Original Article

The Anti-nociceptive Effect and the Reduction of Muscle Hardness of the Combined Extract of *Z. officinale* and *P. amarus* in Animal Model of Myofascial Pain

Sinthuporn Maharan[1], Supaporn Muchimapura[2,3], Wipawee Thukhummee[2,3], Terdthai Thong-Un[2,3], Panakaporn Wannanon[2,3]

[1]Department of Physiology (Neuroscience Program) and Graduate School, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand 40002, +66-43-348394.

[2]Department of Physiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand 40002, +66-43-348394.

[3]Research Institute for Human High Performance and Health Promotion, Khon Kaen University, Khon Kaen, Thailand 40002, +66-43-348394.

Corresponding author : Supaporn Muchimapura, Department of Physiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand 40002, +66-43-348394 supmuc@kku.ac.th

Abstract

Background : *Z. officinale* and *P. amarus* are famous herbs and used as a traditional medicine for a long time. Both herbs have anti-nociceptive and antioxidant properties. However, there is a few data on the development of herbal cream contained both *Z. officinale* and *P. amarus* extract (the combined extract) for dermal application especially in musculofascial condition.

Objective : This study was set up to determine the anti-nociceptive effect and its possible underlying mechanisms of the topical combined extract in hyperalgesic rat.

Material and Methods : 56 male Sprague Dawley rats were randomly divided into the following seven group: 1) control naive, 2) control 3) vehicle 4) positive control (Diclofenac sodium

รับต้นฉบับ 7 มกราคม 2564, ปรับปรุงต้นฉบับ 11 มกราคม 2564, ตอบรับต้นฉบับตีพิมพ์ 13 มกราคม 2564

cream) 5) 25 mg/kg.BW of the combined extract (low dose) 6) 125 mg/kg.BW of the combined extract (medium dose) and 7) 500 mg/kg.BW (high dose) of the combined extract groups. All groups (except control naïve) were induction bilateral hyperalgesic condition via repeated injection of acidic saline pH 4.0 at left gastrocnemius muscle. Then, the three various doses of the combined extract, positive control and vehicle were applied at both left and right gastrocnemius 5 days/week continually for 4 weeks. The nociceptive test was assessed via using mechanical (von Frey filament test), hyperalgesia was evaluated at day 1, 7, 14, 21 and 28. At the end of experiment, left gastrocnemius was collected for evaluate the pathohistological change related oxidative damage occurring in the tissue. All data were express in mean (SD) with p-value < 0.05.

Results : Repeated topical applied of the combined extract 5 times a week, the combined extract could mitigate the effect of acidic saline induced mechanical hyperalgesia in both the ipsilateral (left side) and the contralateral (right side) side when compare with control group. The hyperalgesic rats showed a significantly increase in muscle hardness both side when compared with naïve control group. The combined extract medium dose (500 mg) showed significant decreased muscle hardness after 28 days of treatment when compared with hyperalgesic control rats. After 28 days of treatment period, infiltration of inflammation cells, cell injuries and fibrous tissue were less observed in diclofenac and all doses of the combined extract than the control hyperalgesic group. The hyperalgesic rats were increase in MDA level of left gastrocnemius. While, treated with diclofenac, three doses of the combined extract could significantly decrease the level of MDA. The rat which treated with diclofenac and low dose of the combined extract could significantly increase the level of GPx and SOD scavenging enzymes. The mean level of serum CPK of diclofenac and all doses of the combined extract were significantly reduced mean serum CPK when compared to hyperalgesic control rats.

Conclusion : In conclusion, the innovative topical cream that contain combined extract of *Z. officinale* and *P. amarus* had anti-nociceptive effect against acidic saline induced hyperalgesia in rats. By exert its effect via reduction of tissue injury and restored oxidative status.

Keywords : Anti-nociceptive effect, Muscle hardness, *P. amarus, Z. officinale*

Introduction

Hyperalgesia is one of important abnormal signs that presence in pain conditions including myofascial pain syndrome (MPS) that disturbs the quality of life and decreases the quality of work.[1] Nowadays, there are many therapeutic interventions that have been used for relieving the pain hypersensitization in MPS. Unfortunately, the long term used of medicine such as non-steroidal anti-inflammatory drugs (NSAIDs), muscle relaxants etc. usually produce the gastrointestinal side effects.[2,3] Therefore, numerous complementary and alternative medicines are raised and studied for a decade.

Zingiber officinale (*Z. officinale*) and *Phyllanthus amarus* (*P. amarus*) had been extensively used in traditional medicine. Either *Z. officinale* or *P. amarus* have analgesic effects.[4-8] Thus, both *Z. officinale* and *P. amarus* extract were combined and developed as health product to relieve hyperalgesic condition in myofascial pain syndrome in the form of topical cream. The herbal cream contained the combined extract of *Z. officinale* and *P. amarus* has been proved for its safety, the results showed that the LD_{50} toxicity of both acute and sub-acute dermal is more than 2,000 mg/kg.BW.[9] So, it is highly safe for transdermal application. Therefore, this study aimed to determine the anti-nociceptive, anti-oxidative effect and its possible underlying mechanisms of the combined extract of *Z. officinale* and *P. amarus* in animal model of myofascial pain.

Material and Method

Experimental animal

Adult male Sprague Dawley rats (10-14 weeks old), were used as experiment animals. They were obtained from National Laboratory Animal Center, Salaya, Nakorn Pathom. The weights of the animals on the first day of experiment were 200-350 grams. They were housed 5 rats per cage in the standard metal cages at 22±2 °C on 12:12 h light: dark cycle. All animals were given access to food and water ad libitum. The experiments were performed to minimize animal suffering as followed the experimental protocols approved by the Institutional Animal Care and Use Committee Khon Kaen University, Thailand (Ethic No. MDKKU 3/2559).

The animals were randomized into 7 groups as following; Group I (G I) was naïve control group, Group II (G II) was hyperalgesic rats that received

no-treatment, Group III (G III) was vehicle treated group, Group IV (G IV) was diclofenac treated group which use as positive control, Group V (G V) was low dose of the combined cream extract treated group, Group VI (G VI) was medium dose of the combined extract treated group, Group VII (G VII) is high dose of the combined extract treated group.

All groups (except naïve control) were induced hyperalgesia with the repeated injection (day 0 and day 5) of acidic saline (pH 4.0) at the left lateral gastrocnemius muscle. This method produces bilateral mechanical hyperalgesia of the paw that lasts more than 4 weeks.[10] Then, the assigned treatment cream from each treatment group was applied topically on both left and right legs at volume of 0.1 ml for 5 times per week continuously for 4 weeks.

Testing mechanical hyperalgesia by von Frey filament apparatus

30 minutes after the assigned treatment cream was applied topically on both legs, the rats were subjected to test for mechanical hyperalgesia**.** A series of 15 von Frey filaments start with 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 1.5, 2.5, 3.6 and 4.0 grams (IITC Life Science Inc., USA) were used to evoke paw withdrawal response. The testing starts with the lowest filament for 5 trials per side. Each trial, the filaments were perpendicularly applied to the paw at 10 seconds intervals. The threshold of paw withdrawal response was recorded when the frequency response of the paw withdrawal more than 60%.[11]

The determination of muscle hardness

Muscle hardness meter or commander™ algometry (J-TECH Medical; USA) was used to evaluate the hardness of muscle after repeated injection of acidic saline pH 4.0. Briefly, digital algometry (diameter 0.5 mm^2) was perpendicular applied at both side of lateral gastrocnemius of the rat after irradiation of the Laser for 3 trails. During each trial, the position of both left/right knee and ankle joint of the rat were fixed and maintained in 90° in recumbent by well train physiotherapist (PT license No. 2962, Thailand). The hardness of muscle was shown in real-time with the digital operated numeric from 0-10 LBS after press in muscle. Mean average of the hardness of muscle were conducted to determine.

Histomorphology study

At the end of the experiment, after an anesthesia with sodium pentobarbital (60 mg/kg BW), the animals were perfused with 0.9% normal saline. Injection site of gastrocnemius muscle were collected and immersed sequentially for 24 h each in 10%

formalin. The frozen sample was immersed in a stainless-steel container which is suitable for the tissue size and filled with optimal cutting temperature (OCT) compound. The specimens were frozen rapidly and 5-10 μm thick sections will be made using cryostat and stain with H&E to determine the inflammation cell.

Biochemical assays

Malondialdehyde level

Malondialdehyde (MDA) was determined by quantifying the reaction product with thiobarbituric acid in tissue.[12] The colored end product was read at 540 nm. Results were expressed as nmole MDA/ mg protein.

Scavenging enzymes activity

In this study, the defensive occurring of oxygen free radical species was set up to determine the anti-oxidative role of laser acupuncture in three basically pathway form of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). In brief, the quantifying enzymatic reaction SOD via xanthine/xanthine oxidase method and GPx via were evaluated by 415 nm microplate reader as describe by Weydert and Cullen in 2010.[13] While, CAT scavenge H_2O_2 was determined by 490 nm.[12,13] Results were expressed as unit/mg protein.

Statistical analysis

The Statistical Package for the Social Science (SPSS) version 19.0 (SPSS Inc, Chicago, IL, USA) was used to analyze the parameters. The results were presented as mean ± S.E.M. Statistical significance of differences between groups was detected by one-way ANOVA followed by Tukey and LSD post hoc test. *P*-values lower than 0.05 ($p < 0.05$) were considered significant.

Results

The effect of the combined extract on mechanical hyperalgesia

Repeated topical applied of the combined extract 5 times a week, the combined extract (medium dose) could mitigate the effect of acidic saline induced mechanical hyperalgesia in the ipsilateral side (left side) on day 21st and 28th of treatment (figure 1 A). While the effect of the combined extract (low and medium dose) in the contralateral side (right side), showed a significant improve mechanical hyperalgesia that induced by acidic saline injection on day 14th, 21st and 28th of treatment when compare with control group (figure 1 B).

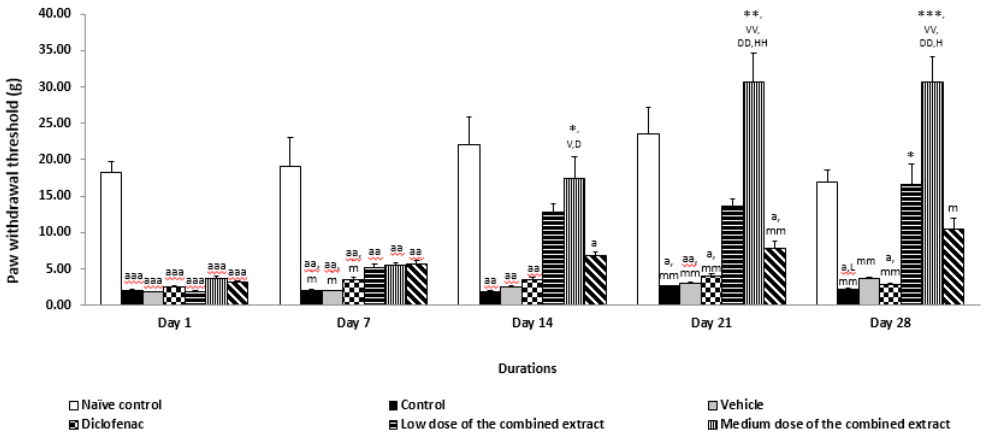

Figure 1-A The effect of the combined extract on mechanical hyperalgesia test in hyperalgesic rats in left side. 30 minutes after the assigned treatment cream was applied topically on both legs, the rats were subjected to test for mechanical hyperalgesia. A series of 15 von Frey filaments start with 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 1.5, 2.5, 3.6 and 4.0 grams were used to evoke paw withdrawal response. [a] $p<0.05$ versus Naïve control group; [aa] $p<0.01$ versus Naïve control group; [aaa] $p<0.001$ versus Naïve control group; * $p<0.05$ versus Control group ; ** $p<0.01$ versus Control group; *** $p<0.001$ versus Control group; [v] $p<0.05$ versus Vehicle group; [vv] $p<0.01$ versus Vehicle group; [D] $p<0.05$ versus Diclofenac group; [DD] $p<0.01$ versus Diclofenac group; [L] $p<0.05$ versus Low dose of the combined extract group; [m, mm, mmm] $p<0.05$, $p<0.01$, $p<0.001$versus Medium dose of the combined extract group repectively; [H] $p<0.05$ versus High dose of the combined extract group.

The effect of the combined extract on the mean muscle hardness.

All groups (except naïve control) were induced hyperalgesia and the assigned treatment cream was applied topically on both left and right legs at volume of 0.1 ml for 5 times per week continuously for 4 weeks. The results in figure 2 revealed that the rat in control group which induction hyperalgesia and received no treatment show a significantly increase in muscle hardness both left and right side when compared with naïve control group ($p<0.05$ and 0.01, respectively). Only the medium dose (500 mg) showed significant decreased muscle hardness after 28 days of treatment when compared with hyperalgesic control rats ($p<0.05$) (figure 2).

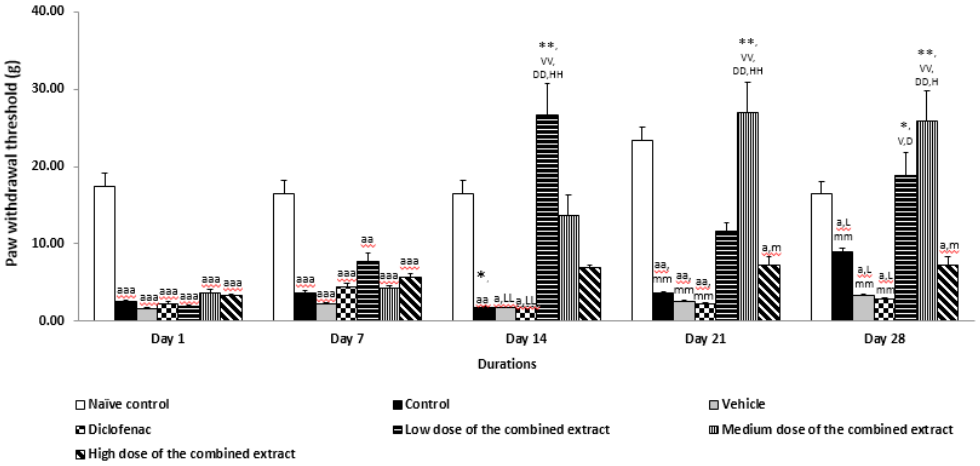

Figure 1-B The effect of the combined extract on mechanical hyperalgesia test in hyperalgesic rats in Right side. 30 minutes after the assigned treatment cream was applied topically on both legs, the rats were subjected to test for mechanical hyperalgesia. A series of 15 von Frey filaments start with 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 1.5, 2.5, 3.6 and 4.0 grams were used to evoke paw withdrawal response. [a] $p<0.05$ versus Naïve control group; [aa] $p<0.01$ versus Naïve control group; [aaa] $p<0.001$ versus Naïve control group; * $p<0.05$ versus Control group ; ** $p<0.01$ versus Control group; *** $p<0.001$ versus Control group; [v] $p<0.05$ versus Vehicle group; [vv] $p<0.01$ versus Vehicle group; [D] $p<0.05$ versus Diclofenac group; [DD] $p<0.01$ versus Diclofenac group; [L] $p<0.05$ versus Low dose of the combined extract group; [m, mm, mmm] $p<0.05$, $p<0.01$, $p<0.001$versus Medium dose of the combined extract group respectively; [H] $p<0.05$ versus High dose of the combined extract group.

Histology of the ipsilateral gastrocnemius

Figure 3 A represented the normal muscle cell with peripheral nuclei in naïve control rat. Whereas figure 3 B-G represented the histology of hyperalgesic rats induced by repeated injection of acidic saline pH 4.0, in various conditions of treatment. Firstly, B was the control hyperalgesic rat that received no treatment. It was revealed that low pH saline injection caused infiltrate of inflammation cells with high magnitude of cell injuries and fibrous tissue presented. C and D were represented of vehicle and diclofenac (positive control) treated group respectively. Lastly E-G were represented of cross section of muscle which treated with low, medium and high dose of the combined extract, respectively. After 28 days of treatment period, infiltration of inflammation cells, cell injuries and fibrous tissue were less observed in diclofenac and all doses of the combined extract than the control hyperalgesic group.

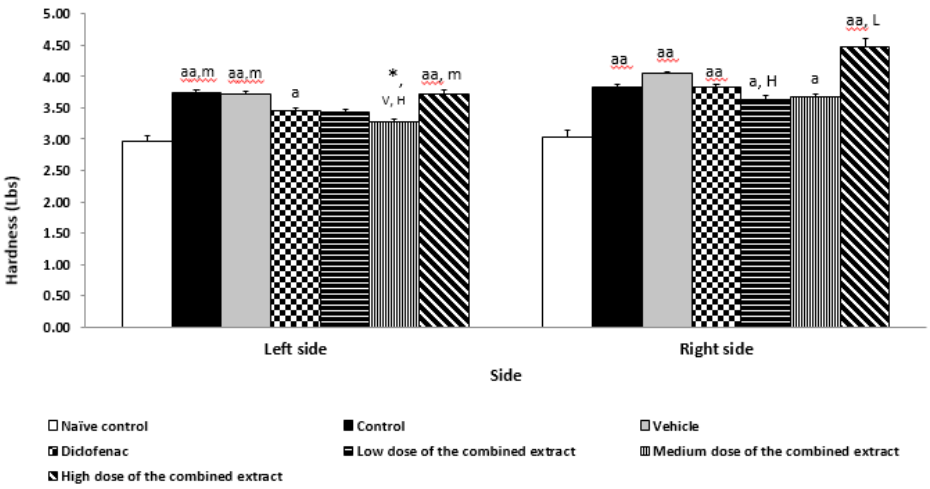

Figure 2 The effect of the combined extract on the mean muscle hardness in hyperalgesic rats on left and right gastrocnemius muscles. After 28 days of treatment of the assigned treatment cream that was applied topically on both legs, the rats were subjected to evaluate muscle hardness. [a, aa] $p<0.05$, 0.01, respectively, versus Naïve control group * $p<0.05$ versus Control group.

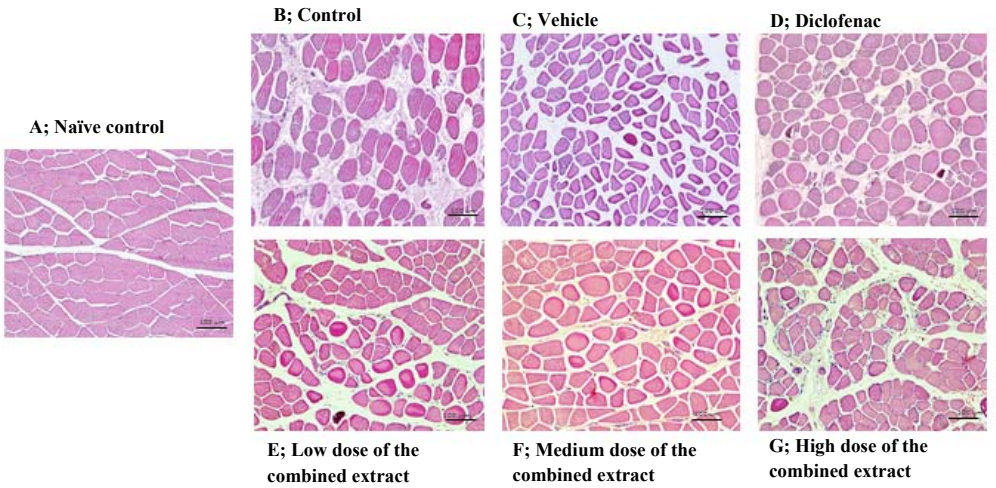

Figure 3 Cross section of the ipsilateral gastrocnemius muscle after treated with the innovative combined extract. A) naïve control rat, B) hyperalgesic control rats, C) hyperalgesic vehicle treated rats, D) hyperalgesic diclofenac treated rats, E)-G) hyperalgesic rats treated with low, medium and high dose of the combined extract respectively. (scale bar 100 μm, 20x of Light microscopy) (ปกหลัง)

Determination of the oxidative status

The effect of the combined extract on the recovery of tissue damage after induction hyperalgesia with low pH injection via measuring of Malondialdehyde -TBARs method. It was found that repeated injection of acidic saline pH 4.0 causes of the significantly increase in MDA level of left gastrocnemius (control group; $p<0.05$). While, treated with diclofenac, three doses of the combined extract could significantly decrease the level of MDA as same as vehicle treated rat (figure 4).

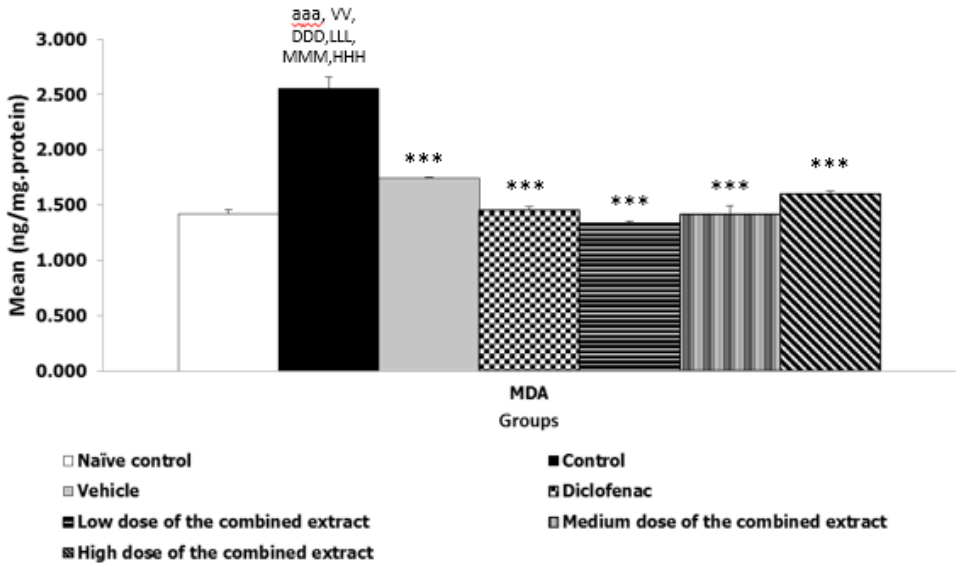

Figure 4 The effect of the combined extract on Malondialdehyde level. All groups (except naïve control) were induced hyperalgesia with the repeated injection (day 0 and day 5) of acidic saline (pH 4.0) at the left lateral gastrocnemius muscle. The assigned treatment cream from each treatment group was applied topically on left leg at volume of 0.1 ml for 5 times per week continuously for 4 weeks. AT the end of experiment, left gastrocnemius was collected and determined MDA level.

Note; ^{aaa} $p<0.010$ versus naïve control group; *** $p<0.001$ versus control group: ^{vv} $p<0.01$ versus Vehicle group; ^{DDD} $p<0.001$ versus Diclofenac group; ^{LLL} $p<0.001$ versus Low dose of the combined extract group; ^{mmm} $p<0.001$versus Medium dose of the combined extract group r; ^{HHH} $p<0.00$ versus High dose of the combined extract group

Table 1 The effect of the combined extracts on scavenging enzymes.

Groups	Types of scavenging enzyme		
	SOD	GPx	CAT
	unit/mg.protein	*unit/mg.protein*	*unit/mg.protein*
Naïve control	0.015±0.000	0.432±0.009	22.336±0.890
Control	0.011±0.000 ^{aaa}	0.332±0.005 ^{a,D,L}	13.855±0.370 ^{aa}
Vehicle	0.012±0.000 ^{aa}	0.355±0.009 ^{aa}	15.698±0.990 ^{a}
Diclofenac	0.012±0.000	0.444±0.020 *	17.957±0.610
Low dose of the combined extract	0.013±0.000 ^{a,*}	0.421±0.005 ^{*,H}	15.789±0.760
Medium dose of the combined extract	0.012±0.000	0.363±0.004	15.034±0.900
High dose of the combined extract	0.012±0.000 ^{aa}	0.359±0.012 ^{D}	18.090±1.660

Note; ^{a} p<0.05 versus naïve control group ^{aa} p<0.01 versus naïve control group; ^{aaa} p<0.001 versus naïve control group; * p<0.05 versus control group: ^{D} p<0.05 versus Diclofenac group; ^{L} p<0.05 versus Low dose of the combined extract group; ^{H} p<0.00 versus High dose of the combined extract group

Table 1 showed the potent of low pH injection on the production of ROS relate defensive working of SOD, GPx and CAT scavenging enzymes. It was found that low pH could significantly decrease the level of three scavenging enzyme production in control rat. However, the rat which treated with diclofenac and low dose of the combined extract could significantly increase the level of GPx scavenging production (p<0.05 both). Moreover, the low dose of the combined extract could also significantly increase the level of SOD scavenging enzyme (p<0.05).

Determination of the mean serum creatinine phosphokinase (CPK) level.

Effect of combined cream treatment on creatinine phosphokinase (CPK), a diagnostic marker for muscle injury showed in figure 5. It was found that the mean level of serum CPK of hyperalgesic control rats had significantly increased when compare with naïve control group (p<0.05). However, the mean different of hyperalgesic control group and vehicle group showed no significant different. While, the mean serum CPK of diclofenac and all doses of the combined extract were significantly reduced mean serum CPK when compared to hyperalgesic control rats (p<0.05).

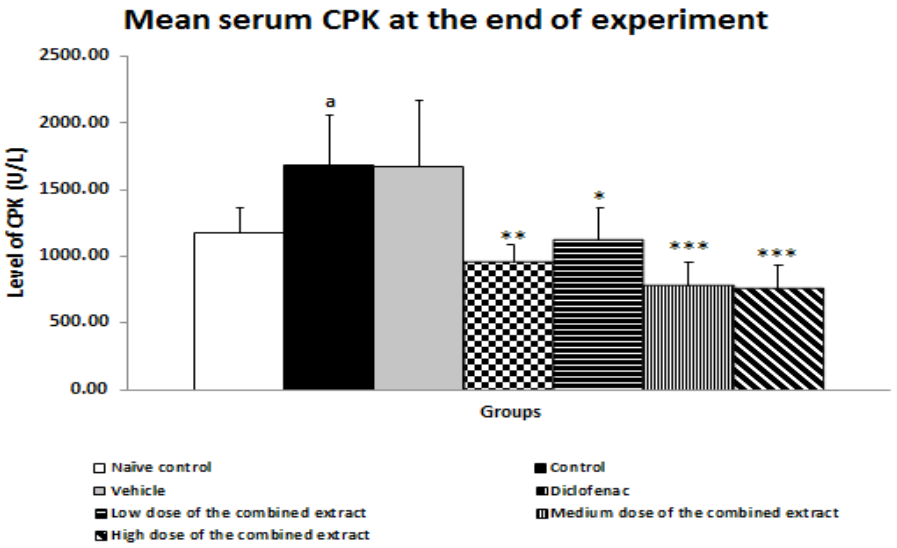

Figure 5 The effect of the combined extract on the mean serum creatinine phosphokinase (CPK) level. All groups (except naïve control) were induced hyperalgesia with the repeated injection (day 0 and day 5) of acidic saline (pH 4.0) at the left lateral gastrocnemius muscle. The assigned treatment cream from each treatment group was applied topically on left leg at volume of 0.1 ml for 5 times per week continuously for 4 weeks. AT the end of experiment, the mean serum creatinine phosphokinase (CPK) level was determined. [a] = $p<0.05$ when compare with naïve control (Mean±SEM). *, **, *** = $p<0.05$, 0.01, 0.001, respectively, when compare with control (Mean±SEM).

Discussion

The combined extract of *Z. officinale* and *P. amarus* has anti-nociceptive and anti-oxidative effects. Its possible underlying mechanisms of the combined extract of *Z. officinale* and *P. amarus* in animal model of myofascial pain were partly via reduction of tissue injury as showed by a reduction of CPK level. Moreover, the combined extract of *Z. officinale* and *P. amarus* was also reduced MDA level and enhanced activity of SOD and GPx. Furthermore, it was also decreased mechanical hyperalgesia and muscle hardness.

The active compounds of the combined extract *Z. officinale* and *P. amarus* are phyllanthin and 6-gingerol. Phyllanthin is one type of lignans that riches in *P. amarus* extract.[14] Previous study reported that *P. amarus* extract showed anti-hyperalgesic activity, as it elevated thermal and mechanical threshold in a model of chronic

musculoskeletal inflammatory pain induced by carrageenan injection.[15] Moreover, the anti-inflammatory properties of *P. amarus* were reported in various conditions.[16-19] However, hexanic extract of *Phyllanthus amarus* containing phyllanthin, niranthin, and 5-demethoxyniranthin failed to show antihypernociceptive activity in experimental autoimmune encephalomyelitis.[20] For the 6-gingerol, Young and coworkers were suggested that 6-gingerol could inhibit writhing response induced by acetic acid injection and formalin-induced licking time (Young et al., 2005). 6-Gingerol also produced an inhibition of paw edema induced by carrageenan.[20] Moreover, ginger oil contained a high content of sesquiterpene hydrocarbons, in particular, 6-gingerol, ar-curcumene, β-bisabolene, and β-sesquiphellandrene, and the monoterpenoids geranial, neral, and camphene in ginger oil,[21] and there was evidence that ginger oil possessed analgesic effect.[22] All of this information supported the efficacy of the combined extract of *Z. officinale* and *P. amarus*, riches in phyllanthin and 6-gingerol, that used in this study. Thus, this innovative topical herbal cream contains the combined extract of *Z. officinale* and *P. amarus* may clinically useful for the treatment of hyperalgesic conditions, such as MFP.

The combined extract of *Z. officinale* and *P. amarus* has anti-oxidative effects as it reduced MDA level and enhanced activity of SOD and GPx. Scavenging enzymes including glutathione, SOD and CAT protect the cell constituents from oxidative damage. Despite these extensive defense systems, biomolecule damage may still occur in hyperalgesic rats induced by acidic saline injection and persist within the muscle. The significant increase in the activities of SOD and GPx suggests a greater level of endogenous antioxidant associated with the combined extract of *Z. officinale* and *P. amarus* treatment resulting in an enhanced free radical scavenging activity. Plants are the sources for a wide variety of compounds like flavonoids and polyphenols. These compounds may be responsible for increasing antioxidant status. These findings were confirmed by the previous studies, *Z. officinale* extracts contain polyphenol compounds, 6-gingerol and its derivatives, which have a high antioxidant activity,[23-25] In addition, *P. amarus* extract potent in Phyllanthin also exhibited antioxidant activity.[15, 26]

It has been generally accepted that raised levels of CK are still closely associated with cell damage, muscle cell disruption, or

disease. These cellular disturbances can cause CK to leak from cells into blood serum.[27,28] After repeatedly injected of acidic saline into left gastrocnemius muscle in this study, acidic condition induced muscle damage thus muscle cell loss its integrity, subsequently raised serum CPK. Treated with the combined extract of *Z. officinale* and *P. amarus* in animal model of myofascial pain, serum CPK level was significantly reduced, this may imply combined extract of *Z. officinale* and *P. amarus* reduced tissue injury.

In conclusion, the innovative topical cream that contain combined extract of *Z. officinale* and *P. amarus* had anti-nociceptive effect against acidic saline induced hyperalgesia in rats. By exert its effect via reduction of tissue injury and restored oxidative status.

References

1. Yazdi-Ravandi S, Taslimi Z, Jamshidian N, Saberi H, Shams J, Haghparast A. prediction of quality of life by self-efficacy, pain intensity and pain duration in patient with pain disorders. Basic and Clinical Neuroscience 2013; 2:117-24.
2. Sostres C, Gargallo CJ, Arroyo MT, Lanas A. Adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs, aspirin and coxibs) on upper gastrointestinal tract. Best Pract Res Clin Gastroenterol 2010;24:121-32.
3. Bjarnason I, Hayllar J, Macpherson AJ, Russell AS. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology1993;104:1832-47.
4. Mishra RK, Kumar An, Kumar AS. Review; Pharmacological activity of Zingiber officinale. International Journal of Pharmaceutical and Chemical Sciences 2012:1:1422-7.
5. Verma S, Sharma H, Garg M. Phyllanthus Amarus: a review. Journal of Pharmacognosy and Phytochemistry 2014;3: 18-32.
6. Afzal M, Al-Hadidi D, Menon M, Pesek J, Dhami MS. Ginger: an ethnomedical, chemical and pharmacological review. Drug Metab. Drug Interact 2001;18: 159-90.
7. Grzanna R, Lindmark L, Frondoza CG. Ginger - an herbal medicinal product with broad anti-inflammatory actions. J Med Food 2005;8:125-32.
8. Iranloye BO. Arikawe AP. Rotimi G. Sobade AO. Anti-diabetic and antioxidant effects of Zingiber officinale on alloxan-induced and insulin resistant diabetic male rats. Nig J Physiol Sci 2011;26:89-96.

9. Maharan S, Muchimapura S, Wattanathorn J, Thukhummee W, Thong-Un T, Wannanon P. Dermal toxicity studies of an herbal cream contained zingiber officinale roscoe and phyllanthus amarus extracts in sprague-dawley rats. Journal of the Medical Association of Thailand 2019;102:52-56.
10. Sluka KA, Kalra A, Moore SA. Unilateral intramuscular injections of acidic saline procedure a bilateral, long-lasting hyperalgesia. Muscle & Nerve 2001;37-46.
11. Yoshinori Hayashi, Kodai Kawaji, Li Sun,1 Xinwen Zhang, Kiyoshi Koyano, Takeshi Yokoyama, Shinichi Kohsaka, Kazuhide Inoue, and Hiroshi Nakanishi. Microglial Ca^{2+}-activated K^{+} channels are possible molecular targets for the analgesic effects of S-ketamine on neuropathic pain. Neurobiology of Disease. The Journal of Neuroscience 2011;31:17370-82.
12. Hou X, Zhang J, Ahmad H, Zhang H, Xu Z, Wang T. Evaluation of antioxidant activities of Ampelopsin and its protective effect in lipopolysaccharide-induced oxidative stress Piglets. Plos one 2014, 9; e108314: 1-10.
13. Weydert, Christine, Cullen, Joseph. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nature protocols 2010;5:51-66.
14. Chopade AR, Sayyad FJ. Pain Modulation by Lignans (Phyllanthin and Hypophyllanthin) and Tannin (Corilagin) Rich Extracts of *Phyllanthus amarus* in Carrageenan-induced thermal and mechanical chronic muscle hyperalgesia. Planta Med 2005;71:721-6.
15. Chopade AR, Sayyad FJ. Antifibromyalgic activity of standardized extracts of *Phyllanthus amarus* and *Phyllanthus fraternus* in acidic saline induced chronic muscle pain. The Journal of Biomedicine and Aging pathology 2014:4;123-30.
16. Pradit W, Chomdej S, Nganvongpanit K, Ongchai S. Chondroprotective potential of *Phyllanthus amarus* Schum. & Thonn. in experimentally induced cartilage degradation in the explants culture model. Cellular & Developmental Biology-Animal 2015;51:336-44.
17. Kandhare AD, Ghosh P, Ghule AE, Zambare GN, Bodhankar SL. Protective effect of *Phyllanthus amarus* by modulation of endogenous biomarkers and DNA damage in acetic acid induced ulcerative colitis: Role of phyllanthin and hypophyllanthin. Apollo Medicine 2013;10:87-97.

18. Mali SM, Sinnathambi A, Kapase CU, Bodhankar SL, Mahadik KR. Anti-arthritic activity of standardized extract of *Phyllanthus amarus* in Freund's complete adjuvant induced arthritis. Biomedicine & Aging Pathology 2011;1:185-90.
19. Chouhan HS, Singh SK. Phytochemical analysis, antioxidant and anti-inflammatory activities of *Phyllanthus simplex*. J Ethnopharm 2011;137:1337-44.
20. Molska GR, Negri G, Paula-Freire LI, Araujo LP, K÷hn DO, Basso AS, Carlini EA. *Phyllanthus amarus* does not affect hypernociception in experimenta autoimmune encephalomyelitis. Planta Medica 2014;80:277-82.
20. Young HY, Luo YL, Cheng HY, Hsieh WC, Liao JC, Peng WH. Analgesic and anti-inflammatory activities of [6]-gingerol. J Ethnopharm 2005; 96: 207-10.
21. Martins DF, Turnes BL, Cidral-Filho FJ, Bobinski F, Rosas RF, Danielski LG, Petronilho F, Santos AR. Light-emitting diode therapy reduces persistent inflammatory pain: Role of interleukin 10 and antioxidant enzymes. Neuroscience 2016;324:485-95.
22. Jia YL, Zhao JM, Zhang LH, Sun BS, Bao MJ, Li FF,Shen J, Shen HJ, Zhao YQ, Xie QM. Analgesic and anti-inflammatory effects of ginger oil. Chinese Herb Med 2011; 3: 150-5.
23. Chen CH, Kuo M, Wu Ch, Ho Ch. Pungent compounds of ginger (Zingiber officinale (L) Rosc) extracted by liquid carbon dioxide. J Agri Food Chem 1986;34:477-80.
24. Herrmann K. Antioxidativ wiksame Pflanzenphenole sowie Carotinoide als wichtige Inhaltsstoffe von Gewürzen. Gordian 1994;94:113-7.
25. Aruoma OI, Spencer JP, Warren D, Jenner P, Butler J, Halliwell B. Characterization of food antioxidants, illustrated using commercial garlic and ginger preparations. Food Chem 1997; 60:49-156.
26. Karuna R, Reddy SS, Baskar R, Saralakumari D. Antioxidant potential of aqueous extract of Phyllanthus amarus in rats. Indian J Pharmacol 2009;41:64-7.
27. Totsuka M, Nakaji S, Suzuki K, Sugawara K, Sato K. Break point of serum creatine kinase release after endurance exercise. J Applied Physio 2002;93:1280-6.
28. Brancaccio P, Maffulli N, Limongelli FM. Creatine kinase monitoring in sport medicine. British Medical Bulletin 2007;81-82:209-30.