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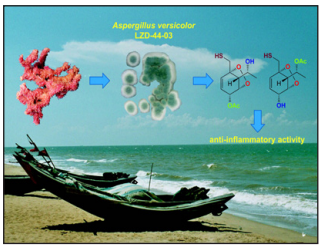

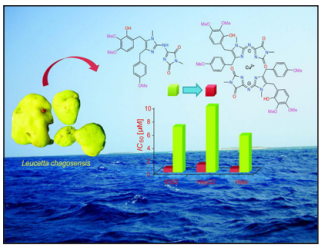

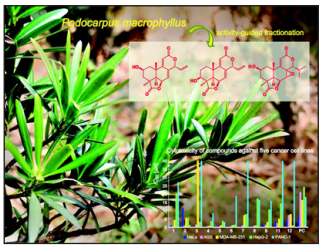

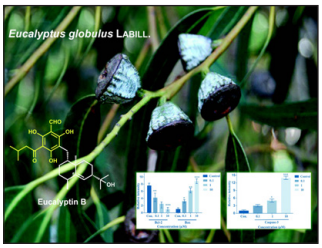

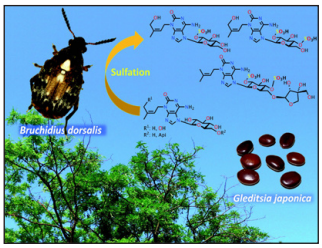

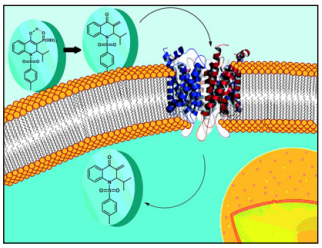

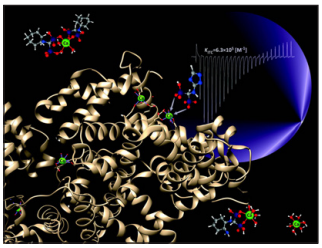

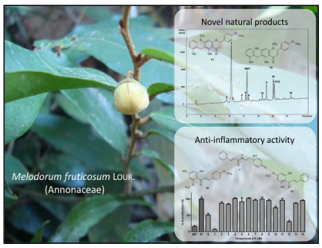

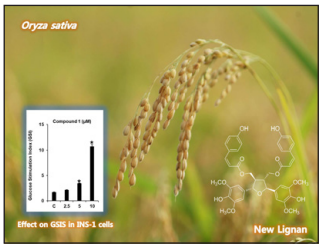

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FULL PAPER

CHEMISTRY & BIODIVERSITY

Activity of Fluorinated Curcuminoids against *Leishmania major* and *Toxoplasma gondii* Parasites

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A new 3,4-difluorobenzylidene analog of curcumin, CDF, was recently reported, which demonstrated significantly enhanced bioavailability and *in vivo* anticancer activity compared with curcumin. For highlighting the antiparasitic behavior of CDF, we tested this compound together with its new *O*-methylated analog MeCDF against *Leishmania major* and *Toxoplasma gondii* parasites. Both CDF and MeCDF were tested *in vitro* against *L. major* and *T. gondii*. In addition, the *in vitro* cytotoxicity against Vero cells and macrophages was determined and selectivity indices were calculated. The DPPH radical scavenging activity assay was carried out in order to determine the antioxidant activity of the test compounds. Both compounds showed high activities against both parasite forms with EC_{50} values in the (sub-)micromolar range (0.35 to 0.8 μM for CDF, 0.31 to 1.2 μM for MeCDF). The higher activity of CDF against *L. major* amastigotes when compared with MeCDF can in parts be attributed to the antioxidant activity of CDF while MeCDF lacking any antioxidant activity was more active than CDF against *T. gondii* parasites. In conclusion, CDF and MeCDF are promising antiparasitic drug candidates due to their high activities against *L. major* and *T. gondii* parasites.

Keywords: curcumin, CDF, neglected tropical diseases, *Leishmania major*, *Toxoplasma gondii*.

Introduction

Leishmaniasis is classified as a neglected tropical disease (NTD) and it is clinically subdivided into cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). The CL form is triggered by several *Leishmania* species such as *L. major*, *L. tropica*, *L. mexicana*, and *L. amazonensis*. CL is the dominant form of leishmaniasis, which is responsible for up to 1 million patients every year and affecting mainly young persons.[1,2] CL often leads to severe and disfiguring skin lesions which poses the problem of stigmatization depressing especially young patients.[3,4] Current treatment options for CL patients comprise toxic antimonials, miltefosine, amphotericin and pentamidine.[2] The emergence of drug-resistant parasite forms as well as the systemic toxicity of clinically applied drugs such as pentavalent antimonials pose severe problems to the clinician and new potent antiparasitic drugs against leishmaniasis are sought. Toxoplasmosis is another infection disease brought about by the globally occurring protozoal *Toxoplasma gondii* parasite. In particular, immune-compromised people are in danger of grave complications upon infection with *T. gondii*, and, thus, the development of new drugs for the treatment of toxoplasmosis is necessary, too.[5] Currently, both CL and toxoplasmosis are endemic diseases in countries of the Middle East.[6]

Curcumin [(1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione] (*Figure 1*) is a biologically active constituent of turmeric, a spice made

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.202100381

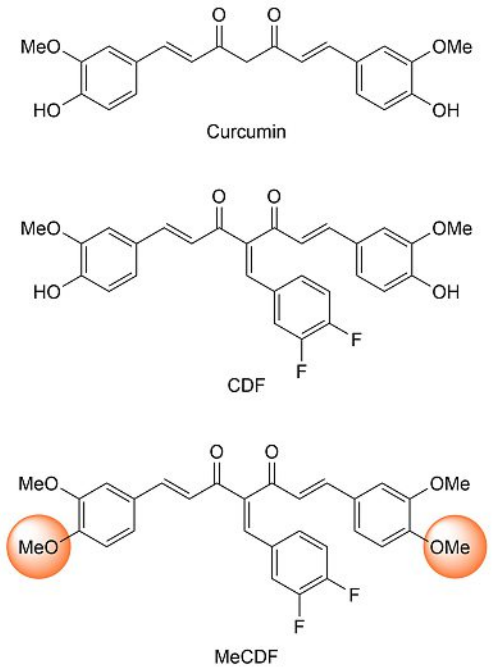


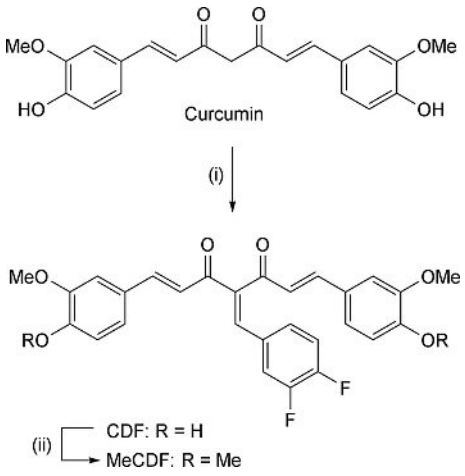
Figure 1. Structures of curcumin and its semi-synthetic derivatives CDF and MeCDF (the new methoxy groups of MeCDF are highlighted by spheres).

from the roots of *Curcuma longa* which is widely applied in Indian cuisine and traditional Indian medicine.[7] Curcumin has shown significant anticancer activity by addressing various cellular targets involved in cancer cell survival and metastasis including inhibition or down-regulation of NF-κB, COX-2, STAT-3, Akt, and MMP-2.[8–12] A phase II trial in patients with advanced pancreatic cancer revealed high tolerability of curcumin in all patients and efficacy of curcumin, orally administered as a monotherapy, in some patients with pancreatic cancer.[13] As a natural product of a tropical plant, curcumin has great potential as a drug against infections and tropical diseases.[14,15] Curcumin was tested against various protozoal parasites and it showed activity against *Leishmania*, *Plasmodium* and *Toxoplasma* species.[16,17] Curcumin and its metal complexes displayed activity against *Leishmania major*.[18] In addition, curcumin inhibited glyoxalase 1 of *Toxoplasma gondii* and the proliferation of this parasite *in vitro*.[19]

The efficacy of curcumin as a therapeutic agent for cancer diseases is often hampered by its low bioavailability. Modified curcumin derivatives with improved bioavailability frequently show a significantly enhanced *in vitro* and *in vivo* activity compared with parent curcumin.[20] Fluorinated curcuminoids like EF24 were reported to be superior to curcumin in the treatment of various cancers.[21,22] Padhye and coworkers developed the difluorobenzylidene derivative (1*E*,6*E*)-4-[(3,4-difluorophenyl)-methylidene]-1,7-bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione, a.k.a. CDF (*Figure 1*).[23] CDF exhibited improved bioavailability and distinct activity against various tumors including prostate and colon carcinomas.[24–26] It was well tolerated by mice and, thus, is an auspicious drug candidate for other human diseases such as leishmaniasis and toxoplasmosis, too. In the present report we disclose first promising results of CDF and of its new *O*-methylated analog MeCDF (*Figure 1*) concerning their activity against *L. major* and *T. gondii* parasites, which may be of relevance for the design of new potent drugs against these parasites.

Results and Discussion

CDF was prepared from curcumin and 3,4-difluorobenzaldehyde according to a literature procedure (*Scheme 1*).[23] The new compound MeCDF was prepared from CDF and methyl iodide via Williamson etherification in high yield (96%). MeCDF was obtained as a yellow solid and analyzed by NMR spectroscopy and mass spectrometry. Four CH_3 groups according to four methoxy groups of MeCDF were identified in the ^{1}H-NMR spectrum. The asymmetric structure of MeCDF due to the 3,4-difluorobenzylidene residue led to distinctly different shifts for each



Scheme 1. Reagents and conditions: (i) 3,4-Difluorobenzaldehyde, piperidine, MeOH, r.t., 48 h; (ii) MeI, K_2CO_3, DMF, r.t., 24H, 96%.

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carbonyl signal in the ^{13}C-NMR spectrum ($\delta = 186.4$ and 197.3 ppm, $\Delta\delta = 10.9$ ppm).

CDF and MeCDF were initially tested against *Toxoplasma gondii* parasites and non-malignant Vero kidney epithelial cells (*Table 1*). Their activities were compared with the activity of curcumin and the known antiparasitic drug atovaquone (ATO) (*Table 1*). Both CDF and MeCDF showed activities against *T. gondii*, with sub-micromolar EC_{50} concentrations, exceeding that of curcumin. MeCDF was especially active here with an EC_{50} value below 0.4 μM and it was more than twice as active as the first-generation derivative CDF. Tests against *L. major* parasites revealed high activities of CDF and MeCDF against promastigotes ($EC_{50} < 0.4$ μM). CDF also exhibited a high activity against *L. major* amastigotes while MeCDF was less active here (*Table 1*). CDF surpassed the activity of the approved antileishmanial drug amphotericin B (AmB) and showed reasonable selectivities for *L. major* promastigotes and amastigotes (SI > 2.4). We also detected an antioxidant activity for CDF ($IC_{50} =$ 52.2 μM) while MeCDF showed no antioxidant activity ($IC_{50} > 300$ μM). These results clearly show that the activities of CDF and MeCDF against *L. major* promastigotes and *T. gondii* are superior those of curcumin by far. The antiparasitic activities of CDF and MeCDF match the efficacy of approved antiparasitic drugs such as AmB and ATO in these parasites. Although the observed selectivities of CDF and MeCDF are lower than the selectivity of ATO for *T. gondii* and AmB for *L. major*, the many other positive effects and properties of CDF suggest it to be a promising, well tolerated, and effective drug candidate for parasitic diseases. The *in vivo* activities of MeCDF remain to be elucidated. The secondary antioxidant effect seems to play only a minor role for the antiparasitic activity of CDF and none at all for MeCDF.

CDF and MeCDF were also tested for activity against macrophages (*Table 2*). MeCDF showed a distinctly lower activity against macrophages than against *L. major* promastigotes with an SI value of 4.4. MeCDF displayed a lower activity against macrophages than against Vero cells, while CDF showed virtually the same toxicity to both cell types. Hence, the SI values of CDF based on Vero cells and on macrophages are roughly the same. The approved reference drugs ATO and AmB were less toxic to the macrophages than were CDF and MeCDF. However, the SI value of AmB taking the promastigotes into account (SI = 9.3) was only twice as high as the corresponding SI value of MeCDF (SI = 4.4). As mentioned above, in vivo activities of MeCDF remain to be elucidated. But since it

Table 1. Inhibitory concentrations IC_{50} (in μM)[a] of test compounds when applied to cells of the Vero (African green monkey kidney epithelial) cell lines and antioxidant DPPH, effective concentrations EC_{50} (in μM)[a] when applied to cells of *Toxoplasma gondii* or *Leishmania major* promastigotes and amastigotes. ATO and AmB were applied as positive controls.

Compounds	*Toxoplasma gondii* (EC_{50})	Promastigotes (EC_{50})	Amastigotes (EC_{50})	Vero (IC_{50})	SI (Vero/*T. gondii*)[b]	SI (Vero/promastigotes)[b]	SI (Vero/amastigotes)[b]	Anti-DPPH
CDF	0.8	0.37	0.35	1.0	1.2	2.7	2.9	52.2
MeCDF	0.33	0.31	1.2	0.9	2.7	2.9	0.8	> 300
Curcumin	38.3[c]	103.2[d]	–	–	–	–	–	–
ATO	0.07	–	–	9.5	136	–	–	–
AmB	–	0.83	0.47	7.8	–	9.6	16.4	–

[a] Values are the means of at least three independent experiments (± SD < 15%). They were derived from concentration-response curves obtained by measuring the percentage of vital cells relative to untreated controls after 72 h. [b] Selectivity index (IC_{50}/EC_{50}) calculated from the corresponding IC_{50} values for the Vero cells and the EC_{50} values for *T. gondii* or *L. major*. [c] Value was taken from Goo et al.[19] [d] Value was taken from Fouladvand et al.[18]

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Table 2. Inhibitory concentrations IC_{50} (in μM)[a] of test compounds when applied to macrophages, selectivity indices (SI).[b]

Compounds	Macrophages	SI (promastigotes)	SI (amastigotes)
CDF	1.02	2.8	2.9
MeCDF	1.35	4.4	1.7
ATO	10.1	–	–
AmB	8.1	9.3	17.2

[a] Values are the means of at least three independent experiments (± SD < 15 %). They were derived from concentration-response curves obtained by measuring the percentage of vital cells relative to untreated controls after 72 h. [b] Selectivity index (IC_{50}/EC_{50}) calculated from the corresponding IC_{50} values for macrophages and the EC_{50} values for *L. major* (*Table 1*).

showed similar or higher selectivities than CDF for the mentioned parasites, it is possible that MeCDF will also be well tolerated and highly bioavailable in laboratory animals.

Conclusions

The antiparasitic activities of the semi-synthetic curcuminoids CDF and MeCDF were characterized by EC_{50} values in the low micromolar range. Both compounds can be considered as highly active against *T. gondii* and *L. major* parasites. When compared with the parent curcumin, its analogs CDF and MeCDF were much more active against these parasites. Both CDF and MeCDF were also more active than the approved drug AmB against *L. major* promastigotes. It is also remarkable that CDF and MeCDF were similarly or more active against *L. major* promastigotes than against *L. major* amastigotes. This is remarkable since AmB showed a higher activity against amastigotes than against promastigotes. Based on these findings, future studies will show if a combination of CDF or MeCDF with AmB can lead to synergy effects.

The antioxidant activity of CDF can be correlated with its phenolic hydroxy groups because the tetramethyl ether MeCDF showed no antioxidant activity. However, the antioxidant activity of CDF was only moderate and, thus, the antiparasitic activity of CDF is likely based on other mechanisms, which remain to be elucidated.

Both CDF and MeCDF can be suitable drug candidates for infection diseases caused by these parasites and further in-depth investigations of their potential as anti-parasitic drugs are warranted given the fact that CDF has already been tested *in vivo* where it was well tolerated and showed higher bioavailability than curcumin.[26] In addition, tests of these promising curcumin derivatives against other protozoal parasites will probably identify further possible fields of application.

Experimental Section

General

Column chromatography: silica gel 60 (230–400 mesh). Melting points (uncorrected), Electrothermal 9100; NMR spectra, Bruker Avance 300 spectrometer (see also *Supporting Information*); chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as internal standard; Mass spectra, Thermo Finnigan MAT 8500 (EI). Elemental analysis, PerkinElmer 2400 CHN elemental analyzer. The preparation of CDF was reported previously.[23,27] All tested compounds were > 98 % pure by elementary analysis. All starting compounds were purchased from the usual retailers and used without further purification.

Synthesis of **(1*E*,6*E*)-4-[(3,4-Difluorophenyl) methylidene]-1,7-bis(3,4-dimethoxyphenyl)hepta-1,6-diene-3,5-dione** (MeCDF) CDF (266 mg, 0.54 mmol) was stirred with K_2CO_3 (746 mg, 5.4 mmol) and iodomethane (3.38 mL, 54 mmol) in DMF (5 mL) at room temperature for 24 h. Ethyl acetate was added and the mixture was washed with water. The organic phase was dried over Na_2SO_4, filtered and the filtrate was concentrated in vacuum. The residue was purified by column chromatography (silica gel 60, ethyl acetate/hexane 1 : 1, v/v). Yield: 268 mg (0.52 mmol, 96 %); R_f = 0.41 (ethyl acetate/hexane, 1 : 1); yellow solid of m.p. 94–96 °C; ν_{max} (ATR) cm^{-1} 3002, 2953, 29363, 2838, 1575, 1508, 1464, 1441, 1422, 1340, 1306, 1260, 1232, 1158, 1137, 1118, 1020, 977, 806, 763, 713; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.83 (3H, s), 3.83 (3H, s), 3.84 (3H, s), 3.85 (3H, s), 6.7–7.3 (11H, m), 7.42 (1H, d, J = 16.2), 7.66 (1H, s), 7.73 (1H, d, J = 15.4); ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ 55.8, 55.9, 109.9, 110.4, 110.9, 111.0, 117.5,

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117.8, 118.5, 118.7, 119.5, 123.5, 123.8, 125.1, 126.6, 126.8, 126.9, 127.3, 130.5, 130.6, 137.5, 141.5, 145.8, 147.9, 148.3, 148.5, 149.1, 149.4, 149.5, 151.7, 151.8, 152.1, 152.9, 186.4, 197.3; EI-MS: *m/z* 520 (36) [M^+], 300 (68), 299 (100), 191 (53), 151 (37).

Toxoplasma gondii culture conditions

Tachyzoites of *T. gondii* RH strain were cultivated in Vero cells according to a published method.[28] Complete RPMI 1640 medium (Invitrogen, USA) with 10% fetal bovine serum (FBS, Invitrogen, USA) was used for culturing Vero cells in 96-well plates (5×10^3 cells/well in 200 μL medium), which were incubated at 37 °C supplied with 5% CO_2 for 24 h followed by washing with phosphate buffered saline (PBS) in order to remove the medium. Then, RPMI 1640 medium with 2% FBS containing *T. gondii* tachyzoites (RH strain) at a ratio of 5 (parasite): 1 (Vero cells) was added. After incubation at 37 °C and 5% CO_2 for 5 h, the cells were washed with PBS and then overlaid with medium containing test compounds or atovaquone (ATO, concentrations of 50, 25, 12.5, 6.25, 3.13, 1.65, 0.75, and 0.37 μg mL^{-1}).

After incubation at 37 °C and 5% CO_2 for 72 h, the cells were stained with 1% toluidine prior to the examination under an inverted photomicroscope (MCD-400, Leica, Japan) in order to determine the infection index (number of infected cells from 200 tested cells) of *T. gondii*. The experiment was done in triplicate.[27]

Leishmania major promastigotes and amastigotes

Promastigote stages of *L. major* were isolated and maintained according to a described method.[29] BALB/c mice were injected at hind footpads with 1×10^6 metacyclic promastigotes of *L. major*. Amastigote stage cells of *L. major* were collected from infected mice. After 8-weeks from inoculation, Schneider's medium (Invitrogen, USA) containing antibiotics and 10% FBS was used to transform isolated amastigotes to promastigotes by incubation at 26 °C. Amastigote-derived promastigotes with less than five *in vitro* passages were only used for infection.

For assessing the activity of the test compounds against *L. major* promastigotes, 10^6 promastigotes/mL were cultured in 96-well plates in Schneider's medium with 10% FBS. Then, the test compounds or amphotericin B (AmB) were added to obtain the final concentrations (50, 25, 12.5, 6.25, 3.13, 1.65, 0.75, and 0.37 μg mL^{-1}). Negative control wells contained cultures with DMSO (1%) only. Plates were incubated at 26 °C for 72 h to evaluate the antiproliferative effect. Colorimetric MTT method was used to count viable promastigotes via microplate absorbance spectrophotometer (xMark, Bio-Rad, USA) at 570 nm. The experiment was done in triplicate.[30]

Macrophages from the peritoneal of female BALB/c mice (6–8 weeks old) were used for culturing amastigote stages of *L. major* in order to assess the activity of test compounds against amastigotes. Briefly, 1 mL of 3% Brewer thioglycollate medium/mouse was injected into the peritoneal cavity. After 4 days, the abdominal skin was removed to expose the peritoneal wall. 3 mL of RPMI 1640 medium was injected before cells were collected by aspiration, whereupon 96-wells plates containing RPMI 1640 medium with 10% FBS were used for culturing 5×10^4 cells/well, which were incubated at 37 °C for 4 h in 5% CO_2. After washing with PBS, RPMI 1640 medium containing 5×10^5 promastigotes were added to each well. Then, the cells were incubated at 37 °C in humidified 5% CO_2 for 24 h to enhance infection and differentiation of amastigotes. Washing with PBS for several times removed free promastigotes. Fresh complete RPMI 1640 medium containing test compounds or AmB at final concentrations (50, 25, 12.5, 6.25, 3.13, 1.65, 0.75, and 0.37 μg mL^{-1}) was added and incubated at 37 °C in humidified 5% CO_2 atmosphere for 72 h. DMSO (1%) only in complete RPMI media was used as negative control. The percentage of infected macrophages was assessed microscopically after the removal of the medium, followed by washing with PBS, fixation with methanol and staining with Giemsa. The reading was done in triplicate.[30] The handling of the laboratory animals followed the instructions and rules of the committee of research ethics, Deanship of Scientific Research, Qassim University, permission number 20-03-20.

Cytotoxicity Assay

MTT colorimetric assay was conducted for assessing the cytotoxicity of the compounds according to a method described previously.[31] Briefly, Vero cells or macrophages were cultured in 96-well plates (5×10^3 cells/well/200 μL) in RPMI 1640 medium with 10% FBS and 5% CO_2 at 37 °C for 24 h. Thereafter, PBS was used for washing the cells. Then, the compounds in RPMI complete medium with 10% FBS (at varying

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concentrations of 50, 25, 12.5, 6.25, 3.13, 1.65, 0.75, and 0.37 $\mu g\,mL^{-1}$) were added to 96-well plates and incubated for 72 h. Cells treated with medium containing only 1% DMSO were used as negative control. After washing, MTT (1 $mg\,mL^{-1}$ in RPMI 1640 medium) was added to each well. The cells were incubated for 4 h followed by the removal of the supernatant. Then, 150 μL DMSO was added and a microplate absorbance spectrophotometer was applied for colorimetric analysis ($\lambda = 540$ nm). Cytotoxic effects were expressed by IC_{50} values (concentration that caused a 50% reduction in viable cells). The experiment was done in triplicate.[27]

DPPH Radical Scavenging Activity Assay

The technique was done according to a published method with slight modifications.[32,33] A solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (1 mg/25 mL ethanol, 100 μL) was added to 5 μL of the compound solution with different concentrations (200, 100, 50, 25, 12.5, 6.25, 3.13, 1.65, 0.75, 3.13 $\mu g\,mL^{-1}$). The mixture was shaken for 15 min. A blank sample of the same volume was prepared from ethanol and DPPH. Then, a spectrophotometer at a wavelength of 517 nm was used to determine the absorbance values. The test was performed in triplicate. The scavenging activities of the tested samples were expressed as percentage of inhibition according to the following equation:

$$\text{Percent of DPPH inhibition} = [(AB - A)/AB] \times 100$$

where AA and AB are the absorbance values of the test sample and of the blank sample, respectively.[32,33] The experiment was done in triplicate.

Statistical Analysis

The results were expressed as averages of three readings. EC_{50} and IC_{50} values were calculated by a linear regression equation using Microsoft excel. The SI (selectivity index) values were calculated by dividing the obtained IC_{50} values from the Vero cells or macrophages over the EC_{50} values from the corresponding parasite inhibition tests.

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Author Contribution Statement

T. A. K. contributed to the writing of the manuscript and supervised the study, W. S. K. designed and supervised the biological studies, I. S. A. N. carried out the biological assays, R. S. contributed to the writing of the manuscript and supervised the study, B. B. prepared the new compound MeCDF and wrote the article.

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