

EVALUATION OF BIOLOGICAL ACTIVITIES FROM THAI HERBAL LOZENGES

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Abstract

UM-MA-RUEK-KA-VA-TEE (UM) is multi-herbal formula as antitussive and expectorant. New UM formula was prepared in lozenge for simply use. The research was proposed to evaluate bioactive constituents and biological activities of lozenge herbal mixture, including liquorice root, Indian gooseberry, coriander seed, gall of chebolic myrobalans, cumin, belleric myrobalan and karanda fruit. Phenolic, flavonoid and saponin contents were determined by spectrophotometric methods. Antioxidant activity was evaluated by DPPH and NO radical scavenging and reduction of lipid peroxidation. Anti-inflammation was evaluated by inhibition of albumin degradation and NO production of LPS-induced macrophages. Cytotoxicity was evaluated against human dermal fibroblasts. Herbal mixture (10%) and other ingredients was performed to lozenge. Reduction of oral microbials was determined in healthy volunteers (N = 30) before and after use lozenges by a modified dip-slide test. Total phenolic, total flavonoid and saponin contents were 188.34±2.96 mg of GAE/g, 19.24±1.59 mg of QE/g and 534.80±34.18 mg of SE/g, respectively. Herbal mixture was strongly inhibited DPPH and NO radicals, and potently reduced NO production from macrophages and without cytotoxicity. Oral microbials, including *Mutans* streptococci and *Candida* sp. were significantly decreased after lozenge use. New UM formula was contained saponins as most bioactive compound, and antioxidant, anti-inflammation and anti-microbial activities was preferable.

Keywords: antitussive, biological activity, expectorant, herbal lozenge, Thai herbal medicine.

INTRODUCTION

Traditional herbal medicine is become important in healthcare units in Southeast Asia including Thailand. The utilization of herbal medicine is highly applied for treatment of chronic diseases, and biological activities of common Thai herbs are prevention or treatment on chronic diseases [1]. “UM-MA-RUEK-KA-VA-TEE”, UM is multi-herbal formula from Thai Ayurveda medicine and including in national list of essential medicine (NLEM) as antitussive and expectorant [2]. There is contain liquorice (*Glycyrrhiza glabra*, *G. uralensis*, or *G. inflata*) root, Indian gooseberry (*Phyllanthus emblica*), coriander (*Coriandrum sativum*) seed, gall (infected leaves and young shoots) of chebolic myrobalans (*Terminalia chebula* Retz.), cumin (*Cuminum cyminum*), belleric myrobalan (*Terminalia bellirica* Roxb.) fruits and *Aristolochia* stem [2].

UM medicines are available in powder and boluses forms and preparing with honey [2]. Recently, UM formula is removed *Aristolochia* stem due to toxicity and carcinogenicity of aristolochic acid contained [3, 4]. Three herbal ingredients, chebolic myrobalan, belleric myrobalan and Indian gooseberry fruits are similar to Indian and Thai Ayurveda formula or “Triphala”, which commonly treatment various chronic diseases, such as cardiovascular disease, hypertension, lipidaemia, liver diseases, asthma, intestinal inflammation and chronic ulcer.

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Several reports on biological activities of Triphala are antimutagenic, radioprotective, antibacterial and antioxidant activity; and bioactive constituents are flavonoids, alkaloids and phenols [5]. UM formula is contained more powerful herbal ingredient, such as liquorice root that exhibit a broad range of biological properties, including antibacterial, anti-inflammatory, antiviral, antioxidant, and antidiabetic activities [6]. However, biological activity of UM formula is reported only antioxidant activity and contain highly phenolic compounds and saponins [2, 7].

Karanda (*Carissa carandas* L.) fruits are possessed various health promoting effects i.e., antioxidant, anti-inflammatory, antidiabetic and antimicrobial activities. Karanda fruits are containing various phytochemical constituents including flavonoids, phenolic acids, steroids, volatile oils, lignans, alkaloids, polysaccharides [8, 9]. Recently, we are developing the new UM formula using karanda fruits replaced to *Aristolochia* sp. and preparing in lozenge form, which is convenient and portable to apply for cough and sore throat relief [10]. Hence, aims of this study were determination of bioactive constituents of herbal mixture in lozenge including phenolic compounds, flavonoids and saponins; and evaluation of its biological activities, such as antioxidant, anti-inflammation, antimicrobial and cytotoxicity activities. These findings can support health promoting benefit for this new Thai herbal lozenge as effective sore throat relief in traditional healthcare aspect.

MATERIALS AND METHODS

Herbal Mixture Preparation

All medicinal plants were purchased from Bangkok and Samut Songkhram, Thailand. Botanical characteristics of herbs were authenticated by senior botanists from Provincial Agricultural Office, Samut Songkram, Thailand. Herbal identification was done according by Saralamp [11] and Thai Pharmacopoeia [12]. Thai herbs including liquorice root (35 g), Indian gooseberry, coriander seed, gall of chebulic myrobalans, cumin seed, belleric myrobalan fruits and karanda fruits (7 g for each) were washed, dried in an oven at 50°C and powdered and macerated with 95% ethanol for 3 days, filtered and concentrated to dryness under pressure and dried by evaporator as herbal mixture.

Evaluation Of Bioactive Compounds

Total phenolic content (TPC) of herbal mixture was determined by Folin-Ciocalteu method. Colorimetric absorbance of reaction mixture was measured at 760 nm. Range of gallic acid concentrations were used as standard curve. TPC of herbal mixture was represented as mg of gallic acid equivalent (GAE) per g [13]. Total flavonoid content (TFC) of herbal mixture was determined by aluminium chloride colorimetric method. Colorimetric absorbance of reaction mixture was measured at 420 nm. Range of quercetin concentrations were used as standard

curve. Flavonoid content was represented mg of quercetin equivalent (QE) per g [13]. Total saponin content (TSC) of herbal mixture was determined by vanillin colorimetric method and reaction was performed with Liebermann-Burchard reagent. Colorimetric absorbance of reaction mixture was measured at 528 nm. Range of saponin concentrations were used as standard curve. Saponin content was represented mg of saponin equivalent (SE) per g [14]. All bioactive compounds were done in triplicate measurements.

Antioxidant Activity Of Herbal Mixture

DPPH radical scavenging activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical in present of herbal mixture (0.01-1 mg/ml) was reduced DPPH absorbance. DPPH radical (6×10^{-5} M) and ascorbic acid were used as negative and positive controls, respectively [15] (Yen and Duh, 1994). Lipid peroxidation inhibition (LPI) activity: lipid peroxidation in present of herbal mixture (0.001-10 mg/ml) was reduced absorbance of ferric iron-thiocyanate complex. α -tocopherol was used as positive control [16]. NO radical scavenging activity: nitric oxide (NO) radical in present of herbal mixture (0.01-1 mg/ml) was reduced absorbance of Griess reagent reaction. Ascorbic acid was used as positive control [17]. Absorbance reaction mixture of all assays were monitored by micro-titer plate reader at maximum absorbance wavelength (λ_{max}). Results was calculated from triplicate measurements and interpreted as 50% inhibitory concentration (IC_{50}) of herbal mixture.

Anti-Inflammatory Activity Of Herbal Mixture

Inhibition of albumin degradation: herbal mixture was dissolved with Tween 20 (20%) and centrifuged (150 rpm) for 5 min. Supernatant was pipetted and diluted (0.01- 100 mg/ml). Each concentration of herbal mixture was incubated with albumin solution at 70 ± 2 °C for 5 min. Reduction of albumin absorbance was monitored by micro-titre plate reader. Diclofenac diethylammonium was used as positive control. Results was calculated and represented as IC_{50} of sample on reduction of albumin degradation [18].

Inhibition of NO production from LPS-induced macrophage cells: mouse macrophage cell (RAW264.7) was obtained from Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand. Cells were seeded in high-glucose Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin) and incubated at 37 °C (containing 5% CO_2) in humidified incubator. After overnight culture in a 24-well plate (1×10^5 cells/well, 500 μ L medium/well), the cells were pre-treated with herbal mixture for 1 h and lipopolysaccharide (LPS) for an additional 24 h, the culture supernatant from each well was collected and used to measure NO production. 50 μ L of culture medium and 100 μ L of Griess reagents were added and incubated in a 96-well plate at room temperature (10 min). Absorbance of reaction mixture was then measured using a microplate reader [17, 19]. Triamcinolone acetonide (0.1 mg/ml) was used as positive control. Results was calculated from triplicate measurements and interpreted as 50%

inhibitory concentration (IC₅₀) of herbal mixture for all anti-inflammatory assays.

Cytotoxicity Test

The resazurin microtiter assay (REMA) was performed to evaluate cytotoxicity with human dermal fibroblasts C-004-5C. Cells were cultured at appropriate condition and resuspended to 2.2-3.3 x 10⁴ cells/ml. Cell suspension (45 µl) was added 50 µl of 5% dimethyl sulfoxide (DMSO) and range of herbal mixture was added in each well of microplate. The mixture was incubated at 37°C in humidified incubator (contained 5% of CO₂). After incubation (3-5 days), 12.5 µl of resazurin (62.5 µg/mL) was added to cell suspension mixture was continued incubation (4 h). Fluorescence signals were measured by microplate reader at excitation and emission wavelength of 530 and 590 nm, respectively. Three-fold serial intensity dilution and the intensity of the cell-restraint extract were calculated for IC₅₀. Sodium lauryl sulphate was used as positive control [20].

Herbal Lozenge Preparation and Anti-Microbial Test

Ingredient of lozenge was Thai herbal mixture (10%), spearmint, avicel, aerosol, xylitol, menthol, microcrystal cellulose and magnesium stearate in a certain ratio (Thai patent application no. 2003003081). Herbal mixture was mixed homogeneously with avicel and aerosol. Former mixture was mixed again with spearmint, menthol, microcrystal cellulose, magnesium stearate. Lozenge mixture was pressed in tablet form and made in 500 mg/each (Fig. 1). Lozenges were kept in sunlight protected container. Anti-microbial test (in vivo) was determined the reduction of oral microbials from volunteers before and after use lozenges (one tablet) within 5 to 10 mins by a modified dip-slide test [21], MU test kit (Mahidol University, Thailand). Healthy volunteers (N = 30) were recruited in this study where were in Clinic of Thai Traditional Medicine, College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram, Thailand during March to May, 2021. Case-control study was conducted on healthy volunteers (N = 30) who were not sore throat/cold-relating symptoms and took any anti-biotics before joining in this study. Oral bacteria in healthy volunteers were reduced after applied lozenges. Microbial count was conducted with *Mutans* streptococci, *Candida* sp. and, *Lactobacilli* sp and done in triplicate for each count. Dependent *t*-test was analysed the different of microbial colonies before and after lozenge usage; and statistically significant was judged at *p*-value <0.05. This study had approved by Suan Sunandha Rajabhat University Ethic Committees, Thailand (COA.1-

6060/2020).

STATISTICAL ANALYSIS

Phytochemical content and biological activities of herbal mixture were analysed by descriptive statistics. Different of microbial colonies before and after lozenge usage was determined by Dependent *t*-test and statistically significant was judged at *p*-value < 0.05.

RESULTS AND DISCUSSION

After extraction and preparation, characteristics of herbal mixture were semi-solid (resin-like), yellowish-brown colour and had special odor. Total phenolic, total flavonoid and saponin contents of herbal mixture were 188.34±2.96 mg of GAE/g, 19.24±1.59 mg of QE/g and 534.80±34.18 mg of SE/g, respectively. Herbal mixture was strongly inhibited DPPH radicals, NO radical (IC₅₀ = 0.06±0.0 and 1.30±0.2 mg/ml), when compared with ascorbic acid (IC₅₀ of 0.03±0.01 and 0.25±0.05 mg/ml, respectively). However, herbal mixture was poorly inhibited lipid peroxidation (IC₅₀ = 502.4±21.97 mg/ml) when was compared with α-tocopherol (IC₅₀ = 0.39±0.1 mg/ml) (Table 1). Anti-inflammation activity of herbal mixture (0.1 mg/ml) was potently reduced NO production (23.0±3.19%) from LPS-induced macrophage cells when compared with 1 mg of triamcinolone acetonide. However, herbal mixture was ineffectiveness anti-inflammation on inhibitory albumin degradation. Herbal mixture was determined cytotoxicity against human skin fibroblast cells. Cell survival incubated with lozenges was 99.6 to 125.5% and interpreted as non-toxic when compared with sodium lauryl sulphate (Table 2). The characteristic of Thai herbal lozenges was tablet form (Fig. 1) and actually dose was oral apply for 1 to 2 lozenges in three times daily [2, 7]. The oral microbials in healthy volunteers were determined before and after herbal lozenge usage by modified dip-slide test. Amount of oral microbial colonies in modified slides were reduced after applied lozenges. Microbial count was conducted on *Mutans* streptococci, *Candida* sp. and, *Lactobacilli* sp. which was reduced from 25.6±4.56 to 6.6±5.32, 32.8±5.74 to 16.6±6.88 and 15.4±8.35 to 12.0±7.14 colonies, respectively. In addition, *Mutans* streptococci and *Candida* sp. were significantly decreased (Table 3). Volunteers were also satisfied on these lozenges.

Table 1 Bioactive compounds and antioxidant activity of herbal mixture contained in lozenge formula *

Test / Assay	TPC	TFC	TSC	DPPH	NO	LPI
Herbal mixture	188.34±2.96	19.24±1.59	534.80±34.18	0.06±0.00	1.30±0.2	502.4±21.97
α-tocopherol	-	-	-	-	-	0.39±0.10
Ascorbic acid	-	-	-	0.03±0.01	0.25±0.05	-

*Antioxidant activity was represented as 50% of inhibitory concentration, IC₅₀ (mg/ml)

Table 2 Anti-inflammation activity and cytotoxicity of herbal mixture contained in lozenge formula

Test / Assay	ADI ^a	LPS ^b	Cytotoxicity ^c
Herbal mixture	ND	23.0±3.19	ND
Diclofenac diethylammonium	0.34±0.01	-	-
Triamcinolone acetonide	-	34.3±3.35	-
Sodium lauryl sulfate	-	-	0.1-1.0 ^d

^a Anti-inflammation was inhibition of albumin degradation (ADI) and represented as 50% of inhibitory concentration, IC₅₀ (mg/ml).

^b Anti-inflammation was inhibition of NO production from lipopolysaccharide (LPS)-induced macrophage and represented as percent of inhibition. Maximum inhibitory concentration of herbal mixture and triamcinolone was 0.1 and 1.0 mg/ml, respectively.

^c Concentration of extract was 0.0001-1.0 mg/l was evaluated for cytotoxicity with human skin fibroblast cells; and toxicity was determined by viability of cells.

^d Toxicity of 0.1 and 1.0 mg/ml of positive control was affected on reduction of cell viability by 48.68±5.65 and 7.84±0.35%, respectively.

ND = not determined.

Table 3: Oral microbial reduction from volunteers before and after apply lozenge by a modified dip-slide test

Condition / Oral microbial	<i>Mutans streptococci</i> ^a	<i>Candida sp.</i> ^b	<i>Lactobacilli sp.</i>
Before apply lozenge	25.6±4.56	32.8±5.74	15.4±8.35
After apply lozenge	6.6±5.32	16.6±6.88	12.0±7.14

^a statistically significant at p <0.001; ^b statistically significant at p <0.01

Recently, the utilizing of natural product formulations is become favor and use of herbal extracts is safer with lower side effects when compared to chemical agents. Medicinal plants were most relied choice in primary healthcare uses [1, 22]. Ingredients of UM is multi-herbal formula from Indian and Thai Ayurveda medicines and these ingredients were three herbs of Triphala formula i.e., chebulic myrobalan, beleric myrobalan and Indian gooseberry. Triphala is possessed preferable antioxidant activity when compare to ascorbic acid and determined by various assays including DPPH radical scavenging, ferric reducing antioxidant power, superoxide dismutase and catalase assays [7]. Our result was found antioxidant activity of UM based herbal mixture against DPPH and NO radicals rather than lipid peroxidation. There is implied that antioxidant activity of herbal mixture was possessed from Triphala herbs and may synergist with another herb ingredient, such as liquorice root [6] and karanda fruit [8, 9, 10]. This herbal extract was contained phenolic compounds, flavonoids and saponins. Saponins were determined in high amount, which are amphipathic glycosides, maybe mono- or poly- desmodic, which are depending on the number of sugar moiety attachment. Potent antioxidant activity of herbal mixture is depended on hydrophilic condition of assay environment and numbers of sugar moieties linked to saponins. Liquorice root is main ingredient of herbal mixture, which is contain saponins and possess expectorant, bacteriostatic and antiviral activities [6]. Ginsenosides are commonly natural saponins, which are represent as immunomodulant and pain-relief properties [23]. In previous study is reported that Triphala formula has not anti-inflammatory activity by inhibitory protein denaturation [5], and our finding in UM-based herbal mixture was corresponded. However, herbal mixture was good agents for inhibiting NO production from

induced-macrophage cells, and there was possessed “indirect” anti-inflammatory action. This result may come from other ingredients of herbal mixture, such as liquorice root [6] and karanda fruit [8, 9, 10] that are possessed anti-inflammation property. Oral Herbal lozenge was inhibited three oral microbial growth i.e., *Mutans streptococci*, *Candida sp.* and, *Lactobacilli sp.*; and *Mutans streptococci*, *Candida sp.* were significantly decreased. It was concluded that herbal mixture can reduced risk of oral cavity and throat infection and dental caries [24, 25]. Antimicrobial activity of this herbal mixture may provide from all of herbal ingredients rather than Triphala herbs [5], liquorice root [6] and karanda fruit [8, 9, 10], such as cumin [26, 27] and coriander seeds [28, 29].

CONCLUSION

Lozenge herbal mixture from new Thai medicinal formula was contained saponins as most of bioactive compounds, and possessed preferable antioxidant, anti-inflammation and anti-microbial activities.

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CONFLICT OF INTEREST

There is no conflict of interest in this study.

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