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Investigation of Flavonoids in *Stereospermum suaveolens* DC Leaves (Patala) Using HPTLC Analysis for Inflammatory Pain, Swelling, and Edema Treatment

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Abstract

Background: As per extensive research study, it was observed that *Stereospermum suaveolens* DC (Patala) is rich sources of flavonoids. These phytoconstituents have played an important role in prevention and maintenance of acute and chronic diseases of pain and inflammation. There is need to explore method for identification and quantification of the presence of flavonoids in *S. suaveolens* DC leaves (SSL) by qualitatively and quantitatively and to establish its mechanism in pain, swelling and edema **Aim**: The aim of the study was to identify and assess role of flavonoids in SSL by high-performance thin-layer chromatography (HPTLC) analysis and animal screening models of inflammatory pain, swelling, and edema. **Materials and Methods**: HPTLC to quantify flavonoid in the ethyl acetate fraction of plant component in comparison with Quarcetin. The inflammatory pain, swelling, and edema method were investigated by acetic acid induced writhing method in mice, histamine-induced edema in Wistar rats, and croton oil-induced ear edema in mice. The test doses 125 mg/kg, 250 mg/kg, and 500 mg/kg oral administration (p.o.) of ethyl acetate fraction of SSL were selected by oral acute toxicity OECD 423. **Results**: HPTLC analytical method estimated presence of flavonoids and estimated amount was 22.64%. However, inhibition in histamine-induced paw edema and reduction inhibition in ear edema significantly ($P < 0.05$) observed by dose-dependent effect and antinociceptive activity detected on acetic acid induced writhing response method in mild to moderately using one-way analysis of variance method. **Conclusion**: It was clear that flavonoids were responsible for direct and indirect release of intermediate inflammatory mediators and promote its role in the treatment of pain and swelling.

Keywords: Edema, Flavonoids, High-performance thin-layer chromatography, *Stereospermum suaveolens* DC

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Introduction

Pain and swelling are two important features of inflammation. The chemical agents such as histamine bradykinin, serotonin, cytokine proteins, and prostaglandins are active key players in inflammatory milieu. The persistent acquaintances of released inflammatory components can roll acute inflammation into injurious health condition leading death of cell. Various steps such as vasodilatation, accumulation of protein rich exudates, and migration of white blood cells are involved in inflammation cascade.[1] The pain producing substance like kinins in inflammatory response is sensitized by prostaglandins. First preferred choice to diminish inflammation and pain reaction is considered to use cyclo-oxygenase (COX)-1 or COX-2 enzyme inhibitors in clinical practice.[2] Nonsteroidal anti-inflammatory class of drugs targets COX-1 and COX-2 enzyme mediated inhibition of prostaglandins synthesis. Prolonged exposure of non-steroidal anti-inflammatory drugs can induce primarily gastrointestinal complications, risk of cardiovascular diseases, and nephrotoxicity. The phytochemical such as flavonoids shows various health promoting effects in acute and chronic inflammatory diseases. These phytoactive compounds decrease release of oxidant and inflammation mediators by enhancing pain relief and tissue healing process. *Stereospermum suaveolens* DC is commonly known as Patala. It is medicinal tree located at various greater parts of India mainly seen in forest and hill side region.[3] Conventionally, various parts of *S. suaveolens DC* (patala) are used for the treatment of pain, inflammation, fever, cancer, virus infection, diabetes mellitus, edema, vomiting, hiccough, and piles.[3-5] *S. suaveolens* DC plant reported for lapachol, p-coumaric acid, Sitosterol, anthraquinone, sterekunthal B, stereochenols A, scutellarein, stereolensin, n-triacontanol, flavones glycosides-6-o-glucosylcutellarein, and dinatin-7-glucuronoids phytoconstituents.[6] Stem and bark of *S. suaveolens* DC (Patala) were reported for prevention free oxygen radicals in stress-induced gastric ulcer and hyperglycemia induced rat model. The stem and bark of this plant were also evaluated for analgesic, antipyretic, and neuroprotective in Parkinson's disease induced model. The root and bark species reported for anti-proliferative, anti-arthritic activity, anti-obesity, antihyperlipidemic activity, and antimicrobial activity.[7-10] The roots of *S. suaveolens* DC are important constituent of Dashmoola preparation a potent herbal mixture studied for treatment of pain and inflammatory conditions. The leaves of Patala reported for the treatment of inflammation, arthritis, and human filarial parasite. The medicinal property of the plant can be explored to estimate phytoconstituents qualitatively and quantitatively. Use of advance analytical technique for justification of scientific validity natural phytocomponents is necessary. High-performance thin-layer chromatography (HPTLC)

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is simple, fast, and reliable instrumental analytical technique. This chromatographic method is applied to ensure purity and efficacy of medicinal product in pharmaceutical industry. It is also helpful to understand plant metabolite profile with rapid analysis of large number of phytoconstituents.[11,12] Therefore, based on traditional claim and reported research articles leaves of *S. suaveolens* DC investigated to find out occurrence of flavonoids by means of HPTLC study and to explore its mechanism of action in the treatment of inflammatory pain and swelling process.

Materials and Methods

Drugs and Chemicals for Experimentation

Histamine and Quercetin were purchased from Dolphin Pharmacy instruments Pvt., Ltd., Mumbai and New Neeta Chemicals, Pune, Maharashtra. All solvents and chemicals of analytical grade standard were used for experimentation.

Instrumentation for HPTLC

Microsyringe (Linomat syringe, CAMAG, Switzerland), linomat-5 applicator, twin trough chamber, WinCATS software (CAMAG, Switzerland), HPTLC plates silica gel 60 F 254 (EMarck KGaA), and TLC scanner (CAMAG) following are the applied parameters for Linomat 5 applicator as spray gas: Inert gas; sample solvent type: Methanol; dosage speed: 150 nl/s; pre dosage volume: 0.2 µl; syringe size: 100 µl; and band length: 6.0 mm.

Plant Material Collection

S. suaveolens DC leaves (SSL) collected from periphery of Junner, District: Pune, Maharashtra. The plant specimen was authenticated by Botanical Survey of India, Pune-01. The herbarium specimen no. BSI/WRC/100-2/Tech/2018/11 was deposited.

Extraction of Leaves

The freshly collected leaves of SSL DC plant were cleaned, washed with water, air dried in shade, and coarsely powdered in the grinder. It is stored for experimentation work. Leaves powder of 150–175 g were extracted with petroleum ether, chloroform and methanol solvent by using Soxhlet apparatus. The crude residue of methanolic extract was obtained with the help of Rotary Vacuum Evaporator (Dolphin-RVE/MCPL/2012). This crude extract was organized for fraction with ethyl acetate solvent and distilled water in 1:1 ratio. Then, ethyl acetate fraction and aqueous fraction separated out. Here ethyl acetate fraction of crude methanolic extract was selected for study.[11,13]

Phytochemical examination

The phytochemical test of ethyl acetate fraction of (SSL) component was performed to identify flavonoids, alkaloids, glycosides, phenolic, and saponins.[14,15]

Study of thin-layer chromatography (TLC)

As per described by Wagner and Baldt, 1996 TLC a type of liquid chromatography study was performed. Here, the solvents toluene: ethyl acetate: formic acid (7:3:0.1) were used as mobile phase for separation of sample mixture. The fluorescent flavonoids visualized under UV chamber and their Rf values were compared with that of standard flavonoids.[16,17]

HPTLC analysis[12,13]

Instrumentation

Microsyringe (Linomat syringe, CAMAG, Switzerland), linomat-5 applicator, twin trough chamber, WinCATS software (CAMAG, Switzerland), HPTLC plates silica gel 60 F 254 (EMarck KGaA), and TLC scanner (CAMAG) following are the applied parameters for Linomat 5 applicator as spray gas: inert gas; sample solvent type: Methanol; dosage speed: 150 nl/s; pre dosage volume: 0.2 µl; syringe size: 100 µl; and band length: 6.0 mm.

Procedure

Standard markers (Quercetin, Luteolin, and Gallic acid), ethyl acetate fractions of *S. suaveolens* DC leaves and aqueous fraction of SSL were applied as 6.0 mm band length by Linomat 5 applicator on 10 cm × 10 cm pre coated HPTLC plates silica gel 60 F 254. The chromatogram was developed for 8 cm using (toluene: ethyl acetate: formic acid) mobile phase. Saturation time for mobile phase was 15 min. Injection volumes for sample application were 10 µL/band and visualization was done at 254 nm.

Animals for research activity

Wistar rats of either sex weighing 150–175 g and female Swiss albino mice of 25–30 g acquired from National Institute of Bioscience Pune-16, Maharashtra for activity. These animals were maintained under well-conditioned animal house at an ambient temperature 25 ± 1°C and light-dark (12 h: 12 h) cycle. The approved protocol number was MCP/IAEC/12/2017 by Institutional Animal Ethics Committee (IAEC) in accordance with Committee for Purpose of Control and Supervision of Experimental Animals.

Oral acute toxicity method

Swiss Albino Mice (25–30 g), ethyl acetate fraction of leaves (SSL) extract oral feeding needle and 1 ml tuberculin syringe used for oral toxicity experiment. The procedure was followed as per OECD guidelines no.423. The toxicity parameters were assessed for 14 days. Selections of test doses of ethyl acetate fraction of SSL extract were 125 mg/kg, 250 mg/kg, and 500 mg/kg finalized for experimentation.[18,19]

Experimental procedure

The Wistar rats (n = 6) per group for histamine-induced edema, Swiss albino mice (n = 6) for writhing method, and ear edema were used as per following group.

- Group 1 (Gr1): Disease control animals treated with Tween 80 5 ml/kg/day; orally
- Group 2 (Gr2): Treated with standard Indomethacin drug 10 mg/kg/day; orally
- Group 3 (Gr3): Treated with test 125 mg/kg Dose/day; orally
- Group 4 (Gr4): Treated with test 250 mg/kg Dose/day; orally
- Group 5 (Gr5): Treated with test 500 mg/kg/day; orally.

Histamine-induced Paw Edema in Wistar Rat

Inflammatory swelling and edema were induced by administrating 0.1 ml of 1% w/w histamine in distilled water at sub-plantar region of the rat hind paw. Test groups, control group, and standard drug group were received their dose at 60 min before swelling and inflammation induction. Then, rat paw volumes in right hind region were measured by Digital Plethysmometer (VJ-001) at 0, 1, 2, 3, 4, and 5 h time interval. Paw edema percentage inhibition was calculated by using formula [1- (Vt/Vc)] × 100. Where, Vt (edema volume in treatment) and Vc (edema volume in control group).[20]

Croton Oil-Induced Ear Edema in Mice

The healthy Swiss Albino mice of either sex or body weight of 15–30 g were used for this experiment. The control group received distilled water 0.5% and Indomethacin drug 5 mg/kg served as standard group. Ethyl acetate fraction of SSL was administered at various doses 125 mg mg/kg, 250 mg mg/kg, and 500 mg mg/kg. Ear edema was induced by topical application of croton oil in acetone (0.03 ml) on the anterior and posterior surfaces of left ear. The standard group and treatment group were administered orally before 30 min of croton oil application. The animals were kept in observation next 2–4 h. Once induction of edema observed. Both ears of the treated mice removed and weighed. The percentage of ear edema was calculated by measuring difference of weight between right ear and left ear.[21]

Table 1: Phytochemical test result

Sr.no.	*Chemical test*	*Ethyl acetate fraction of SSL component*
1.	Flavonoids chemical test	
	*Test: with Lead acetate	+
	*Test: with Sodium hydroxide	+
2.	Tannins chemical test	
	*Test: with 5% Ferric chloride solution	+
	*Test: by Dilute nitric acid use	+
3.	Saponins chemical test	
	*Test: with Foam formation method	+

(+) indicates presence of chemical test for phytoconstituents.
SSL: *Stereospermum suaveolens* leaves

Table 2: Separation of components with rate of flow (Rf) values for different spots

Sr. no.	*Solvent System*	*Drug*	*Number of Spots*	*Rf values*
1	Mobil phase-Solvent Toluene:ethyl acetate:formic acid (7:3:0.1)	Test sample	1	0.19
			2	0.30
			3	0.428
			4	0.58
2			5	0.75
			6	0.96
		Standard Quercetin	1	0.76

Inflammatory Pain Model of Acetic Acid Induced Writhing Method in Mice

The writhing test was performed in mice as per described in previous study. The Peripheral analgesic activity was studied by writhing responses (positive) by evaluating parameters such as extension of hind legs, contraction of the abdomen, and turning of trunk (twist). A solution of acetic acid (0.1 ml, 1% v/v) in distilled water was prepared and injected intraperitoneally. Indomethacin 10 mg/kg Standard drug, SSL - 125 mg/kg, SSL - 250 mg/kg, and 500 mg/kg doses were administered in mice 30 min before injection of acetic acid. The numbers of writhing episodes were recorded up to 20 min. The Percent Analgesic activity was evaluated by analgesic activity = (A-B)/C × 100. Where, A = (average number of writhes in control group), B = (Average no. of writhes in drug treated group), C = (no. of writes in control group).[22]

Statistical Analysis

One-way analysis of variance statistical method with post-test Dunnett's comparison of all groups with disease control group was considered for determination significant activity in experiments. GraphPad prism 5 software was used for calculation. $P < 0.05$ was considered to be statistically significant. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, when compared with control group.[16,22]

Results and Discussion

Phytochemical Examination

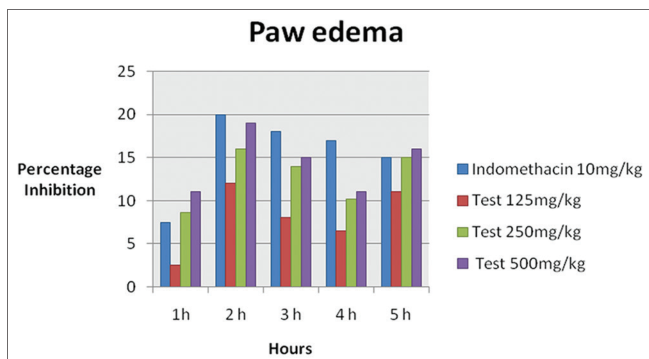
The phytochemical analysis study for ethyl acetate fraction of SSL was examined for the presence of flavonoid, tannis, saponins, etc.

Table 3: Showing peaks with RF values of standard and test sample

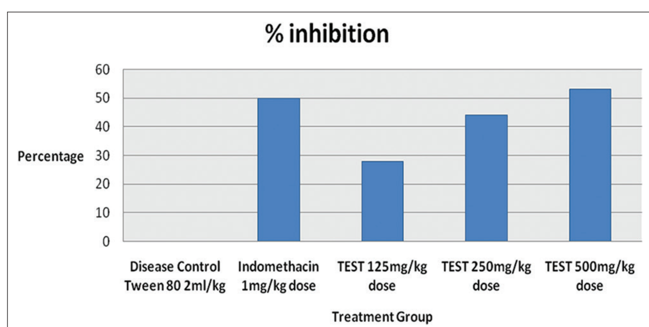
Peak	*Start Rf*	*Start Height*	*Max Rf*	*Max Height*	*Max %*	*End Rf*	*End Height*	*Area*	*Area %*	*Assigned substance*
1	0.33	7.9	0.43	376	48.26	0.46	14.7	14442.4	60	Quercetin
2	0.47	15.5	0.50	63.9	8.21	0.52	34.7	1667	6.91	Unknown
3	0.52	35.3	0.54	75.2	9.66	0.56	66.9	1604.5	6.66	Luteolin
4	0.56	67.1	0.59	158	20.29	0.63	0.3	4133.1	17.15	Unknown
5	0.63	0.2	0.66	82.9	10.65	0.69	20.7	1643.8	6.82	Gallic acid
6	0.69	20.8	0.70	22.9	2.94	0.74	0.5	612.9	2.54	Unknown

Table 4: Paw volume readings

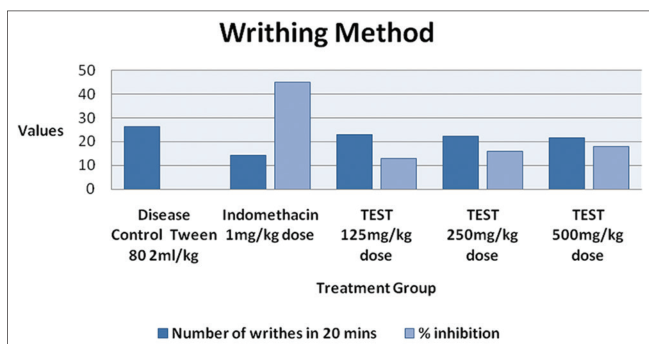
Treatment groups	*0 h*	*1 h*	*2 h*	*3 h*	*4 h*	*5 h*
Control Tween 80 5 ml/kg	0.69±0.012	0.81±0.017	1.23±0.040	1.102±0.020	1.08±0.014	1.00±0.007
Indomethacin 10 mg/kg	0.64±0.009	0.75±0.012	0.88±0.011*	0.90±0.019**	0.90±0.027*	0.85±0.013***
Test 125 mg/kg	0.67±0.014	0.79±0.009	0.91±0.016*	1.015±0.030**	1.01±0.011*	0.89±0.006**
Test 250 mg/kg	0.65±0.013	0.74±0.008**	0.87±0.019**	0.95±0.013**	0.97±0.013**	0.85±0.011***
Test 500 mg/kg	0.64±0.008	0.72±0.008**	0.84±0.012*	0.94±0.010**	0.96±0.014***	0.84±0.010***



Graph 1: Hours versus percentage paw edema inhibition. Statistical analysis as mean ± SEM, (n = 6) using one-way analysis of variance along with post-test of Dunnett. P < 0.05 was used as statistically significant. (*P < 0.05, **P < 0.01 and ***P< 0.001, when it was compared with control group)



Graph 2: Percentage of edema inhibition. Statistical analysis as mean ± SEM, (n = 6) using one-way analysis of variance along with post-test of Dunnett. P < 0.05 was used as statistically significant. (*P < 0.05, **P < 0.01 and ***P < 0.001, when it was compared with control group)



Graph 3: Number of writhes and percentage inhibition. Statistical analysis as mean ± SEM, (n = 6) using one-way analysis of variance along with post-test of Dunnett. P < 0.05 was used as statistically significant. (*P < 0.05, **P < 0.01 and ***P < 0.001, when it was compared with control group)

Table 5: Percentage inhibition at various concentrations of test samples with standard drug in rat paw edema activity

↓Treatment	Percentage inhibition				
Hours→	1 h	2 h	3 h	4 h	5 h
Indomethacin 10 mg/kg	7.5	20	18	17	15
Test 125 mg/kg	2.5	12	8	6.5	11
Test 250 mg/kg	8.6	16	14	10.18	15
Test 500 mg/kg	11	19	15	11	16

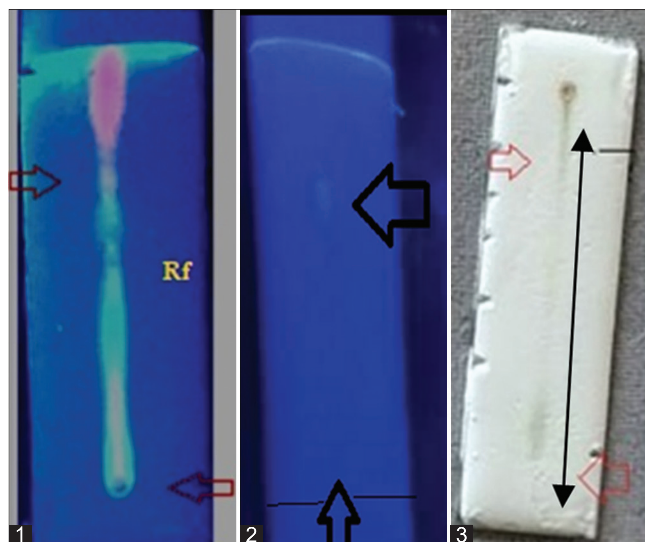


Figure 1: (1) Test sample under ultraviolet cabinet, (2) standard sample, (3) test sample

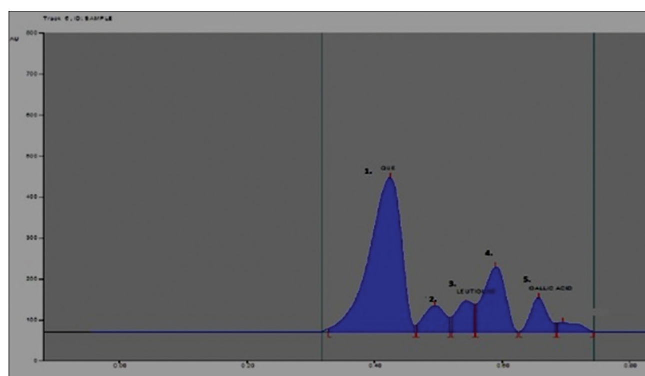


Figure 2: High-performance thin-layer chromatography chromatogram of sample fractions showing peaks of phytoconstituents

Table 6: Ear edema values

Treatment group	Ear edema weight (Mean±SEM)	% inhibition
Disease Control Tween 80 2 ml/kg	37.50±0.76	0
Indomethacin 1 mg/kg dose	18.83±0.60***	50
TEST 125 mg/kg dose	27.00±2.69***	28
TEST 250 mg/kg dose	21.17±1.24***	44
TEST 500 mg/kg dose	17.50±0.84***	53

Table 7: Result showing analgesic activity in acetic acid induced writhing

Treatment group	Number of writhes in 20 min	% inhibition
Disease Control Tween 80 2 ml/kg	26.33±0.88	0
Indomethacin 1 mg/kg dose	14.50±0.92***	45
TEST 125 mg/kg dose	23.00±0.73*	13
TEST 250 mg/kg dose	22.17±1.01**	16
TEST 500 mg/kg dose	21.67±0.71**	18

Study of TLC[13]

The different spots were identified on TLC plate using Toluene: ethyl acetate: formic acid mobile phase to separate secondary plant

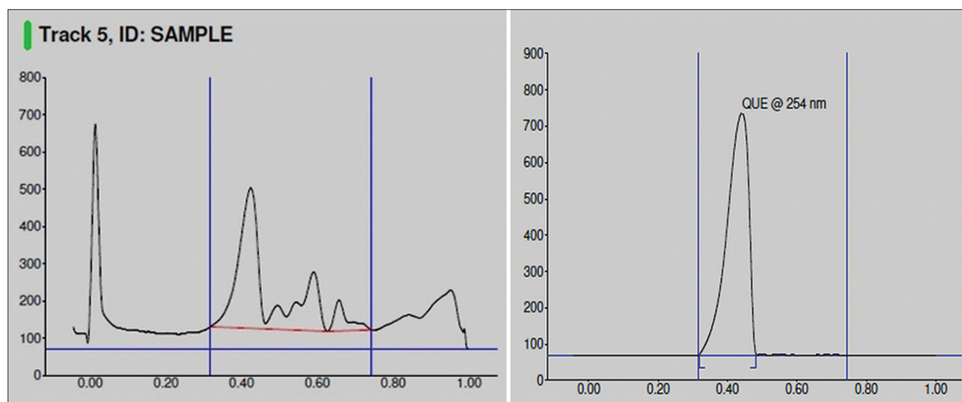


Figure 3: High-performance thin-layer chromatography chromatogram: Peaks and spectral overlay of sample and standard quercetin

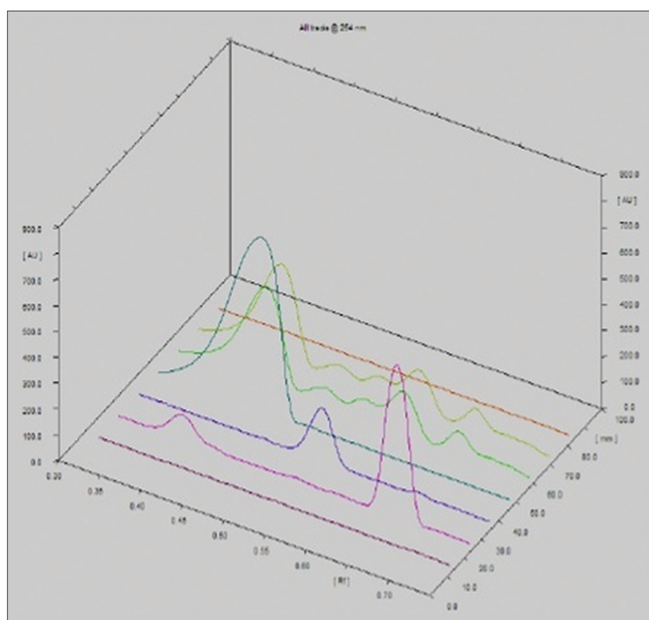


Figure 4: 3D display of high-performance thin-layer chromatography chromatogram of ethyl acetate fraction of *Stereospermum suaveolens* DC

components. The retention factors Rf of SSL were calculated [Table 2]. The Retention factor of test sample was compared with standard quercetin flavonoid marker [Figure 1].

HPTLC Analysis

HPTLC analysis explored to identify and quantify secondary plant metabolites. The following figure 2, 3, 4 represented sample fraction peaks and standard sample peaks.[13] Observation Table 3 showing peaks with RF values of standard and unknown test sample:

Three standard samples such as quercetin, luteolin, and gallic acid used in HPTLC analysis for comparison with test sample. In ethyl acetate fraction of SSL test sample 22.64% w/v of quercetin, 0.685% w/w of gallic acid, and 0.41% w/w of luteolin estimated by calculation, respectively. It was confirmed that the presence of flavonoids in the sample factions of SSL leaves.

Discussion

One of the essential clinical manifestations for any acute to chronic disease is pain and inflammation. The phytochemical component screening is valuable remedy for correction of various inflammatory parameters. Our research work was focused and carried out safe and effective phytochemical drug in the management of pain, swelling, and inflammation cases. Basically inflammation is basic defensive mechanism of human body to protect various noxious components responsible for redness, swelling, pain, vascular permeability, and vasodilatation responses. The progression of inflammation can lead to injury and subsequently death of cell. HPTLC is better separation technique to determine major active phytoconstituents in ethyl acetate fraction of SSL DC. It also assisted in verification of adulterants in selected fraction sample. As per observation table and figure, it was indicated that ethyl acetate fraction of SSL contains flavonoids. It was compared with quercetin, luteolin, and gallic acid standard [Figures 2-4]. Various sample peaks with RF values compared and confirmed presence of flavonoids in the ethyl acetate fraction of SSL [Figure 4]. The phytochemical examination [Table 1] and TLC study also supported confirmation of flavonoids in qualitatively and quantitatively along with HPTLC study. In histamine-induced paw edema method significant paw edema volume inhibition reported on SSL at 125, 250, and 500 mg/kg dose. At 2 h interval maximum and at 5 h $P < 0.05$ as compared to control group paw edema inhibition observed [Table 4 and Graph1]. There is dose-dependent effect higher edema inhibition observed at 500 mg/kg dose. The percentage of edema inhibition was calculated at 2 h 19% and 5 h 16%. The percentage inhibition of paw edema of standard drug indomethacin 10 mg/kg was at 2 h 20% and at 50 h 15% observed [Table 5]. Croton oil is chemical irritant substance. It causes intense reaction of pain and swelling on external application. It contains phorbol esters that are responsible to mortify tissue. The croton oil-induced ear edema in mice screening model significant edema inhibition observed from 28% to 53% at increasing doses of test 125 mg/kg, 250 mg/kg, and 500 mg/kg [Graph2]. The 50% edema inhibition observed also on standard dose of indomethacin 1 mg/kg significantly [Table 6]. These all results were compared with disease control group. It indicted that test drug has remarkable effect in control of inflammatory responses in dose-dependent manner. The peripheral analgesic effect was studied by acetic acid induced writhing in mice [Table 7]. The acetic acid stimulated abdominal inflammation and activation of nociceptor are the major contributors in this model. Average number of writhes and

its percentage inhibition of control group were compared with standard and test drug. It was noted significant writhing inhibition of 45% in standard indomethacin 1 mg/kg dose. The test doses such as 125 mg/kg, 250 mg/kg, and 500 mg/kg were showed 13–18% writhing inhibition as compared with control group [Graph 3]. It was indicated that ethyl acetate fraction of SSL exhibits inhibition of peripheral pain responses. However, it was responsible in inhibition of inflammation induced pain mediators especially peritoneal nociceptors mediated prostaglandin release pathway. Overall research work was investigated mechanism of flavonoid phytoconstituents present in the test sample of ethyl acetate fraction of SSL in inhibition of pain, swelling, and inflammation. Flavonoids are natural plant compounds. They are essential bioactive components in the management of viral, bacterial infection, pain, inflammation, diabetes, hyperlipidemia, aging, etc. Moreover flavonoids identified in the ethyl acetate fraction of SSL were beneficial in the treatment pain, swelling, and inflammation diseases. These flavonoids were reducing inflammation and edema by suppression of free radical generation, release of pro-inflammatory COX-1 and COX-2 enzymes and inhibition of nociceptor pain mediators. It was indicated that flavonoids in ethyl acetate fraction of SSL were associated with number of health beneficial effect.

These flavonoids will be treated as essential phytodrug in the management of pain and inflammation diseases.[22-24]

Conclusion

As per obtained results, it was concluded that available flavonoids in ethyl acetate fraction of SSL can have medicinal properties with anti-inflammatory and analgesic importance. There need to isolate further identified flavonoids to explore its structural elucidation and can become one of the beneficial plant phytoconstituents in the management of pain and inflammation it can be used as herbal drug in Ayurvedic system of medicine or preventive medicine in chronic inflammatory diseases.

Ethical Approval

The approved protocol number was MCP/IAEC/12/2017 by IAEC in accordance with CPCSEA.

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