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Synthesis and pharmacological characterization of new curcumin ester pro-drugs with enhanced anti-inflammatory, anti-ulcerogenic and improved tissue distribution

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ABSTRACT

In this work, curcumin- Non steroidal anti-inflammatory drugs (NSAIDs) conjugates were synthesized by esterification of the phenolic group of curcumin with the acid group of NSAIDs using the Steglich esterification method to enhance the anti-inflammatory activity of curcumin and to reduce the gastrointestinal side effects of NSAIDs. A set of bis C-NSAIDs conjugates was prepared by direct coupling of curcumin with Aceclofenac and Diflunisal respectively in good yield and characterized by Fourier-transform infrared spectra (FTIR) and Proton Nuclear Magnetic Resonance Spectra (1HNMR). In vivo, studies were performed to evaluate the toxicity, anti-inflammatory activity, bio-distribution, and anti ulcerogenic studies of selected conjugates using different animal models. In Vivo studies reveal the enhanced biological activity with better tissue distribution of conjugates than that of parent curcumin and it could be a curcumin-based therapeutic molecule that might be explored further for its efficacy in managing inflammatory diseases.

Keywords: Curcumin, Curcumin-NSAIDs conjugates, Steglich esterification, Anti-inflammatory, Anti- ulcerogenic activity.

INTRODUCTION

Curcumin (CUR), a well-known natural compound derived from Curcuma longa, commonly known as Turmeric, shows many bio-functional qualities including anti-tumour, antioxidant, and anti-inflammatory actions. CUR and its derivatives have drawn a lot of interest in the last 20 years [1-3]. In the form of Turmeric, it was applied topically to alleviate sprains and oedemain Ayurvedic medicine. As an anti-inflammatory agent, it reduces the activity of lipoxygenase, Cyclooxygenase enzyme COX-1 and COX-2, Tumour necrosis factor alpha (TNF-α), Interleukins (IL1, 2, 6, 8, and 12), as well as Monocyte chemoattractant protein (MCP). In addition to affecting the activity of enzymes, growth factor receptors, cofactors, and other molecules, CUR is a highly pleiotropic chemical with a variety of targets and methods of action [4]. However, the clinical application of CUR is limited due to low water solubility, poor absorption, quick metabolism, and rapid elimination, all of which cause poor oral bioavailability, and have impeded its development as a therapeutic agent [5-11]. CUR shows keto enoltautomerism, and there are three sites accessible for drug/ligand attachment to generate new conjugates, namely two phenolic and one active methylene group [12-13].In view of this CUR was conjugated with various amino acids shown to have good permeability as well as improved anti-cancer efficacy [14-15]. CUR when given with piperine enhances the serum level and oral bioavailability in both humans and rats [16].CUR diclofenac conjugates were shown to enhance bioavailability and anti-inflammatory action in conditions of arthritis [12].

NSAIDs are well known to produce gastrointestinal problems including gastric irritation and ulcers mainly because of the presence of the free carboxylic group present in their structures. However, CUR has received little attention in terms of increasing anti-inflammatory benefits and mitigating the negative effects of

NSAIDs (Non-steroidal anti-inflammatory drugs) when used in conjunction with NSAIDs [17].

In this context, we have synthesized bisconjugates of CUR with NSAIDs (Diflunisal and Aceclofenac) via an ester linkage at the site of the phenyl hydroxy group of CUR. Steglich esterification method was adopted for the present work and the ester linkage was made by using Dichloromethane (DCM) as a solvent, Di cyclohexylcarbodiimide (DCC) as a coupling agent, and Dimethyl amino pyridine (DMAP) as a catalyst [18]. Various aspects are considered when choosing NSAIDs. For this research, Diflunisal (DF) was selected due to the presence of small lipophilic group fluorine that also increases stability. Aceclofenac (ACE) wasselected due to its effectiveness in severe pains associated with arthritis and ankylosing spondylitis condition.

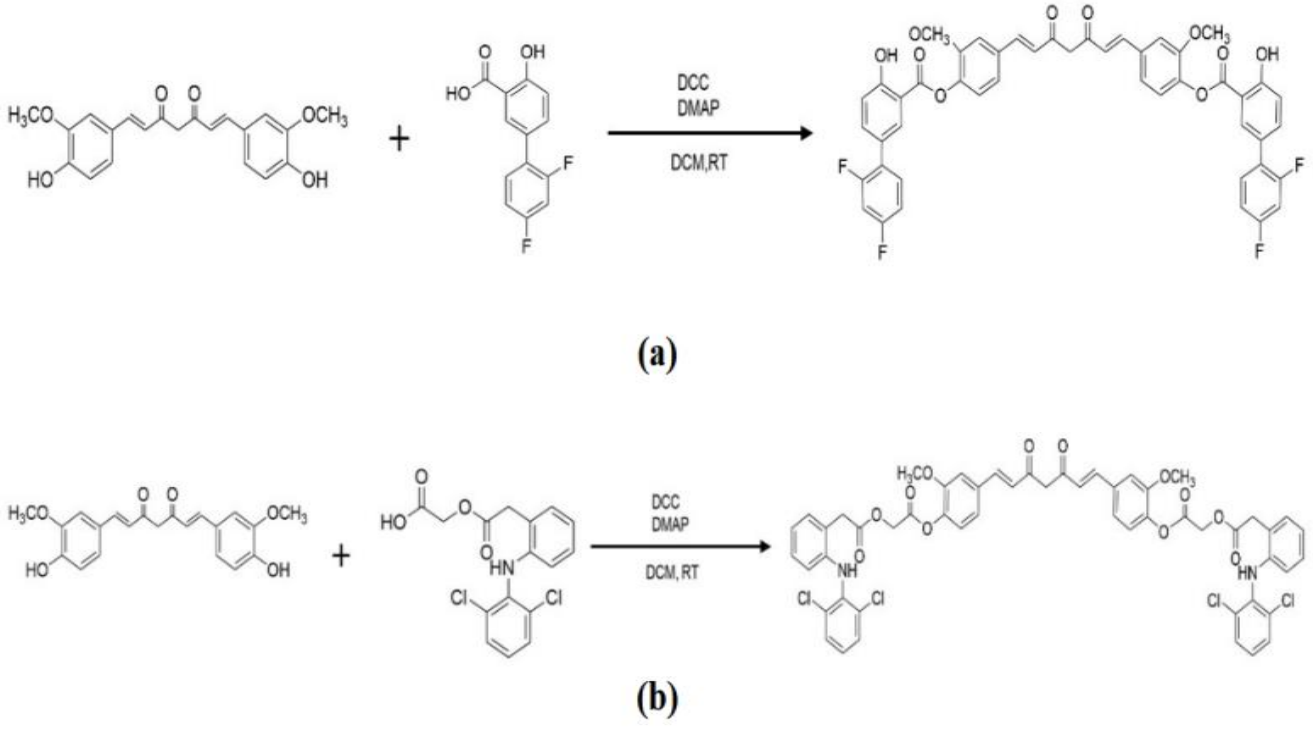
MATERIALSAND METHODS

All reagents and solvents used were analytical grade that was purchased fromHI media and SRL, India. Aceclofenac and Diflunisal were obtained from Stallion Lab. Pvt. Ltd., Gujarat and Prince Scientific, Hyderabad, India. Thin layer chromatography (TLC) was performed by using Silica gel- G plates to check the completion of the reaction. Purification of compounds was done by column chromatography using Silica gel (60-120 mesh). FTIR Spectra of synthesized compounds were taken using PerkinElmer spectra version 10.7.2 from the 4000 cm^{-1} to 450 cm^{-1} range. ^1H NMR Spectra were recorded on BrukerAvance neo-500 MHz NMR spectrometer using $CDCl_3$ as the solvent and chemical shift was recorded as δ (ppm). Melting points (M.P.) were determined by the open capillary tube method using a digital melting point/boiling point apparatus.

Synthesis of Bis CUR-NSAIDs Conjugates

CUR (1 mmol), DCC (1.1 mmol), DMAP (0.1 mmol), and NSAID (2.1 mmol) were taken and dissolved in 30 ml of DCM. The mixture was stirred and allowed to stand for 24 hrs for completing the reaction. TLC evaluation using Ethyl Acetate: Hexane (4:6) of the crude reaction mixture confirmed the formation of bis-adducts (B-CUR-ACE and B-CUR-DF) and unreacted CUR having Rf value 0.75, 0.73, 0.70 respectively. To neutralise the organic phase that might contain the acidic compounds, the crude mixture was washed with a saturated NaHCO3 solution to separate the DCM layer.Then column chromatography was performed to separate the products by using ethyl acetate (40%) in hexane. The solvent was removed from fractions containing the appropriate products, leaving an oil, which was crystallized by dissolving in DCM and adding hexane followed by filtration and drying [18-19] (Yield: B-CUR-ACE: 34.4%, B-CUR-DF: 40.2%)(Figure 1).

Figure 1: a) Reaction scheme of B-CUR-DF (Bis CUR Diflunisal), b) Reaction scheme of B-CUR-ACE (Bis CUR Aceclofenac)



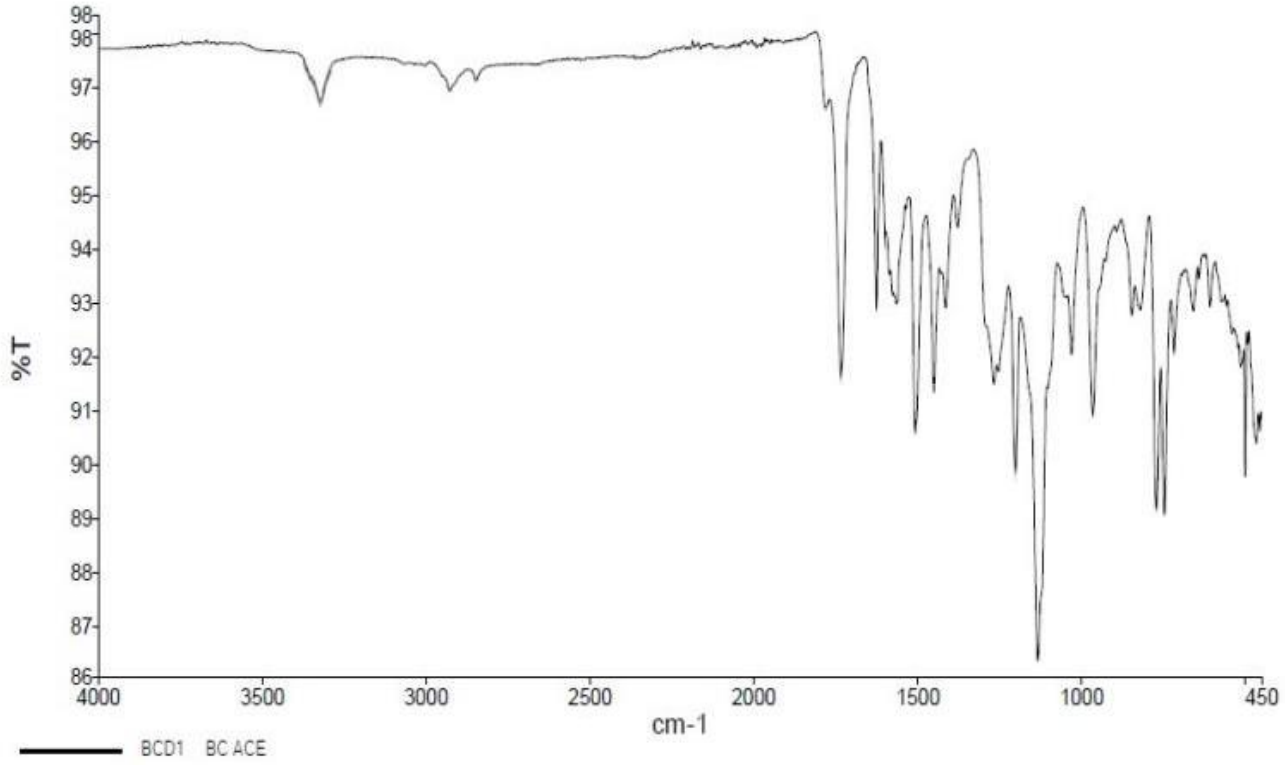
Analysis and Characterization of CUR-NSAIDS Conjugates

B-*CUR-ACE*

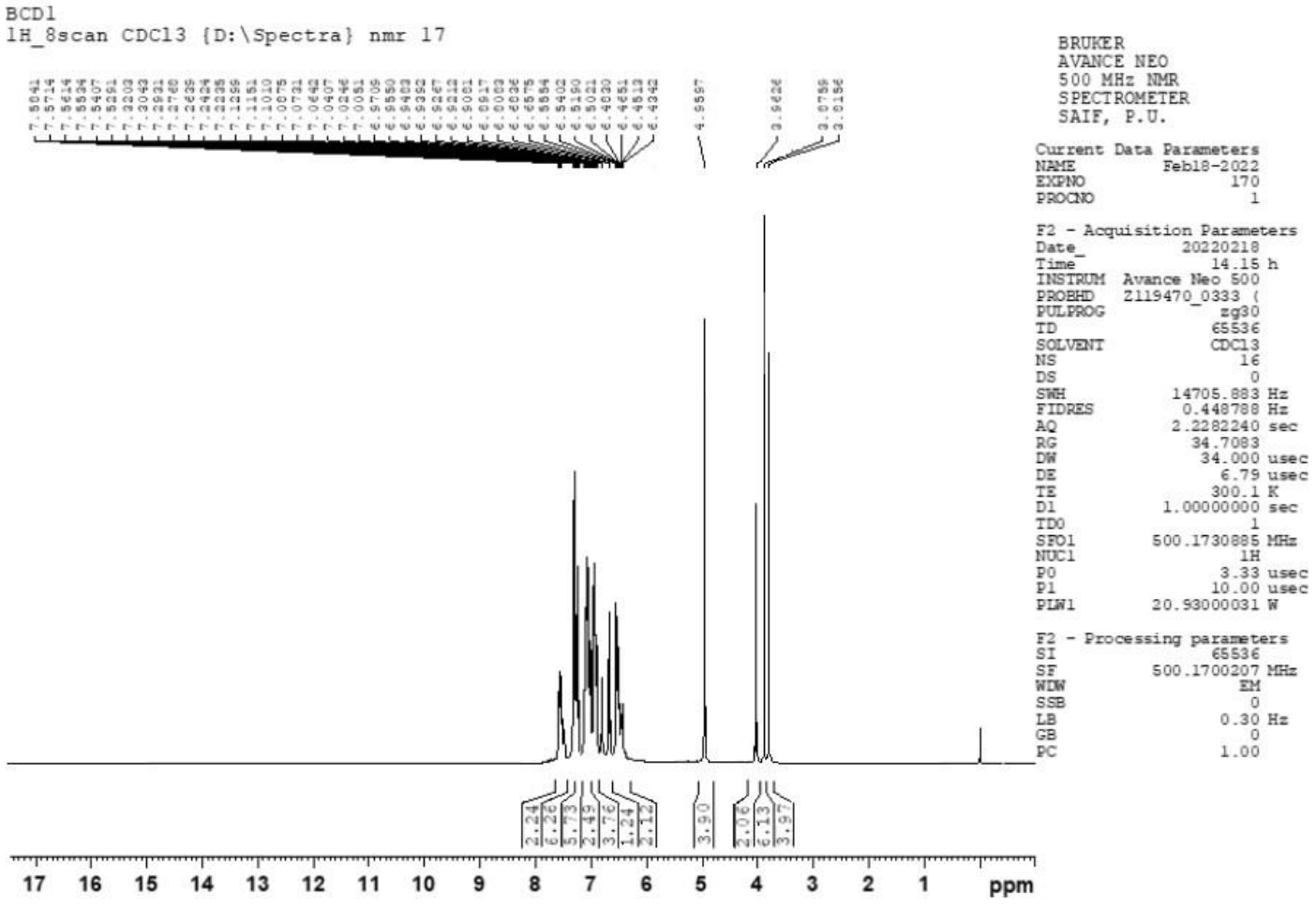
Molecular formula:$C_{53}H_{42}Cl_4N_2O_{12}$, Yield: 34.4%, Yellow solid, M.P. 101°C, Rf (40% ethyl acetate in hexane): 0.61, ^{1}HNMR ($CDCl_3$, 500 MHz): δ 7.59-7.48 (Aromatic -H, 8H, 7.58 (d, J = 15.7 Hz), 7.56 (dd, J = 7.9, 1.6 Hz), 7.52 (dd, J = 8.3, 1.9 Hz)), 7.32-7.22 (Aromatic -H, 6H, 7.32 (dd, J = 1.9, 0.5 Hz), 7.29 (d, J = 16.1 Hz), 7.27 (dd, J = 8.3, 0.5 Hz), 7.24 (ddd, J = 7.9, 1.4, 0.6 Hz), 7.22 (dd, J = 8.3, 0.5 Hz)), 7.11-7.00 (Aromatic -H, 2H, 7.08 (ddd, J = 8.1, 7.5, 1.4 Hz), 7.02 (ddd, J = 8.1, 7.5, 1.4 Hz)), 6.95-6.60 (Aromatic -H, 4H, 6.90 (ddd, J = 7.9, 7.5, 1.2 Hz)), 6.65 (1H, d, J = 15.6 Hz), 6.55-6.43 (Aromatic -H, 2H, 6.50 (ddd, J = 8.1, 1.2, 0.6 Hz), 6.43 (ddd, J = 8.1,

1.2, 0.6 Hz)), 4.95 (-CH_2, 4H, s), 3.96 (-CH_2, 2H, s), 3.90-3.81(-OCH_3, 10H, 3.87 (s), 3.81 (s)). IR: 3380 (-NH), 2950 (-CH), 1750 (-COO), 1600 (Carbonyl C=O), 1507 (C=O), 1252 (-CH_2), 1132 (C-CH), 770-746 (C-Cl) (Figure 2).

Figure 2: (a) FTIR spectra of B-CUR-ACE, (b) NMR spectra



(a)

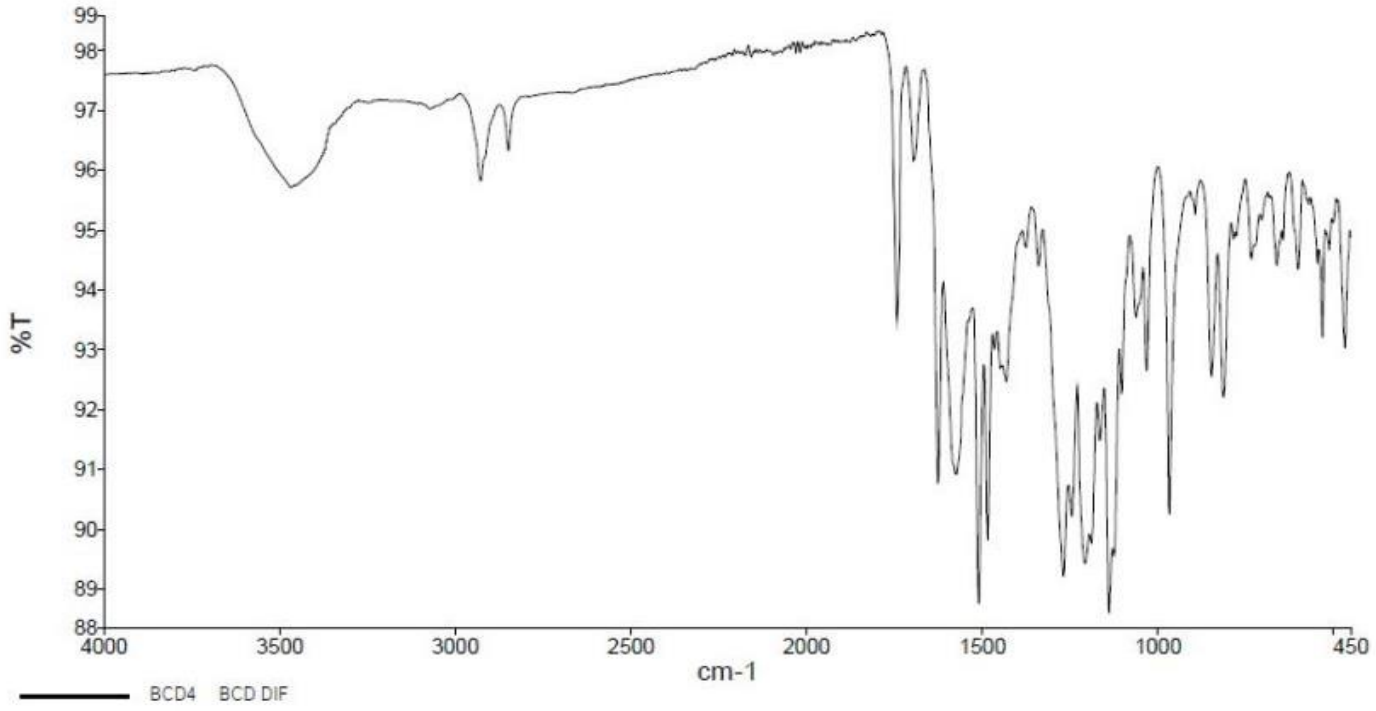


(b)

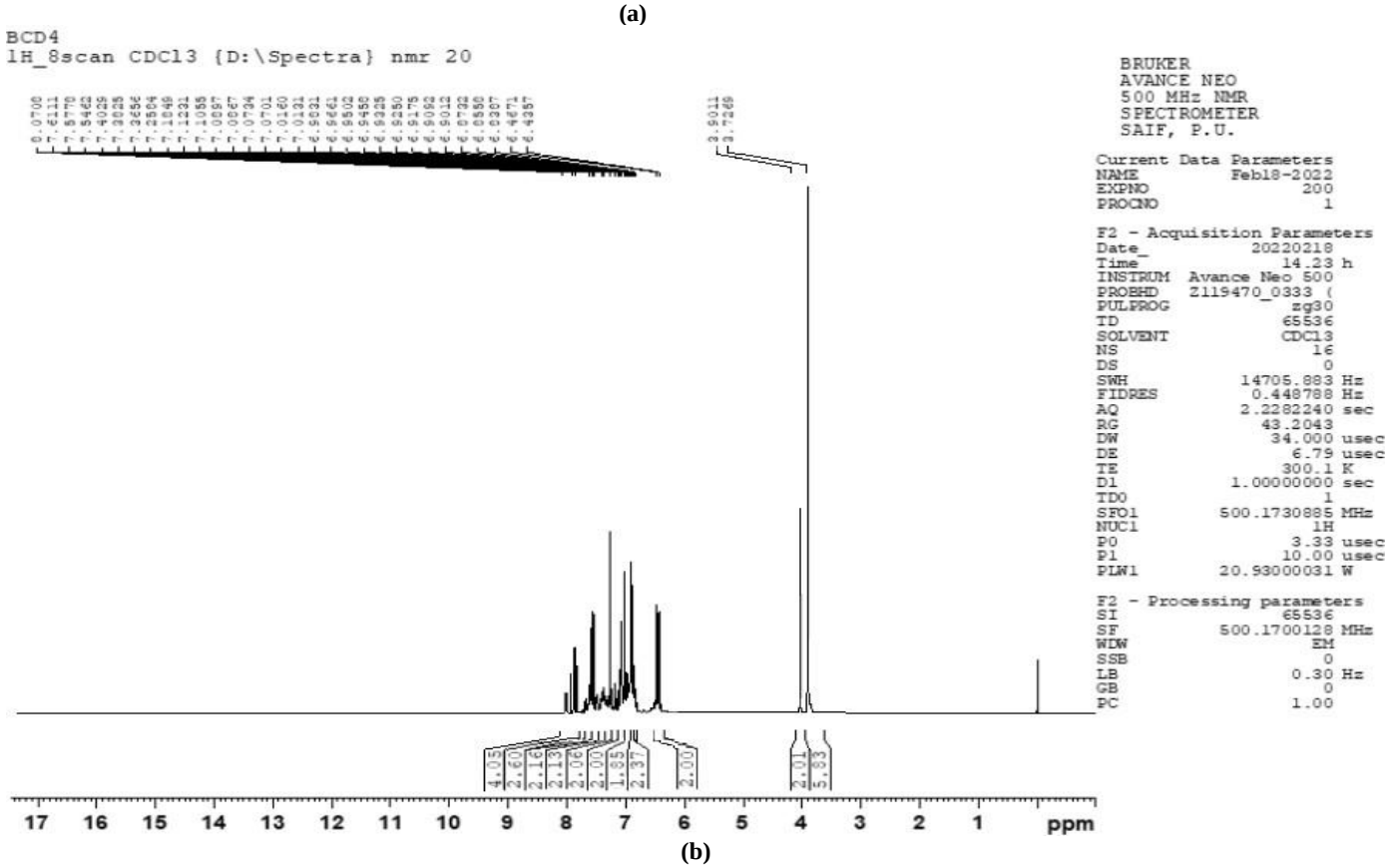
B-CUR-DF

Molecular formula: $C_{47}H_{32}F_4O_{10}$, Yield: 40.2%, Yellow solid, M.P. 110℃, Rf (40% ethyl acetate in hexane): 0.72, ^{1}HNMR ($CDCl_3$, 500 MHz): δ 8.07-7.61 (Aromatic -H, 4H, dd, J = 8.5, 0.5 Hz), 7.54 (Aromatic -H, 2H, d, J = 15.6 Hz), 7.40 (Aromatic -H, 2H, d, J = 15.6 Hz), 7.36 (Aromatic -H, 2H, dd, J = 8.9, 1.8 Hz), 7.25 (Aromatic -H, 2H, dd, J = 8.3, 0.5 Hz), 7.18 (Aromatic -H, 2H, dd, J = 8.9, 0.4 Hz), 7.10 (Aromatic -H, 2H, dd, J = 8.5, 1.7 Hz), 7.01 (Aromatic -H, 2H, dd, J = 8.5, 1.7 Hz), 6.43 (-CH, 2H, d, J = 15.6 Hz), 3.90 (-CH_2, 2H, s), 3.72 (-OCH_3, 6H, s). IR: 3480 (-OH), 2980-2900 (-CH), 1710 (-COO), 1625 (Carbonyl C=O), 1509 (C=O), 1268 (-CH_2), 1206 (C-F), 1138 (C-CH) (Figure 3).

Figure 3: (a) FTIR spectra of B-CUR-DF, (b) NMR spectra of B-CUR-DF



(a)



(b)

Pharmacological Characterizations

The animal tests were carried out after receiving approval from the Ethics Committee of the Department of Pharmacy, Guru Ghasidas Vishwavidyalaya, Bilaspur, C.G. (India), and experiments were carried out in compliance with the standard guidelines set out by the Ethics Committee (Registration no. 994/GO/Re/S/06/CPCSEA). Wistar rats of either sex (Weight 150-200 g) were used in the present *in vivo* studies. The rats were housed in a cage system in a laboratory environment (temp. 25 ± 2 ℃, humidity 37± 2%, 12 h light).Animal feed pellets were given to them. Water was provided ad libitum and food was withdrawn 12 hours prior to the experiment. Animals were chosen at random for different experimental groups (six animals per group) and utilised to investigate *in vivo* evaluation.

Acute Oral Toxicity Study

The acute toxicity test was carried out according to the OECD guideline 420: acute oral toxicity [OECD (The Organisation of Economic Co-operation and Development), 2001]. The female rats were divided into two groups of six each. The test group was treated orally with 300, 500 and 2,000 mg/kg. The control group was given an equal volume of 0.5% Carboxy methyl cellulose. Mortality and signs of toxicity were monitored at 1, 2, 4, and 6 hours after oral administration and then once daily for 14 days after dosing. The body weights of the rats were recorded prior to and at 7 and 14 days after treatment [20].

***In Vivo* Anti-inflammatory activity**

The anti-inflammatory activity of the compounds was performed by using the carrageenan-induced rat paw oedema model. Male or female Wistar rats with body weight150-200 g were taken and divided into groups of six each according to their weight. Test drugs and standard drugs were suspended in 0.5% carboxy methyl cellulose (CMC). The standard group received 300 mg/kg of CUR [21], the test group received 300 mg/kg of Bis-CUR-DF and Bis-CUR-ACE, and the control group received the water orally. After 30 min 0.1 ml of 1% w/v carrageenan was injected into the sub planter side of the right paw subcutaneously. The paw was marked with ink at the planter region and immersed in mercury up to the mark to determine paw volume. Mean normal paw was measured prior to carrageenan injection by using Plethysmometer. The mean increase in the paw volume of standard, control and test groups was measured at 0, 60, 120, and 180 min [22] and the volumes of oedema were obtained in ml. The mean values were obtained by using two-way ANOVA with Dunnett's t-test. Percent inhibition of inflammation was calculated in comparison with a control group using the following formula [23] (Figure 4).

$$\% \text{ Inhibition} = \frac{\text{Mean Oedema of Control Group} - \text{Mean Oedema of Test Group}}{\text{Mean Oedema of Control Group}} \times 100$$

Figure 4: Carrageenan-induced paw oedema study for anti-inflammatory activity, Tissue distribution studies

Rats were not fed after being starved for 24 hours previous to the dose. At various times, the animals were sacrificed after receiving 300 mg/kg of CUR and B-CUR-DF by oral gavage. About 2 ml of blood from the heart was collected. The stomach, small intestine, liver, kidneys, and heart were all removed. Rats that had not received a dosage of CUR were used as controls for the collection of tissue. Except for blood, all tissues were stored frozen at 0°C until analysis. After sample preparation, the samples were subjected to HPLC analysis (Model no. UV 3000 S/N: UV 1506119141)[24].

HPLC Analysis

HPLC method for analysis of CUR and B-CUR-DF was performed using HPLC model no. UV 3000 S/N: UV 1506119141 manufactured by Analytical Technologies Ltd. A gradient mobile phase system consisting of Acetonitrile- Tetrahydrofuran and water (35:20:45 for 0-8 min, 35:20:45 to 50:40:10 for 8-13 min and 50:40:10 for 13-21 min) was employed for sample analysis. The flow rate was set to 1.0 ml/min and the detection wavelength was 424 nm. The sample temperature was kept under control at 4 ℃ while the column temperature was maintained at 30 ℃[25-26].

Ulcerogenicity Study

The animals utilised were Wistar rats (n = 6, 150–180 g). The rats were starved for 12 hours before the drug solution was administered and for 4 hours after the dose. At all other times, food was made available, and water was made freely available throughout the experiment. One group of rats (control) did not receive any drug, while the other groups received either the standard drug (Diflunisal) or the pro-drugs (B-CUR-DF) in 0.5% CMC which were administered orally. The rats were treated with a single dosage and a chronic dose (same dose every day for four days).The rats were sacrificed 4 hours after the single dosage and 24 hours after the final dose of the chronic treatment. Along with the 5 cm of the intestine, the stomach was separated out and cleaned with saline. Using a swab dipped in saline, the stomach and intestine were carefully cleaned after being cut open along the larger curvature. Under a magnifying glass, the mucosal damage was investigated in detail [27]. And Histo pathological studies of gastric tissue were investigated

microscopically [28]. A modified version of a previously reported grading scale was used to determine the severity of the mucosal injury, and the result is indicated in parenthesis. The following ulcer scores were calculated by magnifying the ulcers: 0 for no ulcers, 1 for superficial mucosal erosion, 2 for deep ulcers, and 3 for perforated or penetrated ulcers. The specimen was given an ordinal score according to the grading system based on the degree of mucosal injury. However, the control specimen received a score of 0 because lesions or ulcers had not appeared on it. The scores were tabulated, and the mean score was provided as an indicator of the severity of the drug solution that was administered [29].

RESULT AND DISCUSSION

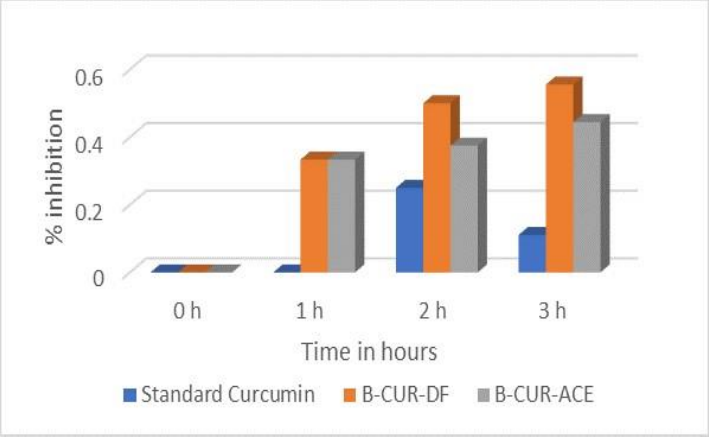
Bis conjugates of CUR with Aceclofenac and Diflunisal (B-CUR-DF and B-CUR-ACE) were synthesized and FTIR and Proton NMR were recorded and found to be similar to the structure of CUR-NSAIDs conjugates. During the acute oral toxicity test, a single treatment of test drugs did not show mortality in any of the tested rats during the period of observation. No signs of toxicity, behavioural changes, or body weight were observed.

Synthesized ester conjugates of CUR were then evaluated for anti-inflammatory potential. Standard and test samples were given orally at a dosage of 300 mg/kg. Ester conjugates of CUR showed higher inhibition of carrageenan-induced inflammation than that of parent CUR. Carrageenan-induced oedema is thought to be biphasic, with the first phase lasting one hour and involving the release of serotonin and histamine, and the second phase lasting more than an hour and being mediated by prostaglandins, which are cyclooxygenase products.

Table 1: Percent inhibition of inflammation after 1, 2 and 3hours

Treatment	% Inhibition after 0 h	% Inhibition after 1 h	% Inhibition after 2 h	% Inhibition after 3 h
Standard CUR	0	0%	25%	11.11%
B-CUR-DF	0	33.33%	50%	55.55%
B-CUR-ACE	0	33.33%	37.5%	44.44%

Figure 5: Graph of percent inhibition of paw oedema



The percent inhibition of standard CUR was maximum at 2 hrs (25%). As expected, B-CUR-ACE and B-CUR-DF significantly decreased paw oedema with an inhibition level of 55.55% and 44.44% respectively at 3 h (Table 1, Figure 5). Therefore, current findings support the theory that curcumin's anti-inflammatory impact involves a decrease in prostaglandins through cyclooxygenase inhibition.

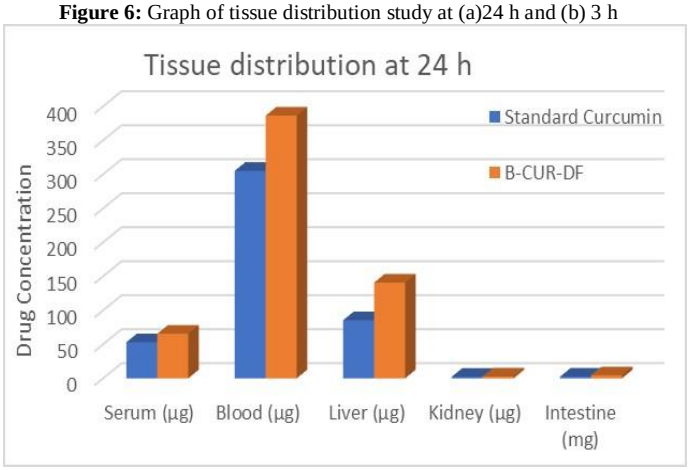
The distribution of standard CUR and B-CUR-DF in serum, blood, liver, kidney and intestine is shown in table 2.

Table 2: Tissue distribution studies at 3 h and 24 h

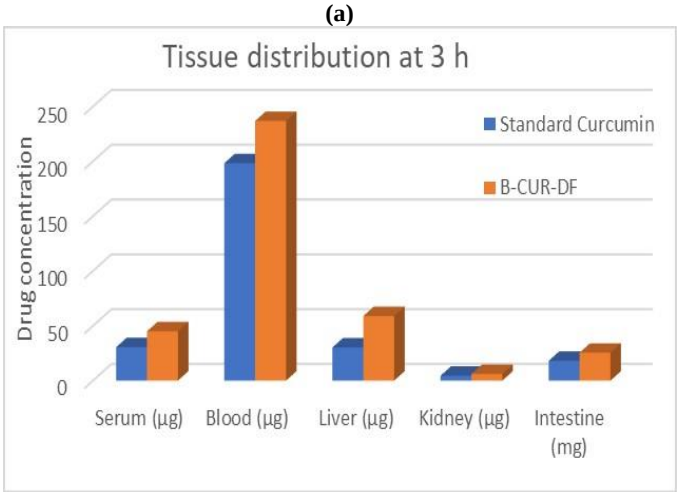
Tissue distribution at 3 h					
Treatment	**Serum (µg)**	**Blood (µg)**	**Liver (µg)**	**Kidney (µg)**	**Intestine (mg)**
Standard CUR	30.52 ±2.28	198.30 ±12.5	30.26 ±3.80	4.50 ±0.58	18.06 ±1.45
B-CUR-DF	45.02 ±3.50	236.85 ±20.74	58.90 ±2.78	6.08 ±1.18	25.46 ±2.08
Tissue distribution at 24 h					
Treatment	**Serum (µg)**	**Blood (µg)**	**Liver (µg)**	**Kidney (µg)**	**Intestine (mg)**
Standard CUR	53.04 ±2.86	305.2 ±15.10	85.5 ±2.02	2.20 ±0.52	2.42 ±0.53
B-CUR-DF	65.57 ±1.84	386.41 ±8.98	140.85 ±6.53	2.74 ±1.08	4.51 ±1.54

Results are expressed as Mean ± SD (n=5)

Figure 6: Graph of tissue distribution study at (a)24 h and (b) 3 h



(a)



(b)

At 3 h, the concentration of CUR from B-CUR-DF in serum, blood, liver, and kidney was 45.02, 236.85, 58.90, 6.08, µg/ml respectively which is significantly higher than that of standard CUR with concentrations of 30.52, 198.30, 30.26, and 4.50 µg/ml (Figure 6). At 3 h and 24 h, amounts of CUR in B-CUR-DF in blood were 237 µg and 386 µg which is about 20% and 26% higher than the standard CUR. CUR concentration in the liver at 3h and 24 h were

about 96% and 66% respectively higher than the standard CUR. The concentration of standard CUR in the intestinal tissue gradually decreased from 18.06 mg at 3 h to 2.42 mg by 24 h. The data obtained indicates the tissue distribution of CUR from B-CUR-DF is greater than the standard CUR.

During the ulcerogenic study, the rats treated with B-CUR-DF shows a significant reduction in ulcers than the standard Diflunisal. In the histo pathological investigation, the gastric tissues were examined under a microscope, and tissue samples from rats in the control group revealed normal histological findings.

Figure 7: Ulcerogenicity studies on standard diflunisal (DF) and its pro-drug B-CUR-DF

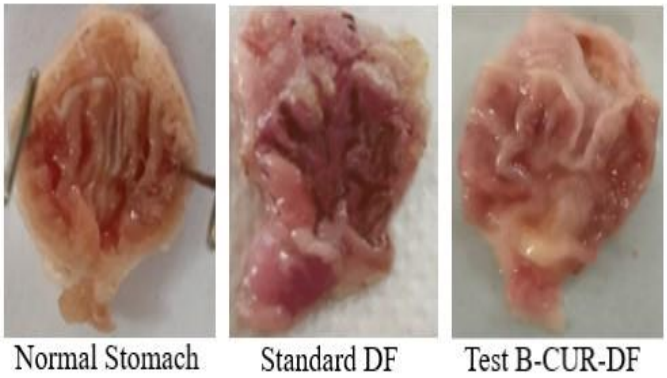
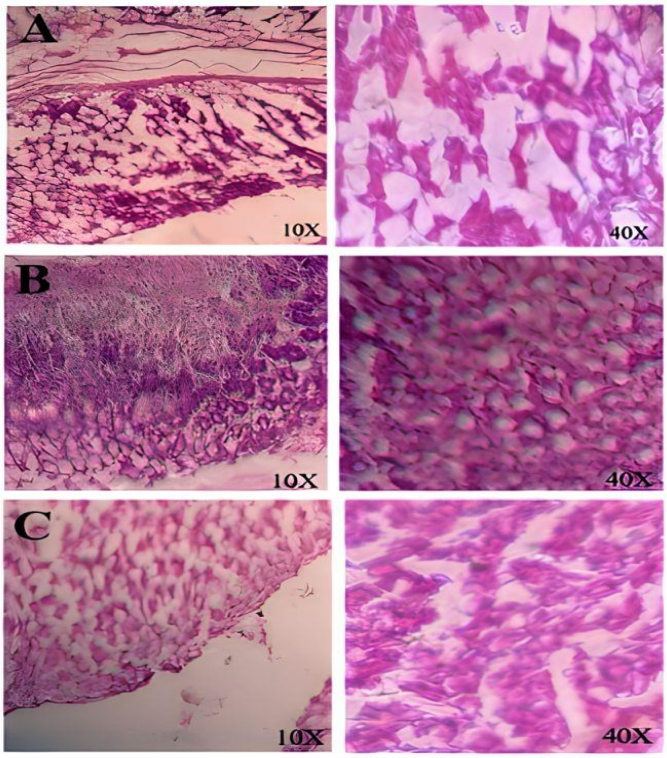


Figure 8: Histological investigations of (A) Control, (B) Diflunisal treated, (C) B-CUR-DF treated groups



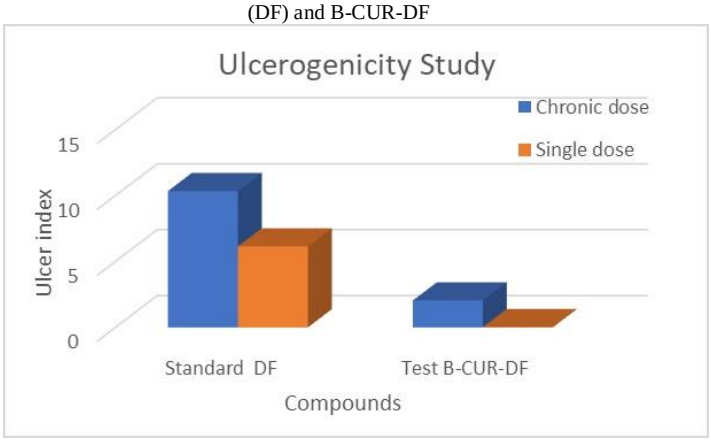
Microscopic examination of Diflunisal disclosed a localised erosive region in the stomach mucosa (Figure 7). In this zone, nuclei of the cells had contracted and become denser, as well as their cytoplasm was stained with dark eosinophilic bodies (Figure 8). Normal histological findings were shown for B-CUR-DF, indicating that the pro-drug did not cause stomach ulcers.

The membrane of rats treated (single dose or chronic dose) with B-CUR-DF showed no significant mucosal damage or ulcers with 99.67% and 80.27% protection from ulcers in single dose treatment and chronic treatment respectively (Table 3, Figure 9).

Table 3: Calculation of ulcer index

Treatment	Standard DF	Test B-CUR-DF
Chronic dose	10.34	2.04
Single dose	6.14	0.02

Figure 9: Ulcer index in rats following a single and chronic dose of diflunisal (DF) and B-CUR-DF



CONCLUSION

In summary, this study shows the synthesis of CUR-NSAIDs conjugates via the Steglich esterification method and is characterized by ^{1}HNMR and FTIR Spectroscopy. Acute oral toxicities and anti-inflammatory activities were investigated where synthesized pro-drugs were found to be more active than that of parent CUR showing higher inhibition in the second phase. Tissue distribution studies prove the enhancement of bioavailability and tissue distribution of CUR given in the form of B-CUR-DF pro-drug. Ulcerogenic studies were carried out in order to lessen the negative effects of NSAIDs. In comparison to the control and Diflunisal-treated groups, the B-CUR-DF pro-drug showed normal results (no ulcers). These CUR pro-drugs can become a medium to overcome all the barriers associated with the delivery of CUR by the oral route and the potential use of these pro-drugs might be an option to treat many diseases in the near future.

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CONFLICTS OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

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