Original Article

Effect of Steaming Process on Chemical Content and Biological Activity of *Curcuma zedoaria* Extract

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Abstract

Introduction: The rhizomes of *Curcuma zedoaria* Roscoe have been used to treat infection and inflammation in the Thai traditional medicine. The traditional preparation of *C.zedoaria* is done performed by steaming the rhizome before drying and using. There has been no information about the effect of the steaming on the chemical components and the activity of *C.zedoaria*. In this report, therefore, we compare the chemical content, anti-inflammatory and antimicrobial activities of steamed, and non-steamed rhizomes of *C.zedoaria*.

Methods: The steamed and non-steamed rhizomes of *C.zedoaria* were extracted by maceration with 50% and 95% ethanol to obtain the ethanolic extracts. Then, the extracts were analyzed for curcuminoid content by HPLC technique, antimicrobial activity and anti-inflammatory activity by nitric oxide (NO) production inhibition assay.

Results: The highest curcuminoid content (152.23 ± 1.80 mg/g of dried extract) was found in the 95% ethanolic extract of the steamed rhizome. Both the ethanolic extracts of the steamed and non-steamed rhizomes exhibited anti-microbial activity against *S.aureus, MRSA* and *C.albicans*. The 95% ethanolic extract of the steamed rhizomes showed the highest inhibition effect on nitric oxide production with IC_{50} value of 11.22 ± 1.21 µg/mL.

Conclusion: From these results, it was concluded that the steaming process can increase the curcuminoid content and biological activities of *C.zedoaria* rhizomes. The best solvent for extraction of this plant part is 95% ethanol which give the highest percent yield of extract and strongest antimicrobial and anti-inflammatory activities.

Keywords: *Curcuma zedoaria*, Curcuminoids, Steamed rhizome, Anti-microbial activity, Antiinflammatory activity

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Introduction

Curcuma zedoaria Roscoe or zedoary is a member in the family Zingiberaceae. It is a perennial rhizomatous herb. The rhizome has a brown bark, with orange-yellow inside. It is native to Southeast Asia and South Asia such as India, Nepal, China, Japan, and Thailand. The Traditional medicine has used this rhizome to treat menstrual disorders, dyspepsia, vomiting, diarrhea, burns, thorns and wound healing.[1]

Curcuminoids are the main chemical constituents found in zedoary, consisting of bisdesmethoxycurcumin, demethoxy-curcumin, and curcumin.[2] Furthermore, the rhizome of zedoary contains sesquiterpenoids, monoterpenoids and essential oils.[3] These substances have extensive pharmacological effects, such as antimicrobial, anti-inflammatory and antioxidant activities.[4-8] Recently, it has been shown that the ethanolic extract of steamed ginger exhibits higher inhibitory effect on prostaglandin E2 and nitric oxide than that of non-steamed ginger.[9] The report suggested that the steaming process can increase biological activities of herbal medicine.

In the case of zedoary, Thai traditional doctors steam the rhizome before using due to their beliefs that the process would increase the biological activities of raw material. However, there has been no scientific report to confirm this, and thus, this prompted us to compare the curcuminoid content, antimicrobial and anti-inflammatory activities of steamed rhizome and non-steamed rhizome of zedoary.

Materials and Methods

1. Plant materials

The rhizomes of *C.zedoaria* were purchased from an organic farm at Nakhon Ratchasima, Thailand in January 2019. They were identified by comparing with the authentic voucher specimens at the Bangkok herbarium, Thailand. The herbarium voucher specimen was BK No. 070333.

2. Chemicals and reagents

Ethanol was purchased from C.M.J Anchor company, Thailand. Curcuminoids standard, ampicillin, vancomycin, amphotericin B and resazurin sodium salt were purchased from Sigma, UK. Muller Hinton broth, nutrient agar, sabouraud dextrose agar were purchased from Difco, USA. Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum, trypsin-EDTA and penicillin-streptomycin were purchased from Gibco, USA. MTT, lipopolysaccharide, N-(1-Naphthyl) ethylenediamine dihydrochloride, phosphoric acid 85%, sulfanilamide and prednisolone were obtained from Sigma, USA. Acetonitrile, methanol and acetic acid were purchased from RCI Labscan, Thailand.

3. Plant extraction

All fresh rhizomes were cleaned and divided into two parts. The first part was steamed at 121°C for 15 minutes in an autoclave while the second part was not steamed. Both steamed and non-steamed rhizomes were dried by hot air oven at 45°C and ground into powder. Then, each rhizome powder (100 mg) was separated into two parts and macerated with 500 mL of 50% or 95 % ethanol for 3 days and filtered through Whatman No.1 paper. The residue was macerated and filtered twice. The solvent was removed by rotary evaporator. The 50% and 95% ethanolic extracts were weighed and calculated the percent yield according to the formula below. All extracts were stored at -20°C until used.

$$\text{Percentage yield} = \frac{\text{weight of dried extract(g)}}{\text{weight of rhizome powder (g)}} \times 100 \quad ...(2.1)$$

4. Determination of curcuminoids content by HPLC

The curcuminoids content in *C.zedoaria* extracts was analyzed with HPLC connected with UV detector (Thermo Separation Product®). The analysis was implemented using an analytical column C18 size 250x4.60 mm (Phenomenex®, Luna 5u) and a security guard cartridge (Phenomenex® kit KJO-4282). Acetonitrile and 0.25% acetic acid in an aqueous solution was used as the mobile phase with gradient elution as follow: 90:10, 0 min; 80:20, 5 min; 55:45, 20 min; 55:45, 55 min; 60:40, 55.1 min; 50:50, 60 min; 5:95, 65 min; 5:95, 65.1 min; 90:10, 70 min; 90:10, at flow rate 1 mL/min, except for 20, 50 and 55 min, flow rate 0.8 mL/min.[2]

The stock solutions of standard curcuminoids were prepared at concentration of 10 mg/mL in

methanol. Standard solution of 10 to 3000 µg/mL were prepared and injected (5 µL) into the HPLC system for calibration plots.

The sample solutions were dissolved with methanol at a concentration of 5 mg/mL. Then, 5 µL of sample solution was injected into the HPLC system. The analysis was performed in triplicate. The concentrations of curcuminoids were calculated using the calibration curves of curcuminoids.

5. Anti-microbial activity

Microorganisms used for anti-microbial tests

Two strains of bacteria, namely *Staphylococcus aureus* ATCC 25923 and methicillin resistance *Staphylococcus aureus* (MRSA) DMST 20651 were streaked onto nutrient agar and incubated at 37°C for 24 hours. *Candida albicans* ATCC 8684 was selected for antifungal activity testing. *C.albicans* was cultured on Sabouraud dextrose agar (SDA) and incubated at 37°C for 48 hours. After incubation, a single colony was transferred into Muller Hinton broth (MHB) for further experiment.

Minimum inhibitory concentration (MIC)

MIC was investigated by using a microtiter plate-based method incorporating resazurin as an indicator.[10] In brief, a serial dilutions of *C.zedoaria* extracts from 0.078-10 mg/mL were prepared in MHB. Then, 50 µL of each concentration was transferred into 96 well plate in triplicate. Bacteria or fungus that was cultured in MHB was adjusted turbidity to 0.5 McFarland standard and diluted 200 times with MHB. The bacteria or fungus culture (50 µL/well) was added into each well of 96 well plate containing *C.zedoaria* extracts and incubated 18-20 hours at 37°C for bacteria and 40 hours for fungus. After incubation, 10 µL of resazurin (1 mg/mL) was added into each well and further incubated at 37°C for 2 hours. The MIC value is the lowest concentration of crude extract that gave unchanged resazurin colour.

Minimum microbicidal concentration (MMC)[11]

The microbial solution from MIC testing that showed unchanged resazurin colour was streaked on to NA and incubated at 37°C for 24 hours while fungus was streaked onto SDA and incubated at 37°C for 48 hours. The MMC value was the lowest concentration that showed no colonies on NA or SDA.

6. Anti-inflammatory activity

The anti-inflammatory activity of *C.zedoaria* extracts was investigated using the inhibition of nitric oxide production in LPS-stimulated RAW264.7 cells.[12] RAW264.7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. These cells were sub-cultured every four days. RAW264.7 cells were seeded into 96-well plate with a concentration of 1×10^5 cells/well (100 µL/well) and incubated for 24 hours at 37°C in a 5% CO_2 incubator. After that, the fresh medium (100 µL/well) containing 10 ng/mL of LPS was replaced into the control and sample wells while the fresh medium without LPS was added into the control blank and sample blank. The extracts were prepared with two-fold serial dilution and added to each well. The final concentration of extract was 3.125-100 µg/mL. Then, plate was incubated at 37°C for 24 hours in 5% CO_2 incubator. After incubation, 100 µL of supernatant was removed to new 96-well plates and 100 µL of Griess reagent was added into each well. The absorbance was measured at 570 nm by using a microplate reader. The percentage of inhibition was calculated using the formula:

$$\text{Percentage inhibition} = \frac{\text{OD control - OD sample}}{\text{OD control}} \times 100 \quad ...(2.2)$$

The IC_{50} values of extract were calculated using the Prism program. After that, 10 µL of MTT solution (5mg/ml) was added into each well of the 96-well plate that contained the cell and incubated for 2 hours at 37°C in 5% CO_2. Finally, all medium was removed and isopropanol containing 0.04 MHCl 100 µL was added to each well to dissolve the formazan. The absorbance was measured with a microplate reader at 570 nm. The percent survival was calculated using the formula:

$$\text{Percentage survival} = \frac{\text{OD sample}}{\text{OD control}} \times 100 \quad ....(2.3)$$

The test samples that showed less than 70% of percent survival were toxic to the cells.

Statistical analysis

All experiments carried out in triplicate. All results are expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed using Prism program and significance (*P* - value < 0.05) was determined by one-way analysis of variance (ANOVA), following Tukey's HSD test.

Results

1. The yield of *C.zedoaria* extracts

The steamed and non-steamed rhizomes of *C.zedoaria* were macerated with 50% ethanol and 95% ethanol. The percent yield of each extract are shown in Table 1. The steamed rhizomes with 95% ethanol provided the highest extractive value with the yield of 20.49%, followed by the 50% ethanolic extract of the steamed rhizomes. The yield of the non-steamed rhizomes extracts was lower than the steamed rhizomes extracts.

2. Determination of curcuminoid content in *C.zedoaria* extracts by HPLC

The HPLC chromatogram of three main components, i.e. bisdemethoxy-curcumin, demethoxycurcumin and curcumin are shown in Figure 1.

The mean correlation coefficient of the curcuminoids calibration curve was 0.9998. The linear calibration curve of curcuminoids was represented by the equations y = 34473x + 534800.

The curcuminoid content of *C.zedoaria* extracts including ZSE50, ZSE95, ZNE50 and ZNE95 were analyzed by HPLC, as shown in Figure 1 and Table 1. The sample of ZSE95 showed the highest curcuminoid content with 152.23 ± 1.80 mg/g of dried extract, followed by those of ZNE95, ZSE50 and ZNE50, respectively. However, the curcuminoid content in *C.zedoaria* extracts could be increased by steaming process. The steamed rhizome extracts was found to contain higher curcuminoid content than non-steamed rhizome extracts (Table 1) .

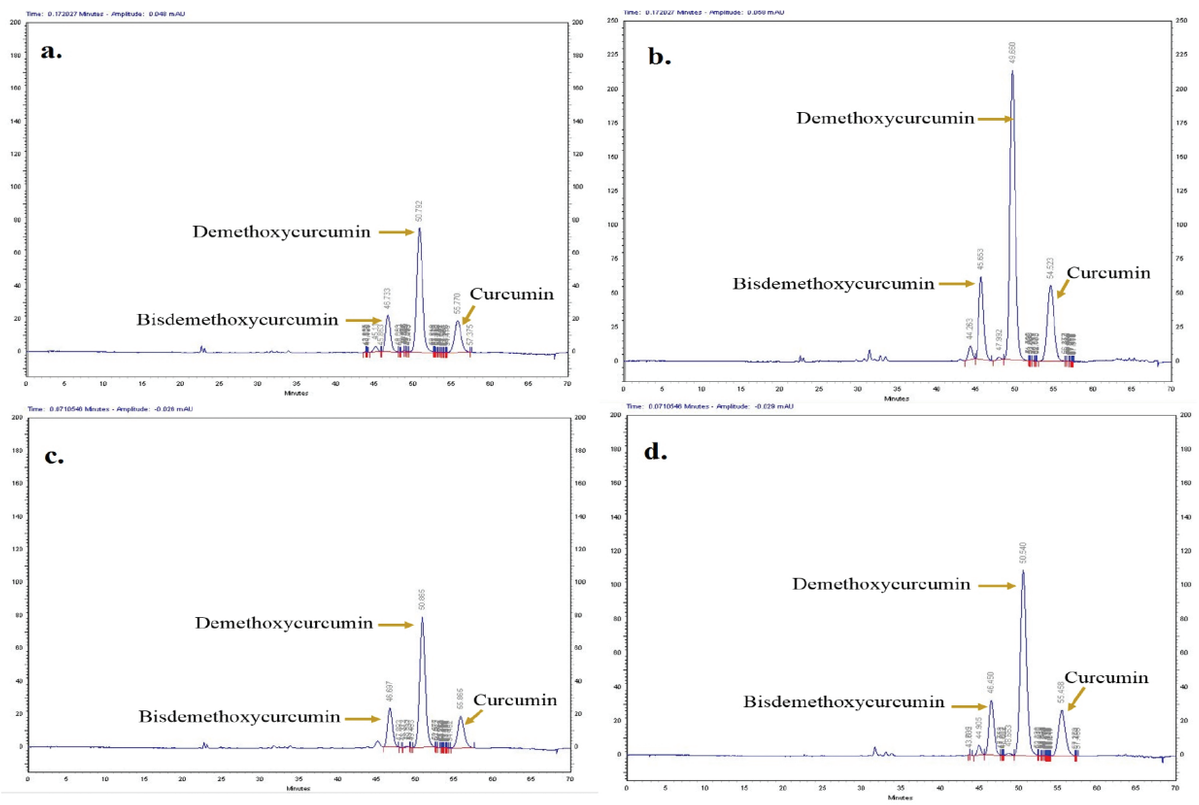

Figure 1 The HPLC chromatograms of curcuminoid content in (a) ZSE50, (b) ZSE95, (c) ZNE50 and (d) ZNE95.

Table 1 The percent yield and Curcuminoids content of *C.zedoaria* extracts

Sample	Solvent (%)	Code	Yield (%)	Curcuminoids content (mg/g of dried extract)
Steamed rhizomes	50 EtOH	ZSE50	14.82	76.16 ± 2.06
	95 EtOH	ZSE95	20.49	152.23 ± 1.80
Non-steamed rhizomes	50 EtOH	ZNE59	11.84	67.51 ± 2.88
	95 EtOH	ZNE95	13.37	91.95 ± 4.14

3. Antimicrobial activity

All ethanolic extracts showed inhibitory effect against *S.aureus* and *MRSA* with the MIC range of 0.3125-0.625 mg/mL and MMC range of 0.625-1.25 mg/mL as shown in Table 2. There was little difference between the steamed rhizome extracts and non-steamed rhizome extracts on antibacterial activity.

Table 2 The MIC and MMC values of *C.zedoaria* extracts and positive control

Sample	Microorganisms					
	S.aureus		*MRSA*		*C.albicans*	
	MIC (mg/ml)	MIC (mg/ml)	MIC (mg/ml)	MIC (mg/ml)	MIC (mg/ml)	MIC (mg/ml)
ZSE50	0.625	0.625	0.3125	1.25	1.25	>5
ZSE95	0.3125	0.625	0.3125	0.625	0.625	0.625
ZNE50	0.625	0.625	0.3125	1.25	1.25	>5
ZNE95	0.625	0.625	0.625	1.25	1.25	>5
Ampicillin	0.00024		NT		NT	
Vancomycin	NT		0.00048		NT	
Amphotericin B	NT		NT		0.0019	

Note: NT = Not tested, MIC = Minimum inhibitory concentration, MMC = Minimum microbicidal concentration

For antifungal activity, MIC range of all extracts were found to be in the range of 0.625-1.25 mg/mL while only ZSE95 had the bactericidal effect against *C.albicans* with MMC value of 0.625 mg/mL. The results indicated that ZSE95 was the most potent extract, it could inhibit the growth of *S.aureus, MRSA* and *C.albicans*. ZSE95 also contained the highest curcuminoid content. However, the highest component of curcuminoids that contained in *C.zedoaria* extract was dimethoxycurcumin, as shown in Figure 1. The 95% ethanolic extract of steamed rhizome contained the highest curcuminoid content, which explained the highest antibacterial activity.

4. Anti-inflammatory activity

All extracts were investigated for the inhibition effect on nitric oxide production in LPS-stimulated RAW264.7 cells. The samples of ZSE50, ZSE95 and ZNE95 showed toxicity against RAW264.7 cells at 100 µg/mL, and therefore their IC_{50} of nitric oxide production inhibition was not calculated. All extracts that showed the inhibitory effect on nitric oxide production are shown in Table 3. The ZSE95 sample had the strongest nitric oxide inhibition with IC_{50} of 11.22 µg/mL, followed by ZNE95 and ZSE50 with the IC_{50} values of 16.34 and 24.82 µg/mL, respectively. The weakest activity was ZNE50 (IC_{50} = 82.80 µg/mL), as illustrated in Figure 2. However, our study focused on curcuminoids which are also the active component

in *C.zedoaria*. We found that the inhibition effect of all *C.zedoaria* extracts was lower than curcuminoids (IC_{50} = 5.70 µg/mL) and prednisolone (IC_{50} = 1.21 µg/mL), as demonstrated in Table 3 and Figure 2. Our results suggested that there was the possible relation between the inhibition of nitric oxide production of *C.zedoaria* extracts and antibacterial activity. The 95% ethanolic extract of the steamed rhizome was the most potent extract with stronger inhibition on nitric oxide production as compared to other extracts. These activities could have been due to the highest curcuminoids content in this extract (Table 1).

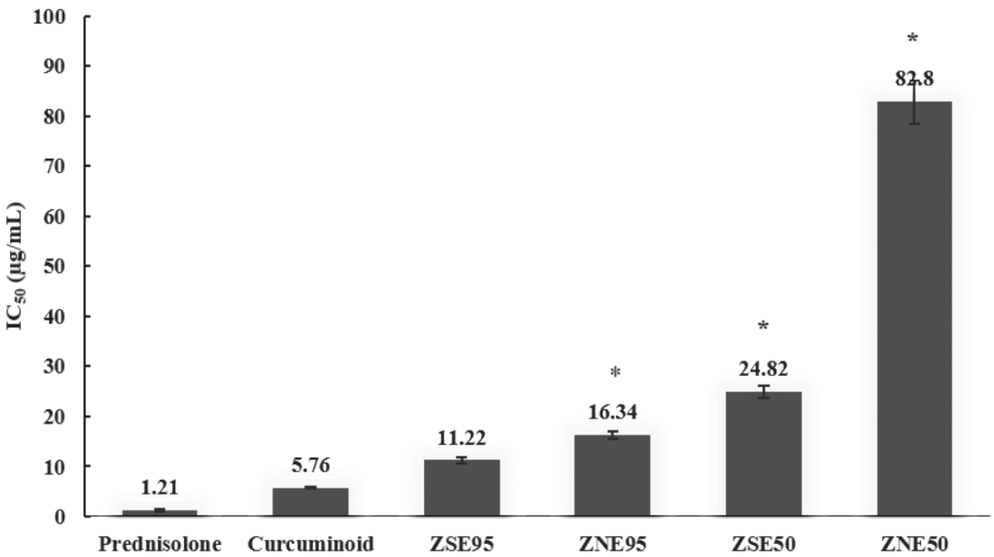

Figure 2 IC_{50} values of *C.zedoaria* extracts, curcuminoid standard and prednisolone on inhibition of nitric oxide production (n = 3), *significant difference ($P < 0.05$) when compared with prednisolone as a positive control.

Table 3 Effect of *C.zedoaria* extracts, curcuminoids and prednisolone on inhibition of nitric oxide production and cytotoxicity on RAW264.7 cells at varous concentrations (n = 3)

Sample	% Inhibition (mean ± SEM) (% Survival, mean ± SEM)					
	100 µg/mL	**50 µg/mL**	**25 µg/mL**	**12.5 µg/mL**	**6.25 µg/mL**	**3.125 µg/mL**
ZSE50	99. ± 710.41 (42.69 ± 0.44)	88.59 ± 2.42 (97.69 ± 3.01)	50.52 ± 4.01 (97.17 ± 4.70)	26.43 ± 4.16 (96.44 ± 4.86)	16.07 ± 4.87 (93.26 ± 2.58)	7.59 ± 2.14 (99.63 ± 3.59)
ZSE95	98.48 ± 0.69 (16.40 ± 4.29)	97.70 ± 1.22 (92.59 ± 3.69)	79.02 ± 2.82 (94.08 ± 3.74)	53.61 ± 3.54 (96.50 ± 3.85)	35.15 ± 2.46 (104.12 ± 4.70)	23.30 ± 2.25 (107.95 ± 2.52)
ZNE50	58.94 ± 4.34 (98.50 ± 1.75)	32.81 ± 3.35 (104.06 ± 1.30)	21.00 ± 4.06 (105.12 ± 4.24)	15.70 ± 3.37 (107.58 ± 3.84)	9.10 ± 1.38 (100.07 ± 1.93)	NT
ZNE95	99.55 ± 1.19 (42.99 ± 2.78)	92.81 ± 2.04 (94.02 ± 4.76)	65.47 ± 0.91 (96.07 ± 4.56)	42.58 ± 1.64 (107.68 ± 6.09)	25.27 ± 6.20 (112.20 ± 5.40)	11.14 ± 1.06 (106.52 ± 4.81)
	50 µg/mL	**10 µg/mL**	**8 µg/mL**	**1 µg/mL**	**0.1 µg/mL**	**0.01 µg/mL**
Curcuminoids	NT	91.91 ± 4.83 (73.05 ± 2.86)	78.01 ± 4.48 (82.33 ± 2.48)	7.67 ± 3.32 (98.42 ± 3.73)	4.54 ± 1.47 (100.48 ± 2.33)	3.10 ± 1.18 (105.88 ± 4.03)
Prednisolone	79.96 ± 2.34 (74.44 ± 5.72)	56.41 ± 4.61 (78.31 ± 6.58)	NT	47.08 ± 4.66 (86.83 ± 6.39)	37.87 ± 4.41 (94.09 ± 6.23)	7.08 ± 3.20 (110.06 ± 7.12)

Note: NT = Not tested

Discussion

Previous studies have shown that the highest composition of curcuminoids in *C.zedoaria* extracts are dimethoxycurcumin.[1] However, the composition of curcuminoids depend on plant species such as *Curcuma longa* showed the highest percentage of curcumin in curcuminoids composition.[13] The percentage of alcohol used in extraction affected curcuminoid content in *C.zedoaria* extracts. The highest yield of curcuminoids in *C. zedoaria* extracts was obtained from maceration in 95% EtOH. This was in accordance with previous report which showed increasing solubility of curcumin by high polarity solvent such as 95% EtOH.[14] Other factors such as the type of solvent, particle size of plant material and extraction time also have effects on the extraction yield of phenolic compounds.[15]

The ethanolic extract of *C.zedoaria* has been previously reported for its antibacterial activity.[5, 16] Similarly, our results showed that ethanolic extracts could inhibit *S.aureus* and *MRSA*. Curcumin has previously been reported that it has an inhibitory effect against *S.aureus* and *MRSA* with MIC values ranged from 125 to 250 µg/mL.[16, 17] Furthermore, the co-incubation of antibiotics with curcumin showed lower MIC values of antibiotics than the single treatment of antibiotics.[16] In addition, *C. albicans* was suppressed by curcumin via the inhibition mechanism of H+extrusion that lead to intracellular acidification and cell death.[18] Dimethoxycurcumin which is present in *C.zedoaria* has been reported to inhibit the growth of *E.coli, S.aureus* and *Shigella dysenteriae* with MIC values of 512, 1,024 and 1,024 µg/mL, respectively.[19] Besides, the essential oil from *C.zedoaria* obtained by heating under 121°C for 20 minutes has been reported to retain its antibacterial activity.[20] From the above findings, it can be concluded that the high temperature in steaming process is in favor of curcuminoid content and antimicrobial activity of *C.zedoaria*.

Another report has shown that the methanolic extract of *C. zedoaria* inhibits the nitric oxide production with IC_{50} value of 23.44 ± 0.77 µg/mL while curcuzedoalide which is its active component also inhibits the nitric oxide production with IC_{50} value of 12.21 ± 1.67 µM.[21] An in vivo study has been that the ethanolic extract of *C.zedoaria* (200 to 500 mg/kg) could inhibit inflammation in rats induced by carrageenan and histamine.[22, 23] Moreover, the polysaccharide from *C.zedoaria* rhizome has been found to increase H_2O_2, nitric oxide and TNF-α production in macrophage.[24]

Three curcuminoids, demethoxy-curcumin, bisdesmethoxy-curcumin and curcumin have been reported on their nitric oxide production inhibition in LPS-based microglia with IC_{50} values of 1.45 µM, 7.12 µM, and 11.53 µM, respectively.[25] Additionally, demethoxy-curcumin and curcumin displayed inhibition effect on nitric oxide production in N9 microglial cells with the IC_{50} values of 1.28 µM and 10.26 µM, respectively.[26] The traditional preparation of plant rhizomes in Ayurveda medicine is usually obtained by boiling before drying prior to use. The boiled-rhizome preparation has been shown to have higher curcuminoid content than the non-boiled one.[27]

In this study, we used autoclave in steaming the rhizomes of *C.zedoaria*. The autoclave was operated by higher heat and pressure than the atmosphere. Prior study of steaming process on turmeric rhizomes also revealed the increasing of curcuminoids content similar to our results.[28] This could be due to the fact that steam and high temperature dissolve starch molecule leading to gelatinization and retrodegradation, after which, starch that acts like gel protect the inside molecule.[29] This would explain the highest curcuminoids content in the steamed rhizome of *C.zedoaria*. As the result, the extract with high curcuminoids content also showed high anti-inflammatory and antimicrobial activities.

Taken together, it can be concluded that the steaming process can increase curcuminoids content and biological activity of *C.zedoaria* rhizomes. The best solvent for the extraction is 95% ethanol which give the highest percent yield of extract and exerted the strongest antimicrobial and anti-inflammatory activities. However, the other biological activities and other active components in steamed *C.zedoaria* should be further investigated to confirm their effects. This is the first report on the preparation of raw material named *C.zedoaria* needed to be steamed before drying, this finding supports its traditional preparation.

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