Published online in http://ijam.co.in ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 15 (3), 2024; 673-679

Simultaneous estimation of catechin hydrate and vanillic acid by HPTLC from *Alternanthera sessilis* plant extract and evaluating their pharmacological activity

Research Article

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Abstract

Objective: The aim of this study was to identify and quantify catechin hydrate and vanillic acid from plant *Alternanthera sessile* and determine their antimicrobial and antioxidant activity. *Method:* By using toluene: ethyl acetate: formic acid (5:5:1v/v) as a mobile phase combination, separation of catechin hydrate and vanillic acid were carried out at 254 nm. Parameters validation of developed method were performed for linearity, precision, robustness, LOD and LOQ. Microorganism's zone of inhibition determine by using agar well diffusion method and antimicrobial activity of these two phytoconstituents were evaluated. DPPH assay method was used for determination of antioxidant activity. *Result:* The Rf values for catechin hydrate and vanillic acid were found to be 0.49 and 0.75 respectively and validation of parameters showed linear and accurate method for analysis. The zone of inhibition was found immensely significant for *S.aureus, B.subtillis* and *C. albicans* which when compared to standard antimicrobial agents and gave promising results. Catechin hydrate and vanillic acid also shows significant antioxidant activity. *Conclusion:* The optimised method was found to be efficient, precise, accurate, specific and economic. Therefore, the method would be useful for qualitative and quantitative routine analysis in pharmaceutical industry. Also, the antimicrobial and antioxidant effect of these two phytochemicals shows excellent result which is useful in many pharmaceuticals and disease conditions.

Keywords: HPTLC, *Alternanthera sessilis*, Catechin hydrate, Vanillic acid, Antioxidant activity, Antimicrobial activity.

Introduction

Phytochemicals present in plant *Alternanthera sessilis (L.) R. Br.* have various pharmacological activities such as antimicrobial, antidiabetic, anticancer, antidepressant, anti-inflammatory and antioxidant(1). Ferulic acid, chlorogenic acid, catechin, vanillic acid, myricetin, gallic acid, ethyl gallate, daidzein and apigenin these are main phytochemicals in plant *Alternanthera sessilis*(2). Antioxidant properties of phytochemical plays important role in plant defence mechanism from UV radiation, against pathogens and pests. When consumed through vegetables, fruits, and other plant-based foods they are very beneficial for human health(3). Meta analysis and epidemiological studies recommend that polyphenols rich plants consumption in diet for long-term give protection against various chronic diseases, including diabetes, cancer, cardiovascular disease, neurological diseases and osteoporosis(4,5). *Alternanthera sessilis* is the annual herb proliferates as a weed across Nepal, India, and Sri Lanka(Fig.1). This plant belongs to family *Amaranthaceae*, which involves 65 genera and 850 species globally(6). Various names such as *Sessile* joyweed and dwarf copper leaf gave to this plant which has adapted to various environments and flourish in various soil conditions(7).

Figure 1: *Alternanthera sessilis* plant

Catechin hydrate comes in the category of flavanols which is also called as taxifolin(8). This compound commonly present in various natural sources like grapes seed, tea leaves, wood and bark of acacia(9). As shown in Fig. 2, catechin hydrate is a trans-2-(3,4-Dihydroxyphenyl)3,4-dihydro-1(2H)-benzopyran-3,5,7-triol trans-3,3′,4′,5,7-Pentahydroxyflavane having molecular formula $C_{15}H_{16}O_7$. This phytochemical is known for its anti-inflammatory, antimicrobial and antioxidative activities. Also, its diverse pharmacological effects encompass to cardio

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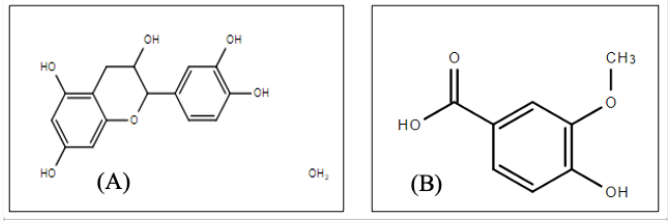
protective, neuroprotective, and anti-cancer properties this makes it to apply in various health conditions(10). Vanillic acid is a benzoic acid derivative (4-hydroxy-3-methoxy benzoic acid) which is an oxidized form of vanillin (Fig.2). This phytochemical use as a flavouring agent as it is aromatic phenolic acid and highly yellow in appearances(11). Higher concentration of vanillic acid found in the roots of *Angelica sinesis* and showed significant activity in various diseases like cardiovascular, liver and gastrointestinal diseases(12–14). Vanillic acid widely used in fruit products, cosmetics, flavourings, polymers and drinks. It is known for various pharmacological activities like anti-inflammatory, neuroprotective properties, antimicrobial and antioxidant (15).

Fig. 2 Catechin hydrate (A) and Vanillic acid (B)



Detection and identification of catechin hydrate and vanillic acid from *Alternanthera sessilis* leaves extract by HPTLC method has not been performed yet with optimal separation and minimal mobile phase. Catechin hydrate and vanillic acid have potent antimicrobial activity and antioxidant activity which may give synergistic effect when combing together. By comparing their activity with commercially available antimicrobial agents, we done in-vitro antimicrobial assay. In-vitro DPPH assay for antioxidant activity of this phytoconstituents were performed which gives promising and satisfactory results.

Materials and methods

Chemicals and reagents

The standard catechin hydrate and vanillic acid were procured from Yucca Enterprises, Mumbai and the silica gel 60F254 TLC plates for HPTLC were purchased from Merck, Germany. The solvents used, namely toluene, ethyl acetate, and formic acid, were of analytical reagent quality. Agar media, DPPH were procured from Molychem, Mumbai, India.

Instrumentation

Micro syringe (Linomat syringe, Hamilton-Bonaduz Switzerland, Camag, Switzerland), automatic sample applicator Linomat 5 (Camag, Muttenz, Switzerland), pre-coated Silicagel 60 F-254 glass plates (10 x 10 cm with 200 µm thickness HPTLC; Merck, Germany), twin trough chamber (10 x 10 cm) (Camag, Muttenz, Switzerland), TLC scanner III (Camag, Muttenz, Switzerland), WinCAT software (Camag, Muttenz, Switzerland), and UV chamber (Camag, Muttenz, Switzerland) were employed for the HPTLC analysis. UV Spectrometer (Jasco V730) for antioxidant assay. Incubator and autoclave for antimicrobial assay.

Microsoft Excel was used to analyse the data statistically.

Preparation of samples for HPTLC

Standard solution of extract

The maceration procedure, which involved precisely weighing 100 g of plant powder and mixing it with 1000 ml of methanol in a closed container with periodic shaking for few days, was employed to extract the plant powder. Following the extraction process, the final product was concentrated using a rotary evaporator at 40°C and filtered by using Whatman filter paper No. 1. The portion of each extract were diluted by 50% in preparation for further HPTLC evaluation.

Standard solutions of catechin hydrate and vanillic acid

Accurately weighed 100 mg of vanillic acid and catechin hydrate, transferred to 100 mL volumetric flask. Methanol was used to dilute the mixture to the appropriate level. 5 mL of each solution was transferred to a 50 mL volumetric flask and diluted with methanol (100 µg/ml concentration) for further dilutions. Subsequently, the mixture was combined and passed through a 0.2 µ filter membrane.

Chromatographic condition

The mobile phase for each chromatographic run comprised of toluene: ethyl acetate: formic acid (5:5:1 v/v). The samples were placed to the plate as 8 mm long bands. The ascending development technique was applied during the 15-minute saturation of the mobile phase in a twin-trough chamber at room temperature (15 ± 2 °C). After allowing the mobile phase on the HPTLC plate to travel up to 8 cm and allowing the plates to fully dry before scanning, additional measurements were carried out using WinCAT software and a TLC scanner III in the 254 nm reflection/ absorption mode. Sample concentrations were calculated using the intensity of the reflected light and a comparison between the sample band's peak area and the standard bands' peak areas.

Method validation

In compliance with ICH-Q2-R1 guidelines, the developed HPTLC technique was validated. In this validation procedure, many factors are evaluated, including robustness, linearity, accuracy, limit of detection (LOD), and limit of quantification (LOQ) (16).

Linearity: Linear correlation was calculated by comparing the peak area and drug concentration over a concentration range indicated in µg/band. For this examination, five measurements were made at six concentrations, from 0.2 to 1.2 µg/band range.

Precision: The terms intra- and inter-day was used to determine precision. Standard solutions containing 0.2, 0.4, 0.6, 0.8, and 1.0, 1.2 µg/mL were utilised to apply bands on the HPTLC plate and measure precision on the same day (n = 3) and over a three-day period (n = 3) in

turn. The acquired peak regions were utilised to calculate the mean and percent RSD.

Limit of detection(LOD) and limit of quantification(LOQ): An essential step in method validation is estimating the amounts of vanillic acid and catechin hydrate. LOD and LOQ was determined independently based on the slope of the calibration curve and standard deviation, denoted as S and σ, respectively, using the formulas LOD = 3.3 σ/S and LOQ = 10 σ/S.

Robustness: Made some intentional variations to the optimised method's parameters in order to assess the produced method's adaptability. This comprises differences of ±15% in the chamber's saturation time and ± 0.1 mL in the composition of the mobile phase. Peak areas and Rf values were taken into consideration while evaluating these alterations, and %RSD was determined for each parameter. These alterations assure accuracy as well as reliability of the developed HPTLC method (17–19).

Antimicrobial Activity

Organism collection

Antibacterial activity of catechin hydrate and vanillic acid were determined against two gram positive bacteria (*Stapphylococcus aureus, Bacillus subtilis)* and antifungal activity of the same drugs were carried out against fungi (*Candida albicans).* All the organisms were collected from microbiology research laboratory of Modern college of pharmacy Pune.

Growth media

Muller Hinton broth was used for determination of antibacterial activity of phytochemicals(20) and for estimation of antifungal activity Sabouraud dextrose agar (SDA) was used(21).

Antibacterial activity

Antibacterial screening is generally performed by agar well diffusion method(22). Sterilized nutrient medium were transferred into one-third volume of the plate. After solidification of culture media, inoculum was spread on that with the help of sterile spreader. 0.2 ml of different concentrations of our drugs were introduced into it. The plates were incubated for 24 hrs at 37^{o}C. The inhibition of bacterial and fungal growth was determined by measuring diameter of zone of inhibition. Clindamycin was used as a reference standard for estimation of antibacterial activity(23).

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) value of both compounds were identified as the lowest concentration causing complete inhibition of bacterial growth after 24 hours of incubation at 37^{0}C(24). In order to determine MIC serial dilution of drug and standard were prepared and results are interpreted as the highest dilution (lowest concentration) of the sample which showed the clear zones. All the tests were performed in triplicates(25).

Antifungal activity

Antifungal activity of polyphenols were studied by agar well diffusion method(26), Sabouraud dextrose broth was used for fungal growth. After 72 hours the cultures grown on Sabouraud's dextrose agar (SDA) were used for inoculation of fungal strain on SDA plates. Inoculums was introduced to molten SDA after poured in to a petri dish by pour plate method. After solidification, the appropriate wells were made on agar plate by using cork borer. Using sterile pippete sample was introduced into it, incubation period of 48 hours at 37^{0}C was maintained for observation of antifungal activity. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the wells. Fluconazole was used as a reference standard for estimation of antifungal activity. The zones of inhibition were measured with scale in mm and the experiment was carried out in triplicates(27).

Antioxidant activity

For estimation of antioxidant activity of phytoconstituents, DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay method was used(28). 50 µl catechin hydrate and vanillic acid in DMSO 10% with various concentrations (3.125µg/ml to 100 µg/ml) were introduced in microplate and added 200 µl DPPH 0.52mmol in DMSO. The mixture was shaken vigorously and incubated at room temperature for 30 min, and then the absorbance values measured at 517 nm. Negative controls used DPPH 250 µl, blank used 250 µl DMSO 10%. The radical scavenging activity of each sample was expressed by the ratio of lowered DPPH absorption (%), relative to the absorption (100%) of DPPH solution in the absence of test sample (negative control)(29). DPPH antioxidant activity calculated by using following formula.

%RSD= Absorbance of control-Absorbance of sample / Absorbance of control*100

Results

Throughout the development of the HPTLC method, a number of experiments were carried out using combinations of various solvents, including non-polar and slightly polar solvents. As demonstrated in Fig. 3 and 4, toluene: ethyl acetate: formic acid (5:5:1 v/v) offered the best results out of all the mobile phase combinations evaluated, offering superior resolution and clear peaks with respectable Rf values of 0.75 and 0.49 for catechin hydrate and vanillic acid, respectively. Fig. 5 and 6 display the combination's HPTLC densitogram under optimal circumstances.

Linearity

For catechin hydrate and vanillic acid, the linear relationship was obtained between peak areas within concentration range of 0.2 to 1.2 µg/mL. For calibration curve, correlation coefficient was obtained to be 0.9987 and 0.9987 respectively as shown in Fig.7.

Fig. 3 Chromatogram of standards vanillic acid

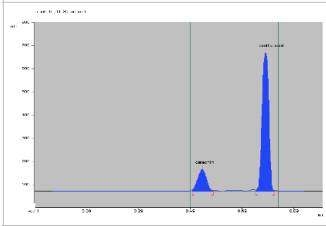


Fig. 4 Chromatogram of *A. sessilis* Extract and catechin hydrate

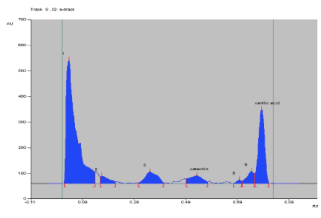


Fig. 5 3D Spectra of catechin hydrate

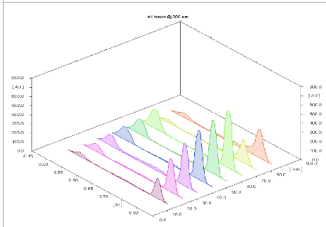


Fig. 6 Overlain spectra catechin hydrate + vanillic acid and vanillic acid

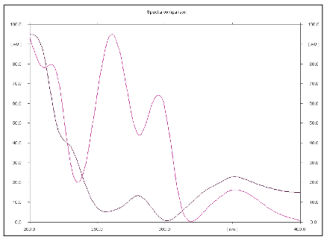
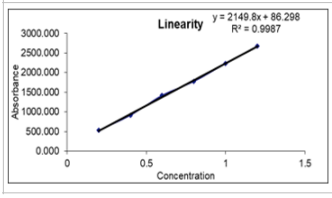
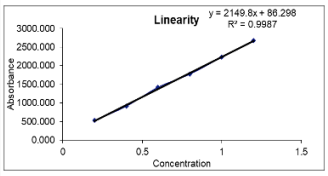


Fig.7 Calibration curve for catechin hydrate (A) vanillic acid (B)





LOD & LOQ

To evaluate the sensitivity for HPTLC methodology limit of detection and limit of quantification were used . LOD and LOQ for catechin hydrate was found to be 1.8710 and 5.6697 ng, and for vanillic acid it was 0.1718 and 0.5206 ng. Above data showed that both the samples were sensitive towards HPTLC.

Precision

Repeatability and reproducibility of the method were found out by using intra-day and interday precision studies. The samples were examined three successive days (inter-day) and three times on same day (intra-day).The results was termed in terms of relative standard deviation (RSD). The resultant RSD for both intra-day and inter-day precision studies was found to be less than 2, gives evidence that proposed method was repeatable and reproducible (Table 1 and 2).

Robustness

By doing small intentional changes in optimised method robustness was studied. Rf values and peak areas of both the samples were examined by changing the mobile phase composition in range of ±0.5ml, duration of chamber saturation with mobile phase of 20, 25 and 30 min . The relative standard deviation for peak areas was found to be less than 2 under all varied method parameters. As there was no significant change in Rf values of both the drugs showed that resolution between catechin hydrate and vanillic acid were not affected. Hence it was proved that the method was robust for determination of catechin hydrate and vanillic acid (Table 3).

Antibacterial activity

The antibacterial activity of catechin hydrate and vanillic acid were compared with the reference standard clindamycin. The zones of inhibition produced were ranged between 21mm to 30 mm diameter (Table 4). The results obtained from the assay showed that both the microorganisms were susceptible to catechin hydrate and Vanillic acid at different concentrations. The present study revealed that catechin hydrate and vanillic acid possessed significant inhibitory activity against *S.aureus, B.subtilis* even at low concentration of 10 mg/ml as shown in Table 4 and Fig.8.

Antifungal activity

The catechin hydrate and vanillic acid also showed significant fungal growth inhibition against *Candida albicans*. Zone of inhibition obtained was in ranged between 23mm to 31mm in the range of 10 mg/ mL to 80 mg/mL concentrations as shown in Table 4 and Fig.8.

Table 1: Precision study of vanillic acid

[2]Conc. (µg/ml)	Intraday			Interday		
	Mean ± [1]SD	[3]Amt Found	% [3]Amt Found	Mean ± [1]SD	[3]Amt Found	% [3]Amt Found
0.6	8479.38± 0.63	0.61	101.46	8479.09 ±0.95	0.61	101.45
0.8	9997.93± 0.58	0.81	100.92	9999.51 ±0.53	0.81	100.94
1	11390.52± 0.68	0.99	98.94	11390.68 ±0.60	0.99	98.95

[1]SD= Standard deviation, [2]Conc. = Concentration, [3]Amt = Amount

Table 2: Precision study of catechin hydrate

[2]Conc. (µg/ml)	Intraday			Interday		
	Mean ± [1]SD	[3]Amt Found	% [2]Amt Found	Mean ± [1]SD	[3]Amt Found	% [2]Amt Found
0.6	1399.37± 0.00	0.61	101.8	1399.37 ±0.00	0.61	101.8
0.8	1785.30± 0.01	0.79	98.79	1785 ± 0.01	0.79	98.79
1	2235.90± 0.01	1	99.99	2235 ± 0.01	1	99.99

[1]SD= Standard deviation, [2]Conc. = Concentration, [3]Amt = Amount

Table 3: Robustness study

Sr no.	Factor	Chromatographic changes	
		Peak-area ± (SD[1])	[2] % RSD
A)	Mobile phase composition: vanillic acid		
	1) 5 : 4.8 :1 v/v	12912.66 ± 1.26	0.01
	2) 5 : 5 :1.3 v/v	12915.62 ± 0.90	0.01
	Catechin hydrate		
	1) 5 : 4.8 :1 v/v	2671.42 ± 0.80	0.03
	2) 5 : 5 :1.3 v/v	2671.57 ± 0.69	0.03
B)	Duration of chamber saturation : Vanillic acid		
	1) 20	12918.05 ± 1.02	0.01
	2) 25	12920.16 ± 0.93	0.01
	3) 30	12920.09 ± 0.75	0.01
	Catechin hydrate		
	1) 20	2672.66 ± 0.79	0.03
	2) 25	2672.44 ± 1.13	0.04
	3) 30	2672.65 ± 0.81	0.03

[1]SD = Standard deviation, [2] %RSD = Percentage relative standard deviation

Figure 8: Antimicrobial activity of catechin hydrate, vanillic acid, clindamycin and fluconazole

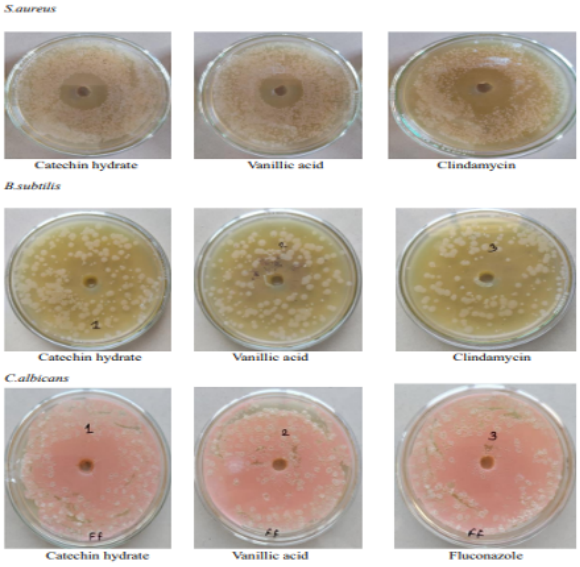


Table 4: Antimicrobial assay result of catechin hydrate, vanillic acid, clindamycin and fluconazole

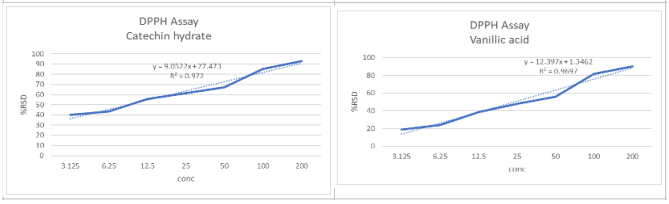
Compound	Concentrations			
	10 mg/	20 mg/mL	40 mg/mL	80 mg/mL
Organism : *Staphylococcus aureus*				
Vanillic acid	14	20	26	44
Catechin	10	16	25	41
Clindamycin	16	28	34	48
Organism : *Bacillus subtillis*				
Vanillic acid	14	18	30	42
Catechin	13	18	26	40
Clindamycin	18	27	37	41
Organism : *Candida albiacans*				
Vanillic acid	16	24	32	42
Catechin	11	18	28	43
Fluconazole	19	23	32	42

Antioxidant activity

The antioxidant activity of catechin hydrate and vanillic acid was assayed by using DPPH scavenging activity. Due to presence of active antioxidant properties in catechin hydrate and vanillic acid free radical scavenging of sample was marked with change in colour from dark purple to pale yellow. IC50 value for catechin hydrate and vanillic acid was found to be 2.488 and 3.924 µg/ml respectively. As smaller the IC50 value higher is the antioxidant activity, highest antioxidant activity in current study was found to be of catechin hydrate as compare to vanillic acid as shown in Fig.9.

Fig.9 Calibration curve for DPPH Assay of

Catechin hydrate (A) **Vanillic acid (B)**



Discussion

We devised a sensitive and trustworthy HPTLC method to precisely identify and determine the quantity of catechin hydrate and vanillic acid in extracts from the *Alternanthera sessilis* plant. This approach showed great precision across both intra-day and inter-day analysis, and it was resilient, yielding consistent outcomes even under slight experimental adjustments. The technique's effectiveness in examining the phytoconstituents of *Alternanthera sessilis* extract is further demonstrated by the strong correlation, linear response, and low detection and quantification limits. This method allows more accuracy and sensitivity than previous studies. For instance, the optimised HPTLC method maintained consistency under different experimental conditions, indicating its resilience and dependability for regular analysis. Vanillic acid and catechin hydrate have simultaneously shown significant antibacterial action against Staphylococcus aureus, Bacillus subtilis, and Candida albicans. These phytoconstituents showed comparable, if not higher, effectiveness when compared to the commercially available antibacterial agent clindamycin and the antifungal agent fluconazole. This is consistent with prior research, but it adds to the understanding as it revealed a synergistic impact when the compounds were combined. Furthermore, satisfactory outcomes were obtained when the antioxidant activity was assessed using the DPPH test method, supporting previous studies on *Alternanthera sessilis* antioxidant qualities. But by more precisely characterising these actions and showcasing their potential for use in therapeutic applications, this investigation makes a greater contribution. The suggested HPTLC approach demonstrates the potential therapeutic properties of catechin hydrate and vanillic acid from *Alternanthera sessilis*, in addition to being a great approach for their identification and quantification. Significant antioxidant

and antimicrobial activity was demonstrated by the determined phytoconstituents, which may help in the development of novel medications and therapies. Therefore, by offering a more thorough and dependable analytical approach and investigating the synergistic effects of these substances, this work presents a substantial advance over prior research, widening possibilities for potential pharmaceutical advances.

Conclusion

The proposed HPTLC method for identification and quantification of catechin hydrate and vanillic acid from the plant *Alternanthera sessile* was proved to be precise, efficient, accurate, specific for qualitative and quantitative routine analysis. The Rf values for catechin hydrate and vanillic acid with their validation parameters showed a linear and accurate analytical method. Analysis of their antimicrobial activity on various microbial species which give significant inhibition of microbial growth and gave promising antimicrobial activity against *S.aureus, B.subtilis* and *C.albicans* when compared to standards. Also, phytoconstituents showed promising antioxidant activity, in comparison to vanillic acid catechin hydrate showed more antioxidant activity that is studied phytochemicals have significant potential in different disease conditions and have various pharmaceutical applications which offering promising scope for future pharmaceutical development.

Conflict of interest: The authors declares that there is no conflict of interest.

Funding: None.

Acknowledgment

We wish to thank Dr. D. Y. PUS's Dr. D. Y. Patil Institute of Pharmaceutical Sciences & Research for providing necessary facilities, Yucca Enterprises, Mumbai, India.

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