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Comparative Study on Biological Activities of Steamed and Non - Steamed Ginger Extracts

Naphatsaran Roekruangrit[1], Nuanjan Jaiarree[1,*], Arunporn Itharat [1,2]
Preecha Wanichsetakul[3], Sumalee Panthong[1],
Sunita Makchuchit[2] and Saovapak Poomirat[1]

[1]*Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand.*
[2]*Center of Excellence on Applied Thai Traditional Medicine Research, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand.*
[3]*Department of Obstetrics and Gynecology, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand.*

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ABSTRACT

Ginger is used for treating motion sickness, nausea and vomiting. The traditional Thai method of preparation is to steam the rhizome before preparing the remedy. There is no report comparing the biological activity and quality of extracts from steamed and non-steamed rhizomes. The objective was to compare the anti-inflammatory activities, cytotoxicity and quality of steamed and non-steamed ginger extracts. All extracts were tested for their potential anti-inflammatory properties via the inhibitory effect on NO and PGE_2 production, and cytotoxic activity via sulforhodamine B assay. Loss on drying, total ash and acid - insoluble ash determinations were used as quantitation evaluation to standardize the ginger extracts. The results showed that the quality of all samples was within standard guidelines. The anti-inflammatory NO inhibition effect was higher in the steamed ginger ethanol extract (AZOE) than in the non-steamed ginger ethanol extract (HZOE), but lower than prednisolone as a positive control, with IC_{50} values of 13.47 ± 0.20, 19.64 ± 0.33 and 1.33 ± 0.02 µg/ml, respectively. The effect of PGE_2 inhibition of AZOE, HZOE and positive control, had IC_{50} values of 0.40 ± 0.06, 0.63 ± 0.02 and 0.066 ± 0.004 µg/ml, respectively. However, the steamed and non-steamed ginger ethanol extracts were not cytotoxic to either SKOV - 3 or HeLa cells. Steamed ginger had a higher inhibition effect on NO and PGE_2 release than non - steamed ginger. These results support the traditional Thai method of steaming the rhizome before preparing the medicine.

Keywords: Ginger; NO; PGE_2; Cytotoxic; Steamed; Non - steamed

***Corresponding author:** nuanjan_j@yahoo.com **doi:** 10.14456/scitechasia.2019.30

1. Introduction

Ginger, *Zingiber officinale* Roscoe, is a herbal remedy in Thai National List of Essential Medicines (Zingiberaceae). It has long been used to relieve flatulence and dyspepsia [1], and in the female formulation [2], nausea and vomiting [3] and primary dysmenorrhea [4] including also in the formulation of cancer drugs. In the past, Thai folk healers have had to steam ginger rhizomes before using them as a medicine [5]. Research on the heating method (e.g. steaming) showed it can affect the chemical profile of vegetables or herbs, leading to changes of bioactivities [6].

Nitric oxide (NO) and prostaglandin E_2 (PGE_2) are involved in inflammation [7]. They are known to act as secondary mediators for pro-inflammatory cytokines, such as tumor necrosis factor (TNF - α), interleukin - 1β (IL - 1β) and interleukin - 6 (IL - 6), which are considered to be important initiators of the inflammatory response and mediators of the development of various inflammatory diseases [8], such as primary dysmenorrhea and menstrual pain which are associated with hypersecretion of prostaglandins due to increased uterine contraction. In Thai traditional medicine, ginger is widely used for pain relief during the menstrual period [9] and research has shown it can reduce menstrual pain [10]. However, there is no report of steaming ginger rhizome affecting nitric oxide and prostaglandin E_2 inhibition including cytotoxicity and quality impacts. Therefore, the objective of this study was to compare the anti-inflammatory activities, cytotoxicity and standardization by quantitative evaluation of steamed and non-steamed ginger extracts.

2. Materials and Methods

2.1 Plant materials

Fresh ginger rhizomes used in this study were obtained from Nam Nao District, Phetchabun province. A specimen voucher (BKF193572) was identified by Mr. M. Poopath, Forest and Plant Conservation Research Office, Department of National Parks, Wildlife and Plant Conservation, 61 Phaholyothin Road, Chatuchak, Bangkok, Thailand.

2.2 Preparation of extracts

One hundred kilograms of ginger rhizomes were cleaned, washed and air dried. All dried rhizomes were divided into two parts. Part one, the steamed ginger, was steamed with an autoclave at 121°C (similar to Thai traditional method) at 15 psi for 15 minutes (AZO), then dried in a hot air oven and ground to powder. Part two, the non - steamed ginger, was oven - dried at temperature of 50 °C and ground to powder (HZO). Both crude powders were extracted by maceration in 95% ethanol for 3 days, being codes AZOE and HZOE. Percentage yields of all the extracts were calculated.

2.3 Standardization by quantitative evaluation of the extract

Standard quality values of the steamed ginger (AZO) and non - steamed ginger (HZO) were determined following THP protocols by loss on drying being not more than 10%, total ash being not more than 8%, acid insoluble ash not more than 1%, ethanol - soluble value not less than 5% and water - soluble not less than 13% [11].

2.3.1 Loss on drying

Loss on drying was determined by an electronic moisture analyzer (Scaltec, Model: SMO 01, Göttingen, Germany). About 2 g of dried sample was accurately weighed and heated at 105°C until sample weight was constant. The moisture content by weight was calculated as below:

$$\% \text{ Moisture content} = \frac{\text{Weight of beginning sample (g)} - \text{Weight of drying sample (g)}}{\text{Weight of beginning sample (g)}} \times 100$$

2.3.2 Total ash content

This method investigated the physiological ash and non-physiological ash or inorganic compounds in the raw material. A crucible was dried until the weight of crucible was stable. Two grams of sample were weighed in the crucible and burned in a muffle furnace at 450 ˚C until the ash was changed to grey or white; then, the crucible was put in a desiccator until cool and then weighed. This process was repeated until the weight was constant. Total ash was calculated compared with the weight before burning, as below:

$$\% \text{ Total ash} = \frac{\text{Weight of beginning sample (g)} - \text{Weight after burning sample (g)}}{\text{Weight of beginning sample (g)}} \times 100$$

2.3.3 Acid - insoluble ash content

This method was continued from the total ash method. First, 10% hydrochloric acid (HCl) was prepared, then boiled with ash for 5 minutes and filtered through Whatman ashless filter paper No. 42. The residue was washed to pH 7 with distilled water. The ashless filter paper was dried and burned at 450 ˚C in the muffle furnace for 9 hours, repeating until the weight was stable. Percentage of acid - insoluble ash was calculated as below:

$$\% \text{ Acid insoluble ash} = \frac{\text{Weight of beginning sample (g)} - \text{Weight after burning sample (g)}}{\text{Weight of beginning sample (g)}} \times 100$$

2.3.4 Extractive value

Extractive value is used to determine the quantity of chemical constituents in the plant. In this study, the dried sample was extracted with ethanol and with water. Methods for determination of ethanol soluble (1) and water soluble (2) extractive values are as follows: 1) 5 g of dried sample was macerated in 100 ml of 95% ethanol in a closed flask for 24 hours. The flask was shaken frequently during the first 6 hours and allowed to stand for 18 hours. It was filtered rapidly as precaution against loss of ethanol and 20 ml of the filtrate was evaporated in a tared, flat-bottom shallow dish, dried at 105˚C and weighed. 2) Water soluble extractive value followed the same process as ethanol soluble but used chloroform: water (2.5:97.5, v/v) instead of 95% ethanol. The percentage of ethanol - soluble and water - soluble extractive values were calculated

$$\text{Extractive value (\%)} = \frac{\text{Weight of the extract (g)}}{\text{Weight of dried powder (g)}} \times 100$$

2.4 Cell culture

Mouse leukemia macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (ATCC TIB-71). This cell line was cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) which was supplemented with 10% heated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), 100 IU/ml penicillin, 100 µg/ml streptomycin and incubated at 37˚C, 95% humidity in 5% CO_2 atmosphere. Cells were subcultured every 3 days.

2.5 Cell viability by MTT assay

Briefly, mouse leukaemia macrophage cell line (RAW 264.7) was seeded in 96 - well plates with 1×10^6 cells/well. After 24 hours incubation with various test samples of 0.1, 1, 10 and 30 µg/ ml, MTT solution (10 µl, 5 mg/ml in PBS) was added to the wells and then incubated at 37˚C at 5% CO_2 atmosphere with 95% humidity for 2 hours. After that the medium was removed and isopropanol containing 0.04 M HCl was added to dissolve the formazan and the absorbance was read using a microplate reader (Biotek model: Power Wave XS, BioTek®, USA) at 570 nm [12 - 13].

2.6 Inhibitory effect on LPS-induced NO production

The RAW 264.7 cells were cultured in RPMI 1640 (BIOCHROM^{AG}) supplemented with 10% heated fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml

streptomycin. The cells were grown at 37°C and 5% CO_2 in humidified air. The cells were seeded in 96 well plates, $1x10^5$ cells/well, and allowed to adhere for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with fresh medium containing 100 µg/ml of lipopolysaccharide (LPS) together with test samples at various concentrations of 0.1, 1, 10 and 30 µg/ml, and then incubated for 24 hours. NO production was determined by measuring the accumulation of nitrite in the supernatant using the Griess's reagent (100 µl) which was added to 96 - well plates and absorbance was read using a microplate reader at 570 nm [12 - 13].

The inhibition of NO production was calculated and IC_{50} values were obtained using the Prism program.

2.7 Inhibitory effect on LPS-induced PGE_2 production

The RAW 264.7 cells were seeded in 96 - well plates at $1x10^5$ cells/well, and 5 µg/ml of LPS was added to stimulate the macrophages and allowed to adhere for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. After incubation, supernatant was collected and the amount of PGE_2 was determined using a PGE_2 Enzyme Immuno-Assay Kit (Cayman Chemical Company). The amount of PGE_2 was measured relative to that of the positive control [12 - 13 - 14].

2.8 Cytotoxicity study on human cell line

Cancer cell lines used in this test were human ovarian adenocarcinoma (SKOV - 3) (ATCC® HTB - 77™), cervical adenocarcinoma (HeLa) (ATCC® CCL-2™) and human keratinocyte cells. HaCaT, SKOV - 3 and HeLa cell lines were grown in an incubator with 5% CO_2 at 37°C in RPMI Medium 1640 (RPMI1640) with L - glutamine, 10% heated fetal bovine serum (FBS), and 1% penicillin - streptomycin (P/S). HaCaT was cultured in DMEM culture medium containing 10% heated fetal bovine serum and 1% of 10,000 IU penicillin and 10 mg/ml streptomycin. According to their growth profiles, the optimal plating densities of each cell line were set at 3×10^3 cells/well for both SKOV - 3 and HeLa to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by SRB assay [15]. The National Cancer Institute guidelines for toxicity of extracts were followed, with IC_{50} value < 30 µg/mL being toxic [16].

2.9 Statistical analysis

Results are expressed as mean ± SEM of four determinations at each concentration for each sample. The IC_{50} values were calculated using the Prism program and the significant difference comparison of each factor by t - test.

3. Results and Discussion

3.1 Standardization by quantitative evaluation of the extract

The results of standard quality values of steamed ginger and non-steamed ginger are shown in Table 1. The standard quality values of steamed ginger and non-steamed ginger were: percentage loss on drying 6.49 ± 1.50% and 8. 45 ± 0. 88%, respectively; percentage total ash of steamed ginger and non-steamed ginger 7.38 ± 0.36% and 7.60 ± 1.08%, respectively; acid insoluble ash of steamed ginger and non-steamed ginger 0.40 ± 0.10% and 0. 37 ± 0. 07%, respectively. The percentages of ethanol and water-soluble extractive values of steamed ginger and non-steamed ginger were 5 ± 0.23%, 5 ± 0.14% for steamed, and 18 ± 0. 34%, 19 ± 0.45% for non-steamed, respectively. All steamed and non-steamed ginger extracts met the standard values of THP by loss on drying being not more than 10%, total ash being not more than 8%, acid

insoluble ash not more than 1%, ethanol - soluble value not less than 5% and water - soluble value not less than 13% [11].

3.2 Inhibitory effect on LPS - induced NO production

The results of inhibitory activity of the extracts and prednisolone positive control against LPS induced NO production are shown in Table 2. The ethanolic extract of the steamed ginger (AZOE) showed higher anti-inflammatory activity than non - steamed ginger ethanolic extract (HZOE) but lower than prednisolone, with IC_{50} values of 13.47 ± 0.20, 19.64 ± 0.33 and 1.33 ± 0.01 µg/ml, respectively. The cytotoxic effect of ginger extracts was also determined using the MTT assay. Ginger extracts up to concentration of 30 µg/ml showed no cytotoxicity (Table 2). There was a significant difference between steamed ginger extracts, AZOE, and non- steamed ginger extracts, HZOE (p - value < 0.05). This result can be used to point out the potential of the extract that may possess anti-inflammatory activity. More study models are required to confirm the anti-inflammatory activity. These results related with the previous study on active compounds of *Zingiber officinale* production of inflammatory mediators (NO and PGE_2) has been inhibited significantly (p < 0.05) and dose-dependently [17] and the dried rhizome of ginger is used in Ayurvedic medicines in the treatment of various diseases that involve inflammation [18].

3.3 Inhibitory effect on LPS-induced PGE_2 production

Results of assay determining inhibitory effect of the ethanolic extract from steamed ginger (AZOE) and non- steamed ginger (HZOE) on LPS-stimulated PGE_2 in RAW 264.7 cells line using the ELISA test kit are shown in Table 3. The ethanolic extract from steamed ginger (AZOE) had higher inhibitory effect than non-steamed ginger ethanolic extract (HZOE) but lower than prednisolone as positive control, with the IC_{50} values of 0.40 ± 0.06, 0.63 ± 0.02 and 0.066 ± 0.004 µg/ml, respectively. There was no significant difference between steamed ginger extract, AZOE, and non-steamed ginger extract, HZOE (p - value > 0.05). However, both extracts showed the potential to act against the PGE_2 production and can be associated with decrease of prostaglandins involved in pain relief during menstrual period [9].

3.4 Cytotoxicity study on human cell line

The results of cytotoxic activity of the 95% ethanol extracts on ovarian cancer cells (SKOV - 3) show that steamed ginger (AZOE) had IC_{50} value of 34.08 ± 0.40 µg/ml and non-steamed ginger (HZOE) had IC_{50} value of 35.06 ± 0.29 µg/ml. In the same way, cytotoxic activity on cervical cancer cells (HeLa) showed that steamed ginger (AZOE) had IC_{50} value of 31.47 ± 0.18 and non-steamed ginger (HZOE) had IC_{50} value of 42.07 ± 2.01 µg/ml. There was no significant difference between steamed ginger extract, AZOE, and non-steamed ginger extract, HZOE (p - value > 0.05), following The National Cancer Institute (NCI) guidelines for extracts with IC_{50} value < 30 µg/mL [16]. Both extracts showed no activity against SKOV - 3, HeLa and HaCaT cell lines.

Table 1. Standardization by quantitative evaluation of steamed ginger (AZO) and non-steamed ginger (HZO).

Sample	Code	%Loss on drying	%Ash contents		% Extractive value	
			Total ash	Acid insoluble ash	Ethanol- Soluble	Water-Soluble
Zingiber officinale Roscoe.	AZO	6.49 ± 1.50	7.38 ± 0.36	0.40 ± 0.10	5 ± 0.23	18 ± 0.34
	HZO	8.45 ± 0.88	7.60 ± 1.08	0.37 ± 0.07	5 ± 0.14	19 ± 0.45
Standard value from THP		≤ 10	≤ 8	≤ 1	≥ 5	≥ 13

Table 2. Inhibitory effect of the ethanolic extract from steamed ginger (AZOE) and non-steamed ginger (HZOE) on LPS induced NO production, cytotoxicity and IC_{50} in RAW 264.7 cells.

Sample and positive control	% inhibition of nitric oxide production (%cytotoxicity) mean ± SEM					IC_{50} (µg/ml)
	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	
AZOE	0.10 ± 0.02 (9.61 ± 3.31)	4.60 ± 0.24 (12.76 ± 5.71)	39.79 ± 0.42 (16.35 ± 4.85)	88.57 ± 0.01 (15.45 ± 6.03)	-	13.47 ± 0.20
HZOE	9.64 ± 0.16 (9.23 ± 2.87)	16.23 ± 0.01 (6.28 ± 3.34)	36.74 ± 0.87 (15.06 ± 4.88)	80.81 ± 1.33 (17.12 ± 3.92)	-	19.64 ± 0.33
Prednisolone	6.43 ± 0.25 (6.15 ± 4.55)	39.41 ± 0.22 (11.63 ± 3.82)	84.61 ± 0.05 (10.97 ± 4.82)	87.23 ± 0.05 (6.12 ± 2.79)	92.08 ± 1.03 (17.34 ± 7.50)	1.33 ± 0.01

Note: "-": not done

Table 3. Inhibitory effect of the ethanolic extract from steamed ginger (AZOE) and non-steamed ginger (HZOE) on LPS-stimulated PGE_2 in RAW 264.7 cells (IC_{50} and percentage of inhibition on PGE_2 release at various concentrations).

Sample and positive control	% inhibition effect of PGE_2 mean ± SEM						IC_{50} (µg/ml)
	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	
AZOE	-	25.30 ± 3.14	97.04 ± 7.65	112.45 ± 8.18	106.86 ± 9.39	-	0.40 ± 0.06
HZOE	-	17.39 ± 2.07	70.99 ± 0.37	108.14 ± 5.30	107.43 ± 6.01	-	0.63 ± 0.02
Prednisolone	52.93 ± 1.34	81.57 ± 0.87	89.16 ± 1.82	87.74 ± 1.50	-	93.20 ± 3.79	0.066 ± 0.004

Note: "-": not done

Table 4. Cytotoxicity against SKOV-3 and HeLa of the ethanolic extract from steamed ginger (AZOE) and non-steamed ginger (HZOE).

Sample and positive control	SKOV-3 IC_{50} (µg/ml ± SEM)	HeLa IC_{50} (µg/ml ± SEM)	HaCaT IC_{50} (µg/ml ± SEM)
AZOE	34.08 ± 0.40	31.47 ± 0.18	38.87 ± 0.08
HZOE	35.06 ± 0.29	42.07 ± 2.01	35.63 ± 0.12

Note: According to National Cancer Institute guidelines extracts with IC_{50} value < 30 µg/mL are plants with cytotoxic activity [16].

4. Conclusion

In summary, the raw materials of steamed ginger powder and non-steamed ginger powder met the quality standards of the Thai Herbal Pharmacopeia (THP). Steamed and non-steamed ginger extracts are not cytotoxic to either cervical or ovarian cancer cells. Steamed ginger had higher effect of inhibition of PGE_2 and NO release than non-steamed ginger. These results support the traditional Thai method of steaming the rhizome before preparing the remedy.

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