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Original Article

HPTLC and FT-IR Guided Phytochemical Characterization and Anti-Inflammatory Evaluation of *Cymbidium aloifolium* (L.) Sw. Leaf Extracts

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ABSTRACT

Background: *Cymbidium aloifolium* (L.) Sw. is traditionally used in indigenous medicine for the treatment of inflammatory conditions, but its anti-inflammatory potential has not yet been thoroughly validated through scientific studies. Therefore, this study aimed to evaluate the *in vitro* and *in vivo* anti-inflammatory activity of *C. aloifolium* leaf extracts and to characterize their putative bioactive constituents using HPTLC fingerprinting and FT-IR spectroscopy. **Materials and Methods:** Successive leaf extracts were assessed for anti-inflammatory activity using egg albumin denaturation and carrageenan-induced paw edema models in Wistar rats. Acute oral toxicity was evaluated according to OECD guideline 423. Phytochemical profiling was performed using HPTLC and FT-IR analysis. Data were analyzed using one-way and two-way ANOVA followed by Tukey's *post hoc* test. **Results:** Among the extracts, the chloroform fraction demonstrated the highest anti-inflammatory activity *in vitro* (IC_{50} = 58.40 µg/mL) and significantly reduced paw edema *in vivo* at 200 mg/kg, comparable to diclofenac. No toxicity was observed up to 2000 mg/kg. HPTLC and FT-IR analyses indicated the presence of caffeine-like alkaloidal constituents and flavonoid-related functional groups. **Conclusion:** *C. aloifolium* leaf extracts exhibit significant anti-inflammatory activity, likely associated with caffeine-like alkaloidal constituents and flavonoids. These findings support its traditional use and warrant further phytochemical characterization.

Keywords: Anti-inflammatory agents, Carrageenan, *C. aloifolium*, Fourier transform infrared spectroscopy, High-performance thin-layer chromatography.

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INTRODUCTION

Inflammation underlies numerous acute and chronic disorders and remains a key target for pharmacological intervention (Libby *et al.*, 2024). Although non-steroidal anti-inflammatory drugs are effective, their long-term use is associated with adverse effects, prompting the search for safer plant-derived alternatives. Medicinal orchids of the family Orchidaceae have been widely used in traditional medicine and are increasingly explored for their pharmacological potential (Choudhary *et al.*, 2023).

Cymbidium aloifolium (L.) Sw., commonly known as the boat orchid, is an epiphytic orchid distributed across South and Southeast Asia (Kim, 2025). Traditional practices report their use in inflammatory conditions, wounds, and skin disorders. Despite these claims, systematic evaluation of its anti-inflammatory activity and phytochemical profile remains scarce.

High-Performance Thin-Layer Chromatography (HPTLC) is a robust analytical tool for quality control and phytochemical fingerprinting of herbal materials (Beressa *et al.*, 2021), while Fourier-Transform Infrared (FT-IR) spectroscopy enables rapid identification of functional groups associated with bioactive constituents (Gong *et al.*, 2024). The present study aimed to evaluate the anti-inflammatory activity of *C. aloifolium* leaf extracts using validated *in vitro* and *in vivo* models and to characterize associated phytochemical features using HPTLC and FT-IR techniques.

MATERIALS AND METHODS

Plant Material and Extraction

Leaves of *C. aloifolium* were collected from the University of North Bengal campus and authenticated by the Botanical Survey of India, Gangtok, Sikkim, with specimen no 09-NBU-2022. Shade-dried leaves were powdered and subjected to successive maceration using petroleum ether, chloroform, ethyl acetate,

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methanol, and water. Extracts were concentrated under reduced pressure and stored at 4°C.

In vitro Anti-Inflammatory Activity

Anti-inflammatory activity was evaluated using the egg albumin denaturation assay. Extracts (50–400 µg/mL) were incubated with egg albumin and phosphate buffer, heated, and absorbance measured at 660 nm. Diclofenac sodium served as the reference drug (Maiti *et al.*, 2024).

Animals and Experimental Design

Male Wistar rats (120–140 g) were randomly assigned to experimental groups ($n = 6$). The sample size ($n = 6$ per group) was selected based on previously published carrageenan-induced paw edema studies, which demonstrated that this group size provides adequate statistical power to detect significant anti-inflammatory effects (Mehallah *et al.*, 2024). All procedures were approved by the Institutional Animal Ethics Committee (IAEC/NBU/2023/68).

Acute Toxicity Study

Acute oral toxicity of the chloroform extract was assessed according to OECD guideline 423 at doses up to 2000 mg/kg. Animals were observed for 14 days for signs of toxicity or mortality (Mehallah *et al.*, 2024).

In vivo Anti-Inflammatory Activity

Carrageenan-induced paw edema was produced by subplantar injection of 1% carrageenan. Extracts (100 and 200 mg/kg) and diclofenac (10 mg/kg) were administered orally one hour prior to carrageenan injection. Paw edema was measured hourly for 6 hr using a digital caliper.

HPTLC and FT-IR Analysis

HPTLC fingerprinting was performed using silica gel 60F254 plates with toluene:ethanol:formic acid (5:4:1) as the mobile phase (Ashraf *et al.*, 2021). FT-IR spectra were recorded between 4000–400 cm^{-1} (Fomina *et al.*, 2023).

Statistical Analysis

Data are expressed as Mean ± SD. Statistical comparisons were performed using one-way or two-way ANOVA followed by Tukey's test. $p < 0.05$ was considered significant.

RESULTS

In vitro anti-inflammatory Activity

The *in vitro* anti-inflammatory effect of petroleum ether, chloroform, ethyl acetate, methanol, and water extract of *C. aloifolium*, with standard Diclofenac was evaluated against the denaturation of egg albumin. Standard diclofenac possesses the lowest IC_{50} value of 49.33 µg/mL, indicating the highest efficacy in inhibiting protein denaturation. All extracts of *C. aloifolium* showed higher IC_{50} values in comparison: chloroform (58.40 µg/mL), petroleum ether (75.07 µg/mL), methanol (77.44 µg/mL), and ethyl acetate (90.79 µg/mL). Tukey's *post-hoc* analysis confirmed that the differences between diclofenac and each extract were statistically significant ($p < 0.05$), with the highest level of significance ($p < 0.001$) observed against PECA, EACA, MECA. The CHCA showed the smallest mean difference from diclofenac (mean diff = -9.067), yet was still statistically significant ($^*p < 0.05$) (Figure 1).

In vivo experimental

Acute toxicity study

An acute toxicity investigation was conducted to assess the toxicological impact of the chloroform extract of *C. aloifolium*, ranging from a low-dose group of 100 mg/kg body mass to a high-dose group of 2000 mg/kg body mass. No signs of toxicity were observed in any of the five dosing groups when experimental indications were tracked from the beginning of treatment through day 14. Additionally, both immediate and delayed mortality were monitored. The results indicated that there were no fatalities in any of the chloroform extract of *C. aloifolium*-treated groups, even at the highest dose of 2000 mg/kg body mass administered orally.

In vivo anti-inflammatory activity

Carrageenan administration to rats caused a increase in paw size shortly after injection. As shown in Table 2, diclofenac and chloroform extracts of *C. aloifolium* significantly influenced carrageenan-induced rat paw edema.

The anti-inflammatory effect of *C. aloifolium* at 100 mg/kg and 200 mg/kg was evaluated against carrageenan-induced paw edema in rats. Diclofenac sodium (10 mg/kg) served as the standard anti-inflammatory agent. Paw edema was measured at hourly intervals up to 6 hr, and the graph provides a summary of the findings (Figure 2).

In the toxic control group, paw edema increased progressively, reaching a peak at 3 hr (72.57% ± 0.98), followed by a gradual decline up to 6 hr (46.56% ± 1.14). Treatment with CA 100 mg/kg significantly inhibited paw edema from the 2nd hr (48.32% ± 0.69) onwards, with a reduction to (36.88% ± 0.84) at the 3rd hr, and a marked inhibition observed at the 5th (14.97% ± 0.81) and 6th hr (4.35% ± 0.51).

More pronounced effects were observed with *C. aloifolium* at 200 mg/kg. At the 3rd hr, paw edema was reduced to 34.59% ± 0.98, and significant inhibition continued through the 4th (16.22% ± 1.01), 5th (4.77% ± 0.19), and 6th (1.29% ± 0.18) hr, showing a strong dose-dependent response.

The standard drug, diclofenac, exhibited potent anti-inflammatory activity, with paw edema reduced to 33.67% ± 0.97 at the 3rd hr,

and nearly complete inhibition at the 5th (3.53% ± 0.35) and 6th (1.21% ± 0.24) hr.

Both CA-treated groups showed statistically significant inhibition in paw edema compared to the toxic control group ($p < 0.05$). Additionally, the 200 mg/kg dose exhibited comparable efficacy to diclofenac in the later stages of inflammation (#$p < 0.05$ vs standard drug).

HPTLC fingerprinting

HPTLC fingerprinting of different leaf extracts of *C. aloifolium* (Pet ether, Chloroform, Ethyl Acetate, Methanol) in White light, UV 254 nm, 366 nm, and white light after Derivatisation with ferric chloride is shown in Figure 3. HPTLC densitometric chromatograms of standard caffeine and selected solvent extracts of *C. aloifolium* recorded at 254 nm are shown in Figure 4. The R_f value of the standard compound Caffeine was found to be 0.446 at 254 nm. Bands corresponding to the R_f value of standard caffeine were observed in the chloroform, ethyl acetate, and methanolic extracts. The R_f values of caffeine in extracts such as chloroform (0.487), ethyl acetate (0.482), and Methanolic (0.481) are closer to the R_f of standard caffeine. The relative peak area percentages of caffeine-like bands in different extracts are shown in Table 1. Hence, the results suggest the putative presence of caffeine-like compounds in the chloroform, ethyl acetate, and methanolic extracts of *C. aloifolium*.

Table 1: *In vitro* anti-inflammatory activity (IC_{50} values) of *C. aloifolium* leaf extracts.

Extract / Standard	IC_{50} (µg/mL)	Statistical significance vs diclofenac
Diclofenac (Std)	49.33	—
Petroleum ether extract (PECA)	75.07	*** $p < 0.001$
Chloroform extract (CHCA)	58.40	* $p < 0.05$
Ethyl acetate extract (EACA)	90.79	*** $p < 0.001$
Methanol extract (MECA)	77.44	*** $p < 0.001$
Aqueous extract (WCA)	>100	*** $p < 0.001$

Values are expressed as mean ± SD ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test.

FT-IR analysis

FT-IR spectra were obtained using an FT-IR Alpha-II BRUKER Spectrophotometer (Germany) between 4000 and 400 cm^{-1} at 4 cm^{-1} resolution and accumulating 24 scans for each spectrum. The background spectrum was established by a blank Potassium Bromide (KBr) pellet. The corresponding spectra of all the functional groups were within the range, and the IR spectra of standard caffeine showed considerable similarity with those of the chloroform, ethyl acetate, and methanol extracts, respectively. In each spectrum, the three different solvent extracts and standard caffeine were very similar and showed characteristic absorption bands that were used for the tentative identification of caffeine-like alkaloidal constituents in the chloroform, ethyl acetate, and methanol extracts. The FT-IR spectra of the extracts showed absorption bands similar to those of standard caffeine, suggesting the presence of caffeine-like alkaloidal constituents. Corresponding IR spectral information of the three different solvent extracts and standard caffeine is given in Table 2. FT-IR functional group assignments are summarized in Table 4.

DISCUSSION

This study applied both *in vitro* and *in vivo* models to explore the anti-inflammatory efficacy of *C. aloifolium* leaf extracts, with the help of phytochemical profiling using FT-IR and HPTLC analysis. Additional knowledge on the pharmacological significance of this long-used Chinese medicinal herb is offered by these combined approaches.

To determine the extracts' effectiveness in preventing protein denaturation, a crucial step in inflammation, the egg albumin denaturation model was employed (Ranaweera *et al.*, 2023). A common NSAID, diclofenac sodium, had the best effectiveness (IC_{50} = 49.33 µg/mL). With the greatest anti-inflammatory efficacy (IC_{50} = 58.40 µg/mL) among the studied extracts, the chloroform extract demonstrated the existence of active

Table 2: Effect of *C. aloifolium* chloroform extract on carrageenan-induced paw edema in rats.

Time (h)	Toxic control (%)	Diclofenac (10 mg/kg) (%)	CA 100 mg/kg (%)	CA 200 mg/kg (%)
1	58.42 ± 1.12	41.36 ± 0.91*	46.88 ± 0.83*	44.12 ± 0.79*
2	66.85 ± 1.04	38.92 ± 0.88*	48.32 ± 0.69*	42.76 ± 0.81*
3	72.57 ± 0.98	33.67 ± 0.97*	36.88 ± 0.84*	34.59 ± 0.98*
4	61.34 ± 1.07	18.44 ± 0.62*	24.26 ± 0.73*	16.22 ± 1.01*#
5	53.19 ± 1.21	3.53 ± 0.35*	14.97 ± 0.81*	4.77 ± 0.19*#
6	46.56 ± 1.14	1.21 ± 0.24*	4.35 ± 0.51*	1.29 ± 0.18*#

*Values are mean ± SD ($n = 6$). $p < 0.05$ vs toxic control; #$p < 0.05$ vs diclofenac (two-way ANOVA followed by Tukey's test).

Table 3: HPTLC R_f values and peak area percentages of caffeine-like compounds in *C. aloifolium* extracts.

Sample	R_f value	Peak area	% Area
Caffeine (Standard)	0.446	—	—
Chloroform extract	0.487	0.03243	26.34
Ethyl acetate extract	0.482	0.03723	24.77
Methanol extract	0.481	0.00402	22.41

HPTLC analysis was performed using silica gel $60F_{254}$ plates with toluene:ethanol:formic acid (5:4:1, v/v/v) as the mobile phase and detection at 254 nm.

Table 4: FT-IR functional group assignments of *C. aloifolium* extracts compared with standard caffeine.

Functional group	Standard caffeine (cm^{-1})	Chloroform extract	Ethyl acetate extract	Methanol extract
O–H stretching	3445	3449	3452	3450
C–H stretching	2955	2955	2954	2955
C=O stretching	1699	1725	1710	1711
C=N stretching	1655	1634	1634	1633
N–H bending	1549	1553	1552	1555
C–N stretching	1358	1382	1379	1381

FT-IR analysis was carried out in the range of 4000–400 cm^{-1} to identify characteristic functional groups.

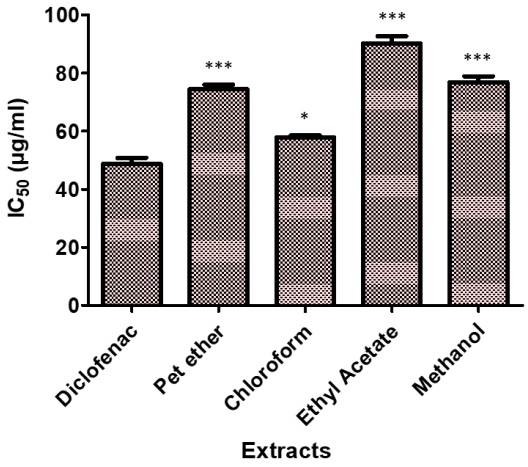

Figure 1: Comparison of anti-inflammatory activity (IC_{50}, µg/mL) of PECA, CHCA, EACA, MECA, and WCA-with diclofenac sodium as the standard drug using the egg albumin denaturation assay. Values represent Mean ± SD (n = 3). Statistical analysis was executed with one-way ANOVA followed by Tukey's multiple comparison test. Asterisk indicates statistically significant differences between diclofenac and the respective extract ($p < 0.05$, $p < 0.01$, $p < 0.001$).

semi-polar components. Methanol, ethyl acetate, petroleum ether, and other extracts showed moderate to poor action.

The model of paw oedema caused by carrageenan is an accepted method for assessing acute inflammation in animal research (Zlatanova-Tenisheva & Vladimirova, 2025). Paw oedema was considerably reduced by *C. aloifolium* extract in a way that was dependent on both time and dosage. At 200 mg/kg, the extract showed anti-inflammatory activity comparable to diclofenac from the 3rd hr onward. This implies possible action against both the early stage of inflammation (mediated by serotonin and histamine) and the late stage (mediated by bradykinin and prostaglandins) (Nguyen *et al.*, 2021). Because of the observed dose-response relationship, the *C. aloifolium* extract may prevent the production or activity of inflammatory mediators. These results are in alignment with observations indicating phytochemicals, including alkaloids and flavonoids, can block the synthesis of cytokines and cyclooxygenase, thus modulating inflammatory pathways (Al-Khayri *et al.*, 2022).

HPTLC analysis suggested the putative presence of caffeine-like compounds, as indicated by R_f values close to that of standard caffeine. Caffeine, a well-documented alkaloid with known anti-inflammatory properties, exerts its effects through multiple mechanisms (Romero-Martinez *et al.*, 2021). The tentative presence of caffeine-like compounds in *C. aloifolium* extracts may partially explain the observed anti-inflammatory activity, especially in the methanolic and chloroformic extracts, which

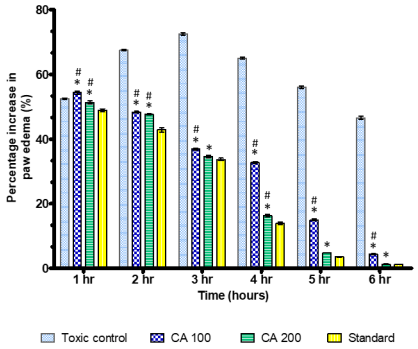

Figure 2: The proportion of increased paw oedema volume. Rats were randomly separated into five groups: Normal control, toxic control, diclofenac (STD), CA (100 mg/kg), CA (200 mg/kg). Data are depicted as Mean ± SD (*n*=6 animals per group). **p* < 0.05 against toxic control (carrageenan) Rats, #*p* < 0.05 against standard (Diclofenac).

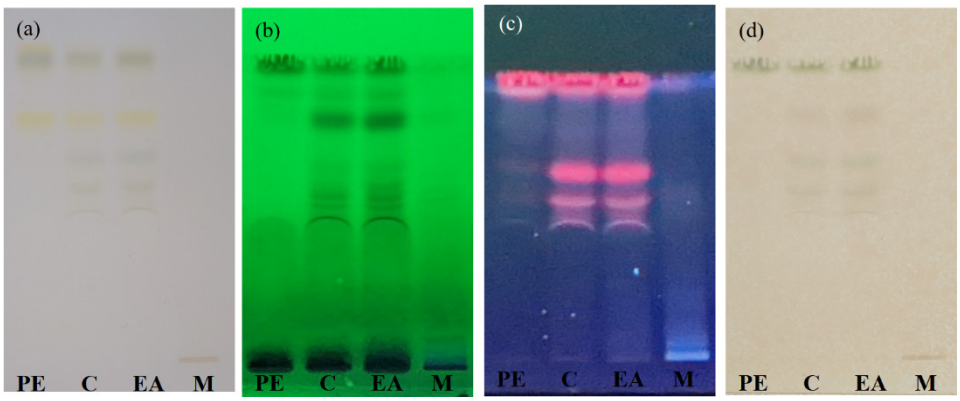

Figure 3: HPTLC fingerprinting of different leaf extracts of *C. aloifolium* with mobile phase, where PE (Pet ether), C (Chloroform), EA (Ethyl Acetate), and M (Methanol). (a) White light, (b) UV 254 nm, (c) 366 nm, (d) White light after Derivatisation with ferric chloride.

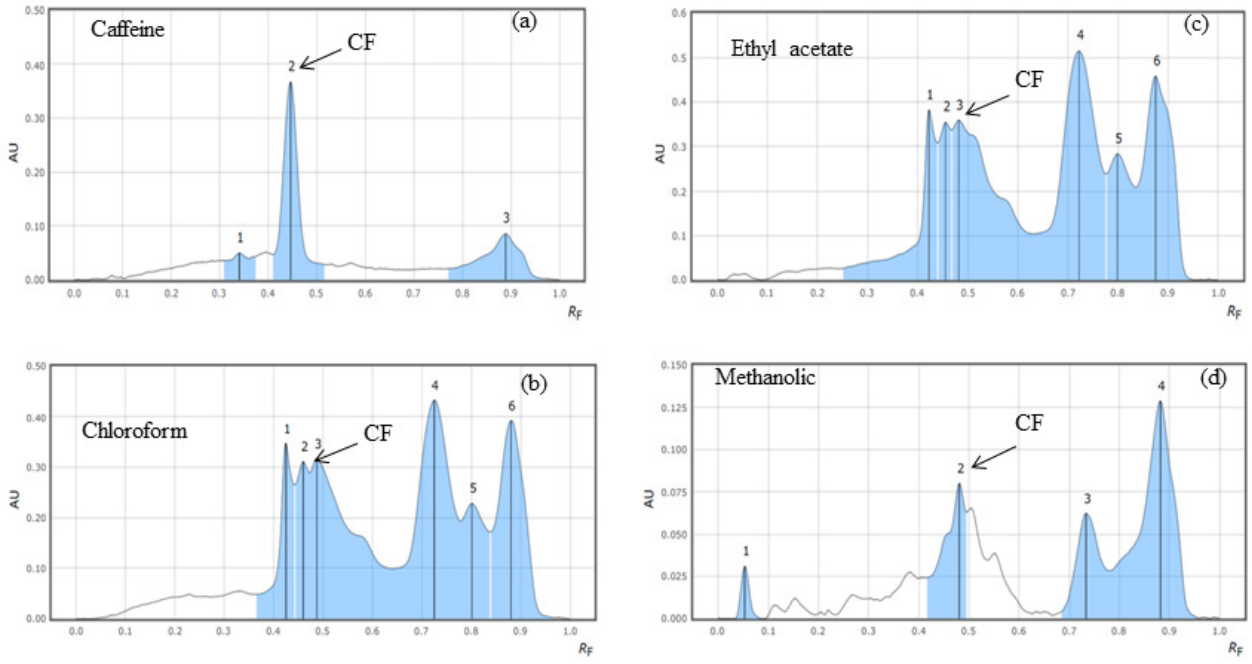

Figure 4: HPTLC densitometric chromatograms of standard caffeine and selected solvent extracts of *C. aloifolium* recorded at 254 nm using Toluene : Ethanol: Formic acid (5:4:1, v/v/v) as the mobile phase.

demonstrated enhanced anti-inflammatory activity in the *in vitro* test.

By detecting functional groups like O-H, C=O, and C-H stretching that are comparable to those found in regular caffeine, FT-IR spectroscopy validated this conclusion (Kwaśniewska-Sip *et al*., 2021). Alkaloids, amides, and phenolic composites-all of which are well-known to have analgesic, anti-inflammatory, and antioxidant properties—are supported by these spectrum signatures. The suggested involvement of caffeine and flavonoids in the reported biological effects is supported by the chemical in accordance between the FT-IR and HPTLC investigations (Hilal *et al*., 2024).

The *C. aloifolium* extract is safe for usage within the studied dose range, according to the acute toxicity. The acute toxicity study showed that the *C. aloifolium* extract was safe up to a dose of 2000 mg/kg, with no signs of toxicity or mortality. This suggests a wide safety margin and supports its potential use in therapeutic applications (Okafor *et al*., 2025).

CONCLUSION

Among the tested extracts, the chloroform extract of *Cymbidium aloifolium* leaves demonstrated the most consistent and significant anti-inflammatory activity in both *in vitro* and *in vivo* models. Additionally, the phytochemicals found in the extracts have been assessed using FT-IR analysis and HPTLC fingerprinting, which suggests the presence of caffeine-like alkaloidal and flavonoid constituents. Numerous pharmacological activities reported in the literature are associated with caffeine-like alkaloidal constituents and flavonoids. Consequently, *C. aloifolium* leaves represent a promising source of bioactive phytoconstituents for further pharmacological investigation. Further studies employing advanced analytical techniques such as LC–MS/MS and NMR are required to convincingly recognize and characterise the bioactive compounds responsible for the observed anti-inflammatory effects.

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ABBREVIATIONS

HPTLC: High-Performance Thin-Layer Chromatography; **FT-IR:** Fourier-Transform Infrared Spectroscopy; **NSAIDs:** Non-Steroidal Anti-Inflammatory Drugs; **OECD:** Organisation for Economic Co-operation and Development; **IAEC:** Institutional Animal Ethics Committee; **ANOVA:** Analysis of Variance; **SD:** Standard Deviation; **IC_{50}:** Half Maximal Inhibitory Concentration; **UV:** Ultraviolet; **R_f:** Retardation Factor (Retention Factor); **KBr:** Potassium Bromide; **LC–MS/MS:** Liquid Chromatography–Tandem Mass Spectrometry; **NMR:** Nuclear Magnetic Resonance.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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