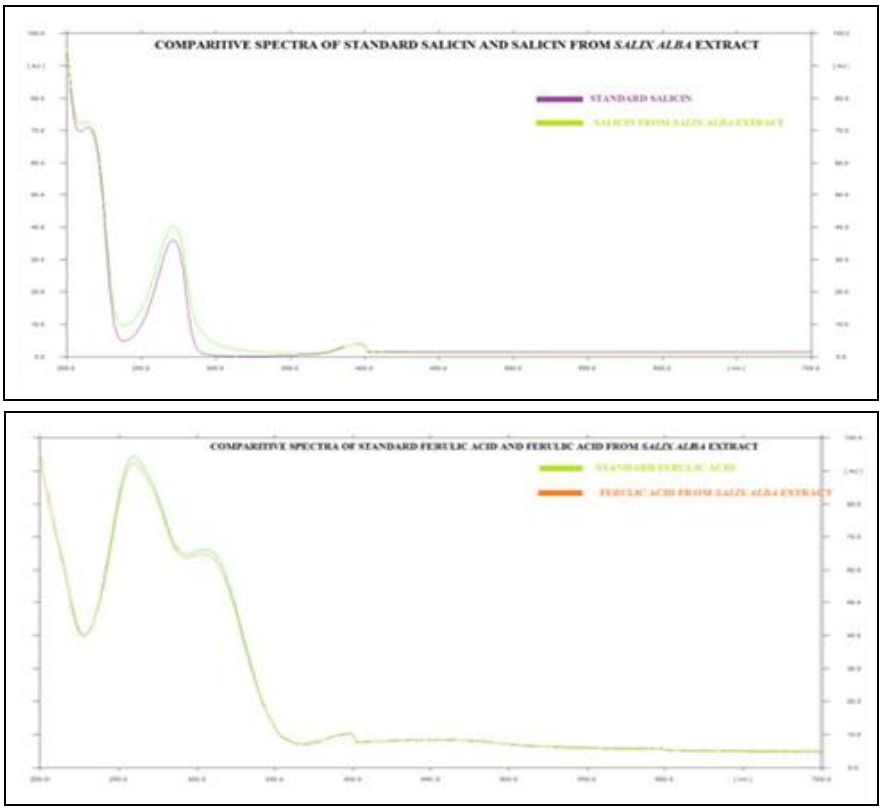


FIG. 4: COMPARATIVE SPECTRA OF SALICIN AND FERULIC ACID

Precision: System, Method, and Intermediate precision of the developed method were expressed in terms of relative standard deviation (RSD) of the peak area. The results showed that the System, Method, and intermediate variation of the results for Salicin and Ferulic acid were within the acceptable range.

The coefficients of variation for System, Method, and Intermediate precision of the method were found to be less than 2.0%. The Salicin and Ferulic acid were also analyzed by two different analysts within the same day, and the results revealed that there is good intermediate precision between analysts **Table 5.**

TABLE 5: METHOD VALIDATION PARAMETERS FOR QUANTITATION OF SALICIN AND FERULIC ACID

S. no.	Parameters	Salicin	Ferulic Acid
1	Specificity	Specific	Specific
2	System precision.(% RSD)	0.98	1.12
3	Method Precision.(% RSD)	1.25	1.37
4	Intermediate precision.(% RSD)	1.57	1.02

Solution Stability: The sample solution was prepared and was kept at room temperature (20 ± 2 °C and 30 ± 2 °C) on a shelf protected from direct light. The solution was analyzed after 1 h, 3 h, 6 h, 12 h, 18 h, and 24 h. Because of the time needed for sonication and filtration, the fastest possible analysis was carried out within 20 min and hence results of the remaining analysis times were compared with it. The average peak area values are presented in **Table 6**. The average peak areas of Salicin and Ferulic acid does not varied significantly from the reference time after 1 day of sample preparation.

TABLE 6: SOLUTION STABILITY STUDY

S. no.	Time of analysis (h)	Peak Area (AU)	
		Salicin	Ferulic Acid
1	0	10104	23087
2	3	10202	22596
3	6	10090	22869
4	12	10078	22731
5	18	10143	22921
6	24	10105	22843

Robustness: The standard deviations of peak areas were calculated for the aforementioned four parameters (variation in the composition of the mobile phase, the volume of the mobile phase, time from spotting to development, and time from development to scanning), and coefficients of variation were found to be less than 2.0% in all cases as shown in **Table 7**. The low RSD values indicate the robustness of the method.

TABLE 7: ROBUSTNESS STUDY FOR THE DEVELOPED METHOD

S. no.	Parameter Studied	Salicin % RSD	Ferulic Acid % RSD
1	Composition of mobile phase	1.37	0.99
2	Volume of mobile phase	1.11	1.38
3	Time from spotting to development (5–60 min)	1.08	0.87
4	Time from development to scanning (5–60 min)	1.30	1.21

Accuracy (Recovery): The recovery studies were carried out at 80%, 100%, and 120% of the test concentration as per ICH guidelines. The percentage recovery of Salicin and Ferulic acid at all three levels were found to be satisfactory **Table 8**. For Salicin and Ferulic acid, the % recovery was found between 98.4 - 100.28%.

TABLE 8: RECOVERY STUDY OF THE METHOD FOR SALICIN AND FERULIC ACID

S. no.	Recovery Level (%)	Salicin	Ferulic Acid
1	80	98.4	99.28
2	100	100.28	101.36
3	120	99.13	100.23
	Average	99.27	100.29

CONCLUSION: The current studies indicate that MESAL exerts a potential antioxidant activity at higher dose comparable to Ascorbic acid. Mesal in a dose-dependent manner exhibited anti-inflammatory activity in the rat paw edema model.

The developed HPTLC/densitometry method was found to be simple, rapid, selective, quite sensitive, and suitable for simultaneous determination of Salicin and Ferulic acid in three different extracts. The method can minimize the cost of reagents and time for analysis. It also utilized the merit of applying several sample spots on HPTLC plates, which may be more advantageous for regulatory quality control laboratories especially to facilitate the post-marketing surveillance program.

In addition, the method is inexpensive and not requires certain types of stationary phases. Thus, it can represent another good alternative for the already existing HPLC methods, especially those using certain types of detectors which are not present in most of the laboratories.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest pertaining to this manuscript.

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