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# Cytotoxic Effect and Permeability Activities of Curcumin Analogue; 2, 6-Bis (2, 5-dimethoxybenzy-lidene) cyclohexanone (BDMC33) in Caco-2 Cell Model

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**ABSTRACT**

Previously, curcumin analogue, 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone (BDMC33) with high anti-inflammatory activity was chemically synthesized in our laboratory to enhance the biological activity of curcumin. In this study, the toxicity and permeability activities of 2,6-bis(2,5-dimethoxybenzy-lidene)cyclohexanone (BDMC33) in Caco-2 cells was investigated. Toxicity effects using MTT assay and apparent permeability coefficient ($P_{app}$), uptake (UR) and efflux (ER) ratios, and mass balance of BDMC33 after permeation in Caco-2 cells for 180 min were evaluated in apical (A) to basolateral (B) and basolateral (B) to apical (A) directions. The similar analyses on 3-(2-fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one; (EF-24) (check control) were also conducted. The 24 hr $LC_{50}$ value for BDMC33 and EF-24 on Caco-2 cells were both 50 µM. The $P_{app}$ value in A→B direction was 3.37 ± 0.47 cm/s (BDMC33) and 2.47 ± 0.15 cm/s (EF-24). Whereas in B→A direction, it was 1.9 ± 0.36 cm/s (BDMC33) and 1.8 ± 0.15 cm/s (EF-24) upon 120 min incubation. The UR and ER ratios calculated were 1.77% and 0.56%, respectively, and the mass balance calculated were 41-44% (BDMC33) and 31-34% (EF-24) in A→B and B→A direction. This study has suggested BDMC33 to be more absorbable than EF-24 in Caco-2 cells. Therefore, BDMC33 could be a leading feature, the anti-inflammatory agent, as it biological activities would be expected outside the intestine.

**Keywords:** Apparent permeability coefficient, Caco-2 cells, Curcumin, Curcumin analogue, Efflux ratio, MTT assay.

## INTRODUCTION

Curcumin (diferuloylmethane) is a polyphenolic compound derived from the dried rhizome (turmeric) of herb (*Curcuma longa* L.). Since time immemorial, curcumin has been used in the Ayurvedic medicine and in the traditional Asian cooking. [1] It has been extensively used in Indian and Chinese traditional medicine to combat many diseases such as respiratory disorders, inflammation, arthritis, and dyspepsia. [2] Curcumin also has been reported to possess chemopreventive effects, proved to be safe and

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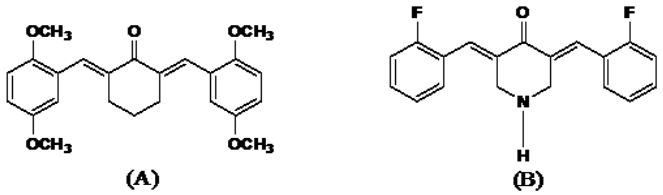
effective in animal and human clinical trials. It was reported that curcumin possess several health promoting activities like antioxidant, anti-bacterial, anti-proliferative, anti-inflammatory, anticarcinogenic, and anti-angiogenic, as indicated in many clinical trials. [3-4] The pharmacological safety and beneficial biological activities of curcumin have made it a recognized therapeutic agent in the treatment of human diseases. [5]




(A) (B)

**Fig. 1: Chemical structure of A) 2, 6-bis (2, 5-dimethoxylidene) cyclohexanone (BDMC33) (MW: 394.46 g/mol), and B) 3-(2-fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one (EF-24) (MW: 317.37 g/mol)**

However, in clinical trials of oral administration of curcumin to human patients, limited efficacy due to its poor systemic availability outside the gut, limited tissue distribution, rapid metabolism, and its rapid elimination from the body were reported. [6] This was evidenced by the fact that after oral administration of curcumin up to 12 g, its level in serum was as low as micromolar level. [7] It was also reported in a clinical study that only 1.3µg/mL was observed in serum after oral doses of 4 - 8 g of curcumin. [5] Also, in human studies the serum levels of curcumin were reported in the low micromolar range. [5] The poor systemic delivery of curcumin was suggested to be due to its structural instability in the system, [8] and this was believed to be attributed to the active methylene group and the β-diketone moiety in curcumin structure. [9-10] Furthermore, studies have suggested that two aromatic regions of curcumin might be critical for potential protein-ligand binding. [11] To circumvent the low bioavailability and keeping the low toxicity of curcumin, our group has adopted the chemical synthesis of a novel curcumin analogue by eliminating the unstable methylene group and β-diketone moiety leading to the formation of 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone (BDMC33), which reported to exhibit high antioxidant and anti-inflammatory properties than the curcumin. [11-13] The synthesized BDMC33 bears two identical aromatic ring region separated by six carbon ring, which could serve as a potential inhibitor (ligand) of protein targets. [11] Higher inhibition of curcumin analogue (BDMC33) on inducible nitric oxide synthase (iNOS) in the anti-inflammatory assay than that of curcumin have been reported. [13] A report on prostaglandin $E_2$ (PGE2) synthesis and cyclooxygenase (COX) expression *in vitro* by BDMC33 have been documented. [12-13] High biological activity and oral bioavailability exhibited by EF-24 than that of curcumin in rats have been documented. [14] Caco-2 cells were used as an *in vitro* system for the intestinal permeation of curcumin analogues, and it was derived from a human colon tumor. [15-16] Thus, the aim of the present study was to compare the *in vitro* toxicity and membrane permeability of 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone (BDMC33) and 3-(2-fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one (EF-24) in Caco-2 cells.

## MATERIALS AND METHODS

### Materials

The Caco-2 (ATCC HTB-37) was purchased from American Type Culture Collection (ATCC, VA 20108 USA). The compound, BDMC33 was chemically synthesized (Figure 1a) at the Institute of Bioscience (IBS), Universiti Putra Malaysia. The 3-(2-fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one (EF-24), Eagle's minimal essential medium (EMEM), nonessential amino acid (NEAA), and Hank's balanced salts solution (HBSS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo, USA). Dimethyl sulfoxide (DMSO), Fetal bovine serum (FBS), phosphate buffered saline (PBS), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide (MTT), and trypsin-ethylenediamine tetra acetic acid (trypsin-EDTA) solution were analytical grade and obtained from our laboratory.

### Sample preparation

The curcumin analogues (BDMC33 and EF-24) were weighed separately, in 1 mL of 100% DMSO to have 50 mM of each compound as the stock solution. Treatment concentrations of each compound (BDMC33 or EF-24) used in this experiment was prepared in 2 mL of Eagle's Minimal Essential Medium (EMEM) by serial dilution.

### Chemical stability test

This was determined according to the method explained by Wahlang *et al.*, [8] Briefly, about 10 mL of Hank's balanced salt solution (HBSS) containing 50 µM of each compound were prepared at different pH values (4.5, 5.0, 5.5, 6.0, 6.5 and 7.4), and kept in a shaker bath at 40 rpm and 37 $^{o}$C. Then, the absorbance of the remaining compounds in the media after 30, 60, 90, 120, 150, 180, 210 and 240 min intervals at 340 nm (curcumin), 420 nm (BDMC33 and 330 nm (EF-24) were read in 96-well Microplates (Corning, USA), separately, using a microplate reader (SpectraMax Plus, Molecular Device Inc. Sunnyvale, CA, USA). The HBSS solutions at pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.4 without the test compound were used as blanks (controls). The unknown concentration of the compound remaining in the mixture at the selected time interval was extrapolated from the standard curve.

### Cell cultures

In this study, Caco-2 (ATCC HTB-37) having cell passage number 18 was used. Cells were grown in 10 mL EMEM containing 20% of FBS, 1% Penicillin-Streptomycin solution in a 75 cm tissue culture flasks (TCF-012-050) and maintained at 37°C in a 5% $CO_2$ incubator with an atmosphere of 95% humidity. After

5-7 days, the cells became 80-85%, confluent, which were harvested using trypsin-EDTA. The cell numbers were counted using haemacytometer in trypan blue under the microscope and the percentages of viable cells showed 85%. The harvested cells were centrifuged at 10,000 ×*g* and 4°C for 30 min and the supernatant was discarded and the pellet was suspended in 10 mL EMEM containing 20% FBS, 1% Penicillin-Streptomycin solution in a 15 mL tube. Then, the suspended cells were seeded and grown on tissue culture treated polycarbonate membrane with 1.12 $cm^2$ diameter inserts of 0.4µm pore size at 37°C and 5% $CO_2$ incubator for 21 days to achieve tight junction monolayers. The seeding density used was 2.6 × $10^5$ cells $cm^2$ (300,000 cells per well) and the growth medium was changed every two days.

**Cell viability test**

The Caco-2 cells were cultured and detached at the confluence, centrifuged, and the pellet suspended in 10 mL EMEM containing 20% FBS, 1% Penicillin-Streptomycin solution in a 15 mL tube. To investigate the cytotoxicity of BDMC33 and EF-24 on Caco-2 cells, MTT assay using the modified method of Lee *et al*., [12] was conducted. Briefly, the suspended cells (2.0 × $10^4$ per well) were seeded in 96-well culture plates, incubated for 24 h in 37°C and 5% $CO_2$ incubator, and then exposed to increasing concentrations (12.5-400µM) of BDMC33 and EF-24, separately, for 17 hours. The control wells were set as methanol (2%) without test compounds. Exactly, 20µL of MTT solution (5 mg/mL in phosphate buffer saline), and 100µL of 20% EMEM was added to each well and incubated for 3-4 hours at 37°C and 5% $CO_2$. Then, the media in each well were discarded and 100µL of 100% DMSO was added to each well and incubated for 30 min. The absorbance was read at 570 nm using a microplate reader (SpectraMax Plus, Molecular Device Inc. Sunnyvale, CA, USA). Cell viability was expressed as a percentage of untreated cells.

**Evaluation of cell monolayer integrity**

The harvested cells were seeded and grown in tissue culture treated polycarbonate membrane with 1.12 $cm^2$ diameter inserts of 0.4µm pore size for 21 days to achieve confluence and tight junction monolayers. Prior to and after the transport experiment, the integrity of cell monolayers was tested using Lucifer yellow reagent. [8] Based on the result of stability test conducted in our laboratory, the solution of HBSS at pH 6.5 was prepared as a medium. Briefly, the apical and basolateral sides were washed three times with HBSS (pH 6.5) at 37°C. Then, 400µL of 100µM Lucifer yellow in HBSS buffer (pH 6.5) was placed on the apical side and 800µL of HBSS buffer (pH 6.5) without Lucifer yellow was placed in the basolateral side of the inserts. The inserts were incubated at 37°C with constant shaking for 2 hours. After 2 h of incubation, about 700µL and 300µL of samples were taken from the basolateral and apical sides of wells, respectively. The solution of HBSS (pH 6.5) was used as blank. A microplate reader (SpectraMax Plus, Molecular Device Inc. Sunnyvale, CA, USA) was used to read the absorbance at 530 nm. The cell monolayers with the permeability of Lucifer dye (A→B) less than 1% were recommended intact for transport studies. [8]

**Permeability experiment using insert wells treated polycarbonate membrane**

The stock solutions of curcumin analogues were prepared separately, in DMSO. The diluted working concentration (50µM) of each compound was prepared in HBSS buffer (pH 6.5), and the final concentration of DMSO was 1%. The methods of Wahlang *et al*., [8] and Dempe *et al*. (17) were used for this experiment. For the apical (A) to basolateral (B) permeability study (A→B), each well (apical site) of the 12- insert well treated polycarbonate membrane plates was seeded with 0.5 mL DMEM medium, that contains 2.6 × $10^5$ cells $cm^2$ (300,000 cells), and each well (basolateral site) was filled with 1 mL DMEM medium alone. Cells were allowed to grow into differentiated monolayers for 21 days and medium in both sites of the wells were renewed every 2 days. Then, the media in both apical (A) and basolateral (B) were discarded and washed 2 times with HBSS buffer solution. Subsequently, 0.5 mL of 50µM BDMC33 or EF-24 in HBSS (pH 6.5) was placed into the apical site (A) and 1 mL of HBSS alone was filled into basolateral site (B). Whereas, for the basolateral (B) to apical site (A) permeability study (A→A), 1 mL HBSS of 50µM of each compound, separately, was filled in bilateral sides and 0.5 ml HBSS buffer alone was placed on the apical side, and all plates were taken for incubation. Then, at a time interval; 30, 60, 90, 120 and 180 min of incubation, 800 and 300µL of samples in the basolateral side and apical side were taken separately for the analysis of A→B and B→A permeability of compounds, respectively. An equal volume of the respective sample taken was replaced with a fresh HBSS solution at each time interval. The absorbance of transport BDMC33 and EF-24 in Caco-2 cells from the apical or basolateral site were read at 330 nm and 420 nm, respectively. [18-19] Apparent permeability coefficients ($P_{app}$), cm/s) on A→B and B→A permeability were calculated using the equation;

$$P_{app}\ (cm/s) = dQ/dt\ (\mu M/mL) \times 1/[A\ (cm^2) \times C_0\ (\mu M/cm^2) \times 60]$$

Where, dQ/dt is the concentration of BDMC33 transported across the cells, A is the surface area of filter membrane and $C_\circ$ is the initial concentration placed in the apical or basolateral site.

Similarly, the apparent permeability coefficients ($P_{app}$) of BDMC33 on Caco-2 cells treated only with verapamil were conducted and calculated using the same procedures and formula stated above.

**Calculation of Uptake and Efflux ratios, and Mass balance**

Then, based on the results of $P_{app}$ (A→B) and $P_{app}$ (B→A) obtained, the uptake ratio (UR) and efflux ratio (ER) were calculated, respectively, [18-20] as follows:. UR

= $P_{app}$ (A→B)/$P_{app}$ (B→A), and UR = $P_{app}$ (BA)/$P_{app}$ (A→B).

Also, the mass balance (recovery) of the quantity of BDMC33 or EF-24 in the apical and basolateral side was calculated after the experiment and expressed as % of the initial dose added to the donor chamber at time zero. This is calculated using the equation:

$$\text{Recovery (\%)} = [(Cr, ft \times Vr + Cd, ft \times VD) / (Cd, ft \times VD)] \times 100$$

Where Cr, ft and Cd, ft is the final concentration measured at the receiver and donor chamber at the end of the experiment, respectively; Vr and Vd is the volume on the receiver and donor side, respectively.

**Permeability experiments in 1α, 25-(OH) $_2$-vitamin D3 treated Caco-2 cells**

The permeability experiments (A→B) for BDMC33 and EF-24, separately, across the Caco-2 cells treated only with 100μM of 1, 25-hydroxyl-vitamin $D_3$ (metabolizing enzyme expressing agent), vitamin $D_3$ with itraconazole (metabolizing enzyme inhibitory agent) alone, and vitamin $D_3$ with verapamil (efflux protein transporter inhibitor), respectively, were performed and compared with the permeability of untreated cells (control)). Briefly, 0.5 mL of DMEM containing 100μM vitamin $D_3$-treated cells (300.000 cells) were filled into each insert well (apical site), and 1 mL of DMEM alone into the basolateral site of the wells. Then, all plates were incubated as previously described in section 2.6. Then, the permeability of BDMC33 or EF-24 in 1, 25-hydroxyl-vitamin $D_3$ treated Caco-2 cells alone, in the present of verapamil (efflux protein inhibitor) and itraconazole (metabolizing enzymes inhibitor), separately, and in Caco-2 cells without vitamin $D_3$ were performed following the procedure described above.

**Statistical Analysis**

The data were expressed as means ± standard deviations. A one-way analysis of variance (ANOVA) was used to carry out the significant differences between the means. Dunnett's Multiple Comparison Test was performed to compare the means of test groups from the control using GraphPad Prism 5. The significant difference was considered at $p$<0. 05, $p$<0.01 and $p$<0.001.

## RESULTS

**Stability of curcumin analogues in Hank's balanced salt solution (HBSS)**

Chemical stability of EF-24 and BDMC33 in HBSS buffer solutions at different pH values were investigated separately, to ascertain the optimal pH condition and the period of their stability in the solution (Fig. 2A and B). The stabilities were expressed as % of each compound remaining after 180 min of incubation at 37°C. At the pH value >6.5 the stability of EF-24 and BDMC33 in HBSS was very low with 38.90μM (38%) and 40.3μM (40%), respectively, which is <50% as their % remaining after 180 min of incubation. However, at the pH values <6.5, the stability values for EF-24 and BDMC33 were greater than 50% with 54.57 and 55.89 μM, respectively, as the % remaining in HBSS after 180 min of incubation (Fig. 2A and B). Therefore, for the first time, this study has suggested HBSS (pH 6.5) as the standard pH condition in which EF-24 and BDMC33 can be incubated for 3 h and still having their chemical stability (% remaining) in the solution >50%.

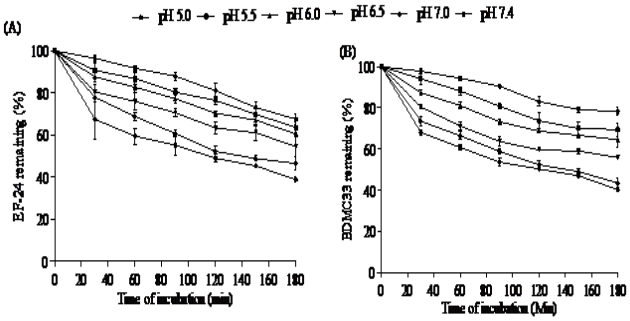



**Fig. 2: Chemical stability of EF-24 and BDMC33 in Hank's buffer salt solution (HBSS) at various pH conditions. All values are expressed in percentage mean ± SD of three independent experiments.**

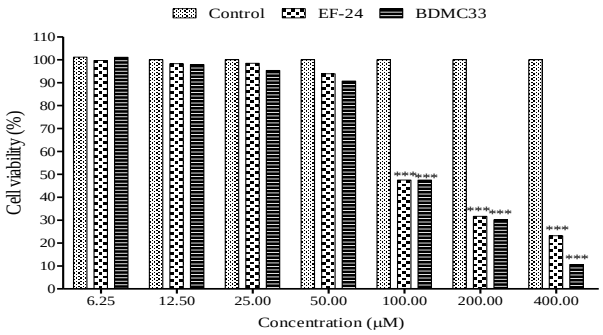



**Fig. 3: Effects of increasing concentrations of BDMC33 and EF-24 on the cell viability of Caco-2 cell monolayers. All values are expressed in percentage mean ± SD of three independent experiments. ***$p$<0.001 was statistically different for BDMC33- or EF-24-treated groups against the control group (First column).**

**Cytotoxicity test of BDMC33**

Cell viability was measured by MTT test to assess the cytotoxicity of BDMC33 in Caco-2 cells and compared with that of EF-24. The cytotoxic concentration of BDMC33 or EF-24 at <50% cell viability was observed at 100μM (Fig. 3). In this study, the approved working concentration (50μM) for BDMC33 showed 90.75%, and EF-24 displayed 93.50% cell viability, which was not significantly different compared to control group after 24 hour of incubation (Fig. 3). That is at the concentrations (6.25 to 50μM) of BDMC33 and EF-24; the cytotoxicity values in Caco-2 cells were not significantly different when compared with the control group. However, at the concentrations (100-400μM), the values of cell viability recorded were differed significantly compared to control groups after 24 hour of incubation (Fig. 3).

**Cell monolayer integrity**

The evaluation of Caco-2 cell monolayer integrity was performed prior and after the experiments using Lucifer yellow (dye). After the permeability of Lucifer yellow in Caco-2 cells before and after the experiment, the quantity received in the basolateral side was

1.26µM and 1.34µM, respectively, which were less than 2% after 3 h of incubation.

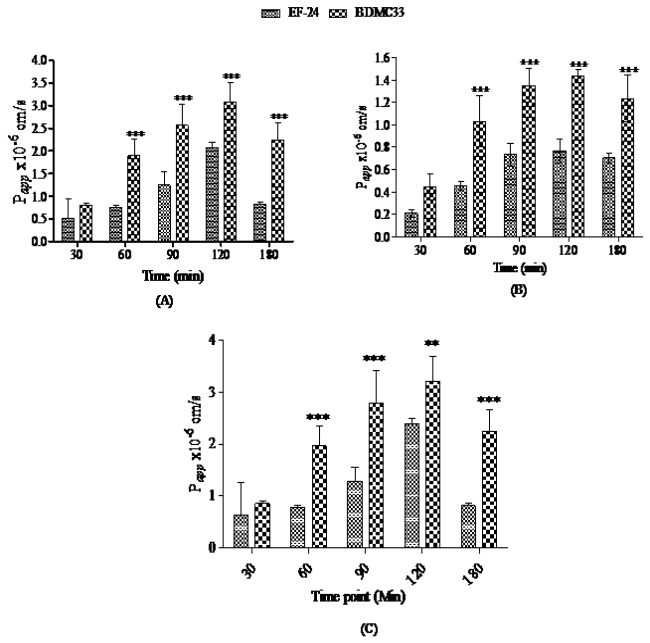



**Fig. 4: Apparent permeability coefficient ($P_{app}$) calculated at different time intervals after exposure of Caco-2 cells to 50µM BDMC33 or EF-24. All values are presented in percentage mean ± SD of three independent experiments. A): $P_{app}$ values in apical to the basolateral site (A→B), B): $P_{app}$ values in basolateral to apical site (B→A), C): $P_{app}$ values in epical to the basolateral site with verapamil (A→B+V) **$p$<0.01, ***$p$<0.001 were statistically different for BDMC3-treated groups against EF-24-treated groups (check control)**

## Apparent permeability coefficient ($P_{app}$) of BDMC33 in Caco-2 cells

The apparent permeability of BDMC33 was determined and compared with that of EF-24 in Caco-2 cell model. Based on our observation on BDMC33 stability in HBSS buffer at different pH values both in the present and without Caco-2 cells (data not shown), the pH values of HBSS for the apical and basolateral sides were maintained at pH 6.5. The apparent permeability coefficient value of the BDMC33 from apical to basolateral (A→B) and basolateral to apical (B→A), and also on the vitamin $D_3$ treated Caco-2 cells in the presence of verapamil (efflux protein inhibitor) and itraconazole (drug-metabolizing enzyme inhibitor), which were compared with that of EF-24 are presented (Fig. 4), respectively. There was a steady increase in $P_{app}$ values (A→B) for BDMC33 right from 30 - 120 min and decreased sharply at 180 min (Fig. 4A). $P_{app}$ values recorded for testing analogues throughout the period of permeability were ranged as: $0.51\times10^{6}$ - $2.1\times10^{6}$ cm/s (EF-24) and $0.80\times10^{6}$ - $3.1\times10^{6}$ cm/s (BDMC33). This result demonstrates that $P_{app}$ values recorded for BDMC33 throughout the period of incubation were significantly higher than that of EF-24 (check control). A higher value of $P_{app}$ was observed at 120 min with $2.1\times10^{6}$ cm/s (EF-24) and $3.1\times10^{6}$ cm/s (BDMC33) compared to other periods of the experiment (Fig. 4A). Then, this study has suggested that 2, 5-bis (2, 5-dimethoxybenzylidene) cyclohexanone (BDMC33) is highly absorbable than 3, 5-bis [(2-fluorophenyl) methylene]-4-piperidinone (EF-24). On the Contrary, $P_{app}$ values for EF-24 and BDMC33, MS65 permeability in a reverse direction (B→A) was calculated to ascertain the contribution of efflux protein transporters (Fig. 4B). Results revealed that $P_{app}$ values recorded for compounds throughout the period of permeability from B→A were ranged as follows; $0.21\times10^{6}$ – $0.77\times10^{6}$ cm/s (EF-24) and $0.4\times10^{6}$ – $1.43\times10^{6}$ cm/s (BDMC33) (Fig.4B). Also, the $P_{app}$ value recorded for BDMC33 in B→A direction was higher significantly, compared to that of EF-24 (Fig. 4B).

This result also demonstrates that $P_{app}$ values for EF-24 and BDMC33 permeation in A→B direction (Fig. 4A) is greater than $P_{app}$ values of the same compounds in B→A direction (Fig. 4B) across the Caco-2 cell monolayers. It was also suggested that $P_{app}$ values for EF-24 and BDMC33 after absorption in Caco-2 cells treated with itraconazole (drug-metabolizing enzyme inhibitor) and verapamil (protein transporter inhibitor), separately, were not differed significantly, compared to that of untreated cells (Fig. 4C).

## Uptake and efflux ratios and mass balance

The uptake ratio (UR) and efflux ratio (ER) of each compound calculated were presented (Fig. 5A and 5B). The UR for EF-24 and BDMC33 at all periods of experiments were ranged as follows; 1.14±0.15 – 1.75±0.20% (EF-24) and 1.30±0.25 - 2.62±0.20% (BDMC33) (Fig. 5A). This finding demonstrates that BDMC33 shows the high value of UR throughout the period of the experiment compared to EF-24. Also, similar to the earlier results of this study, at 120 min of experiment higher UR was observed for EF-24 and BDMC33 compared to other periods of the experiment (Fig. 5A). On the contrary, the efflux ratios (ER) of BDMC33 calculated at all-time points were almost the same compared to that of EF-24 calculated at the respective time points, and the calculated efflux ratios were less than 1% (Fig. 5B). Fig. 5 presents the mass balance (% recovery) calculated for EF-24 and BDMC33 in the apical to basolateral sites (A→B), basolateral to apical site (B→A), and apical to the basolateral site in the presence of verapamil (efflux proteins inhibitory agent).

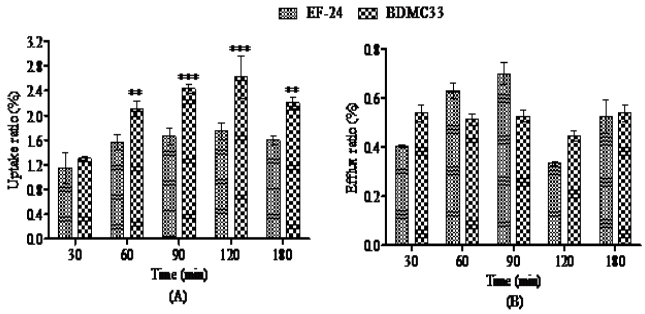



**Fig. 5: The uptake and efflux ratios calculated at different time intervals after exposure of Caco-2 cells to 50µM BDMC33 or EF-24. A) Uptake ratios, B) Efflux ratios. Values are presented in % mean ± SD of three independent experiments. **$p$<0. 05, ***$p$<0.001 were statistically different for BDMC33-treated groups against EF-24-treated group (check control)**

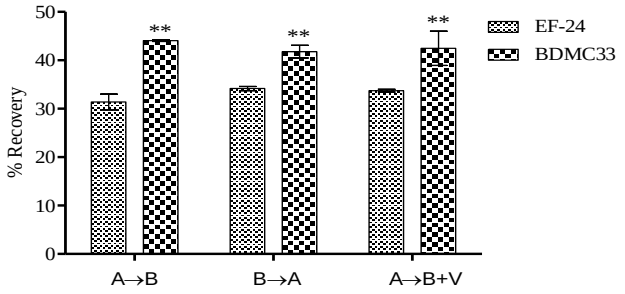



**Fig. 6: Mass balance (% recovery) of BDMC33 and EF-24 at the end of the experiments (180 min). Apical to basolateral site (A→B), Basolateral to apical site (B→A), (A→B+V) Apical to basolateral site of insert well in the present of verapamil. A: Apical site of insert well, B: Basolateral site of insert well, V: Verapamil (Efflux protein inhibitor). Values are presented in percentage mean ± Standard Deviation of three independent experiments. **$p$<0.01 was statistically different for BDMC33-treated groups against EF-24-treated groups (check control)**

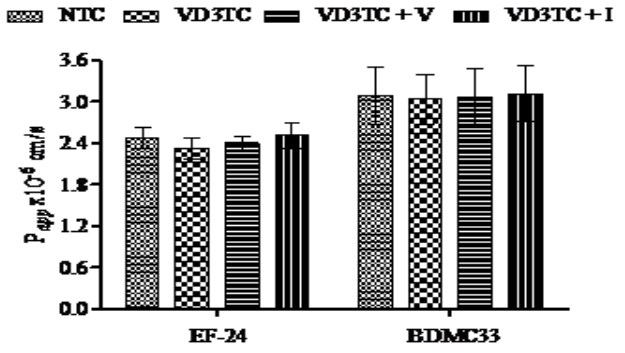



**Fig. 7: Apparent permeability values of test compounds in untreated and 1, 25-hydroxy-vitamin $D_3$-treated Caco-2 cells from apical to basolateral site (A→B). Values are presented in mean ± SD of three independent experiments. Untreated cells (NTC), Vitamin $D_3$ treated cells ($VD_3TC$), Vitamin $D_3$ treated cells with verapamil ($VD_3TC+V$), and Vitamin D3 treated cells with itraconazole (VD3TC + I)**

Mass balance is the calculated ratio of either EF-24 or BDMC33 absorbed and remaining in the apical site (donor site), in relation to the initial doses applied to the apical site. The current study revealed the ranges of % recovery for EF-24 and BDMC33 in apical, basolateral, and apical sites (permeation in the presence of verapamil) after 3 hours of permeability experiments as 31.40±0.20 - 34.17±0.23% (EF-24) and 42.06±0.25 – 44.03±0.25% (BDMC33) (Fig. 6). In both directions of permeability and A→B in the presence of verapamil, BDMC33 recorded higher significant % recovery compared to EF-24 compared to EF-24 (Fig. 6).

**Apparent permeability coefficient values of BDMC33 on 1, 25-hydroxy-vitamin $D_3$ treated Caco-2 cells**

Fig. 7 presents results of $P_{app}$ (A→B) values for EF-24 and BDMC33 in Caco-2 cells treated with 1α, 25-$(OH)_2$ (vitamin D3) (drug metabolizing enzymes expressing agent), Caco-2 cells treated with vitamin $D_3$ in the presence of verapamil (Efflux protein inhibitor), and Caco-2 cells treated with vitamin $D_3$ in the presence itraconazole (Inhibitor of drug-metabolizing enzymes) after 120 min. Apparent permeability coefficient ($P_{app}$) values for EF-24 and BDMC33 in vitamin $D_3$ treated cells ($VD_3TC$), vitamin $D_3$ treated Caco-2 cells with verapamil ($VD_3TC+V$), and vitamin $D_3$ treated Caco-2 cells with itraconazole ($VD_3TC+I$) were ranged as $2.33\times10^6$ - $2.52\times10^6$ cm/s (EF-24) and $3.03\times10^6$ - $3.12\times10^6$ cm/s (BDMC33), which were not differ significantly compared to their non-vitamin $D_3$ treated cells (NTC) with $2.47\times10^6$ cm/s (EF-24) and $3.08\times10^6$ cm/s (BDMC33) (Fig. 7).

## DISCUSSION

Caco-2 cells, a widely accepted *in vitro* systems to investigate human intestinal absorption. [16] In our previous study, BDMC33 with high anti-inflammatory activities than curcumin was chemically synthesized. BDMC33 was reported to inhibit the inducible nitric oxide synthase (iNOS), improve $PGE_2$ synthesis, and expression of COX in *in vitro*. [12-13] Thus, in the present study, we investigated the *in vitro* toxicity and membrane permeability of BDMC33 in Caco-2 cells and compared it with that of check control (EF-24).

It was revealed in this study that both EF-24 and BDMC33 were highly stable in Hank's balanced salt solution (HBSS) at pH <6.5 and 37°C after 180 min of incubation. The high stability of these compounds at the pH levels <6.5 may be explained possibly that their modification in the medium within the period of incubation were limited. The current result is in line with the report of Wahlang *et al*., [8] that the chemical stability of curcumin remaining was higher in HBSS buffer (pH 5.5), with 59.63% of curcumin remaining after 3 hour of incubation. Metzler *et al*., [21] have reported that curcumin is chemically unstable in neutral and highly acidic solutions. The chemical instability of curcumin at neutral pH has been documented. [22] Wang *et al*., [22] have reported that the half-life for curcumin at physiological pH and in a medium (pH 7.2) at 37°C without serum was 10 min. The authors also suggested that the half-life of curcumin increases as the pH of the medium decreases and that pH <6.5 are recommended for high stability of curcumin. Therefore, for the first time, this study has suggested HBSS (pH 6.5) as the standard pH condition in which EF-24 and BDMC33 can be incubated for 3 hour and still having their chemical stability (% remaining) in the solution >50%. Based on this, the time frame selected for the permeability experiments in this study was 4 hour of incubation.

Furthermore, the result of cytotoxicity tests have also demonstrated that EF-24 and BDMC33 at the concentration <50µM did not affects the cell viability of Caco-2 cells, and the cell viabilities recorded at this concentration were >80% after 24 hour of treatment (Fig. 3). Then, 50µM may be proposed as the working concentration for EF-24 or BDMC33 in treating Caco-2 cells for the periods of 24 hrs. This finding was in line with reports of Lee *et al*., [13] that the concentration of BDMC33 at 50µM was not toxic on RAW 264.7 and BV-2 cell lines, with the % cell viability >80% upon incubation for 24 hours. It was also reported that

curcumin at 30µM after 72 h of incubation, 50µM and 170µM after 24 h of incubation did not have an effect on the cell viability of Caco-2 cells. [8, 17, 23-24] Hence, this study has suggested 50µM of EF-24 and BDMC33 as the optimal concentration required for the treatments of Caco-2 cells for 24 hours (Fig.3).

In addition, the integrity of Caco-2 cells was evaluated prior and after the experiment using Lucifer yellow and the quantity of it detected, on the basolateral side was less than 2% after incubation for 3 h, which indicated that the Caco-2 cells was good for the subsequent permeability experiments. A study has shown that Caco-2 cell monolayers with the permeated Lucifer dye from apical to the basolateral site (A→B) <2% were considered intact for permeability study, but if the Lucifer dye permeated across the Caco-2 cell were >2 %, then cells were considered not good for permeability study and discarded. (8) These findings are supported by that of Wahlang *et al*., [8] and Dempe *et al*., [17] that Caco-2 cell monolayers with less than 2% and 1%, respectively, permeation from apical site to basolateral site (A→B) of Lucifer yellow (100µM) after 2 h of incubation were considered for permeability experiments.

Subsequently, the results of this study also revealed that the absorption of BDMC33 across the Caco-2 cells in A→B direction showed high significant values of $P_{app}$ than that of EF-24. The 2 compounds have their calculated $P_{app}$ values greater than $2.0\times10^{6}$ cm/s (Fig. 4A). These results of the current study demonstrate that both EF-24 and BDMC33 may be considered as highly absorbable compounds. Based on the correlation of $P_{app}$ values obtained in Caco-2 cells (*in vitro)* with that of human absorption (*in vivo*), the $P_{app}$ values $>2.0\times10^{6}$ cm/s signify higher (80-100%) permeation, whereas the $P_{app}$ values in between 1.0 and $2.0\times10^{6}$ cm/s indicate moderate (20-80%) and $P_{app}$ values less than $1.0\times10^{6}$ cm/s mean low (0-20%) permeation. [25-26] Then, the higher permeability recorded by the 2 compounds across the Caco-2 cells could be explained probably that BDMC33 and EF-24 went through less chemical transformations during the first-pass metabolism and less chemical degradation in the intestine, which might increase the amounts of free BDMC33 and its conjugates that would reach the blood stream. Though, higher permeability of EF-24 compared to curcumin in everted gut sac of the rats has been documented. [6] These results are not in agreement with the reports of Dempe *et al*., [17] that the lower Papp value of less than $0.1\times10^{-6}$ cm/s was observed on the permeation (A→B) of curcumin. Also, insignificant permeation of curcumin was obtained in the everted gut sac technique. [8] Several clinical trials that focus on the efficacy and safety of curcumin have suggested its limited poor absorption and systemic delivery with lower serum concentration upon oral doses. [27] According to Yang *et al*., [28] and Shehzad *et al*., [27] demonstrated that out of 10 mg/kg intravenous doses of curcumin in rats, only 0.36µg/mL was detected in the serum, and also after 50 times higher doses of curcumin were given orally, only 0.06µg/mL were found in the serum levels. It was also reported by Anand *et al*., [5] and Shehzad *et al*., [27] that the curcumin oral dose (1 g/kg) gave 0.5µg/mL at 45 min of ingestion. Marczylo *et al*., [29] and Shehzad *et al*., [27] documented that 6.5 ± 4.5 nmol of curcumin were observed in the serum level after 50 min of oral doses. Conversely, the $P_{app}$ values for EF-24 and BDMC33 in a reverse direction (B→A) was calculated to ascertain the contribution of efflux protein transporters (Fig. 3B). The results showed that the $P_{app}$ value in the A→B direction for BDMC33 was higher compared to that of B→A direction, indicating that the BDMC33 is not a substrate for active transporters. Also, the $P_{app}$ values for BDMC33 absorption (B→A) in Caco-2 cells at all-time points were significantly different ($p<0.001$) when compared to $P_{app}$ values for EF-24 in the same direction (Fig. 4B). All these results could be explained possibly that active protein transporters were not involved during the absorption of both EF-24 and BDMC33. Wahlang *et al*., [8] and Dempe *et al*., [17] reported that the permeability of curcumin across the Caco-2 cells in A→B direction for 2 hours was greater compared to that in B→A direction, and eventually concluded that active protein transporters were not involved during curcumin absorption across the Caco-2 cell monolayers. Therefore, these results have council out the interference of efflux protein transporters during the absorption of BDMC33 in Caco-2 cells. Though, not all the protein transporters and enzymes are expressed in Caco-2 cells to some extent, then the interference of other protein transporters could be involved. [30] Likewise, $P_{app}$ values of both compounds in the presence of verapamil (protein transporter inhibition) were almost the same with their respective $P_{app}$ in which verapamil was applied (Fig. 4C).

Furthermore, the increase uptake ratios recorded by BDMC33 than that of EF-24 are signifying the higher nature of its rate of permeation through the Caco-2 cell in both A→B and B→A directions as earlier stated in this study than EF-24. Although, there is yet to be any available literature on the permeability of BDMC33 *in vitro* and *in vivo* thus makes it difficult for comparison. On the contrary, the efflux ratios (ER) calculated for EF-24 and BDMC33 throughout the periods of incubation were <1% (Figure 4B). These results indicate that the role of efflux protein transporters was not involved during the period of absorption of the 2 compounds in Caco-2 cells. Wahlang *et al*., [8] have reported that curcumin gave less than 1% as an efflux ratio after absorption in Caco-2 cell monolayers for 3 hours. In addition, the mass balance, which is the ratio of BDMC33 absorbed and remained at the apical site (donor site), in relation to the initial doses applied in the apical site was calculated at the end of the experiment. Results suggested that in both directions of

permeability and A→B in the presence of verapamil, BDMC33 recorded higher significant % recovery compared to EF-24 (Fig. 5). The high mass balance recorded for BDMC33 and EF-24 could be explained probably due to their chemical stability in the medium and less accumulation in the cells during the period of incubation, which may reflect their higher permeation rates as earlier suggested in this study. This result is not in line with that of Wahlang *et al*., [8] that the mass balance of curcumin recorded after apical to basolateral absorption was very low. A study has reported that a mass balance in between 20-80% can be accepted for the high permeation rate. [26] However, in our laboratory, it was reported that the stability of BDMC33, MS65 and EF-24 in HBSS buffer (pH 6.5) were 59.67 ± 0.12%, 61.20 ± 0.12 and 50.12 ± 0.20%, respectively, after incubation for 180 min (data not shown). Although, there is no available information suggesting % recovery of EF-24 and BDMC33 either *in vitro* or *in vivo* studies thus make it difficult for comparison.

In addition, it was also demonstrated in this study that apparent permeability coefficient ($P_{app}$) values for EF-24 and BDMC33 in vitamin $D_3$ treated cells ($VD_3TC$), vitamin $D_3$ treated Caco-2 cells with verapamil ($VD_3TC+V$), and vitamin $D_3$ treated Caco-2 cells with itraconazole ($VD_3TC+I$) were not differed significantly compared to that of their non-vitamin $D_3$ treated cells (NTC). These results may be possibly explained that efflux protein transporters did not involve, and the expression and inhibition of drug-metabolizing enzymes by vitamin $D_3$ and itraconazole, respectively, did not affect the absorption of EF-24 and BDMC33 during the permeation in Caco-2 cells. These findings are in line with the reports that EF-24 and BDMC33 may be considered as highly absorbable compounds as earlier declared in this study. It was also observed in our laboratory that EF-24 and BDMC33 demonstrated a slight decrease but, not differ significantly, compared with normal (control) on the activities of some drug-metabolizing enzymes in Caco-2 cells and zebrafish (data not shown). Though, not all the protein transporters and enzymes are expressed in Caco-2 cells to some extent, then the interference of other protein transporters could be involved. [30] Slight isoforms of CYP enzymes were expressed in Caco-2 cells but, treatment with vitamin $D_3$ before confluence, increases CYP mRNA, NADPH-cytochrome P450 reductase levels and active protein transporters (P-gp) expression. [17, 21]

In this study, we examined the cytotoxicity and permeability of BDMC33 in Caco-2 cell monolayers and compared with that of EF-24 (Check control). This investigation has provided information that can be correlated to the absorption of BDMC33 outside the small intestine of humans. The concentration (50µM) of BDMC33 was found to be related less toxic to Caco-2 cell monolayers upon incubation for 24 hours. The $P_{app}$ values for EF-24 and BDMC33 was greater than 2.0 cm/s, which considered the 2 compounds to be highly absorbable. BDMC33 had higher $P_{app}$ value, uptake ratio, and mass balance than EF-24, and the activities of efflux protein transporters (P-gp) was expected not to be involved, which shows that their mode of transport through Caco-2 cells could be by passive diffusion. All these have suggested BDMC33 to be highly absorbable compound across the Caco-2 cells than EF-24. Thus, BDMC33 could be a potent anti-inflammatory agent that can address the problems of limited bioavailability of curcumin when consumed orally, as its biological benefits might be guaranteed outside the gut. Therefore, further elucidation on the toxicity, permeability, and metabolism of these novel compounds in the experimental animal such as rats is a welcome area of research in the future.

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