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IN VITRO ANTI-OXIDANT, ANTI-INFLAMMATORY AND ANTI-MICROBIAL PROPERTIES OF LOCAL HERBAL RECIPE FOR DIABETIC ULCER TREATMENT

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Abstract: This study aimed to investigate the antioxidant, anti-inflammatory and anti-microbial properties of the diabetic ulcer-treatment recipe of folk healer, Mr. Kitti Nakhun. For preparation, the recipe was extracted by maceration with 80% ethanol and boiling with water then both extracts were freeze-dried to give 80% ethanol extract (KE) and water extract (KW). Then the extracts were tested for antioxidant and anti-inflammatory activities. The results showed that the KE and KW exhibited antioxidant activity by using DPPH assay (IC_{50} were 18.66 ± 1.20 and 58.04 ± 8.12 µg/mL, respectively) and the antioxidant activity was extremely related to phenolic content (r^2 was 0.99). In addition, the extracts were cytotoxic tested in RAW264.7 macrophage cells by using MTT assay (IC_{50} of KE and KW were 470 ± 3.54 and 730 ± 3.28 µg/mL, respectively) and examined the anti-inflammatory effect by measuring nitric oxide (NO) production in the cells using Griess reagent. The results showed that after 24 hr of incubation, the 100 µg/mL concentration of KE and KW inhibited the NO production with 43.89 ± 2.72% and 12.95 ± 1.50%, respectively. Moreover, both of the extracts (400 µg/mL, 24 hr of incubation) revealed anti-microbial activity which can be seen from the inhibition zone on gram positive bacteria, *Bacillus subtilis* and an opportunistic pathogenic yeast, *Candida albicans*. The results support the traditional use of this recipe for treating diabetic ulcer and are particularly useful for product development.

Keywords: anti-oxidation, anti-inflammation, anti-microbial activity, diabetic ulcer

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders with chronic hyperglycemia and reflects a major health complication for the 21st century. DM causes cellular damage and induces cell death in various organs in diabetic patients (Lee and Pervaiz, 2007). Diabetes is characterized by loss of pancreatic β cells function, expression and repression of specific apoptotic proteins and elevation of inflammatory cytokines coupled with insulin deficiency. Despite improvement in the treatment and management of diabetes, the rate of prevalence of diabetes seems to be accelerating drastically particularly in developing countries where access to health facilities is poor and patients cannot afford expensive orthodox medicines (Roy *et al*., 2015). DM is usually accompanied by life threatening complications such as diabetic nephropathy, retinopathy and neuropathy (Ni *et al*., 2019). Recent studies have suggested that persistent hyperglycemia can lead to the generation of reactive oxygen species (ROS) which invariably leads to oxidative stress and inflammation. Furthermore, impairment of the

antioxidant defense systems due to excessive generation of ROS can activate several molecular pathways which can contribute to the progression of the disease (Abou-Hany *et al*., 2018). Under normal physiological conditions, a widespread antioxidant defence system protects the body against the adverse effects of ROS generation. The defence mechanism's efficiency is altered in diabetes and the ineffective scavenging of free radicals may therefore play a crucial role in determining tissue damage in these subjects (Bolajoko *et al*., 2008). People suffering from this chronic metabolic disorder have a 15%-25% chance of developing and suffering from diabetic ulcers during their lifetime (Sarkar *et al*., 2018). Patients with diabetic ulcers often will go through pain, swelling, and irritation of the skin which will intensify due to the microbial infections around the affected area. Usually, microorganisms found are *Psudomonas aeruginosa, Acinetobacter baumannii, Staphylococus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella penumoniae,* and *Entrobacter* spp. (Fu *et al*., 2012). Bacteria exacerbate wound inflammation, especially in diabetic patients with impaired nerves and blood vessels, which promotes slower healing of ulcers and can spread to muscles, bones, superficial fascia and joints (Ogba et al., 2019). Without a proper care, diabetic ulcers might lead to other health problems including infection, gangrene, amputation and even death (Snyder and Hanft, 2009). Therefore, the preventive and therapeutic strategy of diabetic ulcers is globally demanding and continuous challenge.

The diabetic wound healing recipe in this study belongs to Mr. Kitti Nakhun, a folk healer in Ubon Ratchathani Province, who has expertise in treating diabetic ulcers using local herbs. The recipe consisting of *Memecylon edule* Roxb. leaves, *Peltophorum dasyrachis* (Miq.) Kurz bark, and *Elaeodendron glaucum* (Rottb.) Pers. bark. *M. edule* has been reported on biological activity related to diabetic ulcers such as antibacterial, wound healing, and anti-inflammatory effects (Nualkaew *et al*., 2009; Srinivasan *et al*., 2017). The biological activity of the *P. dasyrachis* and *E. glaucum* has not been reported, but the active part of both plants is the tannin-containing bark which has good wound-healing effects. Mr. Kitti has been successful in treating diabetic wounds more than 80%, he is a committee of folk healers of Ubon Ratchathani Province and has passed the selection criteria for the registration of folk healers with the Ministry of Public Health. The researcher considers the importance of promoting wisdom in treating diseases with folk medicine. Being accepted internationally requires scientific research to support the use of herbal recipe. Therefore, in order to support the knowledge of folk healers, the research team intends to study various biological activities related to the treatment of diabetic ulcers including antioxidant, anti-inflammatory, and anti-microbial activities.

MATERIALS AND METHODS

Preparation of the recipe extract

The recipe for treating diabetic ulcers in this study consisting of *Memecylon edule* leaves, *Peltophorum dasyrachis* bark, and *Elaeodendron glaucum* bark in equal proportions was prepared. The plant samples were collected from Pibul-Mungsahan District, Ubon Ratchathani Province, Thailand. The plants were identified and authenticated by Thangthong, J (taxonomist from Faculty of Science, Ubon Ratchathani Rajabhat University). Voucher specimen (CTAM-157, CTAM-158 and CTAM-159) were deposited at the Faculty of Thai Traditional and Alternative medicine, Ubon Ratchathani Rajabhat University. The samples were pulverized and a portion was macerated in 80% ethanol (non polar solvent) for 5 days, then filtered through a thin cloth and centrifuged at 5000 rpm, 25°C for 10 min using a laboratory centrifuge (Kubota, Japan). The clear supernatant was concentrated using a rotary evaporator (EYELA, Japan) at 45-50°C, freeze-dried (Christ, Germany) and then the ethanolic extracts of the recipe was obtained (KE). The other portion of the recipe powder was boiled

with water (polar solvent) for 10 minutes, filtered through a thin cloth, centrifuged, freeze-dried and then the water extract of the recipe was obtained (KW).

Determination of antioxidant activity

Antioxidant activity of the extracts was evaluated using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay (Shimada *et al*., 1992). Briefly, the reaction was performed in 96 well-plates with the extract dissolved in its extraction solvent to various concentrations, then extracts solutions mixed with 20 µl of DPPH solution (1 mM in methanol) and incubated at room temperature in the dark for 30 min. The absorbance was measured at 515 nm using a UV spectrophotometer (Shimadzu, Japan). The DPPH solution alone in methanol was used as a control. L-ascorbic acid (Vit.C), a positive control for the DPPH method, was used as a standard antioxidant. DPPH scavenging activity was calculated using the following equation:

$$\% \text{ scavenging activity} = [(A_{control} - A_{extract})/ A_{control}]*100.$$

The percentage of scavenging activity was plotted against concentration, half maximal inhibitory concentration (IC_{50}) of the extracts and Vit.C were calculated using linear regression analysis from the graph.

Determination of total phenolic content

The total phenolics content was determined by the Folin-Ciocalteu method (Sripanidkulchai and Fangkrathok, 2014). The reaction was performed in 96 well-plates with the sample solution mixed with 25 µl of the 1N Folin-Ciocalteau reagent and 70 µl of 20% sodium carbonate. After mixing and letting stand for 40 minutes at the room temperature, the optical density was measured at 725 nm. The total phenolic contents were expressed as mg gallic acid equivalent (GAE)/g dry basis. The relation between total phenol content and antioxidant activity was calculated through the Excel program using the correlation equation (X-axis was antioxidant activity, IC_{50}; Y-axis was total phenolic content).

Determination of anti-inflammation

***Cell culture*:** The murine macrophage cell line, RAW 264.7 cells was purchased from PromoCell, Germany. The cells were cultured in DMEM media supplemented with 10% heat-inactivated calf serum (HyClone, USA) and 1%penicillin (100U/mL)-streptomycin (100µg/mL) and incubated at 37ºC in a humidified atmosphere with 5%CO_2.

***Cytotoxicity test*:** The viability assay of the extract on macrophage cells was determined by using MTT assay (Mosmann, 1983). Cells were seeded in a 96-well plate at a density of 1 x 10^5 cells per well and allowed to attach for 24 h. Various concentrations of the samples were then added and incubated for 24 hr. After incubation, the MTT solution was added to each well at a final concentration of 0.1 mg/mL, and incubated for 3 h. The solution was removed and dimethyl sulfoxide was added. Finally, the cell proliferation was estimated in terms of absorbance at 570 nm.

***Nitric oxide inhibitory test*:** The nitric oxide (NO) assay was performed as described previously with slight modification (Yang *et al*., 2009). After pre-incubation of RAW 264.7 cells (1.5×10^5 cells/mL) with LPS (1 µg/mL) for 24 h, the quantity of nitrite in the culture medium was measured as an indicator of NO production. Amounts of nitrite, a stable metabolite of NO, were measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent. Subsequently, the mixture was incubated at room temperature for 10 min and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as a blank in every experiment and the NO inhibition was calculated as percentage. And, aminoguanidine was used as a positive control.

Determination of anti-microbial property

Bacteria used in this study were three gram positive strains including *Staphylococcus aureus* TISTR 1466, *S . Epidermidis* TISTR 518*, Bacillus subtilis* TISTR 008, and three negative strains including *Escherichia coli* TISTR 780*, Salmonella enterica* TISTR 1529, and *Pseudomonas aeroginosa* TISTR 781. These bacteria were grown in Mueller Hinton broth. In addition, *Candida albicans*, an opportunistic pathogenic yeast was grown in Sabouraud dextrose broth. Microbial strains were grown in the broth at 37°C for 18-20 h and diluted to 1:100 in normal saline solution to 10^6 cfu/mL. Antibiotic sensitivity of test strains was determined using the disc diffusion method (Chuangchot *et al*., 2017). An overnight microbial culture was diluted to 10^6 cfu/mL and then spread on the surface of Mueller-Hinton agar and Sabouraud dextrose agar for bacteria and yeast, respectively. Antibiotic discs were placed on the inoculated agar surface and then incubated at 37°C for 24 h. All tests were performed in triplicate. The diameter of inhibition zones (mm) were measured and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2011). The antibiotic discs used were ampicillin (1 µg) and amphotericin B (1 µg).

RESULTS AND DISCUSSION

DPPH, a stable free radical, was used to study the radical scavenging effects of the extracts. The results revealed that both ethanolic (KE) and water (KW) extracts of the diabetic ulcer treatment recipe showed antioxidant activity. KE had higher potential than KW for further development as it had higher antioxidant activity and total phenolics content. However, the percentage of extraction (% yield) of KE was lower than that of KW, as shown in Table 1. The anti-oxidative effect of medicinal plants was related to the chemical substance, especially phenolic. Phenolic compounds are a main class of secondary metabolites in plants. Many studies have shown a strong and positive correlation between the phenolic contents and the antioxidant potential of fruits and vegetables (Sarawong *et al*., 2014; Reddy *et al*., 2010)

Table 1. Extraction yield, antioxidative activity and total phenolic contents of KE and KW

Samples	Extraction yield (% w/w)	DPPH* (IC_{50}, µg/mL)	Total phenolic* (eq.gallic acid), mg/g
KE	4.2	18.66±1.20	413.19±38.31
KW	11.7	58.04±8.12	146.12±16.01
Vitamin C	-	6.22±0.30	-

* Values were expressed as mean±SD (n=3)

Macrophage RAW 264.7 cells were treated with various concentrations of the extracts and then incubated at 37ºC in a humidified atmosphere with 5%CO_2 for 24h. Cell viability was analyzed by using MTT assay and the absorbance measured at 570 nm. As shown in Figure 1, the results were calculated for % cell viability and expressed as 50% inhibitory concentration. The results showed that KE showed toxicity on the cells higher than that of KW (IC_{50} of KE and KW were 470 ± 3.54 and 730 ± 3.28 µg/mL, respectively). Based on these results, the extracts at doses 50-200 µg/mL (Concentration for cell viability >70%) were used to evaluate the anti-inflammatory effect by nitric oxide level measurement.

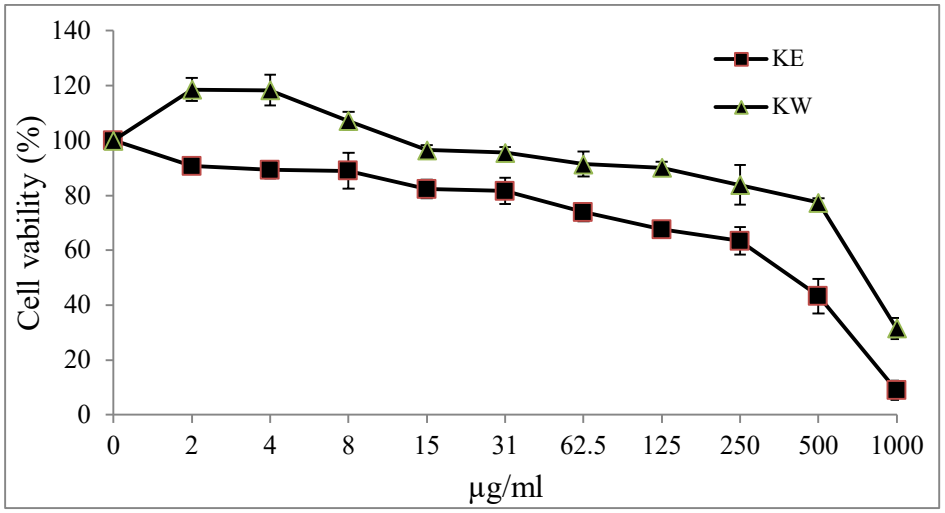

Figure 1. Effect of KE and KW on macrophage RAW264.7 cells viability

Nitric oxide (NO) is a diatomic free radical and generates during the host response against viral and antibacterial infections, and contributes to some pathogenesis by promoting oxidative stress. Oxidative damage may initiate and promote the progression of a number of chronic diseases, including cancer, cardiovascular diseases, Alzheimer's disease, diabetes and inflammation. (Conforti and Menichini, 2011). In this study, the NO inhibitory effect of the extracts was evaluated on LPS-stimulated macrophage RAW264.7 cells. The result found that KE showed NO inhibition greater than that of KW and was very close to the standard substance, aminoguanidine, as shown in Table 2. The results of this research was explained by the relation of NO inhibition and phenolic content in the recipe (Choi *et al*., 2011).

Table 2. Effect of KE and KW on nitric oxide production

Samples	Concentrations (µg/mL)	NO inhibition* (%)
KE	50	34.53 ± 0.83
	100	43.89 ± 2.72
	200	49.67 ± 1.50
KW	50	3.60 ± 2.16
	100	12.95 ± 1.50
	200	27.34 ± 3.81
Aminoguanidine	100	45.32 ± 0.72

* Values were expressed as mean±SD (n=3)

The anti-microbial activity examination showed that most bacteria were sensitive to ampicillin except *P. aeruginosa.* KE and KW had no activity on *S. aureus, S. epidermidis, S. enterica, E. coli* and *P. aeruginosa* while affecting *B .subtilis* which is a gram positive bacteria. The differences in the anti-microbial effect of the extract against gram positive and gram negative bacteria maybe due to the differences in the permeability barriers or compound diffusion properties (Cushnie and Andrew, 2005). Generally, plant extracts have been reported to be more effective against gram positive bacteria than gram negative bacteria (Ahmad and Beg, 2001). In addition, both of the extracts exhibited the anti-mibrobial activity on *C .albicans* especially KE which had an inhibition zone greater than that of KW. DMSO was also effective against *C . albicans* as shown in Table 3. The anti-microbial activity of plant extracts had been attributed to the presence of some constituents in the extracts such as phenolic compounds. The

related mechanisms for the antimicrobial activity of phenolics include an absorption and a disruption of microbial membranes, interaction with enzymes and substrates and metal ion deprivation (Daglia, 2012). The NO inhibitory and antibacterial activity were strongly related to phenolic compounds, especially depending on the position of hydroxyl group in the structure (Karunakaran *et al*., 2018).

Table 3. Anti-microbial activity of KE and KW

Microorganism	Inhibition Zone* (mm)				
	Ampicilin (1 µg)	**Amphotericin B (1 µg)**	**DMSO**	**KE (0.4 mg)**	**KW (0.4 mg)**
S .aureus	51±1.7	ND	NA	NA	NA
S .epidermidis	86.3±1.5	ND	NA	NA	NA
S .enterica	15.0±1.0	ND	NA	NA	NA
E .coli	37.7±0.6	ND	NA	NA	NA
P .aeruginosa	NA	ND	NA	NA	NA
B .subtilis	39.7±2.5	ND	NA	9.7±0.6	7.3±0.6
C .albicans	ND	28.3±0.6	10.0±0.0	10.7±0.6	10.0±0.0

Note: NA =no activity, ND =not determine

* Values were expressed as mean±SD (n=3)

CONCLUSION

Both of the water and ethanol extracts of the diabetic ulcer treatment recipe of Mr. Kitti Nakhun revealed the anti-oxidant, anti-inflammatory and anti-microbial effects. The ethanol extract showed higher in all of the activities and phenolic content than that of the water extract. However, water extract exhibited a higher extraction yield and was safer to macrophage cells than that of the ethanol extract. However, ethanol extract was also biologically active in diabetic ulcers. These results support the traditional use of this recipe for treating diabetic ulcers and are particularly useful for further product development. Further studies are needed to examine more related activities such as wound healing, anti-inflammatory mechanism and also study the active compounds and their machanism of action.

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