ORIGINAL ARTICLE

In vitro anti-inflammatory, mutagenic and antimutagenic activities of ethanolic extract of *Clerodendrum paniculatum* root

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ABSTRACT

Clerodendrum paniculatum L. (Family Verbenaceae) has been used as an antipyretic and anti-inflammatory drug in traditional Thai medicine. This present study investigated the *in vitro* anti-inflammatory, mutagenic and antimutagenic activities of the ethanolic extract of *C. paniculatum* (CPE) dried root collected from Sa Kaeo Province of Thailand. Murine macrophage J774A.1 cells were stimulated by lipopolysaccharide (LPS) to evaluate nitric oxide (NO), tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) production in the anti-inflammatory test while the mutagenic and antimutagenic potential was performed by the Ames test. The outcome of this study displayed that the CPE root significantly inhibited LPS-induced NO, TNF-α, and PGE_2 production in macrophage cell line. In addition, the CPE root was not mutagenic toward *Salmonella typhimurium* strain TA98 and TA100 with and without nitrite treatment. Moreover, it inhibited the mutagenicity of nitrite treated 1-aminopyrene on both strains. The findings suggested the anti-inflammatory and antimutagenic potentials of CPE root.

Key words: Bacterial reverse mutation assay, *Clerodendrum paniculatum*, dried root, murine macrophage anti-inflammatory assay

INTRODUCTION

Inflammation is a complex biological process in response to harmful stimuli as well as a protective attempt to remove the stimuli and initiate the healing process.[1] Inflammation consists of the vascular system, the immune system, inflammatory cells, and chemical mediators within the injured tissue.[2] Macrophages play an important role in inflammatory process by mediating the immunopathological changes, including the overproduction of pro-inflammatory cytokines and chemical mediators such as tumor necrosis factor-α (TNF-α), nitric oxide (NO), and prostaglandin E_2 (PGE_2).[3] Now-a-days, there are many anti-inflammatory drugs which are synthetic compounds and have various side effects such as gastric irritation, antiplatelet effect, acute renal failure, and cardiovascular diseases.[4] Thus, there is a need in searching the herbal medicine which is as effective as the existing product with the lowest adverse effect for the treatment of the inflammatory diseases.

Clerodendrum paniculatum Linn., commonly known as Red Pagoda plant belongs to Verbenaceae family. It is a biennial herb that grows up to 1.5 m in height.[5] It has been used for traditional medicine in India, China and Japan in the treatment of rheumatism, neuralgia, ulcer, inflammation, and healing wounds.[6] For traditional Thai medicine, it has been used as an antipyretic and anti-inflammatory drug. Moreover, it is an essential ingredient of Cha-Tu-Ka-La-Thart remedy which is a remedy notified in traditional Thai medicine textbook named Tumrapaadsard song Khor. Pharmacological investigations have been reported that the extract of *C. paniculatum* root exhibited potent activity against gram-negative and gram-positive bacteria and showed significant antioxidant activity.[7,8] Although there were previous reports of pharmacological activities of *C. paniculatum* but the supporting evidence for safety or toxicity of *C. paniculatum* was still limited. Herbal

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medicines were often assumed to be safe, but there were previous studies revealed the genotoxic effects of herbal medicines.[9,10] Therefore, this present study aimed to investigate the *in vitro* anti-inflammatory, mutagenic and antimutagenic activities of the ethanolic extract of *C. paniculatum* (CPE) root.

MATERIALS AND METHODS

Crude extract preparation

Roots of *C. paniculatum* were collected from Sa Kaeo Province of Thailand. They were collected in December 2011 and were authenticated by Ruangrungsi. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The roots were shade-dried and ground to coarse powders and exhaustively macerated with 95% ethanol. The ethanolic extract was filtered through Whatman No. 4 and evaporated under vacuum. The extract yield was weighed, recorded, and stored at −20°C until use to decrease the possibility of degradation of active compounds.

Cells

Murine macrophages J774A.1 were obtained from American Type Culture Collection. The cells were subcultured 3 times weekly and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37°C in 5% CO_2/95% air.

Cell viability

The viability of the cells was performed to evaluate the cytotoxicity of extracts using the resazurin assay.[11] The 1×10^5 cell/well of J774A.1 murine macrophage cells were treated with the CPE root at the concentrations of 0, 6.25, 12.5, 25, 50, and 100 µg/ml in 96-well plates. 0.2% dimethyl sulfoxide (DMSO) and dexamethasone (7.85 µg/ml) were used as the negative and positive controls, respectively. The cells were stimulated with 0.1 µg/ml lipopolysaccharide (LPS) for 24 h at 37°C. Supernatants were removed from the treated cells to investigate NO scavenging activity. The complete DMEM medium containing 50 µg/ml of resazurin (70 µl) was added in each well and incubated for 2 h at 37°C. The amount of resorufin, the product from resazurin production in viable cells was determined by measuring the absorbance at 570 with wavelength correction set at 600 nm by a microplate reader.

Nitric oxide inhibition assay

Nitric oxide released in the supernatants was investigate using Griess reagent.[12] The supernatants (100 µl) were mixed with 20 µl of 1% sulfanilamide in 5% phosphoric acid, incubated for 10 min at room temperature, 20 µl of 0.1% naphthyl-ethylenediamine dihydrochloride were added and incubated for 10 min at room temperature. After that, the reaction mixture was read the absorbance at 540 nm. Sodium nitrite was used for the calibration curve.

Tumor necrosis factor-α and prostaglandin E2 inhibition assays

J774A.1 murine macrophage cells were seeded at a density of 1×10^5 cell/well and incubated overnight. The macrophage was incubated with the ethanolic extracts at different concentrations (0, 12.5, 25, 50, and 100 µg/ml) for 1 h. LPS (0.1 µg/ml) was added then incubated overnight at 37°C in 5% CO_2/95% air. The supernatants were evaluated for TNF-α and PGE_2 according to the kit manufacturer instructions (ELISA Development Kit, Peprotech, Rocky Hill, USA and PGE_2 ELISA Kit, Thermo Scientific, Pierce Biotechnology, USA). Dexamethasone (7.85 µg/ml) was used as positive control.

Mutagenic assay

Salmonella typhimurium strain for frame-shift mutation (TA98) and strain for base-pair substitution mutation (TA100) were kindly provided by the Biochemistry and Chemical Carcinogenesis Section, Research Division, National Cancer Institute, Bangkok, Thailand. The mutant strains were confirmed for the genotypes of histidine/biotin dependence, *rfa* marker, *uvrB* deletion gene mutations and presence of plasmid *pMK101*.[13] Each mutant strain was incubated in an Oxoid nutrient broth No. 2 and incubated overnight at 37°C in a shaking water bath before use. The preincubation method of Ames test was performed to determine the mutagenic effect of the CPE root on *S. typhimurium* strains TA98 and TA100 under acidic condition.[14] Briefly, the ethanolic extract was dissolved in DMSO to the concentration of 25, 50, 100 and 200 mg/ml. They were acidified to pH 3.0–3.5 by adding 200 µl of each solution to the tube containing 550 µl of 0.2 N hydrochloric acid and adjusted to 1000 µl by 250 µl of DMSO (without nitrite treatment) or 250 µl of 2 M sodium nitrite (with nitrite treatment) then incubated under shaking at 37°C for 4 h. One hundred microliters of the acidified extracts were neutralized with 500 µl of 0.5 M phosphate buffer (pH 7.4) before adding 100 µl of bacterial suspension and incubated at 37°C in shaking water bath for 20 min. Two milliliters of top agar containing 0.5 mM L-histidine and 0.5 mM D-biotin at 45°C were added, mixed well, and poured over the surface of a minimal glucose agar plate. The plates were incubated at 37°C for 48 h and the numbers of his + revertant colonies were counted. DMSO was used as a negative control to determine the spontaneous reversion activity. All tests were performed in triplicate. The mutagenic index (MI) was calculated from the number of revertant colonies of the sample treatment divided by the number of spontaneous revertant colonies. Positive mutagenic effect was considered when the number of induced revertant colonies increases in a dose-response relationship manner, at least two doses were higher than spontaneous revertant (MI ≥1) and at

least one dose gave rise to twice over the spontaneous revertant (MI >2).[15]

Antimutagenic testing

The antimutagenic effect of the extract against 1-aminopyrene treated with sodium nitrite was determined by the preincubation method of Ames test similar to the mutagenic testing. Ten microliters (tested on TA98) or 20 µl (tested on TA100) of 0.075 mg/ml 1-aminopyrene was transferred into the sterile test tube. Then, 740 µl or 730 µl of 0.2 N hydrochloric acid and 250 µl of 2 M sodium nitrite were added to obtain the total volume at 1 ml. The mixtures were shaken at 37°C for 4 h. Stopped reaction for 1 min in an ice bath, then added 250 µl of 2 M ammonium sulfamate and allowed the test tube to stand in an ice bath for 10 min before Ames test. Mixed 25 µl of nitrite treated 1-aminopyrine with 500 µl of 0.5 M phosphate buffer (pH 7.4), added 100 µl of each tester strains that overnight cultured. An aliquot (0, 25, 50, and 75 µl) of CPE roots (200 mg/ml in DMSO) was added, and the final volume was adjusted to 700 µl with DMSO. The mixture was incubated at 37°C in shaking water bath for 20 min, after that, added to 2 ml top agar containing histidine–biotin. The mixture was poured over the surface of a minimal agar plate and incubated for 48 h at 37°C. After incubation, revertant (mutant) colonies were counted. All tests were performed in triplicate. The percent inhibition was calculated by the following formula:[16]

$$\% \text{ Inhibition} = ([A - B]/[A - C]) \times 100$$

Where A was the number of histidine revertant colonies per plate induced by nitrite treated 1-aminopyrene, B was the number of histidine revertant colonies per plate by nitrite treated 1-aminopyrene in the present of extract and C was the number of spontaneous revertant colonies per plate. The percentage of inhibition was classified as strong when it is higher than 60%, moderate ranged from 60% to 41%, weak ranged from 40% to 21% and negligible effect when it was <20%.[17]

Statistical analysis

The mean of the LPS control was normalized to 100% and mean of cell control was normalized to zero. Statistical analysis was performed by ANOVA, followed by a Dunnett's *post-hoc* test. The significant level was chosen at $P < 0.01$ for all statistical analyses.

RESULTS

Cell viability and nitric oxide inhibition assay

The ethanolic extract yield was 7.25% of *C. paniculatum* root. It was found to be non-toxic on J774A cell. NO scavenging activity of the CPE root on LPS-stimulated macrophage cells was shown in a concentration-dependent manner [Figure 1]. At 50 and 100 µg/ml, the CPE significantly inhibited NO production in LPS-stimulated cells at 36% and 97%, respectively. The IC_{50} was 56.28 µg/ml.

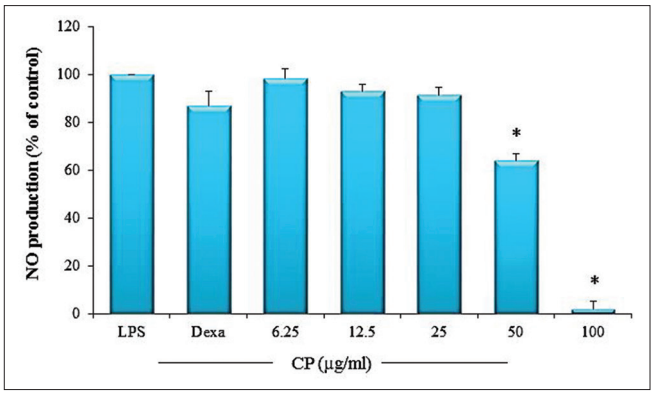

Figure 1: Effect of the ethanolic root extracts of *Clerodendrum paniculatum* on lipopolysaccharide (LPS)-stimulated macrophage inhibition of nitric oxide. Results are presented as the mean ± standard division ($n = 6$). *$P < 0.01$, compared to LPS

Tumor necrosis factor-α and prostaglandin E2 inhibition assay

At 50 and 100 µg/ml, the CPE root significantly inhibited TNF-α level at 25% and 43%, respectively. The significant inhibition of PGE_2 production was demonstrated at 25, 50, and 100 µg/ml of the CPE. Maximum inhibition of PGE_2 production (80%) was observed at 100 µg/ml of the ethanolic extract. Dexamethasone showed 57.53% and 60.67% inhibition of TNF-α and PGE_2 production, respectively [Figures 2 and 3].

Mutagenic and antimutagenic effect

The results of the mutagenic assay of *C. paniculatum* obtained on *S. typhimurium* strains TA98 and TA100 by the Ames test are shown in Table 1. All concentrations (0.4, 0.8, 1.6, and 3.2 mg/plate) of the CPE with and without nitrite treatment were non-toxic and non-mutagenic toward both strains. Sodium nitrite-treated 1-aminopyrene as mutagen exhibited high-mutagenic effect on *S. typhimurium* strains TA98 and TA100 with MI = 18.05 and MI = 4.70, respectively. Furthermore, the CPE had dose-related inhibition effect to the mutagenicity induced by sodium nitrite-treated 1-aminopyrene toward *S. typhimurium* strains TA98 and TA100 in the absence of activating system [Figure 4]. All concentrations showed the strongly inhibitory activity on both strains, only at 5 mg/plate showed moderate inhibitory activity to strain TA98. The CPE exhibited the highest strong inhibitory activity with 100% on strain TA100 and 99.01% on strain TA98 at 15 mg/plate.

DISCUSSION

This study evaluated the inhibitory effect of root extract of *C. paniculatum* on LPS-induced NO, TNF-α, and PGE_2 production in J774A.1 cells. LPS is a gram-negative bacteria which has an endotoxin and a constituent of the outer

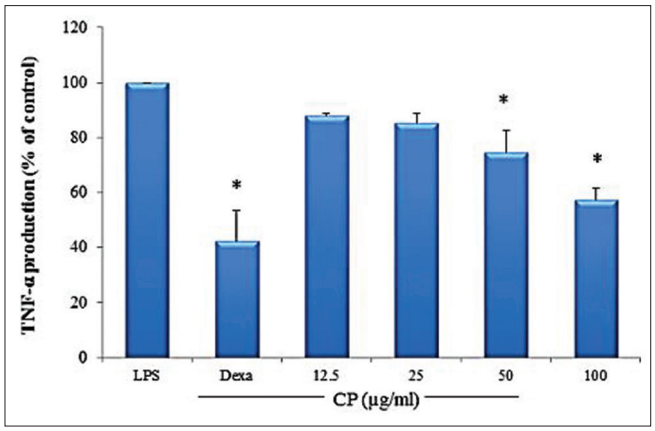

Figure 2: Effect of the ethanolic root extracts of *Clerodendrum paniculatum* on lipopolysaccharide (LPS)-stimulated macrophage inhibition of tumor necrosis factor-α. Results are presented as the mean ± standard division ($n = 6$). *$P < 0.01$, compared to LPS

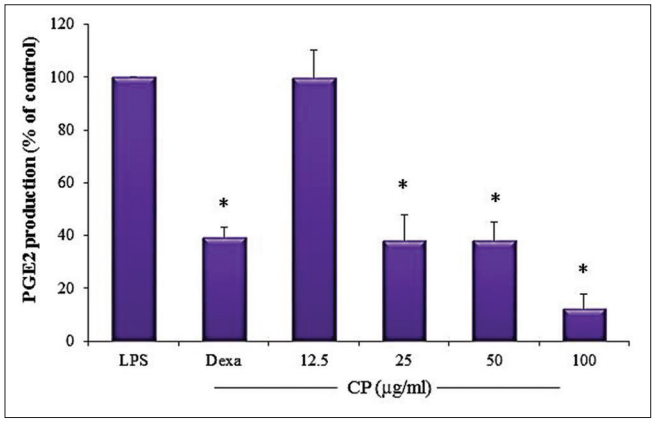

Figure 3: Effect of the ethanolic root extracts of *Clerodendrum paniculatum* on lipopolysaccharide (LPS)-stimulated macrophage inhibition of prostaglandin E2. Results are presented as the mean ± standard division ($n = 6$). *$P < 0.01$, compared to LPS

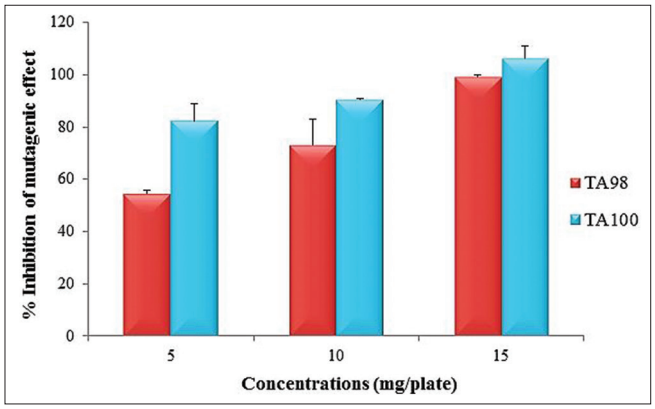

Figure 4: Inhibitory effect of the ethanolic extract of *Clerodendrum paniculatum* on the mutagenicity of sodium nitrite-treated 1-aminopyrene on *Salmonella typhimurium* strains TA98 and TA100 using Ames test

membrane. LPS stimulates innate immunity by regulating the production of inflammatory mediators such as NO, TNF-α, and PGE_2.[1] Examination of the cytotoxicity of the extract in J774A.1 macrophage using the resazurin assay had indicated that all extracts at 0–100 µg/ml did not affect the viability of J774A.1 cells. Thus, the inhibition of LPS-induced mediator inflammation by the extract was not the result of a possible cytotoxic effect on these cells. This study demonstrated that the root extract of *C. paniculatum* significantly reduced the production of NO, TNF-α, and PGE_2. At 100 µg/ml, the root extract of *C. paniculatum* showed the high significant inhibition of NO (98%) and PGE_2 (80%) production, while the inhibition of TNF-α production was moderate (43%). The result suggested that the root extract of *C. paniculatum* might inhibit the chemical mediators better than pro-inflammatory cytokines. This finding agreed with the study of Joseph *et al.* which showed that petroleum ether and chloroform extracts of *C. paniculatum* leaves possessed significant anti-inflammatory activity by *in vitro* and *in vivo* assay.[18]

Table 1: Mutagenic assays for the CPE with and without nitrite treatment using *Salmonella typhimurium* TA98 and TA100 strain

Sample (mg/plate)	TA98		TA100	
	Number of revertant colonies	MI	Number of revertant colonies	MI
CPE without nitrite				
DMSO	22.00±2.53	-	99.33±9.69	-
0.4	27.67±6.71	0.26±0.31	126.00±36.03	0.27±0.36
0.8	25.50±5.89	0.16±0.22	121.67±5.68	0.23±0.06
1.6	33.17±2.64	0.51±0.12	101.17±7.68	0.02±0.08
3.2	31.00±6.69	0.41±0.30	114.83±13.47	0.16±0.14
CPE with nitrite				
DMSO	56.33±8.48	-	134.00±7.04	-
0.4	156.00±9.40	1.77±0.17	174.67±5.05	0.30±0.04
0.8	166.50±10.39	1.96±0.18	190.67±19.66	0.42±0.15
1.6	240.83±16.01	3.28±0.28	272.00±70.54	1.03±0.53
3.2	222.83±61.41	2.96±1.10	171.33±25.03	0.28±0.19

Data are reported as means±SD of three independent replicates and the MI. CPE: Ethanolic extract of *Clerodendrum paniculatum*, MI: Mutagenic index, SD: Standard deviation, DMSO: Dimethyl sulfoxide

For the Ames Salmonella assay, it is short-term *in vitro* screening which has highly efficient in detecting carcinogens and mutagens. It has been tested with a wide variety of carcinogens such as direct alkylating agents, nitrosamines, polycyclic hydrocarbons, fungal toxins, aromatic amines, nitrofuran carcinogens, a variety of antineoplastic agents, and antibiotic carcinogens such as adriamycin, daunomycin, and mitomycin C.[19] In this study, the mutagenic and antimutagenic activities of root extracts of *C. paniculatum* with and without nitrite treatment were tested in absence of metabolic activation and using preincubation method of the Ames test based on *S. tryphimurium* TA98 (detecting

frameshift mutagens) and TA100 (detecting base-pair substitute mutagens) to observe the response of the extracts in an acidic condition which mimicked gastric condition *in vivo*. The results showed that the CPE with and without nitrite treatment was not mutagenic on both strains. For antimutagenic activity, these results indicated that the CPE was strongly antimutagenic against both framshift and base substitution mutation induced by nitrite-treated 1-aminopyrene. Similarly, result was reported on *Clerodendrum* petasites S. Moore by Singharachai *et al.*[20]

CONCLUSION

The overall results of the present investigation suggested the pharmacological potential of *C. paniculatum* dried root crude drug. The root extract of *C. paniculatum* possessed the anti-inflammatory potential by reducing the release of inflammatory mediators (NO, PGE_2) and pro-inflammatory cytokine (TNF-α). Furthermore, the present study indicated that the root extract of *C. paniculatum* was non-mutagenic and capable to inhibit the mutagenicity of nitrite treated 1-aminopyrene mutagen on *S. typhimurium* strains TA98 and TA100.

REFERENCES

1. Liao JC, Deng JS, Chiu CS, Hou WC, Huang SS, Shie PH, *et al*. Anti-inflammatory activities of *Cinnamomum* cassia constituents *in vitro* and *in vivo*. Evid Based Complement Alternat Med 2012;2012:429320.
2. Debprasad C, Hemanta M, Paromita B, Durbadal O, Kumar KA, Shanta D, *et al.* Inhibition of NO (2), PGE (2), TNF-a, and iNOS EXpression by *Shorea robusta* L.: An ethnomedicine used for anti-inflammatory and analgesic activity. Evid Based Complement Alternat Med 2012;2012:254849.
3. Fujiwara N, Kobayashi K. Macrophages in inflammation. Curr Drug Targets Inflamm Allergy 2005;4:281-6.
4. Dugowson CE, Gnanashanmugam P. Nonsteroidal anti-inflammatory drugs. Phys Med Rehabil Clin N Am 2006;17:347-54, vi.
5. Plant Extracts Suppliers; 2012. *Clerodendrum paniculatum*. Available from: http://www.floridata.com/ref/c/cler_pan.cfm. [Last accessed on 2014 May 03].
6. Malaysian Flora; 2010. *Clerodendrum paniculatum* Lin Pagoda flowers. Available from: http://www.mynicegarden.com/2010/06/clerodendrum-paniculatum-pagoda-flower.html. [Last accessed on 2014 May 03].
7. Arun PV, Sachin S, Suganyadevi P. Antioxidant potential of *Clerodendrum paniculatum*. J Pharm Res 2011;4:1796-9.
8. John J, Mathew AJ, Setty MM. Free radical scavenging and anticancer activity of *Clerodendrum paniculatum*. Pharmacologyonline 2008;3:730-43.
9. Manohan R, Palanuvej C, Ruangrungsi N. Genotoxic and cytotoxic effects of Ben-Cha-Moon-Yai remedy and it's ingredients. Res J Pharm Biol Chem 2013;4:1144-55.
10. Wongwattanasathien O, Kangsadalampai K, Tongyonk L. Antimutagenicity of some flowers grown in Thailand. Food Chem Toxicol 2010;48:1045-51.
11. Anoopkumar-Dukie S, Carey JB, Conere T, O'sullivan E, van Pelt FN, Allshire A. Resazurin assay of radiation response in cultured cells. Br J Radiol 2005;78:945-7.
12. Amano F, Noda T. Improved detection of nitric oxide radical (NO) production in an activated macrophage culture with a radical scavenger, carboxy PTIO, and Griess reagent. FEBS Lett 1995;368:425-8.
13. Levin DE, Yamasaki E, Ames BN. A new Salmonella tester strain, TA97, for the detection of frameshift mutagens. A run of cytosines as a mutational hot-spot. Mutat Res 1982;94:315-30.
14. Maron D, Ames BN. Revised methods for the *Salmonella* mutagenicity test. Mutat Res 1983;113:173-215.
15. Mortelmans K, Zeiger E. The Ames *Salmonella*/microsome mutagenicity assay. Mutat Res 2000;455:29-60.
16. Reid KA, Maes J, Maes A, van Staden J, De Kimpe N, Mulholland DA, *et al.* Evaluation of the mutagenic and antimutagenic effects of South African plants. J Ethnopharmacol 2006;106:44-50.
17. Calomme M, Pieters L, Vlietinck A, Vanden Berghe D. Inhibition of bacterial mutagenesis by Citrus flavonoids. Planta Med 1996;62:222-6.
18. Joseph J, Bindhu AR, Aleykutty NA. *In vitro* and *in vivo* antiinflammatory activity of *Clerodendrum paniculatum* Linn. leaves. Indian J Pharm Sci 2013;75:376-9.
19. Ikken Y, Morales P, Martínez A, Marín ML, Haza AI, Cambero MI. Antimutagenic effect of fruit and vegetable ethanolic extracts against N-nitrosamines evaluated by the Ames test. J Agric Food Chem 1999;47:3257-64.
20. Singharachai C, Wongwattanasathien O, Ruangrungsi N. Mutagenicity and anti-mutagenicity of Ben-Cha-Lo-Ka-Wi-Chian remedy using Ames test. J Thail Tradit Alternat Med 2009;7:115.

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