Short Communication:

Synthesis of New Indazole Analogs of Curcumin as Antioxidant and Anti-inflammatory Candidates: An *In Vitro* Investigation

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Received: February 21, 2023
Accepted: June 11, 2024

DOI: 10.22146/ijc.82443

Abstract: *The development of analog curcumin compounds by modifying the structure of monocarbonyl into an analog indazole of curcumin (AIC) is recognized to have a great potential. Still, only a few reports have been available. Rarely occurring in nature, indazole molecules are typically created through chemical synthesis. Therefore, this study aimed to synthesize six new AIC compounds with a particular focus on testing* in vitro *antioxidant activity using the DPPH and FRAP methods, as well as anti-inflammatory activity using the protein denaturation method. The results showed that the compounds formed had high anti-inflammatory activity but low antioxidant activity. All synthesis products produced higher anti-inflammatory activity than standard diclofenac sodium and curcumin compounds. Specifically, compound 3a showed the highest anti-inflammatory activity with an* IC_{50} *= 0.548 ± 0.062 μM. Therefore, it was concluded that compound 3a has the potential to be further studied for anti-inflammatory activity.*

Keywords: *analog indazole curcumin; anti-inflammatory; antioxidant;* in vitro

■ INTRODUCTION

New drugs are constantly being developed, particularly with antioxidant, anti-inflammatory, antiviral, and anticancer activities. In this context, curcumin derivatives represent a leading chemical with promising potential for development towards antiviral, antioxidant, anticancer, and anti-inflammatory effects [1-3]. Curcumin induces anti-inflammatory activity by inhibiting several target molecules such as nuclear factor-κB (NFκB), tumor necrosis factor-α (TNF-α), and cyclooxygenase enzyme 2 (COX-2) [4]. This compound has low stability in aqueous solutions at physiological pH and is easily degraded in a phosphate buffer at pH 7.4, about 90% is degraded within 30 min [5].

According to clinical studies, curcumin nanoparticles showed increased solubility and bioavailability. The therapeutic efficacy increased by applying the drug in different nanoforms [6]. A breakthrough was made in manufacturing food-grade curcumin nanoparticles with decreased crystallinity and maximizing utilization due to increased bioaccessibility [7]. Aside from formulation, the development of curcumin structure also entails modification to achieve increased stability and provide better pharmacological activity.

One of the most recent developments is the modified curcumin analog compounds indazole group (AIC). In previous studies, six AIC have been successfully synthesized, showing good potential as anticancer compounds for colorectal carcinoma [8-9]. Rarely occurring in nature, indazole molecules are typically created through chemical synthesis. The

biological activity of bioactive compounds is markedly enhanced by the creation of indazole rings [10-12]. Several studies on indazole compounds have also shown potential as anti-inflammatory agents, closely related to anticancer activity [13-14].

Reactive oxygen species and reactive nitrogen species are examples of naturally occurring antioxidants that are effective in combating free radicals in the body [15]. Nonsteroidal anti-inflammatory drugs are the most commonly used medications for treating inflammation. On the other hand, the kidneys may experience a number of adverse consequences in addition to major effects on the skin, liver, platelet function, cardiovascular homeostatic processes, and danger of bleeding into the gastrointestinal mucosa [16]. This indicates the need for improved efforts to develop new and safer anti-inflammatory drugs. AIC compounds may be investigated *in vitro* for their anti-inflammatory and antioxidant properties. As potential anti-inflammatory medication options, this study aimed to gather information on the activity of AIC compounds, specifically regarding their antioxidant and anti-inflammatory properties.

■ EXPERIMENTAL SECTION

Materials

(7*E*)-3-(4-Methoxyphenyl)-7-[(4-methoxyphenyl)methylidene] is one of the chemicals utilized in the materials. 4-([(7*E*)-3-(3,4-dimethoxyphenyl)-4,5,6,7-tetrahydro-3a*H*-indazol-7-ylidene]methyl) (compound 3a), 4,5,6,7-tetrahydro-3a*H*-indazole2-methoxyphenol (compound 3b), (7*E*)–3-(3,4-dimethoxyphenyl)-7-(4-methoxybenzylidene)-4,5,6,7-tetrahydro-3a*H*-indazole (compound 3c), (7*E*)-3-(3,4-dimethoxyphenyl)-7-(3,4-dimethoxybenzylidene)-4,5,6,7-tetrahydro-3a*H*-indazole (compound 3d), 1-[(7*E*)-3-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxy-3-methoxybenzylidene)-3,3a,4,5,6,7-hexahydro-2*H*-indazol-2-yl] (compound 5a), and 1-[(7*E*)-3-(4-hydroxyphenyl)-7-(4-hydroxybenzylidene)-3,3a,4,5,6,7–hexahydro–2*H*–indazol2-yl]-ethan-1-one (compound 5b) were used in this experiment. The supplier of sodium diclofenac was PT Kimia Farma in Indonesia, while Merck or Sigma-Aldrich chemical suppliers provided the remaining chemicals and solvents. As explained in previous literature, all compounds have been synthesized and characterized by IR, NMR, and mass spectrometry [8-9].

Instrumentation

A UV-vis spectrophotometer (Shimadzu, Japan) was used to measure absorbance in samples and determine standards for anti-inflammatory antioxidant activity. A personal computer, a Mac mini with Intel® Xeon(R) E5620, was used as the hardware for the drug scan analysis.

Procedure

Drug scan analysis

An online analysis of the drug scan and synthesis accessibility was conducted at (http://swissadme.ch). The ligand file had to be uploaded in smile format for the analysis to be completed, and the findings in Excel format had to be downloaded [17].

Antioxidant assay

DPPH method. By using curcumin as a comparison, the ferric reducing ability potential (FRAP) technique and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging were used to screen the title compounds (3a-d, 5a-b) for antioxidant activity [16,18]. A mixture of title compounds (3a-d, 5a-b) or curcumin solution at 100 µg/mL (0.5 mL) and DPPH solution at 50 µg/mL (2 mL), both in methanol, was maintained at room temperature for 30 min, and then analyzed spectrophotometrically at 517 nm. The inhibition (%) was calculated by Eq. (1);

$$\text{Inhibition } (\%) = \frac{(\text{Acs} - \text{Ats})}{\text{Acs}} \times 100 \quad (1)$$

where Acs = control solution absorbance and Ats = test solution absorbance.

FRAP Method. Following a 6-min incubation period at 37 °C, 0.1 mL of the title compound (3a-d, 5a-b) or curcumin solution at 100 µg/mL in methanol was combined with 0.9 mL of new FRAP reagents. The mixture was then analyzed spectrophotometrically at 595 nm. The Eq. (2) was used to get the Fe^{2+} (Ferrous) equivalent;

$$\text{Ferrous equivalent } (\%) = \frac{Ax}{Ay} \times 100 \quad (2)$$

where Ax = sample's absorbance and Ay = ferrous sulfate (1000 μM) solution's absorbance.

Anti-inflammatory assay

Protein denaturation method. By thermally induced denaturing protein, the title compounds (3a-d, 5a-b) were evaluated as anti-inflammatory agents at 15 μM in methanol for preliminary and at different concentrations (0.15 to 6.0 μM) for IC_{50} determination [19-21]. The mixture was maintained at 37 °C for 15 min, heated to 70 °C for 10 min, cooled, and the turbidity was measured at 660 nm. The 0.5 mL standard diclofenac sodium or test solutions and BSA solution 0.5% (w/v) in tris-buffer saline with pH of 6.3 and volume of 4.5 mL were added. The Eq. (1) was applied in order to determine the inhibition (%). IC_{50} values were determined by the GraphPad Prism software.

■ RESULTS AND DISCUSSION

The structure of AIC compounds is shown in Fig. 1 and Table 1 (6).

Drug Scan Analysis

Lipinski's rule of five was satisfied by the pharmacological scan of six AIC compounds (Table 2). The molecular weight of ligands ranged from 346.42 to 406.47 g/mol, surpassing that of diclofenac but still within the range of Lipinski's rule of five (MW < 500 g/mol). The rule is a collection of *in silico* parameters used in drug discovery to rank compounds that are likely to be absorbed more easily. Lipinski's rule describes the general solubility of specific substances to

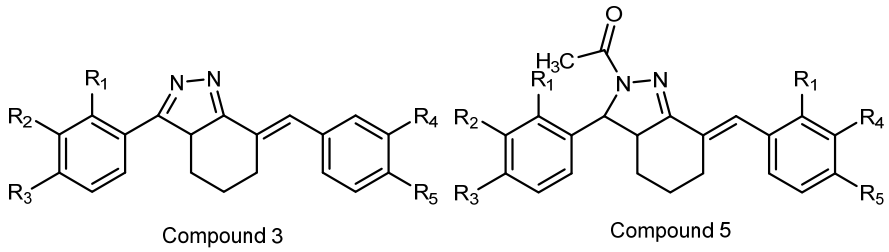

Fig 1. AIC structure of compounds 3 and 5

Table 1. Substituents of AIC structure

No	Structure	Substituent				
		R_1	R_2	R_3	R_4	R_5
1	3a	–H	–H	$–OCH_3$	–H	$–OCH_3$
2	3b	–H	$–OCH_3$	$–OCH_3$	$–OCH_3$	–OH
3	3c	–H	$–OCH_3$	$–OCH_3$	–H	$–OCH_3$
4	3d	–H	$–OCH_3$	$–OCH_3$	$–OCH_3$	$–OCH_3$
5	5a	–H	$–OCH_3$	–OH	$–OCH_3$	–OH
6	5b	–H	–H	–OH	–H	–OH

Table 2. The prediction results are based on Lipinski's rule of five and synthesis accessibility

Compounds code	Prediction using Lipinski's rule of five				
	MW (g/mol)	Log P	Hydrogen bond acceptor	Hydrogen bond donor	TPSA (Å)
3a	346.42	4.25	4	0	43.18
3b	392.45	3.83	6	1	72.64
3c	376.45	4.22	5	0	52.41
3d	406.47	4.18	6	0	61.64
5a	422.47	3.22	6	2	91.59
5b	362.42	3.13	4	2	73.13
Diclofenac	296.15	3.66	2	2	49.33

influence their passive diffusion penetration across cell membranes. As a potential medication, the rule can also be used to forecast the pharmacokinetics of a combination [17]. Oral medications should generally have good water solubility and intestinal permeability profiles with no more than one Lipinski's rule violation [22].

Antioxidant Activity Test with DPPH Method

Two methods were used to assess the antioxidant activity: FRAP and DPPH free radical suppression. The synthesized compounds were tested at a concentration of 100 µg/mL. The technique for DPPH depends on antioxidant substances reducing DPPH• free radicals [19]. The DPPH free radical reacted with antioxidants, resulting in a new bond and a change in the color of the solution from purple to yellow (decoloration). The color changed with an increased concentration of antioxidants happened since DPPH radicals accepted electrons from antioxidants. The decrease in DPPH radicals was monitored by a reduction in absorbance at 517 nm. Fig. 2 displays the findings of the antioxidant activity test using the DPPH method.

When compared to curcumin, five AIC compounds exhibited minimal antioxidant activity, suggesting that the produced compounds' capacity to suppress DPPH free radicals was considerably diminished. Benefits of the DPPH approach include its speed, ease of use, affordability, and capacity to quantify antioxidants in solid and liquid samples and complicated biological systems. Nevertheless, this technique has certain drawbacks, such as its sensitivity to solvents; for example, methanol and acetone often have lower DPPH absorbance than other solvents [19,23-24]. Based on the results, compound 5a provided the most significant %inhibition, of 67.12 ± 0.23%, compared to other new AIC compounds at the same concentration (100 ppm).

Antioxidant Activity Test with FRAP Method

The FRAP method determined the total antioxidant activity by calculating the sample's capacity to degrade Fe^{3+} to Fe^{2+} ions. The concentration of the sample used was 100 µg/mL, while an acetic pH of 3.6 (acid) was used to maintain the solubility of Fe. In the FRAP test, incubation was carried out at a temperature of 37 °C for 4 min. This was significant because the test's redox reaction happened during incubation [25]. Based on the FRAP test results (Fig. 3), compounds 3a-c had a low ability to reduce Fe^{3+} ions, while 3d, 5a-b showed moderate antioxidant activity. The low antioxidant activity in the synthesized compounds was possibly due to the absence of hydroxyl group as an electron donor, offering stability to free radicals. Compounds with a hydroxyl group in the para position tend to have excellent antioxidant activity, such as curcumin [23]. Based on the results, compound 5a produced the most significant total antioxidants, namely 314.47 ± 0.59%, compared to other new AIC compounds at the same concentration (100 ppm).

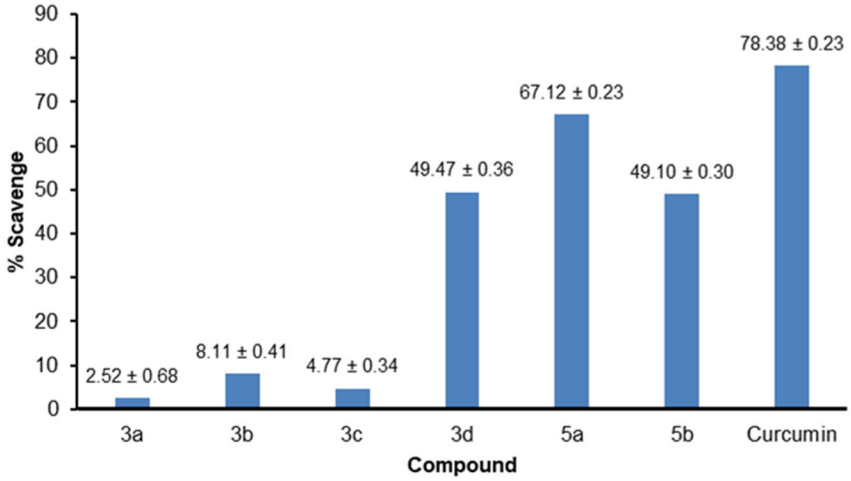

Fig 2. The concentrations and DPPH radical scavenging (%) curve of the synthesized compounds

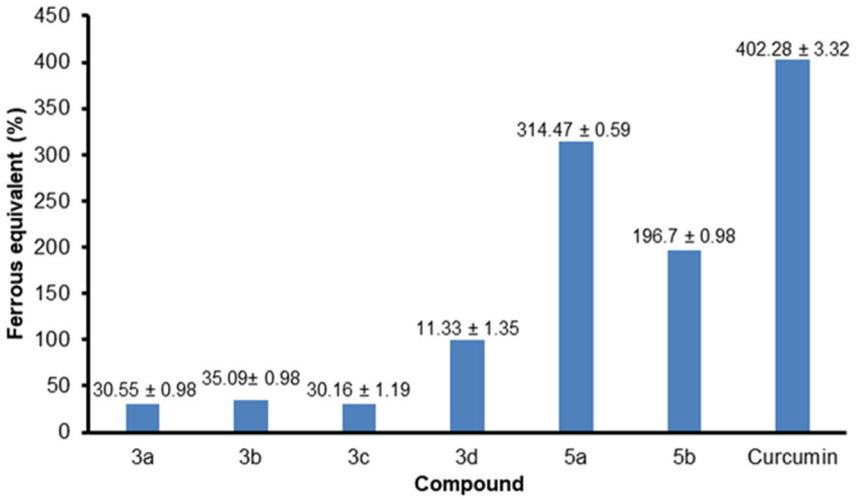

Fig 3. The concentrations and ferrous equivalent (%) curve of synthesized compounds

Anti-inflammatory Activity by the Method of Protein Denaturation

In this study, anti-inflammatory tests were carried out using the protein denaturation method in which proteins lose tertiary and secondary structures due to external stress factors, organic solvents, strong acids or bases, and heat. Proteins lose biological function when denatured, leading to inflammation [26]. Anti-inflammatory tests were conducted on five AIC compounds, with curcumin as the comparison and diclofenac sodium being the standard. The results presented in Table 3 and Fig. 4 showed that AIC compounds had higher inhibition ability than diclofenac sodium and curcumin comparison. According to established standards, a compound that inhibits protein denaturation by more than 20% can be used as a potential anti-inflammatory agent [26]. Based on the results, compound 3a had the lowest IC_{50} value, showing the best activity of 0.548 ± 0.062 µM compared to others. In compound 3a, there was also a methoxy group, which has an anti-inflammatory role based on previous literature [27].

Table 3. IC_{50} value anti-inflammatory activity of AIC compounds

No.	Compound	IC_{50} (µM)
1.	3a	0.548 ± 0.062
2.	3b	0.875 ± 0.055
3.	3c	0.973 ± 0.041
4.	3d	0.941 ± 0.074
5.	5a	1.004 ± 0.062
6.	5b	0.582 ± 0.049
7.	Curcumin	0.706 ± 0.021
8	Diclofenac	0.563 ± 0.017

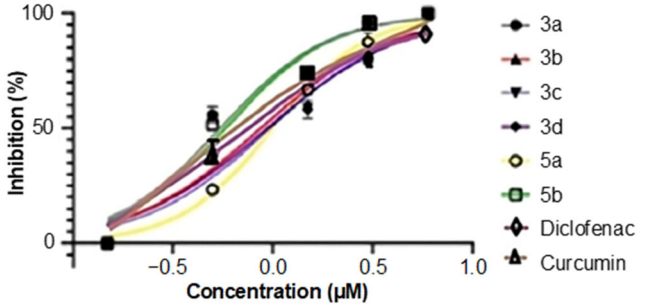

Fig 4. Relationship between test compound concentrations and inhibition of protein denaturation activity

■ CONCLUSION

In conclusion, based on the results of *in vitro* antioxidant assays, compound 5a provided better potential compared to curcumin. However, in the results of *in vitro* anti-inflammatory tests compound 5a provided the best potential compared to diclofenac and curcumin. Compound 5a requires more *in vitro* and *in vivo* assays to validate its antioxidant and anti-inflammatory properties.

■ ACKNOWLEDGMENTS

This work was supported by the Research and Development Department of UHAMKA in 2022 (No. 379/F.03.07/2021), which was gratefully appreciated.

■ CONFLICT OF INTEREST

The authors declare no conflict of interest.

■ AUTHOR CONTRIBUTIONS

Hariyanti and Azminah conducted and carried out the experiments, data acquisition, analysis, interpretation, and participated in drafting the manuscript. Hayun and Arry Yanuar help with the interpretation and conceptualization of the manuscripts.

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