INTRODUCTION
Curcumin, a phenolic compound found in Curcuma spp., exhibits a variety of biological activities, including anti-inflammatory and antioxidant activities [1]. However, because of its low solubility, low stability (both chemically and metabolically), and low bioavailability, its clinical applications have been limited [2,3]. Many curcumin analogs have been synthesized and investigated in attempts to improve its chemical properties and biological activity [4].

Several series of symmetrical monocarbonyl analogs of curcumin (MACs), containing a cyclohexanone or cyclopentanone linker between the two phenyl rings, reportedly have superior anti-inflammatory and antioxidant activity, higher chemical stability, and improved pharmacokinetic profiles compared to curcumin [3,4]. On the other hand, several asymmetrical MAC (AMACs) reportedly exhibit potent anti-inflammatory, antioxidant, and antitumor activities [5-8]. Finally, our group reported that, while AMACs containing a morpholine Mannich base exhibit low antioxidant activity, two compounds exhibit anti-inflammatory, antioxidant, and antitumor activities [5-8]. Finally, our group reported that, while AMACs containing a morpholine Mannich base exhibit low antioxidant activity, two compounds exhibit anti-inflammatory, antioxidant, and antitumor activities [5-8]. Finally, our group reported that, while AMACs containing a morpholine Mannich base exhibit low antioxidant activity, two compounds exhibit anti-inflammatory, antioxidant, and antitumor activities [5-8]. Finally, our group reported that, while AMACs containing a morpholine Mannich base exhibit low antioxidant activity, two compounds exhibit anti-inflammatory, antioxidant, and antitumor activities [5-8].

To understand the effect of Mannich base substitution on the biological activity of compounds, and to discover a new antioxidant and anti-inflammatory compound, we synthesized a series of Mannich base derivatives of one of the AMACs, (2E,6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-(phenylmethylidene)cyclohexan-1-one, and evaluated their antioxidant and anti-inflammatory activity.

MATERIALS AND METHODS
Chemistry
Materials and general procedures
All chemicals and solvents were purchased from commercial sources (Sigma-Aldrich, St. Louis, USA; Merck, Darmstadt, Germany; and Mallinckrodt, St. Louis, USA) and used without further purification. The synthesized compounds’ purity was determined by thin-layer chromatography (TLC) on silica gel 60 F254 plates (Merck, Germany). Melting points were determined via the open-ended capillary method, using the Analogue Melting Point Apparatus SMP11 (Stuart Scientific, UK), and were uncorrected. Infrared (IR) spectra were recorded on an FT-IR spectrophotometer (9400S; Shimadzu, Japan); nuclear magnetic resonance spectra were recorded on an NMR spectrometer (A500a, Agilent, USA) with a DD2 console at 500 MHz for H-NMR and 125 MHz for C-NMR, with d$_6$-DMSO as the solvent for all compounds. High-resolution mass spectra (HR-MS) were recorded using the ESI-TOF LCT Premier XE mass spectrometer (Waters Corp., USA). The synthesized compounds’ purity was determined by thin-layer chromatography (TLC) on silica gel 60 F254 plates (Merck, Germany). Melting points were determined via the open-ended capillary method, using the Analogue Melting Point Apparatus SMP11 (Stuart Scientific, UK), and were uncorrected. Infrared (IR) spectra were recorded on an FT-IR spectrophotometer (9400S; Shimadzu, Japan); nuclear magnetic resonance spectra were recorded on an NMR spectrometer (A500a, Agilent, USA) with a DD2 console at 500 MHz for H-NMR and 125 MHz for C-NMR, with d$_6$-DMSO as the solvent for all compounds. High-resolution mass spectra (HR-MS) were recorded using the ESI-TOF LCT Premier XE mass spectrometer (Waters Corp., USA).

Synthesis of asymmetrical monocarboxyl analogs of curcumin, (2E,6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-(phenylmethylidene)cyclohexan-1-one (1)
Fig. 1: Synthesis scheme for the Mannich base derivatives of (2E,6E)-2-[[4-hydroxy-3-methoxyphenyl]methylidene]-6-(phenylmethylidene)cyclohexan-1-one (2a-d)

Yellow powder, 62.4% yield, mp 108–110°C. FT-IR (KBr) cm$^{-1}$: 2953 (C-H aliphatic), 1669 (C=O), 1572 and 1446 (C=C aromatic), 1269 (C-N), 1155 (C-O).

(2E,6E)-2-[[4-hydroxy-3-methoxy-5-[[1-methylpiperazin-4-yl]methyl]phenyl]methylidene]-6-(phenylmethylidene)cyclohexan-1-one (2c)

Orange powder, 79.5% yield, mp 128–130°C. FT-IR (KBr) cm$^{-1}$: 2951 (C-H aliphatic), 1662 (C=O), 1572 and 1489 (C=C aromatic), 1269 (C-N), 1155 (C-O).

(2E,6E)-2-[[4-hydroxy-3-methoxy-5-[[pyrroolidin-1-yl]methyl]phenyl]methylidene]-6-(phenylmethylidene)cyclohexan-1-one (2b)

Dark red powder, 62.6% yield, mp 120–122°C. FT-IR (KBr) cm$^{-1}$: 2835–2951 (C-H aliphatic), 1716 (C=O), 1562 (C=C aromatic), 1529 (C=N), 1155 (C-O).

**General synthesis of Mannich bases of (2E,6E)-2-[[4-hydroxy-3-methoxyphenyl]methylidene]-6-(phenylmethylidene)cyclohexan-1-one derivatives (2a-d)**

The Mannich base derivatives of (2E,6E)-2-[[4-hydroxy-3-methoxyphenyl]methylidene]-6-(phenylmethylidene)cyclohexan-1-one (2a-d) were prepared via Mannich reactions of compound 1 using the previously reported synthesis method for morpholine Mannich base AMAC derivatives [9]. Compound 1 (2 mmol) was dissolved in ethanol (5 ml) and cooled in an ice bath. Then, we added a corresponding secondary amine (4 mmol) and 37% (v/v) formaldehyde solution (4 mmol) dropwise. The mixture was stirred for 1 h at room temperature (r.t.) and refluxed for 1–10 h until the reaction was deemed complete based on monitoring with TLC. The solvent was evaporated, and the residue obtained was dissolved in methanol (50 ml) and subsequently evaporated. The resulting residue was dissolved in cooled methanol (50 ml) by adding cooled distilled water dropwise. The colored precipitate obtained was filtered, washed with cold methanol, dried at r.t., and purified by column chromatography to obtain pure compounds 2a-d.

(2E,6E)-2-[[4-hydroxy-3-methoxy-5-[[2,6-dimethylmorpholin-4-yl]methyl]phenyl]methylidene]-6-(phenylmethylidene)cyclohexan-1-one (2a)

Yellow powder, 62.4% yield, mp 108–110°C. FT-IR (KBr) cm$^{-1}$: 2852–2933 (C-H aliphatic), 1662 (C=O), 1573 and 1446 (C-C aromatic), 1595 (C=C), 1145 (C-N), 1084 (C-O, CDCl$_3$), 8/9 ppm: 1.17 and 1.25 (two d peaks, 6H, CH$_3$-morpholine), 1.81 and 2.85 (two s and d peaks, 4H, =C-CH$_2$-cyclohexanone), 1.79 (m, 2H, -CH$_2$-cyclohexanone), 3.90 (s, 3H, CH$_3$-cyclohexanone), 53.49 (2C, -CH$_2$-cyclohexanone), 1.86 (4H, -CH$_2$-cyclohexanone), 1.90 (p, CH$_2$-CH$_2$-cyclohexanone), 2.69 (s, 4H, -CH$_2$-cyclohexanone). 1H-NMR (500 MHz, CDCl$_3$) 8/9 ppm: 1.17 and 1.25 (two d peaks, 6H, CH$_3$-morpholine), 1.91 and 2.85 (two s and d peaks, 4H, =C-CH$_2$-cyclohexanone), 2.93 (t, 4H, =CH$_2$-cyclohexanone), 3.90 (s, 3H, CH$_3$-O-Ar), 3.12 (s, 2H, =CH$_2$-cyclohexanone), 4.09 (t, 1H, OH), 6.82 (1H, s, H-Ar), 6.99 (s, 1H, H-Ar), 7.44 (t, 2H, =CH$_2$-cyclohexanone), 7.32 (t, 1H, =CH$_2$-cyclohexanone), 7.45 (d, 2H, =CH$_2$-cyclohexanone), 7.72 and 7.79 (s, 1H, and s, 1H, Ar-CH=C-ethylenic). 13C-NMR (100 MHz, CDCl$_3$) 8/9 ppm: 19.07 (2C, 2,6-d-Ch$_2$-morpholine), 23.15 (1C, CH$_2$-CH$_2$-cyclohexanone), 28.47 and 28.81 (2C, -CH$_3$-cyclohexanone), 58.47 (2C, CH$_2$-N-CH$_2$-cyclohexanone), 56.69 (1C, CH$_2$-O-CH$_2$-morpholine), 113.74, 120.77, 124.10, 127.30, 128.59, and 133.82 (6C, C$_2$), 128.47, and 130.42 (4C, C$_3$), 136.19, 136.39, 136.59, and 137.59 (4C, C=C-ethylenic), 147.84 (1C, CAr-O), 148.31 (1C, CAr-O), 190.18 (1C, C=O). HR ESI-MS (m/z) was 4482421 ([M+H]$^+$), calculated mass for C$_{37}$H$_{43}$NO$_7$ 448.2409 (error: 2.67 ppm).

(2E,6E)-2-[[4-hydroxy-3-methoxy-5-[[1-methylpiperazin-4-yl]methyl]phenyl]methylidene]-6-(phenylmethylidene)cyclohexan-1-one (2b)

Orange powder, 79.5% yield, mp 128–130°C. FT-IR (KBr) cm$^{-1}$: 2750–2931 (C-H aliphatic), 1670 (C=O), 1531 and 1468 (C-C aromatic), 1591 (C=C), 1261 (C-N), 1153 (C-O, H-NMR (500 MHz, CDCl$_3$), 8/9 ppm: 1.79 (m, 2H, =CH$_2$-CH$_2$-cyclohexanone), 2.28 (3H, CH$_3$-N-piperazine), 2.58 (m, 8H, =CH$_2$-N-CH$_2$-cyclohexanone). 2.72 (t, 4H, =CH$_2$-cyclohexanone), 3.09 (s, 3H, CH$_3$-O-Ar), 3.75 (s, 2H, Ar-CH$_2$-N), 6.81 (1H, s, H-Ar), 6.97 (s, 1H, H-Ar), 7.31 (t, 2H, =CH$_2$-cyclohexanone), 7.38 (d, 2H, =CH$_2$-cyclohexanone), 7.44 (t, 1H, =CH$_2$-cyclohexanone), 7.70 and 7.77 (s, 1H, and s, 1H, Ar-CH=C-ethylenic). 13C-NMR (100 MHz, CDCl$_3$) 8/
The synthesized compound (2d) was screened for anti-inflammatory activity via the inhibition of heat-induced albumin denaturation method, previously reported by our research group, using diclofenac sodium and curcumin as the standards [9]. The reaction mixtures consisted of 0.5 ml solutions of the standard or test compounds in methanol, in various concentrations, combined with 4.5 ml of bovine serum albumin (BSA) solution (0.5% w/v, pH 6.3) prepared in Tris-buffered saline. These mixtures were heated for 10 min in a water bath at 70°C ± 2. After cooling to rt, the mixtures' turbidity was measured in triplicate at 660 nm using a UV-Vis spectrophotometer (1601, Shimadzu, Japan). The control was prepared as above but without the test compounds. The percentage (%) of inhibition was calculated using the formula:

\[
\text{% Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test compound}}{\text{Absorbance of control} \times 100}
\]

The test compound's capacity to inhibit denaturation was expressed as an IC\text{\textsubscript{50}} value which was calculated by plotting the percentage inhibition against the concentration of the test compound.

**RESULTS AND DISCUSSION**

**Chemistry**

Various MA\textsubscript{4}s have been synthesized in an attempt to improve the biological activity and other properties [3-8]. The recent study reported that MA\textsubscript{4}s containing a morpholine Mannich base substituted into the phenolic ring and various substituents at para position on another phenyl ring exhibited low antioxidant activity, whereas the compounds containing methoxy or fluoro substituents at the para position on another phenyl ring exhibited potent anti-inflammatory activity that was almost comparable to that of the standard, diclofenac sodium [9]. However, the synthesis and biological activity of various Mannich base AM\textsubscript{4}C derivatives have not yet been reported. In this study, a series of four novel Mannich base derivatives of (2E,6E)-2-{(4-hydroxy-3-methoxyphenyl)methylidene}-6-phenylmethylidene)cyclohexane-1-one (2a-d) were synthesized. The title compounds (2a-d) were synthesized stepwise using the methods summarized in Fig. 1. The synthesized compounds' structures were confirmed by FT-IR, 'H-NMR, 13C-NMR, and HR-MS.

The IR spectra of these compounds showed C-H aliphatic bands at 2750–2992 cm\textsuperscript{-1}. The bands representing the α,β-unsaturated carbonyl groups, C=O aromatic or ethylenic groups, and the C-O-C or C=N moieties appeared between 1611–1716, 1446–1662, and 1084–1269 cm\textsuperscript{-1}, respectively. The 1H-NMR spectra of these compounds exhibited two singlet peaks for the two ethenyl chain protons at 7.70–7.72 and 7.77–7.79 ppm (1H), respectively, indicating the compounds' asymmetrical structures. The two methylene protons connecting the nitrogen of the corresponding amine group to the phenyl ring (2H, Ar-CH=CH\textsubscript{2}) and the three protons of the methoxy groups (3H, Ar-OC\textsubscript{H}\textsubscript{3}) were observed as singlets at 3.69–3.88 and 3.89–3.91 ppm, respectively. The molecular structures were further supported by the 13C-NMR spectra, which provided the number and types of carbons in the compounds, and the HR-MS spectra, which provided the compounds' molecular masses. These values showed complete agreement with the assigned molecular structures [12,13,15].

**Anti-inflammatory and antioxidant activity**

The synthesized compounds (2a–d) were evaluated for anti-inflammatory activity using the inhibition of heat-induced albumin denaturation method. The decline in the test compounds' absorbance, with respect to the control, indicated protein stabilization. Denaturation of protein in vivo is one cause of inflammation; indeed, denaturation in certain rheumatic and arthritic diseases stimulates autoantigen production, which, in turn, drives inflammation [16]. Several anti-inflammatory drugs can inhibit heat-induced albumin denaturation. Therefore, agents inhibiting protein denaturation are worthwhile candidates for anti-inflammatory drugs [17,18]. The compounds showed moderate-to-high inhibition of heat-induced albumin denaturation, which was expressed as IC\text{\textsubscript{50}} values in the range of 1.93–32.75 µM (Table 1 and Fig. 2). In this series, compound 2d, containing the dimethylamine Mannich base moiety, exhibited the most potent activity (IC\text{\textsubscript{50}}=1.93 µM). 2d's activity was comparable to diclofenac sodium (IC\text{\textsubscript{50}}=1.53 µM), four-fold higher than curcumin (IC\text{\textsubscript{50}}=8.43 µM), and 29-fold higher than the parent compound, (2E,6E)-2-{(4-hydroxy-3-methoxyphenyl)methylidene}-6-phenylmethylidene)cyclohexane-1-one (1) (IC\text{\textsubscript{50}}=56.29 µM). A previous study reported that the morpholine Mannich base derivative of AM\textsubscript{4}C compound 1 exhibited anti-inflammatory activity with IC\text{\textsubscript{50}}=41.11 µM [9]. This result is consistent with the results of another study on the introduction of Mannich bases into ibuprofen [19]. Compound 2d should be considered for further study to investigate its action mechanism and toxicity.

The synthesized compounds' (2a–d) antioxidant activities evaluated with the DPPH free radical scavenging assay. This method is fast and
Table 1: Anti-inflammatory activity and antioxidant activity of the synthesized compounds (2a–d)

| Compounds       | Substituent (R) | IC$_{50}$ (μM)±SD | Anti-inflammatory$^a$ | Antioxidant$^b$ |
|-----------------|-----------------|-------------------|----------------------|----------------|
| 1               | H               | 56.29±0.87        | 144.22±0.78          |                |
| 2a              |                 | 10.67±0.01        | 229.6±2.01           |                |
| 2b              |                 | 10.72±0.09        | 57.29±1.14           |                |
| 2c              |                 | 32.75±1.58        | 280.43±0.50          |                |
| 2d              |                 | 1.93±0.07         | 219.22±4.34          |                |
| Curcumin        |                 | 8.43±0.12         | 26.45±0.03           |                |
| Diclofenac sodium |               | 1.52±0.01        | -                    | 27.28±0.48     |
| Quercetin       |                 |                   |                      |                |

$^a$Evaluated with the inhibition of heat-induced albumin denaturation method, (n=3), $^b$Evaluated with the DPPH free radical scavenging assay, (n=3). SD: Standard deviation, DPPH: 2,2-diphenyl-2-picrylhydrazyl.

Fig. 2: The inhibition of heat-induced bovine serum albumin denaturation (IC$_{50}$) by the Mannich base derivatives of (2E,6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-(phenylmethylidene)cyclohexan-1-one (compounds 2a–d). Data represented as mean ± standard deviation (n=3).
the nitrogen atom of the Mannich base derivative of cyclovalone, the higher the DPPH free radical scavenging activity of the compound [14]. However, this relationship was not observed for compounds 2a–d.

CONCLUSION

A series of four novel Mannich bases derivatives (compounds 2a–d) of one AMAC (2E,6E)-2-[(4-hydroxy-3-methoxyphenyl)methylene]-6-(phenylmethylidene)cyclohexan-1-one, was successfully synthesized. The compounds’ anti-inflammatory and antioxidant activities were evaluated using the inhibition of heat-induced albumin denaturation method and the DPPH free radical scavenging assay, respectively. All synthesized compounds (2a–d) showed moderate-to-high anti-inflammatory activity and low-to-moderate antioxidant activity. Compound 2d, containing the dimethylamine Mannich base moiety, showed the highest anti-inflammatory activity, comparable to that of diclofenac sodium. This compound should be studied further to investigate its action mechanism and its toxicity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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