INHIBITION OF LIPOXYGENASES AND CYCLOOXYGENASES BY *Momordica charantia*

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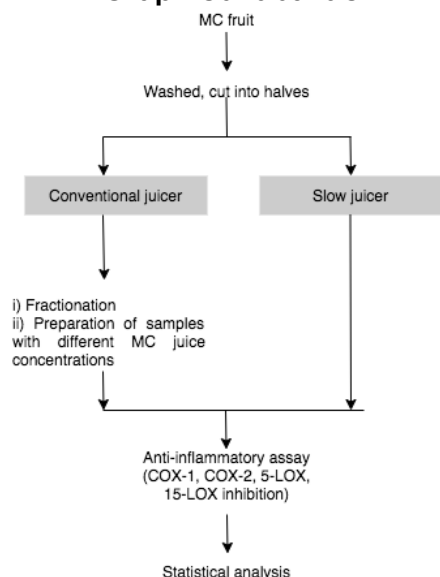[b]School of Chemical & Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

Graphical abstract

Abstract

Momordica charantia (MC) is a climber belongs to the Cucurbitaceae family. While there are accumulating evidences showing its pharmacological activities, the effects of fractionation and processing on its anti-inflammatory activity have not been fully elucidated. In this study, we aimed to investigate the anti-inflammatory effects of MC raw juice and fractions from MC fruit extract and to evaluate the effect of processing methods on its anti-inflammatory activity. First, MC juice was extracted using a conventional juicer (raw juice) and fractionated using a solid phase extraction method into five fractions; strong acid, weak acid, neutral, weak base, and strong base fractions. The raw juice and its fractions then were tested for their anti-inflammatory activities including cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), and 15-lipoxygenase (15-LOX) inhibition assays. The results showed that the COX-1 inhibition activity of MC can be attributed to the neutral metabolites (52.60% inhibition). In contrast, the anti-COX-2 activity of MC was found exerted collectively by metabolites in multiple fractions (strong base, neutral, strong acid fractions). Contrarily, metabolites from all five fractions contributed to the MC inhibition activities on 5-LOX (55.51% inhibition for raw juice) and 15-LOX (55.3% inhibition for raw juice). Additionally, the current findings showed that the juice processing technique influenced the plant bioactivity, where juice extract obtained from slow juicer showed consistently higher anti-inflammatory activities than juice from conventional juicer. In summary, the current study provided evidences of anti-inflammatory action of MC and its fractions, which may contribute towards an improved processing and fractionation strategy.

Keywords: *Momordica charantia*, anti-inflammatory, cyclooxygenase, lipoxygenase

Abstrak

Momordica charantia (MC) merupakan sejenis tumbuhan memanjat daripada keluarga Cucurbitaceae. Biarpun kajian terdahulu telah membuktikan pelbagai aktiviti farmakologi oleh MC, kesan pemecahan dan pemprosesan ke atas aktiviti antiradang masih belum diketahui. Dalam kajian ini, kami menguji kesan kaedah pemprosesan ke atas aktiviti antiradang jus mentah MC dan ekstrak pemecahannya. Jus MC dihasilkan dengan menggunakan alat pemerah jus konvensional (jus mentah). Jus ini seterusnya dipisahkan kepada lima pecahan iaitu pecahan asid kuat, asid lemah, neutral, alkali lemah, alkali kuat, dengan menggunakan kaedah pengekstrakan fasa pepejal. Seterusnya, jus mentah dan pecahannya diuji aktiviti antiradang malalui perencatan enzim cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), dan 15-lipoxygenase (15-LOX). Keputusan ujikaji menunjukkan bahawa aktiviti perencatan COX-1 oleh

MC disumbangkan oleh metabolit neutral (52.60% perencatan). Sebaliknya, aktiviti perencatan COX-2 oleh MC disumbangkan oleh metabolit daripada tiga pecahan (alkali kuat, neutral, asid kuat) secara kolektif. Manakala kelima-lima pecahan menyumbang kepada perencatan enzim 5-LOX (55.51% perencatan bagi jus mentah) dan 15-LOX (55.3% perencatan bagi jus mentah). Tambahan pula, kajian ini menunjukkan bahawa teknik pemprosesan jus mempengaruhi bioaktiviti tumbuhan. Jus yang dihasilkan menggunakan alat pemerah jus perlahan menunjukkan aktiviti antiradang yang lebih tinggi secara konsisten berbanding jus yang dihasilkan menggunakan alat pemerah jus konvensional. Kesimpulannya, kajian ini membuktikan aktiviti antiradang oleh jus MC dan pecahannya yang boleh menyumbang kepada penambahbaikan dalam strategi pemprosesan dan pemecahan untuk MC.

1.0 INTRODUCTION

Inflammation is a condition derived from tissue response to biological, physical and chemical stimulations to eliminate injuring stimuli such as pathogens, irritant and damaged cells [1, 2]. Although inflammation response is important in physiological condition, there are accumulating evidences associating inflammation with diseases including asthma, psoriasis, inflammatory bowel disease, atherosclerosis, insulin resistance, rheumatoid arthritis and cancer [3, 4, 5]. When inflammation occurs, the inflammatory mediators will be released from the activated inflammatory cells (eosinophils, neutrophils, mononuclear phagocytes, macrophages). The released inflammatory mediators are including lipoxygenases (LOX) and cyclooxygenases (COX) enzymes, nitric oxide (NO), prostaglandin E2 (PGE2), cytokines such as tumor necrosis factor (TNF-a) and interleukins (IL), and transcription factor as nuclear factor (NFκB) [6, 7, 8].

Lipid mediators including prostaglandins, thromboxane, leukotrienes, and lipoxins play important roles in biological activities such as the maintenance of normal hemostasis, blood pressure regulation, renal function, reproduction and host defense [9]. COX is the rate-limiting enzyme and responsible for the production of prostaglandins, prostacyclins, thromboxanes from arachidonic acid [10]. COX has two isoforms; cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is a house-keeping enzyme that is constitutively expressed in the body and particularly important for gastrointestinal protection. On the other hand, cyclooxygenase-2 (COX-2) is an inducible cyclooxygenase which is enhanced by cytokines, growth factors and other inflammatory ulcerogenic stimuli [11]. The induction of COX-2 is responsible for the pathological process of several inflammatory conditions including rheumatoid arthritis, cancer, respiratory disorders and Alzheimer's disease [10].

Lipoxygenases (LOX) are oxidative enzymes which play a major role in the regulation of inflammatory responses [12]. The LOX reactions are catalyzed by nonheme iron-containing dioxygenases, which involved the insertion of oxygen into polyunsaturated fatty acids such as arachidonic acid and linoleic acid with one or more cis,cis-1,4-penta-diene moieties in the structure [9, 12]. LOX catalyzed the hydroperoxy eicosatetraenoic acids (HPETEs) formation from arachidonic acid which the HPETEs are subsequently reduced and transformed to form eicosanoids, which are the signaling molecules that plays vital role in the immune responses and other physiological processes [12]. Generally, the LOX can be classified as 5-, 8-, 12-, and 15-LOX depending to the selectivity to oxygenate fatty acids in specific position [12]. Leukotrienes, the metabolic products through 5-LOX activation are responsible in initiating immune cell chemotaxis and actively contributing in inflammatory occurrence such as asthma, allergy, bowel diseases, cancers, cardiovascular diseases (atherosclerosis, heart attack, stroke) [13, 14, 15]. To date, inflammation is typically treated with several anti-inflammatory drugs, which include nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and immunosuppressant drugs. However, many of the drugs are found to be insufficiently effective and may lead to unwanted side effects [16]. There is an increasing interest to search for plant-derived anti-inflammatory compounds which may have several advantages than synthetic compounds due to less toxicity, more accessibility and less expensive [17].

Momordica charantia Linn. (MC) or bitter gourd belongs to Cucurbitaceae family. The fruit contains bitter, cucurbitane-type triterpenoids, including charantin, cucurbitacins and momordicins [18, 19]. The seeds, fruit and leaves of MC had shown a wide range of pharmacological activities, and MC has been traditionally used for its therapeutic activities including anti-diabetic, anti-inflammatory, hypocholesterolemic, hypotriglyceridemic, anti-tumor, hypo-tensive, immunostimulant, anti-viral, anti-inflammatory, anti-oxidant, anti-leukemic, anti-microbial, anthelmintic, anti-mutagenic, anti-ulcer, and insecticidal properties [20, 21, 22]. MC is particularly known for its hypoglycemic activity which leads to reduction in blood glucose level in Type 2 diabetic patients [23, 24] and animal disease models [25, 26]. In addition to its anti-diabetic activity, MC is also recognized as an anti-inflammatory agent. Several reports had consistently shown its anti-

inflammatory activities; Chao and colleagues reported that adding wild MC to diets of sepsis-induced mice inhibited the NF-κB, iNOS and COX-2 expressions, leading to improvement of the inflammation responses [27]. A study by Bao and colleagues showed that freeze-dried MC fruit reduced the adipose tissue inflammation in diet-induced obese mice where the pro-inflammatory cytokine MCP-1 expression was depressed in epicardial adipose tissue and brown adipose tissue. Furthermore, the IL-6 and TNF-α expression in epicardial adipose tissue were also reduced [28]. Another recent study by Dwijayanti and collegues found out that the expression of interleukin-1β mRNA and hepatic lipid accumulation in hepatocytes had been reduced in ob/ob mice type 2 diabetes mellitus model after 7 days of administration with ethyl acetate soluble fraction from MC fruit [18]. In addition, a previous study that involved MC-treated rat model showed that the levels of pro-inflammatory cytokines in the liver, muscle and epididymal fats were significantly down-regulated. Moreover, the activation of NF-κB in the liver and muscle was decreased in MC-treated rat group compared to the non-treated group [29]. These accumulating evidences support MC as a potent anti-inflammatory agent.

Previously, a number of studies had been carried out on solvent fractionation of MC [30, 31, 32]. However, fractionation of MC based on ionic exchange for the recovery of acidic, neutral and basic components from MC, and anti-inflammatory effects of the resulting fractions have yet to be elucidated. It is known that natural products consist of acid-base character which enables compounds to be selectively isolated based on the functional groups through pH manipulation in the fractionation protocol [33]. The fractionation strategy may provide new insight into the anti-inflammatory activities of metabolites in MC. The MC sample used in anti-inflammatory studies is commonly processed through freeze-drying prior to further extraction [31, 32, 34]. While in daily life, consumers of MC usually produce MC fruit juice by using common home appliances such as the sharp-blade conventional juicer or slow juicer. Therefore, a study on the effect of processing methods will be useful for the production of functional beverage of MC with high anti-inflammatory activity. Taken together, a study on fractionation and processing of MC may provide a novel understanding of their effects on anti-inflammatory activities of MC.

2.0 METHODOLOGY

Chemicals and Kits

Strata™ X Polymeric Reversed Phase Strata-X-AW and Strata-X-CW were purchased from Phenomenex, Torrance, California. Lipoxygenase Inhibitor Screening Assay Kit (Item no.: 760700), Lipoxygenase (potato) Screening Enzyme (Item no.: 60401) and COX Colorimetric Inhibitor Screening Assay Kit (Item no.: 701050) were purchased from Cayman Chemical, Ann Arbor, MI, USA.

Plant Material and Preparation of MC Juice

Fresh unripe MC was purchased from local wholesaler in Pagoh, Muar, Johor, Malaysia. The raw material was processed using either a conventional juicer or a slow juicer. 5 kg of MC were used to obtain juice using the conventional juicer while for slow juicer, 4 kg of MC were used. No solvent or water were added during the juice processing. Briefly, the MC fruits were washed, sliced and extracted using a conventional fruit juicer (Kenal EX-362, Malaysia). The produced juice was then centrifuged (Kubota 7000, Japan) at 9,000 rpm, 4 °C for 15 minutes. The supernatant was collected and stored at -20 °C until further use. In addition, MC fruits were processed using a slow juicer (Hurom, H-AA Series, Korea), centrifuged and stored at -20 °C until further process. To study the effect of MC raw juice in different concentration on anti-inflammatory activities, four different concentrations were prepared ranging 25%, 50%, 75% and 100%.

Fractionation of MC Raw Juice using Solid-phase Extraction Method

For this section, solid-phase extraction (SPE) method was used to obtain MC fraction. The Strata-X, Strata-X-AW and Strata-X-CW cartridges (Phenomenex, CA, USA) were used for fractionation, by which a reversed phase functionalized polymeric sorbent provides strong retention of either neutral, acidic or basic compounds under aggressive, high organic wash conditions. Each cartridge served different purpose of collecting fractions which Strata-X was used to obtain neutral fractions, Strata-X-AW for acidic fractions and Strata-X-CW for basic fractions. The fractionation protocol for these cartridges involved conditioning, equilibrating, sample loading, washing and eluting steps. The fractionation process for each cartridge was presented in Figure 1.

All purchased cartridges size was standardized to 100 mg/6 ml which means 100 mg sorbent mass and 6 ml was the volume of the cartridge per load. Each cartridge permitted up to 25 ml of sample to permeate the sorbent for single experiment. Prior the fractionation process, a collection bottle was placed inside the vacuum manifold. Then, the removable cover was placed, and the cartridge was installed on top of the manifold carefully. The stopcock was in lock position to avoid uncontrollable solvent drop. Once the setup was ready, the vacuum pump was turn on and the cartridge was initially conditioned with 3 ml of methanol. The stopcock was adjusted slowly to allow methanol to pass through the sorbent. The flow rate was adjusted at 1 drop/second. After the methanol fully pas through the sorbent, the

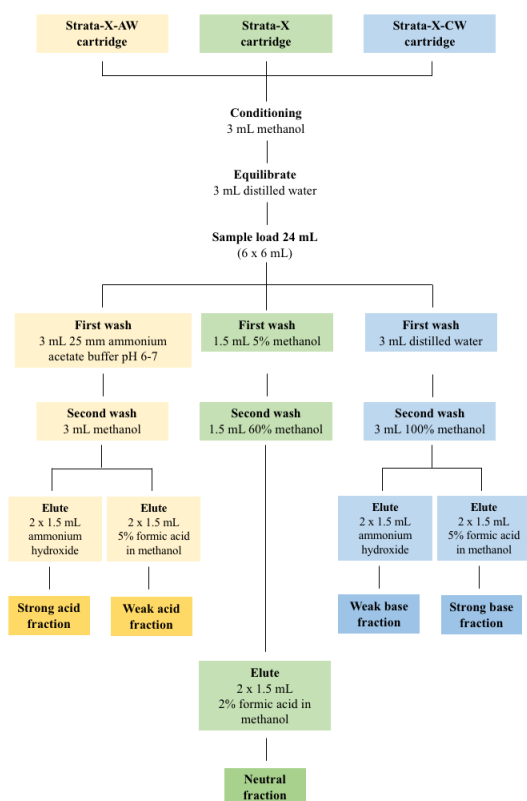cartridge then was equilibrated with 3 ml of distilled water.

Figure 1 Fractionation protocol using cartridge

Anti-inflammatory Study of MC Juice and Its Fractions

For cyclooxygenase essay, the activity was carried out according to instructions provided by the manufacturer. COX Colorimetric Inhibitor Screening Assay Kit (Cayman's Chemical) measures the peroxidase component of COXs, oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). The oxidized TMPD can be assayed colorimetrically and detected at 590 nm wavelength. The kit provides two enzymes; COX-1 (ovine) and COX-2 (human recombinant), for screening the isomer enzyme-specific inhibitors which provides convenience to screen multi-inhibitors simultaneously. Each well final volume was 220 µl. All buffer, enzymes, inhibitors, substrates were prepared prior performing the assay protocol. In background wells, 160 µl assay buffer, 10 µl heme and 10 µl methanol were added to three wells. In initial activity wells, 150 µL assay buffer, 10 µl heme, 10 µl enzyme and 10 µl methanol were added to three wells. In sample inhibitor wells, 150 µl assay buffer, 10 µl heme and 10 µl enzyme and 10 µl sample were added. Blank for each sample was prepared separately by mixing 90 µl assay buffer with 10 µl sample. The plate was placed on a shaker for thirty seconds and incubated at room temperature

for five minutes. Next, 20 µl colorimetric substrate followed by 20 µl arachidonic acid was added to all wells. The plate was carefully shake for 30 seconds and incubated at room temperature for 2 minutes. The plate was read at 590 (Versamax ELISA, Molecular Devices, CA, USA). The pipetting components are presented in Table 1. The results were presented in percentage of inhibitions which was calculated using equation 1.

$$\% \ Inhibition = \left[\frac{Abs_{initial \ activity} - Abs_{sample}}{Abs_{initial \ activity}} \right] \times 100 \quad \text{(Eq. 1)}$$

Table 1 COXs inhibition assay pipetting components

Well/Solution	Background (µl)	Initial activity (µl)	Sample blank (µl)	Inhibitor (MC samples) (µl)
Assay buffer	160	150	160	150
Heme	10	10	10	10
COX enzymes	-	10	-	10
Methanol	10	10	-	-
Sample	-	-	10	10
Colorimetric substance	20	20	20	20
Arachidonic acid	20	20	20	20

Total volume for each well: 220 µl

For lipoxygenase essay, the activity was carried out according to instructions provided by the manufacturer. Lipoxygenase Inhibitor Screening Assay Kit (Cayman's Chemical) quantify the hydroperoxides product from lipoxygenation process using the purified lipoxygenases enzyme. The detection reaction is equally sensitive to hydroperoxides at fatty acid position at any carbon length. Hence, it is a general detection method for LOXs and useful to screen variety of potential LOXs inhibitor compounds. To start the process, all buffer, enzymes, inhibitors, substrates were prepared and diluted according to the protocol. Blanks were prepared for each sample respectively. In this experiment, 5-LOX assay used linoleic acid as substrate while 15-LOX assay used arachidonic acid which performed in a two separate 96-wellplate. In blank wells, 100 µl assay buffer was added into three wells. In positive control wells, 90 µl enzyme and 10 µl assay buffer were added to three wells. In 100% Initial activity wells, 90 µl enzyme and 10 µl methanol were added to three wells. In standard inhibitor wells, 90 µl enzyme and 10 µl nordihydroguaiaretic acid (NDGA) were added into three wells. In Sample Inhibitor Well, 90 µl enzyme and 10 µl sample were added to three wells. Blank sample was provided for each sample by mixing 90 µl assay buffer with 10 µl sample. The plate was incubated at room temperature for five minutes. Next, 10 µl substrate was added to all wells. This is where the initiation process begins. The plate was placed on a shaker for 10 minutes. 100 µl chromogen

was added to each well to stop the process. The plate was placed on the shaker for another five minutes to develop colour reaction. Then, the plate was read at 495 nm (Versamax ELISA, Molecular Devices, CA, USA). The pipetting components are presented in Table 2. The results were presented in percentage of inhibitions which was calculated using Equation 1.

Table 2 LOXs inhibition assay pipetting components

Well/Solution	Blank (µl)	Positive control (µl)	100% initial activity (µl)	Inhibitor (NDGA) (µl)	Inhibitor (MC Samples) (µl)	Sample's blank (µl)
Assay buffer	100	10	-	-	-	90
LOX enzyme	-	90	90	90	90	-
Methanol	-	-	10	-	-	-
Inhibitor	-	-	-	10*	10	10**
Arachidonic acid	10	10	10	10	10	10
Chromogen	100	100	100	100	100	100

Total volume for each well: 220 µl
* NDGA
** MC samples

Data Analysis

The results of anti-inflammatory study were shown as bar graph and the error bars represent standard deviation (SD). All experiments were performed in triplicates. The data were analysed using the Student's t-test, ANOVA followed by Dunnett's post-hoc test. Comparison with $p < 0.05$ is considered significant.

3.0 RESULTS AND DISCUSSION

Cyclooxygenases (COXs) Inhibition Activity

Cyclooxygenases are an essential enzyme that are responsible for the conversion of arachidonic acid, a polyunsaturated fatty acid to prostaglandins (PGs), an inflammatory mediator [35]. There are two COX isomers; COX-1 which plays an essential role in homeostasis and gastrointestinal protection; COX-2, an inducible COX which enhanced by cytokines and is involved in the development of inflammatory events [11].

Effect of MC Raw Juice and Fractions on COXs inhibition Activity

This study investigated the effect of MC fractions against COXs. The juice was initially loaded into the cartridge (Strata-X for Neutral, Strata-X-AW for Acid, Strata-X-CW for Base) and further fractionated

separately to five different fractions namely strong acid, weak acid, neutral, weak base and strong base fractions. Then, the MC Raw Juice and its fractions were tested against COXs inhibition. The data is presented in Figure 2.
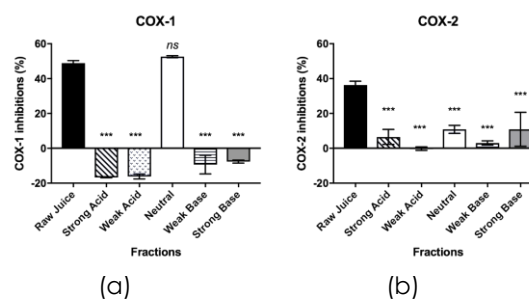

Figure 2 Effect of MC raw juice and fractions on (a) COX-1, (b) COX-2. *** $p < 0.001$ was obtained from ANOVA with Dunnett's post-hoc test comparing data with raw juice (n=3); ns – not significant

As presented in Figure 2a, the neutral fraction and the MC raw juice showed high COX-1 inhibition at 52.60% and 48.89%, respectively. There was no significant between neutral and raw juice, suggesting that the COX-1 inhibitory action of MC can be attributed to the neutral metabolites. Negative inhibitions were observed for strong base (-7.66%), weak base (-9.36%), weak acid (-16.15%) and strong acid (-16.67%) fractions. In contrast, multiple fractions showed inhibitory effects for COX-2 inhibition (Figure 2b): raw juice (36.31%), strong base (10.91%), neutral (10.87%), strong acid (6.52%), and weak base (3.07%). As a summary, MC showed inhibitory effects on both COX-1 and COX-2. The anti-COX-1 effect was mainly due to metabolites in the neutral fraction, while the anti-COX-2 activity of MC was exerted collectively by metabolites in multiple fractions.

Effect of Processing Method on COXs Inhibition Activity

In this section, the effect of processing method on COXs inhibition was investigated. In this study, MC samples were prepared separately through a conventional juicer or a slow juicer. The conventional juicer used sharp-flat blades to reduce the size of sample and separate the juice from the pulp through high-speed centrifugal force. On the other hand, slow juicer used pressing force to extract the juice out through filter with minimal heat and friction. However, the juice extraction rate is relatively slow for a slow juicer compared to a conventional juicer [36]. The COXs inhibition of MC samples produced from different processing methods is presented in Figure 3.
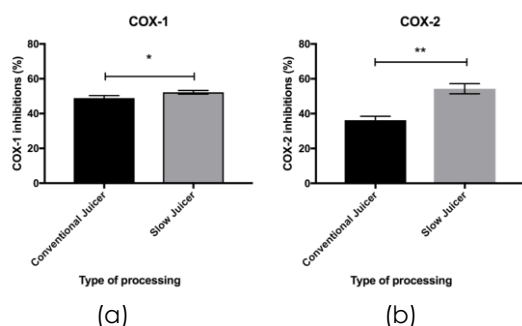
Figure 3 Effect of processing methods on (a) COX-1, (b) COX-2. *p<0.05 and ** p<0.01 were obtained from the Student's t-test (n=3)

For COX-1 inhibitory study, the MC sample produced by the slow juicer gave the highest inhibition (52.25%), followed by conventional juicer (47.23%). A similar order was also found for COX-2 inhibitions where slow juicer showed highest inhibition (54.27%), followed by conventional juicer (36.31%). The current results showed that slow juicer is the preferred method for MC juice preparation. This finding is comparable to previous studies by Kim *et al*. (2015) and Lee *et al.* (2013) and where both studies reported that slow juicer can retain more phytochemicals compared to conventional juicer [36, 37].

A juicer provides a convenient option for the consumer to produce fresh juice from fruits and vegetables. However, nutrient level in the fruits and vegetables can be reduced along the juicing process and lead to destructions of polyphenols and flavonoids [36, 37]. Furthermore, different processing/extraction technique may produce juice with varying amounts of phytochemicals depending on the type of juicer used [38]. In commercial juice industry, pressing methods used may alter the taste of the juice. Notably, high extraction pressure (hard squeeze) produces higher yields. However, a low extraction pressure (soft squeeze) produced less yields but the juice flavor will be more similar to the juice produced by hand manually [39].

Effect of MC Juice Concentrations on COXs Inhibition Activity

The COXs inhibition of MC juice in different concentration was assessed by measuring the peroxidase components of COX. Four different juice concentrations ranging 25%, 50%, 75% and 100% were tested in the current study. From Figure 4, the percentage of COX-1 inhibition showed positive correlation with concentration of MC, increasing from 15.24% inhibition (25% MC) to 50.56% inhibition (75% MC). There was no significant difference in COX-1 inhibition between the 75% and 100% MC samples, indicating that 75% concentration of MC sample was adequate to exert maximum COX-1 inhibition. On the other hand, samples with 25%, 50%

and 100% concentrations showed no significant difference in percentage of COX-2 inhibition, suggesting that at lower concentrations, MC juice contained more potent COX-2 inhibitors than COX-1 inhibitors. As a summary, the COX-1 inhibitory effect of MC juice increased with increasing concentration in general, while COX-2 inhibition ability of MC juice is more consistent for samples with 25%-100% concentration.

A previous study demonstrated that adding MC at the different doses ranging from 1% to 10% in sepsis-induced mouse model diets significantly reduced the formation of PGE2, an inflammatory mediator by reducing the expression of COX-2. The study also found that MC significantly inhibited the expression of other inflammation protein including iNOS, and NF-κB using the same mouse model [40].
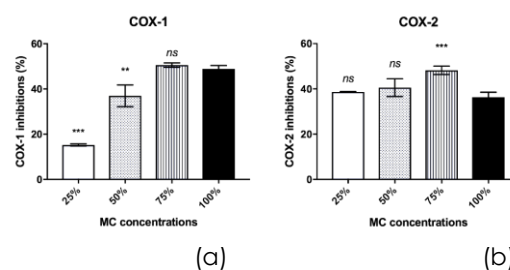

Figure 4 Inhibitory effect of MC juice concentration on (a) COX-1, (b) COX-2. ** p<0.01 and *** p<0.001 were obtained from ANOVA with Dunnett's post-hoc test comparing data with 100% MC juice concentration as controls (n=3); *ns* - not significant

Lipoxygenase (LOXs) Inhibition

The over-expression of lipoxygenases and their leukotrienes product resulted in inflammation events including asthma, atherosclerosis, rheumatoid arthritis, inflammatory bowel diseases, dermatitis, and cancer [12]. A previous research that used obese high-fat-diet-fed mice model demonstrated that leukotrienes B4 (LTB4) was over-expressed in insulin target tissue particularly muscle, liver and adipose tissue. This events directly caused the reduction of insulin sensitivity in hepatocytes and myocytes thus providing a new mechanism connecting inflammation and impaired insulin sensitivity [5]. The present study investigated the potential inhibitory effect of MC on LOXs using Lipoxygenase Inhibitor Screening Assay Kit. The kit detects hydroperoxides produced through lipoxygenation reaction and the absorbance read at 495 nm. The 15-LOX enzyme from soybean is able to catalyze the reactions with both arachidonic acid and linoleic acid as substrate provided with the kit, while 5-LOX enzyme exhibits higher reaction with linoleic acid as substrate.

Effect of MC Raw Juice and Fractions on LOXs Inhibition Activity

This study investigated the effect of different MC fractions on the activities of LOXs. The MC raw juice and its fractions (strong acid, weak acid, neutral, weak base, strong base) were all tested for their potential 5-LOX and 15-LOX inhibitions. Figure 5a presented the 5-LOX inhibition data. The raw juice performed the highest 5-LOX inhibition at 55.51%. The inhibitory effects of MC fractions are as followed (in descending order): strong base (13.93%), strong acid (13.26%), neutral (9.12%), weak acid (5.49%) and weak base (4.36%) fractions. As for 15-LOX, the raw juice resulted in the highest inhibitions at 55.30% followed by strong base (47.23%), weak base (42.00%), strong acid (39.20%), weak acid (21.28%) and neutral (14.64%) fractions (Figure 5b).
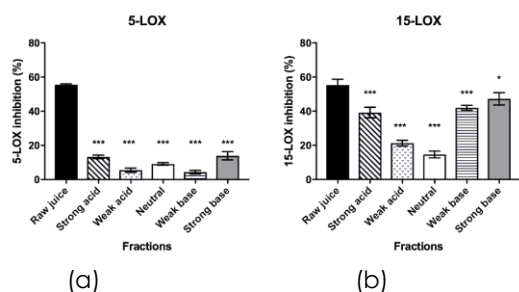

(a)   (b)

Figure 5 Effect of MC Raw Juice and Fractions on (a) LOX-1, (b) LOX-2. * $p < 0.05$ and *** $p < 0.001$ were obtained from ANOVA with Dunnett's post-hoc test comparing data with raw juice (n=3)

Based on these results, raw juice provided the highest 5-LOX and 15-LOX inhibition activity than other fractions. However, the raw juice and the fractions inhibition patterns on both LOXs were slightly different. For 5-LOX, it can be seen that all five fractions showed additive inhibitory effect, and the sum of inhibition exerted by all fractions was almost equal to the percentage of inhibition exerted by the raw juice. For 15-LOX inhibition study, MC fractions gave relatively high inhibitory effect (14.64%-47.23%), and the metabolites from different fractions may interact to give a 55.30% inhibition when the enzyme was treated with the raw juice.

Effect of Processing Method on LOXs Inhibition Activity

This section focuses on the effect of different processing methods (namely conventional juicer and slow juicer) on inhibition of LOXs. Comparable with results on inhibition of COXs, samples from slow juicer consistently showed higher inhibitory effects on LOXs, as compared with conventional juicer (Figure 6).

For 5-LOX inhibition study, the data showed that sample produced by the slow juicer gave higher inhibitory effect (76.46%) than the conventional juicer

(55.51%). A same order was found for 15-LOX where juice from slow juicer gave higher inhibition (97.72%) than the conventional juicer (55.30%). This finding showed that the juice processing technique influenced the bioactivity of the plant. The phytochemical levels in fruit juice are solely depend on the fruit species, germination, the degree of ripeness and seasonal variation. Different post-harvest techniques including storage conditions and processing methods also may affect the phytochemical level in the juice [41]. A previous study suggested that juice extraction techniques by blending and juicing of several kernel fruit (apple, mandarin orange, persimmon and pear) had a significant influence on the phytochemicals and antioxidant property of fruit juices. The researchers also suggest that blending fruit juice that has the highest pulp content may contribute to the higher antioxidant level of the fruit juice [42].
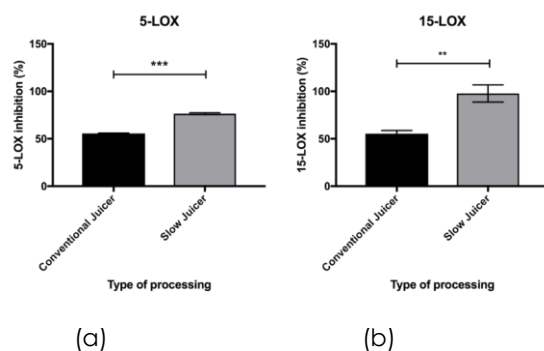

(a)   (b)

Figure 6 Effect of processing methods on (a) 5-LOX, (b) 15-LOX. ** $p < 0.01$ and *** $p < 0.001$ were obtained from Student's t-test (n=3)

Effect of MC Juice Concentrations on LOXs Inhibition Activity

Next, we assessed the LOXs inhibition by MC samples with different MC concentration ranging from 25%, 50%, 75% to 100%. The MC juice obtained from conventional juicer was used in this experiment. The percent of LOXs inhibition was found to have positive correlation with the concentrations of MC juice (Figure 7). For 5-LOX inhibition study, the highest inhibition was achieved by 100% MC juice (55.51% inhibition), followed by 75% MC juice (44.56%), 50% MC juice (27.84%) and 25% MC juice (12.43%). The same order was also found for 15-LOX inhibition, with the percentage of inhibition increased from 4.46% (25% MC juice) to 55.30% (100% MC juice).
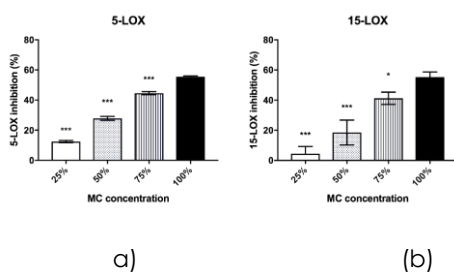
a)   (b)

Figure 7 Effect of MC juice concentration on: (a) 5-LOX, (b) 15-LOX. * p<0.05 and *** p<0.001 were obtained from ANOVA with Dunnett's post-hoc test comparing data with 100% MC juice concentration as the controls (n=3)

4.0 CONCLUSION

The current study focused on the effects of fractionation and processing methods on the inflammatory effects of MC. MC was found to have anti-inflammatory effects on COX-1, COX-2, 5-LOX and 15-LOX activities. Notably, study on the MC fractions showed that the anti COX-1 activity of MC can be solely attributed to neutral metabolites, and selective COX-2 inhibitors may be present in both strong acid and strong base fractions. On the other hand, MC raw juice exerted about 55% inhibitions on both 5-LOX and 15-LOX, but all five fractions contributed collectively to its LOXs inhibitory effect.

In this study, MC juice produced using a slow juicer consistently showed higher inhibitions on COX-1, COX-2, 5-LOX and 15-LOX activities, as compared with juice obtained from conventional juicer. The results supported previous findings that slow juicer can retain bioactivities of food product better than a conventional juicer. This may be due to heat generated by the high-speed blades in a conventional juicer that caused nutrient degradation. Taken together, slow juicer was found to be the preferred method for juicing to preserve anti-inflammatory bio-compounds. Collectively, the current results may offer useful strategy for improved processing and fractionation of MC.

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