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In vitro Antioxidative and Cytotoxicity Effects of *Justicia gangetica* Leaves and Their Application in Cooked Chicken Patties

Viriya Nitteranon[1*], Pawinee Fackthongkum[2], and Tanyarat Pooratanachareonchai[1]

Department of Food Science and Technology, Faculty of Science and Technology Rajamangala University of Technology Tawan-ok, Chonburi, Thailand 20110

*Corresponding Author E-mail: nitteranon@gmail.com

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ABSTRACT

Justicia gangetica is the edible folk vegetable found in tropical area including Thailand. The study was conducted to investigate total phenolic contents, antioxidative and cytotoxic activities of crude extract of *J. gangetica* leaves (JGL) and determined its potential as natural food preservative in chicken patties. The ethanol extract of JGL contained highest total phenolic contents and DPPH-radical scavenging activity ($P \leq 0.05$) compared to JGL water extract. Cell toxicity was evaluated in HT-29, MCF-7 and Vero cells with different concentrations of JGL ethanol extracts using MTT assay. The result showed no cytotoxic effect of JGL extracts at 125-500 µg/ml. Thiobarbituronic acid-reactive substance (TBARS) value was determined during refrigerated storage. The lower TBARS levels were noticed in chicken patties containing JGL and BHT, comparing to control ($P \leq 0.05$). These results indicate that JGL can be used as food preservative in meat product to effectively lower lipid oxidation.

INTRODUCTION

Nowadays, pre-cooked meat products have become increasingly popularity among consumers. Lipid oxidation is a main cause of food spoilage in pre-cooked meat products, developing to off-flavor, color alteration and loss of essential amino acid, fat-soluble vitamins and bioactives in food [1]. Antioxidants have been used to inhibit lipid oxidation and extend shelf-life in meat products. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ) have been successfully used to prevent lipid oxidation in meat industry [2]. However, the use of synthetic antioxidants is limited according to their side-effects as potential hepatotoxic effect [3, 4]. Nowadays, there is a considerable interest in natural products to use as food preservatives. Various plants containing phytochemicals such as phenolics and flavonoids have shown to be effective antioxidants in meat products. For example, broccoli, barley leaf and lotus seed epicarp have been reported to significantly retard lipid oxidation in ground beef, ground pork and Chinese sausage, respectively [5, 6, 7].

Justicia gangetica or Chinese violet (Om-Sap in Thai) is a fast growing and herbaceous ground cover plant. It has green and oval-shaped leaves which contain high amount of protein, amino acids, minerals, sugar, lipid and dietary fibers [8]. They have been used as traditional medicine as anti-inflammatory, antifungal and anthelmintic activities [9]. The major constituents in *J. gangetica* leaves are 5,11- epoxymegastigmane glycoside (asysgangoside), salidroside, apigenin 7-O-neohesperidoside and apigenin 7-O-β-D-glucopyranoside [10, 11].

Therefore, the objectives of this study were to evaluate antioxidative and cytotoxic activities of *J. gangetica* leaves and their application in chicken patties during refrigerated storage for 12 days. There is no previous report on *J. gangetica* leaves used as food preservatives. This is the first paper showing their potential antioxidant in pre-cooked meat.

RESEARCH METHODOLOGY

Chemicals

Folin-Ciocalteu's phenol reagent was purchased from Loba Chemie (Mumbai, India). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), methylthiazolyl diphenyltetrazolium bromide (MTT), 2,6-di-tert-butyl-4-methyphenol (BHT), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA) and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium carbonate, dimethyl sulfoxide (DMSO) and ascorbic acid were

obtained from Fisher Scientific (Pittsburgh, PA, USA). Ethanol and methanol were purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's (DMEM) medium, fetal bovine serum (FBS), L-glutamine, 0.5% trypsin-EDTA and penicillin/streptomycin were obtained from Gibco (Grand Island, NY, USA) All chemicals and reagents used in the study were of analytical grade.

Sample extraction

Fresh leaves of *Justicia gangetica* were collected from Jitsodsai Prathomchai Hydrotech Co., LTD (Chachengsao Province, Thailand). They were air-dried in hot air oven at 40°C for 72 h, ground and stored at 4°C until use. Leave powders were extracted using 100% ethanol and water in 1:10 (w/v) ratio and incubated at 40°C for 2 h. The extracts were then separated by filtration through Whatman® filter paper no. 1. The filtrates were concentrated under reduced pressure at 40°C using a rotary evaporator (Büchi, Switzerland) and freeze dryer (Christ Alpha, UK) to obtain dry extract. All samples were kept at 4°C until further analysis.

A. Total phenolic content determination

The content of total phenolic was measured spectrophotometrically using Folin-Ciocalteu colori- metric method [12] using gallic acid as the standard. In brief, a 0.25 ml aliquot of Folin-Ciocalteu's phenol reagent was added to a test tube containing 0.2 ml of appropriately diluted sample extract. A 2.5 ml of saturated Na_2CO_3 (7% w/v) was added to the mixture. Then, 5.8 ml of ddH2O was added and the mixture was incubated at room temperature for 25 minutes. The absorbance of the mixture was measured at 725 nm using a UV-VIS spectrophotometer (Shimadzu, Japan). All data was determined in triplicate, and the results are expressed as mg gallic acid equivalent (GAE)/g of extract.

Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant activity was measure using DPPH scavenging method [13] using ascorbic acid as the standard. 20 µl of sample extracts were placed in a 96- well microplate. DPPH solution (250 µl, 25mg/L) in methanol was added to each sample. The mixture was incubated for 30 min in the dark at room temperature. The absorbance of the reaction mixtures were measured at 517 nm using Spectra MR microplate reader (Dynex Technologies, USA). The experiment was performed in triplicate. The samples were quantified as µM ascorbic acid equivalent antioxidant capacity (AEAC).

Cell culture

Human colon adenocarcinoma cell line (HT-29), human breast carcinoma cell line (MCF-7) and kidney cells from African green Monkey (Vero cells) obtained from ATCC

(Virginia, USA) were maintained in DMEM medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin/100 µg/ml streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO_2.

In vitro cytotoxicity assay

Cell viability was evaluated using MTT assay [14]. Cells were seeded in 96-well plates in the absence and/or presence of JGL ethanol extract for 24-72 h. At the end of incubation period, 100 µl of an MTT solution (0.05 mg/ml in PBS buffer) was added to each well and the plate was further incubated at 37°C for 4 h. DMSO (200 µl) was added to each well to dissolve tetrazolium dye and after 15 min of agitation at 37°C, the absorbance was determined at 570 nm using microplate reader.

Preparation of ground chicken patties

Fresh chicken thigh was purchased fresh from local market and ground using meat grinder. Ground chicken patties were produced for four different treatments. The negative control was prepared without antioxidants. Ground chicken patties were prepared by mixing with 0.05 and 0.10% (W/W) of JGL extract. Positive control was formulated by adding chicken patties with 0.02% (W/W) of BHA. A 20 g portion of each meat sample was made into patties using a patty mold with 6 cm diameter and 1 cm height. All meat samples were stored in a refrigerator at 4°C for 12 days and TBARS values were investigated at 0, 3, 6, 9 and 12 days of storage.

Lipid peroxidation inhibitory activity

The thiobarbituric acid reactive substances (TBARS) assay was used to measure the antioxidant capacity of food extract against lipid oxidation [15]. Two grams of samples taken from each patty were mixed with 12 ml of trichloroacetic acid (TCA) solution (7.5% TCA, 0.1% propyl gallate dissolved in 3 ml ethanol, and 0.1% disodium ethylenediamine tetraacetic acid (EDTA). Samples were homogenized with a Polytron at medium speed for 30 seconds and filtered. One ml of filtered sample was added to 1 ml of 0.02 M TBA (2.88g/L in water) reagent and mixed. A reagent blank was prepared with 1 ml of TCA and 1 ml of TBA solution. The tubes were heated at 100°C for 10 minutes in heating block. After heating, the samples were cooled in tap water and then centrifuged at 6000 rpm for 20 minutes. 5 ml of the supernatant was measured at 532 nm using UV-VIS Spectrophotometer (Shimadzu, Japan).

Statistical analysis

All experiments were performed 3 times, each in duplicate or triplicate. The results were expressed as the means ± standard deviation (SD). The statistical

significance of different between groups was assessed by a Student's t-test ($P \leq 0.05$).

METHODS AND RESULTS

Total phenolic contents and antioxidant properties of JGL extracts

Ethanol and water are edible solvents that commonly used for bioactive constituents extraction in plants. In this study, the yields of ethanol and water JGL crude extract were 3.9% and 13.7%, respectively from JGL powder on a dry matter basis. The total phenolic contents of JGL ethanol extract was found significantly higher as compared to JGL water extract ($P \leq 0.05$) (Table 1). Phenolic compounds are main ingredients in vegetables, fruits and herbs. It is found that crude methanol extract from *J. gangetica* contains flavonoid and anthraquinone compounds [9, 15].

Generally, the antioxidant activity of phenolic compounds is as a result of their radical scavenging activities. In DPPH assay, ethanol extract from JGL had higher antioxidant capacity than water extract ($P \leq 0.05$) (Table 1). Our results were agreement with the previous finding that 70% ethanolic extract of *J. gangetica* leaves showed potent antioxidant and anti-diabetic activities [16]. Stewart et al., (2013) showed that ethyl acetate *J. gangetica* crude extract have the most potent antioxidant activity using ABTS assay [17]. The results of this study showed a correlation between total phenolic content and free radical scavenging activity. Several studies have determined the relationship between phenolic contents and antioxidant activity. Guo et al. (2008) showed a significant relationship between the antioxidative effects and total phenolic compounds in 16 Chinese medicinal herbs extracts [18].

Table 1. Mean of total phenolic contents of JGL extracts expressed as GAE and DPPH scavenging activity represented as μM AEAC

JGL extracts	Total phenolic contents (mg GAE/g)	Antioxidant activity (μM AEAC/g)
Ethanol	353.75 ± 59.19[a]	1253±40.35[c]
Water	166.56 ± 28.73[b]	832±5.45[d]

Cytotoxic effect of JGL extract

As HT-29, MCF-7 and Vero cells were exposed to 125, 250, and 500 μg/ml of JGL ethanol extract for 24-72 h, they exhibited no cytotoxic effects (data not shown) whereas 1000 μg/ml of JGL ethanol extract showed 85-90% cell viability, when compared to negative control (Fig. 1). There was no significant difference in relative cell viability between treatments was observed

(P > 0.05). There has been no published report on the effect of *J. gangetica* extract on HT-29, MCF-7 and Vero cells so far, but the growth inhibitory effect of the extracts on other human cancer cell lines have been reported by the different researchers. Stewart et al., (2013) demonstrated that *J. gangetica* ethyl acetate extracts showed no cytotoxicity effect on normal fibroblast cells (Hs27) [17]. Therefore, these results indicated that JGL was a non-toxic potential food additive.

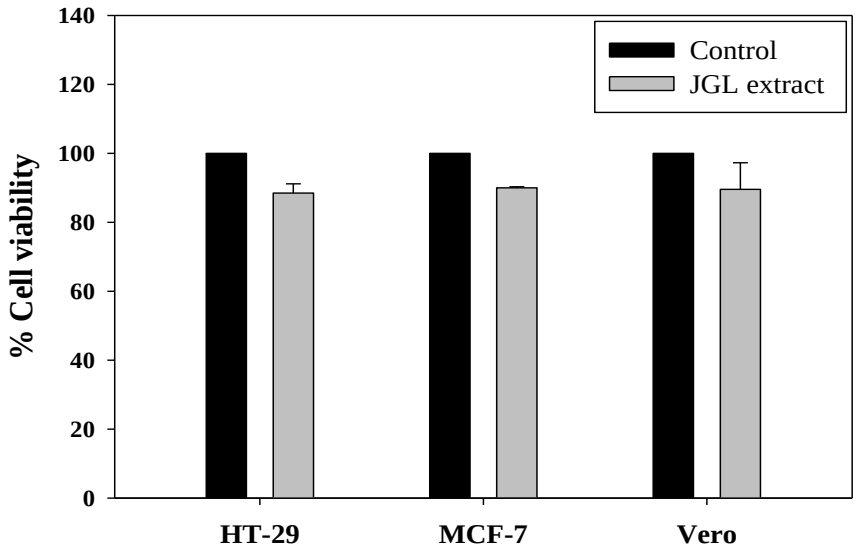

Figure 1. MTT assay of JGL ethanol extract on HT-29, MCF-7 and Vero cells. Cells were treated 1000 µg/ml of JGL ethanol extract for 72 h.

Effect on lipid oxidation in cooked chicken patties

TBARS assay was used to measure secondary lipid oxidation products such as aldehyde and ketone which causes off-flavor in meat [19]. In Fig. 2, the TBARS values of the control sample were dramatically increased during 12 days at 4°C (0.89-16.91 mg MDA/kg), whereas the addition of 0.02% BHA in chicken patties, TBARS values were significantly decreased during 12 days of storage period (0.52-1.98 mg MDA/kg). The similar results were observed in the treatment of chicken patties with JGL extract. The addition of 0.05% and 0.10% JGL extract significantly lower lipid oxidation compared to control sample (P ≤ 0.05) and the effect was a dose-dependent manner. Thus, *J. gangetica* could inhibit lipid oxidation in refrigerated chicken patties and might potentially be used as food preservative. It has been reported that edible plants and herbs such as broccoli (0.10%) and butterbur (0.50%) could retard lipid oxidation in fresh ground beef and extended shelf-life

[5]. In addition, the supplementation of bread with barley leaves contributed to a good source of nutritional quality [20]. Natural antioxidants from edible plants were effective as controlling lipid oxidation and extending the shelf life of meat products. Retardation of lipid oxidation in chicken patties by JGL might due to the presence of phenolic compounds.

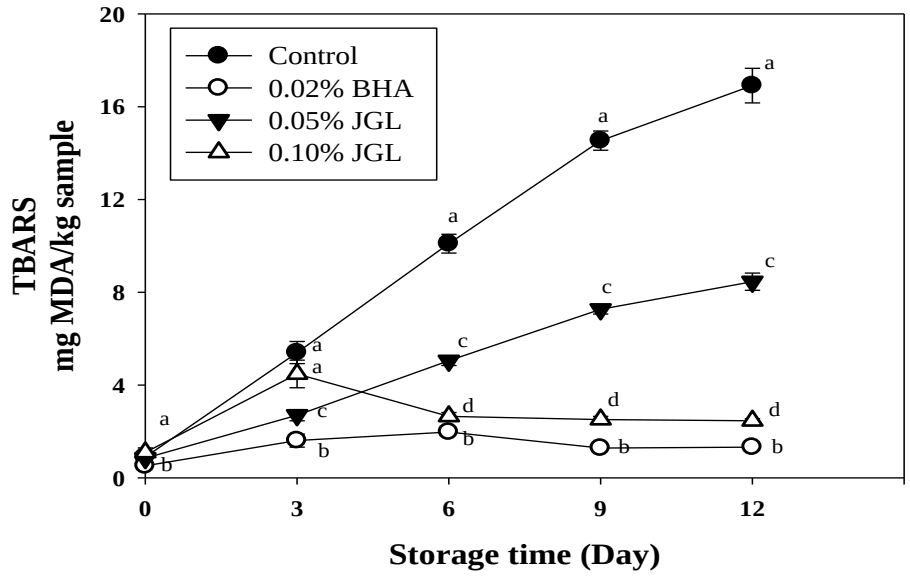

Figure 2. Effect of 0.05%, 0.1% JGL and 0.02% BHT addition on lipid oxidation of chicken patties compared to control patties. Treatments with the same storage time that do not share a common letter were significantly different ($P \leq 0.05$).

CONCLUSION

In summary, this study investigated antioxidant properties of JGL extract. The result indicated that JGL ethanol extract had the highest total phenolic contents and antioxidant activity. Furthermore, the JGL ethanol extract at 125-500 µg/ml demonstrated non-toxic to cells (HT-29, MCF-7, Vero cells) under the experimental conditions. Regarding to their potential antioxidative activity and safety, JGL ethanol extract was selected to study on food preserving properties. The addition of JGL extract in cooked ground chicken patties during refrigerated storage can significantly inhibit lipid oxidation with a dose-dependent response, as evaluated by TBARS values. This is the first study demonstrated that JGL might be a potential candidate for antioxidant substitution in cooked food. As promising of these results, further research will be needed to determine JGL can be used as in vivo health promoting food ingredients and the effect to their nutritive value. Additionally,

sensory evaluation and consumer acceptance testing should be conducted to determine the effect of JGL in chicken patties product.

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