Materials and Methods

3.1 Materials

Curcumin (purity ≥ 95%) was obtained from Himedia, India. All solvents used in this study were Liquid Chromatography-Mass Spectrometry (LC-MS) grade. Dichloromethane and acetonitrile were purchased from Thermo Fisher Chemicals (Mumbai, India). Milli-Q grade water (Millipore, Bedford, MA, USA) was used for the preparation of solution and mobile phase.

3.2 Methods

3.2.1 Synthesis of curcumin di-acetate

Curcumin was added in excess amount of acetic anhydride in the presence of concentrated sulphuric acids. The mixture was stirred for 30 min at room temperature and kept overnight. Next day the aqueous solution was extracted with chloroform followed by the wash of the organic extract with ice cold water. The reaction products were separated by column chromatography (Kieselgel 60, Art 7731, 70-230 mesh, Merk) with dichloromethane as eluting solvents.

3.3 Characterization of curcumin di-acetate

3.3.1 Infrared spectroscopy

FTIR spectra of curcumin di-acetate was recorded using FTIR spectrophotometer (Agilent Cary 630 FTIR Spectrometer). The scanning range was 4000 to 650 cm\(^{-1}\) and the resolution was 8 cm\(^{-1}\).

3.3.2 Mass spectroscopy

Mass spectra (low resolution) of the synthesized derivative, curcumin acetate were recorded at MAT 120 in SAIF, Panjab University, Chandigarh.
3.3.3 $^1$H NMR and $^{13}$C NMR spectra

$^1$H NMR and $^{13}$C NMR spectra of curcumin di-acetate, was recorded on Bruker Advance II 400MHz NMR spectrometers in SAIF, Panjab University (Chandigarh) using tetramethylsilane (TMS) as the internal standard. All spectra were obtained in DMSO and CDCl$_3$ as a solvent. Chemical shifts values are reported as values in ppm relative to TMS as internal standard. Chemical shifts are expressed in δ units; Coupling constants (J) are given in Hertz (Hz).

3.4 Quantification of curcumin and curcumin derivative in rat brain

3.4.1 HPLC system for curcumin

The HPLC system consisted of a chromatographic binary pump (G1312A, Agilent, German), autosampler (G1329A, Agilent, German), diode array detector (G1315D, Agilent, German). For HPLC separation, a reversed-phase C18 column (4.6 × 150 mm, particle size 5µm, Eclipse XDB, Agilent, USA) was used. The mobile phase for curcumin was composed of acetonitrile-10 mM potassium dihydrogen phosphate (pH 4.0 adjusted by acetic acid) (50:50, v/v) at a flow-rate of 0.8 ml/min. The run time for analysis was 20 min and the detection wavelength was set at 425 nm. The mobile phase was filtered through a 0.45µm Millipore membrane filter Nylon 66 and degassed by sonication (Ultrasonic Cleaner-30A) before use. The sample injection volume was 20 µl.

3.4.2 HPLC system for curcumin di-acetate

The mobile phase for curcumin di-acetate was composed of acetonitrile-10 mM potassium dihydrogen phosphate (pH 4.0 adjusted by acetic acid) (40:60, v/v) at a flow-rate of 0.8 ml/min. The run time for analysis was 30 min and the detection wavelength was set at 425 nm. The sample injection volume was 10 µl while all the parameters were same as in the case of curcumin.

3.4.3 Analytical method validation

The stock solution of curcumin in acetonitrile (500 µg/ml) was diluted with 50% acetonitrile to make serial concentrations of the working standard solutions (0.1, 0.25, 1,
2.5, 10 and 25 µg/ml). Calibration standards were prepared by 5 µl of the working standard solution spiked with 45 µl of blank plasma and organ tissue (brain). Extraction procedures followed the sample preparation as described in section sample preparation (3.5.1). The calibration curves were given by: (curcumin/curcumin di-acetate peak area for y-axis and curcumin/curcumin acetate concentration for x-axis). The limit of detection (LOD) and the limit of quantification (LOQ) were defined as a signal-to-noise ratio of 3 and the lowest concentration of the linear regression, respectively. The accuracy and precision of intra-day and inter-day for the curcumin and curcumin di-acetate were assayed. The accuracy (% bias) was calculated as \[(C_{\text{obs}}-C_{\text{nom}})/C_{\text{nom}}\] × 100, where \(C_{\text{nom}}\) represented the nominal concentration and \(C_{\text{obs}}\) indicated the mean value of the observed concentration. Precision as the relative standard deviation (RSD) was calculated from the observed concentrations as follows: % RSD = [standard deviation (SD)/\(C_{\text{obs}}\)] × 100. The % bias and % RSD value for the lowest acceptable reproducibility concentrations was defined as being within ±15%. The recoveries (%) were calculated by comparing the curcumin peak area of the extracted sample with that of the unextracted curcumin standard solution containing the equivalent amount of curcumin in three replicates (at concentrations of 0.1, 0.25 and 2.5 µg/ml).

### 3.5 Animal study

Male Sprague-Dawley rats (210 ± 10 g body weight) were obtained from the laboratory animal center at Shoolini University (Solan, Himachal Pradesh). The experimental protocol was approved by institutional animal ethics committee of Shoolini University (protocol number: IAEC/SU-PHARM/13/027). These animals were specifically pathogen-free and were allowed to adapt to their environmentally controlled quarters (24 ± 1 °C and 12:12 h light–dark cycle). All animal experiments were performed according to the Shoolini University guidelines, principles, and procedures for the care and use of laboratory animals. The animals were acclimatized for a period of 5 days. All the rats had free access to reverse osmosis generated potable water and standard animal diet.

Experimental rats were initially anesthetized by diethyl ether. Then 25 mg/kg of curcumin and of curcumin di-acetate in PEG 400 mixture was intravenously injected via
the tail vein. The brain tissue was excised out surgically at different time interval i.e. 15, 30 and 60 min after curcumin and curcumin di-acetate administration. The brains were removed and transferred into 50 ml tubes.

3.5.1 Sample preparation

The brain were weighed and homogenized with 50% aqueous acetonitrile (1:5, w/v). The brain tissue samples were then centrifuged at 6000 rpm for 10 min at 4 °C and the supernatant was collected and preserved at −20 °C before further sample assay. Each biological sample (50 µl) was vortex-mixed with acetonitrile for protein precipitation. After centrifugation at 12,000 rpm for 15 min, 20 and 10 µl of supernatants were collected filtered through syringe filter nylon (0.2 µm) and analyzed by the HPLC and LC-MS system.

3.6 Metabolite identification by LC-MS

High-accuracy mass spectra were obtained on a Waters Micromass Q-Tof Micro LC-MS System coupled with binary HPLC system, 2777 sample manager, 2996 photodiode array detector, Q-Tof Micro with lockspray, all controlled by MassLynx 4.0 software with the Metabolynx application manager in the SAIF, Panjab University, Chandigarh, India. Identification of metabolites of the synthetic curcumin di-acetate in plasma and brain were evaluated by LC-MS.