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Original Articles

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ORIGINAL ARTICLE

3-(1H-pyrazole-1-yl/1H-1,2,4-triazole-1-yl)-N-propananilide Derivatives: Design, Synthesis and Neuroprotectivity Potential Against 6-OHDA Induced Neurotoxicity Model

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ABSTRACT

Objectives: Excessive amounts of neuroapoptosis are the underlying cause of neurodegenerative diseases. Bax is a pro-apoptotic member of the B-cell lymphoma-2 family that activates caspases which are the members of the cysteine protease family that play a significant role in the initiation and execution phases of apoptosis. The aim of this study was to design and synthesize a group of N-propananilide derivatives bearing pyrazole or 1,2,4-triazole ring were designed and synthesized to analyze the neuroprotectivity potential against 6-hydroxy-dopamine (6-OHDA). Four compounds possessed protectivity at lower doses were subjected to further studies on caspase-3 and Bax pathway.

Materials and Methods: Designed compounds were synthesized by reacting 1H-pyrazole or 1H-1,2,4-triazole with propananilide intermediates in Dimethylformamide. The neuroprotective activity of the title compounds was analyzed against 6-OHDA-6-OHDA-induced neurotoxicity model. Then, caspase-3 and Bax levels were determined for the selected compounds by Western blot study.

Results: All twelve 3-(1H-pyrazole-1-yl/1H-1,2,4-triazole-1-yl)-N-propananilide derivatives possessed neuroprotectivity against the 6-OHDA-induced neurotoxicity model ($p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$). Compounds 7, 10, 11, and 12 were found to be more active at lower doses. They were subjected to further studies and the results revealed that their protecting activity arose from the decreasing levels of Bax, one of the pro-apoptotic proteins, and c expression levels and caspase-3 proteins.

Conclusion: All designed and synthesized derivatives possessed neuroprotectivity against 6-OHDA-induced neurotoxicity in the SH-SY5Y cell line and compounds 7, 10, 11, and 12 revealed that their neuroprotectivity originated from the decreasing Bax expression levels and caspase-3 activation.

Keywords: Neuroprotectivity, caspase-3, Bax protein, 1, 2, 4-triazole, 1H-pyrazole

INTRODUCTION

Physiologically, cell proliferation and death should occur in multicellular organisms.[1] Apoptosis is a programmed cell death process characterized by biochemical and morphological changes that eventually lead to cell death.[2] Central to the execution of apoptosis is a group of proteolytic enzymes known as caspases, which are cysteine proteases. Recently, 14 members of the caspase family have been identified, and 11 of them are found in humans.[3,4] Caspases, directly and indirectly, operate the apoptosis process both directly and

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indirectly.[4] In particular, caspase-3 is a principal effector in the initiation and execution phases of apoptosis. Once caspase-3 is activated in cells, it cleaves a wide range of substrates, leading to biochemical and morphological changes associated with apoptosis.[5]

Proteins of the B-cell lymphoma-2 (Bcl-2) and the mitochondrial pathway are critical intermediates in this pathway, one of the four major pathways that lead to caspase activation.[5] When cells receive apoptotic signals; Bax which is a pro-apoptotic protein that belongs to the Bcl-2 family, undergoes a multistep process to trigger the activation of caspases to execute the apoptotic program leading to cell death.[2,4,5]

Neuronal cells have a different cycle than other cells as they live longer to maintain their routine pathways.[6] Sometimes, redundant amounts and rates of neuronal apoptosis (neuroapoptosis) can occur and trigger neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis (MS), and amyotrophic lateral sclerosis.[3,7]

Based on these findings, it is suggested that apoptosis triggered by increased caspase-3 and Bax plays a significant role in the pathogenesis of neurological disorders. Therefore, inhibition of 6-OHDA-induced neurodegeneration and activation of caspases may be important targets in the treatment of neurodegenerative diseases.

We reported the cholinesterase activity of a group of *N*-propananilide derivatives bearing pyrazole or 1,2,4-triazole rings and investigated the neuroprotective potential of the most active derivatives in our previous studies.[8] Based on the promising results on neuroprotectivity and cholinesterase inhibition, we designed *N*-propananilide derivatives bearing pyrazole or 1,2,4-triazole rings and tested their cholinesterase activity, but the results were negligible. Therefore, we aimed to focus on their neuroprotective activities. We tested the neuroprotective activity of the synthesized compounds in SH-SY5Y neurotoxicity model induced by 6-OHDA, which is a neurotoxic agent used for neurotoxicity assays based on its ability to be autoxidized to yield potentially toxic products and reactive oxygen species.[9] After conducting neuroprotection studies with selecting poteint compounds with more potential, we further analyzed the underlying mechanism *via* immunoblotting studies of the caspase-3 and Bax proteins for the most active ones.

MATERIALS AND METHODS

Thin-layer chromatography was performed on silica gel plates (Merck, Kieselgel 60F254) and detected using 254 nm ultraviolet (UV) light. The structures of the compounds were verified using infrared spectra (Perkin Elmer Fourier transform infrared spectrometer 100 with attenuated total reflectance (ATR) attachment, Perkin Elmer Inc., Massachusetts, USA), mass spectra [atmospheric pressure chemical ionization-electrospray ionization (APCI-ESI), (Thermo MSQ Plus LC/MS, Thermoscientific Inc., San Jose, CA, USA), and NMR spectra (Varian As 400 Mercury Plus nuclear magnetic resonance (NMR), Varian Inc., Palo Alto, CA, USA). Melting points were determined using a melting point apparatus on a Stuart SMP30 and were uncorrected. Elemental analysis was performed using a TruSpec Micro Instrument LECO CHNS 932. All the starting materials and reagents used for the synthesis were commercial products with high-quality properties.

General procedure for the synthesis of the compounds 1a-6a

Substituted aniline (1 eq.) and K_2CO_3 (1 eq.) were dissolved in the mixture of acetone: water (1:2). 3-chloropropionyl chloride (1 eq.) was added dropwise in an ice bath, and the mixture was stirred at room temperature for 2 h. The reaction mixture was then poured into cold water, and the precipitate was filtered and washed with water.[10]

General procedure for the synthesis of the compounds 1-12

ω-Chloro-*N*-propananilides (1 eq.), pyrazole or triazole (1 eq.), and K_2CO_3 (1 eq.) were dissolved in dimethylformamide (DMF) and allowed to react under reflux. After monitoring the end of the reaction, DMF was evaporated under reduced pressure, and the residue was extracted using chloroform and water. The organic phases were evaporated after drying over anhydrous Na_2SO_4.[11] Crude products with various mobile phases were purified by column chromatography.

N-(2-chlorophenyl)-3-(1H-pyrazol-1-yl propanamide (1)

Yield, 30%; *m.p.*, 102 °C; IR (ATR) ν_{max}. (cm^{-1}): 3279 (NH), 1650 (amide I), 1533 (amide II); ^1H NMR ($CDCl_3$, 400 MHz) δ 3.04 (t, 2H, *J*=6.3 Hz, CH_2), 4.53 (t, 2H, *J*=6.3 Hz, CH_2), 6.21 (t, 1H, *J*=2.1 Hz, Py-H), 7.03 (td, 1H, *J*=7.8; 1.6 Hz, Ph-H), 7.22-7.26 (m, 1H, Ph-H), 7.33 (dd, 1H, *J*=8.1; 1.5 Hz, Ph-H), 7.45 (d, 1H, *J*=2.3 Hz, Py-H), 7.54 (d, 1H, *J*=1.5 Hz, Py-H), 7.91 (brs, 1H, NH), 8.27 (d, 1H, *J*=8.3 Hz, Ph-H) ppm; MS (ESI) *m/z* (% intensity): 123 (100), 250 (73) $[M+H]^+$, 252 (23) $[M+2+H]^+$; Anal. Calcd for $C_{12}H_{12}ClN_3O$ (249): C: 57.72; H: 4.84; N: 16.83. Found C: 57.60; H: 4.99; N: 16.42.

N-(3-chlorophenyl)-3-(1H-pyrazol-1-yl)propanamide (2)

Yield, 72%; *m.p.*,76 °C; IR (ATR) ν_{max}. (cm^{-1}): 3236 (NH), 1685 (amide I), 1590 (amide II); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.95 (t, 2H, *J*=6.1 Hz, CH_2), 4.51 (t, 2H, *J*=6.1 Hz, CH_2), 6.25 (t, 1H, *J*=2.1 Hz, Py-H), 7.04-7.07 (m, 1H, Ph-H), 7.19 (t, 1H, *J*=8.0 Hz, Ph-H), 7.26-7.29 (m, 1H, Ph-H), 7.46 (d, 1H, *J*=2.3 Hz, Py-H), 7.57-7.64 (m, 2H, Ph-H and Py-H), 8.53 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 250 (100) $[M+H]^+$, 252 (23) $[M+2+H]^+$; Anal. Calcd for $C_{12}H_{12}ClN_3O$ (249): C: 57.72; H: 4.84; N: 16.83. Found C: 57.38; H: 5.07; N: 16.46.

N-(4-chlorophenyl)-3-(1H-pyrazol-1-yl)propanamide (3)

Yield, 59%; *m.p.*, 151 °C; IR (ATR) ν_{max}. (cm^{-1}): 3246 (NH), 1677 (amide I), 1538 (amide II); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.83 (t, 2H, *J*=6.4 Hz, CH_2), 4.40 (t, 2H, *J*=6.4 Hz, CH_2), 6.09-6.10 (m, 1H, Py-H), 7.09-7.13 (m, 2H, Ph-2H), 7.36-7.42 (m, 4H, Ph-2H and Py-2H), 9.25 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 123 (97), 250 (100) $[M+H]^+$, 252 (32) $[M+2+H]^+$; Anal. Calcd for $C_{12}H_{12}ClN_3O$ (249): C: 57.72; H: 4.84; N: 16.83. Found C: 57.52; H: 4.88; N: 16.50.

N-(2-methoxyphenyl)-3-(1H-pyrazol-1-yl)propanamide (4)

Yield, 62%; *m.p.*, 88 °C; IR (ATR) ν_{max}. (cm^{-1}): 3374 (NH), 1681 (amide I), 1534 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.98 (t, 2H, *J*=6.5 Hz, CH_2), 3.83 (s, 3H, OCH_3), 4.53 (t, 2H, *J*=6.5 Hz, CH_2), 6.19 (t, 1H, *J*=2.1 Hz, Py-H), 6.84 (dd, 1H, *J*=8.1; 1.4 Hz, Ph-H), 6.91-6.95 (m, 1H, Ph-H), 7.03 (td, 1H, *J*=7.8; 1.7 Hz, Ph-H), 7.45-7.46 (m, 1H, Py-H), 7.51-7.52 (m, 1H, Py-H), 7.84 (brs, 1H, NH), 8.29-8.31 (m, 1H, Ph-H) ppm; MS (APCI) *m/z* (% intensity): 123 (100), 246 (86) $[M+H]^+$; Anal. Calcd for $C_{13}H_{15}N_3O_2$ (245): C: 63.66; H: 6.16; N: 17.13. Found C: 63.30; H: 6.48; N: 16.84.

N-(3-methoxyphenyl)-3-(1H-pyrazol-1-yl)propanamide (5)

Yield, 66%; *m.p.*, 96 °C; IR (ATR) ν_{max}. (cm^{-1}): 3262 (NH), 1692 (amide I), 1491 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.94 (t, 2H, *J*=6.1 Hz, CH_2), 3.78 (s, 3H, OCH_3), 4.51 (t, 2H, *J*=6.2 Hz, CH_2), 6.23 (t, 1H, *J*=2.1 Hz, Py-H), 6.62-6.65 (m, 1H, Ph-H), 6.91 (d, 1H, *J*=7.8 Hz, Ph-H), 7.15-7.19 (m, 2H, Ph-2H), 7.45 (dd, 1H, *J*=2.3; 0.7 Hz, Py-H), 7.55-7.56 (m, 1H, Py-H), 8.14 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 246 (100) $[M+H]^+$; Anal. Calcd for $C_{13}H_{15}N_3O_2$ (245): C: 63.66; H: 6.16; N: 17.13. Found C: 63.70; H: 6.24; N: 16.70.

N-(4-methoxyphenyl)-3-(1H-pyrazol-1-yl)propanamide (6)

Yield, 14%; *m.p.*, 127 °C; IR (ATR) ν_{max}. (cm^{-1}): 3297 (NH), 1654 (amide I), 1529 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.91 (t, 2H, *J*=6.2 Hz, CH_2), 3.77 (s, 3H, OCH_3), 4.51 (t, 2H, *J*=6.2 Hz, CH_2), 6.23 (t, 1H, *J*=2.1 Hz, Py-H), 6.82 (d, 2H, *J*= 9.0 Hz, Ph-2H), 7.31 (d, 2H, *J*= 9.0 Hz, Ph-2H), 7.45 (d, 1H, *J*=2.3 Hz, Py-H), 7.55 (d, 1H, *J*=1.9 Hz, Py-H), 7.93 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 178 (100), 246 (91) $[M+H]^+$; Anal. Calcd for $C_{13}H_{15}N_3O_2$ (245): C: 63.66; H: 6.16; N: 17.13. Found C: 63.25; H: 5.98; N: 16.83.

N-(2-chlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (7)

Yield, 7%; *m.p.*, 98 °C; IR (ATR) ν_{max}. (cm^{-1}): 3129 (NH), 1691 (amide I), 1507 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.04 (t, 2H, *J*=6.2 Hz, CH_2), 4.59 (t, 2H, *J*=6.2 Hz, CH_2), 7.03-7.07 (m, 1H, Ph-H), 7.23-7.27 (m, 1H, Ph-H), 7.34 (dd, 1H, *J*=8.1; 1.5 Hz, Ph-H), 7.67 (brs, 1H, NH), 7.95 (s, 1H, Tr-H), 8.17 (s, 1H, Tr-H), 8.24 (d, 1H, *J*=8.4 Hz, Ph-H) ppm; MS (ESI) *m/z* (% intensity): 251 (100) $[M+H]^+$, 253 (30) $[M+2+H]^+$; Anal. Calcd for $C_{11}H_{11}ClN_4O$ (250): C: 52.70; H: 4.42; N: 22.35. Found C: 52.79; H: 4.79; N: 22.18.

N-(3-chlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (8)

Yield, 38%; *m.p.*, 167 °C; IR (ATR) ν_{max}. (cm^{-1}): 3122 (NH), 1690 (amide I), 1540 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.82 (t, 2H, *J*=6.3 Hz, CH_2), 4.42 (t, 2H, *J*=6.3 Hz, CH_2), 6.86-6.89 (m, 1H, Ph-H), 7.05 (t, 1H, *J*=8.1 Hz, Ph-H), 7.24-7.27 (m, 1H, Ph-H), 7.53 (t, 1H, *J*=2.0 Hz, Ph-H), 7.76 (s, 1H, Tr-H), 8.06 (s, 1H, Tr-H), 9.47 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 251 (100) $[M+H]^+$, 253 (29) $[M+2+H]^+$; Anal. Calcd for $C_{11}H_{11}ClN_4O$ (250): C: 52.70; H: 4.42; N: 22.35. Found C: 52.77; H: 4.49; N: 21.98.

N-(4-chlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (9)

Yield, 59%; *m.p.*, 136 °C; IR (ATR) ν_{max}. (cm^{-1}): 3124 (NH), 1697 (amide I), 1547 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.95 (t, 2H, *J*=6.1 Hz, CH_2), 4.57 (t, 2H, *J*=6.1 Hz, CH_2), 7.26 (d, 2H, *J*=8.8 Hz, Ph-2H), 7.38 (d, 2H, *J*=8.8 Hz, Ph-2H), 7.62 (brs, 1H, NH), 7.95 (s, 1H, Tr-H), 8.16 (s, 1H, Tr-H) ppm; MS (ESI) *m/z* (% intensity): 249 (100) $[M-H]^-$, 253 (28) $[M+2-H]^-$; Anal. Calcd for $C_{11}H_{11}ClN_4O$ (250): C: 52.70; H: 4.42; N: 22.35. Found C: 52.79; H: 4.37; N: 22.05.

N-(2-methoxyphenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (10)

Yield, 84%; *m.p.*, 81 °C; IR (ATR) ν_{max}. (cm^{-1}): 3112 (NH), 1688 (amide I), 1537 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.99 (t, 2H, *J*=6.2 Hz, CH_2), 3.84 (s, 3H, OCH_3), 4.58 (t, 2H, *J*=6.3 Hz, CH_2), 6.84 (dd, 1H, *J*=8.1; 1.4 Hz, Ph-H), 6.93 (td, 1H, *J*=7.8; 1.4 Hz, Ph-H), 7.04 (td, 1H, *J*=7.8; 1.7 Hz, Ph-H), 7.75 (brs, 1H, NH), 7.93 (s, 1H, Tr-H), 8.16 (s, 1H, Tr-H), 8.25 (dd, 1H, *J*= 8.1; 1.6 Hz, Ph-H) ppm; MS (ESI) *m/z* (% intensity): 247 (100) $[M+H]^+$; Anal. Calcd for $C_{12}H_{14}N_4O_2$ (246): C: 58.53; H: 5.73; N: 22.75. Found C: 58.11; H: 5.88; N: 22.51.

N-(3-methoxyphenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (11)

Yield, 33%; *m.p.*, 152 °C; IR (ATR) $\nu_{max.}$ (cm^{-1}): 3114 (NH), 1675 (amide I), 1555 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.79 (t, 2H, *J*=6.4 Hz, CH_2), 3.62 (s, 3H, OCH_3), 4.40 (t, 2H, *J*=6.4 Hz, CH_2), 6.44-6.46 (m, 1H, Ph-H), 6.86-6.89 (m, 1H, Ph-H), 7.00 (t, 1H, *J*=8.1 Hz, Ph-H), 7.12 (t, 1H, *J*=2.2 Hz, Ph-H), 7.74 (s, 1H, Tr-H), 8.05 (s, 1H, Tr-H), 9.23 (brs, 1H, NH), ppm; MS (ESI) *m/z* (% intensity): 247 (100) $[M+H]^+$; Anal. Calcd for $C_{12}H_{14}N_4O_2$ (246): C: 58.53; H: 5.73; N: 22.75. Found C: 58.35; H: 5.71; N: 22.41.

N-(4-methoxyphenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (12)

Yield, 49%; *m.p.*, 129 °C; IR (ATR) ν_{max}. (cm^{-1}): 3113 (NH), 1682 (amide I), 1557 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.90 (t, 2H, *J*=6.2 Hz, CH_2), 3.76 (s, 3H, OCH_3), 4.55 (t, 2H, *J*=6.2 Hz, CH_2), 6.81 (d, 2H, *J*=9.0 Hz, Ph-2H), 7.29 (d, 2H, *J*=9.0 Hz, Ph-2H), 7.67 (brs, 1H, NH), 7.93 (s, 1H, Tr-H), 8.15 (s, 1H, Tr-H), 8.25 (dd, 1H, *J*= 8.1; 1.6 Hz, Ph-H) ppm; MS (ESI) *m/z* (% intensity): 178 (29), 247 (100) $[M+H]^+$; Anal. Calcd for $C_{12}H_{14}N_4O_2$ (246): C: 58.53; H: 5.73; N: 22.75. Found C: 58.40; H: 5.72; N: 22.34.

Cell lines and treatments

The SH-SY5Y cell line (human dopaminergic neurons) was obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium; (Biological Ind., Israel) containing 10% fetal bovine serum (Panbiotech, Germany), and 2 mM L-glutamine (Biological Ind., Israel) according to the manufacturer's instructions. Cells were seeded at a density of 2 x 10^4 cells per well for the cell viability assay; for the Western blot (WB) study cells were seeded at 5 x 10^5 per well. The following day, cells were pre-treated with desired concentrations of the synthesized compounds for 12 h and then treated with 50 µm 6-OHDA (Sigma Aldrich, UK) for 12 h. Dimethyl sulfoxide (DMSO)-treated cells were used as a negative experimental control.

While 6-OHDA was prepared in sterile water as a 1000X stock solution, the synthesized compounds were prepared in DMSO. The final DMSO concentration was below 0.1%.

Cell viability assay

The potential neuroprotective properties of the synthesized compounds against 6-OHDA-induced neurotoxicity were assessed using a water-soluble tetrazolium salt (WST) reagent (Roche, Switzerland) according to the manufacturer's instructions. The cell viability assay was performed by replacing the old media with a WST/medium mixture (1:9). The absorbance was measured using a microplate reader at 440 and 690 nm (Varioscan, Thermo Fisher Scientific, US). The cell viability is presented as a percentage of cell viability compared to DMSO-treated cells. This experiment was conducted in triplicate.

WB analysis

Cells were harvested using a Radioimmunoprecipitation Assay buffer containing a protease inhibitor cocktail (Roche, Switzerland). The bicinchoninic acid (BCA, Thermo Fisher Scientific, US) protein assay was used to determine total protein levels according to the manufacturer's instructions. Equal amounts of protein were used in WB studies. After denaturation of protein samples in 4X Laemmli buffer (Bio-Rad, US) at 95 °C for 5 min, the samples were first separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis then transferred to polyvinylidene fluoride membranes (EMD Millipore, Thermo Fisher Scientific, US). Next, the membranes were blocked with blocking buffer (phosphate-buffered saline-0.1% tween-20 with 5% non-fat dry milk). In this study, anti-β-actin (1:10000, Sigma-Aldrich-A5316, UK) was used as a mouse monoclonal antibody, and anti-caspase-3 (1:3000, CST-9665, US) and anti-Bax (1:3000, CST-2774, US) antibodies were used as rabbit monoclonal antibodies. Besides, We used goat anti-rabbit (1.5000, Thermo Fisher Scientific-31460, US) and Goat anti-mouse (1:5000, Thermo Fisher Scientific-31430, US) antibodies as secondary antibodies. Fusion-FX7 (Vilber Lourmat, Thermo Fisher Scientific, USA) and Clarity ECL substrate solution (Bio-Rad-1705061, USA) were used to determine the chemiluminescence signal.

Statistical analysis

Data were shown as means ± standard deviation. The experiment was conducted using three independent biological replicates and two technical replicates. Student's *t*-test was used to determine significant differences between groups ($p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$).

Acetylthiocholine esterase (AChE) activity

AChE enzyme activity was investigated with slight modifications based on the study of Ellman et al.[12,13] Thiocholine, the product of enzymatic hydrolysis, does not have a distinct chromophore for UV detection, therefore 5,5'-Dithiobis(2-nitrobenzoic acid) [Ellman's reagent, DTNB, Sigma-Aldrich, (St. Louis, MO, USA)] was used for the evaluation of enzyme activity. All compounds were tested at 30 μM and 300 μM concentrations. Galantamine was used as a positive control. Each concentration was studied three times. All solutions were adjusted to 20 °C before use. First, 3.0 mL of phosphate buffer (0.1 M; pH 8.0), then 100 *μL* of substrate solution (dissolved in 2% DMSO and then diluted to lower than 0.2% DMSO in the aqueous assay medium), 100 *μL* of enzyme solution [(2.5 units/*μL*) (from electric eel, E.C.3.1.1.7., Type VI-S, Sigma-Aldrich)] were added into the cuvette and incubated for 5 min. After the required fractional amounts of the DTNB solution (0.01 M, 100 ml) and 20 *μL* of the acetylthiocholine (ATC) (0.075 M) were added, the cuvette was mixed immediately and rapidly. The enzymatic hydrolysis of ATC was detected at 412 nm by UV-visible absorption (Shimadzu UV/160-A spectrophotometer). The *in vitro* results for AChE are presented in Table 1.

RESULTS

Chemistry

Synthesis of target compounds was carried out in two steps (1a-6a and 1-12). In the first step, ω-chloro-*N*-propananilide derivatives were obtained.[10] Subsequently, these derivatives were combined with pyrazole or triazole to obtain the final compounds.[11] Column chromatography was used for purification studies. Structure characterization studies of the synthesized compounds were carried out successfully.

The neuroprotective effects of the final compounds against neurotoxicity and cell death

To investigate the potential neuroprotective effects of compounds against neurotoxicity induced by 6-OHDA, we first pre-treated cells with different concentrations of compounds (1, 5, 10, 25, and 50 μM) for 12 h; then treated them with 6-OHDA for additonal12 h. After conducting cell viability experiments, we showed that while 6-OHDA decreased cells' viability 46.30±0.66% compared to the DMSO-treated cells (control cells), the compounds have a potential protective effecs against 6-OHDA induced toxicity. Especially, compounds 1, 5, 7, 10, and 12

Table 1. AChE inhibition results of the title compounds

Compound	Inhibition %	
	30 μM	300 μM
1	22.08±2.63	65.56±1.35
2	12.91±0.68	67.86±0.02
3	14.35±0.02	69.16±0.11
4	16.31±0.9	70.30±1.84
5	15.18±0.65	66.61±1.01
6	25.81±0.16	67.09±0.08
7	29.08±0.68	83.03±0.99
8	17.37±0.67	64.20±1.71
9	13.56±1.65	68.70±1.08
10	23.07±0.47	82.20±0.53
11	18.18±0.46	66.50±0.9
12	30.22±0.56	86.24±0.52
Galantamine	80.7	95.9

AChE: Acetylthiocholine esterase

presented their activities at lower concentration. While 6-OHDA decreased cell viability to 46.30±0.66%, the viability was restored to 40±0.76, and 70.09±1.44% at 1 and 5 concentrations of compound 1, respectively (Figure 1, Supplementary Table 1). While compound 5 enhanced cell viability to 98.78±2.23 (5 μM) and 99.68±4.04% (10 μM), the cell viability with treatment with compound 7 reached up to to 104.33±3.77 and 111.12±3.02% at 1 and 5 μM concentrations, respectively (Figure 1, Supplementary Table 1). The viability of cells with the treatment of complund 10 was found to be increased to to 101.97±3.11 (1 μM) and 107.54±2.25 (5 μM) (Figure 1, Supplementary Table 1). Compound 12, another molecule that shows activity at lower concentrations, increased cells viability up to 10 μM (103.07±2.24; 1 μM , 106.49±2.59; 5 μM, 113.69±2.14%; 10 μM) (Figure 1, Supplementary Table 1). The compounds 2, 6 and 11 presented their activity in a dose dependent manner. For compound 3 and 4, the activity was found to be increased up to 25 μM in a dose dependent manner (Figure 1, Supplementary Table 1). While compound 9 enhanced cell viability to 10 μM in a dose dependent manner, compound 8 presented its activity at higher concentrations as at 10-, 25 and 50 μM (Figure 1, Supplementary Table 1). For this analysis, Student's *t*-test was used for determination of significancy between groups ($p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$). To investigate the potential mechanism for protective effects of selected compounds against 6-OHDA induced toxicity model, we checked some proteins' levels as Bax and cleaved caspase 3 *via* Western blot. The results obtained, showed that while 6-OHDA increased cleaved caspase-3 and Bax proteins' levels, compouns 7, 10, 11, and 12 decreased these proteins levels leadindg to potential neuroprotection.

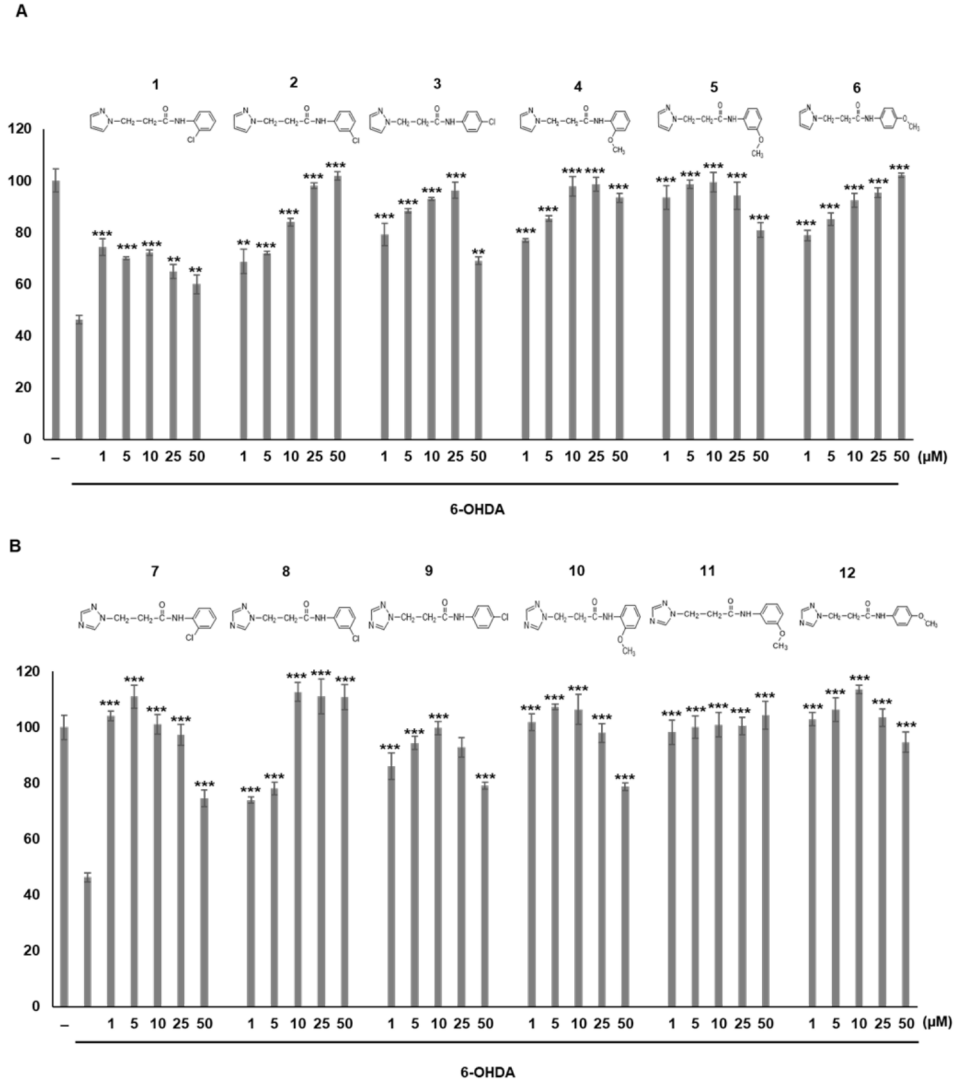
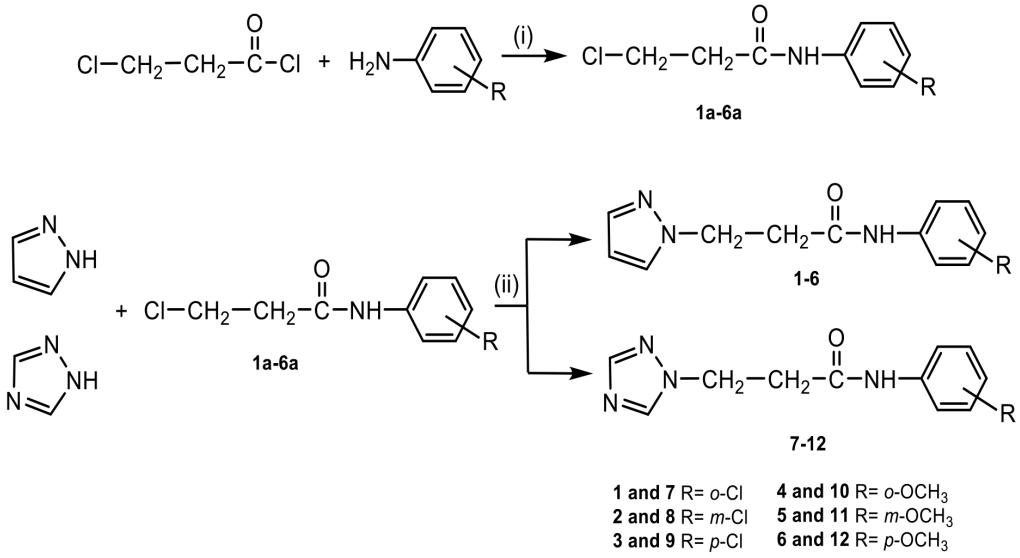


Figure 1. Synthesized compounds protect cells against 6-OHDA-induced toxicity. SH-SY5Y cells were pre-treated with increasing doses of compounds for 12 h and then treated with 6-OHDA for 12 h. The viability of cells was performed by WST-1 reagent. The absorbance of cells treated with DMSO as solvent control was considered 100% and cell viability of the applied concentrations was calculated. The WST-1 assay was performed by triplicate samples. ANOVA analysis with Dunnett's post hoc test was used to determine the significance of the differences compared to the control cells ($^{*}p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$)

6-OHDA: 6-Hidroksi-dopamine, WST: Water-soluble tetrazolium salt, DMSO: Dimethyl sulfoxide, ANOVA: Analysis of variance



Scheme 1. Synthesis pathway of compounds 1-12
DMF: Dimethylformamide

DISCUSSION

Chemistry

In this study, 12 substituted *N*-propananilide derivatives bearing pyrazole or 1,2,4-triazole were synthesized.[10,11] After the acylation of substituted anilines with 3-chloropropionyl chloride (compounds 1a-6a), they were reacted with pyrazole or 1,2,4-triazole in DMF to form 1-6 and 7-12, respectively (Scheme 1). The structures of the final compounds were verified using spectral methods, and purity percentages were determined by elemental analysis. Strong stretching bands were noticed regarding the carbonyl group at 1697-1650 cm^{-1} and 1590-1491 cm^{-1} as amide I and amide II bands, respectively (Supplementary Figures 1-12, Supplementary Table 1). In addition, stretching signals in the H bond region (3374-3112 cm^{-1}) verifying the existence of the amide functional group were observed.[14,15]

The chemical shifts and coupling constants of ^{1}H NMR spectra were consistent with the molecular structures. While the signals of methylene protons neighboring the carbonyl group were observed at δ 2.79 - 3.04, signals of methylene protons neighboring heterocyclic ring systems were observed at δ 4.40 - δ 4.59 region because the deshielding effect of pyrazole/1,2,4-triazole rings is greater than that of the carbonyl functional group.[16] The NH proton of the amide function was noticed as a broad singlet from δ 7.50 to 9.50 depending on the position and type of substituent (Supplementary Figures 1-12).

The mass spectra of the title compounds were recorded using ESI or APCI, and $[M+1]^+$ and $[M-1]^-$ signals were determined according to their molecular weights. Additionally, the expected molecular formulas and purity of the compounds were supported within ±0.4% range according to the elemental analysis results (Supplementary Figures 1-12, Supplementary Table 1).

All molecules except 10 had registry numbers, but no corresponding scientific data was available.

The neuroprotective effects of the final compounds against neurotoxicity

The potential neuroprotective activity of the titles compounds was analyzed in a 6-OHDA-induced neurotoxicity model. First, cells were pre-treated with desired concentrations of compounds (1, 5, 10, 25, and 50 μM) for 12 h; then they were subjected to 6-OHDA treatment for another 12 h. The cell viability assays, performed using WST-1 reagent, showed that all final compounds possessed remarkable neuroprotective effects against 6-OHDA-induced cells, and compounds 1, 5, 7, 10, and 12 reduced the toxic effect of 6-OHDA more effectively at lower concentrations. 6-OHDA treatment decreased cell viability by 46.30±0.66% (Figure 1, Supplementary Table 1). The viability was revitalized to an extent of 74.40±0.76, and 70.09±1.44% at 1 and 5 concentrations of compound 1, respectively. The highest concentration of compound 1 did not reverse cell viability as much as the lowest concentration. For this analysis, Student's *t*-test was used for determination of significancy between groups ($p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$).

Compound 5 treatment increased cell viability to 98.78±2.23 and 99.68±4.04% at 5 and 10 μM concentrations, respectively. The treatment with compound 7 ameliorated cell viability to 104.33±3.77 and 111.12±3.02% at 1 and 5 μM concentrations, respectively; compound 10 increased cell viability to 101.97±3.11 and 107.54±2.25% at 1 and 5 μM concentrations, respectively. Compound 12, another synthesized molecule that increased cell viability at lower doses, enhanced cell viability up to a concentration of 10 μM (103.07±2.24, 106.49±2.59, 113.69±2.14 % at the 1, 5, and 10 μM concentration, respectively). Treatment with compounds 2, 6, and 11 increased cell viability in a

dose-dependent manner which means that the highest doses (50 μM) did not exert any cytotoxic effects on SH-SY5Y cells (101.98±1.97% for 50 μM concentration of compound 2, 102.21±2.26% for 50 μM concentration of compound 6, 104.35±2.26% for 50 μM concentration of compound 11).

Compound 3 increased cell viability up to a concentration of 25 μM in a dose-dependent manner. The viability decreased dramatically to 69.09±0.98% at the highest concentration, which may be due to the cytotoxic effect of the highest dose of this compound. While compound 4 increased cell viability up to a concentration of 25 μM (98.60±3.51%), it was slightly decreased at 50 μM concentration (93.36±3.38%). Furthermore, treatment with compound 9 triggered cell viability in a dose-dependent manner up to a concentration of 10 μM, and it was shown that a minor and moderate decrease at higher concentrations (92.95±0.90% at 25 μM and 79.20±1.95% at 50 μM). Finally, compound 8 did not increase cell viability at lower concentrations, whereas treatments at higher concentrations attenuated 6-OHDA toxicity at higher doses (113.02±4.73%, 111.03±1.48% and 111.20±5.20% at 10-, 25 and 50 μM, respectively) (Supplementary Table 1).

The protective effects of the final compounds against cell death

6-OHDA has been used in neurotoxicity models for Parkinson's both *in vitro* and *in vivo*[17] based on its ability to activate caspase-3 and increase the level of Bax protein in several studies.[18–20]

Based on the significant neuroprotective activity of the final derivatives, we selected derivatives 7, 10, 11, and 12 for further investigation because they also displayed impressive neuroprotectivity at lower concentrations. For this purpose, we used the same experimental conditions used in the cell viability assays. Consistent with the literature, treatment with 6-OHDA alone increased the Bax and cleavage caspase-3 protein levels. This increase in Bax and cleaved caspase-3 was significantly attenuated in cells pre-treated with 7, 10, 11, and 12 (Figure 2). 6-OHDA, a 6-hydroxylated analog of dopamine, causes degeneration of dopaminergic neurons. Treatment with 6-OHDA induces cellular damage through a reaction with nucleophiles, such as protein and DNA, leading to apoptotic cell death.[21] Our results revealed that compounds 7, 10, 11, and 12 protect SH-SY5Y cells against 6-OHDA toxicity by decreasing pro-apoptotic Bax expression and caspase-3 activation.

AChE activity

All compounds were screened for AChE inhibitory potential using Ellman's slightly modified colorimetric method, and galantamine was the standard drug.[12,22] The results demonstrated that all synthesized compounds exhibited weak AChE inhibitory activity (Table 1). As shown in Table 1, the title compounds exhibited 64.4-86.2% inhibition at 300 μM and 13.62-30.22% inhibition at 30 μM on AChE. Among the series, compounds 7, 10, and 12 showed better activity than the other tested compounds at both concentrations. In particular, compound 12, named *N*-(4-methoxyphenyl)-2-(1*H*-1,2,4-triazol-1-yl)propanamide, was found to be the most potent derivative of all the synthesized derivatives, with an 86.2% inhibition at 300 μM and a 30.2% inhibition at 30 μM on AChE (Table 1).

CONCLUSION

In this study, we designed and synthesized twelve substituted *N*-propananilide derivatives bearing pyrazole or 1,2,4-triazole rings based on the neuroprotective results of the compounds from our previous study.[8] In terms of neuroprotective potential, triazole derivatives generally exhibit better activity than pyrazole derivatives. Furthermore, compounds that have potential against AChE have neuroprotective effects. Additionally, compounds 7, 10, 11, and 12 provide neuroprotection against 6-OHDA-induced neurotoxicity by decreasing the pro-apoptotic protein Bax and cleaved caspase-3, which play central roles in cellular apoptosis.

In conclusion, we can state that adding one methylene group to the linker increases the neuroprotective effect of the designed

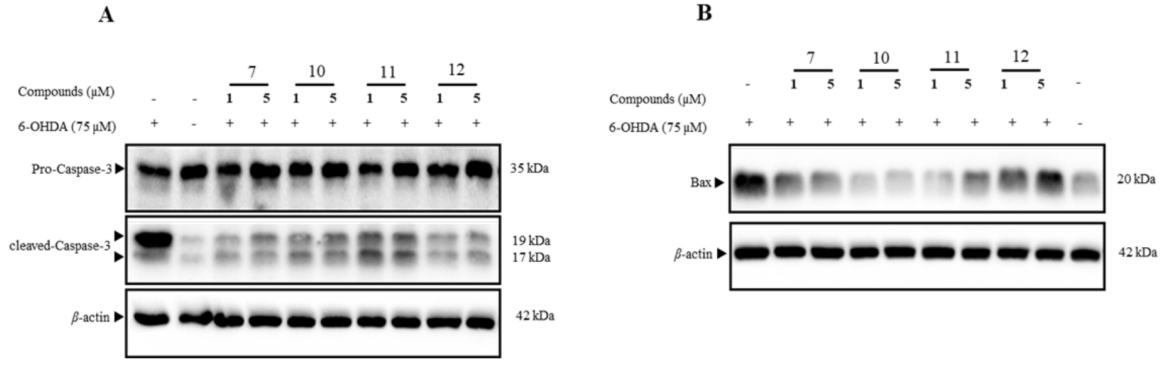


Figure 2. Selected compounds prevent 6-OHDA-induced neurotoxicity. SH-SY5Y cells were first treated with the desired concentration of selected compounds, then treated with 6-OHDA. While DMSO was used as a solvent control; 6-OHDA was used as a positive control which led to activating the cleavage form of caspase-3 and increased the level of Bax. The levels of pro-caspase 3, cleavage-caspase 3, and Bax were determined by WB using antibodies against them. β-actin was used as a loading control. The experiments were repeated three times independently; with one representative result shown

6-OHDA: 6-Hidroksi-dopamine, DMSO: Dimethyl sulfoxide, WB: Western blot

compounds but decreases the AChE inhibitory activity; therefore, adding another methylene group to the intermediate chain to test the neuroprotective activity of the pyrazole/1,2,4-triazole butyramide derivatives will be our future focus.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Footnotes

Authorship Contributions

Surgical and Medical Practices: A.H.T., M.S., S.Y., S.P., P.B., V.A., Concept: A.H.T., M.S., Design: A.H.T., V.A., Data Collection or Processing: A.H.T., M.S., S.Y., S.P., P.B., V.A., Analysis or Interpretation: A.H.T., M.S., S.Y., S.P., P.B., V.A., Literature Search: A.H.T., M.S., S.Y., Writing: A.H.T., M.S., S.Y., S.P., P.B., V.A.

Conflict of Interest: The authors have no conflicts of interest to declare.

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REFERENCES

1. Vitale I, Pietrocola F, Guilbaud E, Aaronson SA, Abrams JM, Adam D, Agostini M, Agostinis P, Alnemri ES, Altucci L, Amelio I, Andrews DW, Aqeilan RI, Arama E, Baehrecke EH, Balachandran S, Bano D, Barlev NA, Bartek J, Bazan NG, Becker C, Bernassola F, Bertrand MJM, Bianchi ME, Blagosklonny MV, Blander JM, Blandino G, Blomgren K, Borner C, Bortner CD, Bove P, Boya P, Brenner C, Broz P, Brunner T, Damgaard RB, Calin GA, Campanella M, Candi E, Carbone M, Carmona-Gutierrez D, Cecconi F, Chan FK, Chen GQ, Chen Q, Chen YH, Cheng EH, Chipuk JE, Cidlowski JA, Ciechanover A, Ciliberto G, Conrad M, Cubillos-Ruiz JR, Czabotar PE, D'Angiolella V, Daugaard M, Dawson TM, Dawson VL, De Maria R, De Strooper B, Debatin KM, Deberardinis RJ, Degterev A, Del Sal G, Deshmukh M, Di Virgilio F, Diederich M, Dixon SJ, Dynlacht BD, El-Deiry WS, Elrod JW, Engeland K, Fimia GM, Galassi C, Ganini C, Garcia-Saez AJ, Garg AD, Garrido C, Gavathiotis E, Gerlic M, Ghosh S, Green DR, Greene LA, Gronemeyer H, Häcker G, Hajnóczky G, Hardwick JM, Haupt Y, He S, Heery DM, Hengartner MO, Hetz C, Hildeman DA, Ichijo H, Inoue S, Jäättelä M, Janic A, Joseph B, Jost PJ, Kanneganti TD, Karin M, Kashkar H, Kaufmann T, Kelly GL, Kepp O, Kimchi A, Kitsis RN, Klionsky DJ, Kluck R, Krysko DV, Kulms D, Kumar S, Lavandero S, Lavrik IN, Lemasters JJ, Liccardi G, Linkermann A, Lipton SA, Lockshin RA, López-Otín C, Luedde T, MacFarlane M, Madeo F, Malorni W, Manic G, Mantovani R, Marchi S, Marine JC, Martin SJ, Martinou JC, Mastroberardino PG, Medema JP, Mehlen P, Meier P, Melino G, Melino S, Miao EA, Moll UM, Muñoz-Pinedo C, Murphy DJ, Niklison-Chirou MV, Novelli F, Núñez G, Oberst A, Ofengeim D, Opferman JT, Oren M, Pagano M, Panaretakis T, Pasparakis M, Penninger JM, Pentimalli F, Pereira DM, Pervaiz S, Peter ME, Pinton P, Porta G, Prehn JHM, Puthalakath H, Rabinovich GA, Rajalingam K, Ravichandran KS, Rehm M, Ricci JE, Rizzuto R, Robinson N, Rodrigues CMP, Rotblat B, Rothlin CV, Rubinsztein DC, Rudel T, Rufini A, Ryan KM, Sarosiek KA, Sawa A, Sayan E, Schroder K, Scorrano L, Sesti F, Shao F, Shi Y, Sica GS, Silke J, Simon HU, Sistigu A, Stephanou A, Stockwell BR, Strapazzon F, Strasser A, Sun L, Sun E, Sun Q, Szabadkai G, Tait SWG, Tang D, Tavernarakis N, Troy CM, Turk B, Urbano N, Vandenabeele P, Vanden Berghe T, Vander Heiden MG, Vanderluit JL, Verkhratsky A, Villunger A, von Karstedt S, Voss AK, Vousden KH, Vucic D, Vuri D, Wagner EF, Walczak H, Wallach D, Wang R, Wang Y, Weber A, Wood W, Yamazaki T, Yang HT, Zakeri Z, Zawacka-Pankau JE, Zhang L, Zhang H, Zhivotovsky B, Zhou W, Piacentini M, Kroemer G, Galluzzi L. Apoptotic cell death in disease-current understanding of the NCCD 2023. Cell Death Differ. 2023;30:1097-1154.
2. Mattson MP. Apoptosis in neurodegenerative disorders. Nat Rev Mol Cell Biol. 2000;1:120-129.
3. Radi E, Formichi P, Battisti C, Federico A. Apoptosis and oxidative stress in neurodegenerative diseases. J Alzheimers Dis. 2014;42 Suppl 3:S125-S152.
4. Friedlander RM. Apoptosis and caspases in neurodegenerative diseases. N Engl J Med. 2003;348:1365-1375.
5. McDonald ES, Windebank AJ. Mechanisms of neurotoxic injury and cell death. Neurol Clin. 2000;18:525-540.
6. Fricker M, Tolkovsky AM, Borutaite V, Coleman M, Brown GC. Neuronal Cell Death. Physiol Rev. 2018;98:813-880.
7. Cacciatore I, Fornasari E, Baldassarre L, Cornacchia C, Fulle S, Di Filippo ES, Pietrangelo T, Pinnen F. A potent (R)-alpha-bis-lipoyl derivative containing 8-hydroxyquinoline scaffold: synthesis and biological evaluation of ıts neuroprotective capabilities in SH-SY5Y human neuroblastoma cells. Pharmaceuticals (Basel). 2013;6:54-69.
8. Saylam M, Tarikogullari AH, Yılmaz S, Ballar Kırmızıbayrak P. Neuroprotective activity studies of some phenylacetamide derivatives bearing 1H-pyrazole or 1H-1,2,4-triazole. Bioorganic Med Chem Reports. 2022;2:1-5.
9. Varešlija D, Tipton KF, Davey GP, McDonald AG. 6-Hydroxydopamine: a far from simple neurotoxin. J Neural Transm. 2020;127:213-230.
10. Ren JL, Zhang XY, Yu B, Wang XX, Shao KP, Zhu XG, Liu HM. Discovery of novel AHLs as potent antiproliferative agents. Eur J Med Chem. 2015;93:321-329.
11. Bonfanti JF, Doublet F, Fortin J, Lacrampe J, Guillemont J, Muller P, Queguiner L, Arnoult E, Gevers T, Janssens P, Szel H, Willebrords R, Timmerman P, Wuyts K, Janssens F, Sommen C, Wigerinck P, Andries K. Selection of a respiratory syncytial virus fusion inhibitor clinical candidate, part 1: improving the pharmacokinetic profile using the structure-property relationship. J Med Chem. 2007;50:4572-4584.
12. Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7:88-95.
13. Kapková P, Stiefl N, Sürig U, Engels B, Baumann K, Holzgrabe U. Synthesis, biological activity, and docking studies of new acetylcholinesterase inhibitors of the bispyridinium type. Arch Pharm (Weinheim). 2003;336:523-540.
14. Robert M. Silverstein, Webster FX, Kiemle DJ, Bryce DL. Spectrometric Identification of Organic Compounds, 8th edition. Published online 2014:2014.
15. Nakanishi K, Solomon PA. Infrared absorption spectroscopy. 2nd Edition. Published online 1977:287.
16. Hesse M, Meier H, Zeeh B. Spectroscopic methods in organic chemistry, 2nd edition. 2007:450.
17. Lou H, Jing X, Wei X, Shi H, Ren D, Zhang X. Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway. Neuropharmacology. 2014;79:380-388.
18. Gomez-Lazaro M, Galindo MF, Concannon CG, Segura MF, Fernandez-Gomez FJ, Llecha N, Comella JX, Prehn JH, Jordan J. 6-Hydroxydopamine

activates the mitochondrial apoptosis pathway through p38 MAPK-mediated, p53-independent activation of Bax and PUMA. J Neurochem. 2008;104:1599-1612.

19. Jordán J, Galindo MF, Tornero D, González-García C, Ceña V. Bcl-x L blocks mitochondrial multiple conductance channel activation and inhibits 6-OHDA-induced death in SH-SY5Y cells. J Neurochem. 2004;89:124-133.

20. Chen JH, Ou HP, Lin CY, Lin FJ, Wu CR, Chang SW, Tsai CW. Carnosic acid prevents 6-hydroxydopamine-induced cell death in SH-SY5Y cells via mediation of glutathione synthesis. Chem Res Toxicol. 2012;25:1893-1901.

21. Drechsel DA, Patel M. Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson's disease. Free Radic Biol Med. 2008;44:1873-1886.

22. Soyer Z, Uysal S, Parlar S, Tarikogullari Dogan AH, Alptuzun V. Synthesis and molecular docking studies of some 4-phthalimidobenzenesulfonamide derivatives as acetylcholinesterase and butyrylcholinesterase inhibitors. J Enzyme Inhib Med Chem. 2017;32:13-19.

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ORIGINAL ARTICLE

Investigation of the Effects of Polyunsaturated Fatty Acid Ratios in Human SH-SY5Y Cells by *in vitro* Methods

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ABSTRACT

Objectives: Neuroblastoma, an embryonic tumor of the sympathetic nervous system, is the deadliest type of cancer, accounting for 6-9% of all childhood cancers. The omega (ω)-6 [linoleic acid (LA)]: ω-3 [eicosapentaenoic acid (EPA)]=13/1 ratio is associated with the presence of chronic disease, and it has been reported that reducing this ratio to 7/1 protects against cancer and cardiovascular diseases. This study aimed to investigate the anticancer effects of different ratios of ω-3 and ω-6 fatty acids in human neuroblastoma cells (SHSY-5Y).

Materials and Methods: SHSY-5Y cells were treated with different ratios of ω-3 and ω-6 fatty acids for 48 and 72 h. The viability of ω-3 and ω-6 fatty acid-treated cells was measured using the methylthiazolyldiphenyl-tetrazolium bromide. The percentage of cell apoptosis was detected using the Fluorescein Isothiocyanate-conjugated Annexin-V/PI assay, and reactive oxygen species (ROS) analysis was performed using flow cytometry. The expression levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and *transforming growth factor beta 1* (TGFβ1) genes were determined using real-time polymerase chain reaction.

Results: EPA and LA separately significantly inhibited the proliferation of SH-SY5Y cells within 48 h (p<0.001). It was found that apoptosis decreased significantly in all groups due to the application of polyunsaturated fatty acids at different ratios, and the most effective dose was ω-3:ω-6 ratio: 1/1. ROS levels were significantly decreased compared with the control group and the lowest ROS level was observed in the ω-3:ω-6 ratio: 1/4 group. Both TNF-α, IL-6, and TGFβ1 mRNA expressions increased significantly after the addition of fatty acid mixtures compared with the control; they were observed to decrease with an increasing ω-6 ratio.

Conclusion: This study is the first to examine the effects of the ω-3:ω-6 ratio on neuroblastoma cancer cells. The application of ω fatty acids decreased apoptosis at all ratios. In contrast, when the ω-3: ω-6 ratio increased, the amount of ROS. Additionally, as the ω-3:ω-6 ratio increases, a decrease in the release of pro-inflammatory cytokines is noted.

Keywords: Apoptosis, IL-6, NF-κB, ROS, SHSY-5Y cell, TGFβ1, ω-3:ω-6 ratio

INTRODUCTION

Neuroblastoma is an embryonic tumor responsible for 6-9% of all pediatric malignancies. Neuroblastoma exhibits a variety of biological and clinical characteristics, ranging from spontaneous remission to exceedingly severe illness with metastatic dissemination.[1] The cure rate for these patients is 50% despite a wide variety of treatment modalities and many relapses due to minimal residual disease.[2] There is a great need for new, effective, and less harmful treatments for patients with neuroblastoma. However, improving current treatment methods may improve overall survival.

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Compared with normal nervous tissue, neuroblastoma, glioma, and meningioma have high levels of omega (ω)-6 [linoleic acid (LA)] fatty acid arachidonic acid (AA) and low levels of ω-3 [eicosapentaenoic acid (EPA)] fat.[3] This proportionate difference may act as an adaptive mechanism in nervous system malignancies.[4] It has been suggested that ω-3 fatty acids contribute to mechanisms that arrest tumor growth progression by accumulating intracellular reactive oxygen species (ROS) and promoting death through antiangiogenic actions.[5]

Clinical trials in adult patients with cancer have shown that ω-3 fatty acids can improve the inflammatory response, nutritional status, and quality of life.[6,7] One study showed that ω-3 supplementation during treatment reduces the side effects of chemotherapy, regardless of the type of drug used.[7-9] ω-3 fatty acids, especially ω-3 (EPA) and docosahexaenoic acid (DHA), have been reported to improve tumor response to treatment, protect against toxicity, and reduce secondary complications when administered as an adjunct to chemotherapy in patients with different types of cancer.[10] Lipid mediators such as prostaglandins derived from ω-6 fatty acids act as pro-inflammatory mediators, whereas the products of ω-3 fatty acids are anti-inflammatory. For example, ω-3 reduces the production of proinflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), and IL-6. At the same time, the ω-6 series exerts indirect anti-inflammatory effects by inhibiting eicosanoid biosynthesis. Given these reciprocal effects, higher ω-6 to ω-3 ratios increase carcinogenesis risk.[11] Numerous ω-6-derived prostaglandins and leukotrienes have been shown to increase tumor growth by promoting angiogenesis, increasing cellular proliferation, suppressing the immune system, and inhibiting apoptosis.[12,13] Studies have shown that inhibition of the enzymes responsible for producing prostaglandins and leukotrienes suppresses tumor growth.[14-16]

Dietary intake of saturated fatty acids instead of polyunsaturated fatty acids (PUFAs) has recently been encouraged. However, high ω-6/ω-3 PUFA ratios in Western diets increase cancer risk.[17] These results have been confirmed in animal studies. Although some studies have shown that high-fat diets rich in monounsaturated fatty acid and ω-6 PUFAs promote tumor growth and induce liver metastasis, in contrast, administration of ω-3 PUFAs, such as EPA or DHA, has been reported to reduce cancer-related symptoms.[18] Further research has revealed that ω-3 PUFA induces ferroptosis in cells through lipid peroxidation and inhibits tumor growth when combined with ferroptosis inducers.[19] In one study, dietary supplementation with DHA and EPA increased the antitumor efficacy of chemotherapy.[20] Dietary programs have also been reported to slow the development of cancer. Among these programs, the ketogenic, low-carbohydrate, and high-fat diets have been reported to reduce tumor growth significantly.[21,22]

Both direct administration of dietary FAs and FA-related modification of dietary patterns have been shown to improve cancer risk. There are no specific nutritional standards or guidelines for cancer treatment. When prescribing dietary therapy, particular attention should be paid to other nutritional deficiencies and the increased risk of disease due to the specific diet; There is no specific recommendation regarding the type of FA to be taken. This study examined the impact of ω-3:ω-6 fatty acid ratio on neuroblastoma cell release and cytokine release. Fatty acids are known to play a role in tumor cell proliferation, metastasis, and drug resistance; thus, targeting fatty acid metabolism is a promising therapeutic strategy. The present study aimed to investigate the anticancer effects of different ratios of ω-3 and ω-6 fatty acids in human neuroblastoma cells (SHSY-5Y).

Given the difficulty of anticancer medications entering the blood-brain barrier, the therapeutic effect of ω-3:ω-6 PUFAs against brain cancer might be an excellent possibility to examine.

MATERIALS AND METHODS

Cell culture

The human neuroblastoma cells (SHSY-5Y) (CRL-2266™) cell line was used in this study. Cells were cultured at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Capricorn Scientific, Germany) with 10% Fetal Bovine Serum (FBS) (Capricorn Scientific, FBS-HI11B, Germany), 100 IU/mL penicillin (Capricorn Scientific, PS-B, Germany), 100 µg/mL streptomycin (Capricorn Scientific, PS-B, Germany), and passaged at 70-80% confluence.

Fatty acids and experimental groups

EPA as the ω-3 fatty acid and LA as the ω-6 fatty acid were used in this study. Six experimental groups were created. The first group was the control group, which was not subjected to treatment. The ω-3:ω-6 ratios were 1/1, 1/2, 1/4, 1/8, and 1/16, respectively.

Administration of EPA and LA

200 µL of EPA (item no: 90110, Cayman) were diluted according to the kit protocol. Then, a 4-mM stock solution was obtained. The stock solution was then serially diluted in DMEM to doses of 5 µM, 10 µM, 25 µM, 40 µM, 50 µM and applied to the cells for 48 and 72 h. 3.241 M LA (PC1003367601, Sigma) was diluted according to the kit protocol to obtain a 4-mM stock solution. As with EPA2, the stock solution was serially diluted in DMEM to doses of 5 µM, 10 µM, 25 µM, 40 µM, and 50 µM and applied to cells for 48 and 72 h.

Methylthiazolyldiphenyl-Tetrazolium Bromide (MTT) assay

Cells were seeded in a 96-well plate at 10^4 cells per well and incubated for 24 h at 37 °C in an atmosphere of 5% CO_2. Cells were treated with a combination of ω-6 and ω-3 fatty acids. After 48 h of incubation, each well received 20 µL of MTT solution and incubated for 3-4 h. After incubation, the supernatant was gently removed. Each well was treated with 100 µL of isopropyl alcohol and incubated for 2 h at room temperature in the dark. The plate was then shaken and read at 570 nm.

Annexin-V analysis

Measurement of Annexin-V bound to the cell surface as an indicator of apoptosis should be performed in conjunction with

a staining test to determine the integrity of the cell membrane. It distinguishes whole cells Fluorescein Isothiocyanate (FITC-/PI-), apoptotic cells (FITC+/PI-), and necrotic cells (FITC+/PI+). To analyze apoptosis, cells ($1x10^5$) were trypsinized after being treated with varying ratios of ω-6 and ω-3 fatty acids after 48 h. The cell pellet was then dissolved in 100 µL binding solution and added to $5x10^5$ cells in 500 mL. Then, 5 µL of Annexin and 20 µL of PI were added, and the mixture was pipetted. After 30 min of incubation in the dark at room temperature, 400 µL of 1X binding buffer was added and the samples were examined using a BD Accuri C6 (BD Biosciences) flow cytometer.

Analysis of ROS levels

The intracellular ROS were determined using a ROS detection kit (THORVACS Biotechnology, Cat#; ROS-100T). Cells prepared in the experimental groups created in the SH-SY5Y cell line were collected by trypsinization method. $1x10^5$ cells were suspended in PBS, centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. Cell pellets were resuspended in 200 µL of 100 mM DCF-DA reagent and incubated at 37 °C for 30 min in the dark. The cells were then centrifuged at 1000 rpm for 5 min, after which the supernatant was discarded. The pellet was resuspended in 100 µL PBS and analyzed using the FL1 channel on a BD Accuri C6 (BD Biosciences) flow cytometer.

Gene expression analysis

Real-time polymerase chain reaction (PCR) was used for quantitative investigation of TNF-α, IL-6, and TGFβ1 mRNA expression levels. After administering fatty acids, cells were cultured for 24 h before the flask was trypsinized. Total RNA was isolated using an RNA isolation kit (Qiagen, Germany). The multi-mode gave purity and quantity measurements using a microbiological reader. The total RNA amounts obtained from the cells were calculated according to the 260/280 nm ratio using a spectrophotometer, and the results were found as ng/µL. cDNA synthesis was performed from the extracted and thawed samples using a transcriptor first strand cDNA synthesis kit (Roche) and a T100 PCR System (Bio-Rad, Hercules, CA, USA) thermal cycler. GAPDH was selected as the housekeeping gene. The real-time PCR heat cycle consisted of 35 cycles: 1 min at 95 °C, 15 s at 95 °C, 1 min at 60 °C, and 30 s at 72 °C. Primary designs were created using NCBI and primer3 sites. Table 1 lists the primers used. National Center for Biotechnology Information GenBank Primer-Blast® was built and evaluated using sequence matching analysis (Table 1).

Statistical analysis

The mean and standard deviation (mean ± standard deviation) were used for continuous data as descriptive statistics. The suitability of continuous data for normal distribution was checked using the Kolmogorov-Smirnov test. The distributions of variables in two groups that were suitable for normal distribution were compared with the Student's *t*-test, and those that were not suitable for normal distribution were compared using the Mann-Whitney *U* test. The distributions of variables that did not comply with normal distribution in three or more groups were analyzed using the Kruskal-Wallis test, and the formula developed by Conover[23] was used as the multiple comparison test. Data were analyzed using the IBM SPSS 23 (IBM SPSS Inc, Chicago, IL) package program. The significance level was set as $p<0.05$.

RESULTS

Determination of cell viability in SHSY-5Y cells after 48-72 h exposure to fatty acid concentrations of 5, 10, 25, 40 and 50 µM via MTT assay

To explore the cell viability of ω-3 and ω-6 fatty acids alone or in combination in the SH-SY5Y cell line, the cells were treated with serial concentrations of EPA and LA (5-50 µM) for 48 and 72 h. Both EPA and LA alone significantly inhibited the proliferation of SH-SY5Y cells within 48 h ($p<0.01$) (Figure 1). Cell viability increased significantly as a result of 72 h of treatment with PUFAs ($p<0.001$) Figure 1.

Determination of cell viability in SHSY-5Ycells after 48 h of exposure to fatty acid ratios of 1/1, 1/2, 1/4, 1/8, and 1/16 via MTT assay

Treatment of SH-SY5Y cells with fatty acid ratios of 1/1, 1/2, 1/4, 1/8, and 1/16 induced a significant decrease in viable cells within 48 h ($p<0.01$) (Figure 2).

Results of the Annexin-V assay

The Annexin-V assay was used to measure the percentage of apoptotic cells. The percentage of live cells in the control group for SHSY-5Y cells is 99.6%. After 48 h of fatty acid treatment at ω-3:ω-6 ratio =1/1, ω-3:ω-6 ratio =1/2, ω-3:ω-6 ratio =1/4, ω-3:ω-6 ratio =1/8, ω-3:ω-6 ratio =1/16 the percentages of viability were 27.4%, 45.4%, 49.7%, 54%, 43.6% respectively (Figure 3).

ROS results

For the ROS assay, the ROS value in the control group was 21%, and the 48th-h fatty acid treatment (ω-3: ω-6 ratio =1/1, ω-3:ω-6 ratio =1/2, ω-3:ω-6 ratio =1/4, ω-3:ω-6 ratio =1/8, ω-3:ω-6 ratio =1/16) for the SHSY-5Y cell lines were 19.8%, 11.5%, 1.5%, 2.7%, and 6.3%, respectively (Figure 4).

Table 1. Forward and reverse primers of genes

GAPDH	F: TTTTGCGTCGCCAGCC	R: ATGGAATTTGCCATGGGTGGA
TNF-α	F: AAAACAACCCTCAGACGCCA	R: TCCTTTCCAGGGGAGAGAGG
IL-6	F: CTTCGGTCCAGTTGCCTTCTC	R: GGCATTTGTGGTTGGGTCAG
TGFβ1	F: ACCTGCCACAGATCCCCTAT	R: GAGCAACACGGGTTCAGGTA

TNF-α: Tumor necrosis factor-alpha, TGFβ1: Transforming growth factor beta, IL-6: Interleukin-6

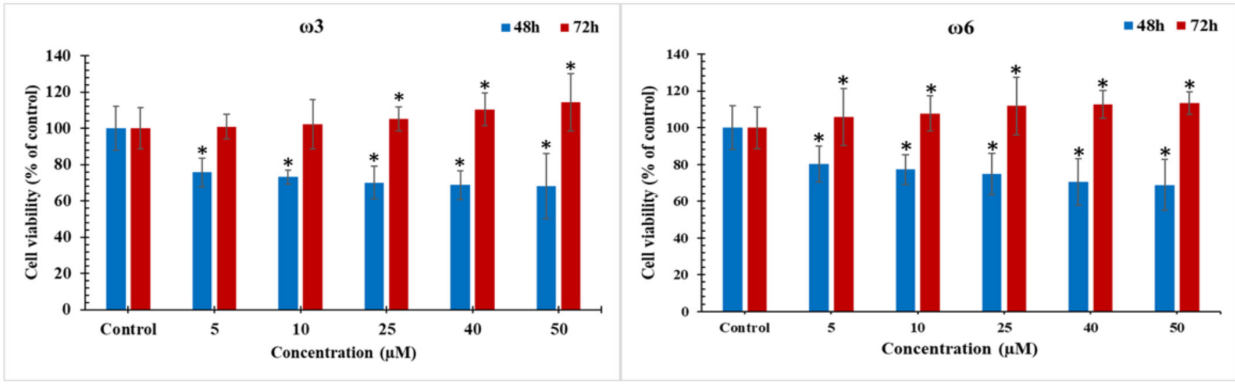

Figure 1. SHSY-5Y cells MTT assay. Fatty acids (5, 10, 25, 40 and 50 µM concentrations were added to SHSY-5Y cell culture. After 48-72 h treatment with fatty acids, MTT tests were performed for each concentration. *p<0.01 significance level obtained by comparing the control group

MTT: Methylthiazolyldiphenyl-tetrazolium bromide

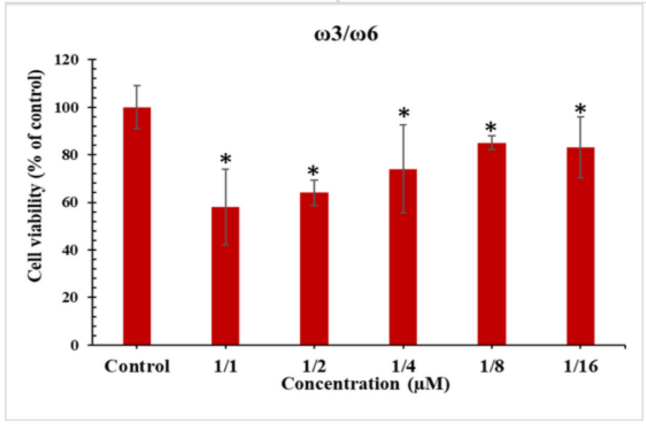

Figure 2. SHSY-5Y cells MTT assay. Fatty acid ratios were added to SHSY-5Y cells. After 48 h of treatment with fatty acids, MTT tests were performed for each concentration. *p< 0.01 significance level obtained by comparing the control group

MTT: Methylthiazolyldiphenyl-tetrazolium bromide

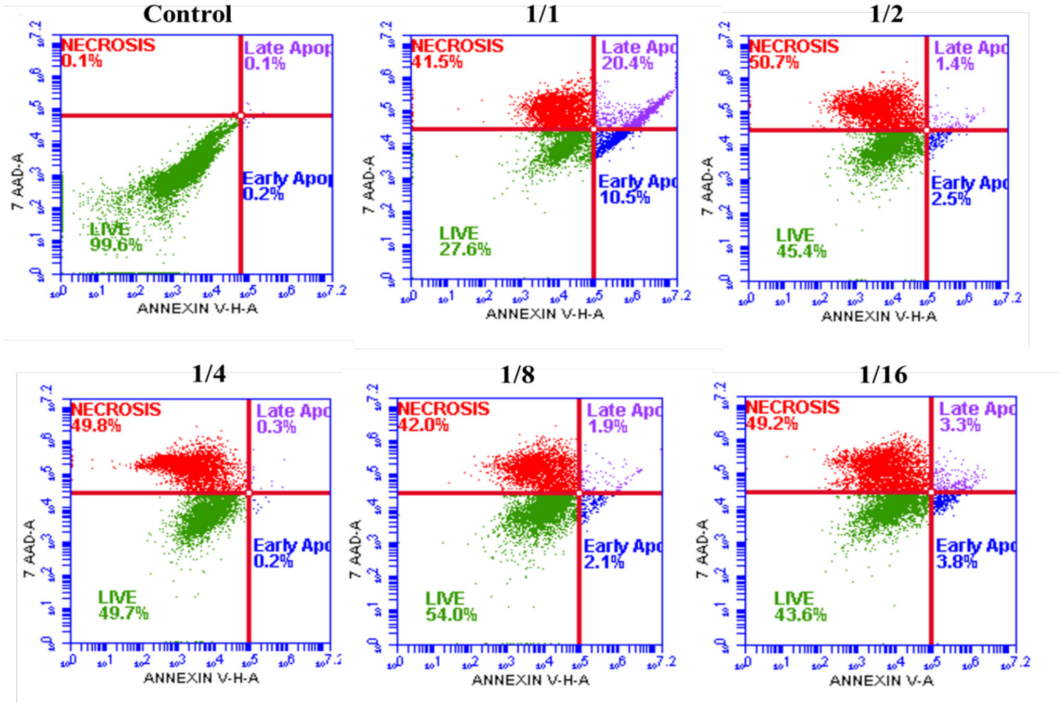

Figure 3. B1 (Annexin -, PI +): Necrotic cells; B2 (Annexin +, PI +): Late apoptosis; B3: Live cells (Annexin -, PI -); B4 (Annexin +, PI -): Early apoptosis

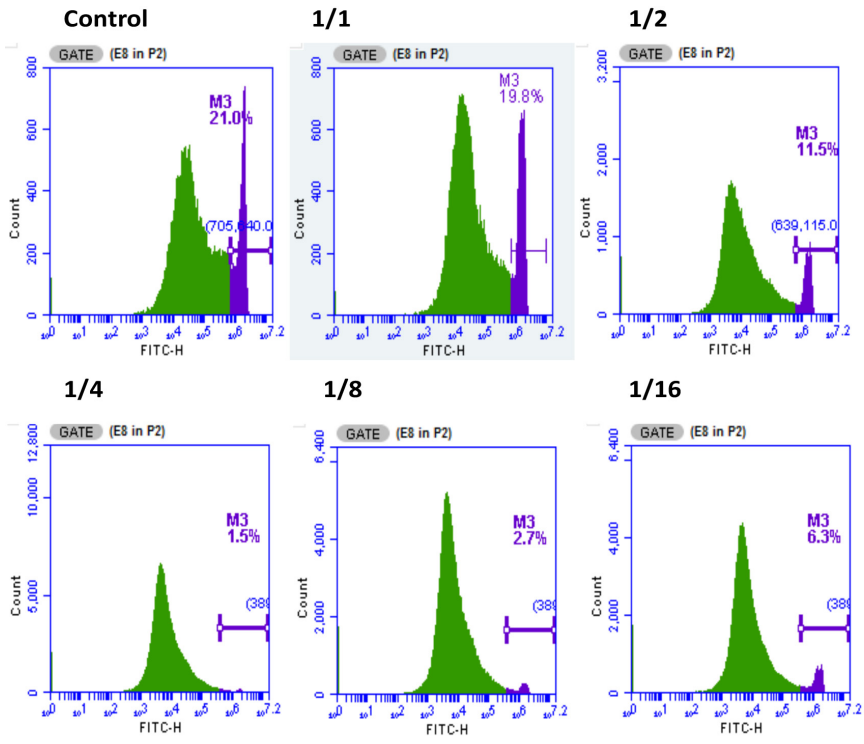

Figure 4. SHSY-5Y cell line ROS results
ROS: Reactive oxygen species

Gene expression results

The mRNA expression levels of TNF-a, IL-6, and TGFβ1 were increased 5.78, 2.14, and 7.3 times at ω-3:ω-6 ratios of 1:4 compared with the control group (p<0.05, p<0.05, p<0.001, respectively) (Figure 5). The mRNA expression levels of IL-6 were increased 2.69, 3.15, and 2.14 times in groups with ω-3/ω-6 ratios of 1:1, 1:2, and 1:4 compared with the control group (p<0.05, p<0.01, and p<0.05, respectively). Compared with the control group, IL-1β expression increased 12.9 and 9.29 times in the 1:4 and 1:16 ω-3:ω-6 ratio (p<0.05 and p<0.005, respectively). TGFβ1 expression increased 7.71 and 6.69 times in the 1:2 and 1:8 groups (p<0.001 and p<0.05, respectively) (Figure 5).

When the ω fatty acid application groups were evaluated within themselves, TNF-a expressions increased 4.77, 4.79, and 5.79 times in the 1:1, 1:2, and 1:4 groups, respectively, and decreased again in the 1:8 and 1:16 groups. TNF-α expression was observed to be statistically significantly lower in the group with a ω-3:ω-6 ratio of 1:8 compared with the group with 1:4 (p<0.01), and it decreased statistically significantly in the group with a ω-3:ω-6 ratio of 1:16 compared with the groups with 1:1, 1:2, and 1:4 (p<0.05, p<0.05 and p<0.005, respectively) (Figure 5).

IL-6 mRNA expression was increased in the group with a ω-3:ω-6 ratio 1:1, and it decreased as the ω-6 ratio increased. IL-6 expression was significantly lower in the group with a ω-3:ω-6 ratio of 1:8 than in the 1:1 and 1:2 groups (p<0.05 and p<0.05, respectively). IL-6 expression was significantly decreased in the group with a ω-3:ω-6 ratio of 1:16 compared with the 1:1, 1:2, and 1:4 groups (p<0.01, p<0.005 and p<0.05, respectively) (Figure 5).

TGFβ1 mRNA expressions were observed to be significantly higher in groups with ω-3:ω-6 ratios of 1:2 and 1:4 than in the 1:1 group (p<0.01 and p<0.05, respectively) and in the 1:16 group (p<0.001 and p<0.001, respectively). TGFβ1 expressions were significantly decreased in the ω-3:ω-6 ratio group compared to the 1:8 group, but there was no statistical significance (p>0.05) expressions significantly decreased in the group with ω-3:ω-6 ratio of 1:16 compared with the 1:8 group (p=0.056) (Figure 5).

DISCUSSION

Western diet contains high amounts of ω-6 PUFAs and small amounts of ω-3 s. The resulting high ω-6:ω-3 ratio is believed to contribute to different diseases, such as inflammation and cancer. In particular, evidence that ω-3 PUFA may be inhibitory on neuroblastoma has been shown in both *in vitro* and *in vivo* studies.[19] In contrast, evidence suggests that a high intake of ω-6 PUFAs is associated with an increased risk of developing cancer.[24] The ω-3:ω-6 ratio is important in maintaining an appropriate level of biological membrane fluidity, which is essential for ion channel function, membrane receptor activity, and the release of neurohormones.[21]

EPA is an ω-3 fatty acid with significant anti-cancer effects by regulating the expression of anti-inflammatory mediators, inhibiting cell proliferation, and modulating cell death pathways.[20] Studies have shown that EPA can activate multiple molecular mechanisms, including both classical and alternative apoptotic

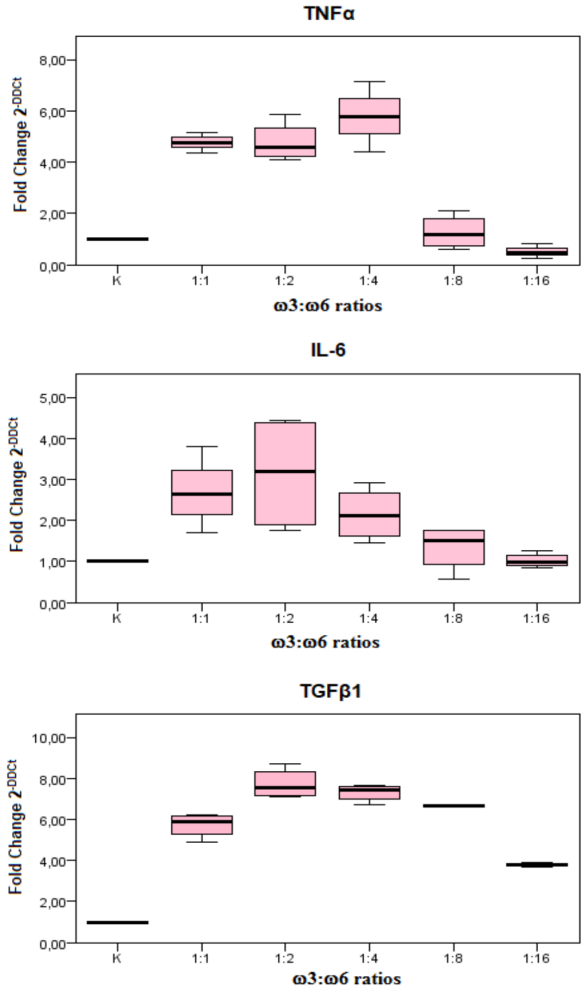

Figure 5. mRNA expression levels of TNF-α, IL-6 and TGFβ1 in the SHSY-5Y cell line

TNF-α: Tumor necrosis factor-alpha, TGFβ1: Transforming growth factor beta, IL-6: Interleukin-6

pathways, and regulate survival and cell growth signalling, ultimately leading to cell death in various *in vitro* and *in vivo* animal models.[22,25] Studies have shown that ω-3 fatty acids reduce neuroblastoma cell proliferation.[26,27] It was revealed that oleic acid treatment significantly reduced SHSY-5Y cells in a time- and dose-dependent manner.[28] In a study of human neuroblastoma LA-N-1 cells, it was reported that ω-3 fatty acids docosahexaenoic acid (DHA) and EPA exert antiproliferative effects depending on time and concentration.[29] In our study, a similar decrease in cell proliferation was observed at 48 h when increasing doses of EPA were administered.

ω-6 PUFAs are also reported to have anticancer activity. In one study, LA as an ω-6, the most abundant PUFA in nature, was reported to suppress cancer cell growth by inducing ROS production and mitochondrial damage.[30] In other studies, unlike this one, they emphasized that LA has a proapoptotic effect on cancer.[31-33] In another study examining the effect of ω-3 and ω-6 fatty acids on human IMR-32 neuroblastoma cells, it was reported that fatty acids significantly reduced the growth rate of cells to different degrees.[34] There are studies suggesting that ω-6 fatty acids have a procarcinogenic effect on increased eicosanoid ratios or, conversely, suppress cancer cell growth by inducing ROS production and mitochondrial damage.[35,36] In our study, we observed a decrease in cell proliferation as a result of increasing doses of LA for 48 h.

The ω-3:ω-6 ratio is an important nutritional parameter. While this ratio was 1:6.4 at the turn of the last century, it changed to 1:10 in the 2000s.[37] The high ω-6:ω-3 ratio in Western diets increases the risk of cancer.[37] The anticancer effects of different ω-3:ω-6 ratios were investigated using *in vitro* methods. In SH-SY5Y cells treated with different ω-3:ω-6 ratios, although similar results were obtained in separate applications of these fatty acids, we observed a significant and greater reduction in cell proliferation in the ω-3:ω-6 mixture at high levels of ω-3 than in the individual applications. This suggests that they are more effective together, and the effect of ω-3 emerges more strongly.

Apoptosis is referred to as programmed cell death during normal development due to cell aging or homeostasis in a cell population. In a study with liver cancer cell lines, DHA and EPA administration was shown to mediate apoptosis by causing caspase 3 and caspase 9 activity.[38] Yamamoto et al.[39] reported that EPA increased the expression of pro-apoptotic (BAX and BCL-XS) and decreased the expression of anti-apoptotic (BCL-2 and BCL-XL) proteins in breast cancer cell lines. On the other hand, the role of ω-6 in apoptosis is more complex, with some studies supporting the activation of apoptosis and others suggesting inhibition.[40] In the present study, we found that apoptosis was induced by increasing the ω-3 fatty acid ratio. The most effective PUFA ratio was found to be 1/1. In addition, our Annexin-V analysis results also support our MTT results.

It is well known that reactive oxygen and nitrogen species are the underlying causes of many diseases, including cancer. Cancer cells produce high levels of ROS because of their rapid metabolism and impaired cellular signalling pathways. In general, the amount of free radicals increases in cancer. A previous study showed that Ppt1-KO neurons treated with a combination of ω-3 and ω-6 had significantly reduced ROS levels compared with the untreated group.[41] However, another study showed that 24 h administration of LA and EPA increased ROS levels in embryonic stem cells.[42] Additionally, a study reported that LA, an ω-6 PUFA, suppresses cancer cell growth by inducing ROS production and mitochondrial damage, and similar to ω-3 PUFAs, ω-6 PUFAs also have anticancer activity.[43] In our study, it is seen that there is a decrease in ROS values 48 h after the application of ω fatty acids. The ROS level is relatively low, especially when the ω-3:ω-6 ratio is 1:4 or 1:8. According to this result, it can be concluded that the use of ω-3:ω-6 in the ratio of 1:4 or 1:8 can make a significant contribution to protecting against free radical damage.

The inflammatory process and cancer development are closely related. Chronic inflammation is a bipolar process that, on the one hand, may stimulate cancer development and progression,

and on the other hand, the recruitment of immunocompetent cells and their activation may cause tumour suppression and apoptosis. Tumour-associated cells are a potent source of immunomodulatory molecules, such as the proinflammatory cytokines IL-1, IL-6, and TNF-α, which are involved in the stimulation of key tumour-promoting factors, such as STAT3 and NF-κB. It has been reported that ω-3 PUFAs modulate the duration and intensity of inflammatory processes, and their increase in the diet reduces proinflammatory cytokines such as IL-1β, TNF-α, and IL-6.[44] In addition, it has been stated that the ω-6 series exerts indirect proinflammatory effects by inhibiting eicosanoid biosynthesis. Considering these mutual effects, higher ω-3:ω-6 PUFA ratios increase carcinogenesis risk.

In a study by Liang et al.,[45] mice inoculated with androgen-sensitive prostate cancer cells were fed a high-fat diet rich in ω-3 fatty acids, and mice in this group were shown to express lower levels of proinflammatory cytokines (IL-6, TNF-α, and IL-10) and chemoattractant protein than animals fed a high-fat diet containing ω-6 fatty acids. While ω-6 fatty acids are known to activate proinflammatory pathways, Lipoxin A4 (LXA4), a common type of lipoxin, is a metabolite derived from endogenous AA in the ω-6 series. LXA4 is reported to be a lipid mediator of endogenous anti-inflammation and resolution.[46] In another study, LipoxinA4 was shown to antagonize TNF-α-stimulated IL-1β and IL-6 synthesis.[47] Another study showed that LXA4 in activated synovial fluid inhibits the synthesis of inflammatory cytokines and MMP and stimulates TIMP production *in vitro*. This result has been interpreted as suggesting that LXA4 is involved in a negative feedback loop that opposes the inflammatory cytokine-induced activation of synovial fluid.[48] In our study, an initial increase in TNF-α and IL-6 expression was observed as the ω-6 fatty acid ratio increased, but a decrease was observed, and similar results were obtained in a repeated study. We believe that this result may be due to an increase in AA-induced lipoxin synthesis due to the increased amount of LA. On the other hand, we observed that as the ω-6 fatty acid ratio increased, the expression of TGFβ1, one of the anti-inflammatory cytokines, decreased. A limitation of our study was that the protein expression of cytokines could not be measured because mRNA expression may not always fully express protein expression.

CONCLUSION

In conclusion, in our study, although significant decreases in apoptosis were generally observed with the application of ω fatty acids, we observed a decrease in the amount of ROS with an increase in the ω-6 ratio. As expected, there was a certain decrease in anti-inflammatory TGFβ1 levels as the ω-6 ratio increased. However, consistent with some studies, the observed decrease in the release of proinflammatory cytokines starting from a 1/4 or 1/8 ω-3:ω-6 ratio was confusing. Considering our findings, further studies are needed to suggest a clear ω-3:ω-6 ratio.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Footnotes

Authorship Contributions

Concept: M.P.E., S.Y.A, Design: M.P.E., S.A., E.M.S., Data Collection or Processing: M.P.E., T.F., S.Ö., Analysis or Interpretation: M.P.E., T.F., S.Ö., Literature Search: M.P.E., S.A., Writing: M.P.E., S.A., E.M.S.

Conflict of Interest: The authors have no conflicts of interest to declare.

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REFERENCES

1. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer. 2003;3:203-216.
2. Maris JM. Recent advances in neuroblastoma. N Engl J Med. 2010;362:2202-2211.
3. Reynolds LM, Dalton CF, Reynolds GP. Phospholipid fatty acids and neurotoxicity in human neuroblastoma SH-SY5Y cells. Neurosci Lett. 2001;309:193-196.
4. Schmitz G, Ecker J. The opposing effects of n-3 and n-6 fatty acids. Prog Lipid Res. 2008;47:147-155.
5. D'Angelo S, Motti ML, Meccariello R. ω-3 and ω-6 polyunsaturated fatty acids, obesity and cancer. Nutrients. 2020;12:2751.
6. Marventano S, Kolacz P, Castellano S, Galvano F, Buscemi S, Mistretta A, Grosso G. A review of recent evidence in human studies of n-3 and n-6 PUFA intake on cardiovascular disease, cancer, and depressive disorders: does the ratio really matter? Int J Food Sci Nutr. 2015;66:611-622.
7. Barnés CM, Prox D, Christison-Lagay EA, Le HD, Short S, Cassiola F, Panigrahy D, Chaponis D, Butterfield C, Nehra D, Fallon EM, Kieran M, Folkman J, Puder M. Inhibition of neuroblastoma cell proliferation with omega-3 fatty acids and treatment of a murine model of human neuroblastoma using a diet enriched with omega-3 fatty acids in combination with sunitinib. Pediatr Res. 2012;71:168-178.
8. Nabavi SF, Bilotto S, Russo GL, Orhan IE, Habtemariam S, Daglia M, Devi KP, Loizzo MR, Tundis R, Nabavi SM. Omega-3 polyunsaturated fatty acids and cancer: lessons learned from clinical trials. Cancer Metastasis Rev. 2015;34:359-80.
9. Zhang Y, Zhang B, Dong L, Chang P. Potential of omega-3 polyunsaturated fatty acids in managing chemotherapy- or radiotherapy-related intestinal microbial dysbiosis. Adv Nutr. 2019;10:133-147.
10. Bayram I, Erbey F, Celik N, Nelson JL, Tanyeli A. The use of a protein and energy dense eicosapentaenoic acid containing supplement for malignancy-related weight loss in children. Pediatr Blood Cancer. 2009;52:571-574.
11. Lavriv DS, Neves PM, Ravasco P. Should omega-3 fatty acids be used for adjuvant treatment of cancer cachexia? Clin Nutr ESPEN. 2018;25:18-25.

12. Skender B, Vaculova AH, Hofmanova J. Docosahexaenoic fatty acid (DHA) in the regulation of colon cell growth and cell death: a review. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2012;156:186-199.

13. Hata AN, Breyer RM. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol Ther. 2004;103:147-166.

14. Baryawno N, Sveinbjörnsson B, Eksborg S, Orrego A, Segerström L, Oqvist CO, Holm S, Gustavsson B, Kågedal B, Kogner P, Johnsen JI. Tumor-growth-promoting cyclooxygenase-2 prostaglandin E2 pathway provides medulloblastoma therapeutic targets. Neuro Oncol. 2008;10:661-674.

15. Johnsen JI, Lindskog M, Ponthan F, Pettersen I, Elfman L, Orrego A, Sveinbjörnsson B, Kogner P. Cyclooxygenase-2 is expressed in neuroblastoma, and nonsteroidal anti-inflammatory drugs induce apoptosis and inhibit tumor growth *in vivo*. Cancer Res. 2004;64:7210-7215.

16. Sveinbjörnsson B, Rasmuson A, Baryawno N, Wan M, Pettersen I, Ponthan F, Orrego A, Haeggström JZ, Johnsen JI, Kogner P. Expression of enzymes and receptors of the leukotriene pathway in human neuroblastoma promotes tumor survival and provides a target for therapy. FASEB J. 2008;22:3525-3536.

17. Hilton C, Ram BS, Shairy K, Agnieszka W, Toru T. The role of functional food security in global health, chapter 14-high omega-6/omega-3 fatty acid ratio diets and risk of noncommunicable diseases: is the tissue, the main issue? Academic Press. 2019;217-259.

18. Senkal M, Haaker R, Linseisen J, Wolfram G, Homann HH, Stehle P. Preoperative oral supplementation with long-chain omega-3 fatty acids beneficially alters phospholipid fatty acid patterns in liver, gut mucosa, and tumor tissue. JPEN J Parenter Enteral Nutr. 2005;29:236-240.

19. Dierge E, Debock E, Guilbaud C, Corbet C, Mignolet E, Mignard L, Bastien E, Dessy C, Larondelle Y, Feron O. Peroxidation of n-3 and n-6 polyunsaturated fatty acids in the acidic tumor environment leads to ferroptosis-mediated anticancer effects. Cell Metab. 2021;33:1701-1715.

20. Berquin IM, Edwards IJ, Chen YQ. Multi-targeted therapy of cancer by omega-3 fatty acids. Cancer Lett. 2008;269:363-377.

21. Lien EC, Westermark AM, Zhang Y, Yuan C, Li Z, Lau AN, Sapp KM, Wolpin BM, Vander Heiden MG. Low glycaemic diets alter lipid metabolism to influence tumour growth. Nature. 2021;599:302-307.

22. Lawrence GD. Dietary fats and health: dietary recommendations in the context of scientific evidence. Adv Nutr. 2013;4:294-302.

23. Conover WJ. Practical Non-parametric Statistics. 2nd edition, John Wiley & Sons, New York. 1980.

24. Montecillo-Aguado M, Tirado-Rodriguez B, Antonio-Andres G, Morales-Martinez M, Tong Z, Yang J, Hammock BD, Hernandez-Pando R, Huerta-Yepez S. Omega-6 polyunsaturated fatty acids enhance tumor aggressiveness in experimental lung cancer model: ımportant role of oxylipins. Int J Mol Sci. 2022;23:6179.

25. Ahmad W. Overlapped metabolic and therapeutic links between Alzheimer and diabetes. Mol Neurobiol. 2013;47:399-424.

26. Mizoguchi K, Ishiguro H, Kimura M, Takahashi H, Sakamoto N, Tanaka T, Takeyama H. Induction of apoptosis by eicosapentaenoic acid in esophageal squamous cell carcinoma. Anticancer Res. 2014;34:7145-7149.

27. Nappo M, Berkov S, Massucco C, Di Maria V, Bastida J, Codina C, Avila C, Messina P, Zupo V, Zupo S. Apoptotic activity of the marine diatom *Cocconeis scutellum* and eicosapentaenoic acid in BT20 cells. Pharm Biol. 2012;50:529-535.

28. Xu JX, Fang K, Gao XR, Liu S, Ge JF. Resveratrol protects SH-SY5Y cells against oleic acid-induced glucolipid metabolic dysfunction and cell injuries via the Wnt/β-catenin signalling pathway. Neurochem Res. 2021;46:2936-2947.

29. Colquhoun A. Mechanisms of action of eicosapentaenoic acid in bladder cancer cells *in vitro*: alterations in mitochondrial metabolism, reactive oxygen species generation and apoptosis induction. J Urol. 2009;181:1885-1893.

30. Calviello G, Palozza P, Piccioni E, Maggiano N, Frattucci A, Franceschelli P, Bartoli GM. Dietary supplementation with eicosapentaenoic and docosahexaenoic acid inhibits growth of Morris hepatocarcinoma 3924A in rats: effects on proliferation and apoptosis. Int J Cancer. 1998;75:699-705.

31. Langelier B, Alessandri JM, Perruchot MH, Guesnet P, Lavialle M. Changes of the transcriptional and fatty acid profiles in response to n-3 fatty acids in SH-SY5Y neuroblastoma cells. Lipids. 2005;40:719-728.

32. Lindskog M, Gleissman H, Ponthan F, Castro J, Kogner P, Johnsen JI. Neuroblastoma cell death in response to docosahexaenoic acid: sensitization to chemotherapy and arsenic-induced oxidative stress. Int J Cancer. 2006;118:2584-2593.

33. Di Loreto S, D'Angelo B, D'Amico MA, Benedetti E, Cristiano L, Cinque B, Cifone MG, Cerù MP, Festuccia C, Cimini A. PPARbeta agonists trigger neuronal differentiation in the human neuroblastoma cell line SH-SY5Y. J Cell Physiol. 2007;211:837-847.

34. So WW, Liu WN, Leung KN. Omega-3 polyunsaturated fatty acids trigger cell cycle arrest and induce apoptosis in human neuroblastoma LA-N-1 cells. Nutrients. 2015;7:6956-6973.

35. Lu X, Yu H, Ma Q, Shen S, Das UN. Linoleic acid suppresses colorectal cancer cell growth by inducing oxidant stress and mitochondrial dysfunction. Lipids Health Dis. 2010;9:106.

36. Zhang C, Yu H, Shen Y, Ni X, Shen S, Das UN. Polyunsaturated fatty acids trigger apoptosis of colon cancer cells through a mitochondrial pathway. Arch Med Sci. 2015;11:1081-1094.

37. Muzio G, Maggiora M, Oraldi M, Trombetta A, Canuto RA. PPARalpha and PP2A are involved in the proapoptotic effect of conjugated linoleic acid on human hepatoma cell line SK-HEP-1. Int J Cancer. 2007;121:2395-2401.

38. Mielczarek-Puta M, Otto-Ślusarczyk D, Chrzanowska A, Filipek A, Graboń W. Telmisartan influences the antiproliferative activity of linoleic acid in human colon cancer cells. Nutr Cancer. 2020;72:98-109.

39. Yamamoto D, Kiyozuka Y, Adachi Y, Takada H, Hioki K, Tsubura A. Synergistic action of apoptosis induced by eicosapentaenoic acid and TNP-470 on human breast cancer cells. Breast Cancer Res Treat. 1999;55:149-160.

40. Burdge GC, Rodway H, Kohler JA, Lillycrop KA. Effect of fatty acid supplementation on growth and differentiation of human IMR-32 neuroblastoma cells *in vitro*. J Cell Biochem. 2000;80:266-273.

41. Kim SJ, Zhang Z, Saha A, Sarkar C, Zhao Z, Xu Y, Mukherjee AB. Omega-3 and omega-6 fatty acids suppress ER- and oxidative stress in cultured neurons and neuronal progenitor cells from mice lacking PPT1. Neurosci Lett. 2010;479:292-296.

42. Taha A, Sharifpanah F, Wartenberg M, Sauer H. Omega-3 and omega-6 polyunsaturated fatty acids stimulate vascular differentiation of mouse embryonic stem cells. J Cell Physiol. 2020;235:7094-7106.

43. Liput KP, Lepczyński A, Ogłuszka M, Nawrocka A, Poławska E, Grzesiak A, Ślaska B, Pareek CS, Czarnik U, Pierzchała M. Effects of dietary n-3 and n-6 polyunsaturated fatty acids in inflammation and cancerogenesis. Int J Mol Sci. 2021;22:6965.

44. Calder PC. Omega-3 fatty acids and inflammatory processes. Nutrients. 2010;2:355-374.

45. Liang P, Henning SM, Schokrpur S, Wu L, Doan N, Said J, Grogan T, Elashoff D, Cohen P, Aronson WJ. Effect of dietary omega-3 fatty acids on tumor-associated macrophages and prostate cancer progression. Prostate. 2016;76:1293-1302.

46. Chen Y, Zheng Y, Xin L, Zhong S, Liu A, Lai W, Liu L, Lin C, Liao C, Zeng J, Zhang L. 15-epi-lipoxin A4 inhibits TNF-α-induced tissue factor expression *via* the PI3K/AKT/ NF-κB axis in human umbilical vein endothelial cells. Biomed Pharmacother. 2019;117:109099.

47. Wu SH, Lu C, Dong L, Zhou GP, He ZG, Chen ZQ. Lipoxin A4 inhibits TNF-alpha-induced production of interleukins and proliferation of rat mesangial cells. Kidney Int. 2005;68:35-46.

48. Sodin-Semrl S, Taddeo B, Tseng D, Varga J, Fiore S. Lipoxin A4 inhibits IL-1 beta-induced IL-6, IL-8, and matrix metalloproteinase-3 production in human synovial fibroblasts and enhances synthesis of tissue inhibitors of metalloproteinases. J Immunol. 2000;164:2660-2666.

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ORIGINAL ARTICLE

Unlocking Urban India's Awareness of Oral Anticoagulation: Implications for Healthcare Education

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ABSTRACT

Objectives: Treatment outcomes for patients with arrhythmias, deep vein thrombosis, prosthetic valves, blood thinning, and cardiac issues/chest pain problems can be affected by knowledge about oral anticoagulant therapy. The primary objective is to assess the knowledge of patients using oral anticoagulants for anticoagulation therapy, and the secondary aim is to identify factors influencing the level of anticoagulation knowledge.

Materials and Methods: This prospective cross-sectional study was conducted at selected community pharmacies. A 33-item, self-administered questionnaire was adopted to evaluate patient understanding of anticoagulant medication in the urban population. Scores were calculated for each part and the association between patients' knowledge. Binary logistic regression analysis was performed to assess variables associated with oral anticoagulation knowledge among participants.

Results: The mean percentage knowledge score of the study population (n=323) was 42.38±12.5. Age has been found to have a negative correlation with anticoagulant therapy knowledge (p=0.01). It was discovered that there were gaps in knowledge regarding critical areas of use and self-management, including the identification of bleeding as a serious side effect of medication, drug-drug interactions, and dose omission.

Conclusion: This research article highlights urban participants' knowledge gaps in oral anticoagulation. Targeted educational interventions by pharmacists are vital for improving patient safety and treatment outcomes. Advancing age was associated with knowledge. Further research could explore the long-term impacts of educational interventions in larger populations.

Keywords: Oral anticoagulant therapy, knowledge assessment, patient knowledge, patient education, pharmaceutical care

INTRODUCTION

Currently, the morbidity and mortality rates are high today. Anticoagulants have been extensively used for a decade for preventing and treating vascular and thromboembolic diseases despite their relatively high risk/safety profile.[1] Anticoagulants are narrow therapeutic range drugs leading to life-threatening complications like bleeding and re-thrombosis, which can occur when patients are over-anticoagulated or under-anticoagulated.[2] If not properly controlled, anticoagulants, which are referred to as "high alert medications," may result in adverse drug events in the inpatient and outpatient healthcare context.[3]

The urban population, in particular, is more likely to be exposed to various risk factors associated with cardiovascular diseases, such as a sedentary lifestyle, unhealthy dietary habits, and increased stress levels.[4] Consequently, anticoagulant medications are frequently prescribed to this population to manage and prevent complications. Several research findings indicate that patients who receive therapeutic education have

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a reduced likelihood of experiencing hemorrhagic accidents and/or thrombotic recurrences 3 months after discharge. Conversely, patients who do not receive therapeutic education are more susceptible to developing complications.[3]

According to previous reports, individuals frequently taking oral anticoagulants are unaware of the risks and consequences associated with their medication.[5] The response of patients to treatment depends on their understanding of oral anticoagulant medication. Patients who are better informed about the benefits and risks of taking oral anticoagulants are more likely to take them consistently. Patients who do not adhere to dose and monitoring regimens not only reduce the likelihood of therapeutic benefits but also put themselves at a higher risk of experiencing adverse events. Up to 36,000 patients are seen in U.S. hospital emergency rooms annually for side effects from anticoagulant therapy.[6]

According to a recent national study, 93% of patients taking oral anticoagulants reported adverse events and other studies have shown that many of these occurrences are caused by avoidable patient mistakes. Some patient errors can likely be attributed to their insufficient understanding of anticoagulant therapy and its associated risks.[7] One of the most important elements in improving treatment and lowering complications for patients receiving oral anticoagulant medication is currently thought to be patient education. Therefore, more efficient patient education regarding anticoagulant therapy is needed. The dissolution of a blood clot it takes about 3 months, so non-adherence to medication provokes the incidence of stroke.[3] Patients taking anticoagulants will benefit from education and information services that lower treatment costs by limiting the risks of bleeding and thromboembolism.[8]

However, it is crucial to note that anticoagulant therapy can be complex and challenging to manage effectively. Patients should have a thorough understanding of the purpose, dosage, potential side effects, and interactions of these medications. Moreover, they must be aware of the importance of regular monitoring and follow-up appointments. Lack of knowledge or misconceptions about anticoagulants can lead to suboptimal outcomes, including increased risk of bleeding or thrombosis. Therefore, in this study, we primarily aimed to assess patient knowledge regarding oral anticoagulant treatment, identify knowledge gaps, and identify patients at risk.

MATERIALS AND METHODS

A prospective cross-sectional study was conducted at selected community pharmacies in Nilgiris, India, between July 2022 and May 2023. In this setting, patients who had medications from various prescribers, many of whom had different clinical problems. Patients who are not willing to participate in the study are excluded.

Sample size estimation

The sample size for the present study was calculated with the help of Cochran's formula, $n=Z^2\ pq/d^2$, where n is the sample size, Z is 1.96, p is the estimated average patient knowledge of anticoagulation at 70%[9], q is 1-p, and there is a 5% margin of error. Therefore, the estimated sample size for this study is approximately 323.

Study instrument

The present study utilized the Oral Anticoagulation Understanding Tool (AKT), a 33-item, self-administered questionnaire for evaluating one's understanding of anticoagulation, by the community pharmacies as the study instrument. The data collection form was derived from a prior study with acceptable validity and reliability.[10] The data collection tool included three major sections:

Section A: Assessment of socio-demographic characteristics: This consists of six questions covering age, sex, level of education, occupation, monthly income, and duration of oral anticoagulant use, which are used to evaluate patients' socio-demographic characteristics.

Section B: Assessment of oral anticoagulation knowledge. This section consists of 20 questions that analyze patients' knowledge about oral anticoagulants.

Assessment of oral anticoagulation knowledge

A study information sheet and a brief explanation of the study's objective were given to each participant. The ethics committee approved the waiver of written informed consent and questionnaire. The responses received from the participants were scored and assessed for their knowledge. Except for questions 18 and 19 in section 2, each response received one point for being correct and zero for being incorrect. For questions 18 and 19, each of the three correct answers to these questions was awarded one point. A cut-off value of more than 50% was considered an adequate knowledge score.

Statistical analysis

Data analysis was performed using SPSS software version 22.0. The participant baseline characteristics were reported using percentages and medians. Using the Shapiro-Wilk test, the normality of continuous variables. Using a linear regression sub-analysis, we assessed whether there was a relationship between mean AKT and age. All analyses were deemed significant at p values of 0.05.

Ethical considerations

This study was approved by the Institutional Review Board of JSS College of Pharmacy (approval number: JSSCP/IRB/10/2022-2023, dated: 11.02.2023).

RESULTS

A total of 323 respondents were included in the analysis. The study revealed tha men are at risk of taking oral anticoagulant drugs compared to women. Maximum of the participants had no formal education (43.1%). The median age of the participants was 59 years. The minimum age of patients was 30 years, and the maximum age was 89 years. Most of the participants were older patients (86%). Of them, 39.4% used oral anticoagulants for less than 3 months. Table 1 presents the demographic characteristics of the study participants and the distribution

Table 1. Demographic characteristics of the study participants

Demographic charactertics	n (%)	Knowledge of anticoagulants (SD)	*p* value
Age			
65 years	237 (73.37)	82 (±12.5)	0.01
65 years and older	86 (26.62)	36 (±8.7)	
Sex			
Female	132 (40.86)	45 (±10.2)	0.77
Male	191 (59.13)	73 (±11.8)	
Highest level of education			
No formal education	152 (43.1)	65 (±9.5)	0.78
High school or equivalent	137 (38.8)	50 (±11.2)	
Technical and vocational skills	5 (1.4)	2 (±1.5)	
College	0	0	
Bachelor's degree	29 (8.2)	13 (±2.8)	
Occupation			
Full-time work	116 (32.9)	42 (±9.9)	0.75
Home maker	121 (34.3)	43 (±10.5)	
Part-time work	0	0	
Unemployed/Retired	86 (24.4)	33 (±8.0)	
Annual income range INR (USD)			
Rs.0-50,000 (0-600$)	156 (44.2)	50 (±9.0)	0.91
Rs.51,000-1,00,000 (612-1200$)	5 (1.4)	1 (±0.5)	
Rs.5,00,000 and later (6000$ and above)	11 (3.1)	4 (±1.1)	
I prefer not to say this	96 (27.2)	35 (±8.2)	
Duration of treatment with oral anticoagulants			
<3 months	139 (39.4)	52 (±10.7)	0.73
1-2 years	68 (19.3)	22 (±6.8)	
3-12 months	104 (29.5)	39 (±9.1)	
>2 years	12 (3.4)	4 (±1.5)	
Not taking anticoagulant medication	0	0	

SD: Standard deviation, INR: International normalised ratio

of knowledge about anticoagulant use among different demographic groups.

The results revealed statistically significant associations between age and knowledge. Other factors like sex, education, occupation, income range, and duration of anticoagulant therapy did not significant association with knowledge scores. Figure 1 presents the results of the multivariate logistic regression. Figure 2 shows a pictorial representation of the knowledge assessment among the elderly and other patient groups. The relationship between the oral anticoagulation knowledge score and the age of respondents (circles) is presented. The straight line is the oral anticoagulation knowledge score.

Responses regarding the use of oral anticoagulants are detailed in Table 2. The results showed that rivaroxaban (48.6%) was the most commonly used anticoagulant, followed by dabigatran (30.3%) and apixaban (21.1%). Regarding the reasons for prescribing anticoagulant medicine, most participants reported taking it for arrhythmias (39.9%), followed by cardiac issues/chest pain (20.1%). Major of respondents were unsure about how the medicine worked in their bodies, and few correctly identified that it prevents blood from clotting (23.3%). Major (42.4%) of respondents were uncertain about the duration needed to take the medicine. Most participants recognized the importance of taking the medicine as prescribed to avoid bleeding

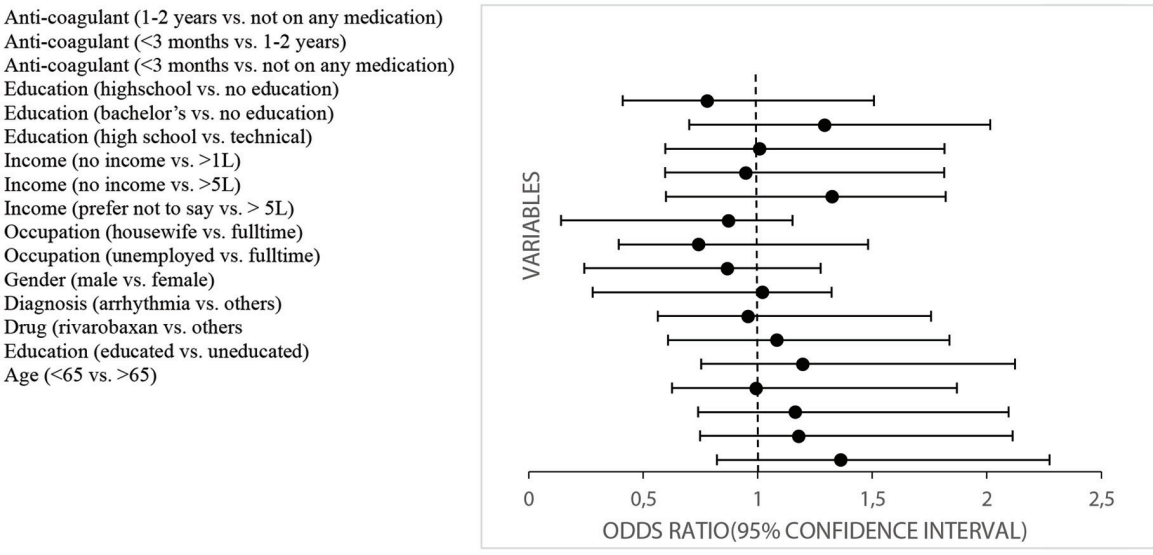

Figure 1. Results of multivariate logistic regression analysis for factors associated with adequate anticoagulant knowledge

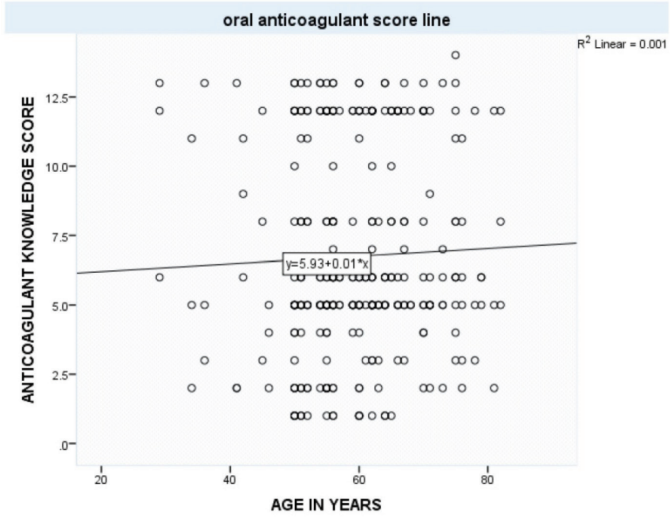

Figure 2. Linear regression plot of oral anticoagulation knowledge and age of the participants

complications (58.5%). Most respondents the significance of taking the medicine at the same time each day (97.2%). The data showed that a large number of respondents did not believe that skipping one dose could worsen their condition (98.5%). The importance of continuing the medication even after feeling better was also addressed, in which a maximum of participants were okay with stopping the medication (94.7%) once they felt better.

When asked about taking more of the medicine than prescribed, respondents were not sure (75.8%) that doing so would have any benefit. Concerning safety and side effects, a vast majority knew that excessive alcohol consumption could increase the risk of side effects (89.7%). Many were uncertain about other safety aspects, such as taking additional doses or taking anti-inflammatory medicines (59.9%) with the anticoagulant. The study explored the respondents' understanding of the most important side effects and the signs to watch out for while taking the medication. Half of the participants identified bleeding (59.4%) as the most important side effect and correctly identified bleeding gums, prolonged nose bleeds, and nose bleeds (59.4%) as signs to watch out for.

DISCUSSION

The findings of this study highlight the need for targeted educational interventions to improve anticoagulant knowledge among urban populations. It is essential to address the identified knowledge gaps to ensure safe and effective anticoagulant therapy. Surveys in several nations, including England,[11] Australia,[12] Hungary,[13] Poland,[14] and Saudi Arabia,[10] showed that patients taking oral anticoagulants had adequate knowledge (>50%), while surveys in others, including London,[15] Brazil,[16] New York City,[17] Iran,[18] Saudi Arabia,[19] Pakistan,[20] Libya,[21] Nepal,[22] and European nations,[23-28] like Switzerland, Germany, France, Denmark, Sweden, Spain, Norway, and Italy, showed that they had inadequate knowledge.

Healthcare providers should play a crucial role in educating patients about their medications and monitoring requirements. Additionally, community-based awareness campaigns and educational materials can enhance the public understanding of anticoagulant therapy.

This study evaluated patients' awareness of anticoagulation in relation to deep vein thrombosis, coronary artery disease, atrial fibrillation, and blood thinning. The majority of the participants had inadequate knowledge of anticoagulation. Overall, there were gaps in our understanding of essential areas such as self-management, including missing doses, drug-drug interactions (DDIs), as well as recognizing bleeding as a significant adverse drug effect. These findings are in line with earlier reports on a variety of demographics in which low oral anticoagulant

Table 2. Assessment of respondents' knowledge about oral anticoagulants (n=323)

Anticoagulant medication knowledge assessment	Response n (%)
Anticoagulant	
Rivaroxaban	157 (48.6)
Apixaban	68 (21.1)
Dabigatran	98 (30.3)
The reason for the prescription	
Arrhythmias	129 (39.9)
Deep vein thrombosis	60 (18.6)
Prosthetic valve	3 (0.9)
Blood thinning	6 (1.9)
Cardiac issue/chest pain	65 (20.1)
Others	60 (18.6)
How does the medication work in the body?	
Lower BP	3 (0.9)
Prevents blood clotting	75 (23.2)
Lowers heart rate	20 (6.2)
Don't know	225 (69.7)
Frequency of medication	
Once	151 (46.7)
Twice	172 (53.3)
Duration of treatment	
3 months	124 (38.4)
6 months	33 (10.2)
1 year	2 (0.6)
Lifelong	27 (8.4)
Don't know	137 (42.4)
The importance of following physician's instructions	
Too much of this can cause bleeding.	189 (58.5)
Don't know	134 (41.5)
Importance of taking medication at the same time daily?	
Yes	314 (97.2)
No	6 (1.9)
Not sure	3 (0.9)
Is it acceptable to double the dose if a dose is missed?	
Yes	0 (0)
No	318 (98.5)
Not sure	5 (1.5)
Does skipping one dose of the medication worsen the patient's condition?	
Yes	3 (0.9)
No	319 (98.5)
Not sure	1 (0.3)
Can we stop taking the medication once the feeling improves?	
Yes	306 (94.7)
No	11 (3.4)
Not sure	6 (1.9)
Can you take anti-inflammatory medicines like ibuprofen in combination with anticoagulant medication?	
Yes	192 (59.9)
No	3 (0.9)
Not sure	126 (39.6)
Can vitamin supplements and herbal medicines with anticoagulant medication be administered without physician's opinion?	
Yes	195 (60.4)
No	2 (0.6)
Not sure	126 (39.0)
Benefits of taking more medication than prescribed?	
Yes	0 (0)
No	78 (24.2)
Not sure	245 (75.8)
Does alcohol consumption affect the side effects of the medication?	
Yes	290 (89.7)
No	0 (0)
Not sure	33 (10.3)
Will you inform health professionals before any surgery?	
Yes	195 (60.4)
No	0 (0)
Not sure	128 (39.6)
Will you inform all health care practitioners about your medications?	
Yes	192 (59.4)
No	0 (0)
Not sure	131 (40.6)
The most important side effect	
Bleeding	192 (59.4)
Others	3 (0.9)
Don't know	128 (40.6)
Three signs of side effects that should be monitored	
Don't know	131 (40.6)
Bleeding gums, prolonged nose bleeding	192 (59.4)
Three ways to reduce the risk of side effects	
Don't know	291 (90.1)
INR monitoring regularly, proper dosing	32 (9.9)
Steps to take if accidentally taking too much	
Consult the doctor	192 (60.1)
Be alert for signs of side effects	129 (39.9)

BP: Blood pressure, INR: International normalised ratio

awareness has been documented on a regular basis. This study considered clinically relevant DDIs. This includes interactions that could significantly affect patient outcomes during anticoagulation therapy.

Our research showed that adults aged 65 years and older had much less knowledge about oral anticoagulants than those younger than 65. The understanding of anticoagulants was identically predicted by age. Several reports have found that aging is inversely connected with knowledge of oral anticoagulants, which have examined the impact of age on oral anticoagulant knowledge in a variety of populations. A known factor in determining drug-related knowledge is the quantity and quality of interactions between patients and medical professionals. An increase in the frequency of patient-healthcare professional interactions will persistently increase patient knowledge. Therefore, it is a great opportunity for healthcare professionals to interact with patients and prevent various side effects and hazards caused by inadequate knowledge regarding anticoagulant use.

Participants cannot effectively participate in shared decision-making or self-management of their condition unless they are informed about anticoagulation. To identify and address awareness gaps, it is imperative that knowledge assessments be incorporated into counseling programs and given to patients at the beginning of their oral anticoagulant therapy and on an ongoing basis thereafter. It is necessary to implement a comparable follow-up session for direct oral anticoagulant users in the absence of routine coagulation monitoring to assess their understanding of oral anticoagulants and other patient-related outcomes.

Anticoagulants are high-risk medications widely used to prevent and treat thrombotic events, resulting in the need for adequate patient education to minimize harm. However, patients should be closely and consistently monitored due to the narrow therapeutic index and potentially fatal side effects. According to previous studies, there is a higher risk of bleeding when the international normalised ratio (INR) is higher than the therapeutic range and a higher risk of thromboembolism when it is lower than 2. Education regarding the management of patients taking oral anticoagulants was identified as a critical component of the Joint Commission International, National Patient Safety Goal guideline for 2014.[7]

Although prior studies have indicated a potential positive relationship between patients' anticoagulant knowledge and achieving INR values within the therapeutic range, the study by Baysal and Midilli[7] did not find a significant correlation between knowledge levels and INR outcomes. An effective education program is therefore required to raise and maintain patients' awareness of anticoagulants. Patients taking anticoagulants will benefit from education and information services that lower treatment costs by lowering the risks of bleeding and thrombosis. Non-adherence to medication can increase the risk of stroke.

Furthermore, the findings of this study have implications for healthcare policies and guidelines. The results can inform the development of educational programs tailored to the specific needs of urban populations. By improving anticoagulant knowledge, healthcare providers can empower patients to actively participate in their treatment plans, resulting in better clinical outcomes.

Study limitations

It is important to acknowledge this study's constraints. The research focused on a specific urban population, and the findings may not be generalizable to rural or other demographic groups. However, patients' knowledge may be poorer if other centers were included. The sample size was limited, which may have affected the representativeness of the results. Consequently, it makes sense to conduct a nationwide survey involving a large sample of patients who are on oral anticoagulants. Moreover, the study relied on self-reported knowledge, which may be subject to recall or social desirability bias. The study did not take into consideration whether the patients were cognitively impaired, psychosocial, or psychotic.

CONCLUSION

In conclusion, this study presents a comprehensive assessment of anticoagulant knowledge among the urban population. The findings indicate both strengths and weaknesses in the level of knowledge, emphasizing the importance of targeted educational interventions to address the identified gaps. Significant knowledge gaps were discovered in this study among urban individuals using oral anticoagulants. Understanding oral anticoagulation was negatively correlated with advancing age. By improving anticoagulant knowledge, healthcare providers can enhance patient safety, adherence, and overall treatment outcomes. This evidence regarding oral anticoagulant medication may increase awareness of patient-related factors that may impact therapeutic outcomes. Future research could focus on evaluating the long-term impact of educational interventions and expanding the study to include a broader population for more robust conclusions.

Ethics

Ethics Committee Approval: This study was approved by the Institutional Review Board of JSS College of Pharmacy (approval number: JSSCP/IRB/10/2022-2023, dated: 11.02.2023).

Informed Consent: Not required.

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Footnotes

Authorship Contributions

Concept: B.V., P.S., Design: B.V., P.S., Data Collection or Processing: S.K., V.H.N., Analysis or Interpretation: S.K., V.H.N., B.V., Literature Search: S.K., V.H.N., B.V., P.S., Writing: S.K., V.H.N., B.V., P.S.

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REFERENCES

1. Fernandes CJ, Alves Júnior JL, Gavilanes F, Prada LF, Morinaga LK, Souza R. New anticoagulants for the treatment of venous thromboembolism. J Bras Pneumol. 2016;42:146-154.
2. Alquwaizani M, Buckley L, Adams C, Fanikos J. Anticoagulants: a review of the pharmacology, dosing, and complications. Curr Emerg Hosp Med Rep. 2013;1:83-97.
3. Amaraneni A, Chippa V, Rettew AC. Anticoagulation Safety. [Updated 2023 Apr 17]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023.
4. World Health Organization: WHO. Urban health [Internet]. 2021. Available from: https://www.who.int/news-room/fact-sheets/detail/urban-health
5. Raccah BH, Erlichman Y, Pollak A, Matok I, Muszkat M. Prescribing errors with direct oral anticoagulants and their impact on the risk of bleeding in patients with atrial fibrillation. J Cardiovasc Pharmacol Ther. 2021;26:601-610.
6. Mazor KM, Baril J, Dugan E, Spencer F, Burgwinkle P, Gurwitz JH. Patient education about anticoagulant medication: is narrative evidence or statistical evidence more effective? Patient Educ Couns. 2007;69:145-157.
7. Baysal E, Midilli TS. Effects of structured patient education on knowledge level and INR control of patients receiving warfarin: Randomized Controlled Trial. Pak J Med Sci. 2018;34:240-246.
8. Hawes EM. Patient education on oral anticoagulation. Pharmacy (Basel). 2018;6:34.
9. Naorem Jiteswori Devi, Dr. Veena Sakhardande. Knowledge regarding anticoagulation therapy among patients attending cardiac clinics. Pharma Innovation 2019;8:125-128.
10. Alajami HN, Alshammari SA, Al-Dossari DS, Alajmi AN, Alsaikhan AS, Alessa MS, Alessa HS, Khalaf Alhothaly S, Alnami MI, Atey TM, Alnajrani RH, Ali S. Knowledge of anticoagulation among saudi patients with atrial fibrillation: a cross-sectional study. Cureus. 2021;13:e19237.
11. Jani YH, Hirani B, Livingstone C. Evaluation of patients' knowledge about oral anticoagulant medicines and use of alert cards by community pharmacists. Int J Clin Pharm. 2021;43:203-211.
12. Obamiro KO, Chalmers L, Lee K, Bereznicki BJ, Bereznicki LRE. Anticoagulation knowledge in patients with atrial fibrillation: an Australian survey. Int J Clin Pract. 2018;72:e13072.
13. Viola R, Fekete H, Csoka I. Patients' knowledge on oral anticoagulant treatment in Hungary. Int J Clin Pharm. 2017;39:1265-1272.
14. Janion-Sadowska A, Sadowski M, Konieczyńska M, Skonieczny G, Metzgier-Gumiela A, Chrapek M, Sobieraj E, Bryk AH, Dębski M, Podolec P, Małecka B, Desteghe L, Heidbuchel H, Undas A. Polish regional differences in patient knowledge on atrial fibrillation and its management as well as in patterns of oral anticoagulant prescription. Kardiol Pol. 2019;77:437-444.
15. Taylor FC, Ramsay ME, Tan G, Gabbay J, Cohen H. Evaluation of patients' knowledge about anticoagulant treatment. Qual Health Care. 1994;3:79-85.
16. Leitão JM, Moreira FMDS, Thiel IE, Spricido IY, Silva RHM, Zétola VF. Alarming lack of knowledge about antithrombotic therapy among patients with atrial fibrillation. Arq Neuropsiquiatr. 2018;76:807-811.
17. Davis NJ, Billett HH, Cohen HW, Arnsten JH. Impact of adherence, knowledge, and quality of life on anticoagulation control. Ann Pharmacother. 2005;39:632-636.
18. Fariborz Farsad B, Dastan F, Salamzadeh J, Moghadamnia Z, Eskandari R, Fahimi F. Assessment of outpatients' knowledge and adherence on warfarin: the impact of a simple educational pamphlet. Iran J Pharm Res. 2019;18:315-320.
19. Al-Shamiri M. Knowledge gaps about oral anticoagulant in Saudi patients. Pharm Res Allied Sci. 2020;9:172-178.
20. Zahid I, Ul Hassan SW, Bhurya NS, Alam SN, Hasan CA, Shah BH, Fatima FB, Ahmed A, Ul Hassan SS, Hayat J, Zulfiqar A, Sheikh R, Aziz M, Siddiqi R, Fatima K, Khan MS. Are patients on oral anticoagulation therapy aware of its effects? A cross-sectional study from Karachi, Pakistan. BMC Res Notes. 2020;13:279.
21. Ahmed H, Saddouh EA, Abugrin ME, Ali AMM, Elgdhafi EO, Khaled A, Tarek A, Elhadi M. Association between patients' knowledge and adherence to anticoagulants, and its effect on coagulation control. Pharmacology. 2021;106:265-274.
22. Shrestha S, Sapkota B, Kumpakha A, Acharya U, Sharma R. Evaluation of patients' knowledge on warfarin in outpatient pharmacy of a tertiary care cardiac center. BMC Res Notes. 2015;8:429.
23. Briggs AL, Jackson TR, Bruce S, Shapiro NL. The development and performance validation of a tool to assess patient anticoagulation knowledge. Res Social Adm Pharm. 2005;1:40-59.
24. Konieczyńska M, Sobieraj E, Bryk AH, Dębski M, Polak M, Podolec P, Małecka B, Pająk A, Desteghe L, Heidbuchel H, Undas A. Differences in knowledge among patients with atrial fibrillation receiving non-vitamin K antagonist oral anticoagulants and vitamin K antagonists. Kardiol Pol. 2018;76:1089-1096.
25. Hernández Madrid A, Potpara TS, Dagres N, Chen J, Larsen TB, Estner H, Todd D, Bongiorni MG, Sciaraffia E, Proclemer A, Cheggour S, Amara W, Blomstrom-Lundqvist C. Differences in attitude, education, and knowledge about oral anticoagulation therapy among patients with atrial fibrillation in Europe: result of a self-assessment patient survey conducted by the European Heart Rhythm Association. Europace. 2016;18:463-467.
26. Chenot JF, Hua TD, Abu Abed M, Schneider-Rudt H, Friede T, Schneider S, Vormfelde SV. Safety relevant knowledge of orally anticoagulated patients without self-monitoring: a baseline survey in primary care. BMC Fam Pract. 2014;15:104.
27. Janoly-Duménil A, Bourne C, Loiseau K, Luauté J, Sancho PO, Ciancia S, Caillet F, Boisson D, Rioufol C, Plauchu MM, Rode G, Jacquin-Courtois S. Oral anticoagulant treatment-evaluating the knowledge of patients admitted in physical medicine and rehabilitation units. Ann Phys Rehabil Med. 2011;54:172-180.
28. Jank S, Bertsche T, Herzog W, Haefeli WE. Patient knowledge on oral anticoagulants: results of a questionnaire survey in Germany and comparison with the literature. Int J Clin Pharmacol Ther. 2008;46:280-288.

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ORIGINAL ARTICLE

Knowledge and Attitude of Iraqi Pharmacists Regarding the Adverse Effects of NSAIDs Based on Years of Experience

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ABSTRACT

Objectives: Informing patients about the adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs) is essential to ensure their safe use. The aim of this study was to determine whether the years of experience of Iraqi pharmacists affect their knowledge of the renal and gastrointestinal adverse effects of NSAIDs, and their attitudes toward informing patients about drug safety.

Materials and Methods: An online cross-sectional survey was conducted between January and October 2023. A convenience sample of Iraqi pharmacists working in hospitals and/or community pharmacies answered a validated questions about demographics, knowledge, and attitudes regarding the adverse effects of NSAIDs.

Results: Of the 309 Iraqi pharmacists who participated, 46% had less than four years of experience. Eighty-five percent had good knowledge of the adverse effects of NSAIDs. Specifically, 83% of participants with good knowledge and ≥4 years of experience were younger than 35 years (p=0.008). Among participants with good knowledge and <4 years of experience, 93% had a bachelor's degree (p=0.008), and 57% worked from six to more than ten hours per day (p=0.045). The dispensing patterns of NSAIDs showed a highly significant association (p<0.001) with participant knowledge regardless of years of experience. Negative attitudes were reported more frequently among pharmacists with fewer years of experience than those with longer years of experience (73% vs. 71%, respectively; p>0.05). Sixty percent of the participants agreed that education about adverse drug reactions (ADRs) increases anxiety and medication nonadherence. Seventy-eight percent agreed that pharmacists and physicians could improve patients' knowledge of ADRs. Pharmacists believed that leaflets reduce patients' medication adherence (57%) but help patients improve their medication knowledge (51%; p<0.05) and monitor and report ADRs (56%; p<0.05).

Conclusion: Despite years of experience, good knowledge and negative attitudes were found regarding safety information for NSAIDs. Pharmacists and physicians play an important role in ensuring appropriate drug use. Leaflets serve as a source of information, but they can also lead to medication nonadherence.

Keywords: Adverse drug reaction, Iraq, medication adherence, NSAIDs, pharmacist

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs), antiplatelets, diuretics, and anticoagulants are four medication classes that contribute to potentially avoidable drug-related hospitalizations.[1] NSAIDs are anti-inflammatory, analgesic, and antipyretic agents commonly used to treat conditions such as arthritis, premenstrual syndrome, headache, and gout.[2-4] Aspirin, an antiplatelet agent, is one of the NSAIDs that is frequently recommended for primary and secondary prophylaxis against ischemic stroke and cardiovascular events.[3] NSAIDs comprise approximately 5-10% of all medications prescribed annually. They are used either alone or in combination with other drugs.[4,5]

The awareness of patients regarding the risks of NSAIDs is lower than expected, which may be due to the ease of their purchase as over-the-counter (OTC) medicines.[6] The drugs are obtained either from a community pharmacy or from other sources, such as supermarkets or websites.[6,7] Thus, pharmacists play a role in ensuring patient safety as part of their job responsibilities.[8] The Beers Criteria of the American Geriatric Society, updated in 2023,[9] strongly recommend avoiding the chronic use of non-selective cyclooxygenase-2 inhibitors and high-dose aspirin for high-risk patients aged >75 years and those on anticoagulants, corticosteroids, and antiplatelet agents. NSAID use increases the risk of gastrointestinal bleeding or peptic ulcer disease; approximately 2-4% of patients experience these issues after 1 year of treatment.[9] Treatment with these drugs can also cause renal vasoconstriction, resulting in decreased renal perfusion and abnormal renal function.[5] In the early stages of treatment, non-aspirin NSAIDs can have adverse cardiovascular effects, and the risk of adverse effects may increase as the treatment progresses. The approximate risk increase ranged from 10% to ≥50%, depending on the drugs and doses studied.[2] These effects include hypertension, myocardial infarction, stroke, and heart failure.[10] Elderly patients (>65 years) with comorbidities such as hypertension, heart disease, diabetes, gastrointestinal, and renal problems may have an increased risk of adverse drug reactions (ADRs) from using NSAIDs.[5,6,11,12] Additionally, polypharmacy use with the risk of drug interactions and the type, duration, and dose of NSAIDs are considered risk factors for NSAID-related ADRs.[5,6,10,13] Alcohol consumption and smoking status may also increase the risk of adverse effects, particularly cardiovascular and gastrointestinal adverse effects.[12]

Adverse drug events (ADEs), including ADRs, are any unintended, adverse medical events or harm following medical intervention unrelated to a patient's medical condition. ADEs can occur even without any errors during prescription, dispensing, or taking the medication.[14] These events are the fifth leading cause of mortality among hospitalized patients.[15] Hussain et al.,[16] found that healthcare professionals (HCPs) in Baghdad, Iraq had a positive attitude toward reporting ADRs, but their knowledge of these reactions remains inadequate. The unavailability of reporting forms was the main reason that discouraged HCPs from reporting and detecting ADRs.[16] Most pharmacists were reported to be inexperienced by the Iraqi Pharmacovigilance Center (IqPhvc).[17]

Since 2010, this center has been a member of the World Health Organization International Monitoring Program of Drugs and is part of the Pharmacy Department of the Directorate of Technical Affairs.[18] The IqPhvc has a center in each healthcare committee, where pharmacists are responsible for monitoring and reporting ADRs. This center verifies and assigns alerts to ADRs in all healthcare settings. The IqPhvc ensures the safety of vaccines, medicines, and herbal and biopharmaceutical products, whether in the private or public sections.[18] To provide effective patient care with the IqPhvc, pharmacists must possess extensive knowledge of drug risks and exhibit a proactive approach to educating patients about drug safety. In Iraq, non-communicable diseases, which account for 55% of deaths, pose a significant health burden. These include cardiovascular disease, stroke, cancer, diabetes, and chronic lung disease.[19] Consequently, due to the scarcity of information on the subject of awareness of the adverse effects of NSAIDs and patient education in Iraq, this study aimed to assess whether years of experience affect pharmacists' knowledge of NSAID-related adverse effects, in addition to their attitudes toward informing patients about the safety of NSAIDs and, more specifically, ADRs.

MATERIALS AND METHODS

A cross-sectional study using an online survey was conducted from January to October 2023. The survey was created using Google Forms and distributed among private Iraqi pharmacist groups on Facebook, Telegram, and WhatsApp. Reminders were sent every two weeks. An online sample size calculator[20] determined the minimum required sample size to be 377 with a 5% margin of error and 95% confidence interval.

Public and private pharmacy colleges in Iraq offer bachelor's degrees in pharmacy sciences after five years of study. After graduation, licensed pharmacists can work in hospitals and/or community pharmacies.

The convenience sampling method was used to recruit pharmacists working in community pharmacies and/or hospitals, excluding those in medical stores or those with invalid responses. The participants provided their consent before answering the questionnaire. This study was approved by the Collegiate Committee for Medical Research Ethics at the University of Mosul (approval number: CCMRE-phA-21-10, date: 25.01.2023).

Survey questionnaire

Three parts of the questionnaire were used to collect data (Appendix I). The questions were in English, which is the language of education in Iraqi pharmacy colleges. The first section included pharmacists' demographic data regarding age, sex, and educational level, as well as their work patterns and NSAID dispensing. The second part was a knowledge scale on NSAID-related kidney and gastrointestinal adverse effects, which was adapted from a validated form by Owusu et al.[21] The scale had ten questions: nine multiple-choice questions with one question requiring either "true," "false," or "I do not know" as an answer. Each correct answer was awarded one

point, resulting in a total of 10 points. The total score was then categorized as poor knowledge (≤4 points) or good knowledge (5-10 points). The third part was a validated attitude scale developed by Phueanpinit et al.,[22] to assess pharmacists' attitudes toward providing drug safety information to patients. The questionnaire consists of 17 questions divided into three subparts: "the roles of pharmacists in providing ADR information to patients" (questions 1 to 8), "usefulness and necessity of patient information leaflets (PILs) for patients" (questions 9-14), and "the roles of drug companies in preparing PILs" (questions 15-17). This score is based on a 5-point Likert scale ranging from strongly disagree =1 to strongly agree =5. The negatively worded questions (questions 7 and 12) in the original scale were transformed into positively worded questions to have the same scale scoring as the other questions. The lowest possible total score was 17; the highest was 85. It was then grouped as poor (17-40), moderate (41-63), and good (64-85). We classified the attitude scores into two groups, negative (<64) and positive (≥64).

Statistical analysis

IBM SPSS version 24 (IBM, New York, NY, USA) was used for statistical analysis. Continuous data were presented as mean, standard deviation (SD), minimum, and maximum, while categorical data were presented as frequencies and percentages (%). A Pearson's chi-square test or Fisher's exact test was used to determine statistical significance at $p<0.05$.

RESULTS

Three hundred nine pharmacists participated in the survey, with a response rate of 82%. The mean (SD) years of experience was 5.2 (±4.8), with a median of 4 years. A total of 45.6% of participants had less than 4 years of experience, whereas 54.4% had four or more years of experience. Table 1 shows that more than two-thirds of the participants were male and aged 23-28 years. Only 17.2% had a higher education beyond a bachelor's degree. Moreover, 57% worked in hospitals and pharmacies, 72.5% had pharmacies located in the city, and 51.8% (156/301) had one or two pharmacists co-working with them in the pharmacy. Nearly half (47%, 143/304) of the workers worked for approximately six to ten hours each day for six days a week.

Most participants (85%) dispensed NSAIDs as OTCs or prescribed medications. The frequently dispensed NSAIDs were diclofenac (76.7%), mefenamic acid (70.9%), and ibuprofen (67.3%). Approximately half of the participants (48%) preferred to instruct their patients about NSAIDs. Almost two-thirds of the participants relied on academic learning and medical textbooks as sources of information on NSAIDs.

On the knowledge scale (Table 2), the participants had a mean (± SD) of 6.8 (±2), and 85.4% had good knowledge about the adverse effects. The number of years of experience was not significantly associated with knowledge ($p>0.05$).

Years of experience were not significantly associated ($p>0.05$) with the answers to the knowledge questions. The lowest percentage of correct answers was for question two regarding NSAID-drug interactions (Figure 1).

Table 3 shows statistically significant associations between age and knowledge of pharmacists with ≥4 years of experience ($p=0.008$). In particular, about 83% of those with good knowledge were younger than 35 years. In addition, knowledge of pharmacists with <4 years of experience showed a significant association with scientific degrees ($p=0.008$); 93% of those with good knowledge had a bachelor's degree. Working hours were significantly associated ($p=0.045$) with knowledge, as 57% of those with <4 years of experience who had good knowledge worked from six to more than ten hours per day for six days a week. The dispensing patterns of NSAIDs showed a highly significant association ($p<0.001$) with participant knowledge.

Table 2 also shows a non-significant association ($p>0.05$) between years of experience and attitudes toward providing drug safety information to patients. Approximately 73% of pharmacists with <4 years of experience and 71% of those with ≥4 years of experience had negative attitudes. Only 28% of the participants exhibited a good attitude.

In Figure 2, 64% of pharmacists with more years of experience agreed to informing patients about ADRs (Q1). About two-thirds (60%) of the participants agreed that informing patients about ADRs might increase their anxiety (Q2), leading to patients discontinuing their medication (Q4). Most participants agreed with the roles of pharmacists (85%) and physicians (70%) in providing patients with ADR information (Q3 and Q6). Regarding question 7, which asked about improving patients' confidence in self-reporting ADRs through education, 47% of pharmacists with fewer years of experience disagreed with this concept. Seventy percent of participants found it necessary to improve patients' knowledge of ADRs (Q8).

Figures 3 and 4 show a significant difference ($p<0.05$) between years of experience and participants' responses to questions 9 and 11 of the attitude scale. Approximately half of the participants (50.8%, $p=0.004$) agreed that PILs improved patients' knowledge about medication (Q9), and 56.6% believed that PILs may reduce patients' adherence to medications (Q10). In contrast, 56% of pharmacists agreed that PILs were useful in helping patients monitor ADRs and improve their confidence in reporting these reactions (Q11, $p=0.019$). Forty-seven percent of pharmacists with many years of experience agreed that PILs are useful in reducing workload by informing patients about ADRs (Q13). Regarding the roles of drug companies in PILs preparation, approximately 60% of the participants agreed (Q15-Q17).

In Table 4, none of the demographic factors showed a significant association with the attitudes of participants based on years of experience. Most participants with negative attitudes were male, young (less than 35 years), had a bachelor's degree, and worked at hospitals and pharmacies, mostly in the city. They had fewer than three co-working pharmacists, worked less than 10 hours daily, had an unspecified dispensing pattern for NSAIDs, and preferred verbal methods to deliver information to patients.

Table 1. Demographical data of pharmacists based on experience years, (n=309)

	Total	Pharmacists with <4 years of experience	Pharmacists with ≥4 years of experience
Factors	**309 (100%)**	**141 (45.6%)**	**168 (54.4%)**
Age (years) (mean ± SD)	28.22 (±4.94)	25.45 (±1.90)	30.55 (±5.48)
23-28	208 (67.3%)	130 (92.2%)	78 (46.4%)
29-34	71 (23.0%)	11 (7.8%)	60 (35.7%)
35-40	16 (5.2%)	0 (0.0%)	16 (9.5%)
More than 40	14 (4.5%)	0 (0.0%)	14 (8.3%)
Gender			
Female	107 (34.6%)	49 (34.8%)	58 (34.4%)
Male	202 (65.4%)	92 (65.2%)	110 (65.5%)
Scientific degree			
Bachelor's degree	256 (82.8%)	126 (89.4%)	130 (77.4%)
Higher than bachelor's level	53 (17.2%)	15 (10.6%)	38 (22.6%)
Working place			
Hospital (general/private)	42 (13.6%)	17 (12.1%)	25 (14.9%)
Private pharmacy	90 (29.1%)	59 (41.8%)	31 (18.5%)
Both	177 (57.3%)	65 (46.1%)	112 (66.7%)
Location of the pharmacy			
In the city	224 (72.5%)	102 (72.3%)	122 (72.6%)
In a rural area	43 (13.9%)	22 (15.6%)	21 (12.5%)
Not working at a private pharmacy	42 (13.6%)	17 (12.1%)	25 (14.9%)
Number of co-working pharmacists (301)			
None	51(16.9%)	26 (18.7%)	25 (15.4%)
Less than three	156 (51.8%)	70 (50.4%)	86 (53.1%)
Equal to or more than three	52 (17.3%)	26 (18.7%)	26 (16.0%)
Not working at a private pharmacy	42 (14.0%)	17 (12.2%)	25 (15.4%)
Working hours per day (304)			
1-5 hours	117 (38.5%)	55 (39.3%)	62 (37.8%)
6-10 hours	143(47.0%)	63 (45.0%)	80 (48.8%)
More than 10 hours	44 (14.5%)	22 (15.7%)	22 (13.4%)
Dispensing patterns of NSAIDs			
OTC medication	22 (7.1%)	8 (5.7%)	14 (8.3%)
The prescribed medication	24 (7.8%)	7 (5.0%)	17 (10.1%)
Both	263 (85.1%)	126 (89.4%)	137 (81.5%)
Names of most frequent dispensed NSAIDs#			
Diclofenac	237 (76.7%)	107 (75.9%)	130 (77.4%)
Mefenamic acid	219 (70.9%)	105 (74.5%)	114 (67.9%)
Ibuprofen	208 (67.3%)	106 (75.2%)	102 (60.7%)
Aspirin	126 (40.8%)	58 (41.1%)	68 (40.5%)
Meloxicam	101 (32.7%)	48 (34.0%)	53 (31.5%)

Table 1. Continued

Factors	Total 309 (100%)	Pharmacists with <4 years of experience 141 (45.6%)	Pharmacists with ≥4 years of experience 168 (54.4%)
Methods of providing patient instructions			
Verbally	148 (47.9%)	66 (46.8%)	82 (48.8%)
Written information by the PILs	15 (4.9%)	7 (5.0%)	8 (4.8%)
Both	139 (45.0%)	63 (44.7%)	76 (45.2%)
None	7 (2.3%)	5 (3.5%)	2 (1.2%)
Sources of information regarding NSAIDs#			
Academic learning	191 (61.8%)	83 (58.9%)	108 (64.3%)
Medical websites	164 (53.1%)	75 (53.2%)	89 (53.0%)
Medical applications	165 (53.4%)	70 (49.6%)	95 (56.5%)
Medical text book	183 (59.2%)	87 (61.7%)	96 (57.1%)
Research articles	85 (27.5%)	38 (27.0%)	47 (28.0%)
Scientific conferences, workshops, webinars	77 (24.9%)	35 (24.8%)	42 (25.0%)

Data are expressed as frequency (%). #Multiple responses were provided, NSAIDs: Non-steroidal anti-inflammatory drugs, PILs: Patient information leaflets, OTC: Over-the-counter, SD: Standard deviation

Table 2. A statistical analysis of pharmacists' knowledge and attitude, (n=309)

Variables	Total 309 (100%)	Pharmacists with <4 years of experience 141 (45.6%)	Pharmacists with ≥4 years of experience 168 (54.4%)	*p* value
The knowledge category				
Poor knowledge (0-4)	45 (14.6%)	20 (14.2%)	25 (14.9%)	0.873*
Good knowledge (5-10)	264 (85.4%)	121 (85.8%)	143 (85.1%)	
Knowledge score				
Mean (± SD)	6.8 (±2.0)	6.77 (±1.92)	6.85 (±2.05)	
Minimum-maximum	1-10	2-10	1-10	
The attitude category				
Poor (17-40)	2 (0.6%)	0 (0.0%)	2 (1.2%)	0.541**
Moderate (41-63)	220 (71.2%)	103 (73.0%)	117 (69.6%)	
Good (64-85)	87 (28.2%)	38 (27.0%)	49 (29.2%)	
Attitude score				
Mean (± SD)	59.5 (±6.4)	59.72 (±6.1)	59.39 (±6.7)	
Minimum-maximum	38.0-77.0	42.0-77.0	38.0 -74.0	

Data are expressed as frequency (%). *p* value is significant at $p < 0.05$. *Based on chi-square test, **based on Fisher's exact test. NSAIDs: Non-steroidal anti-inflammatory drugs, SD: Standard deviation

DISCUSSION

In this study, most Iraqi pharmacists (85.4%) demonstrated good knowledge about NSAID-related renal and gastrointestinal adverse effects. This finding is consistent with the results of Owusu et al.,[21] who reported that 90% of community pharmacists in Qatar had good knowledge. Pharmacists are knowledgeable and skilled in medication-related aspects. This is related to their education, roles, and duties as medication professionals, in addition to other HCPs.[4,8] This is particularly true in remote and rural areas with limited medical services.[23] Pharmacists must be attentive to risk factors while dispensing NSAIDs and be able to reduce complications by screening and monitoring high-risk patients.[4,8]

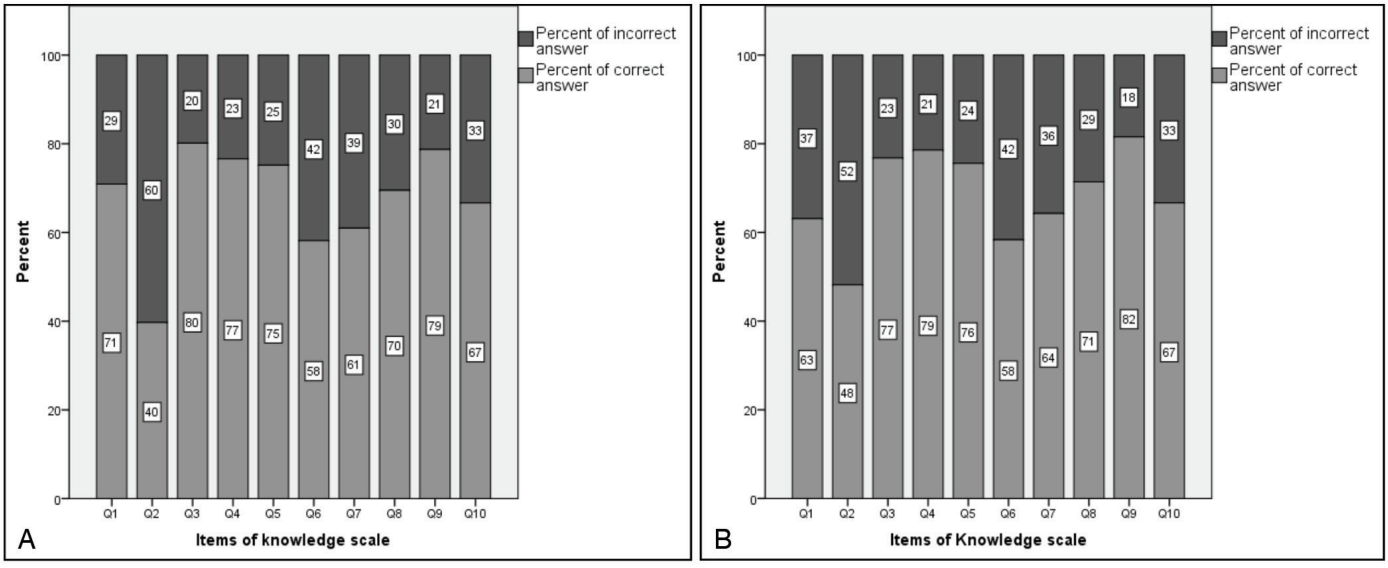

Figure 1. The responses to knowledge scale item (A: Pharmacists with <4 years of experience, n=141, B: Pharmacists with ≥4 years of experience, n=168)

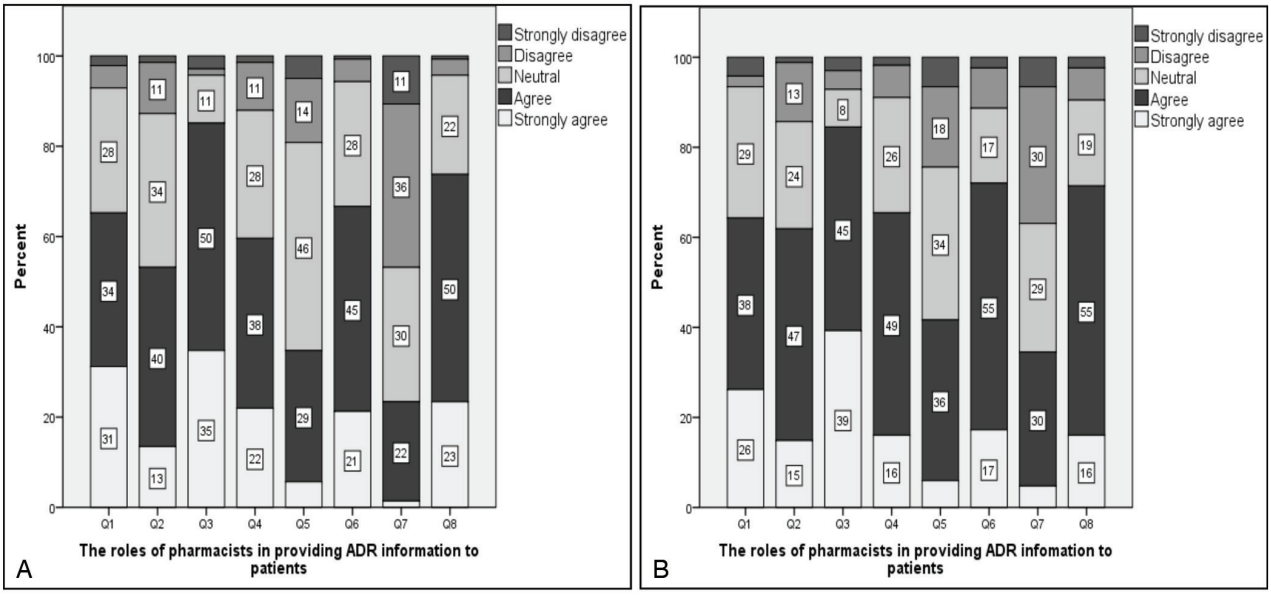

Figure 2. Responses to Q1 to Q8 of attitude scale; the roles of pharmacists in providing ADR information to patients (A: Pharmacists with <4 years of experience, n=141, B: Pharmacists with ≥4 years of experience, n=168)

ADR: Adverse drug reaction

Years of experience were unrelated to participants' knowledge and attitude (p>0.05). A significant association was found between the knowledge of pharmacists with ≥4 years of experience and their age and the knowledge of pharmacists with <4 years of experience and their scientific degree (p<0.05). Younger Iraqi pharmacists and those with a bachelor's degree were more attentive and proactive and tended to depend on their knowledge of academic learning and medical textbooks. Other pharmacists preferred online sources to update their knowledge of NSAIDs' adverse reactions.[21] More knowledgeable pharmacists can provide better patient advice, leading to increased confidence in their careers.[4] Perhaps this is why our participants with long working hours had good knowledge. Patients often prefer to consult pharmacists about ADRs because such consultations are free and readily available, and they form part of their professional duties.[24]

For proper patient management, continuous medical education on updated NSAID safety information is needed.[21,25] In addition, more ADR education topics should be included in undergraduate healthcare education programs.[26] Technology, on the other hand, plays a crucial role in healthcare and should be carefully adopted. For example, support systems contain alerts for drug interactions and reminders for drug monitoring.

Table 3. The association between different demographic factors and pharmacists' knowledge based on experience years (n=309)#

Factors	Pharmacist knowledge <4 years of experience; n=141		Pharmacist knowledge ≥4 years of experience; n=168	
	Poor knowledge n=20	Good knowledge n=121	Poor knowledge n=25	Good knowledge n=143
Age (years)				**(*p*=0.008)***
23-28	19 (95.0%)	111 (91.7%)	17 (68.0%)	61 (42.7%)
29-34	1 (5.0%)	10 (8.3%)	3 (12.0%)	57 (39.9%)
35-40	-	-	1 (4.0%)	15 (10.5%)
More than 40	-	-	4 (16.0%)	10 (7.0%)
Gender				
Male	17 (85.0%)	75 (62.0%)	15 (60.0%)	95 (66.4%)
Female	3 (15.0%)	46 (38.0%)	10 (40.0%)	48 (33.6%)
Scientific degree		**(*p*=0.008)***		
Bachelor's degree	14 (70.0%)	112 (92.6%)	21 (84.0%)	109 (76.2%)
Higher than bachelor's level	6 (30.0%)	9 (7.4%)	4 (16.0%)	34 (23.8%)
Working place				
At the hospital	3 (15.0%)	14 (11.6%)	5 (20.0%)	20 (14.0%)
At a private pharmacy	8 (40.0%)	51 (42.1%)	6 (24.0%)	25 (17.5%)
Both	9 (45.0%)	56 (46.3%)	14 (56.0%)	98 (68.5%)
Location of the pharmacy				
In the city	13 (65.0%)	89 (73.6%)	18 (72.0%)	104 (72.7%)
In a rural area	4 (20.0%)	18 (14.9%)	2 (8.0%)	19 (13.3%)
Not working at a private pharmacy	3 (15.0%)	14 (11.6%)	5 (20.0%)	20 (14.0%)
Number of co-working pharmacists (301)				
None	1 (5.3%)	25 (20.8%)	2 (9.5%)	23 (16.3%)
Less than three	10 (52.6%)	60 (50.0%)	9 (42.9%)	77 (54.6%)
Equal to or more than three	5 (26.3%)	21 (17.5%)	5 (23.8%)	21 (14.9%)
Not working at a private pharmacy	3 (15.8%)	14 (11.7%)	5 (23.8%)	20 (14.2%)
Working hours per day (304)		**(*p*=0.045)***		
1-5 hours	3 (15.8%)	52 (43.0%)	10 (43.5%)	52 (36.9%)
6-10 hours	13 (68.4%)	50 (41.3%)	13 (56.5%)	67 (47.5%)
More than 10 hours	3 (15.8%)	19 (15.7%)	0 (0.0%)	22 (15.6%)
Dispensing patterns of NSAIDs		**(*p*<0.001)***		
OTC medication	4 (20.0%)	4 (3.3%)	8 (32.0%)	6 (4.2%)
The prescribed medication	4 (20.0%)	3 (2.5%)	3 (12.0%)	14 (9.8%)
Both	12 (60.0%)	114 (94.2%)	14 (56.0%)	123 (86.0%)
Methods of providing patient instructions				
Verbally	7 (35.0%)	59 (48.8%)	11 (44.0%)	71 (49.7%)
Written information by the PILs	2 (10.0%)	5 (4.1%)	4 (16.0%)	4 (2.8%)
Both	9 (45.0%)	54 (44.6%)	10 (40.0%)	66 (46.2%)
None	2 (10.0%)	3 (2.5%)	0 (0.0%)	2 (1.4%)

*Based on Fisher's exact test. #*p* values of all other associations were >0.05 based on chi-square test or Fisher's exact test as appropriate. Data are expressed as frequency (%). NSAIDs: Non-steroidal anti-inflammatory drugs, PILs: Patient information leaflets, OTC: Over-the-counter

Table 4. The association between different demographic factors and pharmacists' attitudes based on experience years (n=309)#

Factors	Pharmacists' attitudes toward <4 years of experience; n=141		Pharmacists' attitudes toward ≥4 years of experience; n=168	
	Negative attitude n=103	Positive attitude n=38	Negative attitude n=119	Positive attitude n=49
Age (years)				
23-28	97 (94.2%)	33 (86.8%)	58 (48.7%)	20 (40.8%)
29-34	6 (5.8%)	5 (13.2%)	38 (31.9%)	22 (44.9%)
35-40	-	-	13 (10.9%)	3 (6.1%)
>40	-	-	10 (8.4%)	4 (8.2%)
Gender				
Male	64 (62.1%)	28 (73.7%)	78 (65.5%)	32 (65.3%)
Female	39 (37.9%)	10 (26.3%)	41 (34.5%)	17 (34.7%)
Scientific degree				
Bachelor's degree	94 (91.3%)	32 (84.2%)	90 (75.6%)	40 (81.6%)
Higher than bachelor's degree	9 (8.7%)	6 (15.8%)	29 (24.4%)	9 (18.4%)
Working place				
At the hospital	11 (10.7%)	6 (15.8%)	19 (16.0%)	6 (12.2%)
At a private pharmacy	42 (40.8%)	17 (44.7%)	24 (20.2%)	7 (14.3%)
Both	50 (48.5%)	15 (39.5%)	76 (63.9%)	36 (73.5%)
Location of the pharmacy				
In the city	79 (76.7%)	23 (60.5%)	85 (71.4%)	37 (75.5%)
In a rural area	13 (12.6%)	9 (23.7%)	15 (12.6%)	6 (12.2%)
Not working at a private pharmacy	11 (10.7%)	6 (15.8%)	19 (16.0%)	6 (12.2%)
Number of co-working pharmacists (301)				
None	17 (16.7%)	9 (24.3%)	19 (16.8%)	6 (12.2%)
Less than three	57 (55.9%)	13 (35.1%)	58 (51.3%)	28 (57.1%)
Equal to or more than three	17 (16.7%)	9 (24.3%)	17 (15.0%)	9 (18.4%)
Not working at a private pharmacy	11 (10.8%)	6 (16.2%)	19 (16.8%)	6 (12.2%)
Working hours per day (304)				
1-5 hours	43 (42.2%)	12 (31.6%)	47 (40.2%)	15 (31.9%)
6-10 hours	41 (40.2%)	22 (57.9%)	55 (47.0%)	25 (53.2%)
More than 10 hours	18 (17.6%)	4 (10.5%)	15 (12.8%)	7 (14.9%)
Dispensing patterns of NSAIDs				
OTC medication	7 (6.8%)	1 (2.6%)	12 (10.1%)	2 (4.1%)
The prescribed medication	4 (3.9%)	3 (7.9%)	14 (11.8%)	3 (6.1%)
Both	92 (89.3%)	34 (89.5%)	93 (78.2%)	44 (89.8%)
Methods of providing patient instructions				
Verbally	50 (48.5%)	16 (42.1%)	57 (47.9%)	25 (51.0%)
Written information by the PILs	5 (4.9%)	2 (5.3%)	8 (6.7%)	0 (0.0%)
Both	43 (41.7%)	20 (52.6%)	53 (44.5%)	23 (46.9%)
None	5 (4.9%)	0 (0.0%)	1 (0.8%)	1 (2.0%)

Data are expressed as frequency (%). #*p* values of all associations were >0.05 based on chi-square test or Fisher's exact test as appropriate. NSAIDs: Non-steroidal anti-inflammatory drugs, PILs: Patient information leaflets, OTC: Over-the-counter

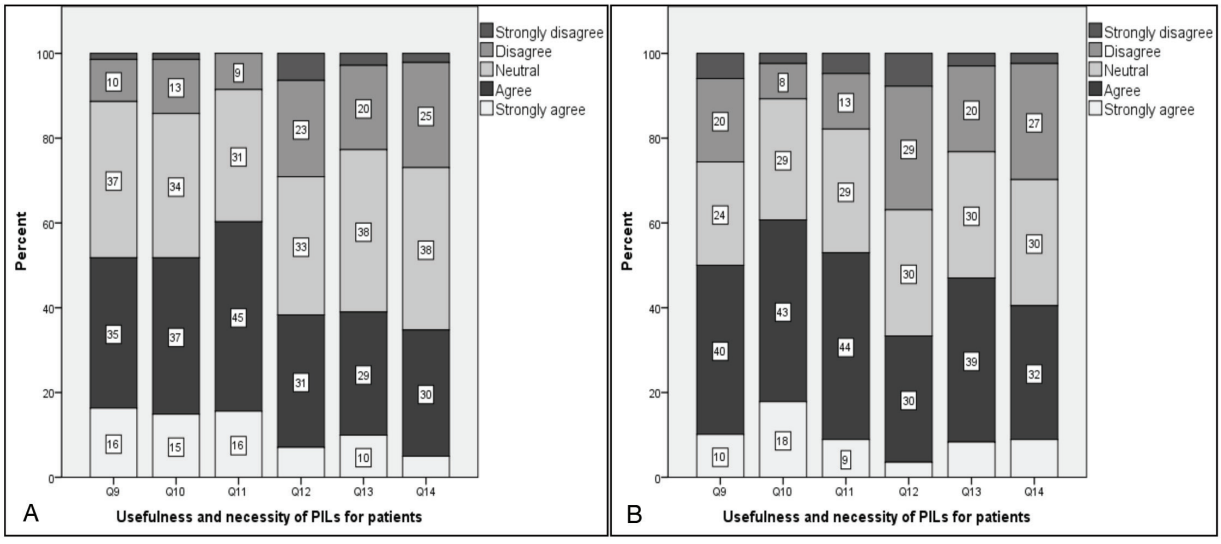

Figure 3. Responses to Q9 to Q14 of attitude scale; the usefulness and necessity of PILs for patients. (A: Pharmacists with <4 years of experience, n=141, B: Pharmacists with ≥4 years of experience, n=168)

PILs: Patient information leaflets

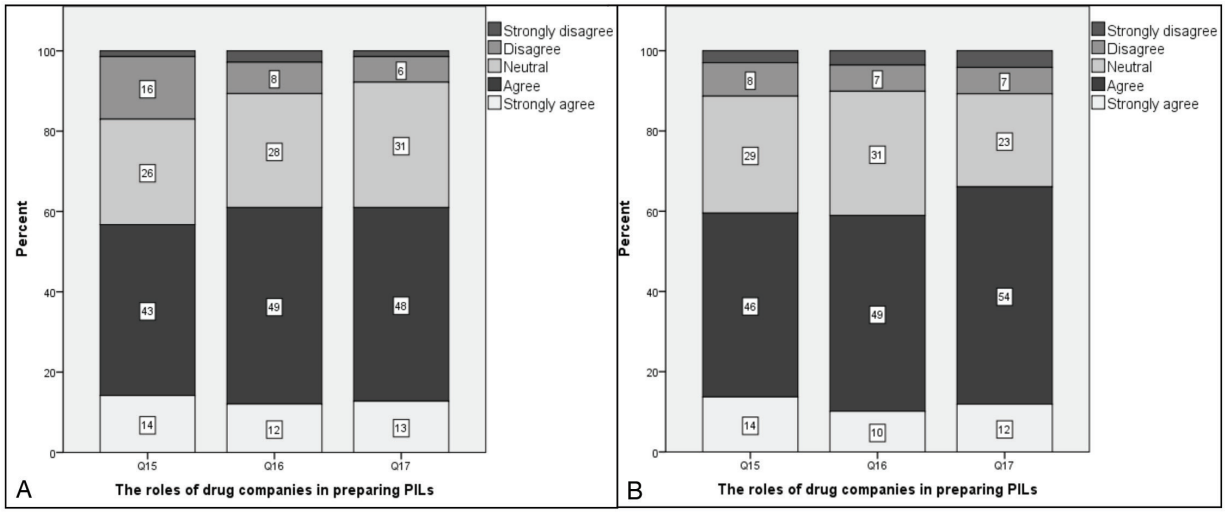

Figure 4. Responses to Q15 to Q17 on the attitude scale; the roles of drug companies in preparing PILs (A: Pharmacists with <4 years of experience, n=141, B: Pharmacists with ≥4 years of experience, n=168)

PILs: Patient information leaflets

Through this system, physicians and pharmacists can also share patient information to guide their prescription decisions.[25] Accessing artificial intelligence (AI) applications can enhance pharmacovigilance activities, but caution should be exercised to ensure accurate information.[27]

A highly significant association (p<0.001) was observed between the dispensing patterns of NSAIDs and the participants' knowledge. These drugs are available as prescribed and OTC medicines.[28,29] The increase in their use as OTC medicines may lead to unintentional overuse and therapeutic repetitions.[30] Most NSAID overuses lacked adequate information or counseling from HCPs.[4] In particular, these drugs are frequently prescribed for elderly patients, those with comorbidities, and those with polypharmacy.[11,31] Community pharmacists may be better positioned to educate patients with minimal health literacy[30] and advise on OTC drugs.[32,33] Patients often believe NSAIDs are safe and are unaware of risks such as drug interactions or abuse/misuse potential.[32] On the other hand, hospital pharmacists should review medications for high-risk patients,[8,26] for greater safety awareness, therapeutic individualization, and follow-up.[8] Those high-risk patients included elderly, use multiple medications, low therapeutic index, and unsuitable drugs.[8]

Only approximately one-third of the participants had a good attitude toward educating patients about drug safety information. This is in contrast to the findings of Owusu et al.,[21] and nearly similar to the good attitudinal results reported among 42.1% of hospital pharmacists[22] and 36% of orthopedic physicians[29] in Thailand using the same attitude scale. Pharmacists with less

than four years of experience had more negative attitudes than those with more years of experience (73% vs. 71%). These findings are similar to the results reported by others[4,22] and in contrast to Kopciuch et al.,[24] who showed that pharmacists with longer professional experience were less willing to report ADRs. Therefore, regardless of whether years of experience influence pharmacists' attitudes, continued awareness of the importance of patient education regarding ADRs remains essential.

Although 85% of the participants agreed that pharmacists play a role in educating patients about ADRs, 70% believed that physicians also play a similar role. Likewise, a study reported that Iraqi physicians had a role in reporting ADRs (78%) and monitoring drug safety (96%) as part of their duty.[34] Physicians play an essential role in providing risk information regarding NSAIDs and desire to share patient education responsibilities with pharmacists.[30] By recognizing the risk factors before prescribing, adverse effects during treatment can be minimized.[29] It also allows patients to weigh the risks and benefits before starting treatment with any class of NSAIDs.[4,29]

Health literacy presents a challenge, as patients often lack medical vocabulary, knowledge, and understanding of new information.[30] In this study, 64% of pharmacists with more experience agreed to inform patients about ADRs, and 70% of participants found it necessary to improve patients' knowledge of these reactions. In addition, approximately half of the less experienced pharmacists disagreed with the ability of education to improve patients' confidence in self-reporting ADRs. However, participants' concerns (60%) increased regarding patients' anxiety and medication nonadherence, similar to others' findings.[22,29] There is uncertainty about whether NSAID users have adequate information about their therapeutic risks and benefits,[30] and all essential NSAID issues, such as risk information, may not be fully explained to all patients.[28] Pharmacists and physicians tend to inform patients about common NSAID adverse effects, such as gastrointestinal adverse effects.[22,29,30] However, patients may lack information about the severe, perhaps less common, cardiovascular, and renal adverse effects of NSAIDs.[5,22,30] This may render patients with inadequate understanding more vulnerable to the risks of NSAID therapy.[30] Therefore, balanced, individualized information is needed based on patients' cases to use NSAIDs without fear or concern, similar to others' opinions.[4,25,28]

Various methods, including teach-back techniques and written materials, can be used to effectively convey information to patients.[30] Using PILs is useful in saving time, improving patient education, and increasing their confidence in reporting and monitoring ADRs, as agreed upon by approximately 50% to 56% of our participants and supported by other studies.[22,29] The FDA also mandates the use of approved medication guides as written information to clarify treatment risks and benefits.[30] However, concerns have been raised about the impact of PILS on patient care, as the content may not be suitable or understandable for all patients.[4] This may affect their willingness to use the drug again if they are aware of its adverse effects on their health. Approximately 57% of the participants had similar concerns. Phueanpinit et al.,[22] suggested the availability of user-friendly leaflets to help patients who extensively use a high-risk drug with the potential for serious ADRs. These leaflets should be easy to read and understandable for patients with minimal health literacy.[30] Technology can support both pharmacist and patient education, but multimedia education should be limited to a supplementary role, not a replacement for HCP education.[7]

Several factors can influence the rate and type of education on drug safety information, such as patient-related factors like age, understanding ability, type, and duration of NSAIDs used.[11,28,30] Pharmacist-related factors include age, gender, workload, number of co-workers, working hours, experience, and educational level.[21,24,28,30,35] Pharmacists may not have sufficient time to counsel patients adequately in busy pharmacies with limited staff.[32] According to Kopciuch et al.,[24] younger pharmacists, those with higher educational degrees (master's and doctoral degrees), and those with less experience had a strong sense of duty regarding drug safety.

Online surveys offer cost-effective, easy-to-implement, and nationwide participant access. However, the low response rate may limit the generalizability of the study results. The response rates were influenced by the participants' desirability and interest in responding to the survey. Our study's response rate, although slightly lower than the target rate, was higher than that reported by others.[21,22,33] A cross-sectional design does not allow for the assessment of causal relationships, and self-reporting raises concerns about recall bias, social desirability bias, and the trust issue between participants and researchers.

In this study, it was difficult to distinguish between counseling and education regarding new or repeated prescriptions of NSAIDs. The knowledge questions did not assess other adverse effects of NSAIDs, and perhaps the participants were more interested in the gastrointestinal and renal adverse effects of NSAIDs, which resulted in higher knowledge scores. The results should be exercised when interpreting the results. Nonetheless, the study can still benefit pharmacists in terms of improving their attitudes toward informing patients about drug safety information and encouraging pharmacists to stay up-to-date with the latest information.

CONCLUSION

Regardless of years of experience, a good degree of knowledge was found among most Iraqi pharmacists regarding the renal and gastrointestinal adverse effects of NSAIDs. Knowledge was significantly associated with the dispensing patterns of NSAIDs, participants' age, their scientific degrees, and working hours. Most participants had negative attitudes toward providing information on drug safety, which was not significantly related to years of experience. Patient education requires the efforts of both pharmacists and physicians to ensure proper drug use with minimal risks. Leaflets can be helpful as a reliable source of information but can also be a reason for nonadherence to the therapeutic regimen.

Ethics

Ethics Committee Approval: This study was approved by the Collegiate Committee for Medical Research Ethics at the University of Mosul (approval number: CCMRE-phA-21-10, date: 25.01.2023).

Informed Consent: The participants provided their consent before answering the questionnaire.

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Footnotes

Authorship Contributions

Concept: H.F.K., Design: H.F.K., A.İ.S., Data Collection or Processing: H.F.K., A.İ.S., H.A., Analysis or Interpretation: H.F.K., Literature Search: H.F.K., Writing: H.F.K.

Conflict of Interest: The authors have no conflicts of interest to declare.

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REFERENCES

1. Howard RL, Avery AJ, Slavenburg S, Royal S, Pipe G, Lucassen P, Pirmohamed M. Which drugs cause preventable admissions to hospital? A systematic review. Br J Clin Pharmacol. 2007;63:136-147.
2. U.S. Food and Drug Administration. FDA strengthens warning that non-aspirin non-steroidal anti-inflammatory drugs (NSAIDs) can cause heart attacks or strokes. 2015. Available at: https://www.fda.gov/downloads/Drugs/DrugSafety/UCM453941.pdf [Accessed 12 Dec. 2023].
3. Al-Azayzih A, Al-Azzam SI, Alzoubi KH, Jarab AS, Kharaba Z, Al-Rifai RH, Alnajjar MS. Non-steroidal anti-inflammatory drugs utilization patterns and risk of adverse events due to drug-drug interactions among elderly patients: a study from Jordan. Saudi Pharm J. 2020;28:504-508.
4. Malebari AM, Khayyat AN, Mahdali RA, Alamoudi JS, Alsayed BY, Alrasheed SA. Evaluation of the community pharmacists' performance in the screening of non-steroidal anti-inflammatory drugs risks in Saudi Arabia. Saudi Med J. 2020;41:849-857.
5. Wongrakpanich S, Wongrakpanich A, Melhado K, Rangaswami J. A comprehensive review of non-steroidal anti-inflammatory drug use in the elderly. Aging Dis. 2018;9:143-150.
6. Phueanpinit P, Pongwecharak J, Krska J, Jarernsiripornkul N. Evaluation of community pharmacists' roles in screening and communication of risks about non-steroidal anti-inflammatory drugs in Thailand. Prim Health Care Res Dev. 2018;19:598-604.
7. Perrot S, Cittée J, Louis P, Quentin B, Robert C, Milon JY, Bismut H, Baumelou A. Self-medication in pain management: the state of the art of pharmacists' role for optimal over-the-counter analgesic use. Eur J Pain. 2019;23:1747-1762.
8. Mansur JM. Medication safety systems and the important role of pharmacists. Drugs Aging. 2016;33:213-221.
9. By the 2023 American Geriatrics Society Beers Criteria® Update Expert Panel. American Geriatrics Society 2023 updated AGS Beers Criteria® for potentially inappropriate medication use in older adults. J Am Geriatr Soc. 2023;71:2052-2081.
10. Brune K, Patrignani P. New insights into the use of currently available non-steroidal anti-inflammatory drugs. J Pain Res. 2015;8:105-118.
11. Jarernsiripornkul N, Phueanpinit P, Pongwecharak J, Krska J. Experiences of and attitudes towards receiving information about non-steroidal anti-inflammatory drugs: a cross-sectional survey of patients in Thailand. Expert Opin Drug Saf. 2016;15:417-426.
12. Gómez-Acebo I, Dierssen-Sotos T, de Pedro M, Pérez-Gómez B, Castaño-Vinyals G, Fernández-Villa T, Palazuelos-Calderón C, Amiano P, Etxeberria J, Benavente Y, Fernández-Tardón G, Salcedo-Bellido I, Capelo R, Peiró R, Marcos-Gragera R, Huerta JM, Tardón A, Barricarte A, Altzibar JM, Alonso-Molero J, Dávila-Batista V, Aragonés N, Pollán M, Kogevinas M, Llorca J. Epidemiology of non-steroidal anti-inflammatory drugs consumption in Spain. BMC Public Health. 2018;18:1134.
13. Moore N, Pollack C, Butkerait P. Adverse drug reactions and drug-drug interactions with over-the-counter NSAIDs. Ther Clin Risk Manag. 2015;11:1061-1075.
14. Foppe van Mil JW, Westerlund T, Brown L, Chen TF, Henman M, Hersberger K, McElnay J, Schulz M. Medical care and drug-related problems: do doctors and pharmacists speak the same language? Int J Clin Pharm. 2016;38:191-194.
15. Alfahmi AA, Curtain CM, Salahudeen MS. Assessment of knowledge, attitude and practices of the hospital and Community Pharmacists in Saudi Arabia (Jeddah) towards Inappropriate Medication Use in Older Adults. Int J Environ Res Public Health. 2023;20:1635.
16. Hussain SA, Abbas AN, Habeeb SA, Abd-Ali AK, Abdulrahman ZS. Healthcare personnel's experience of reporting adverse drug reactions in Baghdad city: cross-sectional study. Int J Clin Pharm. 2019;41:1307-1313.
17. Allela O. Explore Adverse Drug Reactions (ADRs) reporting by clinical and community pharmacists in Duhok, Kurdistan region-Iraq: hampered and perspective. Pharmacia. 2022;69:1057-1062.
18. Al-Jumaili AA, Younus MM, Kannan YJA, Nooruldeen ZE, Al-Nuseirat A. Pharmaceutical regulations in Iraq: from medicine approval to postmarketing. East Mediterr Health J. 2021;27:1007-1015.
19. The invisible burden of antibiotic resistance in Mosul, Iraq, MSF. 2019. Available at: https://www.msf.org/invisible-burden-antibiotic-resistance-mosul-iraq [Accessed 13 Dec. 2023].
20. Sample Size Calculator by Raosoft, Inc. Available at: http://www.raosoft.com/samplesize.html [Accessed 13 Nov. 2023].
21. Owusu YB, Elkhalifa WH, Awaisu A, Kheir N. Assessment of Qatar community pharmacists' competence and practices related to renal and gastrointestinal adverse effects of non-prescription NSAIDs. Saudi Pharm J. 2022;30:1396-1404.
22. Phueanpinit P, Jarernsiripornkul N, Pongwecharak J, Krska J. Hospital pharmacists' roles and attitudes in providing information on the safety of non-steroidal anti-inflammatory drugs in Thailand. Int J Clin Pharm. 2014;36:1205-1212.
23. Morrison C, Beauchamp T, MacDonald H, Beattie M. Implementing a non-steroidal anti-inflammatory drugs communication bundle in remote and rural pharmacies and dispensing practices. BMJ Open Qual. 2018;7:e000303.
24. Kopciuch D, Zaprutko T, Paczkowska A, Ratajczak P, Zielińska-Tomczak Ł, Kus K, Nowakowska E. Safety of medicines-Pharmacists' knowledge, practice, and attitudes toward pharmacovigilance and

adverse drug reactions reporting process. Pharmacoepidemiol Drug Saf. 2019;28:1543-1551.

25. Hwong WY, Lim YMF, Khoo EM, Sivasampu S. High-risk nonsteroidal anti-inflammatory drugs prescribing in primary care: results from National Medical Care Survey Malaysia. Int J Clin Pharm. 2020;42:489-499.
26. Cheema E, Almualem AA, Basudan AT, Salamatullah AK, Radhwi SO, Alsehli AS. Assessing the impact of structured education on the knowledge of hospital pharmacists about adverse drug reactions and reporting methods in Saudi Arabia: an open-label randomized controlled trial. Drugs Ther Perspect. 2019;35:296-300.
27. Praveen J. Empowering pharmacovigilance: unleashing the potential of generative AI in drug safety monitoring. J Innov Appl Pharm Sci. 2023;8:24-32.
28. Jarernsiripornkul N, Phueanpinit P, Pongwecharak J, Krska J. Practices of healthcare professionals in communicating with non-steroidal anti-inflammatory drug users in Thailand: a qualitative study. Int J Pharm Pract. 2019;27:362-369.
29. Phueanpinit P, Pongwecharak J, Sumanont S, Krska J, Jarernsiripornkul N. Physicians' communication of risks from non-steroidal anti-inflammatory drugs and attitude towards providing adverse drug reaction information to patients. J Eval Clin Pract. 2017;23:1387-1394.
30. Schmitt MR, Miller MJ, Harrison DL, Farmer KC, Allison JJ, Cobaugh DJ, Saag KG. Communicating non-steroidal anti-inflammatory drug risks: verbal counseling, written medicine information, and patients' risk awareness. Patient Educ Couns. 2011;83:391-397.
31. Phueanpinit P, Pongwecharak J, Krska J, Jarernsiripornkul N. Knowledge and perceptions of the risks of non-steroidal anti-inflammatory drugs among orthopedic patients in Thailand. Int J Clin Pharm. 2016;38:1269-1276.
32. Ylä-Rautio H, Siissalo S, Leikola S. Drug-related problems and pharmacy interventions in non-prescription medication, with a focus on high-risk over-the-counter medications. Int J Clin Pharm. 2020;42:786-795.
33. Halila GC, Junior EH, Otuki MF, Correr CJ. The practice of OTC counseling by community pharmacists in Parana, Brazil. Pharm Pract (Granada). 2015;13:597.
34. RMS M, YA A. Physicians' knowledge about pharmacovigilance in Iraq. J Pharmacovigil. 2016;4:13.
35. AlShammari TM, Almoslem MJ. Knowledge, attitudes, and practices of healthcare professionals in hospitals towards the reporting of adverse drug reactions in Saudi Arabia: a multi-center cross-sectional study. Saudi Pharm J. 2018;26:925-931.

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ORIGINAL ARTICLE

Exploring the Nexus of Professional Commitment, Emotional Labor, and Self-Efficacy Among Community Pharmacists: Implications for Healthcare Delivery

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ABSTRACT

Objectives: This study aimed to explore the relationship between emotional labor, professional commitment, and self-efficacy among community pharmacists. Specifically, this study examined how self-efficacy influences professional commitment and the mediating role of emotional labor strategies in this relationship.

Materials and Methods: A cross-sectional survey design was used to collect data from 396 community pharmacists. The study used a convenience sampling method and included standardized measures of emotional labor, professional commitment, and self-efficacy. Descriptive statistics were used to examine the levels of these variables among the participants. Multiple regression analyses were conducted to assess the interdependencies and mediating effects of emotional labor strategies.

Results: General self-efficacy was positively correlated with emotional commitment (β=0.275, p<0.05) and continuance commitment (β=0.364, p<0.05), explaining 5% and 8% of their variances, respectively. A normative commitment was influenced by self-efficacy (β=0.464, p<0.05) and deep emotional labor (β=0.134, p<0.05), explaining 11% of its variance. Self-efficacy and deep emotional labor positively affected overall professional commitment (β=0.368, p<0.05), accounting for 15% of the variance.

Conclusion: The results highlight the crucial role of self-efficacy in managing the emotional demands of the pharmacy profession and in fostering stronger professional commitment. Enhancing pharmacists' self-efficacy and emotional management skills can improve their job satisfaction and commitment to the profession. These findings have clinical implications for the development of training interventions aimed at supporting pharmacists in coping with the emotional aspects of their work and improving their overall professional well-being.

Keywords: Emotional labor, professional commitment, self-efficacy, community pharmacists, pharmacy practice

NOTE: this study is the article of the author's doctoral thesis.

INTRODUCTION

The healthcare sector is a pivotal part of the service industry and is tasked with meeting the population's health needs.[1] Central to healthcare services is a commitment to preserving and enhancing human health through sustained engagement between professionals and patients.[2] This relational aspect reflects the broader dynamics of the service industry, where employees are expected to align with societal values and standards.[3]

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Healthcare professionals, including physicians, nurses, and pharmacists, are integral components of a system that supports society's health.[4] Understanding their multifaceted roles and interactions is crucial for patient outcomes and satisfaction. As healthcare evolves with technological advancements, changing demographics, and new challenges, the ability of professionals to adapt while maintaining professionalism is essential.[5] This adaptation involves fostering trust, empathy, and effective communication with patients and their families.[6]

Community pharmacists play a crucial role in the healthcare ecosystem, offering accessible consultation services and guidance beyond dispensing medications.[7] Comprehensive education and experience allow healthcare professionals to address a spectrum of challenges, making them key players in patient care and health outcomes.[8] Community pharmacists are involved in patient education, chronic disease management, medication management, and preventive health services, positively impacting healthcare delivery.[9] This expanded role highlights the importance of professional commitment, emotional labor, and self-efficacy in community pharmacy.[10] This study explores these dynamics to provide insights into how these factors influence community pharmacists' practices and their impact on healthcare.

Community pharmacists' interactions with patients emphasize their engagement and dedication. Factors shaping professional commitment, emotional labor, and self-efficacy are essential yet underexplored in healthcare research. Although the literature addresses these themes across various sectors, specific exploration within community pharmacy is limited.[11,12]

This study aimed to explore the relationship between emotional labor, professional commitment, and self-efficacy among community pharmacists. Specifically, this study examined how self-efficacy influences professional commitment and the mediating role of emotional labor strategies in this relationship.

The findings of this study can enhance the understanding of community pharmacists' contributions to healthcare by emphasizing their commitment, emotional resilience, and confidence in fostering a positive healthcare environment.

MATERIALS AND METHODS

Participants and procedures

This cross-sectional study was designed to examine the effects of community pharmacists' emotional labor behaviors and general self-efficacy perceptions on their levels of professional commitment. The study was conducted from June 2018 to May 2019 and focused on a target population of 1,992 community pharmacists registered with the Ankara Chamber of Pharmacists.

Due to practical challenges such as time and cost constraints, a decision was made to sample a portion of the population. A simple random sampling technique was used to select a representative sample of pharmacists from the population list provided by the Ankara Chamber of Pharmacists. Each pharmacist had an equal chance of being selected. The sample size was determined using the formula:

$n_o=[(t\times S)/D]^2$, $n=[n_o/(1+(n_o/N))]$

where:

- t represents the z-score for the desired confidence level,
- S is the estimated population standard deviation,
- D denotes the acceptable margin of error, and
- N is the total population size.

Based on these calculations, the initial sample size was determined as $n_o=384.16$. After applying the finite population correction factor, the final adjusted sample size was n=322.

After randomly selecting the pharmacists, invitations to participate in the survey were sent, and the pharmacists who agreed to participate did so voluntarily. In total, 402 pharmacists participated in the face-to-face survey. After data cleaning (removing incomplete or erroneous responses), the final dataset comprised responses from 396 pharmacists. Voluntary participation was essential for ensuring ethical compliance and participant willingness, although the initial selection process was random.

Instruments

The initial section of the measurement tool consists of five questions designed to gather demographic information from the participants. The foundational data serves to contextualize the subsequent analyses by providing insights into the diverse backgrounds of the study population.

The measurement tool's second section incorporates the 18-item, three-dimensional Professional Commitment Scale originally developed by Meyer et al.[13] Adapted for the Turkish context through factor analyses by Tak and Çiftçioğlu[14], this scale is designed to evaluate the complex construct of professional commitment, encapsulating three distinct factors: emotional, continuance, and normative commitment. The scale's comprehensive approach to assessing professional commitment is further validated by its overall reliability, which is underscored by a Cronbach's alpha coefficient of 0.85, confirming its efficacy in capturing the nuanced dimensions of professional commitment among Turkish professionals.

The third section incorporates the 10-item general self-efficacy scale developed by Schwarzer and Fuchs,[15] with Turkish validity and reliability established by Aypay.[16] This scale, characterized by its unidimensional structure, evaluates an individual's belief in their capacity to cope with a broad range of demanding or novel situations. The reliability of the general self-efficacy scale was confirmed by a Cronbach's alpha coefficient of 0.83, highlighting its consistency in measuring self-efficacy among Turkish participants.

The fourth section of the measurement tool employs the Emotional Labor Scale, a 13-item instrument initially developed by Diefendorff et al.[17] and subsequently validated for the Turkish context by Basım and Beğenirbaş.[18] Contrary to the initial three-factor structure, this scale effectively distills emotional labor into two core dimensions, Surface Behavior and Deep Behavior, providing a focused exploration of the emotional labor dynamics encountered in the workplace. The reliability of this scale, as evidenced by a Cronbach's alpha coefficient of 0.80,

underscores its capacity to accurately reflect the complexities of emotional labor among Turkish professionals, ensuring its applicability and relevance in examining workplace emotional dynamics.

Informed consent and institutional review board (IRB) approval

Informed consent was obtained from all participants. This study was approved by the Ankara University Health Sciences Ethics Sub-Board (approval number 143, date: 25.06.2018).

Statistical analysis

Using the robust capabilities of the Statistical Package for the Social Sciences and Analysis of Moment Structures, the data underwent a comprehensive analysis through two pivotal phases. Initially, Exploratory Factor Analysis (EFA) was employed to ascertain the dimensionality and construct validity, followed by Confirmatory Factor Analysis (CFA) to validate the scales' structure and assess model fit, employing maximum likelihood estimation as recommended by Anderson and Gerbing.[19]

The Kaiser-Meyer-Olkin (KMO) measure, a testament to sampling adequacy, yielded favorable results across the board. The Professional Commitment Scale revealed a KMO value of 0.802, coupled with a significant chi-square test result (χ^2=2559.694, p<0.05), underscoring the data's fitness for factor analysis. General self-efficacy and emotional labor scale followed suit, registering KMO values of 0.900 and 0.821, respectively, with both scales demonstrating statistically significant chi-square test results, thus validating the preparedness of the dataset for nuanced factor analysis. The EFA for the Professional Commitment Scale has a three-dimensional structure, which, after refinement, comprises 17 items distributed across three factors. This restructured scale accounted for 54.461% of the total variance and exhibited high reliability with a Cronbach's alpha coefficient of 0.816. General Self-Efficacy Scale had a unidimensional structure, encompassing 10 items that explained 49.326% of the variance and achieved a Cronbach's alpha of 0.884, indicating exemplary reliability. The emotional labor scale, upon further analysis, presented a two-factor structure. The final configuration, a 10-item scale, elucidated 64.861% of the variance and exhibited a Cronbach's alpha of 0.816, affirming its reliability. In the process of validating the constructs involved in this study, CFA was meticulously applied to the scales representing professional commitment, general self-efficacy, and emotional labor. The aim of this study was to corroborate the structures unearthed during the exploratory phase, ensuring their statistical robustness and relevance to the professional dynamics of community pharmacists. The CFA was instrumental in affirming the three-dimensional construct of the Professional Commitment Scale, as initially identified. The model fit indices revealed a commendable alignment with the theoretical model, with the chi-square to degrees of freedom ratio (χ^2/df) at 3.35, a marker of good model fit. The Goodness-of-Fit Index (GFI) and the Comparative Fit Index (CFI) registered values of 0.90, underscoring a substantial model fit. Additionally, the Root Mean Square Error of Approximation (RMSEA) was 0.08, further solidifying the scale's capacity to accurately represent the facet of professional commitment among the pharmacists surveyed. The analysis of the general self-efficacy scale through CFA highlighted its unidimensional structure, complemented by persuasive fit indices that underscored the scale's reliability and construct validity within the study's framework. The χ^2/df ratio was noted at 2.85, indicative of a favorable model fit. Exceptional GFI and CFI values of 0.96 and 0.97, respectively, confirmed the model's satisfactory alignment with the hypothesized structure. The RMSEA index quantified at 0.07, validated a close fit, further confirming the scale's ability to gauge self-efficacy perceptions among participants. Emotional labor scale underwent CFA to validate its factorial structure, with the resultant model fit indices robustly supporting the scale's construct validity. The χ^2/df ratio achieved a commendable value of 3.18, indicating a good model fit. Noteworthy GFI and CFI values of 0.96 and 0.97, respectively, demonstrate exceptional data fit. Furthermore, an RMSEA value of 0.07 was within the acceptable range, affirming the scale's efficacy in capturing the nuanced dimensions of emotional labor pertinent to the community pharmacy context. These analyses are summarized in Table 1.

The validation of the constructs was meticulously undertaken by examining both convergent and discriminant validity. This critical evaluation was achieved through the application of several key metrics, namely average variance extracted (AVE) and composite reliability (CR). Furthermore, the study's AVE values were observed to lie between 0.52 and 0.71, thereby exceeding the accepted benchmark of 0.5. This indicates a satisfactory level of variance explained by the constructs relative to the measurement error. CR values ranged from 0.69 to 0.89, well above the standard criterion of 0.6 recommended by Bagozzi and Yi,[20] attesting to the reliability and internal consistency of the constructs. The assessment of discriminant validity further reinforced the constructs' distinctiveness. This was evidenced by the square roots of the AVE values, which were found to be greater than the correlations among the constructs. This result substantiates the discriminant validity of the measurement model, affirming that each construct indeed captures a unique phenomenon, distinct from the others within the study. Collectively, these findings lend substantial support to the construct validity of the study's measurement instruments, thereby affirming the reliability and accuracy of the underlying research framework.

RESULTS

A total of 396 community pharmacists participated in the study after data cleaning. A total of 24 participants were excluded due to incomplete or inconsistent data. The sex distribution of the participants was nearly balanced, with 52.5% identifying as male (n=208) and 47.5% as female (n=188). The age range of the participants was diverse, with the most significant proportion (26.0%, n=103) falling within the 31-40 years age group. Other age groups were also represented, reflecting the broad spectrum of age among community pharmacists. A comprehensive summary of characteristic of responders is presented in Table 2.

A significant majority of the participants were married, accounting for 74.5% (n=295) of the sample. Regarding educational qualifications, 81.3% of the pharmacists (n=322) held a bachelor's degree, indicating a high level of educational attainment across the sample. Additionally, participants varied in terms of their professional experience, with 29.0% (n=115) having over 26 years of experience. Others had a range of shorter durations in practice, contributing to a comprehensive representation of community pharmacists in the study.

Table 1. Characteristics of responders

Characteristic	Frequency (n)	Percentage (%)
Sex		
Male	208	52.5
Female	188	47.5
Age group		
21-30 years	82	20.7
31-40 years	103	26.0
41-50 years	97	24.5
51+ years	114	28.8
Marital status		
Married	295	74.5
Single/Other	101	25.5
Educational qualification		
Bachelor's degrees	322	81.3
Other degrees	74	18.7
Professional experience		
0-5 years	65	16.4
6-15 years	98	24.7
16-25 years	118	29.8
26+ years	115	29.0

Table 2. Descriptive statistics of scale scores

Variable	Mean	SD	Skewness	Kurtosis
Emotional commitment	4.28	0.66	-1.513	2.589
Continuance commitment	3.69	0.74	-0.705	0.894
Normative commitment	3.37	0.88	-0.448	-0.014
Professional commitment	3.78	0.54	-0.687	1.736
General self-efficacy	3.25	0.52	-0.455	-0.409
Surface behavior	3.07	0.85	-0.083	0.365
Deep behavior	3.71	0.84	-0.711	0.711
Emotional labor	3.39	0.64	-0.446	0.883

SD: Standard deviation

As shown in Table 3, the mean score for emotional commitment was 4.28, with a standard deviation of 0.66, indicating a relatively high level of emotional attachment to the profession among the participants. Continuance commitment had a mean score of 3.69 and a standard deviation of 0.74, suggesting a moderate level of commitment based on the costs associated with leaving the profession. The normative commitment had a mean score of 3.37 with a standard deviation of 0.88, reflecting a moderate sense of obligation to remain within the profession. The overall professional commitment yielded a mean score of 3.78 and a standard deviation of 0.54. This suggests that the participants have a strong commitment to the pharmacy profession. General self-efficacy scale had a mean of 3.25 with a standard deviation of 0.52, indicating a positive belief in one's capability to execute necessary actions in one professional role. Emotional labor constructs were also examined, with surface behavior recording a mean score of 3.07 and a standard deviation of 0.85, which denotes the frequency of surface acting among participants. Deep behavior had a higher mean score of 3.71 and a standard deviation of 0.84, suggesting greater engagement in deep-acting strategies. The combined emotional labor scale had a mean of 3.39 and a standard deviation of 0.64, highlighting the overall emotional labor efforts of community pharmacists.

In this research, regression analysis was employed to explore the impact of general self-efficacy and the subdimensions of emotional labor on various aspects of professional commitment among community pharmacists. The analysis demonstrated that general self-efficacy positively influences emotional commitment (β=0.275, p<0.05), explaining 5% of its variance. This suggests that pharmacists' belief in their abilities contributes to their emotional attachment to their profession. Similarly, general self-efficacy was found to positively affect continuance commitment (β=0.364, p<0.05), accounting for 8% of its variance. This indicates that self-efficacy beliefs also play a role in pharmacists' evaluation of the costs associated with leaving their profession. In terms of Normative Commitment, both general self-efficacy (β=0.464, p<0.05) and Deep Behavior

Table 3. Model fit indices of the constructs involved in the study

Acceptable fit index	Calculated fit indices		
	Professional commitment	Self-efficacy	Emotional labor
$\chi 2/df<5$	3.355	2.848	3.180
GFI>0.90, indicating	0.903	0.962	0.955
AGFI>0.90	0.866	0.925	0.921
CFI>0.90	0.894	0.968	0.965
RMR<0.08	0.087	0.021	0.052
RMSEA<0.08	0.077	0.068	0.074

GFI: Goodness-of-fit index, AGFI: Adjusted goodness of fit index, CFI: Comparative fit index, RMR: Root mean square residual, RMSEA: Root mean square error of approximation

(β=0.134, p<0.05) from the emotional labor subdimensions positively influenced this aspect, while Surface Behavior had a negative effect (β=-0.104, p<0.05). These findings illustrate that pharmacists' self-efficacy and deeper engagement in emotional labor positively contribute to their feeling of obligation to continue in their profession, with 11% of normative commitment variance explained.

The regression analysis further revealed that general self-efficacy and deep behavior positively impact overall professional commitment (β=0.368 for general self-efficacy and β=0.062 for deep behavior, p<0.05), explaining 15% of its variance. This underscores the significant role of pharmacists' confidence in their professional abilities and their depth of emotional engagement in fostering a strong commitment to their field.

The study also examined the effect of general self-efficacy on emotional labor as a whole. It was found to positively influence emotional labor (β =0.131, p<0.05), albeit explaining a smaller portion of its variance (1%). This highlights the nuanced impact of self-efficacy on how pharmacists manage their emotions in the workplace.

These findings support the hypothesis that general self-efficacy significantly influences various dimensions of professional commitment and emotional labor processes among community pharmacists. Specifically, the positive effect of general self-efficacy on both deep emotional labor strategies and normative commitment sheds light on the critical interplay between pharmacists' self-belief, approach to emotional regulation, and adherence to professional norms and obligations. Conversely, the lack of significant effect of general self-efficacy on Surface Behavior suggests a more complex relationship between self-efficacy and superficial emotional labor tactics. Collectively, these results offer a comprehensive understanding of the factors contributing to professional commitment and emotional labor in the context of community pharmacy, emphasizing the importance of fostering self-efficacy to enhance professional engagement and effective emotional management. For a detailed examination, the regression analysis results for each construct are presented in Table 4.

DISCUSSION

This study thoroughly explores the intricate interconnections among emotional labor, professional commitment, and self-efficacy within the field of community pharmacy, drawing on a strong theoretical framework supported by existing literature. It carefully examines how its findings align with and expand upon previous research by incorporating concepts from Bandura's[21] self-efficacy theory and emotional labor theory to clarify the complex dynamics at play in the professional lives of pharmacists.

The positive influence of high self-efficacy on quality of life and satisfaction, as demonstrated in numerous studies,[21,22] underscores the pivotal role of self-efficacy in fostering a resilient professional demeanor among pharmacists. This study revealed that pharmacists with higher self-efficacy perceive challenging conditions not as obstacles but as opportunities for excellence, highlighting their capacity to perform at optimal levels when facing adversity. This finding aligns with Bandura's[21] assertion that individuals with high self-efficacy exhibit distinct cognitive, emotional, and behavioral responses to tasks or challenges, further emphasizing the critical importance of self-efficacy in professional commitment and emotional labor management.

Furthermore, this study explores the effects of emotional labor behaviors and self-efficacy on professional commitment among community pharmacists, revealing nuanced insights through correlation and regression analyses. The validity of the scales used in the data collection tool, as established through factor analyses, attests to the reliability and relevance of the study's methodological approach.

Interestingly, the study's regression analyses revealed that neither surface nor deep acting significantly influences emotional commitment, challenging some existing assumptions in the emotional labor literature. However, deep acting positively affects normative commitment, whereas surface acting negatively impacts it, suggesting that the quality and authenticity of emotional labor are crucial determinants of professional commitment. These findings resonate with limited prior research linking emotional labor factors with professional

Table 4. Regression analysis results

Dependent variable	Independent variable	Coefficient (β)	t	p	R^2
Emotional commitment	General self-efficacy	0.275	4.394	<0.05	0.052
Continuance commitment	General self-efficacy	0.364	5.215	<0.05	0.084
Normative commitment	General self-efficacy and emotional labor subdimensions	0.464 (GSE), 0.134 (Deep behavior), -0.104 (Surface behavior)	5.712 (GSE), 2.602 (Deep behavior), -2.091 (Surface behavior)	<0.05	0.114
Professional commitment	General self-efficacy and Deep behavior	0.368	7.593	<0.05	0.154
Emotional labor	General self-efficacy	0.131	2.147	<0.05	0.012

GSE: General self-efficacy

commitment, yet they provide a fresh perspective by highlighting the significant positive impact of deep acting on professional commitment. This contrasts with studies like Yıldırım[23] and Giderler et al.[24], who reported varied effects of emotional labor dimensions on different aspects of professional commitment across diverse professions.

This research also identified a significant positive impact of general self-efficacy on deep acting in emotional labor, suggesting that individuals with higher self-efficacy are more inclined toward genuine emotional engagement. This finding is consistent with findings from Lee and Van-Vlack[25] and Alev[26], who indicated that high self-efficacy fosters a preference for deep, over-surface acting in emotional labor, potentially reducing emotional dissonance and burnout. Durak-Buz[27] further supports this, noting the significant influence of general self-efficacy on emotional labor dimensions.

Crucially, this study demonstrated a meaningful positive effect of general self-efficacy on professional commitment among community pharmacists, which is consistent with Bandura's[21] insights on the relationship between high self-efficacy, success, and personal fulfillment. This reinforces the notion that self-efficacy not only enhances individuals' confidence in facing uncertain and challenging situations, and supports their adaptation to continually changing life conditions, as noted by Karadağ et al.[28]

The practical implications of this study extend valuable insights into the management of community pharmacies and the broader healthcare sector, emphasizing the intertwined roles of emotional labor, professional commitment, and self-efficacy in enhancing workplace dynamics. A pivotal suggestion is the enhancement of pharmacists' self-efficacy through continuous professional development programs, mentorship opportunities, and constructive feedback mechanisms. By strengthening pharmacists' confidence in their professional abilities, organizations can foster increased commitment levels, improved job performance, and potentially reduce turnover rates.

Addressing the nuanced impacts of surface and deep acting on professional commitment, this study underscores the necessity for targeted training in emotional labor strategies. By equipping pharmacists with the skills to engage more deeply in their emotional labor through genuine emotional expression and empathy-building techniques, organizations can facilitate more authentic interactions with patients and colleagues, thereby enhancing normative commitment. The creation of a supportive work environment that recognizes the emotional challenges faced by pharmacists is also crucial. Promoting a culture of emotional support where employees feel valued and understood can mitigate the negative effects associated with surface acting and encourage a healthier emotional engagement with work.

The interplay between self-efficacy and emotional labor strategies suggests a strategic approach to task and role assignments in pharmacies. Aligning pharmacists' roles with their levels of self-efficacy and capacity for emotional labor can enhance job satisfaction and minimize emotional exhaustion. This approach extends to strategic human resource practices, including recruitment, performance management, and career development, all of which should support the cultivation of professional commitment and effective management of emotional labor.

Finally, the emphasis on promoting work-life balance reflects the acknowledgment of the emotional toll associated with pharmacy work. Implementing policies that support flexible scheduling, wellness programs, and initiatives aimed at reducing work-related stress can help pharmacists manage the emotional demands of their roles more effectively. This holistic approach not only contributes to higher levels of professional commitment and job satisfaction but also elevates the quality of care provided to patients. Collectively, these practical implications provide a comprehensive framework for community pharmacies and healthcare organizations to enhance employee well-being and organizational effectiveness by addressing the emotional and cognitive aspects of healthcare work.

Study limitations

One of the primary limitations of this study stems from its cross-sectional design, which restricted the ability to infer causality among the examined constructs. Longitudinal or experimental studies could offer a deeper understanding of how changes in self-efficacy influence emotional labor strategies and professional commitment over time. Additionally, reliance on self-reported measures, while practical, may introduce bias and does not capture the dynamic nature of emotional labor and professional commitment in real-time work settings. Future studies could incorporate observational methods or diary entries to provide a more nuanced picture of these phenomena.

The sample drawn from community pharmacists in a specific geographical region limits the generalizability of the findings. Subsequent research could broaden the scope to include pharmacists from diverse practice settings and geographical locations to enhance the external validity of the results. Moreover, exploring these constructs among other healthcare professionals could provide comparative insights and highlight the profession-specific dynamics of emotional labor and commitment.

Future investigations could incorporate additional psychological constructs, such as job satisfaction, stress, and burnout, to provide a more comprehensive understanding of the factors influencing professional commitment in the pharmacy sector. Additionally, the impact of organizational culture and support on the management of emotional labor and professional commitment requires further exploration.

CONCLUSION

The comprehensive exploration undertaken in this study sheds light on the intricate dynamics between emotional labor, professional commitment, and self-efficacy among community pharmacists, with significant implications for both theory and practice. A rigorous statistical analysis revealed that a higher sense of self-efficacy among pharmacists positively influences

their professional commitment and how they manage emotional labor, particularly favoring deeper, more authentic emotional engagements over superficial ones. A key takeaway from the findings is the critical role of self-efficacy in enhancing professional commitment. Pharmacists with strong beliefs in self-efficacy tend to exhibit greater emotional, continuance, and normative commitment. This underscores the importance of self-confidence in individuals' abilities, not only in executing professional tasks but also in navigating the emotional complexities inherent in pharmacy practice. Moreover, the study highlighted the nuanced effects of emotional labor strategies on professional commitment. Deep acting, characterized by genuine emotional expressions, positively impacts normative commitment, suggesting that authentic emotional engagement fosters a stronger sense of obligation and loyalty to the profession. Conversely, surface acting or superficial emotional management did not significantly affect professional commitment, indicating that mere compliance with expected emotional displays may not be sufficient to foster a deeper sense of professional belonging. The findings also point to the necessity of addressing emotional labor in pharmacy practice, emphasizing the benefits of deep-acting strategies for both pharmacists and patient care. Training and development initiatives that enhance pharmacists' emotional intelligence and capacity for genuine emotional engagement can lead to more fulfilling professional experiences and higher-quality patient interactions.

Ethics

Ethics Committee Approval: This study was approved by the Ankara University Health Sciences Ethics Sub-Board (approval number 143, date: 25.06.2018).

Informed Consent: Informed consent was obtained from all participants.

Footnotes

Authorship Contributions

Concept: Y.Ö., G.Ö., Design: Y.Ö., G.Ö., Data Collection or Processing: Y.Ö., Analysis or Interpretation: Y.Ö., Literature Search: G.Ö., Writing: Y.Ö., G.Ö.

Conflict of Interest: The authors have no conflicts of interest to declare.

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REFERENCES

1. Berry LL, Bendapudi N. Health care: a fertile field for service research. J Serv Res. 2007;10:111-122.
2. Ludovichetti FS, Zuccon A, Lucchi P, Signoriello AG, Stellini E, Mazzoleni S. Survey on oral health education knowledge of family members and health workers dedicated to patients with disabilities. Eur J Dent. 2023;17:1325-1329.
3. Darzi MA, Islam SB, Khursheed SO, Bhat SA. Service quality in the healthcare sector: a systematic review and meta-analysis. LBS J Manag Res. 2023.
4. Søvold LE, Naslund JA, Kousoulis AA, Saxena S, Qoronfleh MW, Grobler C, Münter L. Prioritizing the mental health and well-being of healthcare workers: an urgent global public health priority. Front Public Health. 2021;9:679397.
5. Alowais SA, Alghamdi SS, Alsuhebany N, Alqahtani T, Alshaya AI, Almohareb SN, Aldairem A, Alrashed M, Bin Saleh K, Badreldin HA, Al Yami MS, Al Harbi S, Albekairy AM. Revolutionizing healthcare: the role of artificial intelligence in clinical practice. BMC Med Educ. 2023;23:689.
6. Chen W, Chung JOK, Lam KKW, Molassiotis A. Patients', families' and healthcare providers' perspectives on end-of-life communication in Chinese hospital settings: a qualitative study protocol. PLoS One. 2023;18:e0296342.
7. Tinelli M, Ryan M, Bond C. Patients' preferences for an increased pharmacist role in the management of drug therapy. Int J Pharm Pract. 2009;17:275-282.
8. Rajiah K, Sivarasa S, Maharajan MK. Impact of pharmacists' interventions and patients' decision on health outcomes in terms of medication adherence and quality use of medicines among patients attending community pharmacies: a systematic review. Int J Environ Res Public Health. 2021;18:4392.
9. George PP, Molina JA, Cheah J, Chan SC, Lim BP. The evolving role of the community pharmacist in chronic disease management-a literature review. Ann Acad Med Singap. 2010;39:861-867.
10. Kılıçdağı Y. Investigation of the effect of emotional labor behaviors and general self-efficacy perceptions of community pharmacists on their professional commitment: ankara province sample. Doctoral dissertation, Ankara University. 2020.
11. Yong FR, Hor SY, Bajorek BV. Australian community pharmacy service provision factors, stresses and strains: a qualitative study. Explor Res Clin Soc Pharm. 2023;9:100247.
12. Yıldız A, Gayır GB. The relationship between the emotional labor behaviors of sales staff and their life satisfaction: a research in free pharmacies in TRB-1 region in Turkey. Kahramanmaraş Sütçü İmam Univ J Soc Sci. 2023;20(1):300-319.
13. Meyer JP, Allen NJ, Smith CA. Commitment to organizations and occupations: extension and test of a three-component conceptualization. J Appl Psychol. 1993;78:538-551.
14. Tak B, Çiftçioğlu BAA. Testing three dimensional occupational commitment scale on Turkish sample: An empirical investigation. J Dokuz Eylul Univ Fac Bus. 2009;10:35-54.
15. Schwarzer R, Fuchs R. Self-efficacy at different stages of the health behavior change process. In: Rodriguez-Marin J, ed. Health Psychology and Quality of Life Research: Proceedings of the 8th Annual Conference of the European Health Psychology Society, Vol. I. University of Alicante; 1995:64-66.
16. Aypay A. The Adaptation Study of General Self-Efficacy (GSE) Scale to Turkish. Inonu Univ J Fac Educ. 2010;11:113-132.
17. Diefendorff JM, Croyle MH, Gosserand RH. The dimensionality and antecedents of emotional labor strategies. J Vocat Behav. 2005;66:339-357.

18. Basım HN, Beğenirbaş M. Emotional labor in work life: a study of scale adaptation. J Manag Econ. 2012;19:77-90.
19. Anderson JC, Gerbing DW. Structural equation modeling in practice: a review and recommended two-step approach. Psychol Bull. 1988;103:411-423.
20. Bagozzi RP, Yi Y. Specification, evaluation, and interpretation of structural equation models. J Acad Mark Sci. 2012;40:8-34.
21. Bandura A. The explanatory and predictive scope of self-efficacy theory. J Soc Clin Psychol. 1986;4:359-373.
22. Luszczynska A, Gutiérrez-Doña B, Schwarzer R. General self-efficacy in various domains of human functioning: evidence from five countries. Int J Psychol. 2005;40:80-89.
23. Yıldırım D. Duygusal emek ve mesleki bağlılık ilişkisi: Avukatlar üzerine bir araştırma [Master's thesis]. Muğla: Muğla Sıtkı Koçman Üniversitesi, Sosyal Bilimler Enstitüsü; 2019. Available from: tez.yok.gov.tr
24. Giderler C, Baran H, Kirmizi C. Does emotional labour affect occupational commitment? A study on academicians. Res J Bus Manag. 2016;3:194-206.
25. Lee M, Van Vlack S. Teachers' emotional labour, discrete emotions, and classroom management self-efficacy. Educ Psychol. 2018;38:669-686.
26. Alev S. Öğretmenlerin genel öz yeterlilik algıları ile duygusal emek davranışları arasındaki ilişkinin incelenmesi: izlenim yönetimi taktiklerinin aracılık rolü [Master's thesis]. Gaziantep: Gaziantep Üniversitesi, Eğitim Bilimleri Enstitüsü; 2018.
27. Durak-Buz D. Öz-yeterliğin duygusal emek üzerindeki etkisi ve bu süreçte örgütsel desteğin düzenleyici rolüne ilişkin bir çalışma [Doctoral dissertation]. İstanbul: İstanbul Üniversitesi, Sosyal Bilimler Enstitüsü; 2019.
28. Karadağ E, Aksoy Derya Y, Ucuzal M. The self-efficacy-sufficiency levels of a health college student. Maltepe Univ J Nurs Sci Art. 2011;4:13-20.
29. Hochschild AR. The managed heart: commercialization of human feeling. In: the production of reality: essays and readings on social interaction. University of California Press; 1983.

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ORIGINAL ARTICLE

Formulation and Characterization of Etoricoxib Suppositories for the Management of Hemorrhoids

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ABSTRACT

Objectives: This study aimed to formulate and evaluate etoricoxib suppositories to improve patient compliance and drug efficacy in the management of hemorrhoids.

Materials and Methods: Suppositories were prepared using glycerin and gelatin. The prepared suppositories were evaluated for content uniformity, homogenization, hardness, weight variation, disintegration time, texture analysis, and *in vitro* drug release.

Results: Hardness, weight variation, disintegration time, and content uniformity values were found in the range of 4.00±0.50 to 7.50±0.50 kg/cm^3, 1.20±0.03 to 1.31±0.01 g, 11.00 to 19.05 min, and 66.98±0.86 to 80.76±3.60%, respectively. The SB3 gave 91.47±17.74% drug release in 6 h, whereas the SB1 gave 99.08±3.40% drug release in 12 h. Drug release from all formulations of suppositories was supported by zero-order, first-order, and Higuchi plots, except for SB4. The mechanism of drug release from all suppositories was fickian diffusion-based. The SB2 results were found to be more appropriate than those of the other batches.

Conclusion: These results confirm that the prepared formulation has a future scope and should be further explored in *in vitro* cell lines and animal studies.

Keywords: Etoricoxib, suppositories, hemorrhoids, texture analysis

INTRODUCTION

Hemorrhoids, often known as piles, indicate bulging veins in the lower part of the rectum and anus that resemble varicose veins. Hemorrhoids grow on the inner side of the rectum, which is recognized as interior or internal hemorrhoids, whereas beneath the skin surface around or surrounding the anus, which is recognized as exterior or external hemorrhoids.[1] The internal hemorrhoids occur above the dentate line and are covered by columnar epithelium, whereas the external hemorrhoids occur under the dentate line and are covered by squamous epithelium.[2-6] It commonly occurs as an inflammatory process of the hemorrhoidal plexus.[1,7] Hemorrhoids may be asymptomatic or symptomatic. The common symptoms associated with hemorrhoids are pain, the passage of bleeding in stool, itching around the anus, swelling, discomfort, lumps inside or around the anus, incomplete bowel emptying, and mucus discharge from the anus.[1,5,7] Long-term constipation, extreme pressure on the rectum, diarrhea, obesity, aging, pregnancy, and excessive use of laxatives contribute to the development of hemorrhoids owing to the irritates and enlarged blood vessels inside and around the anus.[1,7,8] Although hemorrhoids have low morbidity, they have a significant negative influence on quality of life. Available treatments for the management of symptomatic hemorrhoids include non-surgical medical procedures (like lifestyle modification and medications) and surgery. Patients try to avoid surgery, which is also expensive; however, this is also preferred by physicians as a last option for treatment. Lifestyle changes are recommended in the primary stage, which includes diet control and regular exercise.[9] Medications are preferred in the primary stage if diet control and regular

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exercise do not work well. Medicine-based treatment is associated with pain relief, control of inflammation, reduction of swelling, control of excessive blood loss, and reduction of diarrhea and constipation.

In intolerable pain conditions, patients expect immediate pain relief before other treatments. To tackle this problem, ointments, creams, and suppositories with non-steroidal anti-inflammatory drugs (NSAIDs) with or without antibiotics and anesthetic agents are available.[10,11] The suppositories are ideal for internal hemorrhoidal conditions, where they are inserted into the anus after bowel passage, whereas ointments and creams are ideal for external hemorrhoidal conditions. Furthermore, suppositories can have an immediate or prolonged effect compared to ointments and creams.[1] All these dosage forms are suitable for local application as well as can also provide immediate relief and avoid first-pass metabolism with minimal demerits of leakage and stains on clothes.

For intolerable pain relief before or after surgery and waning inflammation, NSAIDs are mostly prescribed. NSAIDs primarily work by inhibiting the two isoforms of cyclooxygenase (COX) enzymes. The COX enzymes play an important role in the production of prostaglandins, which are elements in the body that contribute to pain and inflammation. Conventional, non-selective NSAIDs suppress both COX-1 and COX-2 enzymes. However, COX inhibitors have gastrointestinal (GI) side effects after oral administration. The major side effects of COX inhibitors extend from mild GI tract irritation to severe GI bleeding and perforation.[12-14] Other associated side effects include peptic ulcers, heartburn, dyspepsia, and nausea. The side effects of upper GI are reported more frequently.[14,15] This upper GI intolerance can be alleviated by avoiding contact with the gastric mucosa.[13]

Etoricoxib is a selective COX-2 inhibitor that is used as an anti-inflammatory painkiller. Although it is prescribed for osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, for a limited duration in gout, and so on. It inhibits COX-2 in a dose-dependent manner without blocking COX-1 over the therapeutic dose range. Etoricoxib exhibits 106-fold COX-2 selectivity. Etoricoxib has fewer cardiovascular side effects compared to another coxib with a sulfone moiety, *i.e.*, rofecoxib.[16,17]

By considering the intriguing and pertinent characteristics of suppositories and identifying the upper GI intolerance to COX inhibitors, this study aimed to prepare and characterize etoricoxib suppositories for pain relief and anti-inflammatory response in hemorrhoids. In this study, etoricoxib suppositories were prepared using gelatin and glycerin as the bases, and the formulated suppositories were evaluated for homogeneity, weight variation, content uniformity, hardness, texture analysis, and drug release.

MATERIALS AND METHODS

Chemicals

Sanofi India Ltd., Verna, Goa, India, gifted Etoricoxib for research work. Gelatin powder was purchased from SRL Lab Pvt. Ltd. MIDC, Taloja, India. Glycerol was purchased from Merck Life Science Pvt. Ltd., Mumbai, India. Milli Q water was freshly prepared in our lab using MERK Millipore, ABC Enterprises, West Bengal, India.

Instrumentation and apparatus

Ultraviolet-visible spectroscopy (UV-vis) spectrophotometer (UV-1900i, Shimadzu Corporation, Kyoto, Japan), Dissolution apparatus (DS 14000 Smart, LAB INDIA, Thane, Maharashtra, India), Disintegration Tester (DT 1000, LAB INDIA, Thane, Maharashtra, India), Hardness Tester (Monsanto), Texture Analyzer (XCTX0000000S00, Ametek Brookfield, USA), Magnetic Stirrer (10 MLH, REMI, Kolkata, India), and electronic balance (Valency Lab, Kolkata, India).

Drug-excipient compatibility

Attenuated total reflectance-fourier transform infrared spectroscopy (FTIR) (Spectrum Two, PerkinElmer, UK) was used to identify etoricoxib, glycerin, and gelatin as well as to assess the drug-excipient compatibility. The drug and excipients were used at a ratio of 1:1 as a physical mixture. The FTIR spectra were logged in the infrared (IR) range of 4000 and 400 cm^{-1}. The test sample results obtained in the form of IR spectra using the PerkinElmer spectrum IR v10.7.2 software were compared with standard drug IR spectra.[18]

Method for preparing etoricoxib suppositories

In a glass beaker, glycerin was added to the required volume, and heating was applied to maintain the temperature at 120 °C. Then, a precisely weighed amount of gelatin was added, followed by continuous stirring until the gelatin was completely solubilized in glycerin. A constant temperature was maintained during the preparation. Then, the calculated amount of drug and water was continuously added until a homogeneous mixture. The obtained mixture was decanted in pre-cleaned and greased (with glycerin) suppository molds. The molds containing the formulations were then stored in a refrigerator at 4±0.5 °C. After two hours, the prepared suppositories were collected, wrapped in parchment paper, and stored in a cool, dry place.[18] The compositions of the different batches of suppositories are shown in Table 1.

Physical appearance

The color and shape of the formulated etoricoxib suppositories were assessed with the naked eye against a clear background, and observations were recorded.[18,19]

Homogeneity test

The prepared suppositories were assessed using the naked eye to determine the drug distribution pattern. The dryness

Table 1. Composition of etoricoxib rectal suppositories

Drug/Excipients	Formulation composition (*w/w*%)			
	SBI	SB2	SB3	SB4
Etoricoxib	3.00	3.00	3.00	3.00
Glycerin	60.00	60.00	65.00	65.00
Gelatin	20.00	25.00	20.00	25.00
Purified water	17.00	12.00	12.00	7.00

and/or roughness of the suppositories were also confirmed. Three suppositories from each batch were collected and cut longitudinally. The observations were recorded against a clear background.[18,20]

Weight variation

Twelve etoricoxib suppositories were randomly selected from each batch, and the suppository weight was recorded. Average weight and weight variations were calculated to compare with official limits.[18,19-21]

Content uniformity

The suppository was dissolved in 10 mL of methanol under mild heating. The final volume was made up to 100 mL with a phophate buffer (pH 7.4). The resultant solution was passed through the Whatman filter paper, and the filtrate was collected. The drug was estimated in the filtrate after appropriate dilutions with phosphate buffer at pH 7.4 using a UV-vis spectrophotometer (UV-1900i, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 283 nm.[18] The study was repeated three times, and the average drug content was reported.

Hardness

The Monsanto tester was used to determine the hardness of the etoricoxib rectal suppositories. Three suppositories were selected from each batch, and the hardness was measured at room temperature.[22,23]

Disintegration time

Six suppositories were randomly selected from each batch and placed in cylindrical glass tubes [having a United States Pharmacopoeia (USP) type A basket with 10 mesh] of the USP tablet disintegration tester (DT 1000, LAB INDIA, Thane, Maharashtra). Suppositories holding glass tubes were allowed to be immersed in 900 mL of phosphate buffer (pH 7.4) and taken in a 1000 mL beaker while maintaining a dip speed of 30±1 DPM. The phosphate buffer temperature was maintained at 37±0.2 °C. The time taken by the suppositories to eliminate debris particles from the perforated ends of the glass tubes was recorded.[18,21]

Texture analysis

The adhesiveness, adhesive force, and stringiness of the formulated etoricoxib suppositories as mechanical properties were analyzed using a texture analyzer (XCTX0000000S00, Ametek Brookfield, USA), which was connected to a 1500 g load cell. The probe (TA39) and TA-RIF fixture were used in the analysis. The trigger load, distance, and holding time were 50.00 g, 5.00 mm, and 2.00 s, respectively. The probe traveled at a rate of 1 mm/s until the surface of the suppositories was detected.

Dissolution studies and drug release kinetics

The dissolution studies of the prepared etoricoxib suppositories were performed using a USP Type 2 Dissolution apparatus (DS 14000 Smart, LAB INDIA, Thane, India) in 250 mL of phosphate buffer (pH 7.4) by maintaining 25 rpm paddle speed. The dissolution media temperature was maintained at 37±0.5 °C using a thermostat. The 4.0 mL of sample was collected at predefined time points, and the equivalent volume of the replacement buffer was exchanged at regular time points. Collected samples were filtered through Whatman filter paper, and the drug was estimated in the filtrate after appropriate dilutions with pH 7.4 phosphate buffer using the UV-Vis spectrophotometer (UV-1900i, Shimadzu Corporation, Kyoto, Japan) at 283 nm wavelength. The study was repeated in triplicate, and the percent cumulative drug release was reported.[24] The drug release data were further assessed for *in vitro* drug release kinetics and release mechanisms. Zero-order kinetics, first-order kinetics, and the Higuchi plot were applied to evaluate the drug release kinetics, whereas the Korsmeyer-Peppas model was applied to evaluate the release mechanisms. The release mechanism was evaluated by applying the model to an initial 60% cumulative drug release.[24, 25]

Differential scanning calorimetry (DSC)

The chemical modifications in the polymeric structure, temperature transition changes, and stability of the formulations at 30 °C to 120 °C to 30 °C, 30 °C to 120 °C to 4 °C, and 30 °C to 150 °C to 30 °C were ensured by DSC studies. The study was performed using a DSC 2500, Discovery Series [TA Instruments Division, Waters (India) Private Limited, Bangalore] connected to a refrigerated cooling system 90. The 4-6 mg of drug containing suppositories were weighed, crimped in an aluminum pan, and analyzed with a heating rate of 10 °C/min and a nitrogen flow of 50 mL/min.[26]

Physical stability studies

The physical stability of the prepared suppositories was evaluated by storing them at 4±1 °C. The stability of the formulations was assessed by analyzing their homogeneity and color.

Statistical analysis

The results were calculated and presented as mean ± SD of independent assessments (n=3 or n=12). The Excel office 2019 was used for the calculation of data.

RESULTS

Drug excipient compatibility

The FTIR spectra of etoricoxib, glycerin, gelatin, and physical mixture (etoricoxib + both gelatin and glycerin in a 1:1 ratio) are shown in Figure 1A-D, respectively. Figure 1A shows the etoricoxib spectra with the characteristic absorbance bands at 3050.8 cm^{-1}, 1594 cm^{-1}, 1428.7 cm^{-1}, 1390 cm^{-1}, 1292.7 cm^{-1}, 1139.9 cm^{-1}, 1090 cm^{-1}, 1008 cm^{-1} (in-plane: C-H bend), 955.7 cm^{-1} (in-plane: C-H bend), and 769.42 cm^{-1} for aromatic C-H stretching, stretching of C=C, aliphatic bending (asymmetric) of C-H, aliphatic bending (symmetric) of C-H, stretching vibration (asymmetric) of O=S=O, stretching vibration (symmetric) of O=S=O, aromatic stretching of C-Cl, in-plane C-H bend (aromatic), and out-of-plane C-H bend (aromatic), respectively.[27,28] The characteristic absorbance bands in the obtained spectra confirm that the present sample is etoricoxib.

Figure 1B depicts the glycerin spectra with absorbance bands of 3283.1 cm^{-1} for OH stretching, 2931.5 cm^{-1} to 2821.3 cm^{-1} for C-H stretching, 1414 cm^{-1} for C-O-H bending, 1108 cm^{-1} and 1035.2 cm^{-1} for C-O stretching, and 993.37 cm^{-1} for O-H bending.[29-31] The presence of the above-mentioned absorbance bands reveals that the present sample is glycerin.

Figure 1C illustrates the IR spectra of gelatin with characteristic absorbance bands of 2300 cm^{-1} to 3600 cm^{-1}, for amide A, 1600 cm^{-1} to 1620 cm^{-1} for amide 1 (C=O) stretching, 1560 cm^{-1} to 1300 cm^{-1} for amide 2 (NH bending), 1240 cm^{-1} to 2929.4 cm^{-1} and 670 cm^{-1} for amide 3 (C-N stretching).[32] The obtained band confirms that the given sample is gelatin.

Figure 1D shows the physical mixture of etoricoxib with both glycerin and gelatin in a 1:1 ratio of the drug and excipients. The presence of characteristic absorbance bands at 1595 cm^{-1} for C=C aromatic benzene, 1294 cm^{-1} for O=S=O stretching vibration (asymmetric), 1139 cm^{-1} for O=S=O stretching vibration (symmetric) of etoricoxib, for glycerin bands at 3297.8 cm^{-1} for OH stretching, 2929.4 cm^{-1} and 2875 cm^{-1} for C-H stretching, and for gelatin absorbance bands are observed. FTIR studies confirm the absence of interactions between etoricoxib and excipients.

Physical appearance

Table 2 lists the physical characteristics of the prepared suppositories. There were no observable differences in the suppositories among the batches. Suppositories were slightly yellowish and opaque. All the suppositories were conical with no breakage.

Homogeneity test

As shown in Table 2, the SB2 suppositories were found to be homogeneous, smooth, and free from dryness. The remaining batches of suppositories were found to be almost homogeneous, smooth, and free from dryness.

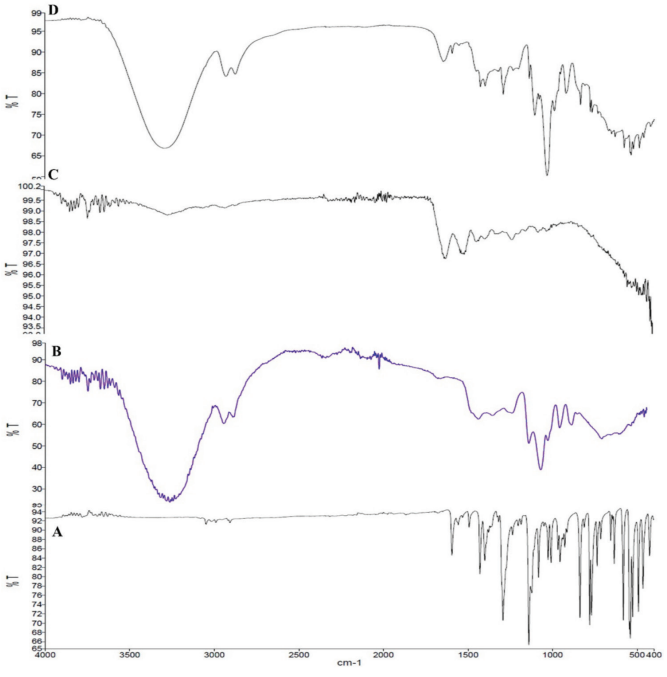

Figure 1. FTIR spectra: (A) Etoricoxib; (B) Glycerin; (C) Gelatin; (D) PM
FTIR: Fourier transform infrared spectroscopy, PM: Physical mixture

Hardness

Different hardness values were recorded for different batches of suppositories. Batch SB1 suppositories showed the lowest hardness values, *i.e.*, 4.00±0.50 kg/cm, SB2 suppositories showed the highest hardness values, *i.e.*, 7.50±0.50 kg/cm^3, whereas SB3 and SB4 presented almost the same hardness values, *i.e.*, between 5.9±0.36 and 5.5±0.50 kg/cm^3 (results are reported in Table 2). The SB2 sample had the highest hardness, which may be attributed to the higher concentration of gelatin in this batch.

Weight variation

The weight variation values (shown in Table 2) were within the acceptable weight variation limits, with values between 0.00 and 1.09%. SB2 exhibited the highest content uniformity, *i.e.*, 80.76±3.6%.

Content uniformity and disintegration time

The content uniformity (results are depicted in Table 2) for all batches was found to be between 66.98 and 80.76% with a very low standard deviation. As reported in Table 2, the disintegration time was noticed in the following order: SB2>SB4>SB1>SB3. SB2 exhibited a longer disintegration time, which may be due to the presence of a higher gelatin concentration in this batch. SB3 showed the least disintegration time, which may be due to the lowest concentration of gelatin in these suppositories.

Texture analysis

The texture analysis of the formulated suppositories is presented in Table 3. Suppositories SB1 and SB3 showed the highest adhesive forces, *i.e.*, 4.70±1.20 and 3.90±2.80 g, respectively, compared with the other batches of suppositories, which may be due to the presence of a higher glycerin-to-gelatin ratio, *i.e.*, 3.00 and 3.25, respectively. These two batches of suppositories also gave higher standard deviation values than the SB2 and SB4 suppositories. The SB2 suppositories showed the least adhesive force, *i.e.*, 3.73±0.90 g) among all remaining batches. The standard deviation values of SB2 and SB4 were also lower.

The SB1 and SB3 suppositories (with adhesiveness values of 0.07±0.04 and 0.14±0.01 mJ, respectively) also showed the highest adhesiveness compared with the SB2 and SB4 suppositories (with adhesiveness values of 0.06±0.01 and 0.09±0.01 mJ, respectively).

Stringiness was found to decrease with increasing glycerin concentration but to increase with increasing gelatin concentration. SB2 showed the highest stringiness (1.36±0.24 mm), whereas SB3 showed the lowest stringiness (0.20±0.15 mm).

Dissolution studies and drug release kinetics

Figure 2 shows the *in vitro* drug release profile of the drug from the prepared batches of suppositories in phosphate buffer (pH 7.4) to mimic the physiological condition of the rectal. SB3 gave the fastest drug release (*i.e.*, 91.47±17.74% in 6 h) because it had a gelatin-to-glycerin ratio of 0.31. SB1

induced complete drug release within 12 h (*i.e.*, 99.08±3.40%). The drug release from SB2 and SB4 was found to be almost the same, (*i.e.*, 98.14±13.50% and 97.12±26.66%, respectively) within 10 h.

The obtained drug release data was assessed for drug release kinetics and mechanisms (the data is reported in Table 4). The drug release from all the formulated batches of suppositories except SB4 was appropriately supported by zero-order and first-order drug release kinetics. The release of drugs from SB4 was noticed to be first-order-based. The release of drugs from the suppositories was also supported by the Higuchi plot.

Differential scanning calorimetry

To confirm the changes in the polymeric structure of gelatin between 30 °C and 120 °C, the prepared suppositories underwent DSC analysis. The study was executed by scanning the samples between 30 °C and 120 °C and then reversing the thermal cycle from 120 °C to 30 °C at a rate of 10 °C/min. The absence of modifications was recorded in the thermogram during the study, as reported in Figure 3A, confirming that gelatin retains its polymeric structure at the mentioned temperature range.

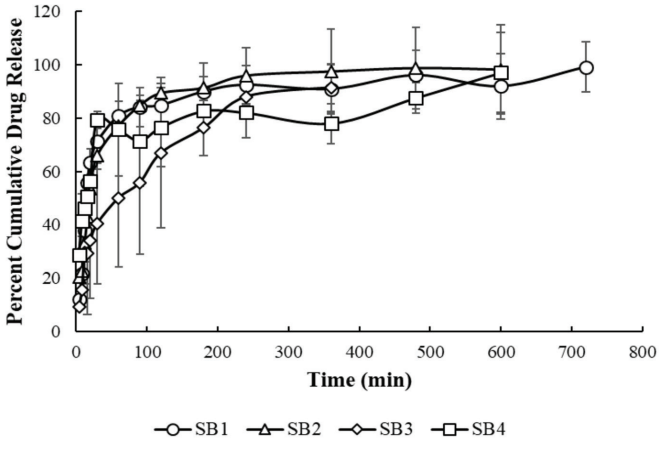

Figure 2. In vitro drug release profile of formulated suppositories. *Above data are presented as mean ± SD, n=3

SD: Standard deviation

To assess the stability of the prepared suppositories at 4 ºC, the DSC runs were recorded in the thermal cycle from 30 to 120 °C and then reversed from 120 to 4 ºC. The absence of modifications in the thermogram was observed (data presented in Figure 3B), indicating the stability of the prepared suppositories between 4 °C and 120 ºC.

The DSC thermogram shown in Figure 3C confirms the stability of the active molecule over the temperature range of 30 °C to 150 ºC.

Physical stability studies

The prepared suppositories exhibited good homogeneity over a period of 21 days (Figure 4). No observable differences in homogeneity or physical appearance were recorded during storage at 4 °C. However, a slight color change was observed in the formulated suppositories.

DISCUSSION

As reported in the literature,[33,34] etoricoxib has good permeability, with a log P of approximately 2.8. However, its solubility was reported to be 0.0245, 0.0103, 0.0772, and 0.0785 mg/mL in water, acetate buffer pH 1.2, phosphate buffer pH 6.8, and phosphate buffer pH 7.4, respectively.[35] Hence, the drug solubility was better at the rectal pH, *i.e.*, between pH 7 and 8, compared to all other regions. Another reason is that because the drug has a pKa value of 5,[33,34] it can also have better absorption at rectal pH.

Long-term constipation contributes to the development of hemorrhoids owing to the presence of irritates and enlarged blood vessels inside and around the anus.[36,37] Glycerin in suppositories is also most commonly used in the treatment of constipation.[38] Hence, the presence of glycerin in the suppositories for the management of constipation-based hemorrhoids may be preferable. Another reason is that the addition of glycerin to the suppositories enhances their lubrication and imparts their moisturizing properties, which further, in contact with the minimal amount of water from the surrounding tissue, enhances the dissolution of the suppositories.[39,40] On the other hand, the presence of gelatin in the suppositories dissolves slowly and

Table 2. Physical characteristics of etoricoxib rectal suppositories

Physical characteristics	Formulation batch			
	SB1	SB2	SB3	SB4
Color and opacity	Slight yellowish and opaque	Slight yellowish and opaque	Slight yellowish and opaque	Slight yellowish and opaque
Shape*	Conical	Conical	Conical	Conical
Homogeneity*	Almost homogeneous and smooth with no dryness	Homogeneous and smooth with no dryness	Almost homogeneous and smooth with no dryness	Almost homogeneous and smooth with no dryness
Hardness* (kg/cm^3)	4.00±0.50	7.50±0.50	5.90±0.36	5.50±0.50
Weight variation (g)#	1.20±0.03	1.30±0.02	1.31±0.01	1.25±0.02
Content uniformity (%)*	66.98±0.86	80.76±3.60	74.34±5.70	78.66±1.48
Disintegration time (min.)	13.45	19.05	11.00	18.00

*Results present as mean ± SD, n=3, #Results present as mean ± SD, n=12. min.: Minimum, SD: Standard deviation

maintains long-term drug release at the site.[38] The combination of glycerin and gelatin is complementary to each other to maintain sustained drug release.

Furthermore, drugs that are not completely soluble and have been dispersed in suppositories with opposing properties encourage the drug to leave the fluid.[38,41,42] Considering this useful fact, lipophilic drugs (such as nifedipine, diclofenac sodium, *etc.*) have been incorporated in hydrophilic bases for better delivery on the application site.[39,43]

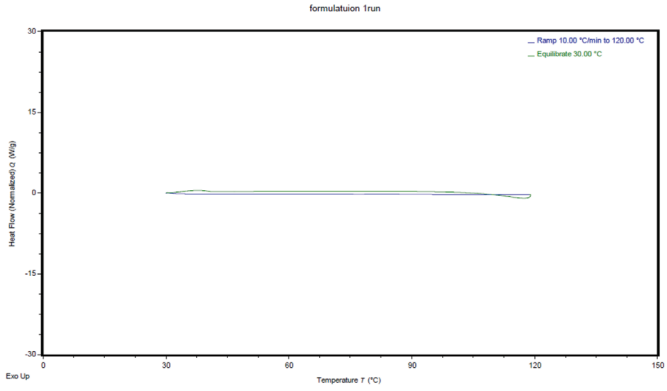

Figure 3A. DSC thermogram of suppositories from 30 °C to 120 °C and 120 °C to 30 °C

DSC: Differential scanning calorimetry

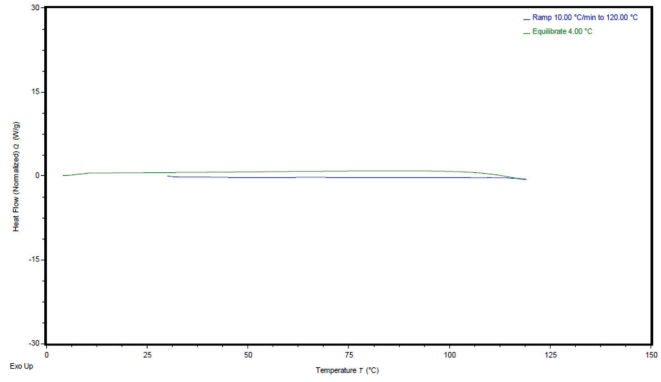

Figure 3B. DSC thermogram of suppositories from 30 °C to 120 °C and 120 °C to 4 °C

DSC: Differential scanning calorimetry

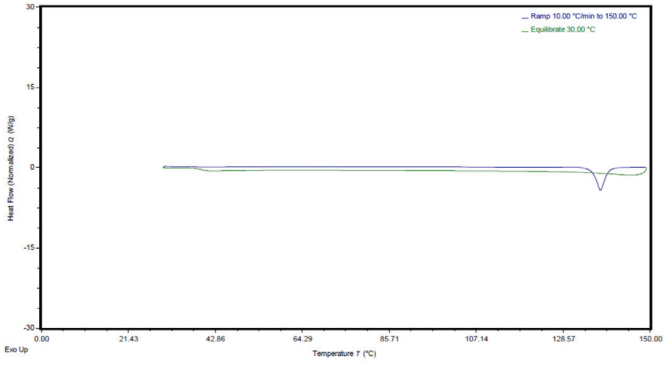

Figure 3C. DSC thermogram of suppositories from 30°C to 150°C and 150°C to 30°C

Abbreviations: DSC: Differential scanning calorimetry

USP 1151 recommends a glycerin: gelatin: water ratio of 70:20:10 for suppositories. However, this study was planned with slight modifications to the above-mentioned ratio to obtain a suitable sustained-release formulation. The study used glycerin: gelatin: water in ratios of 60 to 65:20 to 25:7 to 17. The study was planned to sustain the release of the drug from the formulated suppositories for a period of 8-10 h, as this is preferred during bedtime.

FTIR spectroscopy was conducted to confirm etoricoxib and its compatibility with the excipients planned in the study. The study showed all characteristic absorbance bands positioned as per the literature[27-32] in the obtained spectra, which confirms that the present sample is etoricoxib and also ensures its compatibility with the selected polymers. This study is in line with the earlier findings reported[24] for other molecules such as gelatin and glycerin.

The formulated suppositories were wrapped in parchment paper, which is a cellulose-based paper with properties such as non-stickiness, grease resistance, humidity resistance, and heat resistance. This paper was used to wrap the suppositories to prevent moisture loss during storage at 4 ºC.

Although color, size, and shape have negligible effects on the effectiveness of the suppositories,[38] they are considered parameters for their attractive appearance. This study demonstrated that the use of glycerin and gelatin in the suppositories not only maintains their shape but also helps in the homogeneous distribution of the drug.

The hardness of the suppositories has been given the least importance in the literature; however, this parameter is assumed to be important as it may influence drug release, therapeutic response, and packaging and transportation hazards. It is assumed that as the hardness of the material increases, drug release and response decrease. The formulation with glycerin: gelatin: water in a ratio of 60:25:12 produced uniform suppositories with optimal hardness, least weight variation, and sufficient disintegration time. This combination is considered suitable and anticipated to be useful for sustaining the release of the formulation due to the slightly higher concentration of the gelatin.[38]

The highest adhesive force was noted with a higher glycerin-to-gelatin ratio. The findings are similar to earlier research in this area, in which researchers showed an increase in bio-adhesive force with glycerin.[44] Glycerin enhances the bioadhesive strength by enhancing the degree of penetration of the polymer chains.

Both SB2 and SB4 contained a higher concentration of gelatin, which retarded drug release from formulated suppositories compared with the other batches. On the other hand, these studies showed that glycerin enhances drug release with an increase in its concentration owing to its wetting properties.[18] SB2 can provide complete drug release throughout 8 to 10 hours, which is essential for a patient to achieve complete drug release during bedtime. According to the reported n-value

Table 3. Texture analysis of etoricoxib suppositories

Formulation	Adhesive force (g)	Adhesiveness (mJ)	Stringiness (mm)
SB1	4.70±1.20	0.07±0.04	1.10±1.30
SB2	3.73±0.90	0.06±0.01	1.36±0.24
SB3	3.90±2.80	0.14±0.01	0.20±0.15
SB4	4.20±0.20	0.09±0.01	0.25±0.05

All results represent as mean ± SD, n=3. SD: Standard deviation

Table 4. Drug release kinetics

Formulation code	Zero-order		First-order		Higuchi plot	Korsmeyer-Peppas	
	r^2	K	r^2	K	r^2	r^2	N
SB1	0.92±0.05	13.50±0.98	0.92±0.03	0.24±0.03	0.92±0.03	0.77±0.14	0.20±0.04
SB2	0.85±0.14	9.44±1.61	0.89±0.12	0.16±0.02	0.93±0.08	0.87±0.08	0.10±0.01
SB3	0.88±0.07	7.85±3.20	0.83±0.13	0.12±0.05	0.88±0.07	0.93±0.05	0.12±0.03
SB4	0.57±0.26	3.53±0.99	0.84±0.13	0.13±0.01	0.85±0.09	0.82±0.06	0.065±0.01

*Above data are presented as mean ± SD (n=3), r^2: Correlation coefficient, and K: Reflecting rate constant, SD: Standard deviation

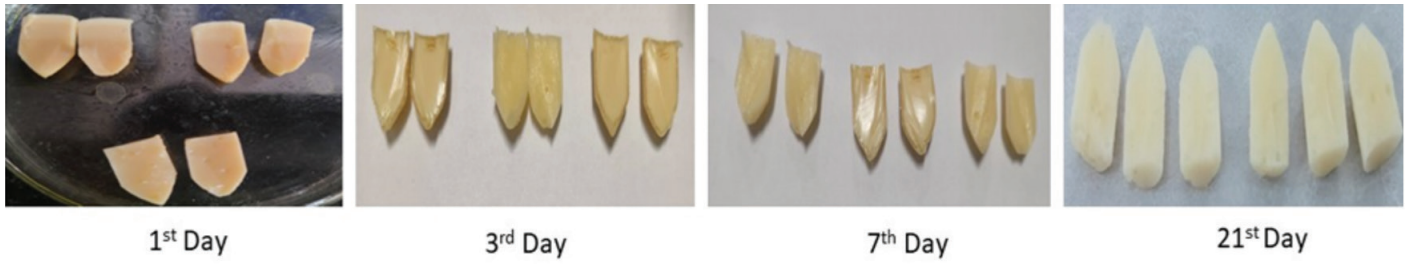

Figure 4. Appearance of formulated suppositories at 4 °C for a period of 21 days

(release exponent), all batches of the formulated suppositories showed the Fickian diffusion mechanism.[24,25,45]

The formulations are physically stable; this may be due to the presence of gelatin, which may contribute to maintaining the shape and size of the suppositories. However, chemical stability studies (especially for the estimation of drug content) are preferred for these formulations in the future.

CONCLUSION

This study was initiated to develop novel etoricoxib rectal suppositories to provide instant pain relief and anti-inflammatory activity in hemorrhoids. Drug-containing suppositories were formulated using glycerin and gelatin as a suitable base. From the evaluation studies of the formulated suppositories, we conclude that the SB2 suppositories have a very good physical appearance with optimum hardness, disintegration time, and weight variation. Further, from the texture analysis studies, we also concluded that the formulated suppositories have good adhesive force and adhesiveness, demonstrating their suitability for preventing rectal leakage and entry of the drug into the colon. The elasticity of the formulated suppositories, as stringiness, was also found to be suitable. The *in vitro* drug release, as an important study to predict the fate of formulated suppositories *in vivo,* showed a desirable release pattern with etoricoxib release of 98.14±13.50% in 10 h, and this drug release was diffusion-based, which is expected to be suitable for this type of formulation. Therefore, based on the *in vitro* evaluations of the formulated suppositories, we conclude that the present work has future scope and should be explored for further investigation of their activities in cell lines and *in vivo*.

Ethics

Ethics Committee Approval: This work does not involve any studies or research that needs approval from any review or ethics board.

Informed Consent: Not required.

Authorship Contributions

Concept: L.K., Design: L.K., Data Collection or Processing: B.S., Y.K., N.M., S.K., Analysis or Interpretation: B.S., Y.K., N.M., S.K., Literature Search: B.S., Writing: B.S., L.K.

Conflict of Interest: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study received no financial support.

REFERENCES

1. Bartels DA, Johnson R, Bayor MT, Ainooson GK, Ossei PPS, Etuaful RK, Buamah R. Formulation of suppositories of alum produced from bauxite waste in ghana for the treatment of hemorrhoid. ScientificWorldJournal. 2021;2021:6667562.
2. Yng HK. Anal anatomy. In: Hyung Kyu Yng; editor. Hemorrhoids. Berlin Heidelberg: Springer-Verlag: 2014. pp. 5-14.
3. Kibret AA, Oumer M, Moges AM. Prevalence and associated factors of hemorrhoids among adult patients visiting the surgical outpatient department in the University of Gondar Comprehensive Specialized Hospital, Northwest Ethiopia. PLoS One. 2021;16:e0249736.
4. Trompetto M, Clerico G, Cocorullo GF, Giordano P, Marino F, Martellucci J, Milito G, Mistrangelo M, Ratto C. Evaluation and management of hemorrhoids: Italian society of colorectal surgery (SICCR) consensus statement. Tech Coloproctol. 2015;19:567-575.
5. Sun Z, Migaly J. Review of hemorrhoid disease: presentation and management. Clin Colon Rectal Surg. 2016;29:22-29.
6. Rubbini M, Ascanelli S. Classification and guidelines of hemorrhoidal disease: present and future. World J Gastrointest Surg. 2019;11:117-121.
7. Riss S, Weiser FA, Schwameis K, Riss T, Mittlböck M, Steiner G, Stift A. The prevalence of hemorrhoids in adults. Int J Colorectal Dis. 2012;27:215-220.
8. Nassa YG, Danjuma A, Ayuba SB, Yahaya SA, Inusa B, Yakubu I. Prevalence and predictors of hemorrhoids among commercial motorcyclists in Kaduna state, Nigeria. World J Prevent Medicine. 2016;4:1-4.
9. Alonso-Coello P, Mills E, Heels-Ansdell D, López-Yarto M, Zhou Q, Johanson JF, Guyatt G. Fiber for the treatment of hemorrhoids complications: a systematic review and meta-analysis. Am J Gastroenterol. 2006;101:181-188.
10. Davids JS, Ridolfi TJ. Hemorrhoids. In: Beck DE, Roberts PL, Saclarides TJ, Senagore AJ, Stamos MJ, Wexner SD. (editors). The ASCRS textbook of colon and rectal surgery. Springer Cham. 2011. pp. 209-229.
11. Tjandra JJ, Tan JJ, Lim JF, Murray-Green C, Kennedy ML, Lubowski DZ. Rectogesic (glyceryl trinitrate 0.2%) ointment relieves symptoms of haemorrhoids associated with high resting anal canal pressures. Colorectal Dis. 2007;9:457-463.
12. Yamazaki R, Kawai S, Matsuzaki T, Kaneda N, Hashimoto S, Yokokura T, Okamoto R, Koshino T, Mizushima Y. Aceclofenac blocks prostaglandin E2 production following its intracellular conversion into cyclooxygenase inhibitors. Eur J Pharmacol. 1997;329:181-187.
13. Kilor VA, Sapkal NP, Awari JG, Shewale BD. Development and characterization of enteric-coated immediate-release pellets of aceclofenac by extrusion/spheronization technique using kappa-carrageenan as a pelletizing agent. AAPS PharmSciTech. 2010;11:336-343.
14. Harirforoosh S, Asghar W, Jamali F. Adverse effects of non-steroidal antiinflammatory drugs: an update of gastrointestinal, cardiovascular and renal complications. J Pharm Pharm Sci. 2013;16:821-847.
15. Qureshi O, Dua A. COX inhibitors. [Updated 2023 Mar 2]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan. Available from: https://www.ncbi.nlm.nih.gov/books/NBK549795/
16. Croom KF, Siddiqui MA. Etoricoxib: a review of its use in the symptomatic treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and acute gouty arthritis. Drugs. 2009;69:1513-1532.
17. Riendeau D, Percival MD, Brideau C, Charleson S, Dubé D, Ethier D, Falgueyret JP, Friesen RW, Gordon R, Greig G, Guay J, Mancini J, Ouellet M, Wong E, Xu L, Boyce S, Visco D, Girard Y, Prasit P, Zamboni R, Rodger IW, Gresser M, Ford-Hutchinson AW, Young RN, Chan CC. Etoricoxib (MK-0663): preclinical profile and comparison with other agents that selectively inhibit cyclooxygenase-2. J Pharmacol Exp Ther. 2001;296:558-566.
18. Reddy RS, Kumar L, Pydi CR, Reddy MS, Verma R. Development of fluconazole suppositories for the treatment of candida infection of genitourinary tract. Indian J Pharmaceut Edu Res. 2018;52;S16-S22.
19. Sah ML, Saini TR. Formulation development and release studies of indomethacin suppositories. Indian J Pharm Sci. 2008;70:498-501.
20. Jawahar N, Jayaprakash S, Maria N, Rajan G, Nagarajan M, Moorthi DD, Jubie S, Manivannan R. Design and evaluation of sustained release suppositories of nimesulide. Indian J Pharmaceut Sci. 2005;67:558-561.
21. Mahjabeen S, Hatipoglu MK, Chandra V, Benbrook DM, Garcia-Contreras L. Optimization of a vaginal suppository formulation to deliver sheta2 as a novel treatment for cervical dysplasia. J Pharm Sci. 2018;107:638-646.
22. Ghorab D, Refai H, Tag R. Preparation and evaluation of fenoterol hydrobromide suppositories. Drug Discov Ther. 2011;5:311-318.
23. El-Majri MA, Sharma RK. Formulation and evaluation of piroxicam suppositories. Int J Drug Deliver. 2010;2:108-112.
24. Kumar L, Reddy MS, Shirodkar RK, Pai GK, Krishna VT, Verma R. Preparation and characterisation of fluconazole vaginal films for the treatment of vaginal candidiasis. Indian J Pharm Sci. 2013;75:585-590.
25. Chatterjee A, Kumar L, Bhowmik BB, Gupta A. Microparticulated anti-HIV vaginal gel: *in vitro-in vivo* drug release and vaginal irritation study. Pharmaceut Dev Technol. 2011;16:466-473.
26. Chevala NT, Dsouza JA, Saini H, Kumar L. Design and development of tranexamic acid loaded film-forming gel to alleviate melasma. J Cosmet Dermatol. 2022;21:6863-6874.
27. Silverstein RM, Webster FX. Infrared Spectroscopy. In: Silverstein RM, Webster FX (Eds.). Spectrometric identification of organic compounds. John Wiley & Sons, Inc. Authorized reprint by Wiley India (P.), Ltd., Daryaganj, New Delhi. Printed at: Pashupati Printers Pvt. Ltd., Delhi. 6th Edition. 6th reprint, 2008. pp. 71-143.
28. Srinivasan S, Elhassan GO, Janakiraman AK, Kayarohanam S, Dey T, Nachiya RJ, Nath UU, Mohamed JMM. Preparation and characterization of etoricoxib ternary complex for the enhancement of solubility. J Pharmaceut Negative Result. 2023;14:1703-1712.
29. Guimarães JL, Trindade Cursino AC, Ketzer Saul C, Sierrakowski MR, Ramos LP, Satyanarayana KG. Evaluation of castor oil cake starch and recovered glycerol and development of "green" composites based on those with plant fibers. Materials. 2016;9:76.
30. Dixit V, Tewari JC, Cho BK, Irudayaraj JM. Identification and quantification of industrial grade glycerol adulteration in red wine with fourier transform infrared spectroscopy using chemometrics and artificial neural networks. Appl Spectrosc. 2005;59:1553-1561.
31. Gómez-Siurana A, Marcilla A, Beltrán M, Berenguer D, Martínez-Castellanos I, Menargues S. TGA/FTIR study of tobacco and glycerol-tobacco mixtures. Thermochimica Acta. 2013;573:146-157.
32. Irfanita N, Jaswir I, Mirghani ME, Sukmasari S, Ardini YD, Lestari W. Rapid detection of gelatin in dental materials using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). J Phys: Conf Ser. 2017;884:Article ID: 012090.
33. Gonzalez-Alvarez I, Bermejo M, Tsume Y, Ruiz-Picazo A, Gonzalez-Alvarez M, Hens B, Garcia-Arieta A, Amidon GE, Amidon GL. An *in*

vivo predictive dissolution methodology (iPD Methodology) with a BCS Class IIb drug can predict the *in vivo* bioequivalence results: etoricoxib products. Pharmaceutics. 2021;13:507.

34. Ifrah S, Porat D, Deutsch M, Dahan A. Quantification of etoricoxib in low plasma volume by UPLC-PDA and application to preclinical pharmacokinetic study. Pharmaceuticals (Basel). 2024;17:507.
35. Sapkal SB, Adhao VS, Thenge RR, Darakhe RA, Shinde SA, Shrikhande VN. Formulation and characterization of solid dispersions of etoricoxib using natural polymers. Turk J Pharm Sci. 2020;17:7-19.
36. Nassa YG, Danjuma A, Ayuba SB, Yahaya SA, Inusa B, Yakubu I. Prevalence and predictors of hemorrhoids among commercial motorcyclists in Kaduna state, Nigeria. World J Prevent Medicine. 2016;4:1-4.
37. Bartels DA, Johnson R, Bayor MT, Ainooson GK, Ossei PPS, Etuaful RK, Buamah R. Formulation of suppositories of alum produced from bauxite waste in ghana for the treatment of hemorrhoid. ScientificWorldJournal. 2021;2021:6667562.
38. Ham AS, Buckheit RW Jr. Designing and developing suppository formulations for anti-HIV drug delivery. Ther Deliv. 2017;8:805-817.
39. Kurosawa IN, Owada IE, Ito K. Bioavailability of nifedipine suppository in healthy subjects. Int J Pharm. 1985;27:81-88.
40. Shende S, Meshram B, Karemore H, Gaikwad P, More H, Devhare L, Srivastava A. Development and characterization of glycerol-gelatin suppositories for enhanced efficacy. European J Pharma Medical Res. 2023;10:522-528.
41. Jannin V, Lemagnen G, Gueroult P, Larrouture D, Tuleu C. Rectal route in the 21st century to treat children. Adv Drug Deliv Rev. 2014;73:34-49.
42. Hua S. Physiological and pharmaceutical considerations for rectal drug formulations. Front Pharmacol. 2019;10:1196.
43. Sultan T, Hamid S, Hassan S, Hussain K, Ahmed A, Bashir L, Naz S, Maqbool T. Development and evaluation of immediate release diclofenac sodium suppositories. Pak J Pharm Sci. 2018;31:1791-1795.
44. Choi H, Lee M, Kim M, Kim C. Effect of additives on the physicochemical properties of liquid suppository bases. Int J Pharm. 1999;190:13-19.
45. Bhaskaran NA, Salwa, Fernandes AV, Volfová G, Pydi CR, Kumar L, Verma R, Marques SM, Shirodkar RK. Development of cream to enhance the antifungal activity and reduce the side effects of fluconazole for the treatment of *Candida albicans*. Tenside Surfact Det. 2011;59:231-239.

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ORIGINAL ARTICLE

Preliminary Study on the Development of Orodispersible Film Containing Desloratadine

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ABSTRACT

Objectives: Orodispersible films (ODFs) are new-generation dosage forms that increase patient compliance, providing ease of drug administration in many patient groups, such as pediatric, geriatric, and patients with physiological and psychiatric disorders. The aim of this study was to conduct preliminary studies to develop ODF containing the poorly water-soluble and oxidation-sensitive drug desloratadine (DL).

Materials and Methods: In this study, the formulation and process parameters, as well as the characterization method were investigated using 20 film formulations manufactured by the solvent casting method. The films were characterized in terms of their appearance, mechanical properties, thickness, disintegration time, and content uniformity. Various strategies have been applied to increase the chemical stability of DL in the formulations and, therefore to choose suitable antioxidants, and morphological and compatibility studies using differential scanning calorimetry were performed. For increasing drug loading, different film compositions were also evaluated.

Results: Among the preliminary formulations tested with a casting height of 400 µm, homogeneous, good mechanical properties with tensile strength values between 6.21-10.34 MPa, flexibility, and ODFs with a disintegration time of less than 60s ODFs were developed. By increasing the solubility of DL in the formulation with the selected components, the drug loading capacity was increased to 3% by the desired level.

Conclusion: One of the enabling formulations, F20, was particle-free with a suitable thickness uniformity (relative standard deviation =4.6%) and content uniformity (acceptance values =5) films were developed.

Keywords: Orodispersible film, solvent casting method, desloratadine

INTRODUCTION

Oral administration is the most preferred route. However, some problems may occur in pediatric, geriatric, and special patient groups with limited swallowing ability in terms of treatment with conventional liquid and solid dosage forms.[1] Orodispersible films (ODFs) are appropriate dosage forms not only for patients who have difficulty swallowing due to physical and cognitive disorders and are at risk of choking but also for those who do not cooperate to take the medication.[2] ODFs offer another advantage in that they enable rapid treatment of various conditions such as allergies, migraines, and nausea without the need for water. On the other hand, one of the most important disadvantages of ODFs is their limited drug-loading capacity.[3]

ODFs are defined in the European Pharmacopoeia (Ph. Eur.) 11.4 as single- or multi-layer strips made of suitable material that disintegrate rapidly when placed in the mouth.[4] Whereas there are no standardized methods or guides for the quality control and characterization of films, it is stated in Ph. Eur. "In the manufacture of ODFs, measures are taken to ensure that they possess suitable mechanical strength to resist handling without being damaged." The tensile strength (TS) is an often used parameter in evaluating the mechanical properties of thin films.[5] The type and concentrations of film-forming polymers that form the main component of orally disintegrating films are largely responsible for producing films with appropriate mechanical strength and integrity.[5,6] Films are manufactured

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using different methods such as hot melt extrusion, electrospinning, and solvent casting method.[3] Among these methods, the solvent casting method is the most widely used in the pharmaceutical industry due to its simple production process and low cost.[7] Polymers used in ODFs can basically be classified as cellulose derivatives, starch derivatives, synthetic, and semi-synthetic polymers. Starch derivatives are among the most preferred polymers among all natural biopolymers because of their low price, widespread availability, and biodegradability. Modified starches used in oral films include maltodextrin (Maltrin®, Maltodextrin®), hydroxypropyl pea starch (Lycoat®), pregelatinized starch (Instant Pure Coat®), and Pullulan.[6] Of the cellulose derivatives, hydroxypropylmethylcellulose (HPMC) and hydroxypropyl cellulose (HPC) are most commonly used as film-forming polymers. Other commonly used excipients in the production of ODF include plasticizers [glycerol, propylene glycol (PG), sorbitol, polyethylene glycol], fillers (maltodextrin, mannitol), saliva enhancers (citric acid), solvents, color, flavor, stabilizer, surfactants, and various solubility enhancing agents (CD derivatives, Kleptose® linecaps) depending on the product's quality target product profile and the active ingredient used.[1,2,6,8]

Another factor that may affect mechanical strength is morphological changes in the films, such as crystal formation caused by the active ingredient.[9] Therefore, for mechanical strength in films, in addition to the film-forming materials, different factors, such as the type and amount of active substance in the film, the thickness, and the manufacturing process must be carefully controlled.[5]

Desloratadine (DL) is a 2nd generation H1 antihistamine that is widely used in the treatment of allergic rhinitis and urticaria. The recommended oral dose is 5 mg for adults and adolescents. For the pediatric population from 1 to 5 years old, 1.25 mg of DL can be administered once a day, whereas children aged between 6 and 11 years may be administered 2.5 mg of DL once daily.[10] DL is currently available in the market in the form of a 5 mg film-coated tablet, as well as an oral solution suitable for use at lower doses in pediatric patients and certain patient groups. The development of an ODF formulation for DL provides several advantages over traditional formulations, including enhanced patient compliance, decreased risk of choking, mitigation of stability issues associated with liquid formulations, and accurate dosing. With the growing focus on personalized medicine, ODFs offer the advantage of dose-specification. These formulations can be tailored in terms of dose and size to suit individual age and physiological conditions, facilitating individualized treatment and improving therapeutic outcomes. Given the advantages of ODFs and the necessity to overcome the issue of low drug-loading capacity in this dosage form, we aimed to develop a formulation containing 5 mg of DL that aligns with the highest recommended dosage and ensures therapeutic efficacy.

DL is practically insoluble in water.[11] Moreover, due to its molecular structure, DL is prone to degradation and is especially sensitive to oxidation.[12,13]

Considering the properties of the active substance, the present study aimed to evaluate the appropriate formulation composition, process determination, and characterization methods through preliminary formulation development studies for DL-containing ODF.

Materials and Methods

Materials

DL was a gift sample from Nobel İlaç. Citric acid anhydrous (10024) was obtained from Merck. Pregelatinized hydroxypropyl pea starch (Lycoat RS 780 and Lycoat RS 720), pea maltodextrin (Kleptose Linecaps), and maltodextrin (Glucidex IT6) were kindly donated by Roquette Pharma. HPMC E15 and HPMC E5 (Methocel E15 LVP, Methocel E) were supplied by Colorcon. HP-β-CD (Cavasol W7 HP Pharma) was gifted by Ashland. Sodium Metabisulfite, ascorbic acid, and EDTA were procured as gift samples from Drogsan. Propylgallate was kindly supplied by Ali Raif Pharmaceuticals. Polyvinylpyrrolidone (PVP) (Kollidon® 30 LP), PEG 400, and poloxamer (Kolliphor® P188) were obtained from BASF. Ethanol absolute (Merck) and PG (Merck Emsure) were purchased from local vendors. All other reagents and solvents were of analytical grade.

Method

Compatibility study

Differential scanning calorimetry (DSC) analyses were performed on 2 mg samples of DL, excipient, and drug: excipient in a ratio of approximately 1:1 (*w/w*) and were weighed and placed in aluminum sample containers. After closing the aluminum cover and compressing it with pressure, the cover was placed in the heating cell of the instrument (Shimadzu, DSC-60, Japan). Measurements were performed in the temperature range of 25 °C-300 °C at a heating speed of 10 °C/min under a nitrogen atmosphere.

ODF preparation

ODF was prepared using the solvent casting method. The quantitative compositions of the formulations are listed in Table 1. The ODF preparation steps are illustrated in Figure 1. According to the procedure for the preparation of the polymer solution, film-forming enhancing agents and plasticizers were first added to a measured amount of water, which was heated to 90 °C when using HPMC polymer, and then mixed until a homogeneous solution was obtained. In a separate beaker, solubility-enhancing agents, antioxidants, ethanol, DL, and other excipients were dissolved in a measured volume of water. The mixture was stirred at 1000 rpm for 30 min. The polymer solution was then gradually added to the beaker containing the active ingredient mixture. The resulting mixture containing the active ingredients was stirred using an overhead stirrer (High-Speed Digital, R1042 Dissolver, Ika Eurostar 20) for a total of 30 min, following a stepwise mixing protocol: 10 min at 750 rpm, 10 min at 1000 rpm, and 10 min at 1500 rpm. The bulk wet film was left to degass overnight. Wet film masses were cast using an automated film applicator equipped with a quadruple-layer film applicator (Coatmaster 510, Erichsen). The ODFs were cast at a casting height of 400-1500 µm at a speed of 6 mm/s. Subsequently, the films were dried at room temperature for 24

h and then cut into desired sizes, each containing 5 mg of DL, for further analysis. The prepared films were heat-sealed with polyethylene terephthalate/aluminum sachet foil as the primary packaging.

Characterization of ODFs

ODFs were visually examined for appearance based on the following parameters: homogeneity (absence of insoluble particles and uniform texture), peelability (removability of ODFs from the surface), brittleness, and color alterations. In this respect, following the evaluation of wet mass and films, applicable formulations and casting heights were selected, and further characterization studies were carried out with F17-F20 formulations.

Thickness

The thickness was measured from various regions of the film using a digital micrometer (precision ±0.001 mm Mitutoyo, Japan).

Tensile strength

The TS of the film formulations was measured by attaching a miniature tensile grip accessory to a TA-XT Plus Texture Analyzer (Stable Micro Systems, UK). The distance between the upper and lower handles of the films (2x1 cm) was set to 10 mm. While the upper handle part of the apparatus, whose lower handle part is fixed, moves upwards at a speed of 5 mm/min, the TS is calculated by the device software by dividing the force (N) required to break the film by the cross-sectional area (mm^2).[14]

Table 1. Quantitative composition of formulations for examining drug load and development stable orodispersible film

Ingredients (%)	Formulation code																			
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16	F17	F18	F19	F20
Lycoat RS720	18.0	18.0	18.0	20.0	20.0	20.0	20.0	-	20.0	19.25	-	-	-	-	-	-	-	-	-	-
Lycoat RS780	-	-	-	-	-	-	-	-	-	-	-	-	-	5.0	4.0	4.0	5.0	-	-	-
HPMC E15	-	-	-	-	-	-	-	15.0	-	-	12.0	12.0	12.0	10.0	8.0	8.0	10.0	10.0	10.0	9.0
HPMC E5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.0	3.0	3.0
PVP 30LP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.0	4.0
DI	-	-	-	-	-	-	-	-	-	0.3	0.5	0.5	0.5	3.0	2.4	2.4	3.0	3.0	3.0	3.0
Ethanol	-	14.0	14.0	14.0	7.0	7.0	7.0	-	7.0	7.0	7.0	7.0	7.0	7.0	5.6	5.6	7.0	7.0	10.25	10.25
PEG	7.5	7.5	10.0	7.5	-	-	-	-	7.5	7.5	7.5	-	-	7.5	6.0	6.0	7.5	6.5	6.5	7.0
Glycerol	-	-	-	-	7.5	7.5	3.0	5.0	-	-	-	-	-	-	-	-	-	-	-	-
PEG 400	-	-	-	-	-	-	-	-	-	-	-	7.5	7.5	-	-	-	-	-	-	-
Glucidex® IT6	5.0	5.0	5.0	5.0	5.0	3.0	5.0	5.0	5.0	5.0	-	-	-	-	-	-	-	-	-	-
HP-β-CD	-	-	-	-	-	-	-	-	-	-	3.0	3.0	-	5.0	4.0	4.0	-	-	-	-
Pea maltodextrin	-	-	-	-	-	-	-	-	-	-	-	-	3.0	-	-	-	-	-	-	-
Poloxamer 188	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.0	5.0	4.0	3.0
Citric acid	-	-	-	-	-	-	-	-	0.5	0.5	0.2	0.2	0.2	0.2	0.4	0.4	0.2	0.2	0.2	0.2
Ascorbic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	-	-	-	-	-
Sodium metabisulphite	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.5	0.5	0.5	0.5
EDTA	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Distilled water to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Assesment remarks																				
Peelability	+/-	+/-	+/-	+/-	-	-	-	+	+/-	-	+	+	+	ND[b]	+	+	+	+	+*	+*
Brittleness	+/-	-	-	-	ND[a]	ND[a]	ND[a]	+	-	+/-	+	+	+	ND[b]	+	+	+/-	+	+*	+*
Homogeneity	+	+	+	+	ND[a]	ND[a]	ND[a]	+	+	-	+	+	+	ND[b]	+/-	+/-	+/-	+	+*	+*

Evaluations obtained from experimental observations: + Desired; +/- Moderate; - Not desired; +*: Best casting solutions.
ND[a]: Not detected because the films could not be removed from the surface, ND[b]: Not detected because the films could not be cast, HPMC: Hydroxypropylmethylcellulose, PVP: Polyvinylpyrrolidone, PEG: Polyethylene Glycol, EDTA: Ethylenediaminetetraacetic acid

Disintegration time

Disintegration times were evaluated using the Petri dish method, and the slide frame method proposed for ODFs in the literature.[15] In the Petri method, a film is placed on the surface of the water in a Petri dish containing 2 mL of distilled water, and the time until the strip disappears completely is recorded. In the slide frame method, films cut in 5x2 cm dimensions were placed on the slide frame. The slide frame was placed on a beaker, and 200 µL of 37 °C distilled water was dropped into the middle of the film using a pipette. The time at which the film dissolved when the first drop fell into the beaker was recorded.

Uniformity of content

DL content was determined by spectrophotometry at 280 nm and was validated according to the International Conference on Harmonisation Q2 (R1) guidelines. The film samples were completely dissolved in 0.1 N hydrochloric acid and diluted to a final concentration 10 µg/mL. Content uniformity was determined by calculating acceptance values (AV) according to the Ph. Eur. 2.9.40.[16]

Statistical analysis

All statistical data were analyzed using Microsoft Excel (Microsoft Office). The Student's *t*-test was used to perform statistical comparisons between two different levels. Results for thickness uniformity results are expressed as mean with relative standard deviation (RSD)%, while mechanical properties and disintegration tests are expressed as mean ± standard deviation (SD).

RESULTS

The preliminary formulations of placebo and DI-containing films and their characteristics are presented in Table 1. The formulation development studies began with the development of orodispersible placebo films (F1) using starch-derived film-forming polymers. Ethanol was added to the F1 formulation to reduce bubble formation. An increase in film brittleness was observed in F2. With the F3 formulation, in which PG amount was increased to reduce brittleness, no improvement in brittleness was achieved, and even increased stickiness was observed. Increasing the amount of PG promoted a significant decrease (p<0.05) in TS. Results of physico-mechanical properties are presented in Figure 2. To support the production of a more cohesive and durable film, the hydroxypropyl pea starch content increased in F4, and an inadequate improvement was observed. Glycerol was tested as a different plasticizer in F5, F6, and F7 with a starch-based film-forming polymer to improve brittleness, and the films could not be removed from the surface. The ODF formulation containing HPMC as a film-forming polymer along with Glycerin in the F8 was easy to remove, non-brittle, and exhibited good mechanical integrity. Placebo F9 films were prepared to evaluate the impact of citric acid on the starch-based films; an increase in brittleness was observed. It was detected that the peelability of the film from the surface became difficult. In F10, the addition of an active ingredient further negatively affects the removability of the film from the surface. The films formed HPMC were flexible, homogenous, and easy to remove from the substrate. F12 and F13 containing PEG 400 showed an improvement in the morphological and mechanical properties of the films as the TS increased. The color of the aqueous casting solutions and films changed to slightly pink. It was intended to contain 3% DI, the amount of HP-β-CD was increased to enhance the water solubility of the active ingredient in the formulation. In F14, due to the presence of an excessive amount of solid mass in the formulation, wetting could not occur, resulting in the formulation could not be cast. To increase the water content and ensure wetting, all excipient ratios except citric acid, as well as DI amount were reduced in F15 and F16. The formulations exhibited high viscosity due to the presence of significant amounts of HP-β-CD, resulting in the entrapment of air bubbles.

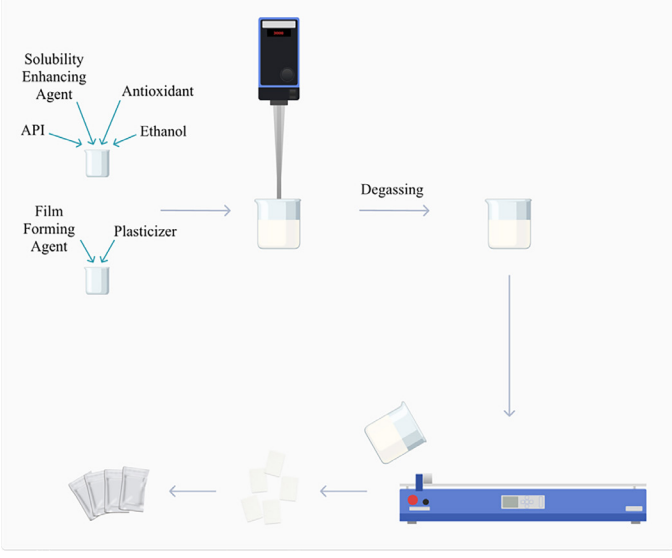

Figure 1. Schematic view of solvent casting procedure of orodispersible film containing DI

DI: Desloratadine

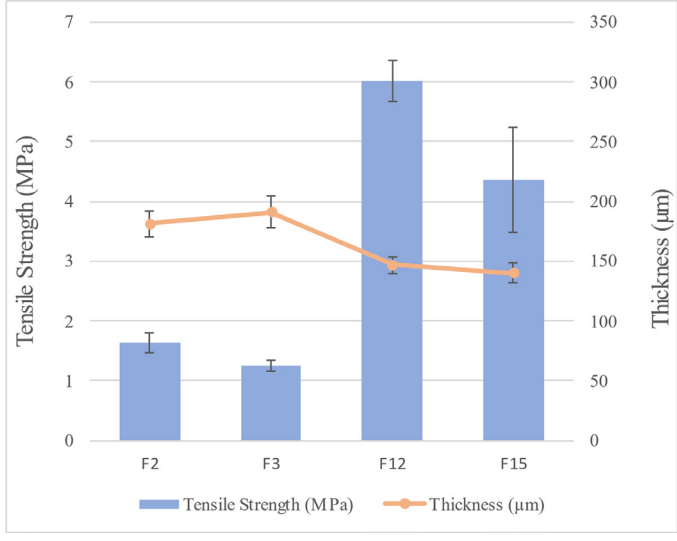

Figure 2. Comparison of TS and dry film thickness characteristics between formulations incorporating HPMC and starch based compositions

HPMC: Hydroxypropylmethylcellulose, TS: Tensile strength

In the preliminary stage, the appropriate antioxidants were investigated by DSC compatibility study. DSC thermograms of DI, antioxidant constituents, and DI: antioxidant in a ratio of 1:1 (*w/w*) are presented in Figure 3. The melting endothermic peak of DI disappeared in the DI: propilgallate binary mixture (Figure 3B). In the DSC thermogram illustrating the 1:1 ascorbic acid mixture, there was a reduction in the peak intensity of the DI, and the peak corresponding to Ascorbic acid disappeared entirely (Figure 3C). To confirm that the formulation containing Ascorbic acid was prepared (F15), it was also observed that the color of DI and ascorbic acid containing casting dispersion changed to light pink (Figure 3E). The influence of casting height on the TS is compared by comparing the disintegration time using the Petri Dish and Slide Frame Method and thickness using F16. These results are shown in Table 2.

Based on the F14 formulation, formulation 17 containing Poloxamer 188 at the same concentration of the film-forming polymer was prepared. It was observed that there was an increase in brittleness and air bubble formation. The particles observed in the wet mass and film surface thickness uniformity (RSD%) were 17.0%. The disintegration time, TS, and thickness measurements of F17-F20 formulations prepared using 400 µm casting height are shown in Table 3. With formulation 18 starch-based polymers excluded, HPMC E5 was added to increase the HPMC ratio in the formulation without further increasing the viscosity to improve the solubility of DI. The amount of particles in the wet mass and on the film surface has decreased significantly. In F19 and F20 formulations with the addition of PVP and Poloxamer, which acts as a film-forming agent and plasticizer, to increase solubility, no particles were observed both in wet mass and homogenous, flexible ODFs were obtained.

DISCUSSION

The preliminary studies of formulation development were conducted to evaluate formulation factors, select the final excipients, determine the process, and choose an appropriate characterization method because no pharmacopeial method has been described and no acceptable limit has been specified.

ODFs typically contain one or a combination of suitable film-forming agents, which constitute a backbone for incorporating drug substances and various excipients.[6,17] A variety of hydrophilic polymers have been extensively investigated in

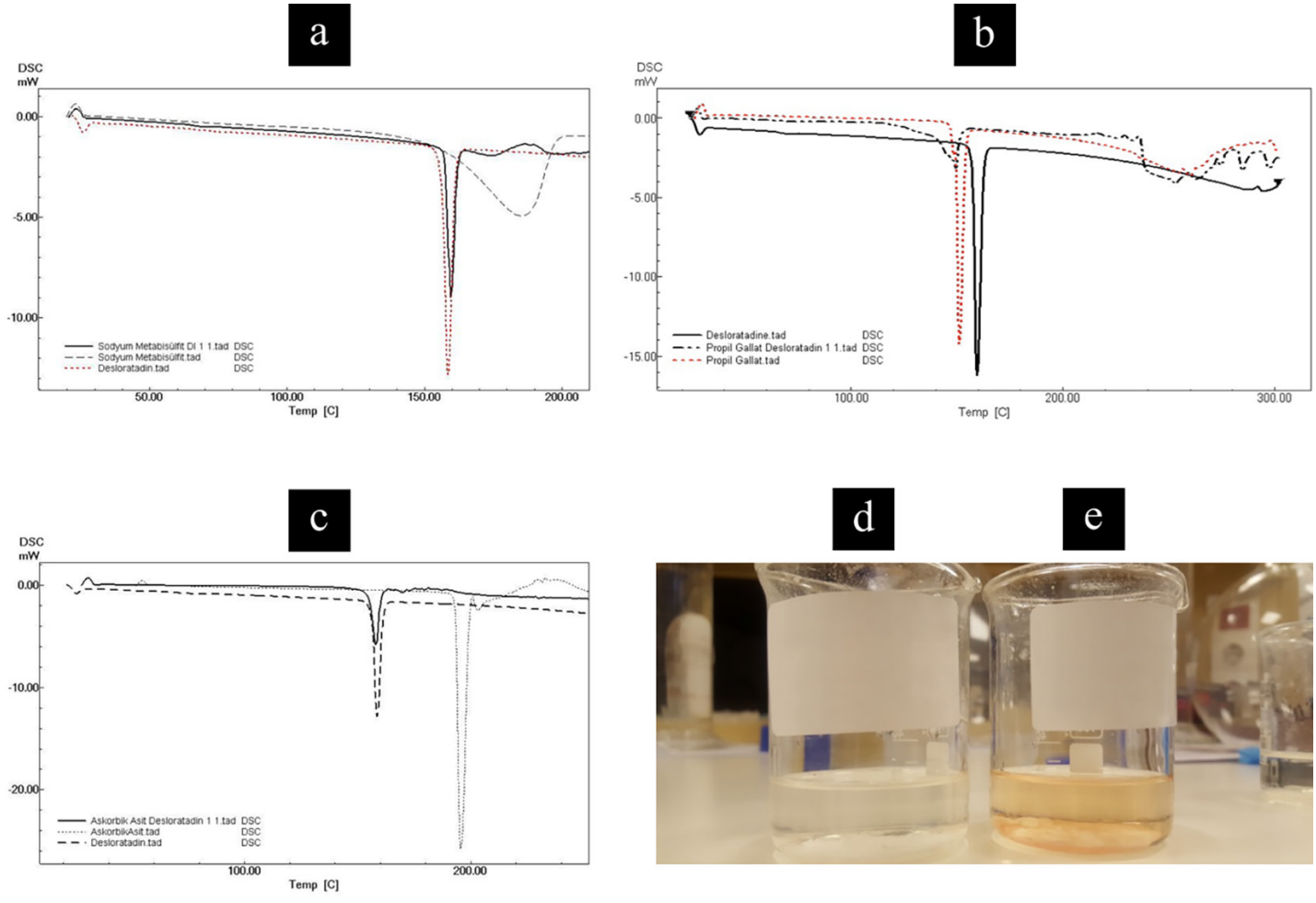

Figure 3. Selected DSC curves of (A) DI, sodium metabisulphite and DI: sodium metabisulphite. (B) DI, propil gallate and DI: propil gallate. (C) DI, ascorbic acid and DI: Ascorbic acid. Wet film formulations containing sodium metabisulfite (D) and ascorbic acid (E) where color change is observed

DI: Desloratadine, DSC: Differential scanning calorimetry

Table 2. Film thicknesses, tensile strengths, and disintegration times measured by the Petri Dish and Slide frame method for prepared orodispersible films with casting heights of 400 µm, 1000 µm and 1500 µm

Formulation code	Casting height (µm)	Dry thickness (µm)	Disintegration time (sec.)		TS (MPa)
			Slide frame	Petri	
F16	400	41.5±8.0	25.3±2.3	31.3±6.1	8.4±0.6
	1000	138.9±8.0	212.3±6.9	380.0±40.0	4.5±0.9
	1500	183.9±8.5	363.0±8.9	483.3±75.1	3.3±1.3

TS: Tensile strength, sec.: Second

Table 3. Characterization of physical and mechanical parameters and disintegration time measured by the sliding frame method for orodispersible films with a casting height of 400 µm

Formulations	Disintegration time (sec)	Thickness (µm)	TS (MPa)	AV
F17	44.3±9.0	68.7±17.0	ND	ND
F18	44.66±3.5	53.9±3.4	10.3±1.7	13.4
F19	44.5±4.5	64.7±4.3	9.1±3.2	6.8
F20	40.5±4.8	56.5±4.6	6.2±3.3	5.0

ND: Not detected, TS: Tensile strength, AV: Acceptance value

the preparation of ODFs, including HPMC; HPC; pregelatinized hydroxypropyl pea starch; PVP and maltodextrin.[17] The different types of HPMC and HPC differ in terms of the degree of substitution and viscosity. It is stated in the literature that appropriate films can be formed by combining different grades of polymers with PVP, HPMC, or starch derivatives.[6,18,19] Although maltodextrin alone can act as film-forming polymers, it has been stated that film properties can be improved by mixing them with other polymers, one of which is modified starches.[6] It was noted that maltodextrin with low dextrose equivalents reduces the brittleness of films.[20] In our study, to improve the mechanical properties of films prepared with Lycoat, such as reducing brittleness and improving removability from the surface, experiments were carried out with the use of Glucidex, different plasticizers, and their ratios, and different film-forming polymer combinations, as shown in Table 1. It was observed that film-forming polymers have a notable effect on mechanical properties. To demonstrate these effects, F2 and F3, containing only Lycoat, F12, containing only HPMC, and F15, containing a combination of Lycoat and HPMC, were selected. As shown in Figure 2, the TS of HPMC-produced films is higher than that of starch-based films. Additionally, when the results obtained from Table 1 in terms of brittleness and film removability were examined, it was found that the major effect in terms of improvement in the mechanical properties of films was obtained with the HPMC polymer. Further studies on HPMC-based films, and the results of formulations of F17-F20 are presented in Table 3. TS values were found to be 1.47%-33.91 MPa in the study evaluating the mechanical properties of commercial ODFs.[21] Our measurement results were within this range, and as the polymer concentrations of HPMC and PVP increased within F19 and F20, flexible films and TS increased as desired; thus, films suitable for handling were obtained. Visser et al.[22] investigated the mechanical properties of polymer films and reported TS values above 2 MPa, with films containing a higher percentage of HPMC exhibiting the greatest TS and being the most preferred.

The results of the investigation of the impact of wet film thickness on TS, disintegration time, and dry thickness show that, in Table 2, the decrease in wet film thickness is associated with an increase in TS and, as expected, a decrease in disintegration time. Since the F16 formulation prepared with a wet film thickness of 400 µm was thin, easy to remove, and flexible with a disintegration time of less than 30s for both methods, a casting height of 400 µm was found appropriate for further studies. Due to the lack of standardized characterization methods for ODFs, the objective of this study was not only to develop DI-containing film formulations but also to assess and compare various characterization techniques. In this context, in addition to evaluating the effect of wet mass thicknesses, different disintegration methods were comparatively evaluated as there is no formal disintegration test for orodispersible films. Disintegration times were evaluated using the Petri dish method and the slide frame method proposed for ODFs in the literature.[1,23] The results are presented in Table 2, and when the Petri dish method was applied, the difference between the disintegration times of the formulations could not be distinguished precisely; therefore, the SD values were found to be higher. In addition, measurement results can vary between individuals. With the Slide Frame method, the endpoint could be easily determined, and the repeatability was high. It is clear from the obtained results that the Slide-frame method is more precise and sensitive than the Petri dish method. An additional advantage of the slide frame method is its simplicity and minimal equipment requirements. The test setup only requires the use of a beaker, slide frame, and small volume of liquid, making it a cost-effective and straightforward technique.[24] However, the slide frame method does not fully correlate with in vivo conditions. Under physiological conditions, the oral film is wetted from both directions, reflecting a more complex and dynamic interaction between the film and saliva. In contrast, this method only involves wetting the film in one direction. In addition, adhesion to the oral mucosa and the force exerted by the tongue are not taken into account.[24, 25] From this point of view, it can be inferred that the disintegration times found with the slide frame method may be longer than physiological conditions, which can effectively simulate the worst-case

scenario. This hypothesis could be further validated through additional studies in the future. The measurement results of the disintegration times of F17-F20 formulations with the Slide Frame method were found to be lower than the 60s (Table 3), and the results obtained were significantly lower than the 180s specified in Ph. Eur. 11.4 for orally disintegrating tablets.[26]

For ODFs, the thickness depends on the wet mass thickness, formulation components, and solid mass content. In the literature, the thickness of 9 commercial preparations was measured, and the results were found to be between 40 and 140 µm.[21] In another study, it was reported that the ideal thickness of buccal films was between 50 and 100 µm.[27] In our study, with the selected formulation content and casting height of 400 µm, homogeneous and suitable films with dry film thicknesses ranging between 50 and 70 µm were obtained (Table 3). Because the thickness uniformity is directly related to the amount of drug in the film, it is important for content uniformity. The RSD% value used in the thickness uniformity evaluation for F19 and F20 was found to be lower than 5%. Another important issue in ensuring content uniformity in ODFs is the homogeneous distribution of the active ingredient in the film. The fact that the film contains particles poses a risk in terms of both content uniformity and mechanical strength. The interaction between the polymer and the crystalline active substance can harden the surface of the film, disrupt its homogeneity, and make it brittle.[28-30] The choice of a film-forming polymer in ODFs is not only important for the mechanical properties and disintegration time but also plays an important role in the dissolution of the drug in the polymer.[31] Studies have shown that some film-forming polymers such as PVP and HPMC increase the solubility of poorly water-soluble drugs by acting as crystallization inhibitors.[32-34] Using these polymers, the crystallization that may occur in the films due to active pharmaceutical ingredients can be reduced or completely prevented during the production and storage of films. It has also been reported in the literature that recrystallization of some active substances, such as Dimenhydrinate, is prevented by the use of maltodextrin and cyclodextrins.[35] Aim of this part of the study was to dissolve Dl in the film to prevent the formation of crystal lumps in ODF. In our previous study, we found that HP-β-CD increased its water solubility by forming a 1:1 stoichiometric complexation with Dl.[36] However, when Dl and HP-β-CD were incorporated into the film formulation, since a very high amount of HP-β-CD was required to form a soluble complex, it was not found suitable for ODF containing Dl. Similarly, in the literature, it was stated that ODFs containing high amounts of CDs negatively affected the mechanical properties.[35] As aimed in this part of our study, Dl could be dissolved in the film at a rate of 3% with the combination of HPMC, PVP, and the surfactant poloxamer P188, and F19 and F20-particle-free homogeneous films were obtained.

In the selection of excipients in addition to their usage purposes, the chemical compatibility between excipients and active ingredients is also critical in the early formulation development stage. Compatibility studies are the first step toward eliminating incompatible excipients.[37] Using DSC as a screening technique, the results showed incompatibility between ascorbic acid and propylgallate, which was also confirmed by further formulation development studies by observing a color change in the bulk formulation containing ascorbic acid (Figure 3E).

The trace amounts of reactive impurities in excipients can cause drug instability. The most common reactive impurities in excipients are peroxides.[38] It is known that peroxides consist of very weak O-O bond and can readily form hydroxyl and alkoxy radicals. Hydroperoxides are commonly formed by the degradation of excipients such as PG and PVP.[38] Formaldehyde and formic acid formed by oxidative degradation are involved in the N-methylation and N-formylation of amine-containing active drug ingredients.[38] The chemical reactions of reactive impurities of formaldehyde and formic acid, majorly formed by the degradation of PG, particularly with amine-containing active drug ingredients, have been investigated extensively.[39-42] Formic acid is often responsible for the formation of N-formyl impurities in active drug ingredients containing primary and secondary amino groups.[39,42,43] It is known that the main degradation product of Dl is N-formyl-Dl.[44] Very small amounts of the degradation product N-formyl desloratadine were found to cause discoloration of Dl.[45] Since an orange-yellow color was observed in the PG-containing formulations and was attributed to oxidation triggering by the mentioned mechanism, PG was excluded from the study. Among the plasticizers tested, PG, one of the solvents in which Dl dissolves well,[46] was found suitable in terms of plasticizing effect, considering the sensitivity of the active substance to oxidation and the need to increase its solubility to increase the amount of drug loading in the film. In the early stages of drug development, understanding the type and degree of degradation of a drug candidate is crucial. As metals found in excipients can catalyze oxidation in drugs at residual levels[38,] in addition to sodium metabisulfite, which is used as an antioxidant, EDTA, which acts as an antioxidant synergist,[47] was also added to the formulations.

CONCLUSION

In this study, it was demonstrated that Dl-containing ODF was successfully developed by taking into account the drug loading and chemical stability of the active substance in combination with selected excipients and process parameters. As a result of preliminary studies, thin, homogeneous, flexible, fast disintegrating (40s), particle-free films were developed with F20, which was found suitable for further studies. The fact that the formulations met the criteria for AV below 15 confirmed that the active ingredient was distributed homogeneously. Based on the obtained promising results, further optimization studies were conducted to develop a generic ODF product of Dl for the effective treatment of allergy and to improve patient compliance.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Footnotes

Authorship Contributions

Concept: Ö.Ç., Z.Ş.T., Design: Ö.Ç., Z.Ş.T., Data Collection or Processing: Ö.Ç., Z.Ş.T., F.N.T.D., Analysis or Interpretation: Ö.Ç., Z.Ş.T., F.N.T.D., Literature Search: Ö.Ç., Writing: Ö.Ç., Z.Ş.T., F.N.T.D.

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REFERENCES

1. Preis M, Pein M, Breitkreutz J. Development of a taste-masked orodispersible film containing dimenhydrinate. Pharmaceutics. 2012;4:551-562.
2. Hoffmann EM, Breitenbach A, Breitkreutz J. Advances in orodispersible films for drug delivery. Expert Opin Drug Deliv. 2011;8:299-316.
3. Musazzi UM, Khalid GM, Selmin F, Minghetti P, Cilurzo F. Trends in the production methods of orodispersible films. Int J Pharm. 2020;576:118963.
4. European Pharmacopoeia Commission. Oromucosal preparations. European Pharmacopoeia 11.4. 2024:985-7.
5. Karki S, Kim H, Na S-J, Shin D, Jo K, Lee J. Thin films as an emerging platform for drug delivery. Asian J Pharm Sci. 2016;11:559-574.
6. Borges AF, Silva C, Coelho JF, Simões S. Oral films: Current status and future perspectives: I-Galenical development and quality attributes. J Control Release. 2015;206:1-19.
7. Allen LV Jr. Basics of Compounding: Clinical Pharmaceutics, Part 1. Int J Pharm Compd. 2016;20:389-396.
8. Palezi SC, Fernandes SS, Martins VG. Oral disintegration films: applications and production methods. J Food Sci Technol. 2023;60:2539-2548.
9. Preis M, Knop K, Breitkreutz J. Mechanical strength test for orodispersible and buccal films. Int J Pharm. 2014;461:22-29.
10. European Medicines Agency (EMA). Aerius (Desloratadine)-EPAR product information. 26/06/2009 [updated: 25/06/2024; cited: 11.2024]. Available from: https://www.ema.europa.eu/en/documents/product-information/aerius-epar-product-information_en.pdf.
11. European Pharmacopoeia Commission. Desloratadine. European Pharmacopoeia, 11.3. 2024:2487.
12. Rao DD, Satyanarayana NV, Malleswara Reddy A, Sait SS, Chakole D, Mukkanti K. A validated stability-indicating UPLC method for desloratadine and its impurities in pharmaceutical dosage forms. J Pharm Biomed Anal. 2010;51:736-742.
13. Walash MI, Belal F, El-Enany N, Eid M, El-Shaheny RN. Stability-indicating micelle-enhanced spectrofluorimetric method for determination of loratadine and desloratadine in dosage forms. Luminescence. 2011;26:670-679.
14. Dixit RP, Puthli SP. Oral strip technology: overview and future potential. J Control Release. 2009;139:94-107.
15. Garsuch V, Breitkreutz J. Comparative investigations on different polymers for the preparation of fast-dissolving oral films. J Pharm Pharmacol. 2010;62:539-545.
16. European Pharmacopoeia Commission. Uniformity of Dosage Units (2.9.40.). European Pharmacopoeia, 11.0. Strasbourg, 2017:421-423.
17. Turković E, Vasiljević I, Drašković M, Parojčić J. Orodispersible films-pharmaceutical development for improved performance: a review. J Drug Deliv Sci Technol. 2022;75:103708.
18. Schobel AM, Vangala SS. Solid dosage form containing a taste masked active agent. United States Patent and Trademark Office (USPTO), 2015; US8986735B2.
19. Liew KB, Tan YT, Peh KK. Effect of polymer, plasticizer and filler on orally disintegrating film. Drug Dev Ind Pharm. 2014;40:110-119.
20. Dzija MR, Barkalow DG, Chapdelaine AH, Zyck DJ. Edible film formulations containing maltodextrin. United States Patent and Trademark Office (USPTO), 2003; US6656493B2.
21. Borges AF, Silva C, Coelho JF, Simões S. Outlining critical quality attributes (CQAs) as guidance for the development of orodispersible films. Pharm Dev Technol. 2017;22:237-245.
22. Visser JC, Dohmen WM, Hinrichs WL, Breitkreutz J, Frijlink HW, Woerdenbag HJ. Quality by design approach for optimizing the formulation and physical properties of extemporaneously prepared orodispersible films. Int J Pharm. 2015;485:70-76.
23. Garsuch V, Breitkreutz J. Comparative investigations on different polymers for the preparation of fast-dissolving oral films. J Pharm Pharmacol. 2010;62:539-545.
24. Speer I, Steiner D, Thabet Y, Breitkreutz J, Kwade A. Comparative study on disintegration methods for oral film preparations. Eur J Pharm Biopharm. 2018;132:50-61.
25. Krampe R, Sieber D, Pein-Hackelbusch M, Breitkreutz J. A new biorelevant dissolution method for orodispersible films. Eur J Pharm Biopharm. 2016;98:20-25.
26. European Pharmacopoeia Commission. Tablets. European Pharmacopoeia, 11.4. Strasbourg, 2024.
27. Nair AB, Kumria R, Harsha S, Attimarad M, Al-Dhubiab BE, Alhaider IA. In vitro techniques to evaluate buccal films. J Control Release. 2013;166:10-21.
28. Gaisford S, Verma A, Saunders M, Royall PG. Monitoring crystallisation of drugs from fast-dissolving oral films with isothermal calorimetry. Int J Pharm. 2009;380:105-111.
29. Kianfar F, Chowdhry BZ, Antonijevic MD, Boateng JS. Novel films for drug delivery via the buccal mucosa using model soluble and insoluble drugs. Drug Dev Ind Pharm. 2012;38:1207-1220.
30. Garsuch V, Breitkreutz J. Novel analytical methods for the characterization of oral wafers. Eur J Pharm Biopharm. 2009;73:195-201.
31. ElMeshad AN, El Hagrasy AS. Characterization and optimization of orodispersible mosapride film formulations. AAPS PharmSciTech. 2011;12:1384-1392.
32. Broman E, Khoo C, Taylor LS. A comparison of alternative polymer excipients and processing methods for making solid dispersions of a poorly water soluble drug. Int J Pharm. 2001;222:139-151.
33. Marsac PJ, Konno H, Taylor LS. A comparison of the physical stability of amorphous felodipine and nifedipine systems. Pharm Res. 2006;23:2306-2316.
34. Konno H, Handa T, Alonzo DE, Taylor LS. Effect of polymer type on the dissolution profile of amorphous solid dispersions containing felodipine. Eur J Pharm Biopharm. 2008;70:493-499.

35. Krampe R, Visser JC, Frijlink HW, Breitkreutz J, Woerdenbag HJ, Preis M. Oromucosal film preparations: points to consider for patient centricity and manufacturing processes. Expert Opin Drug Deliv. 2016;13:493-506.

36. Çakmakyapan Ö, Tuğcu Demiröz F, Teksin Z. Evaluation and comparison of ß-Cyclodextrin derivatives on aqueous solubility of desloratadine. 13th International Symposium on Pharmaceutical Sciences (ISOPS), 2021.

37. Wu Y, Levons J, Narang AS, Raghavan K, Rao VM. Reactive impurities in excipients: profiling, identification and mitigation of drug-excipient incompatibility. AAPS PharmSciTech. 2011;12:1248-1263.

38. Robnik B, Naumoska K, Časar Z. A Novel testing approach for oxidative degradation dependent incompatibility of amine moiety containing drugs with PGs in solid-state. Pharmaceutics. 2020;12:37.

39. Waterman KC, Arikpo WB, Fergione MB, Graul TW, Johnson BA, Macdonald BC, Roy MC, Timpano RJ. N-methylation and N-formylation of a secondary amine drug (varenicline) in an osmotic tablet. J Pharm Sci. 2008;97:1499-1507.

40. Hoaglund Hyzer CS, Williamson ML, Jansen PJ, Kopach ME, Scherer RB, Baertschi SW. Mechanistic studies of the N-formylation of Edivoxetine, a secondary Amine-containing drug, in a solid oral dosage form. J Pharm Sci. 2017;106:1218-1238.

41. Gibala P, Douša M, Kalužíková A, Tkadlecová M, Štefko M, Kalášek S, Břicháč J. Identification and structure elucidation of a new degradation impurity in the multi-component tablets of amlodipine besylate. J Pharm Biomed Anal. 2019;162:112-116.

42. Colgan ST, Zelesky TC, Chen R, Likar MD, MacDonald BC, Hawkins JM, Carroll SC, Johnson GM, Space JS, Jensen JF, DeMatteo VA. Use of activated carbon in packaging to attenuate formaldehyde-induced and formic acid-induced degradation and reduce gelatin cross-linking in solid dosage forms. J Pharm Sci. 2016;105:2027-2031.

43. Robnik B, Likozar B, Wang B, Stanić Ljubin T, Časar Z. Understanding and kinetic modeling of complex degradation pathways in the solid dosage form: the case of Saxagliptin. Pharmaceutics. 2019;11:452.

44. European Medicines Agency (EMA). Aerius (Desloratadine)-Public Assessment Report. 2004. Available from: https://www.ema.europa.eu/documents/scientific-discussion/aerius-epar-scientific-discussion_en.pdf

45. Yogananda, Chaitanya, Gujjar Shimoga. Pharmaceutical composition comprising desloratadine. European Patent Office (EPO), 2011; EP2269586B1(09008618.2).

46. United States Pharmacopeial Convention. Description and relative solubility. United States Pharmacopeia (USP). Rockville, MD, 2023. 2024:16.

47. European Medicines Agency (EMA). Guideline on excipients in the dossier for application for marketing authorisation of a medicinal product. 2007:10.

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ORIGINAL ARTICLE

A Green Microwave-Assisted Extraction of *Cannabis sativa* L. Extract and Its Cytotoxic Activity Against Cancer Cells

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ABSTRACT

Objectives: This study aimed to explore the use of D-limonene and some vegetable oils with different amounts of saturated and unsaturated fatty acids as alternative green solvents for microwave-assisted extraction (MAE) of cannabis (*Cannabis sativa* L.). A standardized cannabis extract was selected to evaluate its potential as a chemopreventive agent.

Materials and Methods: Alternative green solvents, powder-to-solvent ratios, and irradiation cycles were determined to optimize the MAE conditions. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to assess the cytotoxic effects against human breast cancer (MCF-7), liver cancer (HepG2), and mammary epithelium (hTert-HME1) cell lines.

Results: The extracts obtained from D-limonene and palm oil contained the highest concentrations of cannabidiol (CBD) and D-tetrahydrocannabinol (THC). A standardized D-limonene extract of cannabis (DEC) containing 0.03% *w/w* CBD and 1.37% *w/w* THC was selected for the evaluation of cytotoxic activity compared with CBD and THC. The results revealed that CBD and THC exhibited significant cytotoxic effects ($p<0.05$) against MCF-7 and HepG2, with the 50% inhibitory concentration (IC_{50}) values of 18.5 and 12.37 µg/mL for CBD and 24.21 and 4.30 µg/mL for THC, respectively, whereas DEC exhibited moderate cytotoxicity against MCF-7 (IC_{50} of 488.85 µg/mL). However, CBD and THC exhibited significant cytotoxicity ($p<0.05$) against hTert-HME1 (IC_{50} values of 35.61 and 25.63 µg/mL, respectively), whereas DEC exhibited low cytotoxicity against hTert-HME1 (IC_{50} of 1.537.03 µg/mL).

Conclusion: DECs containing appropriate levels of THC and CBD have the potential to be candidates for cancer treatment. However, further investigations are required to improve the efficacy and safety profiles.

Keywords: Cannabis, cancer, limonene, microwave extraction, vegetable oil

INTRODUCTION

Globally, cancer is a significant cause of morbidity and mortality, resulting in a large disease burden. According to Global Cancer Statistics 2020, breast cancer, with the largest number of 2.3 million new cases, accounted for 11.7% of all cancers, followed by lung cancer (11.4%), colorectal cancer (CRC) (10.0%), while lung cancer was the main cause of cancer death (1.8 million deaths, 18%), followed by CRC (9.4%) and liver cancer (8.3%).[1] Conventional cancer treatment includes surgery, chemotherapy, and radiotherapy. Although traditional treatments like chemotherapy and radiotherapy are effective, they have limitations, such as severe side effects and the development of multidrug resistance in cancer cells. Medical cannabis is gaining attention as a treatment option for various

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diseases. Currently, the Food and Drug Administration of Thailand has approved cannabis for specific conditions like nausea and vomiting, intractable epilepsy, and neuropathic pain. However, previous studies have explored the potential benefits of medical cannabis for various medical conditions, including cancer. Cannabis contains cannabinoids that interact with specific endogenous cannabinoid receptors, as well as other receptors, resulting in the expectation of anti-cancer effects.[2] The cannabis industry now favors microwave-assisted extraction (MAE) because of its superior extraction efficiency compared to traditional methods.[3] Generally, organic solvents, including hexane, chloroform, and methanol, are commonly used for cannabis extraction.[3] However, most of these compounds are toxic to the human body,[4] which limits the industrial applications of these cannabis extracts. Therefore, the need for an alternative green solvent for cannabis extraction is a pressing concern because it can enhance the safety of the cannabinoid extraction process. Vegetable oils have higher cannabinoid content and a slower rate of cannabinoid degradation in cannabis extract than ethanol.[3] Furthermore, other natural compounds, such as D-limonene, are potential candidates primarily due to their non-polar properties. D-limonene not only aids in the extraction process but also possesses inherent anti-cancer properties,[5] which may synergistically enhance the anti-cancer effects of the cannabis extract itself. This dual benefit supports the rationale for using D-limonene as an alternative green solvent. This study aimed to investigate the potential of D-limonene and various vegetable oils, such as olive oil, sunflower oil, soybean oil, palm oil, and coconut oil, as green solvents for cannabis MAEs. The MAE conditions were optimized to yield cannabinoid-enriched extracts. Furthermore, we selected a standardized cannabis extract for cytotoxicity studies against human breast and liver cancers. We compared it with normal human cells to assess its potential as a chemopreventive agent.

MATERIALS AND METHODS

Plant materials

Dried *Cannabis sativa* inflorescences were obtained from the Faculty of Natural Resources, Prince of Songkla University, Thailand. The inflorescences were dried in a hot air oven and reduced to powder using an electric blender. The powder was then passed through a sieve to ensure its homogeneity.

Chemicals and materials

The purification and acquisition of tetrahydrocannabinol (THC) were accomplished using the method previously outlined.[6] The cannabidiol (CBD) compound was acquired from Chemfaces, a company based in Wuhan, China. Methanol, ethanol, and hexane were acquired from RCI Labscan (Bangkok, Thailand). D-limonene was procured from Krungthepchemi (Bangkok, Thailand). Sunflower oil, soybean oil, and palm oil were procured from Lam Soon (Thailand) Public Company situated in Samut Prakarn, Thailand. The acquisition of coconut oil was madeby Ampol Food Processing, located in Nakornpathom, Thailand. The acquisition of olive oil was made by Sino-Pacific Trading, a company based in Bangkok, Thailand. The acquisition of rice bran oil was made by Oleen, a company in Samut Sakhon, Thailand. A Luna® C-18 column was obtained from Phenomenex (Bangkok, Thailand). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma Chemical, Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), modified Eagle's medium (MEM), and fetal bovine serum (FBS) were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA).

Cell cultures

Human mammary epithelium (hTERT-HME1; ATCC CRL-4010™), human liver cancer (HepG2; ATCC HB-8065™), and human breast cancer (MCF-7; ATCC HTB-22™) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). hTERT-HME1 and HepG2 cells were cultured in DMEM, whereas MCF-7 cells were cultured in MEM. All cell lines were kept in a 5% CO_2 humidified incubator at 37 °C. DMEM and MEM media were supplemented with 10% FBS, 1% L-glutamine (2 mM), 1% penicillin (100 IU/mL), and 100 µg/mL streptomycin.

Identifying alternative green solvents and MAE conditions

The powders of cannabis inflorescences were extracted with vegetable oils, D-limonene, ethanol, and hexane using MAE under the optimal conditions (for D-limonene and vegetable oils: microwave power: 900 W, irradiation time: 60 sec, and temperature: 115-120 °C; for ethanol and hexane: microwave power: 450 W, irradiation time: 35 sec, and temperature: 65-70 °C). The extracts were filtered, and the yields were recorded. The content of THC and CBD was determined using quantitative high-performance liquid chromatography (HPLC). The cannabis powders were subsequently extracted with suitable solvents using MAEs with different powder-to-solvent ratios and irradiation cycles. The extracts were then filtered, and the yields were recorded. The content of THC and CBD was determined using quantitative HPLC. All experiments were performed in triplicate.

Quantitative HPLC of THC and CBD

We used the previously described HPLC method with some modifications to determine the content of CBD and THC in the cannabis extracts. Briefly, the analysis was performed using a UFLC Shimadzu model equipped with a photodiode-array detector and autosampler (Shimadzu, Japan) at a wavelength of 220 nm. A 4.6x250 mm, 5 µm Luna® C18 column (Phenomenex, Thailand) was eluted with a mobile phase consisting of 85% *v/v* methanol in water at a flow rate of 1 mL/min. Calibration curves for THC and CBD were established using six concentrations (from 6.25 to 200 mg/mL based on linear regression; the calibration curves of CBD and THC were $Y=72615X+72146$ ($r^2=0.9998$), and

$Y=54467X+77267$ ($r^2=0.9999$), respectively.

The samples (2.0 mg) were accurately weighed and diluted with methanol to 10 mL in a volumetric flask. Before HPLC analysis, the sample solutions were filtered through a 0.45 µm membrane filter. The experiments were performed in triplicate.

Determination of anticancer activity

Anti-cancer activity was determined using the MTT assay.[7] Briefly, HepG2, MCF-7, and hTERT-HME1 cells were seeded into a 96-well microplate at a density of $1x10^4$ cells per well and then incubated in a 5% CO_2 humidified incubator at 37 ℃ for 24 h. The cells were treated with sample solutions at various concentrations, including CBD (3.12, 6.25, 12.5, 25, and 50 µg/mL), THC (3.12, 6.25, 12.5, 25, and 50 µg/mL), D-limonene extract of cannabis (DEC) (125, 250, 500, 1000, and 2000 µg/mL), D-limonene (125, 250, 500, 1000, and 2000 µg/mL), and 5-Fluorouracil (5-FU) (1, 5, 10, 50, and 100 µg/mL), and then incubated at 37 ℃ for 24 h. The media was removed, and the cells were treated with MTT solution (500 µg/mL) and incubated for 2 h at 37 ℃. The formazan product was solubilized with DMSO, and the intensity of solutions was measured at 570 nm using a microplate reader (Biotek, Winooski, VT, USA). 5-FU was used as a positive control. The percentage of cell viability relative to non-treated cells was presented as a negative control. The selectivity index (SI) was calculated by dividing the 50% inhibitory concentration (IC_{50}) values of the samples by those of cancer and normal cells.

Statistical analysis

The results are expressed as the mean ± standard deviation. A statistically significant difference was evaluated using one-way analysis of variance, followed by Duncan's multiple range test ($p<0.05$).

RESULTS

Identifying an alternative solvent for extraction

This study determined D-limonene and some vegetable oils containing different ratios of unsaturated to saturated fatty acids (SFAs) as alternative green solvents for the extraction of THC and CBD from *C. sativa* inflorescences using the MAE method and compared them to conventional solvents such as ethanol and hexane. Based on an HPLC analysis (Figure 1), ethanol and hexane provided the extracts with the highest cannabinoid concentrations, especially the THC levels (Table 1).

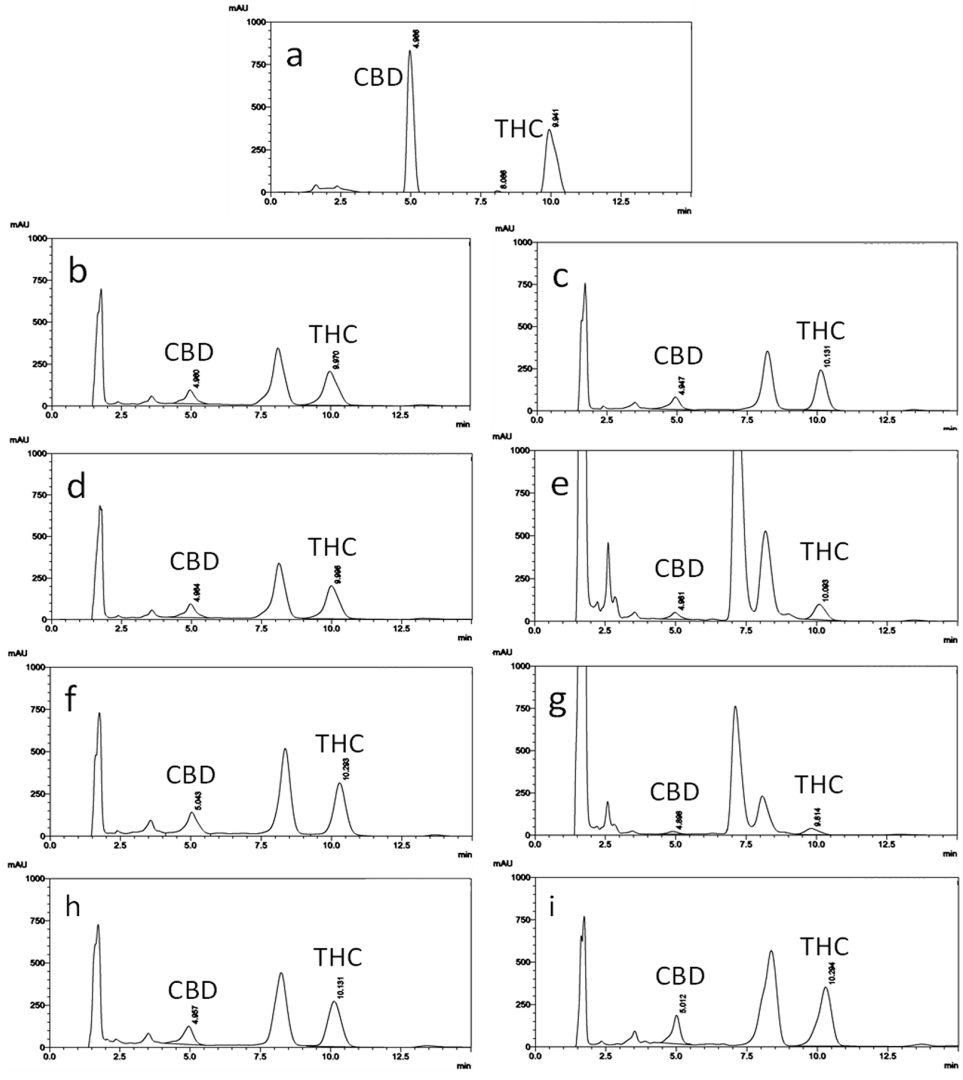

Figure 1. High performance liquid chromatography chromatograms of the cannabidiol and tetrahydrocannabinol standard (a) and cannabinoids extracts using sunflower oil (b), olive oil (c), soybean oil (d), palm oil (e), coconut oil (f), D-limonene (g), ethanol (h), and hexane (i) as the extraction solvents

The results of this study indicate that D-limonene, coconut oil, and palm oil have non-polar properties similar to those of CBD and THC.

Optimization of the extraction conditions

This study evaluated the effects of varying amounts of cannabis powder (1, 2, 4, and 6 g) per 20 mL of solvent extracted using the MAE method. The results revealed that a powder-to-solvent ratio of 4 g per 20 mL produced the cannabis extracts with the highest total yields of CBD and THC for palm oil and D-limonene (Table 2). The concentrations of both THC and CBD increased as the powder content increased. The extraction yields of the extracts were markedly reduced at ratios greater than 4 g per 20 mL due to solvent adsorption by cannabis powders, which resulted in a decrease in total yields of both THC and CBD. In this study, the irradiation cycles up to three cycles (one cycle was 70-sec power-on and 50-sec power-off) resulted in a significant increase in total yields of THC and CBD for both palm oil and D-limonene cannabis extracts (Table 3). These MAE conditions increased the extraction temperature to 110 ˚C. Increased irradiation cycles of more than 3 cycles, which resulted in a higher extraction temperature, did not significantly increase the total yields of cannabinoids in either extract.

Determination of the anticancer activity of cannabinoids

As shown in Table 4, the MTT assay revealed that both THC and CBD had strong cytotoxicity against MCF-7 and HepG2. They also had strong cytotoxicity against HTERT-HME1. DEC containing 0.03% *w/w* CBD and 1.37% *w/w* THC exhibited moderate cytotoxicity against MCF-7 and low cytotoxicity against HepG2. DEC exhibited very low cytotoxicity against hTERT-HME1. D-limonene demonstrated reduced cytotoxic effects on MCF-7 cells and showed no cytotoxicity toward HepG2 and hTERT-HME1.

DISCUSSION

The superior efficiency and environmental benefits of MAE make it suitable for extracting cannabinoids from cannabis.

Table 1. Cannabinoid content and yields of *Cannabis sativa* inflorescence extracts obtained using various extraction solvents

Solvents	Mean ± SD				
	Extraction yield (mL)	CBD concentration (mg/mL)	THC concentration (mg/mL)	Total yield of CBD (mg/2 g powders)	Total yield of THC (mg/2 g powders)
Sunflower oil	11.83±0.14^{a}	0.04±0.00^{a}	2.04±0.19^{a,b}	0.45±0.02^{a}	24.24±2.47^{a}
Olive oil	12.75±0.50^{a}	0.04±0.00^{a}	1.87±0.12^{a}	0.51±0.01^{a}	23.81±0.71^{a}
Soybean oil	12.08±0.88^{a}	0.05±0.00^{b}	2.01±0.12^{a,b}	0.62±0.04^{b}	24.32±0.70^{a}
Palm oil	13.08±0.52^{a}	0.06±0.01^{c}	2.25±0.06^{b,c}	0.77±0.07^{c,d}	29.45±1.32^{b}
Coconut oil	12.33±1.26^{a}	0.06±0.00^{c}	2.43±0.02^{c}	0.73±0.08^{c}	29.96±3.10^{b}
D-limonene	12.67±0.29^{a}	0.07±0.00^{d}	2.34±0.05^{b,c}	0.85±0.05^{d}	29.61±0.73^{b}
Ethanol	9.75±0.87^{b}	0.05±0.00^{b}	2.99±0.46^{d}	0.50±0.06^{a}	28.85±2.21^{b}
Hexane	8.33±0.38^{c}	0.09±0.00^{e}	2.98±0.07^{d}	0.71±0.05^{c}	24.82±1.73^{a}

Values with non-identical letters in the same column are significantly different with statistic values $p<0.05$. Total yield of cannabinoids = extraction yield (mL) x concentration (mg/mL). CBD: Cannabidiol, THC: Tetrahydrocannabinol, SD: Standard deviation

Table 2. Effects of powder-to-solvent ratios on CBD and THC content and yields of *Cannabis sativa* inflorescence extracts containing palm oil and D-limonene

Solvent	Powder content (g)	Mean ± SD				
		Extraction yield (mL)	CBD conc. (mg/mL)	THC conc. (mg/mL)	Total yield of CBD (mg)	Total yield of THC (mg)
Palm oil	1	14.50±0.43^{a}	0.02±0.00^{a}	1.03±0.02^{a}	0.35±0.03^{a}	15.23±0.38^{a}
	2	13.08±0.14^{b}	0.06±0.01^{b}	2.13±0.06^{b}	0.74±0.03^{b}	28.26±0.83^{b}
	4	8.75±0.66^{c}	0.08±0.01^{c}	4.10±0.12^{c}	0.67±0.08^{b,c}	34.87±1.03^{c}
	6	4.92±0.38^{d}	0.11±0.01^{d}	6.41±0.26^{d}	0.56±0.03^{c}	27.60±1.32^{d}
D-limonene	1	14.75±0.25^{a}	0.01±0.00^{a}	0.63±0.05^{a}	0.14±0.00^{a}	9.07±0.74^{a}
	2	13.18±0.16^{b}	0.11±0.01^{b}	2.13±0.02^{b}	1.34±0.17^{b}	27.18±0.30^{b}
	4	8.42±0.52^{c}	0.27±0.01^{c}	5.51±0.11^{c}	2.18±0.07^{c}	44.11±0.88^{c}
	6	5.25±0.25^{d}	0.34±0.00^{d}	7.19±0.10^{d}	1.63±0.01^{b}	33.74±0.45^{d}

Values with non-identical letters in the same column differ significantly for each solvent (statistical values $p<0.05$). Total yield of cannabinoids = extraction yield (mL) x concentration (mg/mL) CBD: Cannabidiol, THC: Tetrahydrocannabinol, SD: Standard deviation

Compared with conventional methods such as heat reflux extraction, soxhlet extraction, supercritical fluid extraction, and ultrasound-assisted extraction, MAE consistently achieves the highest extraction yields of cannabis cannabinoids.[8] This method is more effective and sustainable, requires significantly less solvent, and requires a shorter time frame. These advantages make MAE an optimal choice for cannabinoid extraction.

According to reports, THC and CBD are non-polar compounds that display nearly identical lipophilicity, with log P values of 5.41 and 5.42, respectively. However, their water solubility differed slightly, with log values of 5.93 and 5.41, respectively.[9] Therefore, non-polar solvents should extract both CBD and THC with high efficiency. Based on the chemical structures of D-limonene and vegetable oils, which exhibit non-polar properties, they can be used as an alternative green solvent for extracting naturally occurring active compounds with non-polar properties. The major component of vegetable oils is triglycerides, which are esters of fatty acids and glycerol. Different types of fatty acid composition affect the physical and chemical properties of triglycerides, resulting in different extraction efficiencies for vegetable oils.[10] For example, coconut oil and palm oil contain higher levels of SFAs than the others. The major SFA in coconut oil is lauric acid, whereas palm oil contains palmitic acid as the predominant SFAs.[11] However, among the alternative solvents, D-limonene, palm oil, and coconut oil produce the highest total yields of cannabinoid content, which are not significantly different from those of the ethanol and hexane extracts. Because ethanol and hexane have a low boiling point, they are highly volatile in the MAE. Thus, although these two solvents produced the extract with higher concentrations of both cannabinoids, they produced lower extraction yields and, therefore, produced slightly lower total yields of cannabinoids than those extracted from D-limonene, palm oil, and coconut oil. As a result, they can be an alternative green solvent to extract

Table 3. Effects of irradiation cycles on CBD and THC content and yields of *Cannabis sativa* inflorescence extracts containing palm oil and D-limonene

Solvent	Irradiation cycle	Mean ± SD				
		Extraction yield (mL)	CBD conc. (mg/mL)	THC conc. (mg/mL)	Total yield of CBD (mg/2 g powders)	Total yield of THC (mg/2 g powders)
Palm oil	0.5	12.40±0.53^{a}	0.04±0.00^{a}	1.74±0.06^{a}	0.53±0.04^{a,b}	22.59±0.83^{a}
	1	12.23±1.08^{a}	0.04±0.00^{a}	1.81±0.03^{a}	0.56±0.03^{b,c}	23.53±0.37^{a}
	2	12.08±0.14^{a}	0.04±0.01^{a}	1.87±0.10^{a}	0.49±0.06^{a}	22.43±1.14^{a}
	3	12.68±0.08^{a}	0.04±0.00^{a}	2.11±0.08^{b}	0.52±0.02^{a,b}	26.93±1.00^{b}
	4	12.47±0.06^{a}	0.04±0.00^{a}	2.15±0.07^{b}	0.55±0.02^{a,b,c}	26.84±0.86^{b}
	5	12.54±0.46^{a}	0.05±0.00^{a}	2.15±0.04^{b}	0.60±0.01^{c}	26.82±0.46^{b}
D-limonene	0.5	12.65±0.37^{a}	0.06±0.00^{a}	2.08±0.11^{a}	0.73±0.01^{a}	27.00±1.39^{a}
	1	12.24±0.28^{a}	0.06±0.00^{b}	2.22±0.01^{a,b}	0.81±0.01^{b}	27.81±0.27^{a,b,c}
	2	12.30±0.09^{a}	0.07±0.00^{c}	2.25±0.05^{b}	0.81±0.01^{b}	27.51±0.57^{a,b}
	3	12.67±0.10^{a}	0.07±0.00^{d}	2.35±0.02^{b}	0.87±0.01^{c}	29.39±0.21^{d}
	4	12.65±0.32^{a}	0.07±0.00^{c}	2.24±0.07^{b}	0.86±0.01^{c}	29.14±0.91^{c,d}
	5	12.70±0.60^{a}	0.07±0.00^{c}	2.21±0.03^{a,b}	0.86±0.01^{c}	28.75±0.01^{b,c,d}

Values with non-identical letters in the same column differ significantly for each solvent (statistical values $p<0.05$). Total yield of cannabinoids = extraction yield (mL) x concentration (mg/mL). CBD: Cannabidiol, THC: Tetrahydrocannabinol, SD: Standard deviation

Table 4. Cytotoxic activities of DEC, CBD, and THC against MCF-7 and HepG2 cancer cells and hTERT-HME1 normal cells

Compounds	IC_{50} (µg/mL)			Selectivity index	
	hTERT-HME1	MCF-7	HepG2	MCF-7	HepG2
CBD	35.61	18.46	12.37	1.93	2.88
THC	25.63	24.21	4.30	1.06	5.96
DEC	1537.03	488.85	1336.97	3.14	1.15
D-limonene	N/A	1150.9	N/A	N/A	N/A
5-FU	N/A	1.9	99.83	N/A	N/A

hTERT-HME1: Human mammary epithelium, MCF-7: Human breast cancer cells, HepG2: Human liver cancer, DEC: Standardized D-limonene extract of cannabis, IC_{50}: 50% inhibitory concentration, DEC: D-limonene extract of cannabis, CBD: Cannabidiol, THC: Tetrahydrocannabinol, 5-FU: 5-Fluorouracil, N/A: Selectivity index is not available. Not active for 2000 µg/mL D-limonene and 100 µg/mL 5-FU

cannabinoids. However, for nutraceutical applications, coconut oil consumption considerably increases the levels of low-density lipoprotein cholesterol and total cholesterol compared with palm oil, which may increase the risk of cardiovascular disease.[12] Therefore, D-limonene and palm oil are considered suitable alternative green solvents for cannabinoid extraction, to produce functional food products.

In addition to solvent polarity, powder-to-solvent ratios, and microwave irradiation cycles are common factors that affect MAE efficiency. According to mass transfer principles, during the solid-liquid extraction process, the powder-to-solvent ratio has a significant impact on the concentration gradient between the solute in the powder and the solvent at the surface of the raw material.[13] The increasing diffusion rate of the compounds from the extracted powder into the solvent depends on the concentration gradient, which increases with increasing powder-to-solvent ratio. However, the concentration gradient does not continue to increase once equilibrium is reached, which is characterized by the relationship between the amount of powder and solvent used that gives the maximum yields.[13,14] Additionally, researchers typically perform the MAE method under several irradiation cycles to prevent overheating or bumping during herbal extraction. Furthermore, the number of irradiation cycles in MAE has a significant impact on extraction time and temperature. Time and temperature are critical extraction conditions because they affect the solubility, mass transfer, and stability of natural compounds. However, prolonged extraction and extreme temperatures may lead to the degradation of bioactive compounds.[14]

Recent reports indicate that D-limonene inhibits anti-cancer activity through various mechanisms of action.[15] Accordingly, cannabis extraction using D-limonene has attracted attention and has the potential to be a novel anticancer nutraceutical. The cytotoxicity categorization[16] classifies DEC as having moderate cytotoxicity (IC_{50}: 100-500 g/mL, for herbal extract) against MCF-7 and THC and CBD as potentially toxic substances with moderate cytotoxic activity (IC_{50}: 20-100 µM, for pure compounds) against MCF-7 and HepG2 cell lines. However, only THC exhibited very strong cytotoxicity against HepG2 cell lines (IC_{50}: 1-20 µM, for the pure compound). Calculating the SI value is crucial for evaluating the anticancer activity of herbal drugs. A SI value >3 is classified as a prospective anti-cancer sample.[17] According to these standards, the SI data of DEC was specifically toxic to MCF-7 cells, whereas CBD and THC were not selectively toxic to cells. Although DEC contained only 1.37% *w/w* of THC and 0.03% *w/w* of CBD, it also showed potential cytotoxicity against MCF-7 with higher selectivity than CBD and THC. Nevertheless, using D-limonene as an alternative green solvent for the preparation of a cannabis extract may improve its anti-cancer effects. However, the enhancement of DEC's anticancer properties necessitates careful consideration of cannabis strain selection, specifically those characterized by an ideal balance between THC and CBD. This critical factor plays a pivotal role in the production of cannabis extracts with maximized anticancer potential.

CONCLUSION

The present study identified D-limonene and palm oil as promising alternative green solvents for extracting cannabinoids from cannabis inflorescences under MAE optimal conditions. The MAE method offers several advantages, including reduced time and energy consumption. In this study, DEC exhibited moderate cytotoxicity against MCF-7 cells with higher selectivity than CBD and THC. Therefore, DEC containing an appropriate amount of THC and CBD may exhibit a more satisfying anticancer effect and be a promising candidate for cancer treatment. However, additional research is required to understand the mechanisms of anticancer activity and to investigate additional efficacy and safety profiles.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

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Footnotes

Authorship Contributions

Concept: P.P., Design: P.P., W.S., Wa.S., Data Collection or Processing: W.S., Analysis or Interpretation: P.P., W.S., Wa.S., Literature Search: W.S., Writing: W.S., P.P.

Conflict of Interest: The authors have no conflicts of interest to declare.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71:209-249.
2. Suttithumsatid W, Panichayupakaranant P. Narrative review: phytocannabinoids and their potential use as a phytochemotherapy. Int J Pharmacogn Chinese Med. 2020;4:000209.
3. Al Ubeed HMS, Bhuyan DJ, Alsherbiny MA, Basu A, Vuong QV. A comprehensive review on the techniques for extraction of bioactive compounds from medicinal cannabis. Molecules. 2022;27:604.
4. National Research Council, Committee on Acute Exposure Guideline Levels Committee on Toxicology, Nineteenth interim report of the

committee on acute exposure guideline levels: Part A, National Academies Press, 2011.

5. Machado TQ, da Fonseca ACC, Duarte ABS, Robbs BK, de Sousa DP. A narrative review of the antitumor activity of monoterpenes from essential oils: an update. Biomed Res Int. 2022;2022:6317201.
6. Suttithumsatid W, Kara J, Lomlim L, Nualsri C, Panichayupakaranant P. Acetylcholinesterase inhibitory activity of standardized cannabinoids-rich fractions. Pharm Biomed Res. 2023;9:173-182.
7. Suttithumsatid W, Sukketsiri W, Panichayupakaranant P. Cannabinoids and standardized cannabis extracts inhibit migration, invasion, and induce apoptosis in MCF-7 cells through FAK/MAPK/Akt/NF-κB signaling. Toxicol in Vitro. 2023;93:105667.
8. Chang CW, Yen CC, Wu MT, Hsu MC, Wu YT. Microwave-assisted extraction of cannabinoids in hemp nut using response surface methodology: optimization and comparative study. Molecules. 2017;22:1894.
9. Suttithumsatid W, Shah MA, Bibi S, Panichayupakaranant P. α-Glucosidase inhibitory activity of cannabidiol, tetrahydrocannabinol and standardized cannabinoid extracts from *Cannabis sativa*. Curr Res Food Sci. 2022;5:1091-1097.
10. Yara-Varón E, Li Y, Balcells M, Canela-Garayoa R, Fabiano-Tixier AS, Chemat F. Vegetable oils as alternative solvents for green oleo-extraction, purification and formulation of food and natural products. Molecules. 2017;22:1474.
11. Annisa AN, Widayat W. A review of bio-lubricant production from vegetable oils using esterification transesterification process. MATEC Web Conf. 2018;156:06007.
12. Jayawardena R, Swarnamali H, Lanerolle P, Ranasinghe P. Effect of coconut oil on cardio-metabolic risk: a systematic review and meta-analysis of interventional studies. Diabetes Metab Syndr. 2020;14:2007-2020.
13. Wong B, Tan C, Ho C. Effect of solid-to-solvent ratio on phenolic content and antioxidant capacities of "Dukung Anak" (*Phyllanthus niruri*). Int Food Res J. 2013;20:325-330.
14. Tchabo W, Ma Y, Kwaw E, Xiao L, Wu M, T. Apaliya M. Impact of extraction parameters and their optimization on the nutraceuticals and antioxidant properties of aqueous extract mulberry leaf. Int J Food Prop. 2018;21:717-732.
15. Mukhtar YM, Adu-Frimpong M, Xu X, Yu J. Biochemical significance of limonene and its metabolites: future prospects for designing and developing highly potent anticancer drugs. Biosci Rep. 2018;38:BSR20181253.
16. Indrayanto G, Putra GS, Suhud F. Validation of *in vitro* bioassay methods: Application in herbal drug research. Profiles Drug Subst Excip Relat Methodol. 2021;46:273-307.
17. Weerapreeyakul N, Nonpunya A, Barusrux S, Thitimetharoch T, Sripanidkulchai B. Evaluation of the anticancer potential of six herbs against a hepatoma cell line. Chin Med. 2012;7:15.