STUDIES OF THE ANTI-INFLAMMATORY ACTIVITIES OF

PALMITOYLETHANOLAMIDE (PEA) AND POTENTIAL SYNERGISTIC

COMBINATIONS WITH PLANT POLYPHENOLS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2020

Major Subject: Food Science and Technology

ABSTRACT

The endogenous compound palmitoylethanolamide (PEA), has significant anti-inflammatory and immune modulating properties. As a nutraceutical, PEA assists in prevention and treatment of chronic inflammatory diseases, acting at several molecular targets. This study hypothesized a potential synergistic interaction between PEA, quercetin and curcuminoids in mitigating the inflammatory response. The interactive effects of their combination against inflammation in RAW264.7 macrophages were investigated and combinations demonstrated ability to reduce the expression of proinflammatory markers related to the NF-kB pathway, such as IL-1b, TNF-α and IL-10. The synergistic nature of the interactions was demonstrated in mRNA expression of IL-1b. Simulated CI values identified, for the first time, a range of effect levels in which combinations are synergistic. PEA reduced IL-1b and TNF-α expression via activation of the peroxisome proliferator-activated receptor (PPAR-α), whereas quercetin and curcuminoids effects were not affected by PPAR-α blockage. LPS-stimulation decreased expression of PEA-degrading enzymes, while treatment with PEA increased enzyme expression. PEA exhibited good stability under cell culture conditions, as 82% and 78% of added PEA and d4-PEA, respectively, were found in the supernatant after 12h. Presence of quercetin and curcuminoids did not change d4-PEA cellular uptake. Additional studies are recommended to understand the role of polyphenols in the PEA uptake process.

A combined formulation containing the three agents (PCQ) reduced in vitro expression of pro-inflammatory markers, as well as reactive oxygen species and nitric oxide release in RAW264.7 cells. When given as a dietary supplement for 8-weeks to family-owned osteoarthritic dogs, PCQ reduced concentration of disease associated pro-inflammatory markers in some patients. Improvement in pain scores from Canine Brief Pain Inventory (CBPI was seen in 3 out of 6 dogs and force plate analysis indicated that 4 dogs exhibited improvement in both peak vertical force (PVF) and vertical impulse (VI). Outcomes from this pilot study provided insight on the potential benefits of this combination in an already established disease and how the supplementation can help in pain management, activity and improved gait, as well as in reduction of OA-associated inflammation. The novel formulation exhibits high applicability as a dietary supplement to be used alone or as an adjuvant to standard of care in chronic inflammatory conditions.

DEDICATION

To my mom and dad, Lourdinha and Ross, for always believing in me and for the

unconditional love and support.

ACKNOWLEDGEMENTS

roommate, for the conversations, laughter, reiki sessions and all the fun stuff. Thank you, Glenn, for being such a great friend!

To my husband, Vinicius, thank you, thank you, thank you!! For your infinite patience and understanding when I would come home feeling down, for your support in the most difficult moments, for lifting me up when I needed a push, for your love, for being so kind and always believing in me, even when I didn't. Thank you for everything!

Finally, the biggest of all thanks to my family, my mom and dad, Lourdinha and Ross, for their encouragement, unconditional love and support. To my brother Cheney, for his funny stories and comments, always making me laugh. I love you all!

Thank you!

CONTRIBUTORS AND FUNDING SOURCES

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CHAPTER I

INTRODUCTION

A growing body of evidence suggests that chronic, unresolved inflammation,
together with the sustained production and release of inflammatory mediators in the
body, has an important role in the induction and maintenance of inflammatory chronic
diseases such as osteoarthritis (Medzhitov, 2008; Okin and Medzhitov, 2012). These
findings support alternative prophylactic and therapeutic strategies against chronic
inflammation based on a sustained reduction of inflammatory process.

Current standard of care for inflammation and chronic inflammatory diseases is
based on the long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs). These
drugs contribute to symptom control and pain management, but do not lead to resolution
of the disease. Prolonged use of NSAIDs can also result in drug tolerance and increased
incidence of adverse events, being gastro-intestinal bleeding and ulceration the most
common reported adverse effect (Innes *et al.*, 2010). Though several oral drug
formulations are available, there is an increasing trend towards the use of dietary
supplements and nutraceuticals as preventative or adjuncts to conventional therapies.

A potential compound to be used for that purpose is palmitoylethanolamide
(PEA). PEA is a biologically active fatty acid amide belonging to the family of the N-
acylethanolamines. This molecule was first isolated from egg yolk, soybean lecithin and
peanut meal, and later found to be an endogenous molecule detected in the lipid fraction
of rat brain, liver and skeletal muscle (Kuehl *et al.*, 1957; Bachur *et al.*, 1965). PEA is
produced and released on demand from cell membrane phospholipid precursors, as a

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mechanism to maintain and restore homeostasis in response tissue injury and stress (Ueda *et al.*, 2010a; Iannotti *et al.*, 2016; Petrosino and Di Marzo, 2016). It has been reported to downregulate many pro-inflammatory markers including IL-1b, Cox-2, iNOS, IL-6 and IL-10 in vitro and in animal models (Ross *et al.*, 2000; Esposito *et al.*, 2014; Alhouayek *et al.*, 2017; Gabrielsson *et al.*, 2017), in addition to being effective at reducing clinical signs and pain in human clinical trials (Marini *et al.*, 2012; Cremon *et al.*, 2017). Direct activation of the peroxisome proliferator-activated receptor-α (PPAR-α) is believed to be the main mechanism through which PEA exerts it anti-inflammatory activity (Lo Verme *et al.*, 2005), although other mechanisms have also been proposed (Petrosino and Di Marzo, 2016). Furthermore, given the broad spectrum of its pharmacological activities and absence of side effects in animal and clinical trials (Paladini *et al.*, 2016; Nestmann, 2017), PEA has become a promising multi-target molecule to be used as a dietary supplement.

Considerable research has been done in elucidating bioactive plant polyphenols' potential health effects. The well-known flavonol quercetin, as well as curcuminoids from turmeric can modulate pro-inflammatory pathways, down-regulating key pro-inflammatory mediators such as IL-1b, TNF-α, IL-6, IL-10, while also exerting antioxidant properties, through free radical scavenging and/or enhancement of the endogenous antioxidant system (Tsao, 2010; Kim *et al.*, 2017; Ma *et al.*, 2017). Considering that PEA and polyphenols are effective in reducing inflammation when used individually, there is a potential in identifying interactions that are able to enhance their beneficial health effects. Providing evidence of bioactive compounds' combination

significant superiority compared to the single agents, is of particular interest, as

synergistic interactions enables increased efficacy, reduced dose and toxicity, as well

lower production costs (Ting-Chao Chou, 2010).

These studies therefore investigated PEA's anti-inflammatory activity and the

potential synergistic effect when combined with quercetin and curcuminoids in vitro and

in a pilot-study involving osteoarthritic dogs. We also assessed the uptake of PEA and

the effect of quercetin and curcuminoids in maximal uptake by macrophages. Our

purpose was to screen for compounds that can act synergistically towards an improved

anti-inflammatory activity, define an optimized formulation and confirm its effects

in osteoarthritic dogs. This will contribute to advancement in the use of combined

compound supplementation as a more natural way to prevent and complement standard

of care therapies in chronic inflammation.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of the inflammatory response

Inflammation is a protective cellular response and part of the body's defense mechanism. During the inflammatory response, the immune system recognizes harmful stimuli such as invading pathogens or endogenous signals such as damaged cells and initiates the healing process (Medzhitov, 2008). Acute inflammatory responses are crucial for health maintenance and tissue repair, involving complex mechanisms that results in resolution and restoration of homeostasis (Kotas and Medzhitov, 2016). While the acute inflammatory response contributes to tissue healing and recovery, prolonged, non-resolved inflammation eventually becomes destructive, leading to chronic inflammatory diseases development and progression. Diabetes, cardiovascular diseases, arthritis and joint diseases, allergies, inflammatory bowel diseases and cancer are conditions resulting from homeostatic imbalance of the chronic inflammatory process (Nathan, 2002; Medzhitov, 2008).

The body's initial response to inflammation is to activate locally residing cells (endothelial cells, macrophages, dendritic cells) that will release chemokines and other soluble mediators, creating a chemotactic gradient that changes vascular permeability, leading to specialized cell recruitment to the site of injury (Fujiwara and Kobayashi, 2005). This tightly regulated mechanism results in neutrophil accumulation, which will

later be replaced by mononuclear cells that eventually differentiate into macrophages (Medzhitov, 2008).

Macrophages play a central role in all inflammatory response stages. Upon activation by inflammatory stimuli, they secrete a wide array of mediators, which affect tissue resident and recruited macrophage functions, consequently modulating the inflammatory response and aiding in tissue repair. Pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukins-1, 6, 8 and 12 (IL-1, IL-6, IL-8, and IL-12), chemokines, leukotrienes and prostaglandins are examples of these mediators (Dunster, 2016).

Upon infectious agents and/or inducers elimination, a resolution and repair phase take place, characterized by an active shift towards expression of anti-inflammatory cytokines such as IL-10 and IL-13, as well as lipid mediators such as lipoxins and resolvins, which are important for inflammation resolution and mediating wound healing (Serhan and Savill, 2005; Medzhitov, 2008). When acute inflammatory response fails to eliminate the inducer, the inflammatory process will persist, modifying the profile of mediators and resulting in tissue necrosis, tumors and chronic inflammatory diseases development. Therefore, a valuable tool for evaluating the severity of inflammatory process is the determination of cytokine expression in biological systems.

2.2 Osteoarthritis as an inflammatory disease

Osteoarthritis (OA) is the most common degenerative joint disease and a leading cause of disability, affecting 10-15% of all adults aged over 60 (World Health Organization, 2018). It is also a concerning problem among companion animals, as it is estimated that 20% of dogs over one year of age suffer from OA (Comblain *et al.*, 2017). Osteoarthritis is characterized by cartilage breakdown and synovial inflammation, with clinical symptoms including joint pain, swelling, stiffness, synovitis and inflammatory pain, resulting in functional disability and reduced quality of life (Goldring and Otero, 2014). The disease is multifactorial and dependent on genetic components, but it can be exacerbated by systemic factors such as age, gender and race/ethnicity, as well as environmental factors such as diet, exercise levels and obesity (Zhang and Jordan, 2010).

Over the past decade, OA stopped being seen as a non-inflammatory degenerative disease resulting from normal bodily wear, but as a disorder in which chronic low-grade inflammation has a central role (Robinson *et al.*, 2016). In the progression of OA, the immune system participates by infiltrating T cells, B cells and macrophages in the joint tissue. Those cells secrete cytokines and chemokines, leading to release of matrix metalloproteins (MMPs), prostaglandin E2 (PGE2) and activation of the complement system, resulting in further cartilage damage. Macrophage-derived IL-1b and TNF-α are the major cytokines participating in the cartilage breakdown in OA (Haseeb and Haqqio, 2013). Levels of IL-6 were found to be higher in synovial fluid of OA patients compared to healthy (Richardson *et al.*, 2008). Robinson et al. 2016 found

increased numbers of inflammatory cells and higher level of TNF-α in synovial fluid (SF) from OA knees compared to contralateral joints in dogs. Thus, as accumulating evidence supports the association between OA and inflammation, it is natural to target inflammatory markers as a therapeutic route to slow the disease progression and prevent or reduce its pathological signs.

In addition to inflammation, oxidative stress is closely related to the progression of OA, as overproduction of reactive oxygen species (ROS) in the joint tissue induces several structural damages to biological membranes and extracellular matrix proteins (Panahi *et al.*, 2016). Articular chondrocytes produce ROS at low levels, which participate in maintenance of cartilage homeostasis. Nonetheless, when the endogenous antioxidant system is not able to control the production of these reactive species, ROS will contribute to the inflammatory-related tissue degradation in OA. The signaling pathways involved are very complex and require further investigation (Lepetsos and Papavassiliou, 2016), but one approach to prevention and management of chronic inflammatory states, including OA, is by the supplementation with natural antioxidants.

It is important to mention that because there is no cure for OA, early intervention has the greatest potential for progressive deterioration cycle interruption and providing effective disease management.

2.3 Standard of care and use of dietary supplementation as an alternative to control chronic inflammation and osteoarthritis

The standard of care for most chronic inflammatory diseases relies on symptom and pain management using nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs mediate their anti-inflammatory activity by inhibiting cyclooxygenase (COX)-1 and COX-2, which catalyzes the conversion of arachidonic acid to prostaglandin (PG) (Díaz-González and Sánchez-Madrid, 2015). Continuous use of the pharmacological agents can potentially lead to drug tolerance and increased adverse events incidence, being gastro-intestinal bleeding and ulceration the most commonly reported (Innes *et al.*, 2010). Pharmacological therapies for OA rely mostly on the long-term use of NSAIDs such as carprofen, meloxicam and firocoxib, to control pain and inflammation. Non-pharmacological treatments include exercising, physical therapy, changes in lifestyle, exercise and diet, aiming an improvement in general mobility, function and well-being (Laev and Salakhutdinov, 2015).

The interest in safe agents that are effective in reducing inflammation and improving quality of life has grown substantially. Glucosamine and chondroitin sulfate are aminosaccharides, and are one of the alternative nutraceuticals used for management of OA, as it has been shown that they can stimulate synthesis or reduce degradation of cartilage matrix molecules (Lippiello *et al.*, 2000). Participation of oxidative stress in OA progression creates potential for natural antioxidants incorporation in dietary supplements. Turmeric (curcuminoids), avocado/soybean unsaponifiable (ASU) oils and

Boswellia serrata extracts are popular antioxidants incorporated in OA supplements (Grover and Samson, 2016), although several other phytochemicals and herbal extracts have been tested and are currently commercially available. A dietary supplement containing glucosamine-chondroitin-quercetin glucoside (GCQG) reduced chondroitin 6-sulphate (C6S) concentration and molecular weight of HA in the synovial fluid of OA patients, in addition to improvements in the walking and pain scores (Matsuno *et al.*, 2009). In contrast, Alves et al. (2017) found no differences in pain scores between treatment and carprofen (control) groups (measured by the canine brief pain inventory (CBPI), when evaluating the effectiveness of a commercially available dietary supplement containing glucosamine HCl (400 mg), chondroitin sulphate (300 mg), and hyaluronic acid (15 mg) in OA dogs. In humans, a recent clinical trial also indicated a lack of superiority of glucosamine and chondroitin sulfate agents over placebo in joint pain reduction and functional impairment in patients with symptomatic knee OA over 6 months (Roman-Blas *et al.*, 2017).

It is safe to say that the oral joint supplements use is a popular option in the management of joint disease and related pain in humans and companion animals, whereas there is still a lack of effective agents exhibiting consistent results in the improvement of OA clinical signs and molecular indicators.

2.3.1 Bioactivity of quercetin and curcuminoids in inflammation and osteoarthritis

Plant polyphenols are a group of naturally occurring compounds functioning as plant secondary metabolites. They exert a range of bioactivities and have great potential to be used as preventative agents and as alternative therapies. Quercetin ($C_{15}H_{10}O_7$) is a flavonoid, more specifically a flavonol (Figure 1), often found in a variety of foods including onions, Brassica vegetables, apples and berries as well as many seeds, nuts, flowers, barks, and leaves (Li *et al.*, 2016). Curcuminoids are another type of plant secondary metabolite, found in turmeric root (*Curcuma longa* L.) and classified as diarylheptanoids. The main curcuminoid types in turmeric are curcumin (77%), demetoxycurcumin (DMC; 17%) and bis-demetoxycurcumin (BDMC; 3%) (Figure 1) (Goel *et al.*, 2008). When sold as a dietary supplement, the product is usually standardized to an extract containing 80-95% curcuminoids, mainly curcumin (Daily *et al.*, 2016). Currently, both quercetin and curcuminoids extracts are marketed as ingredients for dietary supplements, or as dietary supplements per se, with several structure function claims, allowed by the Dietary Supplement Health and Education Act

of 1994 (DSHEA).
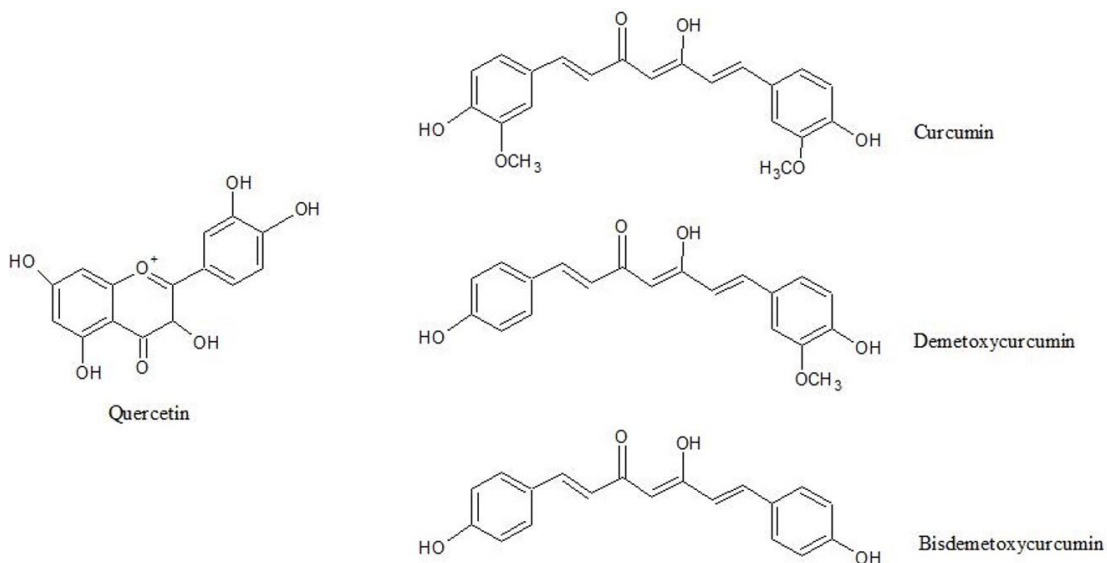
Figure 1. Chemical structures of quercetin and the curcuminoids curcumin, demetoxycurcumin and bisdemetoxycurcumin.

Numerous papers reported great antioxidant and anti-inflammatory potential of quercetin and curcuminoids against inflammation. Lesjak et al. (2018) demonstrated a concentration-dependent inhibitory potential of quercetin towards the inflammatory mediators 12-HHT (12-Hydroxyheptadecatrenoic acid), TXB2 (thromboxane B_2) and PGE2 (prostaglandin E_2) using an ex vivo human platelet assay, and such inhibition was comparable with aspirin. In another study, quercetin inhibited NF-κB (nuclear factor-κB), Erk1/2 (extracellular signal-regulated kinase ½) and JNK (c-Jun N-terminal kinase) signaling, reduced nitric oxide (NO) production and IL-6 protein levels in RAW264.7 macrophages (Kim *et al.*, 2017).

In cartilage explant models, curcumin inhibited production of nitric oxide (NO) and expression of PGE2, IL-6, IL-8, and MMP-3 production by human chondrocytes in

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a concentration-dependent manner (Mathy-Hartert *et al.*, 2009). More recently, Ma et al. (2017) found decreased secretion of IL-6 and TNF-α by LPS-stimulated RAW264.7 macrophages treated with 5, 10, and 15 µM of curcumin at different time points.

In addition to modulating general inflammatory response, quercetin and curcuminoids have been reported as an effective supplement and potential alternative treatment for joint pain and diseases, being investigated alone or in combination with other polyphenols and herbal extracts. A human clinical trial with osteoarthritic patients evaluated oxidative stress biomarkers upon supplementation with curcuminoids extract (1500 mg/day) and piperine (to enhance bioavailability) versus a placebo. Results showed oxidative stress biomarkers improvement following a 6-week supplementation with the curcuminoids-piperine combination (Panahi *et al.*, 2016). In osteoarthritic dogs, curcumin supplementation resulted in significant down regulation of TNF-α, CXCL8 (IL-8), NFKB1 (nuclear factor kappa B subunit 1) and Cox-2 (cyclooxygenase-2) after 60 days of supplementation (Sgorlon *et al.*, 2016). Similarly, (Colitti *et al.*, 2012) reported that curcumin regulated inflammatory response molecular targets in OA dogs. In addition to improvement in inflammatory biomarkers and oxidative stress, a combination of curcuminoids extract, hydrolyzed collagen and green tea extract given as a dietary supplement to dogs was able to improve pain at manipulation after 3 months (Comblain *et al.*, 2017). Very recently, quercetin was shown to up-regulate superoxide dismutase (SOD), an important endogenous antioxidant enzyme, and tissue inhibitor of metalloproteinase-1 (TIMP-1) in a rabbit model of knee OA. This is relevant since TIMPs inhibits the activity of matrix-degrading enzymes such as matrix

metalloproteinases (MMPs) in synovial fluid. In this same study, there was no significant difference in serum and synovial fluid MMP-13 levels between celecoxib treated and quercetin treated groups, but both were lower than the untreated group (Wei *et al.*, 2019).

Most publications investigating the use of dietary supplements for OA focus on formulations containing more than one bioactive compound, and this is most probably due to the multifactorial nature of OA pathogenesis. For example, promising results were observed in human subjects with symptomatic knee OA consuming a glucosamine-chondroitin-quercetin glucoside supplement for 16 weeks, in which they observed lower pain score compared to a placebo (Kanzaki *et al.*, 2012). In a mouse model of OA, an ultra-micronized formulation containing palmitoylethanolamide (PEA) and quercetin was shown to improve pain scores and reduce serum concentrations of tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1b), metalloproteases1,3 and 9 (MMP-1, MMP-3, MMP-9) and nerve growth factor (NGF) (Britti *et al.*, 2017). This last study is the first report on the anti-inflammatory compound palmitoylethanolamide (PEA) associated with quercetin. It makes sense to investigate combined therapies instead of individual compounds, as a multi-target approach can lead to improved effectiveness, possibly with the use of lower doses.

2.4 Background on the N-acylethanolamine family

Fatty acid amides (FAAs) are biologically active molecules that can be grouped into two major classes, N-acylethanolamines (NAEs) and the fatty acid primary amides (Ezzili *et al.*, 2010). N-acylethanolamines (NAEs) consists of long-chain fatty acids coupled to an ethanolamine molecule by an amide bond. They are endogenous bioactive lipid molecules differentiated by the length and unsaturation degree of their acyl chain (Alhouayek *et al.*, 2017). Despite their structural similarity, NAEs can bind to several different receptors and exert numerous biological effects.

Anandamide (N-arachidonoyl ethanolamide or AEA), one of the most studied NAEs, is derived from arachidonic acid and it was the first endogenous agonist of the cannabinoid receptors CB1 and CB2 to be discovered, acting as a potent analgesic and anti-inflammatory (Iannotti *et al.*, 2016). Other important NAEs do not bind to the cannabinoid receptors, but exert a variety of biological actions via several other receptors (Rahman *et al.*, 2014).

Oleoylethanolamide (OEA) is believed to interact with peroxisome proliferator-activated receptor-α (PPAR-α), causing a feel of satiety and decreasing meal frequency (Piomelli, 2015). N-stearoylethanolamine (SEA) is a less investigated saturated NAE, shown to reduce inflammation in vitro and in vivo (Dalle Carbonare *et al.*, 2008). SEA's effect is suggested to occur via modulation of the proxisome proliferator-activated receptor gamma (PPARγ) (Berdyshev *et al.*, 2015). More recently, N-docosahexaenoylethanolamine (DHEA), which is an ω-3 fatty acid-derived

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ethanolamide, has been reported to inhibit COX-2-derived eicosanoid production in murine macrophages (Meijerink *et al.*, 2015) and to exert potent anti-inflammatory effects through cAMP/PKA signaling and inhibition of NF-κB activation in microglia cells (Park *et al.*, 2016). Palmitoylethanolamide (PEA) is the N-acylethanolamine derived from palmitic acid and it will be described in detail in the next sections.

2.5 Palmitoylethanolamide (PEA)

2.5.1 Discovery, synthesis and metabolism of palmitoylethanolamide (PEA)

Palmitoylethanolamide (PEA) ($C_{18}H_{37}NO_2$) is a biologically active fatty acid amide belonging to the family of the N-acylethanolamines (NAEs) (Figure 2). The molecule was first isolated from egg yolk, soybean lecithin and peanut meal by Kuehl et al., in 1957, who demonstrated its anti-inflammatory activity in guinea pigs (Kuehl *et al.*, 1957). Later, PEA was found to be an endogenous molecule found in the lipid fraction of rat brain, liver and skeletal muscle (Bachur *et al.*, 1965). Pharmacological properties of PEA began to be investigated in animal models of inflammation, however, since no mechanism of action was identified at the time, research with the newly discovered compound faded and interest only emerged again around the 1990's, after Professor Rita Levi-Montalcini published a paper proposing that lipid amides such as PEA are able to modulate mast cell activation in vivo via an "autacoid local inflammation antagonism" (ALIA) (Aloe *et al.*, 1993). In this theory, the lipid amides

are synthesized in response to injury or inflammation and controls metabolism locally. Further research has proposed more detailed mechanisms of action for PEA and those will be reviewed later in the text.
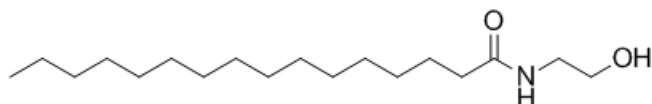
Figure 2. Chemical structure of Palmitoylethanolamide (PEA)

Endogenous PEA and related NAEs are not stored in the cells, but rather produced and released on demand from cell membrane phospholipid precursors, as a mechanism to maintain and restore homeostasis in response tissue injury and stress. Their levels in cells and tissues is strictly regulated by enzymes responsible for their synthesis and degradation (Ueda *et al.*, 2010a; Iannotti *et al.*, 2016; Petrosino and Di Marzo, 2016).

The biosynthetic pathways for different NAEs are analogous and have been extensively reviewed (Di Marzo *et al.*, 1996; Lambert *et al.*, 2002; Ueda *et al.*, 2010b; Iannotti *et al.*, 2016). Different NAEs are be formed based on the fatty acid chain esterified at sn-1 position of the precursor glycerophospholipid (Ueda *et al.*, 2010b). Therefore, palmitoylethanolamide (PEA) is synthesized from a glycerophospholipid containing palmitic acid (C16:0) at the sn-1 position. Briefly, the fatty acyl chain from membrane glycerophospholipids is transferred to the nitrogen atom of phosphatidylethanolamine (PE), forming the intermediate N-acylphosphatidyl-

ethanolamine (NAPE). This reaction is mediated by a Ca^{2+}-dependent N-acyltransferase (Ca-NAT). The second step involves the hydrolysis of NAPE to the correspondent NAE and phosphatidic acid, through the action of the enzyme N-acyl-phosphatidyl-ethanolamine-selective phospholipase D (NAPE-PLD) (Okamoto *et al.*, 2004).

Two enzymes have been consistently reported to participate in the hydrolysis of NAEs to their corresponding fatty acid and ethanolamine, terminating their biological activities: fatty acid amide hydrolase (FAAH) and N-acylethanolamine-hydrolyzing acid amidase (NAAA) (Alhouayek and Muccioli, 2014; Bottemanne *et al.*, 2018). FAAH is a membrane-associated serine hydrolase that is active at neutral and alkaline pH. It is able to hydrolyze several NAEs, but has highest affinity for anandamide (AEA) (Ueda *et al.*, 2010a). NAAA, on the other hand, is a lysosomal cysteine amidase that hydrolyzes NAEs, but it is only active at acidic pH and it has a preference for PEA (Ueda *et al.*, 2001, 2013). Both FAAH and NAAA are expressed in human and rodent tissues, with FAAH being abundant in brain and liver (McKinney and Cravatt, 2005) and NAAA being predominantly expressed in macrophages (Giang *et al.*, 1997; Sun *et al.*, 2005).
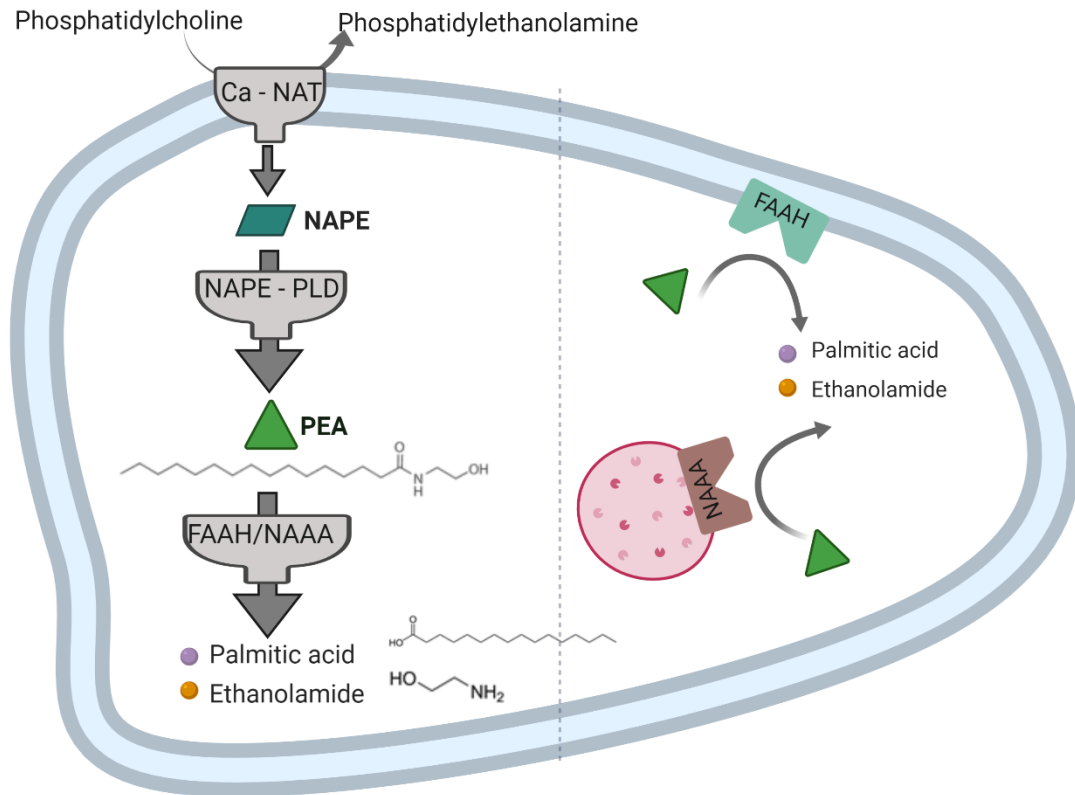
Figure 3. Synthesis and degradation of palmitoylethanolamide (PEA). The fatty acyl chain from membrane glycerophospholipid phosphatidylcholine (PC) is transferred to the nitrogen atom of phosphatidylethanolamine (PE), forming N-acylphosphatidyl-ethanolamine (NAPE), mediated by Ca^{2+}-dependent N-acyltransferase (Ca-NAT). Hydrolysis of NAPE to PEA occurs through the action of the enzyme N-acyl-phosphatidyl-ethanolamine-selective phospholipase D (NAPE-PLD) PEA can be then degraded to palmitic acid and ethanolamine by fatty acid amide hydrolase (FAAH) or N-acylethanolamine acid amidase (NAAA) (Ueda *et al.*, 2010b; Iannotti *et al.*, 2016; Petrosino and Di Marzo, 2016).Created with BioRender.com.

Endogenous PEA levels are tightly regulated and highly variable, especially in situations associated with inflammatory processes, although how endogenous concentration is regulated during inflammation is not exactly clear. In vitro, the LPS-stimulation of J774 murine macrophages did not change the endogenous levels of PEA after 8h and 24h (Alhouayek *et al.*, 2017), while in two other studies, LPS-stimulation of

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RAW264.7 murine macrophages caused a reduction in cellular levels of PEA (Solorzano *et al.*, 2009; Zhu *et al.*, 2011). This last author also reported that the reduction in PEA levels occurs due to reduction in the expression of the PEA-synthesizing enzyme, NAPE. In contrast, Gabrielsson et al. (2017) reported that in RAW264.7 murine macrophages, the hydrolysis of added [^{3}H]-PEA was lower (~0.6%) in the LPS + IFNγ cells compared to untreated cells following a 30 minutes incubation. It is interesting to mention that in all these studies, the use of FAAH and NAAA inhibitors contributed to the reduction in PEA hydrolysis. In chronic inflammatory osteoarthritis, it has been demonstrated that high levels of PEA can be found in the synovial fluid of normal human subjects, while levels were greatly reduced in OA patients (Richardson *et al.*, 2008). Another human study reported increased level of PEA and other NAEs in multiple sclerosis subjects plasma compared to control (Jean-Gilles *et al.*, 2009), leading to believe that the nature of the inflammatory stimulus is determinant on endogenous PEA concentrations (Balvers *et al.*, 2013).

Numerous chemically synthesized inhibitors of NAAA and FAAH have been described in the literature (Ahn *et al.*, 2009; Yang *et al.*, 2015; Pędzińska-Betiuk *et al.*, 2017; Zhou *et al.*, 2019). Efforts to identify potential NAAA inhibitor molecules – to increase PEA levels and counteract inflammation - focuses of different strategies, such as utilizing PEA as a starting point for chemical modulation and searching for covalent modifiers of the catalytic cysteine (Piomelli *et al.*, 2020). Researchers found that NAAA inhibition by systemically administered AM9053 increases PEA levels in the colon and reduces colon inflammation in a mouse model of colitis, while FAAH inhibition did not

increase PEA levels in the colon (Alhouayek *et al.*, 2015). This same inhibitor lead to increased NAE levels in J774 murine macrophages (Alhouayek *et al.*, 2017). Recently, Zhou *et al.*, (2019) reported increased NAAA levels in synovial membrane in OA rats and demonstrated that intraperitoneal and intra articular injection of the NAAA inhibitor F215 (Figure 4) significantly prevented cartilage damage, attenuated synovial inflammation and alleviated pain via modulating PEA levels in joints. This effect was prevented by a PPAR-α antagonist, showing the PPAR-α mediated effect of increased PEA levels on OA. The structure of other NAAA inhibitors used in vitro and in animal studies is shown in Figure 4. Although literature on the topic is extensive and several effective inhibitors are available for mechanistic research purposes, data on safety and use of these inhibitors in humans particularly limited to a few papers about FAAH (Pawsey *et al.*, 2016; D'Souza *et al.*, 2019).
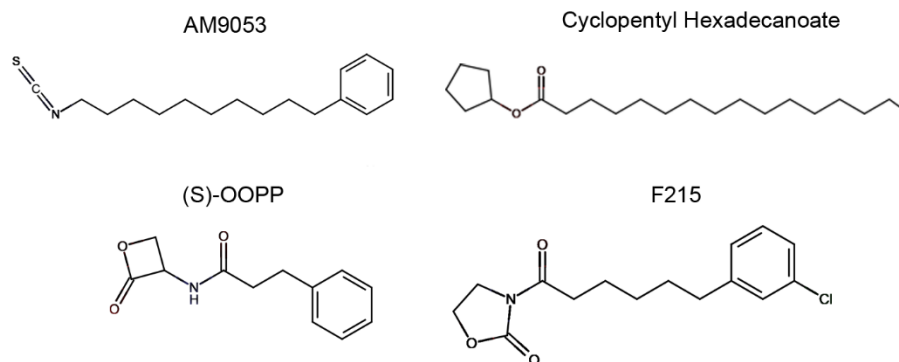
Figure 4. Examples of N-acylethanolamine-hydrolyzing acid amidase (NAAA) inhibitors (Bottemanne *et al.*, 2018).

Since PEA is synthesized from the precursor fatty acid in membrane phospholipids, it is natural to question if endogenous PEA levels could be modulated by dietary intake of palmitic acid. However, the few studies addressing the topic report that dietary fatty acid intake have minimal effects on the composition of membrane phospholipids and tissue levels of PEA and other NAEs (Hansen and Artmann, 2008; Hansen, 2013).

2.5.2 Pharmacokinetics and safety of exogenous palmitoylethanolamide (PEA)

Due to its lipophilic nature and poor solubility, the therapeutic use of PEA can become limited. A frequently used technique to overcome that is the micronization, which consists on particle size reduction down to the micron range (<10 μm) to enhance dissolution and bioavailability (Impellizzeri *et al.*, 2014). Micronized particle size has consistently shown better clinical results compared to the non-micronized molecule (Impellizzeri *et al.*, 2014; Noli *et al.*, 2015; Petrosino *et al.*, 2018) and is considered to be safe. The lethal dose (LD_{50}) for micronized PEA was shown to be greater than 2000 mg/kg body weight (bw) in a toxicity study with Sprague-Dawley rats (Nestmann, 2017). Despite the lack of studies investigating PEA's safety in humans, a frequent observation in clinical trials using micronized PEA is the absence of side effects related to the intervention, as reviewed by (Paladini *et al.*, 2016; Nestmann, 2017).

Comprehensive pharmacokinetic data is still lacking for exogenous PEA, although some experimental data has been published and demonstrated rapid absorption

followed by decrease to basal levels. Upon oral administration of PEA (100mg/kg to Wistar rats), the highest plasma concentration was observed after 15 min, corresponding to a 20-fold increase of its basal values (Vacondio *et al.*, 2015). In Beagle dogs, micronized PEA reached maximal plasma concentration – in the order of pmol/mL - after 1-2h of oral administration (30 mg/Kg), representing a five-fold increase in its basal plasma levels (Cerrato *et al.*, 2012). Similarly, Petrosino et al. 2016 reported maximal concentration in plasma after 1-2h, after a single 30 mg/Kg oral dose of micronized PEA to Beagle dogs. In that same study, a single oral dose of micronized PEA (300 mg) was given to human subjects and resulted in a maximum two-fold increase in baseline levels after 2h. Interestingly, the anti-inflammatory effects of PEA are long-lasting and usually outlasts its plasma levels, indicating that the rapid decline is not tied to the pharmacological effect.

2.5.3 Mechanisms of action

Since the discovery of PEA as a potent anti-inflammatory molecule, the compound has been exploited in different biological systems and pathological conditions such as endometriosis (Lo Monte *et al.*, 2013; Caruso *et al.*, 2015; Di Paola *et al.*, 2016), arthritis (Impellizzeri *et al.*, 2013), osteoarthritis (Richardson *et al.*, 2008; Britti *et al.*, 2017; Valastro *et al.*, 2017), joint pain (Marini *et al.*, 2012; Bartolucci *et al.*, 2018), retina (Matias *et al.*, 2006; Hesselink *et al.*, 2015; Puglia *et al.*, 2018), dermatitis (Petrosino *et al.*, 2010; Noli *et al.*, 2015; Vaia *et al.*, 2016; Abramo *et al.*, 2017), irritable

bowel syndrome (IBS) (Cremon *et al.*, 2017), depression (Ghazizadeh-Hashemi *et al.*, 2018), and also models of neuroinflammation and chronic pain (Costa *et al.*, 2008; Impellizzeri *et al.*, 2014; Crupi *et al.*, 2016; Parrella *et al.*, 2016; Germini *et al.*, 2017; Seol *et al.*, 2017). With so much evidence on its efficacy, PEA is commercially available as a nutraceutical for chronic pain and some veterinary formulations for skin conditions.

PEA is believed to be a multi-target molecule, and several mechanisms of action has been proposed. The first proposed mechanism was the previously mentioned "Autacoid Local Inflammation Antagonism" (ALIA) hypothesis (Aloe *et al.*, 1993), although more recent findings point out the "entourage effect" and the direct receptor-mediated mechanism (Iannotti *et al.*, 2016) as more relevant. It is known that PEA does not bind to cannabinoid CB1 and CB2 receptors (Lo Verme *et al.*, 2005), but is able produce cannabinoid receptor-mediated effects via increased concentrations of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) which are true cannabinoid agonists (Petrosino *et al.*, 2016). This occurs through competition of PEA for FAAH, resulting in higher AEA concentrations and indirect sensitization of transient receptor potential vanilloid type 1 (TRPV1) receptor system (Ho *et al.*, 2008). More recently, Petrosino *et al.*, (2019) reported for the first time that PEA has a stimulatory effect on 2-AG biosynthesizing enzymes (diacylglycerol lipases - DAGLs α and β), leading to elevation of cellular 2-AG concentrations in mast-cells, corroborating the "entourage effect" theory. The direct receptor-mediated mechanism of action of PEA was first reported by (Lo Verme *et al.*, 2005), who demonstrated in a series of in vitro and in vivo experiments that PEA directly activates PPAR-α. Boccella et al., (2019) demonstrated a

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PPAR- α dependent effect of PEA in ameliorating pain and cognitive function in mice. PPARs can regulate gene networks responsible for controlling pain and inflammation via the inhibition of the nuclear factor-kappa B (NF-κB) cascade, a key element in the transcription of genes leading to the synthesis of pro inflammatory mediators (D'Agostino *et al.*, 2009). When PEA bounds to PPAR-α, heterodimers are formed with retinoic acid receptors (RXR), which act as a transcription factor that will result in downregulation of nuclear-factor kB (NF-kB) and subsequent downstream signaling cascades (Daynes and Jones, 2002). In HeLa cells, PEA directly engages PPAR-α receptors, while palmitic acid and ethanolamine were ineffective under same conditions. PEA also inhibited inflammation and induced the expression of PPAR-α mRNA in mouse inflamed skin. Most importantly, PEA did not elicit anti-inflammatory effects in mutant PPAR-α null mice (PPAR-α-/- mice). PEA's antinociceptive effects are also considered to be mediated by PPAR-α activation and TRPV1 channels desensitization (Aldossary *et al.*, 2019). Considering all the proposed mechanisms, it is believed that the potent biological effects of PEA are a consequence of its multiple-target actions.

2.5.4 Pharmacology of PEA against inflammation

Macrophages are intimately involved in the responses to inflammation and tissue repair, and exogenous PEA exerts useful effects upon their function. A reduction in nitric oxide (NO) production was reported in response to stimulation with endotoxin lipopolysaccharide (LPS) in PEA treated RAW264.7 cells (Ross *et al.*, 2000). More

recently, Alhouayek *et al.*, (2017) reported a decrease in mRNA expression of pro-inflammatory markers (Cox-2, IL-1b and IL-6) in LPS-stimulated murine macrophages (J774) treated with PEA (10 µM). Additionally, PEA treatment (10 µM) was shown to reduce production of Cox-2 derived oxilipins, such prostaglandins D2 and E2 by RAW264.7 macrophages following LPS + IFNγ-stimulation. The same authors also observed that these effects were maintained when the hydrolysis of PEA to palmitic acid was blocked (Gabrielsson *et al.*, 2017).

The effect of exogenous PEA on inflammation-related diseases is also demonstrated in animal studies. In a murine model of colon inflammation, PEA decreased expression and release of iNOS, COX-2, GFAP in a dose dependent matter (Esposito *et al.*, 2014). In dogs suffering from atopic dermatitis, pruritus levels were decreased back to normal and animals had improved lesion scores after treatment with ultramicronized-PEA (10 mg/kg) (Noli *et al.*, 2015).

When evaluating PEA as a potential treatment for arthritic and osteoarthritic inflammation and pain, Bartolucci et al. (2018) reported significant reduction of TMJ damage, pain and macrophage activation in rats upon administration of micronized PEA (10 mg/kg). Treatment with PEA (10 mg/kg) or PEA + luteolin (1 mg/kg) improved clinical signs of Type II collagen-induced arthritis (CIA) arthritis in mice. Additionally, the treatment was able to significantly reduce levels of TNF-α, IL-1b, and IL-6 (Impellizzeri *et al.*, 2013). A recent study tested a co-ultramicronized formulation containing PEA and quercetin in a CAR-induced paw edema model in rats and reported improvement in pain score and inflammatory markers, with superior effects compared to

meloxicam, which is the standard NSAID for treatment of arthritis (Britti *et al.*, 2017). A

human clinical trial comparing the effects of PEA versus ibuprofen for pain relief in

temporomandibular joint (TMJ) osteoarthritis demonstrated higher pain reduction in

subjects taking PEA. In accordance to other clinical studies, no adverse effects were

reported in the PEA group, while subjects taking ibuprofen reported stomachache

(Marini *et al.*, 2012).

2.6 Determining synergistic interactions between bioactive compounds

Phytochemicals' effects on health promotion and disease prevention has been

extensively studied. Isolated compounds are effective in preventing and treating

pathological conditions, although it has been proposed that the bioactivity of many

fruits, vegetables, and herbal extracts results from the interaction of several constituents

instead of individual phytochemicals (Williamson, 2001). Compounds can act in an

additive or synergistic manner, although undesired antagonistic effects can also be

observed. An additive effect is observed when the effect is equivalent to the sum of

individual components, while a synergism is when the effect exceeds the additive effect.

On the other hand, when the sum of the effects is less than would be predicted from

individual components, the interaction is considered antagonistic (Chou, 2006).

The concept of synergy has been extensively explored by the drug industry, and

more recently, it has been applied in food and dietary supplements development.

Possible mechanisms underlying synergistic interactions are the possibility of targeting

multiple mechanisms at the same time and improved pharmacokinetics (better solubility, stability and bioavailability) (Wagner, 2011). As a consequence, formulations may present increased efficacy, reduced dose and toxicity, possibility of targeting multiple targets, as well lower production costs (Ting-Chao Chou, 2010). Thus, screening for synergistic interactions among phytochemicals to be used as dietary supplements represent a great potential.

Studies combining PEA with other bioactive compounds are relatively recent and have been restricted to luteolin (a flavonoid) (Impellizzeri *et al.*, 2013; Parrella *et al.*, 2016), and polydatin, a natural glucoside of resveratrol (Cremon *et al.*, 2017; Gugliandolo *et al.*, 2017). Only recently, a study came out reporting the combination of PEA and quercetin in a mouse model of induced arthritis (Britti *et al.*, 2017). This study reported improved effects compared to agents alone but did not investigate the nature of the interaction and synergy was not mentioned.

A well-established method to determine the nature of compound interactions is the Combination Index (CI) and related Isobologram. The method is based on the Loewe additivity principle and derived from 4 basic equations in biomedical sciences pioneered by Henderson-Hasselbalch, Michaelis-Menten, Hill, and Scatchard, which are described in detail elsewhere (Tallarida, 2001, 2006; Chou, 2006). Briefly, the CI corresponds to the sum of the ratio of the amount of drug A used in combination to the amount of drug A required as a single agent for a specific effect (usually 50%), plus the corresponding ratio for drug B for the same effect. It can be calculated by $CI = D_1/(Dx)_1 + D_2/(Dx)_2$, whereas D_1 and D_2 are the IC_{50} of the combination between compound 1 and compound

2 and $(Dx)_2$ and $(Dx)_2$ are the IC_{50} of each individual compound. $CI < 1$ indicates synergy; $CI = 1$ additive effect and $CI > 1$ antagonism (Chou, 2006). The isobologram is the resulting graphic representation of the interaction between compounds. The coordinates display the dose required for individual compounds to achieve a specific effect level (for example, the IC_{50}). The line of additivity is constructed by connecting these two points and lastly, the concentrations of the two drugs used in combination to provide the same effect (IC_{50}) are placed in the same plot. The points falling below the additivity line are considered a synergistic interaction. (Chou, 2006).

CHAPTER III

ANTI-INFLAMMATORY ACTIVITY OF PALMITOYLETHANOLAMIDE (PEA)

QUERCETIN AND CURCUMINOIDS IN RAW264.7 MACROPHAGES: EFFECT OF

COMBINED FORMULATIONS

3.1 Introduction

Acute inflammatory responses are crucial for health maintenance and tissue repair, involving complex mechanisms that results in resolution and restoration of homeostasis (Kotas and Medzhitov, 2016). Chronic, unresolved inflammation, accompanied by the sustained production and release of inflammatory mediators in the body has an important role in the induction and maintenance of inflammatory chronic diseases (Okin & Medzhitov, 2012). Current standard of care includes the use of non-steroidal anti-inflammatory drugs (NSAIDS). However, their long term use is frequently correlated with multiple adverse effects in the gastrointestinal, renal, hepatic and cardiovascular systems (Daily *et al.*, 2016). Thus, identifying novel, effective and safe strategies based on a sustained reduction of the inflammatory process is of interest.

Macrophages play a central role in all inflammatory response stages, and represents one of the main in vitro models for cell culture studies. Upon activation by inflammatory stimuli, they secrete a wide array of mediators, which affect tissue resident and recruited macrophage functions, consequently modulating the inflammatory response. Pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukins-1, 6, 8 and 12 (IL-1, IL-6, IL-8, and IL-12) and chemokines resulting from misregulation

of the NF-kB pathway, as well as leukotrienes and prostaglandins are examples of these mediators (Dunster, 2016; Liu *et al.*, 2017).

Palmitoylethanolamide (PEA) is a biologically active fatty acid amide, produced and released on demand from cell membrane phospholipid precursors, as a mechanism to maintain and restore homeostasis in response tissue injury and stress (Ueda *et al.*, 2010a; Iannotti *et al.*, 2016; Petrosino and Di Marzo, 2016). PEA has been reported to downregulate many pro-inflammatory markers including IL-1b, Cox-2, iNOS, and IL-6 in vitro and in animal models (Ross *et al.*, 2000; Esposito *et al.*, 2014; Alhouayek *et al.*, 2017; Gabrielsson *et al.*, 2017), and to reduce symptoms and pain in human clinical trials (Marini *et al.*, 2012; Cremon *et al.*, 2017). Direct activation of the peroxisome proliferator-activated receptor-α (PPAR- α) is believed to be the main mechanism through which PEA exerts it anti-inflammatory activity (Lo Verme *et al.*, 2005), although other mechanisms have also been proposed (Petrosino and Di Marzo, 2016). Given the broad spectrum of its pharmacological activities and absence of side effects in animal and clinical trials (Paladini *et al.*, 2016; Nestmann, 2017), PEA has become a promising multi-target molecule to be used as a dietary supplement.

Considerable research has been done in elucidating the potential health effects of plant bioactive polyphenols. The well-known flavonol quercetin, as well as curcuminoids from turmeric can modulate pro-inflammatory pathways, down-regulating key pro-inflammatory mediators such as IL-1b, TNF-α, IL-6, IL-10, while also exerting antioxidant properties (Tsao, 2010; Kim *et al.*, 2017; Ma *et al.*, 2017). Studies combining PEA with polyphenols are relatively recent and have been restricted to

luteolin (a flavonoid) (Impellizzeri *et al.*, 2013; Parrella *et al.*, 2016), and polydatin, a natural glucoside of resveratrol (Cremon *et al.*, 2017; Gugliandolo *et al.*, 2017). More recently, a study on the combination of PEA and quercetin in a mouse model of induced arthritis (Britti *et al.*, 2017) was published and reported improved effects compared to compounds alone, although the nature of the interaction was not investigated.

Considering that PEA and polyphenols are effective in reducing inflammation when used individually, there is a potential in identifying synergistic interactions that are able to enhance their beneficial health effects, contributing to increased efficacy as well as lower doses and toxicity (Ting-Chao Chou, 2010). This study was designed to assess the anti-inflammatory effects of PEA, quercetin and curcuminoids alone and in combination and the potential synergistic interaction among these compounds in reducing inflammation in an in vitro model.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Fetal bovine serum (FBS), Phosphate Buffer Saline (PBS), Dulbecco's Minimum Essential Medium (DMEM) culture medium and penicillin streptomycin combination were purchased from Gibco (Fisher Scienctific, Waltham, MA, USA). All analytical standards, quercetin, primers for real-time quantitative PCR (RT-qPCR), the beads for protein expression analyses, dimethyl sulfoxide (DMSO) and resazurin were purchased

from Sigma (MiliporeSigma, MA, USA). All reagents had the highest possible purity. Micronized palmitoylethanolamide (PEA) from Wuxi Cima Science (98% pure) and the curcuminoids extract (80%) were kindly donated by NaturPro Scientific.

3.2.2 Cell Culture

RAW 264.7 murine macrophage cell line was purchased from American Type Culture Collection (ATCC-TIB71). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, stable glutamine and sodium pyruvate, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin mix. Cells were maintained in an incubator at 37 °C with 5% CO_2 and the medium was changed every 2 days. All the tests were conducted on cells between the 5th and the 15th passage. All treatments with PEA and other polyphenols were diluted in complete culture media immediately before use.

3.2.3 Characterization of the Curcuminoid extract

The target compounds in the curcuminoid extract were detected on a triple quadrupole mass spectrometer (Altis, Thermo Scientific, Waltham, MA) coupled to a binary pump HPLC (Vanquish, Thermo Scientific). MS parameters were optimized for the target compounds under direct infusion at 5 µL min^{-1} to identify the SRM transitions (precursor/product fragment ion pair) with the highest intensity in positive mode as

309.15-119.07 m/z for bisdemethoxycurcumin (BDMC), 339.1-147.05 m/z for demethoxycurcumin (DMC) and 369.1-177 m/z for curcumin (CUR). Samples were maintained at 4 °C on an autosampler before injection. The injection volume was 10 µL. Chromatographic separation was achieved on a Hypersil Gold 5 µm 50 x 3 mm column (Thermo Scientific) maintained at 30 °C using a solvent gradient method. Solvent A was water (0.1% formic acid). Solvent B was acetonitrile (0.1% formic acid). The gradient method used was 0-4 min (20% B to 80% B), 4-4.1 min (80% B to 95% B), 4.1-6 min (95% B), 6-6.5 min (95% B to 20% B) and 6.5-8 min (20% B). The flow rate was 0.5 mL min^{-1}. Sample acquisition and analysis was performed with TraceFinder 3.3 (Thermo Scientific).

3.2.4 Cell Viability

Cell viability was evaluated according to the methodology described by Wang & Mazza (2002) with minor modifications. Murine RAW 264.7 macrophage cells (4×10^4 cells per well) were seeded onto 96-well plates and incubated overnight to allow cell attachment. The cultures were exposed to palmitoylethanolamide (PEA), quercetin (Q) and to the curcuminoids extract (C) (0.25 – 3 µg/mL), as well as to their combinations for 1h, followed by LPS (10 ng/mL) stimulation for a total of 9 and 24 hours. DMSO was used as a vehicle for all treatments, with maximum concentration of 0.2%. Hydrogen peroxide (H_2O_2) at 1 mM was used as positive control to demonstrate toxicity towards cells. Untreated wells were also included (control). Resazurin solution (200µL,

10%) was added to each well, and the plates were incubated at 37°C for 3 hours. The fluorescence intensity was analyzed using a CLARIOstar® Plus microplate reader (BMG Labtech Inc. Durhan, NC, USA) at 560 nm excitation and 590 nm emission. Relative cell viability was quantified with the fluorescence intensity in the control group considered as 100%.

3.2.5 Determination of Reactive Oxygen Species (ROS) and Nitric Oxide (NO) release

Cellular ROS levels were evaluated with the cell permeant probe DCFDA (2′,7′-Dichlorodihydrofluorescein diacetate). DCFDA is nonfluorescent until intercellular esterases remove the acetate groups and oxidation occurs within the cell (Myhre *et al.*, 2003). RAW264.7 cells (2.5 × 104/well) were seeded in flat bottom black 96-well plates and incubated overnight to allow attachment. Cells were pre-treated for 1h with P, Q or C alone or in combination, at 1 or 3 µg/mL. Following treatment of the cells for 18h, cell culture plates were loaded with 10 µM DCFDA for 30 min, washed once with phosphate buffer saline (PBS), and analyzed for ROS levels using CLARIOstar® Plus microplate reader (BMG Labtech Inc. Durhan, NC, USA) set at 480 nm excitation and 525 nm and emission wavelengths. Nitrite concentration was used as an indication of NO production. The procedure for NO determination was based on the Griess reaction, following manufacturer's instructions. Fifty microliters of culture supernatant or sodium nitrite standard (0 - 100 µM) was mixed with an equal volume of Griess reagent and allowed to

react at room temperature. After 15 minutes, the absorbance at 540 was measured using a CLARIOstar® Plus microplate reader (BMG Labtech Inc. Durhan, NC, USA).

3.2.6 Lipopolysaccharide (LPS) induced inflammatory assays

Murine RAW 264.7 macrophage cells (1.5×10^5 cells per well) were seeded in 12-well plates and incubated overnight to allow cell attachment. Cells were pre-treated with palmitoylethanolamide (PEA) (0.5 – 2.5 µg/mL) , quercetin (0.25 – 1.25 µg/mL) and curcuminoids extract (1.0 – 3.0 µg/mL) for 1 hour and then lipopolysaccharide (LPS), a major activating agent of macrophages, was added for a final concentration of 10 ng/mL for 8 h. For the combination experiments, two experimental designs were tested. In the non-fixed ratio design, PEA at 0.5 and 1.0 µg/mL was combined with quercetin or curcuminoids at 0.5, 0.75 and 1.0 µg/mL. In the fixed ratio design, several concentrations were tested at a fixed ratio. PEA and quercetin were combined at a 2:1 ratio, while PEA and curcuminoids were combined at a 1:2 ratio. The ratios were selected based on results obtained in the non-fixed ratio experiments. Negative (culture media only), positive (LPS) and vehicle controls (0.2% DMSO + LPS) were also included.

3.2.7 RNA extraction and quantitative real-time PCR analysis of mRNAs

Total RNA was isolated and purified using a RNeasy mini kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's protocol. mRNA quality and quantification were assessed with NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was performed with a iScript reverse transcription supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. RT-qPCR reactions were performed using iTaq Universal SYBR Green Supermix (Applied Biosystems, Foster City, CA, USA) and a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). mRNA expression of cyclooxygenase-2 (COX-2), interleukin-1 beta (IL-1b), tumor-necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10) and inducible nitric oxide synthase (iNOS) were analyzed using beta actin (ACTB) as a reference gene. Primer sequences are listed in Table 1. The levels of transcripts were calculated relatively to the control group by $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer sequences used for qRT-PCR

Target	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
ACTB	GATGTATGAAGGCTTTGGTC	TGTGCACTTTTATTGGTCTC
Il-1b	GGATGATGATGATAACCTGC	CATGGAGAATATCACTTGTTGG
Cox-2	ACTCATAGGAGAGACTATCAAG	GAGTGTGTTGAATTCAGAGG
iNOS	CATCAACCAGTATTATGGCTC	TTTCCTTTGTTACAGCTTCC
TNF-α	AGGCAGTCAGATCATCTTC	TTATCTCTCAGCTCCACG
IL-6	AAGAAATGATGGATGCTACC	GAGTTTCTGTATCTCTCTGAAG
IL-10	CAGGACTTTAAGGGTTACTTG	ATTTTCACAGGGGAGAAATC
NAAA	CTGGTGATGAACGAGATAAAG	AATAAACGTCAGCAATTGAGG
FAAH	CCCTACTTTGTGAGGATTTG	AACTTCTGTAGATCTCCTCC

3.2.8 Combination Index (CI), Isobologram and Dose Reduction Index (DRI)

To formally confirm whether PEA and each of the tested polyphenols interact synergistically, the combination index (CI) was calculated and an isobolographic analysis was performed using the Compusyn software 1.0 (Combosyn Inc., Paramus, NJ, USA), based on the Chou–Talalay method (Chou and Talalay, 1984). This method is based on the median effect principle of the mass action law, which provides the theoretical basis for the combination index (CI)-isobologram equation that allows quantitative determination of compound interactions (Chou and Talalay, 1977; Ting-Chao Chou, 2010). The median-effect equation (Chou, 1976, 1977) describes dose-effect relationships in simple terms, and it is described by:

$$f_a/f_u = \left(D/D_m\right)^m \quad (1)$$

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where D is the dose of a compound, f_a is the fraction affected by D (effect or percentage of inhibition) and f_u is the fraction unaffected by D ($f_u = 1 - f_a$). D_m is the median-effect dose that inhibits the system under study by 50% (i.e. IC_{50}) and m is the coefficient that determines the shape of the dose-effect relationship, where $m = 1, > 1$ and < 1 indicate hyperbolic, sigmoidal and flat-sigmoidal dose-effect, respectively.

Rearranging equation (1), it yields:

$$D = D_m \left[\left(f_a / (1 - f_a) \right) \right]^{1/m} \qquad (2)$$

The median-effect plot is determined plotting $x = \log(D)$ versus $y = \log(f_a/f_u)$ based on the logarithm form of equation (1) (Chou, 1976), which linearizes the dose-effect curves.

$$\log(f_a/f_u) = m\log(D) - m\log(D_m) \qquad (3)$$

In the median-effect plot, m is the slope and D_m is the antilog of the x-intercept, which is given by the following equation:

$$D_m = 10^{-(y-\text{intercept})/m} \qquad (4)$$

According to (Chou, 2006) the median-effect for a single compound can be extended to multiple compounds. Therefore, the median-effect equation to account for synergistic effects between the combination of two different compounds is:

$$\left(\frac{f_{a_{1,2}}}{f_{u_{1,2}}}\right)^{1/m} = \left(\frac{f_{a_1}}{f_{u_1}}\right)^{1/m} + \left(\frac{f_{a_2}}{f_{u_2}}\right)^{1/m} = \frac{D_1}{D_{m_1}} + \frac{D_2}{D_{m_2}} \quad (5)$$

where subscripts 1 and 2 indicate compounds 1 and 2.

When two compounds are combined and subjected to serial dilutions, the combined mixture of the two drugs behaves as the third drug for the dose-effect relationship. Thus, $y = \log[f_{a1,2}/f_{u1,2}]$ versus $x = \log[D_1 + D_2]$ will give $m_{1,2}$ and $D_{m1,2}$, values (Chou, 2006). Based on Equation (5), the Combination Index (CI) can be obtained by:

$$CI = \frac{D_1}{D_{x_1}} + \frac{D_2}{D_{x_2}} = \frac{D_1}{D_{m_1}\left[\left(f_a/(1-f_a)\right)\right]^{1/m_1}} + \frac{D_2}{D_{m_2}\left[\left(f_a/(1-f_a)\right)\right]^{1/m_2}} \quad (6)$$

where D_1 and D_2 are the IC_x of the combination between compounds 1 and 2, and $(D_x)_1$ and $(D_x)_2$ are the IC_x of each compound when used individually. CI < 1 indicates synergy; CI = 1 additive effect and CI > 1 antagonism (Chou, 2006).

Different experimental designs can provide different information about the nature of compound combinations. A fixed ratio experiment can provide a computer simulation of CI values at all effect levels and a classic isobologram, while a non-fixed ratio design only provides CI values for the actual tested concentrations and a dose-normalized isobologram. In this graph, the doses are normalized by the Dx to unity on both -x and -y axis. A non-fixed ratio design is extremely useful when screening for the optimal combination ratio for maximal synergy (Chou, 2006). Both experimental designs were tested in this study.

Data was derived from mRNA expression of the pro-inflammatory cytokine IL-1b. CI values for each combination were calculated using the Compusyn software. The Dose Reduction Index (DRI) represents how many folds the concentration of each compound in a synergistic combination may be reduced at a given effect level compared with the doses of each compound alone (Chou, 2006). DRI for each compound was also calculated by the CompuSyn software, using the following equation:

$$DRI = \frac{D_x}{D} \quad (7)$$

were Dx is the dose of the compound when used individually and D is the dose of the compound used in combination in order to get the same effect.

3.2.9 Multiplex bead assay

RAW264.7 cells were seeded in 6-well plates and incubated for 24 hours at 37°C. Cells were pre-treated with PEA, quercetin and curcuminoids extract (1.0 µg/mL alone or in combination) for 1h, then LPS (10 ng/mL) was added to induce inflammation (positive control) for 8h. Supernatants were collected for protein analysis. Protein expression assays were performed using a high sensitivity mouse cytokine/chemokine magnetic bead panel (MiliporeSigma, MA, USA) in 96-well plates. Cell culture supernatants (25 µL per well) were mixed with beads for TNF-α, IL-1b, IL-10 and IL-6 for 16 hours at 4 °C. Detection antibody was added to each well, and the plate was incubated for 1 hour at room temperature. Streptavidin– phycoerythrin was added, and the plate was incubated again for 30 minutes at room temperature. After successive plate washing steps, the beads were suspended in sheath fluid and analyzed with a Luminex 200 flow cytometer using Luminex xPONENT software (Luminex Corporation, Austin, TX, USA).

3.2.10 Statistical analysis

All experiments were performed with at least triplicates. Data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's, Sidak's or Tukey's posttest using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA), according to specific experiments. Data were considered significantly different when $p < 0.05$.

3.3 Results and Discussion

3.3.1 Tentative characterization of the curcuminoids extract

Collectively called curcuminoids, curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) are the three main curcuminoids present in turmeric. Curcuminoids are analogues and differ in methoxy substitution on the aromatic ring. Curcumin has two symmetric *o*-methoxy phenols linked through the α,β-unsaturated β-diketone moiety, BDMC, also symmetric, is deficient in two *o*-methoxy substitutions, and DMC has an asymmetric structure with one of the phenyl rings having *o*-methoxy substitution (Anand *et al.*, 2008). The curcuminoids extract used in this study consisted of a standardized 80% curcuminoids extract, containing a mixture of the three compounds and 20% exipients (maltodextrin and starch). The three main curcuminoids were detected and tentatively identified based on a comparison of their molecular weights, molecular formulas and MS/MS fragment ions with those published in the literature for curcuminoids from *Curcuma* species (Jiang *et al.*, 2006; Shen *et al.*, 2013; Verma *et al.*, 2013; Liu *et al.*, 2016). A curcuminoids extract solution was prepared at 50 µg/mL in methanol and analyzed. Chromatograms showed a retention time of 3.65, 3.71 and 3.76 minutes for bisdemethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin (CUR) respectively (Figure 5).
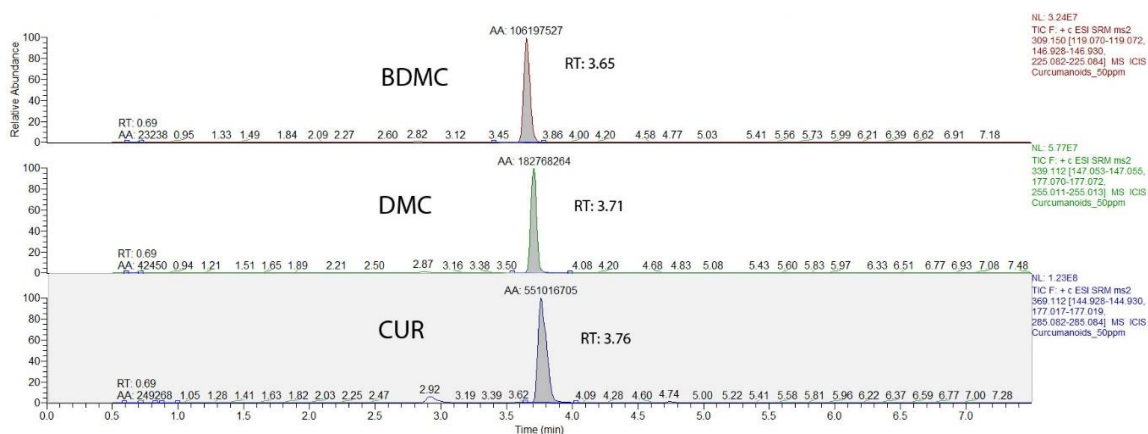
Figure 5. Extracted ion chromatograms for curcumin (CUR), demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) from a standardized curcuminoids extract.

MS-MS analysis of the curcuminoids extract revealed a mass spectra of major product ions of 369.112 [M+H]$^+$ to the product ions 285.08, 177.02 and 144.93 (Figure 6). The differences in the mass of the parent ion and its main fragment ions (m/z 285.08, 177.02) were 84.02, 192.09, which corresponded to a loss of $C_4H_4O_2$, $C_{11}H_{12}O_3$, with a furher breakdown of 177.02 to 144.93, with the loss of CH_4O (224.182 Da). Based on comparison with data from the literature, this compound was tentatively identified as curcumin. The mass spectra of the major product ions for what was identified as demethoxycurcumin was m/z 339.112 [M+H]$^+$ to product ions 255.01, 177.07 and 147.05, and for bisdemethoxycurcumin, 309.150 [M+H]$^+$ to product ions 225.08, 146.93 and 119.07. The relative proportions between CUR, DMC and BDMC in commercially available curcuminoid extracts is usualy around 75-77% CUR, 17-20% DMC, and 3-5% BDMC (Anand *et al.*, 2008; Angel-Morales *et al.*, 2012). Standardized curcuminoids extracts are frequently used as ingredients for dietary supplements, since DMC and BDMC also posess potent anti-inflammatory, antioxidant and anti-carcinogenic effects.

It is believed that presence of DMC and BDMC can enhance the biological effects of curcumin (Sandur *et al.*, 2007; Anand *et al.*, 2008).
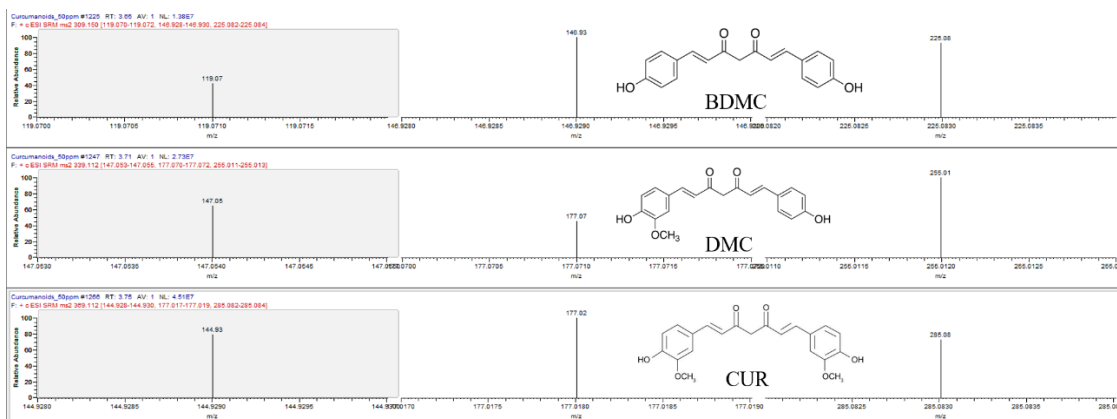
Figure 6. Product ion spectra of curcumin (CUR), demetoxycurcumin (DMC) and bisdemetoxycurcumin (BDMC) showing fragment ion transitions.

3.3.2 Effect of palmitoyletanolamide (PEA), quercetin and curcuminoids extract on cell viability of RAW264.7 macrophages

The effect of PEA, quercetin and curcuminoids extract on viability of RAW264.7 cells was investigated by the Resazurin assay. Viability was determined following pre incubation with different concentrations (0.25 – 3.0 µg/mL) of each compound for 1h followed by LPS stimulation (10 ng/mL) for 8h and 23 h. Cell viability of non LPS-stimulated cells was also investigated. Either in presence or absence of LPS, treatment with concentrations up to 2.5 µg/mL for PEA and quercetin and 3.0 µg/mL of curcuminoids extract did not reduce viability of cells after 9h or 24h incubation (Figure 7).
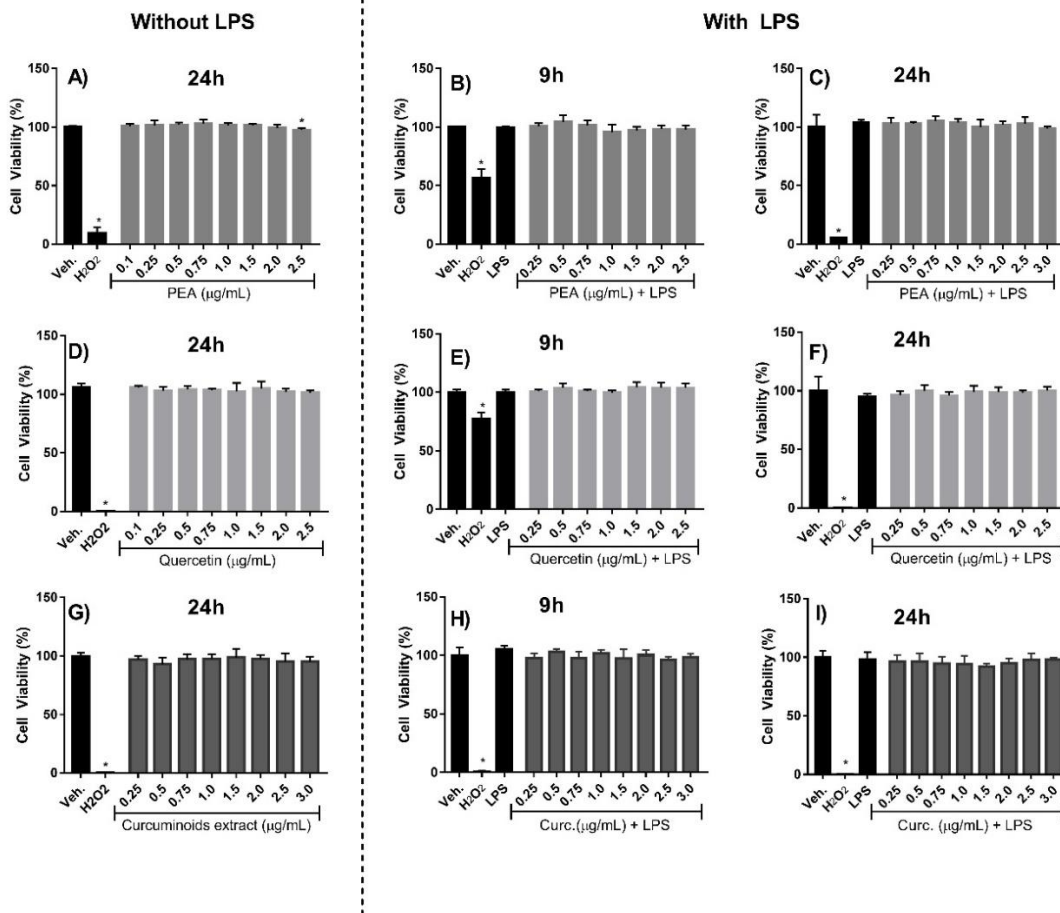
Figure 7. Cell viability of RAW264.7 cells treated for 9h or 24h with palmitoylethanolamide (PEA), quercetin, and curcuminoid extract, with and without LPS stimulation (10 ng/mL). * indicate significant difference from the Veh ($p < 0.05$; ANOVA-Dunnett). Values are means ± SD of at least three replicates

Given compounds alone did not elicit any citotoxicity, cell viability of PEA+QUER and P+CUR was tested to ensure combinations would also not exert any cytotoxic effect in the model used. Cell viability was not reduced after 9h and 24h incubation with combinations between PEA and quercetin and PEA and cucuminoids in presence of LPS at the concentrations tested (Figure 8). Theferore, these concentrations were selected to be used in the subsequent in vitro experiments.

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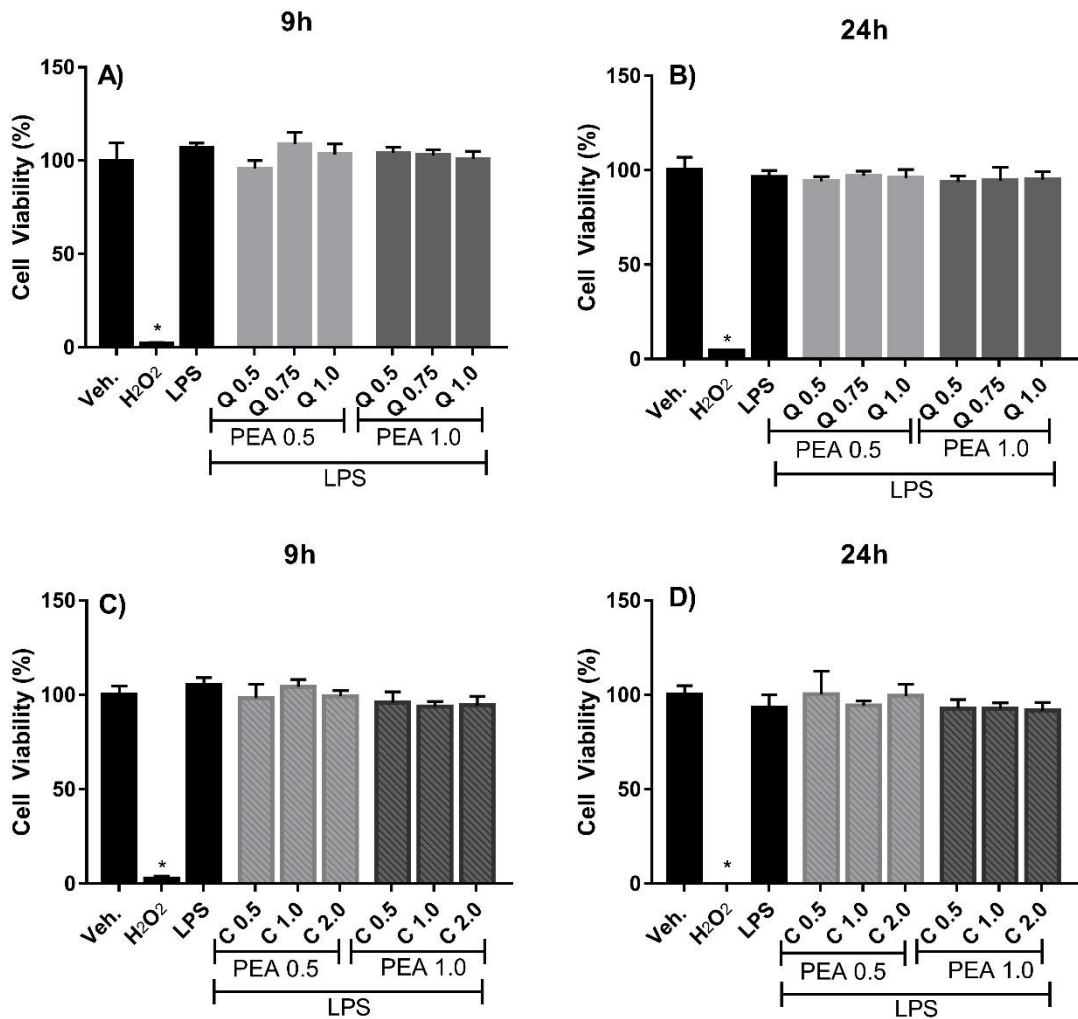
Figure 8. Cell viability of RAW264.7 cells pre-treated for 1h with compound combinations: palmitoylethanolamide (PEA) + quercetin (Q) (A and B), and palmitoylethanolamide (PEA) + curcuminoids extract (C) (C and D) followed by 23h of LPS stimulation (10 ng/mL). * indicate significant difference from Veh. ($p < 0.05$; ANOVA-Dunnett). Values are means ± SD of seven replicates.

3.3.3 The RAW264.7 murine macrophage cell line as a model for LPS-induced inflammatory response

Experiments aimed at optimizing LPS concentrations used in the RAW264.7 model and assessing their response to PEA treatment. LPS exerted a dose-dependent effect on macrophage activation, with 10 ng/mL being the lowest concentration to significantly increase the mRNA expression of Il-1b and Cox-2 (Figure 9A and B). Therefore, this concentration (10 ng/mL) was selected to be used in the following experiments, as it led to significant increase in the inflammatory response, similar to what is found in published literature for this cell line (Mosser *et al.*, 2008; Gabrielsson *et al.*, 2017). Macrophages play a significant role in immune reactions and are predominantly involved in the inflammatory response, representing a well-established model for studying inflammation (Murray and Wynn, 2011). Lipopolysaccharide (LPS) is a component of the cell wall of gram-negative bacteria, known to activate cellular signals in macrophages and other cell types (Guha and Mackman, 2001), leading to production of several inflammatory mediators. PEA at 2.0 µg/mL was able to significantly decrease the expression of the inflammatory cytokines Il-1b and Cox-2 after LPS-stimulation (10 ng/mL), indicating the effectiveness of the model and PEA treatment (Figure 9C and D).
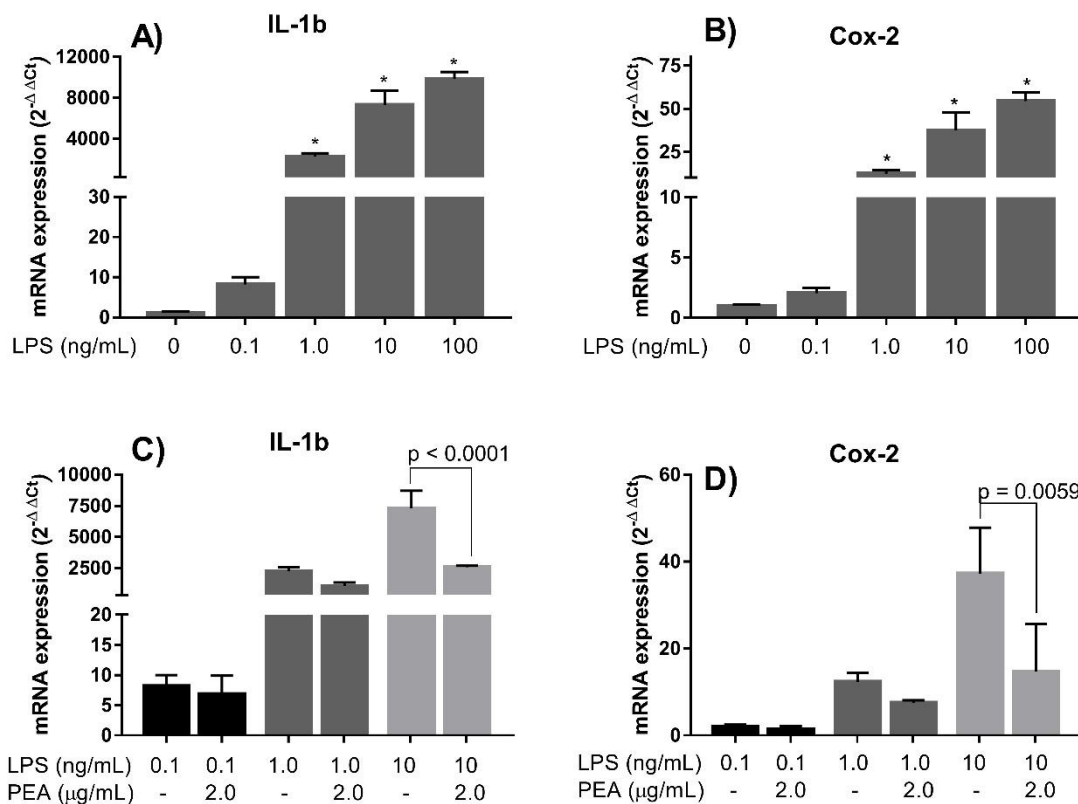
Figure 9. Effect of different LPS concentrations (ng/mL) (A and B) and anti-inflammatory activity of PEA (2.0 µg/mL) (C and D) in murine RAW264.7 macrophages. (* significantly different from negative control (no LPS) at $p < 0.05$, ANOVA-Dunnett's Test; indicated p values are significantly different from each other at $p < 0.05$, ANOVA-Sidak's Test). Data are means ± SD of three replicates.

Since PEA, quercetin and curcuminoids are not water-soluble, stock solutions for the treatments were prepared in DMSO. The effect of DMSO on expression of selected pro-inflammatory markers was tested in order to discard any possible influence of the solvent in further experiments. The presence of 0.2% DMSO did not exert any effect in the expression of IL-1b, Cox-2 and TNF-α when compared to the negative control (NC) (cell culture media) and did not prevent upregulation of gene expression by LPS (Figure

10). Therefore, DMSO concentration was kept at a maximal concentration of 0.2% and used as vehicle control (Veh.) in all experiments.
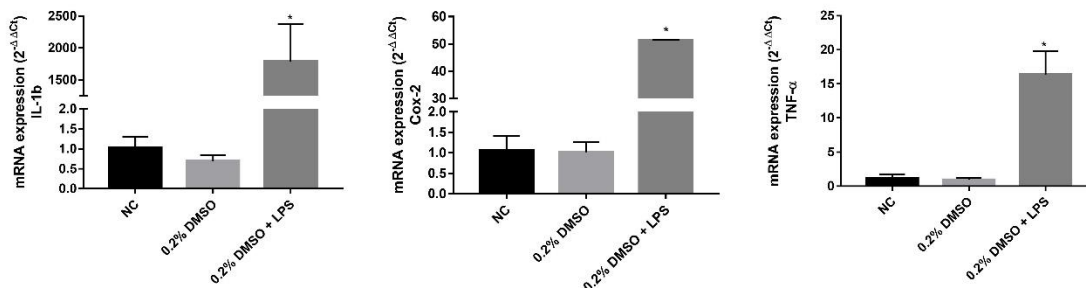
Figure 10. Effect of 0.2% DMSO treatment (used as vehicle) in mRNA expression ($2^{-\Delta\Delta Ct}$) of IL-1b, Cox-2 and TNF-α in RAW264.7 cells after LPS stimulation (10ng/mL) for 8 hours. NC = cell culture media only. * significantly different from NC at $p < 0.05$, ANOVA-Dunnett. Data are means ± SD of three replicates.

3.3.4 Effect of palmitoyletanolamide (PEA), quercetin and curcuminoids extract against LPS-induced reactive oxygen species (ROS) and nitric oxide (NO) generation

To optimize LPS concentration and incubation time for ROS and NO release experiments, RAW264.7 cells were incubated with LPS at 20 ng/mL or 100 ng/mL for up to 24h. ROS production started being statistically different from NC after 4h and 1h incubation when 20ng/mL and 100 ng/mL LPS was used, respectively (Figure 11). LPS at 100 ng/mL resulted in higher ROS production by cells. At 20 ng/mL LPS, peak ROS production was detected after 18h incubation. For both LPS concentrations, NO release also peaked at 18h. Therefore, LPS at 20 ng/mL and 18h incubation were selected to be used in subsequent experiments.

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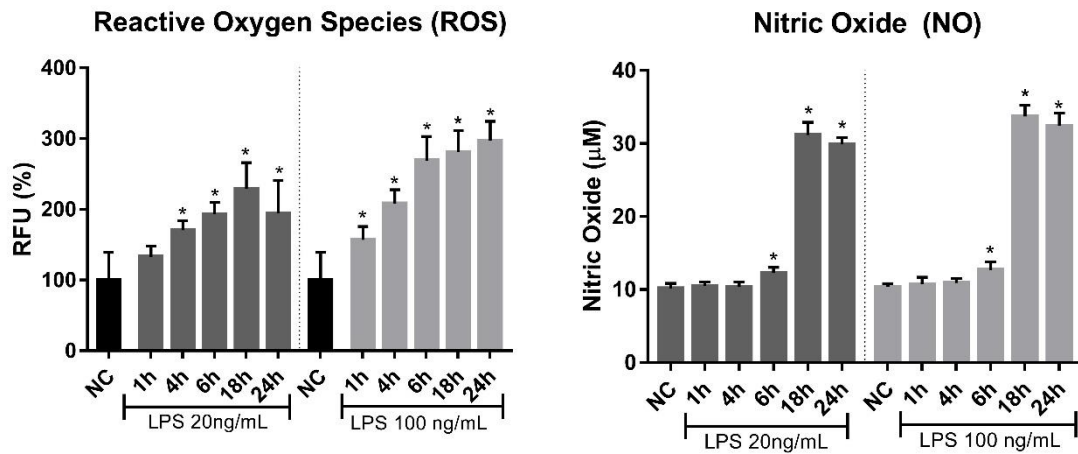
Figure 11. Effect of LPS concentration and incubation time on ROS generation and NO release by RAW264.7 cells. (*) indicate significance compared to NC at $p < 0.001$ (ANOVA- Dunnett); Values are means ± SD of seven replicates.

Pre-treatment with PEA, quercetin and curcuminoids extract alone at 1 µg/mL did not prevent the LPS-induced ROS production by RAW264.7 cells (Figure 12A and C). Combination of PEA and curcuminoids at 1 µg/mL each resulted in a 26% reduction in ROS compared to LPS. Interestingly, treatment with PEA or curcuminoids alone at 3 µg/mL reduced ROS to the same levels as treatment with their combination at 1 µg/mL each (around 23% reduction compared to LPS). Furthermore, the combination between PEA and quercetin at 3 µg/mL of each reduced 78% of ROS production compared to LPS, while PEA and quercetin alone at that same concentration reduced 46% and 21% respectively. When PEA was used in combination with quercetin, ROS production was 60% (2.46 times) lower than treatment with PEA alone. For PEA+CUR, ROS levels were 20% (1.24 times) lower than PEA alone. Overproduction of reactive molecular species derived from oxygen and nitrogen can change the cellular and tissue redox

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status, which can induce activity of pro-inflammatory enzymes and lead to alteration in cell signaling pathways. A primary example is the activation of the NF-κB pathway due to excessive ROS generation and oxidative stress (Forrester *et al.*, 2018).
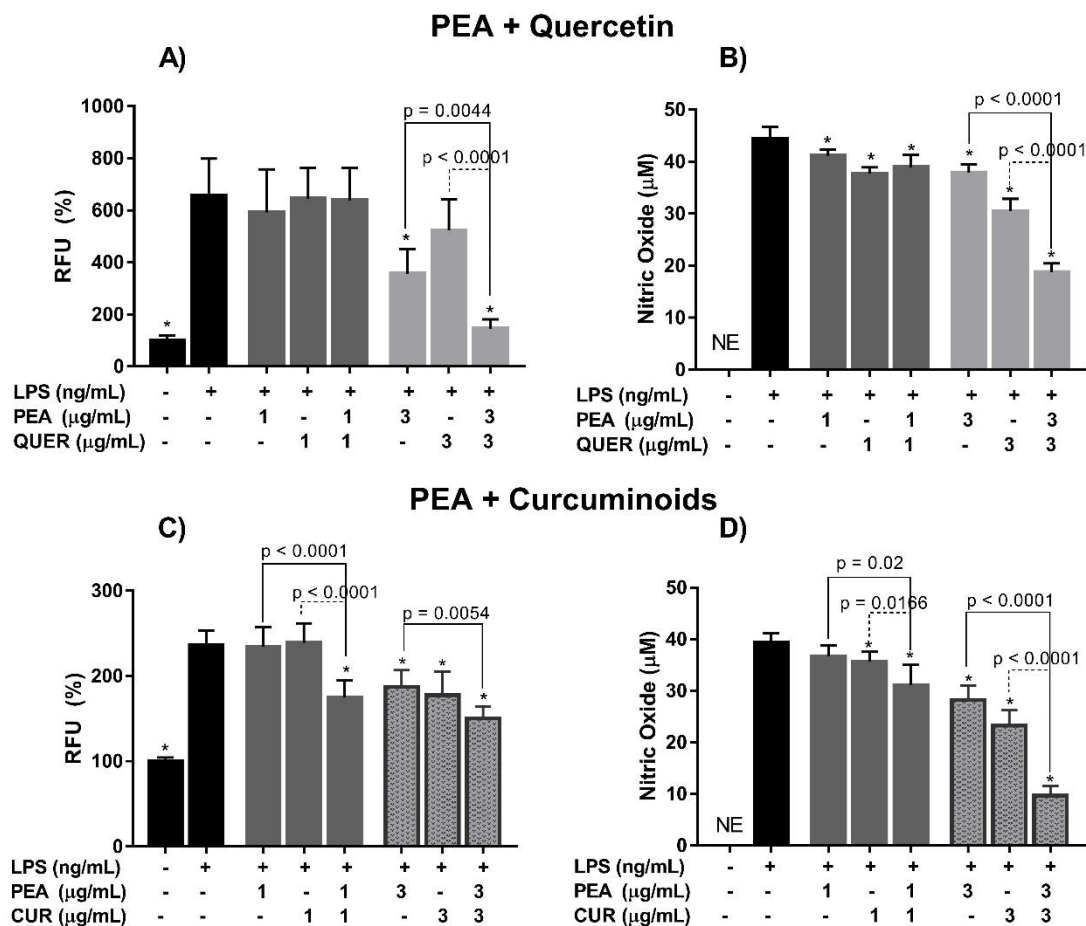
Figure 12. Effect of PEA, quercetin and curcuminoids on LPS-induced ROS generation (A and C) and Nitric Oxide (NO) (B and D) production in RAW264.7 cells. Cells were pretreated for 1h with each compound alone or in combination at either 1μg/mL or 3μg/mL, followed by 18h LPS stimulation (10 ng/mL). (*) indicate significance compared to LPS at $p < 0.0001$ (ANOVA- Dunnett); Indicated p values are significant by ANOVA-Sidak multiple comparison. Values are means ± SD of seven replicates.

NO release after 18h was mitigated by PEA and quercetin individually at 1 µg/mL, although effects were more pronounced at 3 µg/mL (Figure 12B). At 3 µg/mL, while PEA alone reduced 17% of NO compared to LPS, when combined with quercetin, NO release was reduced by 58%. Compared to treatment with quercetin alone, PEA+QUER was also more efficient in reducing NO ($p < 0.0001$). Similarly, curcuminoids extract at 3 µg/mL reduced NO by 41% compared to LPS, and when combined with PEA, NO release was reduced by 76%, demonstrating a potential synergistic effect between PEA and these polyphenols in mitigating NO release (Figure 12D).

When cells were treated with different concentrations of PEA+QUER or PEA+CUR, expression of iNOS was reduced in a dose dependent manner (Figure 13). This agrees with the observed reduction in NO release observed in cells treated with PEA+QUER and PEA+CUR combinations. Inducible nitric oxide synthase (iNOS) is an enzyme that converts L-arginine to NO when induced by bacterial products and inflammatory cytokines in macrophages and several other cells (Kim *et al.*, 2017). Under normal physiological conditions, NO has important role in cardiovascular system and as an inflammatory mediator. However, under abnormal situations it can be overexpressed and lead to further oxidative stress and tissue damage (Sharma and Parvathy, 2007). PEA lacks a direct antioxidant activity, and therefore was not expected to reduce ROS production in the cells. However, it does have the ability to reduce expression of iNOS and other proinflammatory markers related to the acute inflammatory response (Ross *et al.*, 2000). Hence, a possible explanation for PEA alone reducing ROS is that is does so

by modulating the inflammatory response towards a more normal physiological condition, resulting in lower ROS and NO levels.
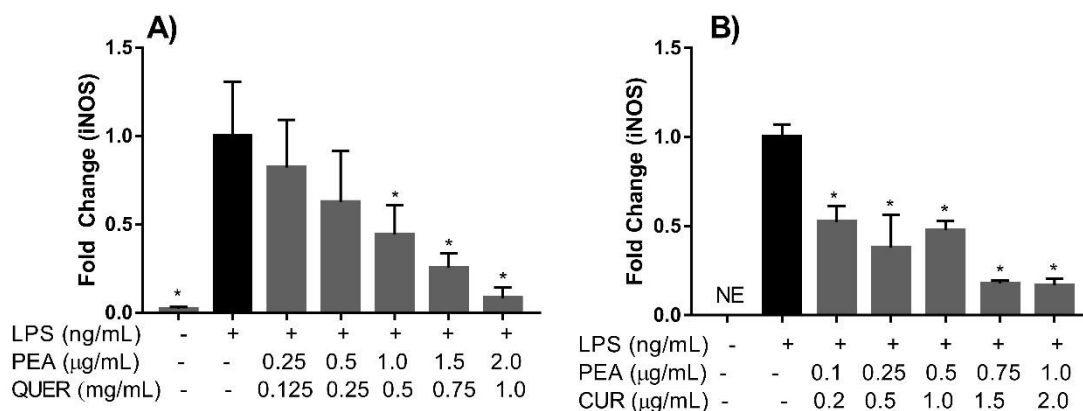
Figure 13. Effect of combined treatment of PEA and quercetin and curcuminoids in the mRNA expression of iNOS in RAW264.7 cells pre-treated with compounds for 1h followed by 8h LPS stimulation (10 ng/mL). (*) indicate significance compared to LPS at $p < 0.05$ (ANOVA- Dunnett).

Polyphenols are able to protect against oxidative damage through various mechanisms, including direct ROS scavenging properties and reducing the catalytic activity of enzymes involved in ROS generation (Hussain *et al.*, 2016). In RAW264.7 cells, quercetin is reported to reduce ROS production, iNOS expression and consequently NO release (Kim *et al.*, 2017; Cui, 2019). Similarly, curcuminoids are able to significantly decrease ROS levels and attenuate oxidative stress by increasing activity of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH- PX) (Lin *et al.*, 2019). Although there are no studies on the association between PEA and quercetin/curcuminoids, a micronized composite including PEA and luteolin is reported to reduce inducible nitric oxide synthase (iNOS) expression

and NO release in spinal cord slice cultures from mouse (Paterniti *et al.*, 2013).

Interestingly, in that same study, PEA and luteolin alone did not elicit the same effect.

Taken together, these results demonstrate that the association between PEA and

polyphenols exerting antioxidant effects may provide a multi-target approach to

mitigating oxidative stress.

3.3.5 Anti-inflammatory activity of palmitoyletanolamide (PEA), quercetin and curcuminoids extract in LPS-stimulated macrophages

While the acute inflammatory response lasts short periods of time and is essential

for health maintenance and restoring homeostasis, chronic unresolved inflammation

persists for longer periods and it is characterized by sustained production and release of

inflammatory mediators in the body. This chronic, low-grade inflammation is believed to

play critical role in the induction and maintenance of inflammatory chronic diseases

(Okin & Medzhitov, 2012). Tumor necrosis factor alpha (TNF-α), inducible nitric oxide

synthase (iNOS), interleukins-1, 6, 8 and 12 (IL-1, IL-6, IL-8, and IL-12), and

chemokines resulting from activation of the NF-kB pathway, as well as leukotrienes and

prostaglandins are examples of these mediators (Dunster, 2016; Liu *et al.*, 2017).

Macrophages modulate the inflammatory response at all stages. Once activated, they

induce the expression of the mentioned pro-inflammatory factors, representing one of

the main in vitro models for cell culture studies (Dunster, 2016). To ensure that the

normal physiological inflammatory response is adequately resolved and a chronic

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inflammatory state is not established, a balance needs to be maintained between the activation and down-regulation of relevant biomarkers (Okin and Medzhitov, 2012).

The mRNA expression of inflammatory cytokines was significantly up-regulated upon LPS stimulation (Figure 14), and treatment with PEA 1 µg/mL alone reduced expression of IL-1b and iNOS, by 27% and 36%, respectively, while increasing IL-10 by 45% compared to LPS. For Cox-2, PEA alone at 1 µg/mL also reduced its expression by 28%, although it did not reach statistical significance. Quercetin downregulated IL-1b, iNOS and IL-10, by 69%, 49% and 53% respectively, while curcuminoids at that same concentration had a lowering effect in Il-1b levels (60% reduction).
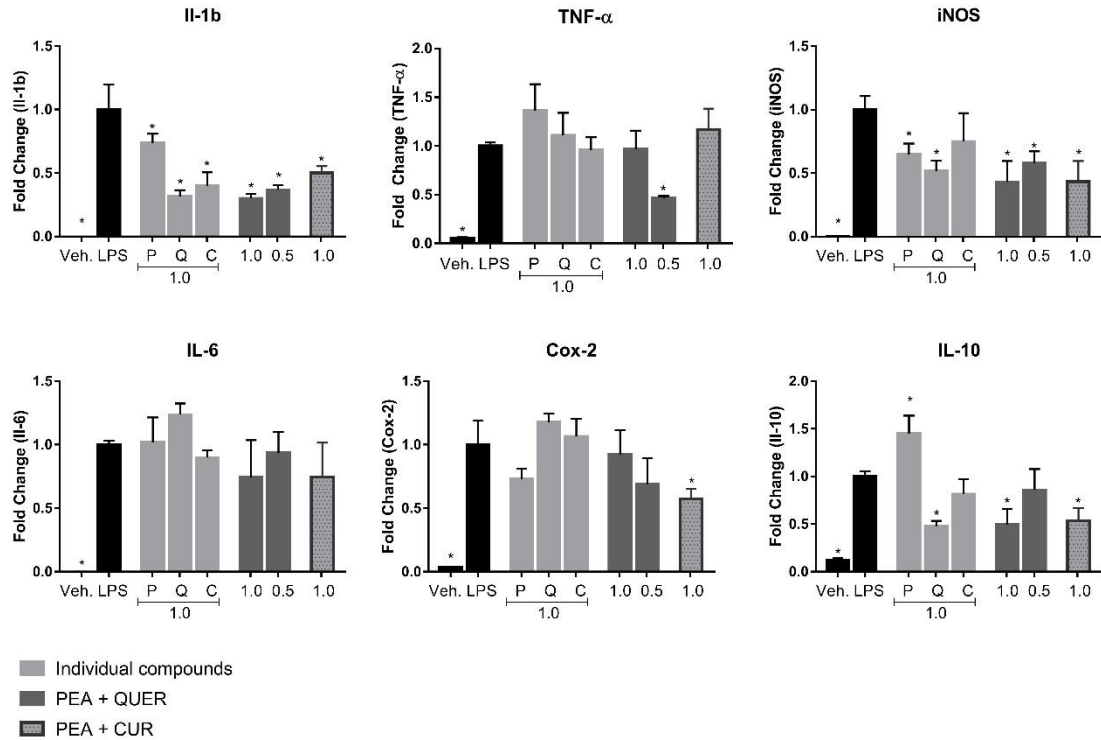
Figure 14. Effect of PEA, quercetin and curcuminoids extract alone and in combination in the mRNA expression of pro-inflammatory biomarkers Il-1b, TNF-α, iNOS, IL-6, Cox-2 and IL-10 in LPS-stimulated RAW264.7 macrophages. * indicate statistical difference from LPS ($p < 0.05$; ANOVA-Dunnett). Data are means ± SD of three replicates.

PEA and QUER alone did not reduce expression of TNF-α, although upon association (0.5 µg/mL each), downregulation of TNF-α became significant (53% reduction compared to LPS) (Figure 14). Combination of PEA with QUER and CUR slightly reduced expression of iNOS compared to compounds alone, however, the effect was not statistically significant. Although treatment with compounds alone or association with quercetin had no effects in Cox-2, association with curcuminoids reduced its expression by 43%. There was no effect of compounds alone or in combination in expression of IL-6.

At the protein level, PEA alone slightly reduced expression of IL-1b, not reaching statistical significance (Figure 15A). The same was observed for TNF-α, although for this marker, reduction was more evident (49% reduction compared to positive control) (Figure 15B). The high standard deviation in the positive control samples may be responsible for the lack of significance. Quercetin alone significantly reduced IL-1b, TNF-α and IL-10 levels. Upon combination, PEA+QUER at 1.0 µg/mL of each compound was more effective in reducing IL-1b expression compared to PEA alone (48% versus 12%) (Figure 15A). PEA+Q at both concentrations tested (0.5 and 1.0 µg/mL of each compound) significantly decreased TNF-α compared to LPS and to PEA alone, resulting in levels that were not different from unstimulated cells (Figure 15B). IL-10 levels were reduced by 58% by QUER alone, and effect was not different when PEA+QUER at 1.0 µg/mL was used (Figure 15C). Similar to what was observed at the mRNA levels, there was no effect of treatments on IL-6 levels (Figure 15D).
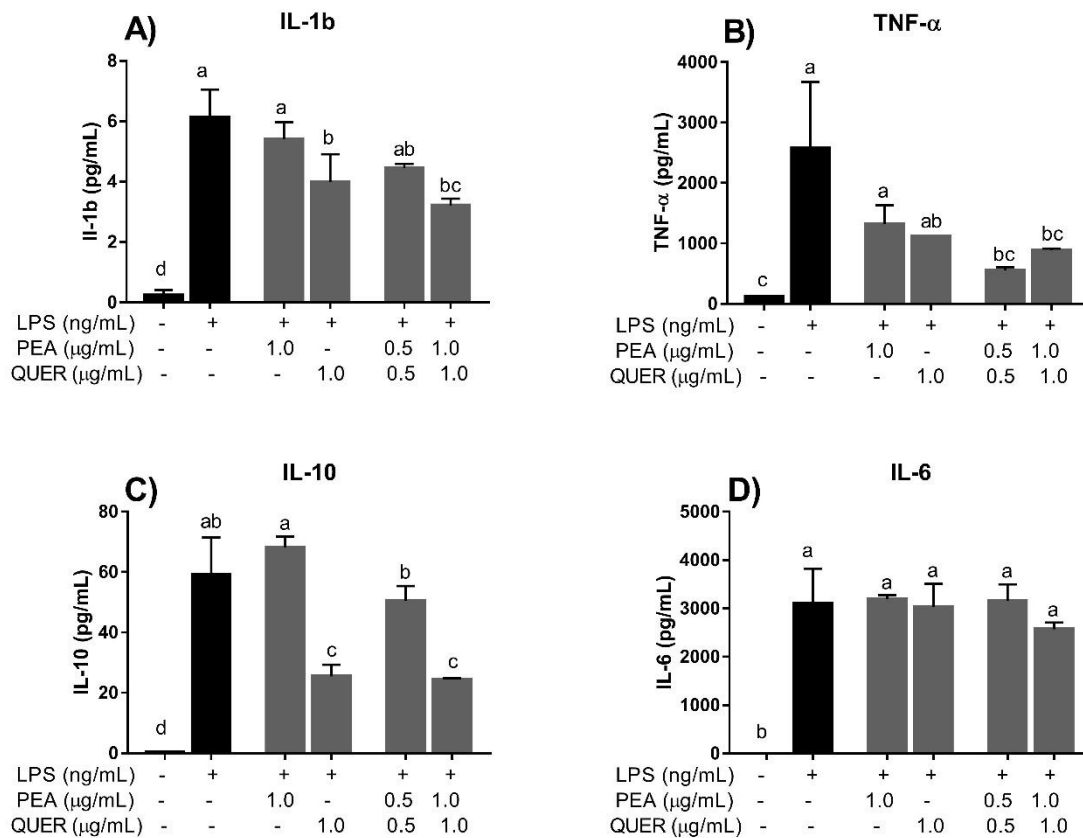
Figure 15. Effect of PEA and quercetin alone and in combination on the protein levels of pro-inflammatory biomarkers Il-1b, IL-6, IL-10 and TNF-α in RAW264.7 macrophages pre-treated for 1h followed by LPS-stimulation (10 ng/mL) for 8h. Different letters indicate significant differences between groups ($p < 0.05$; ANOVA-Tukey). Data are means ± SD of three replicates.

Reduction of IL-1b protein levels was superior upon combined treatment with PEA+CUR (43% reduction) compared to the reduction observed when cells were treated with compounds alone (12% for PEA and Q alone) (Figure 16A). The difference at the protein level was more evident than what was observed at the mRNA levels. On the other hand, TNF-α protein levels were significantly lower when PEA or curcuminoids were used alone compared to the combined treatment (Figure 16B). This is also in

agreement with the observed at the mRNA levels for this treatment. Curcuminoids alone or combined with PEA at 1 µg/mL had the same effect on IL-10, reducing its expression by around 23% (Figure 16C). As observed at the mRNA levels, there was no effect of compounds alone or in combination on the protein levels of IL-6 (Figure 16D).
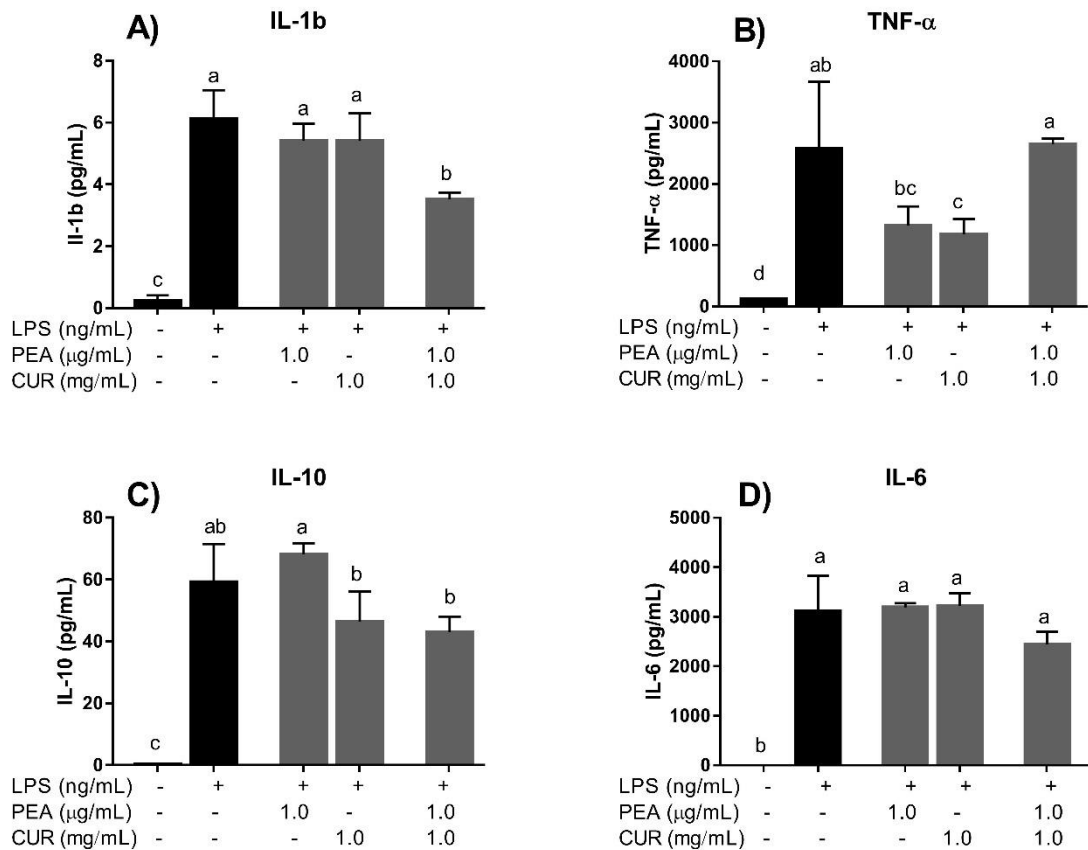
Figure 16. Effect of PEA and curcuminoids extract alone and in combination in the protein levels of pro-inflammatory biomarkers Il-1b, IL-6, IL-10 and TNF-α, in RAW264.7 macrophages pre-treated for 1h followed by LPS-stimulation (10 ng/mL) for 8h. Different letters indicate significant differences between groups ($p < 0.05$; ANOVA-Tukey). Data are means ± SD of three replicates.

These results indicate an overall ability of compounds alone to mitigate the expression of pro-inflammatory cytokines in activated macrophages and an enhancement of the effect upon combination. The effect of anti-inflammatory compounds in the expression of pro-inflammatory cytokines can vary greatly and modulation might occur in some markers, while others remain unchanged or increased. (Alhouayek *et al.*, 2017), for example, observed different effects of PEA treatment on inflammatory biomarkers in J774 macrophages. Treatment with PEA at 10 µM led to downregulation of COX-2, IL-1b and IL-6, and increased iNOS expression. In the present study, no effect was observed in IL-6 and Cox-2, while both studies agree on the potent effect of PEA in reducing IL-1b expression. In a study evaluating the effects of quercetin in RAW264.7, Tang *et al.*, (2019) reported downregulation on TNF-α and IL-1b. Similarly, curcuminoids inhibited LPS-induced cytokines TNF-a and IL-6 in mouse macrophages (Ma *et al.*, 2017).

Polyphenols in general do not have a specific receptor binding activity and can exert anti-inflammatory activities via multiple mechanisms (Hussain *et al.*, 2016). Curcuminoids' pleiotropic effects originates from its ability to modulate numerous signaling molecules such as pro-inflammatory cytokines, apoptotic proteins, NF-kB, cyclooxygenase-2, activation of PPAR-γ, among others (Jacob *et al.*, 2007; Hassanzadeh *et al.*, 2020). PEA is also a multitarget compound, with ability to indirectly reduce inflammation via entourage effect, indirect activation of TRPV1 channels and GPR55 (Iannotti *et al.*, 2016). The most well studied and elucidated mechanism is direct PPAR-α activation (Lo Verme *et al.*, 2005). By binding to PPAR-α, PEA allows formation of

heterodimers with retinoic acid receptors (RXR), which act as a transcription factor that will result in downregulation of nuclear-factor kappa B (NF-kB) (Daynes and Jones, 2002) and inhibition of a cascade of macrophage proinflammatory cytokines (Clark, 2002). The ability of these compounds to mitigate inflammation via different mechanisms make them an interesting option as ingredients in multicomponent dietary supplement formulations aiming to mitigate chronic inflammation.

3.3.6 Concentration-response curves for the effect of PEA, quercetin and curcuminoids on mRNA expression of selected pro-inflammatory biomarkers

Since treatment with PEA and selected polyphenols were able to consistently modulate Il-1b at both the mRNA and protein levels, and considering the important role this cytokine has in sustaining the chronic inflammatory response (Ren and Torres, 2009), IL-1b was selected to be further investigated as a potential target to PEA-polyphenol combinations. Concentration-response curves for the effect of PEA, quercetin and curcuminoids in the mRNA expression of IL-1b were generated based on treatment of cells with each compound alone. PEA alone was significantly effective at downregulating IL-1b expression and at concentrations as low as 0.5 µg/mL, expression was reduced by 28% (Figure 17A). For this reason, this concentration, as well as 1 µg/mL were consistently used in several of the following experiments.

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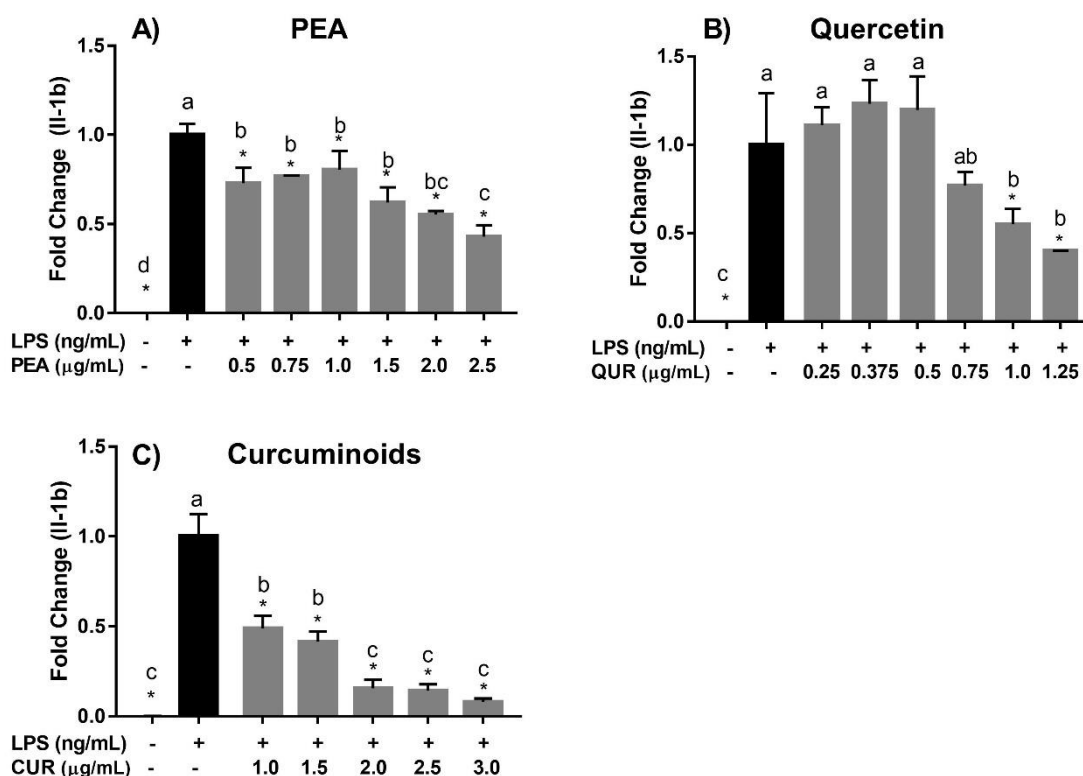
Figure 17. mRNA expression of Il-1b in RAW264.7 murine macrophages pre-treated with PEA (A), quercetin (B) and curcuminoids extract (C) for 1h followed by LPS stimulation (10 ng/mL) for 8 hours. (*) significantly different from LPS at $p < 0.05$; ANOVA-Dunnett's Test; Different letters represent significant difference by ANOVA-Tukey). Data are means $\pm$ SD of three replicates.

Quercetin and curcuminoids extract decreased IL-1b expression in a dose-dependent manner, starting at 1.0 µg/mL (Figure 17B and C). At this concentration, QUER and CUR reduced 45% and 57% of Il-1b mRNA expression, respectively. The calculated IC_{50} for the three compounds were 2.55 µg/mL for PEA, 1.08 µg/mL for quercetin and 1.07 µg/mL for curcuminoids extract. In a study using J774 macrophages, PEA at 10 µM (~ 3 µg/mL) reduced around 30% of IL-1b mRNA expression (Alhouayek *et al.*, 2017). The effect of PEA in downregulating protein levels of Il-1b

was also demonstrated in an in vivo colon inflammation model in mice (Alhouayek *et al.*, 2015). Results demonstrated substantial effect of all tested compounds against Il-1b mRNA expression. This mediator is a highly active pro-inflammatory cytokine secreted by monocytes, macrophages and dendritic cells, that lowers pain thresholds and damages tissues (Dinarello *et al.*, 2012). Overproduction of Il-1b is involved in the pathophysiological changes during several chronic diseases such as inflammatory bowel disease, rheumatoid arthritis (RA), osteoarthritis, chronic asthma, and atherosclerosis (Braddock and Quinn, 2004). Thus, identifying compounds able to markedly reduce IL-1b and ultimately natural compounds in combined formulations able to target IL-1b is of great interest in the context of chronic inflammation and pain.

Effect of increasing concentrations of PEA on mRNA expression of Cox-2 and TNF-α was also tested and it is shown in Figure 18A. PEA treatment had no effect in mRNA expression of Cox-2, while TNF-α was mildly downregulated by PEA at 0.5, 0.75 and 2 µg/mL (Figure 18B). In a study with J774 murine macrophages, PEA at 3 µg/mL was able to reduce COX-2 mRNA expression (Alhouayek *et al.*, 2017). On the other hand and in line with results obtained herein, Gabrielsson et al., (2017) did not observe significant effect of PEA treatment (3 µg/mL) on gene expression or protein levels of Cox-2 in LPS+IFN-γ stimulated RAW264.7 macrophages. Based on individual anti-inflammatory effects observed for these compounds, particularly against IL-1b, and lack of studies investigating the effects of combined formulations containing PEA-QUER and PEA-CUR, the interactions were tested in RAW264.7 cells to determine a potential synergistic relationship between these compounds.
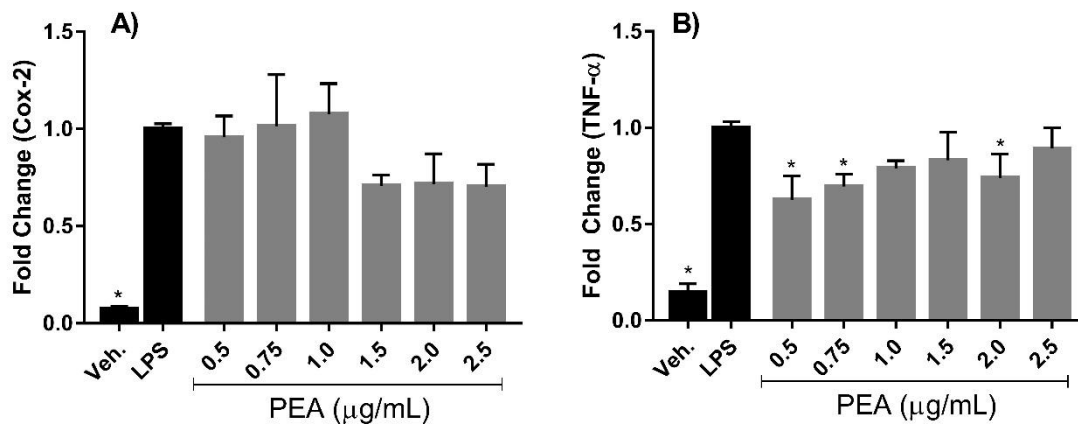
Figure 18. mRNA expression of Cox-2 and TNF-α in RAW264.7 murine macrophages pre-treated with with PEA for 1h followed by LPS stimulation (10 ng/mL) for 8 hours. (*) significantly different from LPS at $p < 0.05$; ANOVA-Dunnett's Test). Data are means ± SD.

3.3.7 Effect of the combination between palmitoylethanolamide (PEA), quercetin and curcuminoids extract on mRNA expression of IL-1b and Cox-2

Given the significant anti-inflammatory effects of individual compounds, PEA was tested in combination with quercetin and curcuminoids extract to investigate a potential synergistic interaction. In the non-fixed ratio experimental design, fixed PEA concentrations (0.5 and/or 1.0 µg/mL) were combined with different QUER and CUR concentrations. Combinations were more potent in downregulating LPS-induced IL-1b and Cox-2 expression than compounds alone (Figure 19), with effects in IL-1b being more pronounced than in Cox-2. As previously mentioned, PEA and QUER were able individually reduce Il-1b mRNA expression in RAW264.7 cells at concentrations of 0.5

µg/mL and 1.0 µg/mL, respectively. When combined at 0.5 µg/mL of each, mRNA

expression of IL-1b was significantly reduced by 70% and Cox-2 by 27% compared to

LPS (Figure 19A and B). Furthermore, combination with quercetin starting at 0.5 µg/mL

PEA + 0.75 µg/mL QUER resulted in significantly lower levels of Cox-2 compared to

LPS, as opposed to PEA alone, which did not reduce Cox-2 at concentrations up to 2.5

µg/mL. Similar trend was observed for the PEA+CUR association at 0.5 µg/mL each,

which reduced Cox-2 expression by 30% compared to LPS (Figure 19D).
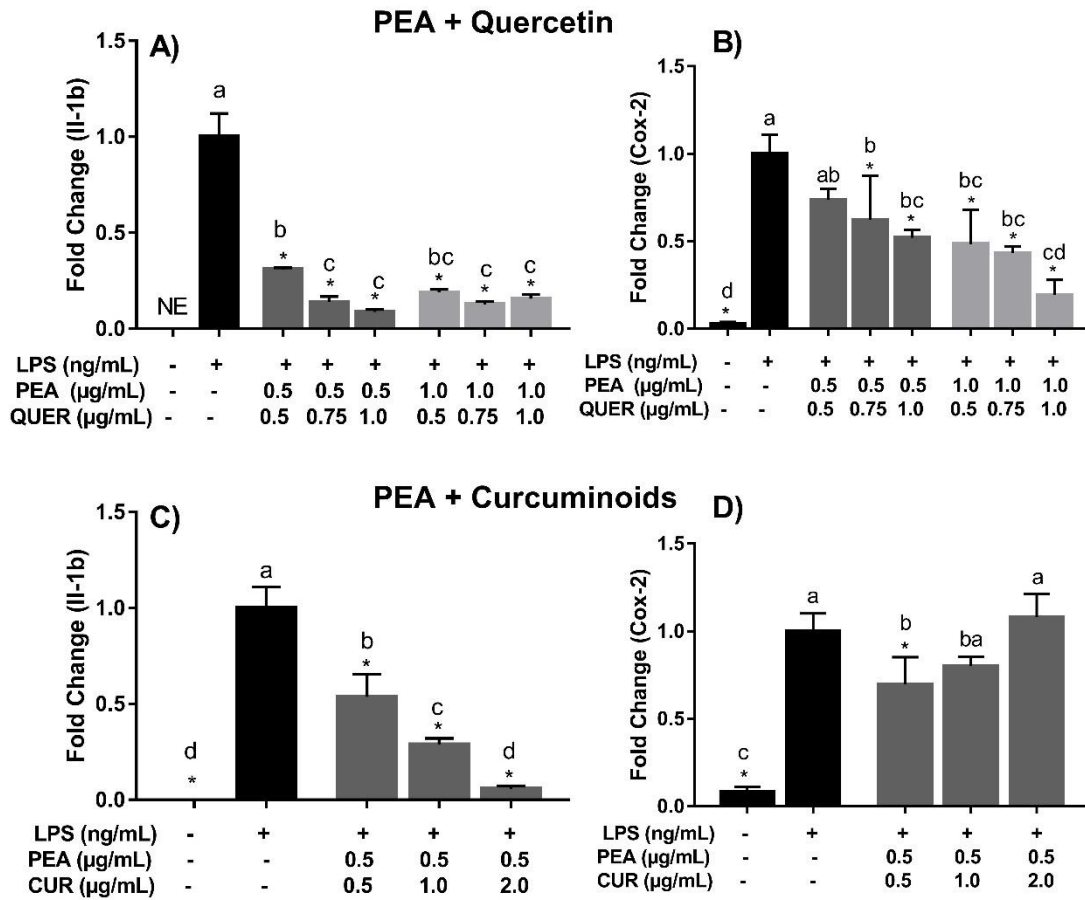
Figure 19. mRNA expression of LPS-activated (10ng/mL) murine macrophages (RAW264.7) treated with different combinations of PEA-QUER (A and B) and PEA-CUR (C and D) at different ratios. PEA was fixed at 0.5 or 1.0 µg/mL and combined with increasing concentrations of quercetin or curcuminoids extract. (* significantly different from LPS at $p < 0.05$; ANOVA-Dunnett's Test; Different letters represent significant difference by ANOVA-Tukey). Data are means ± SD of three replicates.

Combination between PEA and CUR also downregulated Il-1b at the lowest concentration tested (0.5 µg/mL of each compound) (Figure 19C), with a 46% reduction at that concentration and even superior effects at higher concentrations. When looking at the effects of compounds used individually or in combination, it is observed that the lowest concentration to significantly reduce the expression of IL-1b was 0.5 µg/mL for

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PEA, 0.75 µg/mL for QUER and 1.0 µg/mL for CUR. When compounds were used in combination, at the lowest concentration tested (0.5 µg/mL of each compound), a reduction of 70% was observed for PEA+QUER and of 47% for PEA+CUR. This emphasizes the potential synergistic interaction between these compounds against inflammation and more specifically, at reducing IL-1b expression.

The same compounds were tested in a fixed ratio experimental design setting, to allow deeper investigation about synergistic effects. Reduction in mRNA expression in the fixed ratio experimental design was still observed, although effects were not as strong as the observed for the non-fixed ratio. IL-1b expression was reduced by PEA+QUER starting at 0.5 µg/mL PEA + 0.25 µg/mL QUER, and maximal reduction (83%) was observed at highest concentration tested (2.0 µg/mL PEA + 1.0 µg/mL QUER) (Figure 20A). A 46% reduction in Cox-2 expression was observed upon treatment with PEA+QUER at 2.0 µg/mL PEA + 1.0 µg/mL QUER, while lower concentrations did not exert statistically significant effects (Figure 20B). In the non-fixed ratio experiment, a similar reduction (48%) in this marker was observed at lower dose (0.5 µg/mL PEA + 1.0 µg/mL QUER). Treatments at a fixed ratio between PEA and CUR also led to reduction in IL-1b expression (Figure 20C). At 0.25 µg/mL PEA + 0.5 µg/mL CUR, mRNA expression was reduced by 41%, although it did not reach statistical significance. High standard deviation in the positive control samples (LPS) may be leading to the lack of statistical significance, whereas as a 41% reduction can be considered a notable reduction. Similar to results in the non-fixed ratio experiment, effect of combined compounds in Cox-2 expression was less evident than in IL-1b. Cox-

2 levels were reduced by 74% only by treatment with 0.75 µg/mL PEA + 1.5 µg/mL CUR (Figure 20D).

PEA + Quercetin

PEA + Curcuminoids
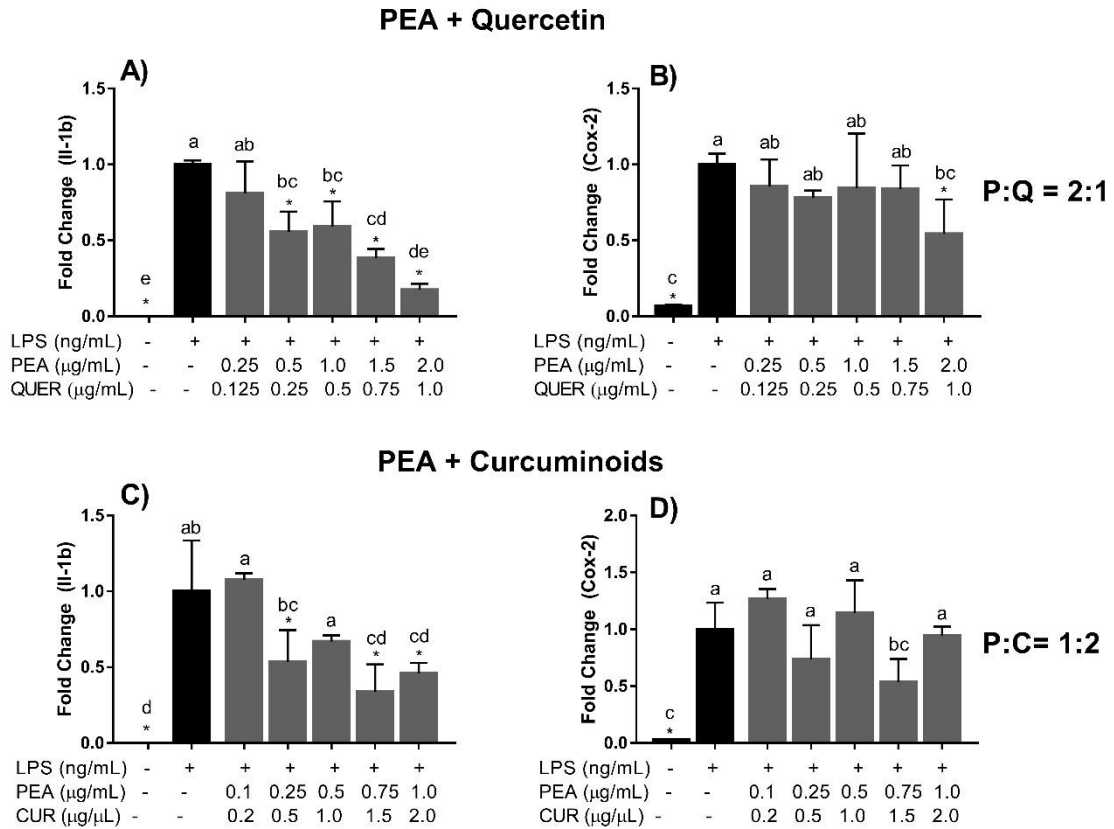
Figure 20. mRNA expression of LPS-activated (10ng/mL) murine macrophages (RAW264.7) treated with different combinations of PEA-QUER (A and B) and PEA-CUR (C and D) at fixed ratios. (P:QUER = 2:1; P:CUR = 1:2) (* significantly different from LPS at $p < 0.05$; ANOVA-Dunnett's Test; Different letters represent significant difference by ANOVA-Tukey). Data are means ± SD of three replicates.

The enhanced effect of combined plant extracts and purified polyphenols on inflammatory markers has been investigated in several studies (Mertens-Talcott and Percival, 2005; Agah *et al.*, 2017; Güran *et al.*, 2019). However, the effect of PEA and

polyphenols association has been investigated mainly with polydatin (Esposito *et al.*, 2016; Cremon *et al.*, 2017) and luteolin (Crupi *et al.*, 2016; Parrella *et al.*, 2016; Cordaro *et al.*, 2017) in disease specific models. A single report is available on the use of PEA and quercetin in a rat model of induced osteoarthritis, reporting valuable effect of PEA-Q treatment in reducing serum levels of TNF-α and IL-1b (Britti *et al.*, 2017). In both experimental settings (non-fixed and fixed ratios), our results suggest that PEA-QUER and PEA-CUR associations may be synergistically interacting in the reduction of IL-1b mRNA expression, contributing to enhanced anti-inflammatory action.

The variation in the magnitude of effects of combined treatment with PEA, QUER and CUR in RAW264.7 cells from different batches is believed to be attributable to differences in cell batches such as cell passage number and metabolic state, although a short range of passage numbers were used in these studies (from 5th to 15th). This highlights the importance of testing different cell batches and cautiously interpreting results, always taking into consideration the variation that might occur between cell batches. In this study, although the magnitude of effects in one setting was superior than the other, the overall observation remained unchanged: combined compound treatment reduced expression of IL-1b in RAW264.7 macrophages in what appears to be an enhanced manner compared to single compound treatment. To confirm this hypothesis, the nature of the interaction as additive, synergistic or antagonistic between compounds was tested in the following section.

3.3.8 Synergistic interaction between PEA and selected polyphenols

Based on the individual concentration-response curves and the promising results from combination experiments in downregulating IL-1b mRNA, we hypothesized that the nature of the interaction between PEA and each of these phenolic compounds could be synergistic. In order to prove the suggested synergism, Il-1b mRNA data from individual compounds and their respective combinations were loaded into the CompuSyn software (Combosyn Inc., Paramus, NJ, USA). The Combination Index (CI) was calculated and the respective isobologram for each combination was generated. A CI < 1 indicates that the concentrations producing a given effect in combination are lower than the expected concentrations from additivity and can therefore be interpreted as synergy (Chou and Talalay, 1984).

- **Non-fixed ratio experimental design**

The combination index (CI) for all tested combinations was lower than 1, indicating synergistic interaction between compounds. The smallest CI and therefore strongest synergy for PEA+QUER was observed at PEA 1.0 + QUER 0.5 µg/mL, while for PEA+CUR, the best combination was PEA 0.5 + CUR 2.0 µg/mL (Table 2).

Table 2. Combination Index (CI) for association between PEA and quercetin and curcuminoids extract in the non-fixed ratio experimental design

PEA + Quercetin		PEA + Curcumin	
Concentrations (µg/mL)	CI	Concentrations (µg/mL)	CI
PEA 0.5 + QUER 0.5	0.43406	PEA 0.5 + CUR 0.5	0.72958
PEA 0.5 + QUER 0.75	0.41116	PEA 0.5 + CUR 1.0	0.69643
PEA 0.5 + QUER 1.0	0.45195	PEA 0.5 + CUR 2.0	0.55813
PEA 1.0 + QUER 0.5	0.36346	-	-
PEA 1.0 + QUER 0.75	0.41885	-	-
PEA 1.0 + QUER 1.0	0.59522	-	-

For the non-fixed ratio experimental design, the normalized isobologram was generated by normalizing the dose of each compound in combination by the Dx of individual compounds and placing it in both x- and y-axis. The isobologram also indicated that all combinations between PEA and QUER were synergistic, as all treatments fell under the additivity line (Figure 21). Similarly, isobolographic analysis for PEA+CUR demonstrated synergistic effect for all the tested combinations (Figure 21).
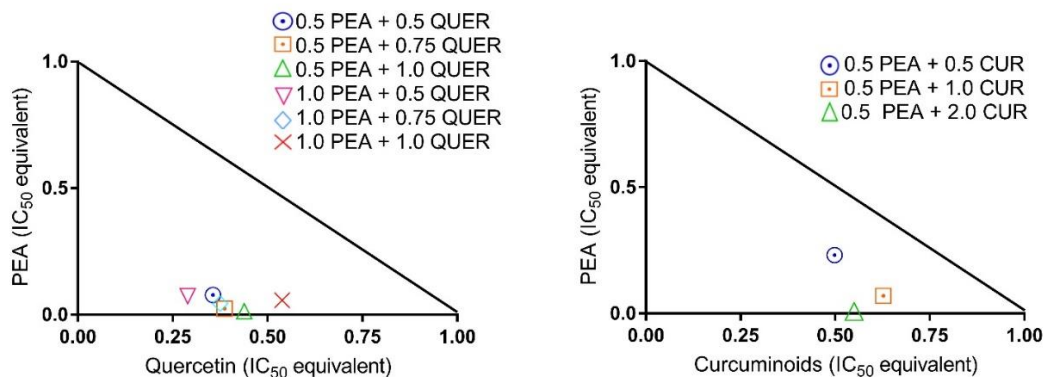
Figure 21. Normalized isobolographic analysis based on Il-1b mRNA expression in murine macrophages (RAW264.7) cells treated for 9h with PEA, quercetin and curcuminoids alone and their combinations. Isobologram was generated by normalizing the dose of each compound in combination by the IC_{50} of individual compounds.

One of the primary goals of finding synergistic compound combinations is to reduce the dose of the compounds used, hence reducing potential toxicity, while maintaining efficacy. The non-fixed ratio experimental design allows the determination of the dose reduction index (DRI) at each effect level observed with the actual experimental concentrations. DRI values were favorable for all tested combinations, as they were all > 1 (Table 3). To reach an effect of 0.31 (i.e. 69% reduction), 6.43 µg/mL of PEA alone or 1.40 µg/mL of QUER would be needed. However, in combination, 12.85-fold less PEA and 2.81-fold less QUER was needed to reach the same effect, supporting the benefits of combining PEA+QUER.

Table 3. Dose Reduction Index (DRI) for from tested combinations between PEA and quercetin in the non-fixed ratio design

Effect (compared to LPS = 1)	PEA (µg/mL)	QUER (µg/mL)	DRI PEA	DRI QUER
0.31	6.43	1.40	12.85	2.81
0.14	20.54	1.94	41.07	2.59
0.09	36.84	2.28	73.68	2.28
0.19	13.53	1.73	13.53	3.45
0.13	22.91	2.00	22.91	2.67
0.16	17.60	1.86	17.60	1.86

For the PEA-CUR association, all calculated DRI were also >1 (Table 4). At the effect level of 0.54, for example, a reduction of 4.33-fold and 2.01-fold is possible for PEA and CUR, respectively, when used in combination. Together with calculated CI and DRI for quercetin, results demonstrate synergism and the enhanced effects of combined treatments compared to single agents in reducing Il-1b mRNA expression in RAW264.7 macrophages.

Table 4. Dose Reduction Index (DRI) for from tested combinations between PEA and curcuminoids extract in the non-fixed ratio design

Effect (compared to LPS = 1)	PEA (µg/mL)	CUR (µg/mL)	DRI PEA	DRI CUR
0.54	2.17	1.00	4.33	2.01
0.29	7.22	1.59	14.45	1.59
0.06	61.47	3.64	122.94	1.82

- **Fixed ratio experimental design**

To compare synergistic effect parameters in the two different experimental designs the same calculations were performed using data from the fixed ratio

experiment. This specific design enables the CompuSyn software to simulate the combination index (CI) at other effect levels rather than only the experimentally obtained, allowing identification of a "synergistic range" (if present), as well as to generate a conventional isobologram with actual concentrations (not normalized). The CIs obtained for experimental PEA+QUER combination data points using the fixed ratio design were all < 1, except for the PEA 1.0 + QUER 0.5µg/mL concentration (Table 5). CIs for PEA+CUR association were mostly close or higher than 1, except for the PEA 0.25 + CUR 0.5 µg/mL treatment (CI = 0.6124), which indicates that at the concentrations tested, the combination was additive or moderately antagonistic.

Table 5. Combination Index (CI) for association between PEA and quercetin and curcuminoids extract in fixed-ratio experimental design

PEA + Quercetin (2:1)		PEA + Curcuminoids (1:2)	
Combination concentrations	CI	Combination concentrations	CI
PEA 0.25 + QUER 0.125	0.68827	PEA 0.25 + CUR 0.5	0.61240
PEA 0.5 + QUER 0.25	0.50098	PEA 0.5 + CUR 1.0	1.71973
PEA 1.0 + QUER 0.5	1.11328	PEA 0.75 + CUR 1.5	1.17258
PEA 1.5 + QUER 0.75	0.93318	PEA 1.0 + CUR 2.0	2.05966
PEA 2.0 + QUER 1.0	0.69043	-	

Despite the apparent weaker synergistic effect obtained in this second set of combination experiments for PEA+QUER, most concentrations at the 2:1 ratio were synergistic (CI <1) and conventional isobolograms at the IC_{50} and IC_{20} were generated (Figure 22). To reach 50% inhibition of IL-1b expression, the concentration of PEA and quercetin needed if used alone was 2.4 µg/mL and 1.09 µg/mL, respectively. When combined at a 2:1 ratio, to reach the same inhibition, only 0.82 µg/mL PEA and 0.41

µg/mL QUER were needed. Similarly, concentrations needed to reach 20% inhibition are in combination are lower than individual compounds (0.45 µg/mL PEA and 0.70 µg/mL Q alone, versus 0.25 µg/mL and 0.13 µg/mL QUER in combination).
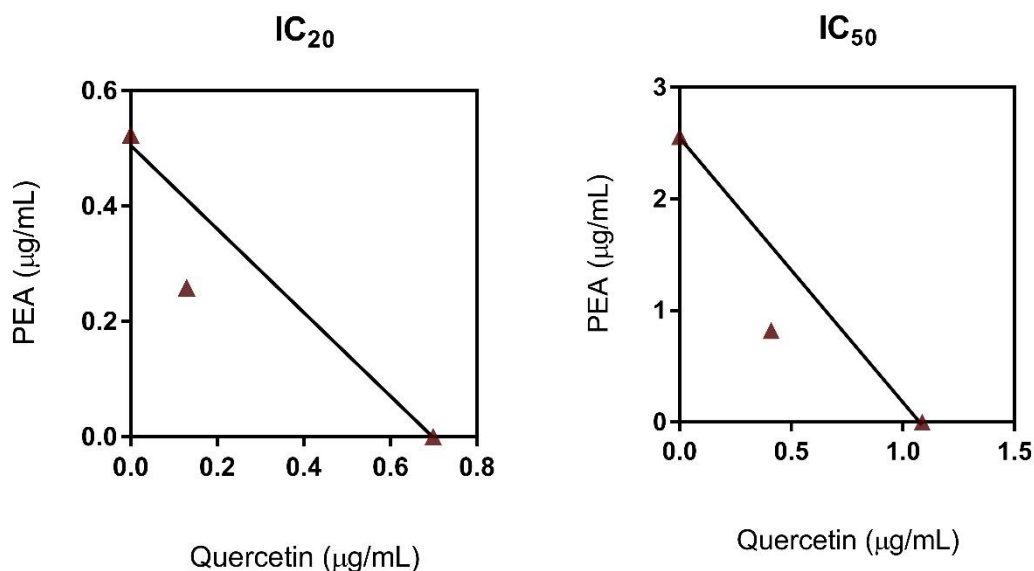
IC_{20}

IC_{50}

Figure 22. Classic isobologram for fixed ratio combination between PEA+QUER at the IC20 and IC50 levels.

Figure 23 demonstrates that at the IC_{20}, the PEA+CUR combination was strongly synergistic (CI = 0.13), requiring a dose of PEA and QUER 20.5- and 11.3-fold lower, respectively, in combination compared to when compounds were used alone (Appendix A for DRIs). At the IC_{50}, however, PEA+CUR exerted an antagonistic effect, with a CI = 1.248.
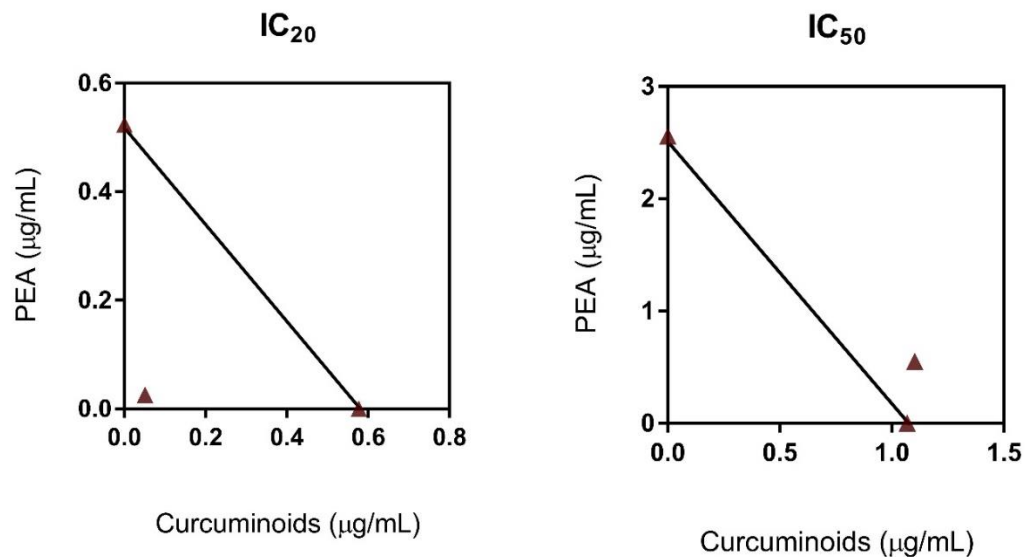
Figure 23. Classic isobologram for fixed ratio combination between PEA+CUR at the IC20 and IC50 levels.

Although the actual experimental concentrations tested did not reveal synergy for PEA+CUR association at the IC50, by calculating CIs at different effect levels and plotting CI versus effect, it was possible to identify a range in which both combinations are synergistic (Figure 24). PEA+QUER combination was synergistic at effects equal or higher than 0.25 (inhibition ≤ 75%), although at higher effect levels, CIs were closer to 1, indicating weak synergy. In a practical application, too high effect levels are not of interest, as it means poor inhibition of Il-1b. Hence, a practical synergistic range for PEA+QUER can be defined between effect levels 0.25 and 0.8 (75% and 20% inhibition) (Figure 24A), which can be achieved by treatment with total concentrations between 0.39 µg/mL (0.26 µg/mL PEA + 0.13 µg/mL QUER) and 3 µg/mL (2 µg/mL PEA + 1 µg/mL QUER).
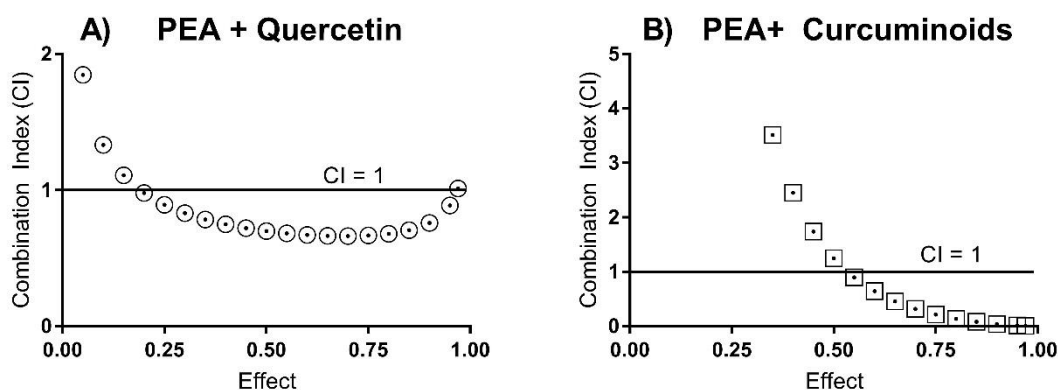
Figure 24. Simulated CI-effect plot for PEA+Q and PEA+CUR combinations at fixed ratios.

Synergy was also observed for PEA+CUR combination, although not at all effect levels. Synergy was warranted only at effect levels higher or equal to 0.55 (inhibition ≤ 45%) (Figure 24B). A practical synergistic range is identified between the effects of 0.55 and 0.8 (45% and 20% inhibition), reachable upon treatment with total concentrations between 0.08 (0.025 µg/mL PEA + 0.054 µg/mL C) and 1.06 (0.35 µg/mL PEA + 0.71 µg/mL C). Despite the limitation found at certain inhibition levels, from Figure 24 it is possible to demonstrate that PEA+QUER and PEA+CUR association is synergistic within a determined range and the synergism between PEA+CUR is warranted within a shorter range and it is not as strong as for PEA+QUER.

Studies investigating association between compounds usually report results in terms of enhancement of effects, without providing information about the nature of the interaction (Csaki *et al.*, 2009; Güran *et al.*, 2019). When synergy is claimed, one of the two experimental designs is defined (fixed or non-fixed ratio), CI is calculated and isobolograms are often reported as well (Mertens-talcott *et al.*, 2003; Mertens-Talcott

and Percival, 2005; Zhang *et al.*, 2016; Agah *et al.*, 2017). In this study, besides calculating CIs and generating the isobolograms, two different experimental designs were tested, which allowed a deeper data interpretation and detection of limitations in the synergistic nature of the interactions. Exploration of the simulated CIs at different effect levels was valuable in order to determine a range of effects and concentrations in which PEA+QUER and PEA+CUR combinations were synergistic. To the extent of our knowledge, this is the first time a synergy is reported between PEA and quercetin and PEA and curcuminoids and ultimately, that CIs are reported at experimental and simulated values for combinations including PEA. Further studies are recommended to extend the investigation to other inflammatory markers in vitro, to better characterize the immune-modulatory effect of the associations. Dose reduction index (DRI) for both experimental designs also demonstrated dose reduction potential when PEA is used in association with either quercetin and curcuminoids.

3.3.9 Effect of palmitoylethanolamide (PEA), quercetin and curcuminoids on fatty acid amide hydrolase (FAAH) and n-acylethanolamine acid amidase (NAAA) expression in LPS-induced RAW264.7 macrophages

Fatty acid amide hydrolase (FAAH) and N-acylethanolamine acid amidase (NAAA) are the enzymes responsible for hydrolysis of PEA and other bioactive NAEs (N-acylethanolamines) (Alhouayek *et al.*, 2017), being expressed by several cells and tissues, including macrophages (Sun *et al.*, 2005). The metabolism of NAEs can be

altered by inflammatory stimuli, which in turn, may modify the progression of the inflammatory process (Pontis *et al.*, 2015). Although FAAH is able to hydrolyze PEA, its affinity for anandamide (AEA) is much higher and NAAA is considered the main PEA degrading enzyme (Ueda *et al.*, 2010a). RAW264.7 murine macrophages expressed both FAAH and NAAA. Expression of NAAA was higher than FAAH, as evidenced by higher delta Ct values for FAAH (the smaller the ΔCt number, higher the expression) (Figure 25). While FAAH can be found in the outer face of mitochondria and endoplasmic reticulum of most mammalian cells, NAAA is mainly localized in the lysosomal compartment of macrophages (Tsuboi *et al.*, 2007b) and it has high affinity for PEA (Sun *et al.*, 2005; Piomelli *et al.*, 2020). NAAA is the primary enzyme to metabolize PEA into palmitic acid and ethanolamine, terminating its anti-inflammatory actions.
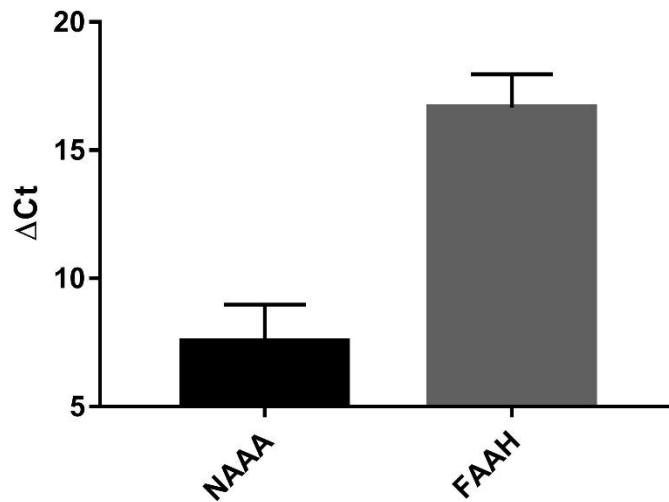
Figure 25. Expression of NAAA and FAAH in murine macrophage cell line RAW264.7. Smaller ΔCt values indicate higher mRNA expression.

- **Effect of LPS and PEA on the mRNA expression of PEA-degrading enzymes fatty acid amide hydrolase (FAAH) and N-acylethanolamine-hydrolyzing acid amidase (NAAA)**

In the present study, stimulation of RAW264.7 macrophages with LPS at 10 ng/mL for 8h led to marked downregulation of NAAA mRNA expression, whereas the effect on FAAH was less pronounced and did not reach statistical significance (Figure 26A and B). Similarly, Alhouayek *et al.*, (2017) found that in J774 mouse macrophages, FAAH mRNA expression was unchanged after 8h of LPS stimulation (100 ng/mL), while NAAA expression was decreased after 8 h and 24 h.
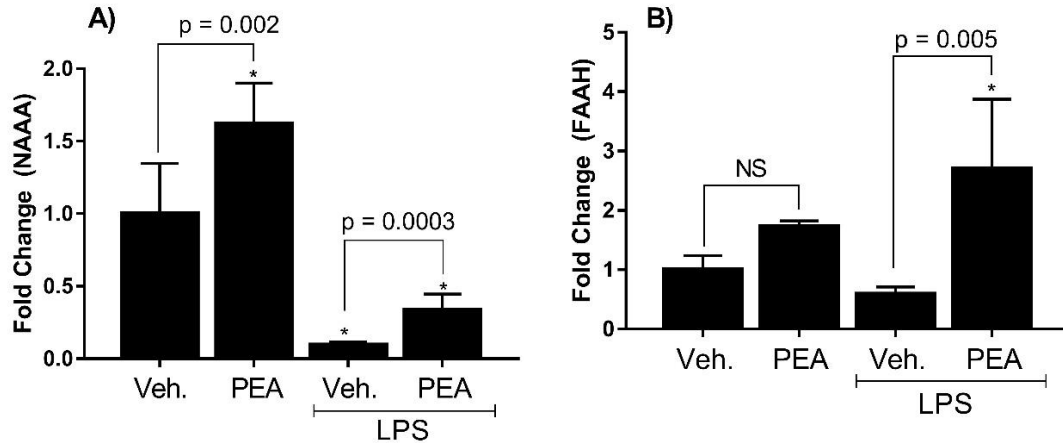
Figure 26. mRNA expression of N-acylethanolamine-hydrolyzing acid amidase (NAAA) and fatty acid amide hydrolase (FAAH) in RAW264.7 murine macrophages pre-treated with PEA (2 µg/mL) for 1h, with and without LPS stimulation (10ng/mL) for additional 8h. Data is normalized to Veh (Veh = 0.1% DMSO); * significantly different from Veh. at $p < 0.05$; ANOVA-Dunnett's Test. Indicated p values are significant by ANOVA-Sidak multiple comparison. Values are means ± SD of three replicates.

Although in the present study the reduction in FAAH expression due to LPS was less pronounced, studies do report a strong downregulation of this enzyme upon LPS treatment. Maccarrone *et al.*, (2001) reported downregulation of FAAH mRNA levels after LPS stimulation (100 µg/mL) in primary lymphocytes, leading to enhanced concentrations of anandamide (AEA). AEA is an anti-inflammatory NAE able to activate endocannabinoid receptors. Additionally, administration of LPS to mice significantly reduced mRNA expression and protein levels of FAAH in peripheral blood mononuclear cells (PBMCs) (Wolfson *et al.*, 2013). Interestingly, in a study by Zhu *et al.*, (2011), LPS-stimulation of RAW264.7 macrophages did not significantly affect FAAH and NAAA expression, although it did reduce expression of PEA synthesizing

enzyme, NAPE-PLD, leading to lower endogenous PEA levels. These results highlight the complexity of the mechanisms that control intracellular PEA levels.

Moreover, treatment with PEA at 2 µg/mL without LPS stimulation led to marked increase in NAAA expression (1.62-fold increase, $p = 0.002$) (Figure 26A) and although not statistically significant, expression of FAAH was also increased by 1.72-fold compared to non-treated cells (Figure 26B). In the presence of LPS, PEA was still able to increase expression of NAAA and FAAH, even with the robust downregulating effect caused by LPS. Given that the main function of NAAA and FAAH is to regulate intracellular levels of NAEs and ensure appropriate tissue response to injury and inflammatory conditions (Ueda *et al.*, 2000; Tsuboi *et al.*, 2007a; Gorelik *et al.*, 2018), an increase in substrate concentration might lead to increased gene expression of these enzymes, aiming to regulate intracellular PEA levels and maintain its normal metabolism. (Alhouayek *et al.*, (2017) observed reduction in both NAAA expression and activity when J774 macrophages were stimulated with LPS for 8h, but the effect of PEA treatment on enzyme activity was not investigated. To understand the effects of LPS and exogenous PEA on PEA metabolism, as well as the relationship between the increased mRNA expression and enzyme activity, the specific activities of NAAA and FAAH should be further investigated under these same conditions.

- **Effect of quercetin and curcuminoids on the mRNA expression of PEA-degrading enzymes NAAA and FAAH**

Besides evaluating the anti-inflammatory and antinociceptive effects of exogenous PEA on several inflammatory diseases, another strategy currently being investigated is the inhibition of FAAH and NAAA (Solorzano *et al.*, 2009; Yang *et al.*, 2015; Pędzińska-Betiuk *et al.*, 2017; Bottemanne *et al.*, 2018; Danandeh *et al.*, 2018). This may provide additional therapeutic strategies for the treatment of diseases in which higher PEA levels are desired. Based on the observed enhanced anti-inflammatory activity observed when PEA was combined with quercetin and curcuminoids, it was hypothesized that these polyphenols could be exerting an inhibitory effect on NAAA and/or FAAH, resulting in higher intracellular PEA levels and consequently, better modulation of the inflammatory response.

Treatment of RAW264.7 cells with quercetin or curcuminoids at 2 µg/mL did not significantly affect the mRNA levels of NAAA (Figure 27). Curcuminoids demonstrated slight tendency to increase NAAA levels, but did not reach statistical significance. FAAH levels, on the other hand, were upregulated by treatment with curcuminoids extract ($p = 0.0004$). Similar to previous results, LPS-stimulation led to marked downregulation of enzyme expression and treatment with polyphenols did not alter the results.
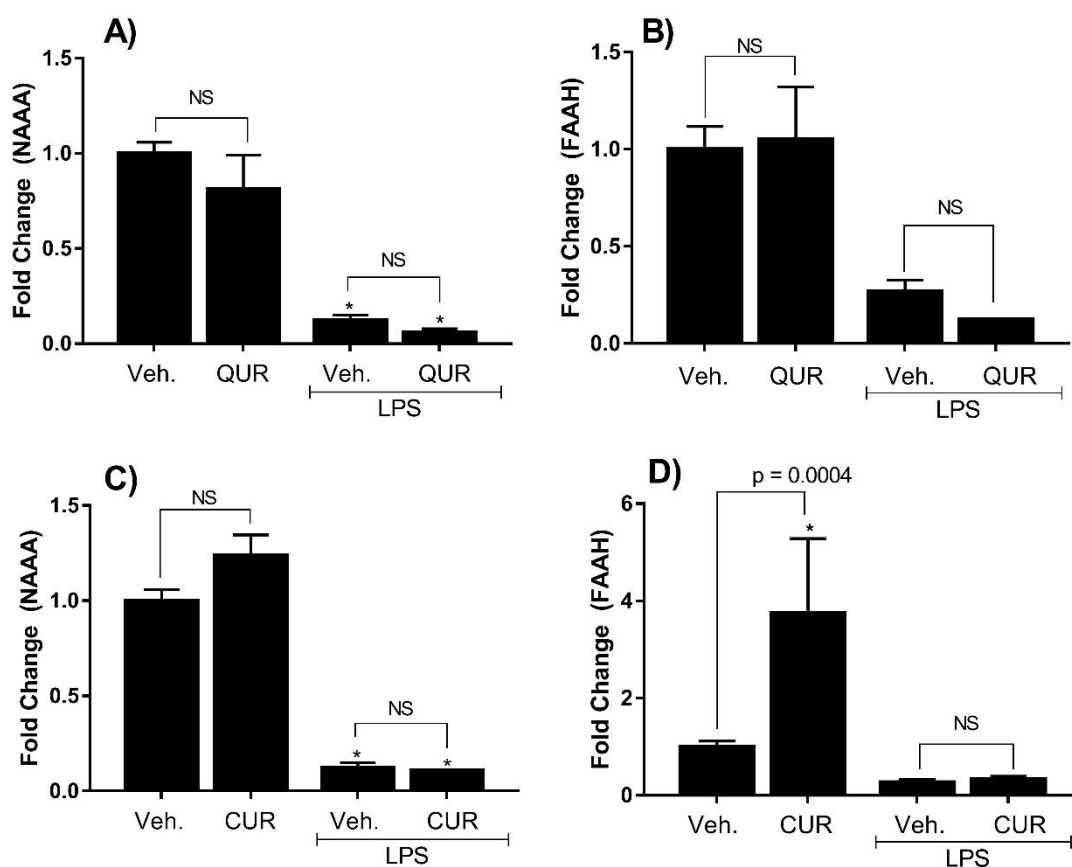
Figure 27. mRNA expression of N-acylethanolamine-hydrolyzing acid amidase (NAAA) (A and C) and fatty acid amide hydrolase (FAAH) (B and D) in RAW264.7 murine macrophages treated with quercetin or curcuminoids (2 µg/mL), with and without LPS stimulation (10ng/mL) for 8h. Data is normalized to Veh. (Veh. = 0.1% DMSO). * significantly different from Veh. at $p < 0.05$; ANOVA-Dunnett's Test. Indicated p values are significant by ANOVA-Sidak multiple comparison. Values are means ± SD of three replicates.

Numerous chemically synthesized inhibitors of NAAA and FAAH have been described in the literature (Ahn *et al.*, 2009; Yang *et al.*, 2015; Pędzińska-Betiuk *et al.*, 2017; Zhou *et al.*, 2019). However, apart from one study reporting the inhibitory effect of kaempferol and other polyphenols on hydrolysis of anandamide (AEA) by FAAH (Thors *et al.*, 2008), very little information is available on the effect of polyphenols on

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expression or activity of FAAH and no information was found regarding effects of polyphenols on NAAA. In the mentioned study, authors found that 7-hydroxyflavone was the most active flavonoid towards FAAH inhibition in rat brain homogenates. Kaempferol, had the best inhibitory activity among commonly occurring flavonoids ($IC_{50} = 6.2$ µM), while quercetin, had a much lower inhibitory effect ($IC_{50} = 35$ µM). Overall, the authors reported the presence of a hydroxy substituent at the position 7 of the benzopyran-4-one ring as the most important structural feature for enzyme inhibition. Further studies focusing on specific polyphenols and its effects on enzyme expression and activity are needed to elucidate the potential use of polyphenols as FAAH and NAAA inhibitors. Finding safe compounds that have an inhibitory action against these enzymes might be a potential strategy to boost the effects of exogenously administered PEA. Albeit preliminary results shown in this study did not support the downregulation of NAAA and FAAH gene expression by quercetin and curcuminoids, further investigation of potential effects in enzyme activity is suggested to distinguish effects in mRNA versus enzyme activity.

3.3.10 Effect of PPAR-α inhibition on expression of pro-inflammatory markers upon treatment with PEA and polyphenols

The most researched mechanism of action of PEA is through direct PPAR-α activation (Lo Verme *et al.*, 2005). The peroxisome proliferator-activated receptor alpha (PPAR-α) is a ligand-activated transcription factor that participates in multiple

regulatory functions, including the control of macrophage activity and inflammation (Clark, 2002). Upon ligand-binding, PPAR-α heterodimers are formed with retinoic acid receptors (RXR), which act as a transcription factor that will result in downregulation of nuclear-factor kappa B (NF-κB) and subsequent downstream signaling cascades (Daynes and Jones, 2002). To understand the effect of PPAR-α inhibition on the expression of pro-inflammatory markers in RAW264.7 cells, the irreversible PPAR-α inhibitor GW6471 was used (IC_{50} = 240 nM). Cells were pre-treated for 1h with GW6471 (2uM), followed by treatment with PEA (1h) and LPS (8h). GW6471 alone had no effect on the mRNA expression of the evaluated biomarkers (Figure 28). Without GW6471, PEA treatment was able to reduce expression of IL-1b by 42% (p = 0.0035) and TNF-α by 40% (p = 0.0195). No effect was observed on IL-6. Upon PPAR-α inhibition, the effect of PEA in Il-1b and TNF-α was suppressed, as shown in Figure 28A and B. This confirms the reported direct action of PEA on PPAR-α receptors in reducing inflammation and more specifically the role of PPAR-α activation in murine macrophage cell line RAW264.7 inflammatory response.
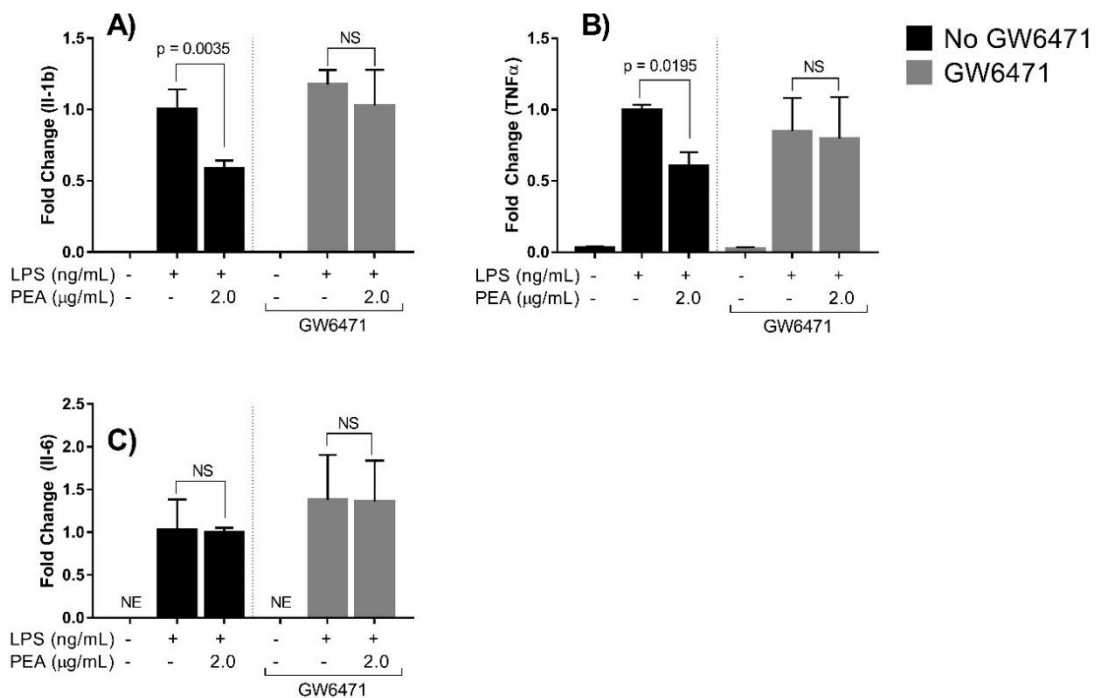
Figure 28. Effect of PPAR-α inhibitor GW6471 (2 uM) on expression IL-1b, TNF-α and IL-6 in RAW264.7 cells pre-treated with PEA (2 µg/mL) for 1h followed by LPS-stimulation (10 ng/mL) for 8h. Indicated p values are significant by ANOVA-Sidak multiple comparison. Data are means ± SD of three replicates.

When cells were treated with a combination between PEA and quercetin or PEA and curcuminoids, expression of IL-1b was significantly reduced by 67% and 72%, respectively (Figure 29A), compared to LPS. As opposed to what was observed for PEA alone, when GW6471 was used, the combination treatment was still able to reduce expression of IL-1b. This might indicate that once PPAR-α receptors are blocked, PEA's activity against IL-1b is suppressed, but quercetin and curcuminoids can still reduce expression of this marker, suggesting they are able to downregulate IL-1b via PPAR-α independent mechanisms.
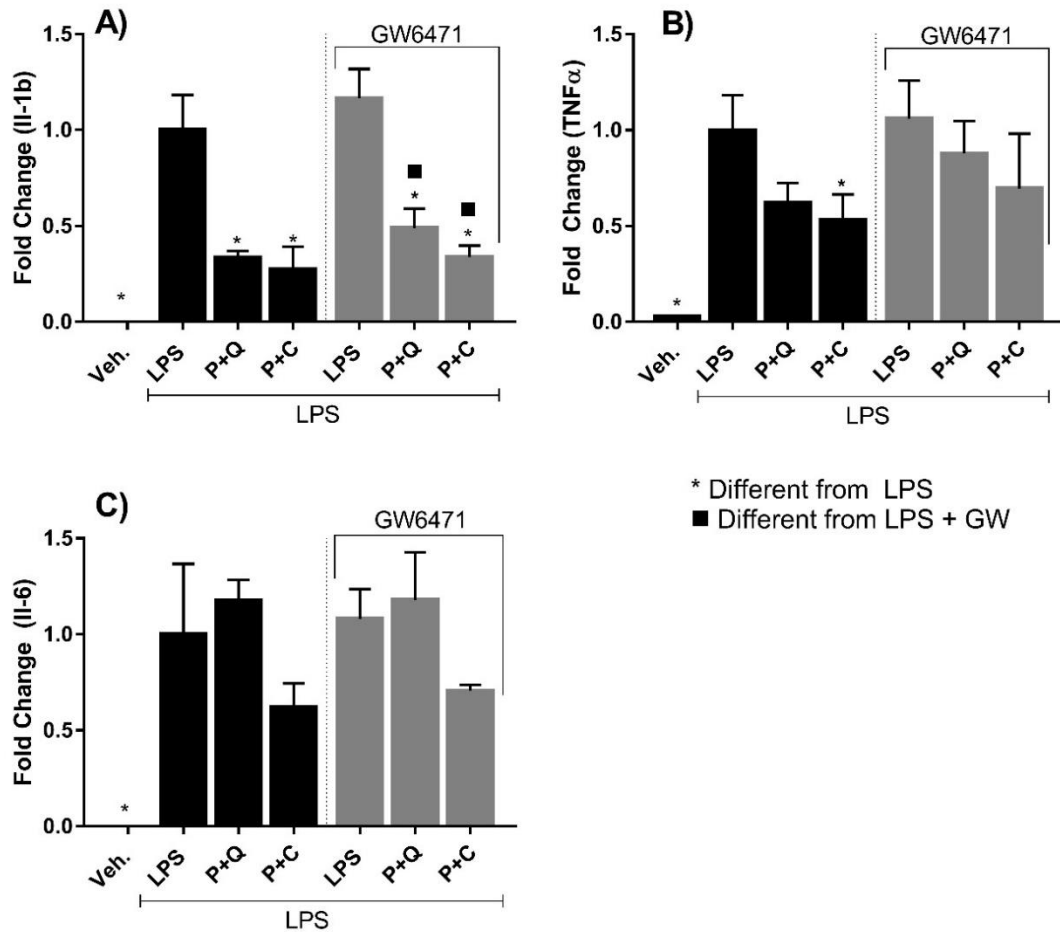
Figure 29. Effect of PPAR-α inhibition with GW6471 (2 uM) on expression IL-1b, TNF-α and IL-6 in RAW264.7 cells pre-treated with PEA+QUER(2 µg/mL each) and PEA+CUR (2 µg/mL each) for 1h followed by LPS-stimulation for 8h. * significantly different from LPS and ■ is significantly different from LPS + GW6471 at p < 0.05; ANOVA-Dunnett's Test). Data are means ± SD of three replicates.

TNF-α expression was also downregulated by the combined treatments (Figure 29B). The effect of PEA+QUER and PEA+CUR on TNF-α expression followed the same trend in the presence and absence of GW6471, although the magnitude of the effects was attenuated by PPAR-α blockage, indicating a weaker effect of quercetin and curcuminoids in this specific marker. PEA+CUR treatment led to 38% reduction of IL-6

expression, although it did not reach statistical significance (Figure 29C). This was different from PEA treatment alone (Figure 28C), which did not elicit any changes in IL-6, indicating possible enhancement of anti-inflammatory activity when PEA is combined with curcuminoids. GW6471 did not elicit any changes in the effect of treatments on IL-6 expression in RAW264.7 cells. Both quercetin and curcuminoids are reported to inhibit the activation of NF-kB pathway, which contributes to lower secretion of pro-inflammatory cytokines and enzymes (Jacob *et al.*, 2007; Kim *et al.*, 2017; Tang *et al.*, 2019). Upregulation and activation of PPAR-γ is described as one of the underlying mechanisms involved in both quercetin and curcuminoids' inhibition of NF-κB pathway, while no direct effects on PPAR-α are described (Jacob *et al.*, 2007; Lee *et al.*, 2013; Li *et al.*, 2016; Mazidi *et al.*, 2016). The search for a multi-target approach for pharmacological and nutraceutical development is rapidly rising, considering that the pathogenesis of many diseases is more likely to have a multi-factorial nature rather than a single cause. Since PEA works primarily through PPAR-α activation, its combination with polyphenols able to exert anti-inflammatory effects by other mechanisms is encouraged, especially given the results obtained herein indicating the synergistic interaction between PEA-QUER and PEA-CUR in reducing expression of pro-inflammatory markers.

3.4 Conclusions

The anti-inflammatory effect of PEA, quercetin and curcuminoids extract in RAW264.7 macrophages was demonstrated. Compounds alone and in combination were able to mitigate oxidative stress by reducing ROS and NO production, as well as to downregulate expression of inflammatory markers such as Cox-2, iNOS and TNF-α at the mRNA and protein levels. Most importantly, a synergistic effect was demonstrated between PEA and quercetin and PEA and curcuminoids in downregulation of IL-1b mRNA expression. A practical synergistic range was identified and dose reduction indexes (DRI) were calculated for both combinations. The downregulation of IL-1b by PEA in RAW264.7 cells was demonstrated to occur via PPAR-α activation, while the effects of quercetin and curcuminoids do not appear to be mediated by the same receptor. This indicates that the synergism observed in these combinations may arise from activation of different biochemical pathways and should be the scope of a future mechanistic investigation. LPS-stimulation decreased expression of the PEA-degrading enzymes FAAH and NAAA to almost zero, while treatment with PEA increased enzyme expression, possibly in an attempt to regulate intracellular levels of PEA upon inflammatory stimuli. Quercetin and curcuminoids extract did not exert significant effects in the expression of these enzymes, demonstrating that the synergistic mechanism is not arising from transcriptional inhibition of these enzymes by the polyphenols. Further studies should assess effects of compounds in the enzymatic activity, in addition

to enzyme expression, to elucidate a possible inhibitory activity of quercetin and curcuminoids in these enzymes.

Chronic inflammation is the leading cause of life-debilitating chronic diseases and identifying novel formulations able to reduce the inflammatory state can play a major role in preventing and acting as adjuvants in disease treatment. This research provides foundation for future mechanistic studies, as well as clinical trials using a synergistic combined formulation able to mitigate inflammation at lower doses with increased efficacy.

CHAPTER IV

A PILOT INVESTIGATION INTO THE EFFECT OF QUERCETIN AND

CURCUMINOIDS ON PALMITOYLETHANOLAMIDE (PEA) LEVELS IN

RAW264.7 MACROPHAGES

4.1 Introduction

Palmitoylethanolamide (PEA) is an endogenous fatty acid amide belonging to the

N-acylethanolamine (NAE) family of endogenous signaling molecules (Hesselink *et al.*,

2014). PEA is synthesized on-demand from cell membrane phospholipid precursors, as a

mechanism to maintain and restore homeostasis in response to tissue injury and stress,

acting as a potent anti-inflammatory and immunomodulatory agent (Ueda *et al.*, 2010a;

Iannotti *et al.*, 2016). Given its potent biological effects and lack of adverse effects, PEA

is currently being investigated as a more natural way to counteract the inflammatory

response, being sold as a nutraceutical across the United States and Europe (Hesselink *et

al.*, 2013).

Comprehensive pharmacokinetic studies are still lacking for exogenously

administered PEA, although some experimental data has been published and

demonstrated a rapid absorption followed by decrease to basal levels in plasma of rats

(20-fold increase after 15 minutes, dropping to basal levels after 2 hours) (Vacondio *et

al.*, 2015). After a single oral administration of PEA (30 mg/kg) to dogs, plasma PEA

levels were increased by 5-fold after 1 hour and returned to basal levels after 4 hours

(Petrosino *et al.*, 2016). In that same study, PEA concentration in human plasma peaked after 2 hours, returning to basal levels after 4 hours. Once it gets into systemic circulation, PEA is transported into cells and tissues, where it will exert its biological effects by multiple mechanisms (Lo Verme *et al.*, 2005; Petrosino and Di Marzo, 2016). Inside the cells, PEA is metabolized into palmitic acid and ethanolamine by fatty acid amide hydrolase (FAAH) and N-acylethanolamine acid amidase (NAAA), which terminates its biological actions (Tsuboi *et al.*, 2007b). It has been demonstrated that macrophages synthesize PEA (Zhu *et al.*, 2011; Pontis *et al.*, 2015; Jin *et al.*, 2020) and that treatment with exogenous PEA counteracts the inflammatory response in different macrophage-like cells (Ross *et al.*, 2000; Alhouayek *et al.*, 2015; Rinne *et al.*, 2018). Limited information is available about cellular transport of PEA in different types of cells. Studies from Bisogno et al., (1997) and Jacobsson and Fowler (2001), have shown cellular uptake of PEA to occur in a similar way in neuronal and immune cells, penetrating the cells by passive diffusion due to its high lipophilicity, and also through a facilitated transport system.

Although available data on PEA uptake and degradation by cells is limited, a study from Luongo *et al.*, (2014) has reported that upon treatment of neuroblastoma cells (SH-SY5Y) with PEA (3 µM), the maximal intracellular concentration was seen after 15 minutes of incubation at 37°C, followed by quick reduction, demonstrating the rapid hydrolysis by intracellular enzymes. In the mentioned study, a prodrug of PEA was synthesized in order to increase cell membrane entry of PEA, which resulted in higher intracellular concentrations. Furthermore, it is believed that increased PEA local levels

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play protective and pro-homeostatic roles (Re *et al.*, 2007; Hesselink and Hekker, 2012). Specific inhibitors of PEA's degrading enzymes have also been studied as tools to increase intracellular PEA concentrations (Solorzano *et al.*, 2009; Alhouayek *et al.*, 2015; Bottemanne *et al.*, 2018). Interestingly, Thors *et al.*, (2008) have reported that the polyphenol kaempferol was able to inhibit the hydrolysis of anandamide (AEA) (another biologically active compound from the NAE family), by rat brain homogenates, while quercetin and other flavonoids had weaker FAAH inhibition. However, it is not known if polyphenols have the potential to inhibit PEA hydrolysis in RAW264.7 macrophages. Thus, understanding the cellular uptake and metabolism of PEA by macrophages and exploring strategies that allow prolonged exposure of cells to exogenously administered PEA can assist in the development of formulations able to improve compound life span, intracellular concentration and hence potentiate its biological effects.

Given the enhanced anti-inflammatory effects observed when PEA was combined with quercetin and curcuminoids, a hypothesis was tested that the improved effect may arise not only from pharmacodynamic mechanisms led by their association, but also from possible alteration of PEA's uptake and metabolism process, resulting in higher intracellular PEA concentrations. The aim of this study was to screen the cellular absorption of PEA by RAW264.7 macrophages and determine if intracellular levels of PEA are changed upon simultaneous treatment with quercetin and a curcuminoid extract.

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

Quercetin (≥95%, HPLC) was purchased from Sigma (MiliporeSigma, MA, USA), deuterated PEA (d4-PEA) was purchased from Cayman Chemicals (MI, USA). Methanol, acetonitrile, Fetal bovine serum (FBS), Phosphate Buffer Saline (PBS), Dulbecco's Minimum Essential Medium (DMEM) culture medium and penicillin streptomycin combination were purchased from Gibco (Fisher Scienctific, Waltham, MA, USA). Micronized palmitoylethanolamide (PEA) from Wuxi Cima Science (98% pure) and the curcuminoids extract were kindly donated by NaturPro Scientific.

4.2.2 Cell culture

RAW 264.7 murine macrophage cell line was purchased from American Type Culture Collection (ATCC-TIB71). Cells were routinely cultured as in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, stable glutamine and sodium pyruvate, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin mix. Cells were maintained in an incubator at 37 °C with 5% CO_2 and the medium was changed every 2 days. All the tests were conducted on cells between the 5th and the 15th passage. All treatments with PEA, d4-PEA and other polyphenols were diluted in complete culture media immediately before use.

For experiments, cells were seeded into 10 mm cell culture dishes (55 cm²

surface area) at a starting density of 2×10^6 cells per dish and allowed to grow for 48h.

This cell density was determined in order to obtain a 50mg cell pellet upon cell

collection. Treatments with d4-PEA alone or combined with quercetin or curcuminoids

extract were prepared in fresh medium to a final concentration of 500 ng/mL of each

compound, and 10mL were added to each plate. After 3h incubation at 37°C, 300 µL of

supernatant from each plate was collected for supernatant extraction (described in

section 4.2.3). For cell collection, remaining treatment was removed and the plate was

washed with 5mL PBS, followed by 5mL of sterile nanopure water. Cells were scraped

in the presence of 1mL of sterile nanopure water and collected into Precellys 24 lysing

tubes. Tubes were centrifuged for 15 minutes at 8000 rpm (4°C), the supernatant was

discarded and cell pellets were weighed. Methanol (800µL) was added to each tube and

samples were stored at -80°C for following extraction.

4.2.3 LC-MS conditions for quantification of PEA and d4-PEA

The target compounds in samples (PEA and d4-PEA) were detected and

quantified on a triple quadrupole mass spectrometer (Altis, Thermo Scientific, Waltham,

MA) coupled to a binary pump HPLC (Vanquish, Thermo Scientific). MS parameters

were optimized for the target compounds under direct infusion at 5 µL min^{-1} to identify

the single reaction monitoring (SRM) transitions (precursor/product fragment ion pair)

with the highest intensity as 300.2-62.03 m/z for palmitoylethanolamide (PEA) and

304.4-66.14 m/z for the stable isotope, d4-palmitoylethanolamide (d4-PEA) (Figure 30).

Samples were maintained at 4 °C on an autosampler before injection. The injection

volume was 10 μL. Chromatographic separation was achieved on a Hypersil Gold 5 μm

50 x 3 mm column (Thermo Scientific) maintained at 30 °C using a solvent gradient

method. Solvent A was water (0.1% formic acid) and solvent B was acetonitrile (0.1%

formic acid). The gradient method used was 0-1 min (20% B to 80% B), 1-4 min (80%

B), 5-5.1 min (80% B to 20% B), 5.1-6 min (20% B). The flow rate was 0.5 mL min^{-1}.

Limit of detection (LOD) was 0.5 ng/mL and Limit of Quantification (LOQ) was 1

ng/mL. Sample acquisition and analysis was performed with TraceFinder 3.3 (Thermo

Scientific).
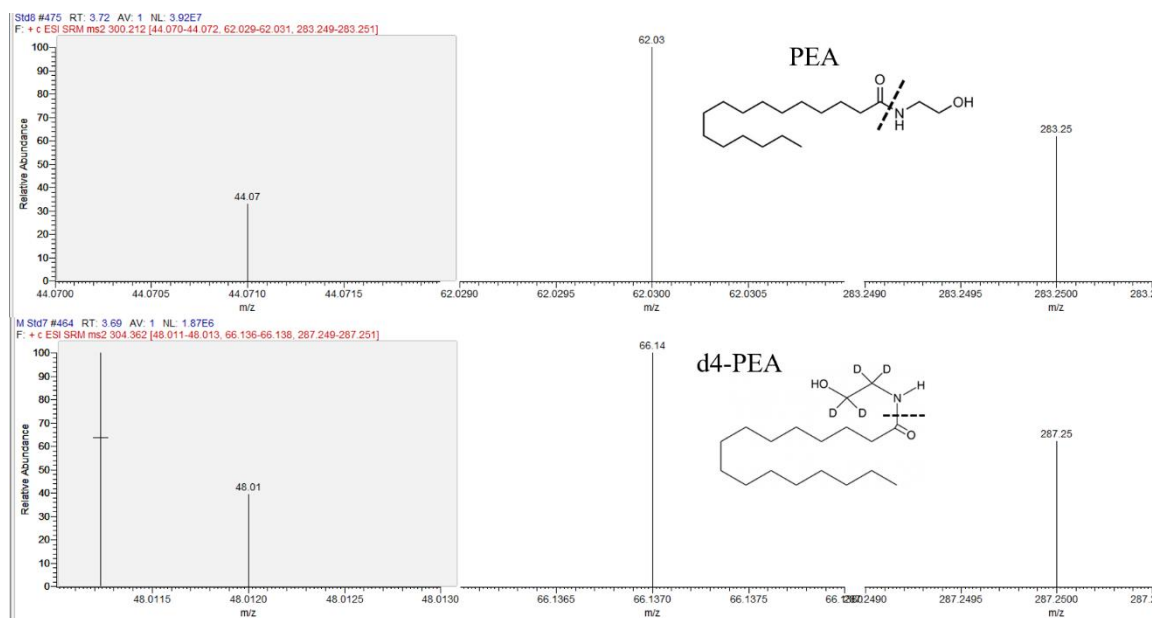
Figure 30. Structure and product ion mass spectrum for PEA (300.2-62.03 m/z) at RT = 3.72 minutes and d4-PEA (304.4-66.14 m/z) at RT= 3.69 minutes, following dissociation.

4.2.4 Sample preparation

4.2.4.1 Extraction of PEA from cell culture media

In order to determine the best solvent to extract PEA from cell culture media, methanol and acetonitrile were tested as extraction solvents. Palmitoylethanolamide (PEA) was added to cell culture media at a final concentration of 2000 ng/mL. After homogenizing the samples by gently inverting the tubes 20 times, 300uL of treated media was combined with 4x volume of appropriate solvent (1.2 mL) in an Eppendorf tube. Samples were vortexed for 10 seconds and centrifuged for 15 minutes at 8000 rpm, 4°C. Supernatants were collected, filtered through 0.45nm PTFE filters and stored at -80°C until HPLC-MS analysis.

4.2.4.2 Extraction of PEA from cells

Eight hundred microliters of ice-cold methanol were added to approximately 50 mg cells in pre-weighed and pre-chilled Precellys 24 tube. The cell samples were then homogenized on the Precellys 24 Homogenizer for 30 seconds on intensity "6000". After a 5-minute incubation in ice these were centrifuged at 15,000 g for 5 min at 4 °C to pellet beads and cell debris. Supernatant was transferred to a clean microcentrifuge tube. An additional 800 µL methanol was added to cells in Precellys tube and again homogenized for 30 seconds on intensity "6000" and incubated on ice for 5 minutes.

These were then centrifuged at 15,000 g for 5 min at 4 °C to pellet beads and cell debris and the supernatants were transferred and pooled from both rounds of extraction. The supernatant was then filtered through a 0.2 µm nylon spin filter by centrifuging at 10,000 g for 1 minute and 500 µL of the filtrate was concentrated in a 3K MWCO Amicon Ultra concentrator at 10,000 g for 30 min at 4 °C. The isolated, filtered, and concentrated cellular extract was used for intracellular PEA determination by LC-MS.

4.2.5 Stability of PEA and d4-PEA in cell culture media

Stability of d4-PEA and PEA in cell culture media (DMEM) at 37°C was determined over a 12h and 24h incubation period, respectively. A PEA working solution was prepared at 20 µg/mL, by diluting the PEA stock (2000 µg/mL) in media. A volume of 100 µL of working solution was added to 900 µL of cell culture media, to a final concentration of 2000 ng/mL. Similarly, for d4-PEA, a working solution was prepared at 2000 ng/mL, by diluting the d4-PEA stock (1 mg/mL) in media. A volume of 750 µL of media was spiked with 250 µL of the d4-PEA working solution to yield a final concentration of 500 ng/mL. Samples were gently inverted 20 times for complete homogenization and incubated at 37°C for up to 24h. After appropriate incubation times (1h, 3h, 5h and 12h), 300 µL of media was collected and combined with 1.2 mL of acetonitrile. Samples were vortexed for 10 seconds and centrifuged for 15 minutes at 8000 rpm, 4°C. Supernatants were collected, filtered through 0.45 µm PTFE filters and stored at -80°C until HPLC-MS analysis.

4.2.6 Matrix Effect

The suppression or enhancement of the analytical ion count signal for d4-PEA due to matrix effect was investigated using the post-extraction spike method. Ion suppression effects were studied at a low, a medium and a high concentration (50, 250 and 500 ng/mL) and determined by adding known amounts of d4-PEA standards to extracted cell culture media (supernatants) and cell lysates. The ion suppression percentage was calculated by comparing the peak areas obtained for each sample in the respective matrix with the peak areas of d4-PEA spiked in pure solvents, at the same concentrations (acetonitrile for supernatants and methanol for cell lysates). Ion suppression was calculated using the formula: [1 – (peak area in matrix/peak area in pure solvent)] *100.

4.2.7 Recovery

Extraction recovery from cell culture media was studied at three concentration levels (50, 250 and 500 ng/mL) by adding d4-PEA to cell culture media samples before (pre-spike) and after extraction (post-spike). Percentage recovery was calculated using the formula: % Recovery = ((Peak Area of Pre-Spike/Peak Area of Post-Spike) x 100).

4.2.8 Statistical Analysis

All experiments were performed in triplicates. Data were analyzed by one-way analysis of variance (ANOVA) with Tukey's posttest using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Data were considered significantly different when $p < 0.05$.

4.3 Results and Discussion

4.3.1 Extraction Recovery and Matrix Effect

To initially identify a suitable solvent to extract PEA from cell culture media, methanol and acetonitrile were tested as extraction solvents after spiking cell culture media with 2000 ng/mL of PEA. Extraction yield was calculated by dividing the PEA concentration obtained from each solvent extraction by the total PEA concentration added (2000 ng/mL), and expressed as extraction yield (%). Both solvents resulted in a satisfactory extraction of PEA, however, percentage recovery of PEA in acetonitrile (95% ± 3.72 SD) was superior compared to methanol (82.7% ± 1.61 SD) (Figure 31). The data obtained is in accordance with previous studies aiming optimization of NAE extraction from human plasma and rat brain, which demonstrated acetonitrile as a better solvent, as it allowed optimal recovery of several NAEs, while effectively precipitating proteins and minimizing the co-extraction of highly abundant triglycerides and

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cholesterol esters that could interfere in ion suppression (Balvers *et al.*, 2009; Liput *et al.*, 2014).
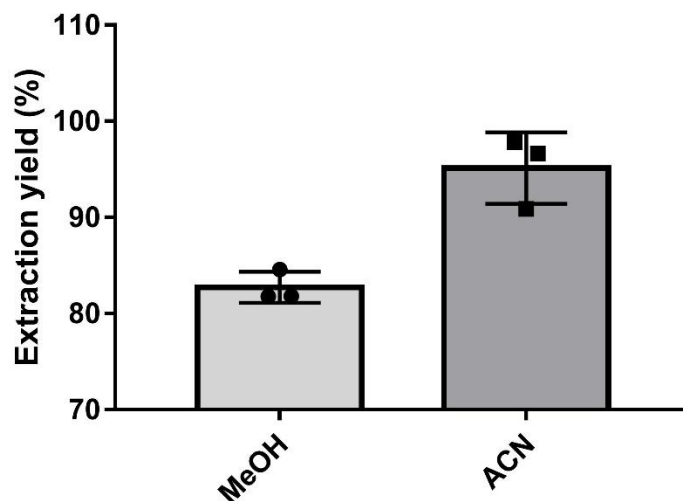
Figure 31. Extraction yields (%) acquired with methanol (MeOH) and acetonitrile (ACN) used for solvent extraction of PEA from cell culture media. Values are means ± SD of three replicates.

Deuterated PEA (d4-PEA) was used in following experiments involving intracellular determination of PEA in order to allow analysis of exogenously added PEA, as opposed to the endogenously present compound. Recovery of d4-PEA from cell culture media ranged from 56.59% to 76.49% within the three concentrations tested. Highest recovery (76%) was observed at 50 ng/mL, followed by recovery at 500 ng/mL (71%) (Table 6).

Table 6. Extraction recovery and matrix effect of d4-PEA in cell culture media and cell lysates

Concentration (ng/mL)	Recovery from media (%)	Matrix Effect (% suppression)	
		Supernatant	Cells
50	76.49 ± 12	19.30	6.91
250	56.59 ± 6.82	8.11	0.84
500	71.64 ± 1.85	9.16	-3.60

[1] Percentage recovery was calculated using the formula: % Recovery = (Peak Area of Pre-Spike/Peak Area of Post-Spike) x 100.

[2] Ion suppression was calculated using the formula: % Suppression = [1 – (Peak area in matrix/Peak area in pure solvent)] *100.

The effect of the different matrices (cell culture supernatant and cell lysates) on PEA ionization was evaluated and higher ion suppression was seen in supernatants, compared to cell lysate matrix (Table 6). As shown in Table 6, d4-PEA had a decrease in ion efficiency from 8 to 19% for supernatant and 0 to 6.9% for cells. At 5000 ng/mL, there was an ionization enhancement of 3.6%. For both matrices, the matrix effect was more evident at lower concentrations. However, even the 19% suppression observed for supernatant at 50 ng/mL is not considered high if compared to what is usually reported for more complex matrices such as plasma (26%) and tissues (29%) (Balvers *et al.*, 2009; Ottria *et al.*, 2014). Since most published studies reporting extraction and quantification of PEA and related NAEs in cells use methods that were developed and validated for more complex matrices such as plasma and tissues (Petrosino *et al.*, 2016; Alhouayek *et al.*, 2017, 2019; Gabrielsson *et al.*, 2017), direct comparison of matrix effect of cell culture media on PEA ionization becomes challenging. It is usually accepted that matrix effects results from the ionization competition between the different

compounds co-eluting from the column (Souverain *et al.*, 2004), leading to enhancement or suppression of the analyte ionization. Therefore, it can be expected that the matrix effect will be more evident at lower analyte concentrations, as it was seen in this study. Even though the matrix effect on d4-PEA ionization was not as significant as what it is usually reported for plasma and tissues, all calibration curves for d4-PEA were prepared in the respective matrices to account for the calculated suppression.

4.3.2 Chemical stability of PEA and d4-PEA in cell culture media

A stability time-course graph was generated for PEA and d4-PEA in cell culture media at 37°C, starting at 1h, to determine when the compound starts being chemically degraded and to establish optimum incubation times for following experiments. PEA concentrations remained stable up to 3h incubation (Figure 32A), followed by a 9% decrease at 8h. At 12h, 82% of initial PEA was still present and at 24h, 70% of PEA was still found. For d4-PEA 89% and 78% d4-PEA was still present in the media after 5h and 12h, respectively (Figure 32B). Vacondio *et al.*, (2015) determined chemical stability of PEA in PBS (pH = 7.4) and found 95% to still be present after 6h incubation at 37°C, although no data was available for longer incubation times. For further experiments, the 3h time-point was selected to capture effects before any significant chemical degradation.
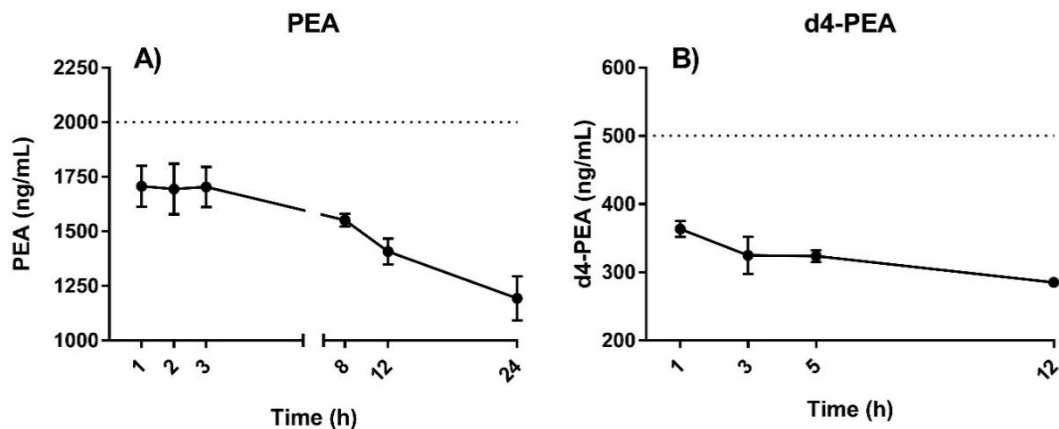
Figure 32. Stability of A) PEA for up to 24h and B) d4-PEA for up to 12h in cell culture media. PEA and d4-PEA were spiked at 2000 ng/mL and 500 ng/mL, respectively. Values are means ± SD of three replicates.

4.3.3 PEA concentration in cell culture supernatants

After determining stability of PEA in cell culture media alone, the PEA concentration in cell culture supernatants was also investigated. Upon treatment of cells with 2000 ng/mL of PEA and incubation at 37°C for up to 3h, a 11.6% decrease in PEA concentration in the supernatant was seen after 30 minutes (Figure 33). From 1 to 2h, concentrations remained stable, followed by another decrease at 3 hours. At this time-point, 58.6% of the initial PEA concentration was still found in cell culture supernatant. Compared to PEA incubated for 3h in the absence of cells, there was a 36.7% loss. Since both PEA-degrading enzymes (NAAA and FAAH) are intracellular, PEA needs to be transported into the cell before it can be hydrolyzed and inactivated (Bisogno *et al.*, 1997; Fowler and Jacobsson, 2002). Therefore, the 36.7% loss observed in the presence of cells may be attributed to cellular uptake.

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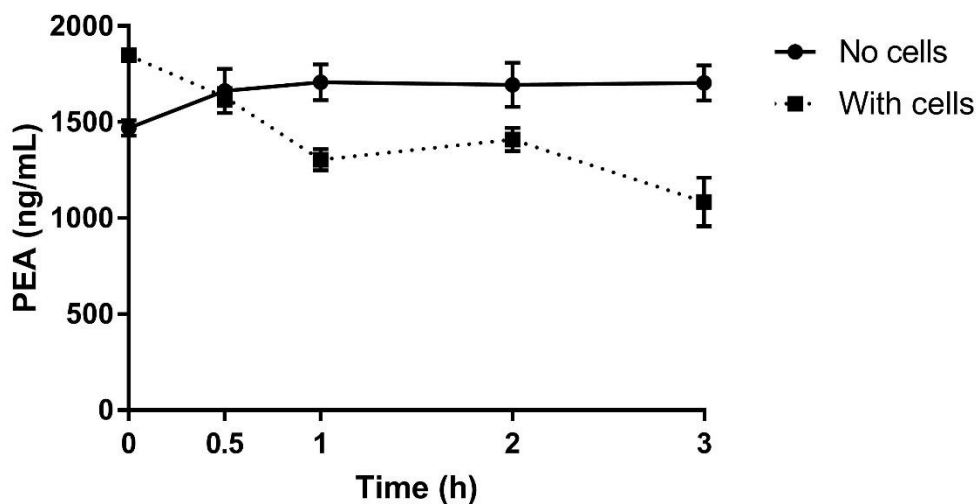
Figure 33. PEA concentration in cell culture media in the presence and absence of RAW264.7 cells. PEA was added at a final concentration of 2000 ng/mL and incubated for up to 3h min at 37°C. Values are means ± SD of three replicates.

4.3.4 PEA and d4-PEA concentrations after simultaneous treatment of RAW264.7 cells with d4-PEA, quercetin and curcuminoids extract.

To determine the amount of d4-PEA found inside cells after treatment and if the presence of quercetin and curcuminoids extract interferes with the cellular uptake and metabolism, the concentrations of d4-PEA in the supernatant and inside the cells was investigated after 3h incubation. After incubation with d4-PEA alone, 30% of initially added compound was still found in supernatant (Figure 34). A small part of this 70% loss may be accounted to chemical degradation in media, as observed in time-course stability (Figure 32B), while most of the PEA decrease from supernatant is believed to be due to cellular uptake. d4-PEA levels in the supernatant were the same for all

treatments, indicating that the presence of quercetin and curcuminoids did not lead to any chemical degradation. Intracellular d4-PEA concentration after 3h incubation was 0.66 ± 0.10 ng/mL/mg of cells (Figure 34). PEA-hydrolyzing enzymes are believed to metabolize PEA immediately upon cellular absorption and may relate to a maintenance of homeostatic balance (Alhouayek and Muccioli, 2014; Bottemanne *et al.*, 2018). Thus, there is a simultaneous process of d4-PEA being absorbed by the cells and the compounds being enzymatically metabolized into palmitic acid and ethanolamine so the net intracellular PEA determination is difficult to determine.
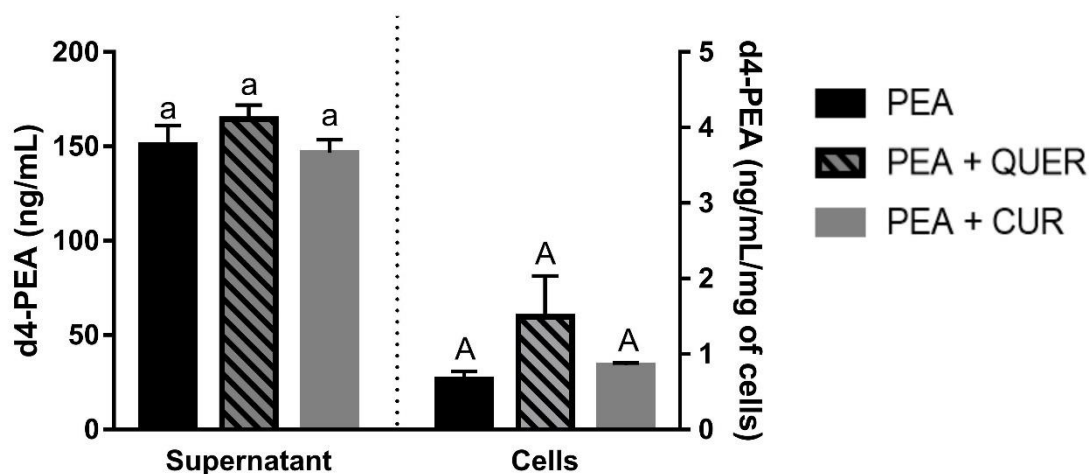
Figure 34. Concentration of d4-PEA in cell culture supernatant (left y-axis) and inside cells (right y-axis) after 3h incubation (37°C) with 500 ng/mL d4-PEA or d4-PEA combined with quercetin and curcuminoids extract. Same letters within groups indicates no statistical difference among treatments (p < 0.05; ANOVA-Tukey). Values are means ± SD of three replicates.

When cells were treated with a combination of PEA+QUER at 500 ng/mL of each compound, or with PEA+CUR at the same concentrations, no difference was observed on d4-PEA concentrations in the cell culture supernatant or inside the cells

(Figure 34). These results indicated there was no influence of co-incubation of d4-PEA with quercetin and curcuminoids in the amount of PEA that is found in the cells, at least after 3h. This initial experiment aimed at determining if after an established incubation time, the presence of quercetin and curcuminoids extract would change the intracellular d4-PEA concentrations. Since neither the extracellular or intracellular concentrations changed, at this point, it is not possible to determine if there is an effect of the polyphenols on the PEA transport into the cells or in the rate of hydrolysis by endogenous enzymes. To further clarify these questions, experiments using enzyme inhibitors should be conducted, to determine if the presence of polyphenols is able to increase intracellular PEA by interfering with PEA diffusion and transport. Given the challenge that is measuring intracellular PEA due to rapid hydrolysis, assays evaluating enzymatic activity of FAAH and NAAA in the presence of these polyphenols may also provide information about weather combined treatment is able to reduce PEA hydrolysis and consequently increase intracellular PEA levels.

In this study, endogenous PEA concentration in untreated cells was 0.13 ± 0.01 ng/mL/mg of cells and these concentrations did not significantly change upon treatment with d4-PEA or d4-PEA-polyphenol combinations (Figure 35). Although no published studies reporting effect of polyphenols on endogenous PEA levels were found, a study from Thors *et al.*, (2008) reported the flavonol kaempferol to have inhibitory effect on FAAH and to reduce hydrolysis of anadamide, a PEA congener. Additionally, Alhouayek *et al.*, (2017a) reported that upon treatment of J774 macrophages with synthetic inhibitors of FAAH and NAAA for 8h, endogenous PEA levels doubled,

demonstrating that upon inhibition of hydrolyzing enzymes, it is possible to obtain higher intracellular PEA levels. As a result, anti-inflammatory effects of PEA were potentiated (Alhouayek *et al.*, 2017), even without exogenous PEA addition. Further investigation of the potential effect of different polyphenols on inhibition of PEA degradation are of interest, given they have already been shown to exert anti-inflammatory effects and their use is considered to be safe.
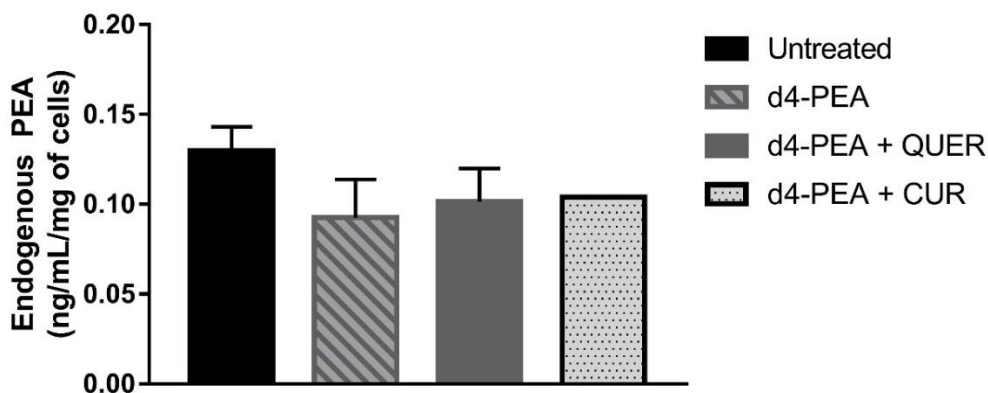
Figure 35. Endogenous PEA concentrations in untreated and treated RAW264.7 cells. Cells were incubated at 37°C for 3h with or without treatments (500 ng/mL of each compound). Concentrations were not statistically different from each other (ANOVA-Tukey, $p < 0.05$). Values are means ± SD of three replicates.

4.4 Conclusions

The present pilot investigation work described some method development parameters used for extraction and quantification of PEA in cell culture media and cell lysates and basic characteristics of PEA stability and uptake in a cell culture system.

Acetonitrile was better than methanol to extract PEA from cell culture media, and maximum recovery of PEA from supernatant was 76.49%. The highest matrix effect observed was 19% for cell supernatant at 50 ng/mL, which is lower than what it is usually reported for other complex matrices. When evaluating stability in media, 82% (PEA) and 78% (d4-PEA) of initial concentrations were still found in the supernatant after 12h, indicating good chemical stability. After 3h incubation in the presence of cells, there was a 37% difference between d4-PEA concentrations in media only versus cell culture supernatant, suggesting cellular uptake. In addition, d4-PEA levels in the supernatant were the same for all treatments, demonstrating that the presence of quercetin and curcuminoids did not lead to any chemical degradation. Intracellular d4-PEA concentrations were also not affected by simultaneous treatment with quercetin and curcuminoids extract. Endogenous PEA levels were not altered by the presence the polyphenols and d4-PEA. At this point, it was not possible to determine if there is an effect of the polyphenols on the actual transport into the cells or in the rate of hydrolysis by endogenous enzymes. Further experiments should focus on the use of PEA-degrading enzyme inhibitors to determine total PEA uptake by cells, as well as on enzymatic assays to determine if there is any effect of the studies polyphenols in enzyme activity. These investigations will allow to determine if the enhanced effects seen when RAW264.7 cells are treated with a combination between PEA, quercetin and curcuminoids arise solely from pharmacodynamic mechanisms or if these polyphenols have any effect on uptake and pharmacokinetic parameters.

CHAPTER V

IN VITRO ANTI-INFLAMMATORY ACTIVITY AND EFFECTS OF A DIETARY

SUPPLEMENT CONTAINING PALMITOYLETHANOLAMIDE, QUERCETIN AND

CURCUMINOIDS ON INFLAMMATION AND CLINICAL SIGNS OF

OSTEOARTHRITIS IN DOGS

5.1 Introduction

Osteoarthritis (OA) is a chronic, degenerative and inflammatory joint disease that affects humans and animals worldwide. It is estimated that 20% of dogs over the age of one suffer from OA (Comblain *et al.*, 2017). The pathogenesis of OA is characterized by cartilage breakdown and synovial inflammation with clinical symptoms including joint pain, swelling, stiffness, synovitis and inflammatory pain resulting in functional disability and reduced quality of life (Goldring and Otero, 2014). More recently, OA is no longer being seen as a non-inflammatory degenerative disease resulting from normal bodily wear or acute injury, but as a disorder in which chronic low-grade inflammation has a central role in its pathogenesis (Robinson *et al.*, 2016). To date, there is no cure for OA, therefore, early intervention and a multimodal approach have the greatest potential to disrupt or delay the progressive deterioration and provide effective disease management.

The current standard of care for OA in companion animals centers on the continuous use of non-steroidal anti-inflammatory drugs (NSAIDs) such as carprofen,

meloxicam and firocoxib for attenuation of clinical signs (Laev and Salakhutdinov, 2015). However, their continuous use often leads to drug tolerance and increased incidence of adverse effects such as gastro-intestinal bleeding and ulceration (Innes *et al.*, 2010). Therefore, there is an increasing interest in identifying safer and more efficient alternatives or adjuncts to NSAIDs for OA management.

Clinical and pilot trials evaluating nutraceuticals for improvement of OA in dogs often reveal promising outcomes, although clinical efficacy is still variable (Scott *et al.*, 2017). Multicomponent formulations with multiple targets are frequently explored, in the attempt to elicit optimal activity and potentially reach synergistic interactions (Alves *et al.*, 2017; Comblain *et al.*, 2017; Martinez *et al.*, 2017; Scott *et al.*, 2017). Curcuminoids from turmeric (*Curcuma longa*) are often included as an ingredient in nutraceutical formulations for OA due to reported anti-inflammatory activities and radical scavenging properties (Colitti *et al.*, 2012; Moreau *et al.*, 2014; Panahi *et al.*, 2016; Sgorlon *et al.*, 2016; Musco *et al.*, 2019). Similarly, quercetin is being used as an anti-inflammatory ingredient in nutraceutical formulations for OA in dogs (Britti *et al.*, 2017; Crovace *et al.*, 2017). Quercetin has been shown to weaken the oxidative stress and to mitigate the degradation of extracellular matrix of the cartilage in rabbits (Wei *et al.*, 2019), down-regulate pro-inflammatory cytokines IL-1b (interleukin 1 beta) and TNF-α (tumor-necrosis factor alpha) production via the Toll-like receptor-4/ Nuclear Factor kappa beta (TLR-4/NF-kB) pathway (Zhang *et al.*, 2019), as well as to exert immunomodulatory effects in synovial macrophages in a rat model of OA (Hu *et al.*, 2019). Based on the reported effects of the use of quercetin and curcuminoids in dietary

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supplements together with other herbals and natural compounds, there is potential to explore both polyphenols together in novel formulations, potentially including other less explored compounds.

Palmitoylethanolamide (PEA) is an endogenous fatty acid amide with anti-inflammatory properties, belonging to the family of the N-acylethanolamines (NAEs) (Lo Verme *et al.*, 2005). PEA has emerged as a disease-modifying agent in several conditions, including joint pain and osteoarthritis (Marini *et al.*, 2012; Impellizzeri *et al.*, 2013; Bartolucci *et al.*, 2018). Multiple mechanisms of action have been proposed for PEA (Petrosino and Di Marzo, 2016) and the direct activation of the peroxisome proliferator-activated receptor-α (PPAR-α) is believed to be the main mechanism through which PEA exerts its anti-inflammatory activity. Activation of PPAR-α leads to downregulation of nuclear-factor kB (NF-kB) and subsequent downstream signaling cascades (Daynes and Jones, 2002). Studies investigating the potential anti-inflammatory and pain relieving effects of PEA in dogs is currently limited to skin diseases such as atopic dermatitis and cutaneous allergic inflammatory responses (Cerrato *et al.*, 2012; Abramo *et al.*, 2014; Noli *et al.*, 2015). It's effect in joint inflammation and osteoarthritis has been reported in human and other animal models. PEA was shown to significantly reduce temporomandibular joint (TMJ) damage, pain and macrophage activation in rats upon administration of micronized PEA (10 mg/kg) (Bartolucci *et al.*, 2018). A human clinical trial comparing the effects of PEA versus ibuprofen for pain relief in temporomandibular joint (TMJ) osteoarthritis demonstrated superior pain reduction in subjects taking PEA. Treatment with PEA (10 mg/kg) or PEA + luteolin (1 mg/kg)

improved clinical signs of Type II collagen-induced arthritis (CIA) in mice and additionally, the treatment was able to significantly reduce levels of TNF-α, IL-1b, and IL-6 (Impellizzeri *et al.*, 2013). Nevertheless, the precise effect of PEA and the contribution of other compounds in joint disease-related pain and inflammation remains to be fully understood.

Apart from a study that has tested a co-ultramicronized formulation containing PEA and quercetin in a carrageenan-induced paw edema model in rats (Britti *et al.*, 2017), there are no reports of combination of PEA with quercetin or curcuminoids available and more importantly, the effect of a combined formulation using PEA and polyphenols has not been investigated in canine osteoarthritis. To date, this study is the first to assess the effects of PEA in dogs with osteoarthritis and moreover, the first to propose its combination with polyphenols for this purpose.

Considering that OA is a multimodal disease that involves both the inflammatory response and oxidative stress of joint tissues, we hypothesized that a dietary supplement combining PEA, quercetin and curcuminoids extract may represent a potential adjunct to managing associated pain and inflammation in canine OA. Therefore, the aim of this case study was to evaluate the effects of the oral consumption of a dietary supplement containing PEA, quercetin and curcuminoids in reducing inflammation and clinical signs of osteoarthritis in six family-owned companion dogs.

5.2 Materials and Methods

5.2.1 Dietary supplement formulation and dosage

The dietary supplement formulation used in this pilot study was developed based on in vitro experiments in RAW264.7 cells (shown in Chapter III) that exhibited a potentiated anti-inflammatory effect upon combination of palmitoylethanolamide (PEA) and quercetin and/or PEA with a curcuminoid extract (95%). The formulation used in this study consisted of a mixture of PEA, curcuminoids, and quercetin dihydrate (PCQ) at a 5:2:1 ratio that was mixed and bottled as a powder (PCQ + Ultra (TM) (PCQ), Vital Pet Sciences, Ojai, California). All ingredients conform to dietary supplement or food standards according to FDA stipulations, which is that they must be safe for human consumption and free of any unreasonable hazard.

5.2.2 Cell culture experiments

The combined formulation containing PCQ was evaluated in RAW 264.7 murine macrophage cells to determine cytotoxicity by the Resazurin method, ability to prevent reactive oxygen species (ROS) production, by using the DCFDA as fluorescent probe, and release of nitric oxide, by the Griess method (detailed procedures are described in sections 3.2.4 and 3.2.5, Chapter III). The anti-inflammatory activity of the PCQ formulation against expression of proinflammatory cytokines was also tested by PCR.

All in vitro experiments were conducted as described in the LPS-induced inflammatory

assay section in Chapter III and results are presented in the following Results and

Discussion section, under Part I.

5.2.3 Dietary Supplementation Dosage

A double-sided measuring scoop and an instruction sheet was provided to dog

owners on how to administer the supplement in the dog's food. For the first two weeks,

the total loading dose was 10 mg PCQ/lb a day, given as 5mg PCQ/lb twice per day. For

the following 6 weeks, a maintenance dose of 5 mg/lb was given once daily. Table 7

below contains the appropriate dose based on the animal's weight. A heaping small

scoop described in the table has 60 mg of PCQ, while a heaping large has 180 mg of

PCQ.

Table 7. Instructions to owners on dosing the dietary supplement based on the animal's weight

Weight of your Dog	Loading Dose for 14 days	Maintenance Dose starting after 14 days
Under 12 lbs	1 heaping small scoop **twice daily**	1 heaping small scoop **once daily**
12 – 23.9 lbs	2 heaping small scoop **twice daily**	2 heaping small scoop **once daily**
24 – 35.9 lbs	1 heaping large scoop **twice daily**	1 heaping large scoop **once daily**
36- 47.9 lbs	1 heaping small scoop + 1 heaping large scoop **twice daily**	1 heaping small scoop + 1 heaping large scoop **once daily**
48- 59.9 lbs	2 heaping small scoop + 1 heaping large scoop **twice daily**	2 heaping small scoop + 1 heaping large scoop **once daily**
60 – 71.9 lbs	2 heaping large scoop **twice daily**	2 heaping large scoop **once daily**
72-111.9 lbs	3 heaping large scoops **twice daily**	3 heaping large scoops **once daily**
112 lbs and more	3 heaping large scoops+ 1 level large scoop **twice daily**	3 heaping large scoops + 1 level large scoop **once daily**

Dog owners did not incur any costs for participation and were provided with a supply of the test dietary supplement during the study.

5.2.4 Pilot Study Design

This pilot study has been approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC) (TAMU IACUC# 2018-0193 CA). All dog owners received a detailed description of the protocol and signed and informed consent

form prior to the screening process. In this study, a formulation containing palmitoylethanolamide (PEA), curcuminoids extract and quercetin (5:2:1) was given as a dietary supplement to dogs (n = 6) affected by osteoarthritis (OA) over an 8-week period. The dietary supplement was given together with regular dog food. Assessments were done at baseline (day 0) and after 8 weeks of supplementation.

5.2.5 Inclusion/Exclusion criteria

The study population consisted of free-living dogs in a home-setting that were initially screened at the Texas A&M University Veterinary Clinics for evaluation of osteoarthritis. Dogs of any breed were considered for inclusion in the study if they had a minimum body weight of 20 kg (44 lbs), were between 2 and 10 years of age, exhibited consistent and persistent signs of OA in the shoulder, elbow, hip joint and/or knee, with one joint obviously more affected than others. The inclusion criteria were determined based on each dog's medical history, a clinical examination, and radiographs of the joints. Dogs were also subjected to a force plate gait analysis at the screening visit to determine if they presented weight bearing differences between left and right sides. Dogs with <6% difference were not enrolled in the study. Exclusion criteria was based on e dogs that were receiving or have received treatment with NSAIDs within 2 weeks of enrollment, were receiving or have received a glucosamine-based joint supplement, or prescription joint diet within 1 month of enrollment, were pregnant, had neurological or musculoskeletal abnormalities, have had orthopedic surgery performed within 1 year of

enrollment, had an increased risk for adverse events during sedation (heart and kidney disease), and those that appeared overly stressed or exhibit aggressive behavior with physical screening.

5.2.6 Physical/Orthopedic Examination and Radiographic Analysis

A general physical examination followed by a complete orthopedic examination were performed by a board-certified veterinary orthopedic surgeon at the screening visit and after the 8-week intervention to allow for evaluation of the affected joint(s). Radiographic examination of the most affected joint was performed under sedation.

5.2.7 Canine Brief Pain Inventory (CBPI) questionnaire

The Canine Brief Pain Inventory (CBPI) is a validated questionnaire that allows owners to rate the severity of their dog's pain and the degree to which that pain interferes with daily function. The first four items access the pain intensity in the dogs during the last seven days. Zero indicates "no pain" and 10 represents "extreme pain". The remaining six items covers the degree to which the owners rate the pain interference with function for their dog (Brown *et al.*, 2009). Owners were asked to complete the CBPI questionnaire addressing the dog's osteoarthritis pain and function prior to enrolment and at the follow-up visit after 8 weeks of supplementation. The same owner was asked to complete the questionnaire at each time-point.

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5.2.8 Force plate gait analysis

Kinetic analysis was performed prior to treatment (baseline) and 8 weeks post-treatment. Gait analysis was performed on a force platform embedded in a runway (Advanced Medical Technology Inc, Newtown, MA, USA) in a dedicated canine gait lab. Dogs were acclimated and trained to trot across the force platform prior to data acquisition. All dogs were evaluated at a trot. Data logging was triggered by a force of 5N on the force platform. Five successful trials at a velocity of 1.7-2.1 m/s and acceleration of 0 ± 0.5 m/s^2 were recorded for each limb. Data generated were peak vertical force (PVF) (Newtons/Newtons $_{Body\ Weight}$) and vertical impulse (VI) (Newtons per second/Newtons $_{Body\ Weight}$). The mean value from the five successful trials for each limb were determined for each limb. Due to the fact that PVF and VI values vary substantially between thoracic and pelvic limbs and that fact that enrollment criteria for the present study allowed for the assessment of either thoracic or pelvic limbs, each animal's 8-week PVF and VI data were compared to the pre-treatment values using a "percent change from baseline" method so as to assess a response to treatment across all enrolled dogs.

5.2.9 Synovial fluid (SF) collection and analysis

For joint fluid collection, dogs were sedated with a combination of dexmedetomidine (125 mcg/m²) and either hydromorphone (0.1 mg/kg) or methadone

(0.2 mg/kg) administered IV. Joint fluid was aspirated under aseptic conditions from the affected joints. Samples were submitted to the clinical pathology laboratory for evaluation of joint fluid by automated cell counts. If sufficient quantities were obtained, joint fluid differential cell counts were performed. Smears were prepared by Giemsa staining and percentages of large mononuclear cells (macrophages), small mononuclear cells (lymphocytes), and neutrophils were determined by a licensed clinical pathologist.

5.2.10 Blood collection and analyses

Blood samples were collected from the jugular vein at the baseline visit and after the 8-week intervention. Ten to 12 milliliters of blood were transferred into tubes lithium heparin and EDTA for routine chemistry and hematology, and to tubes with no anticoagulant for serum preparation. Tubes for serum preparation were allowed to incubate at room temperature for 30 minutes prior to centrifugation for 10 minutes at 3000 rpm. The supernatant (serum) was transferred to 1.7 ml tubes and stored at -80C for synovial fluid cytology, differential cell count and analysis of inflammatory markers. Routine hematology (complete blood count and differential) and chemistry panel were performed on anticoagulated blood.

5.2.11 Multiplex cytokine immunoassay and ELISA

A Milliplex® MAP magnetic bead panel based on Luminex® xMAP® technology (CCYTOMAG-90K, Millipore) was used to measure 5 different cytokines in canine serum: granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFNγ), tumor necrosis factor (TNF)-α, interleukin 6 (IL-6), interleukin 10 (IL-10) and the chemokine monocyte chemoattractant protein-1 (MCP-1). The method was performed according to the manufacturer's instructions using a Luminex L200 analyzer system and data was analyzed using the Luminex xPONENT® software version 3.1 (Luminex Corporation, Austin, TX, USA). The observed concentration of each analyte for each sample was calculated using a standard curve generated from six standards and a blank provided in the kit. The minimum detectable concentrations in pg/ml according to the manufacturer were 9.2 (GM-CSF), 3.7 (IL-6), 8.5 (IL-10), 6.1 (TNF-α), 21 (MCP-1) and 10.5 (IFN-γ). Levels of interleukin 1 beta (Il-1b) were measured with a canine-specific ELISA kit (Invitrogen, Carlsbad, CA, USA) with analytical sensitivity of 10 pg/mL.

5.2.12 Statistical analysis

Data were analyzed by paired t-tests or nonparametric Wilcoxon sign rank test, one-way (ANOVA) with Sidak's posttest using GraphPad Prism 6.0 (GraphPad

Software, La Jolla, CA, USA), according to specific experiments. Data were considered significantly different when $p < 0.05$.

5.3 Results and Discussion

PART I – In vitro anti-inflammatory activity of a multicomponent dietary supplement formulated with PCQ.

5.3.1 Effect of PCQ formulation on cell viability of RAW264.7 macrophages

Based on the favorable results obtained with the combination of P+Q and P+C against inflammation in vitro (Chapter III), a formulation containing the three bioactive compounds was developed. PEA, curcuminoids extract and quercetin dihydrate were combined at a ratio of 5:2:1, in the form of a loose powder, without any other excipients, to be used in the in vivo portion of this study. This formulation was also tested in vitro to access cytotoxicity against RAW264.7 macrophages, prevention of ROS formation and NO release, as well as the effective concentrations needed to reduce the expression of selected pro-inflammatory markers. The treatments were prepared by dilution of the stock solution with cell culture media (DMEM), resulting in the concentrations presented in Table 8.

Table 8. Final concentrations of each individual compound in the mixed powder treatments (µg/mL)

PCQ formulation concentration	PEA	Curcuminoids Extract	Quercetin
0.4	0.25	0.1	0.05
0.8	0.5	0.2	0.1
1.6	1.0	0.4	0.2
2.4	1.5	0.6	0.3
3.2	2.0	0.8	0.4
4.0	2.5	1.0	0.5
4.8	3.0	1.2	0.6

Although PEA, quercetin and curcuminoids alone did not elicit any cytotoxicity against RAW264.7 cells at concentrations up to 3 µg/mL (reported in Chapter III), the effect of combined formulation was also tested to ensure treatment does not reduce cell viability. Treatment with PCQ for 24h in the presence of LPS did not change cell viability of RAW264.7 cells up to the concentration of 4.8 µg/mL. At 5.5 µg/mL, cell viability was reduced to 72% ($\pm$4.78) and therefore was not used for further experiments (Figure 36).
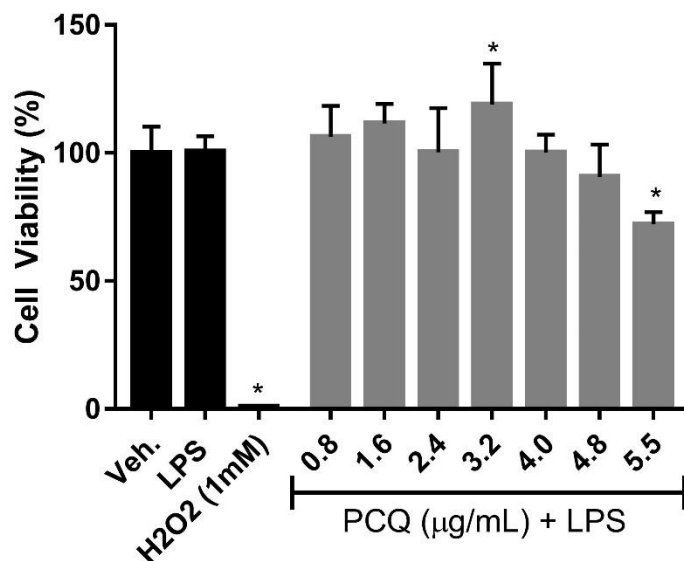
Figure 36. Cell viability of RAW264.7 cells pretreated treated for 1h with the PCQ formulation followed by LPS stimulation (10 ng/mL) for 24h. * indicate significant difference from Veh. ($p < 0.002$; ANOVA-Dunnett). Values are means ± SD of seven replicates.

5.3.2 Effect of PCQ formulation in Reactive Oxygen Species (ROS) production and nitric oxide (NO) release in RAW264.7 cells

Increased intracellular ROS production may contribute to activation of NF-kB pathway and further release of downstream associated proinflammatory biomarkers (Forrester *et al.*, 2018). Therefore, the ability of the combined formulation to reduce ROS production and nitric oxide release may be an important factor related to its potential to act via different mechanisms to downregulate inflammatory pathways. During the first 4.5h of exposure to LPS + treatments, there was a steady increase in ROS production for all treatments (Figure 37A), with LPS starting to be significantly

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higher than vehicle control after 240 minutes. Starting at 150 minutes, all tested

concentrations of PCQ were also significantly lower than LPS, remaining lower until

270 minutes. There was no difference among different concentrations tested.
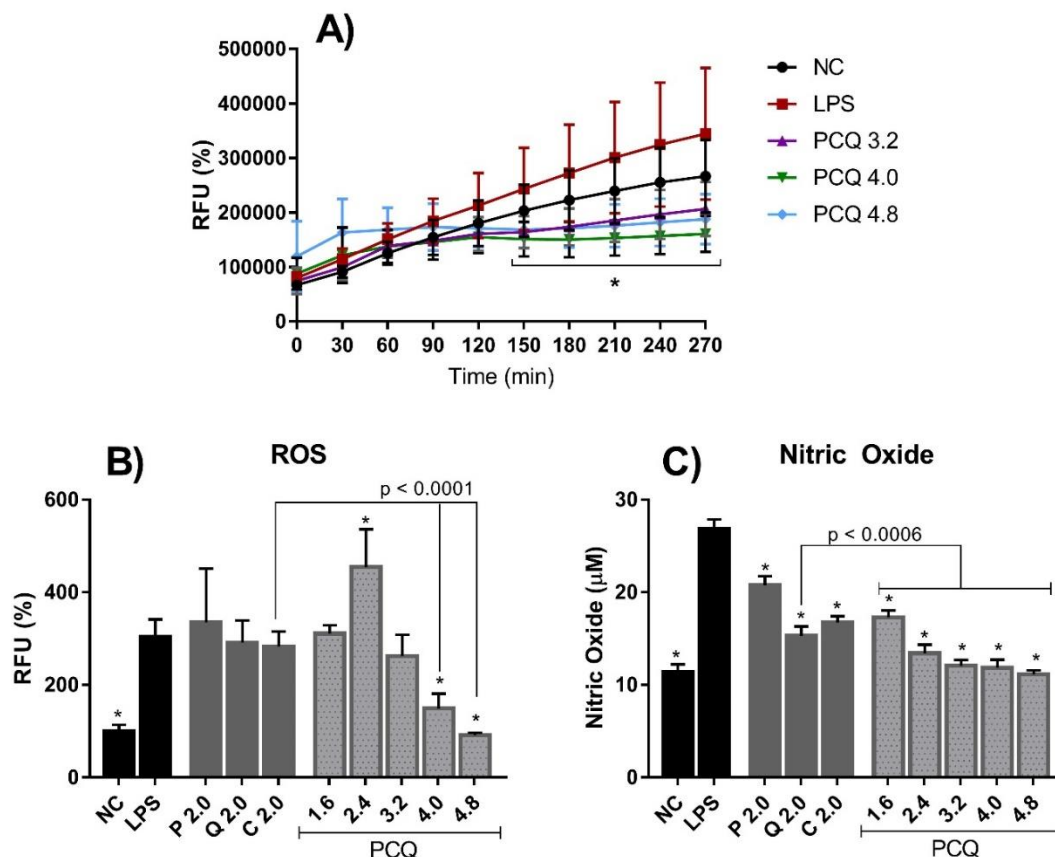
Figure 37. Effect of different PCQ formulation concentrations on LPS-induced (20 ng/mL) ROS generation and NO release in RAW264.7 cells. A) Intracellular reactive oxygen species (ROS) production up to 270 minutes of treatment with compounds + LPS (20 ng/mL) simultaneously. B) Intracellular ROS production and C) NO release after 1h pre-treatment with compounds + 23h LPS (20 ng/mL) stimulation. RFU = Relative Fluorescence Units. (*) indicate significance compared to LPS at $p < 0.05$ (ANOVA- Dunnett); Indicated p values are significant by ANOVA-Sidak multiple comparison. Values are means ± SD of 7 replicates.

Results from previous experiments in this cell line showed that the peak ROS production upon LPS stimulation occurs from 18 to 24h (chapter III, Figure 11). Therefore, ROS production and NO release were also evaluated after 24h of incubation (Figure 37B and C). Positive control (cells challenged with 20 ng/mL LPS) showed a significant increase in ROS production and NO release. There was no effect of PEA at 2 µg/mL on ROS production. PCQ at 4.0 and 4.8 µg/mL were able to mitigate increase in ROS production compared to LPS (51% and 70% reduction, respectively). These concentrations also resulted in 47% and 68% reduction in ROS compared to 2 µg/mL of curcuminoids alone. On the other hand, the effect in NO release was seen at concentrations as low as 1.6 µg/mL, with a 36% reduction when compared to LPS, and all tested concentrations were more effective than quercetin treatment alone ($p < 0.0006$). These results indicate effectiveness of the PCQ formulation in the reduction in NO release.

It is well established that quercetin and curcuminoids exert antioxidant effects in vitro and in vivo, contributing to oxidative stress control. This occurs through modulation of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX) and by increasing protein levels of Nrf2, as well as direct radical scavenging properties (Granado-Serrano *et al.*, 2012; Lin *et al.*, 2019). Considering the anti-inflammatory and antioxidant activity of quercetin and curcuminoids and the results obtained herein, the combination of PEA with these polyphenols is a potential way to achieve a multi-target approach to mitigating activation of proinflammatory pathways.

5.3.3 Effects of PCQ on the expression of inflammatory markers in RAW264.7 cells

The combined formulation was effective in reducing the mRNA expression of Il-1b, TNF-α, Cox-2, iNOS, and IL-10 in a dose-dependent manner as shown in Figure 38. For Il-1b, which is the biomarker previoulsy selected to confirm synergistic effect due to it's consistent results, the minimal effective concentration was 2.4 µg/mL of mixed powder (P 1.5: C 0.6: Q 0.3 µg/mL), which reduced 42% of the mRNA expression. Reduction in TNF-α and Cox-2 was also observed.
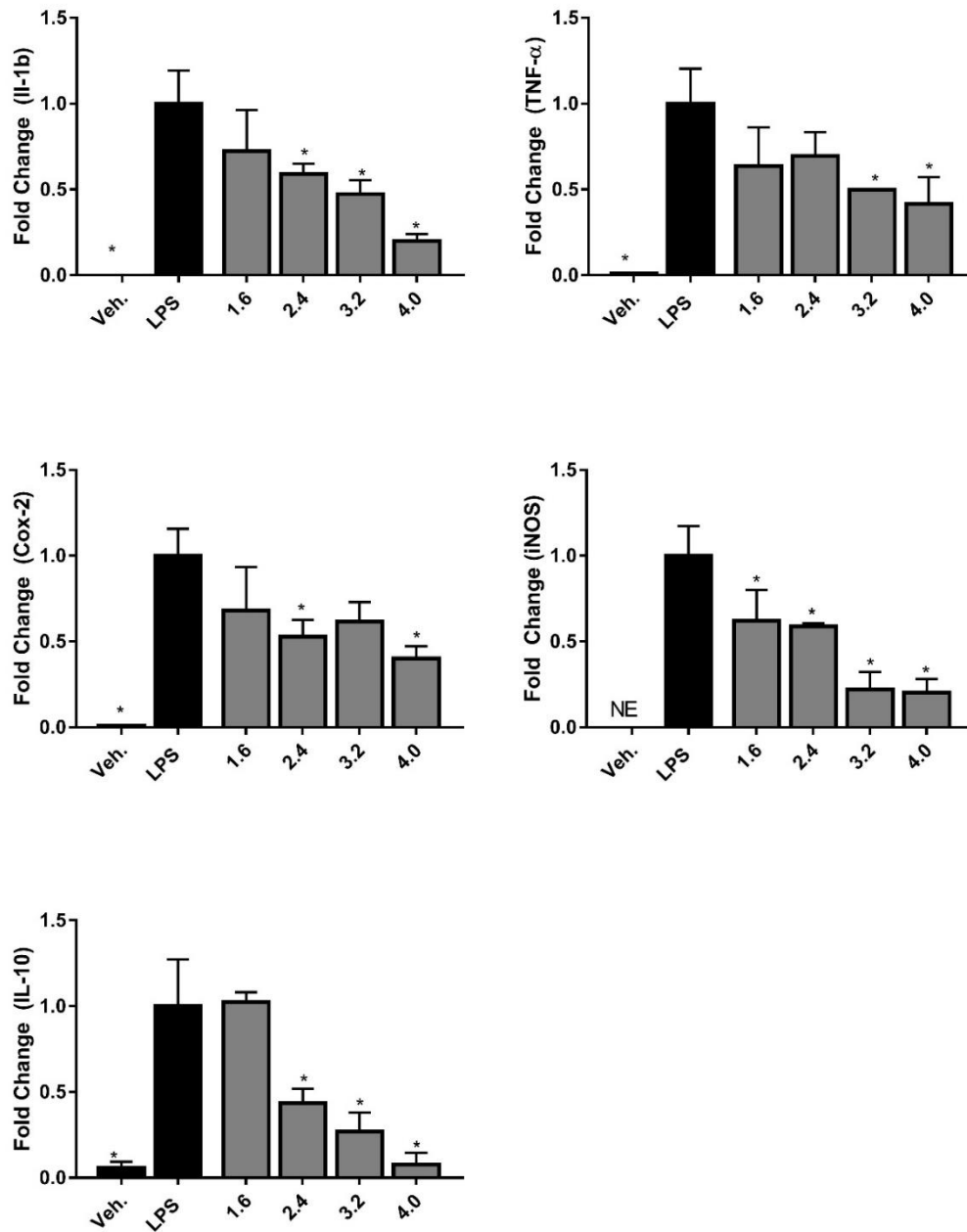
Figure 38. mRNA expression of Il-1b, TNF-α, Cox-2, iNOS and IL-10 in LPS-activated (10ng/mL) murine macrophages (RAW264.7) treated with increasing concentrations (µg/mL) PCQ formulation for 9 hours. (Veh.= 0.1% DMSO; NE = not expressed; * significantly different from LPS at $p < 0.05$; ANOVA-Dunnett's Test). Data are means ± SD of three replicates.

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Nitric oxide synthase (NOS) is an enzyme that can produce nitric oxide (NO) from L-arginine. The inducible form of the enzyme (iNOS), is induced by bacterial products and inflammatory cytokines in macrophages and several other cells (Kim *et al.*, 2017). Although NO exerts important role under normal phyiological conditions, high expression and activity during the inflammatory response can lead to excessive NO release, promoting oxidative stress and tissue injury (Wang and Mazza, 2002). Expression of iNOS was significantly decreased upon treatment with all tested concentrations, compared to the LPS-treated positive control (Figure 38). The lowest concentration (1.6 µg/mL) was able to significanlty reduce 38% of iNOS mRNA expression. This is consistent with the reduction in nitric oxide release observed in cells treated for 24h with the same formulation (Figure 37C). All the single compounds present in the formulation have the ability to reduce iNOS expression and consequently the NO release in vitro (Ross *et al.*, 2000; Bisht *et al.*, 2010; Kim *et al.*, 2017). Results from this study confirmed that the combined formulation developed is able to reduce iNOS expression and NO release.

LPS stimulation increased expression of IL-10 in RAW264.7 cells. Macrophages are a major source of this anti-inflammatory cytokine, which is generated in response to Toll-like receptor (TLR) signaling as feedback mechanism to restrict the inflammatory response (Iyer *et al.*, 2010). Studies suggest that the LPS-induced production of IL-10 is mediated by activation of the Akt pathway (Pengal *et al.*, 2006) and that IL-10 secretion reduces production of TNF-α and Il-1b (Mollazadeh *et al.*, 2019). There was no clear association between IL-10 and TNF- α/Il-1b levels in this study. Interestingly, starting at

2.4 µg/mL, PCQ treatments led to a significant reduction in IL-10 expression, counteracting the effect induced by LPS. Quercetin and curcuminoids have been reported to increase expression of this cytokine in RAW264.7 cells (Mollazadeh *et al.*, 2019). During the systemic inflammatory response, the crosstalk between pro- and anti-inflammatory cytokines can be disrupted and from this experiment it is not possible to correlate decreased expression of TNF-α and Il-1b to an increase in IL-10. Overall, the combined formulation used in the pilot trial exerted anti-inflammatory and antioxidant effects in RAW264.7 cells, which allows to hypothesize that the formulation has the potential to exert these effects in vivo, contributing to mitigation of systemic inflammation resulting from of osteoarthritis.

PART II – Effect of PCQ on inflammation and clinical signs of osteoarthritis in companion dogs

5.3.4 Demographics

The study population consisted of six free-living dogs in a home-setting. Table 9 shows the demographic data for the 7 enrolled dogs that finished the 8-week supplementation period. Dog #5 was screened and met study eligibility criteria, but was later diagnosed with an advanced tumor and was excluded from the study. Based on inclusion/exclusion criteria and compliance, Dog #8 was also excluded from analysis

due to compliance failure. The average age and weight of enrolled dogs was 7.6 years

and 29.2 kg.

Table 9. Demographic data of 7 dogs with osteoarthritis initially enrolled in the PCQ
pilot study

Dog #	Breed	Weight (kg)	Age (years)	Gender	Affected Limb
Dog 1	Pitbull	26.5	10	Female	Left elbow
Dog 2	German Shepard	27.6	6	Female	Right hip
Dog 3	Labrador	44.1	4	Male	Right elbow
Dog 4	Boxer	22	8	Female	Right Knee
Dog 6	English Springer Spaniel	20.2	10	Female	Right elbow
Dog 7	Blue Lacy	24.8	6	Male	Right elbow
Dog 8	Boxer	39.4	9	Female	Right knee

5.3.5 Joint fluid analysis

Joint fluid was collected from the enrolled dogs at baseline and follow up visit.

Usually aspiration of joint fluid from dogs yields less than 0.1 to 0.25mL and often,

joints do not offer sufficient SF volume to be collected for analysis (Macwilliams and

Friedrichs, 2003). In those cases, results were reported as *NC (not collected) or *qns

(quantity not sufficient). According to Table 10, the nucleated cell counts for all enrolled

dogs are within the range considered normal ($< 3000/\mu L$) and the red blood cell counts

(RBC) also fell within reference intervals (Raskin and Meyer, 2016; Valenciano and

Cowell, 2020).

Table 10. Joint fluid cytology for 7 enrolled dogs

Dog	Nucleated cell count (/µL)	Nucleated cell count (/µL)	Red Blood Cells (RBC) (/µL)	Red Blood Cells (RBC) (/µL)
	Baseline	Follow-up	Baseline	Follow-up
Dog 1	1400	1593	150000	19494
Dog 2	qns	qns*	qns	qns
Dog 3	1230	qns	bld	qns
Dog 4	986	qns	928	qns
Dog 6	1504	1924	1143	qns
Dog 7	qns	2155	qns	4605
Dog 8	898	1332	1531	2121

*qns: quantity not sufficient; NC: not collected; *bld: below limit of detection

Differential cell counts were also performed when sufficient joint fluid was obtained. According to results (Table 11) and pathology report, there were no cytologic abnormalities and joint fluid for most dogs were considered "normal-appearing". Appearance of normal synovial fluid should be colorless to light yellow and have high viscosity (Valenciano and Cowell, 2020). Per pathologist comments, Dogs 3, 4, 6 and 7 presented a mildly increased proportion of large mononuclear cells, although levels still within normal range. This could be an indicator of degenerative diseases of the joint, which is consistent to their history of osteoarthritis. There were no marked changes on differential cell count in synovial fluid from baseline versus the follow-up visit.

Table 11. Differential cell count in joint fluid

	Large mononuclear cells (macrophages) (%)		Small mononuclear cells (lymphocytes) (%)		Non-degenerate neutrophils (%)	
	Before	**After**	**Before**	**After**	**Before**	**After**
Dog 1	70	76	30	16	-	8
Dog 2	NC	NC	NC	NC	NC	NC
Dog 3	68	70	30	30	2	rare
Dog 4	75	89	25	10	rare	1
Dog 6	89	91	11	8	<1	1
Dog 7	NC	62	NC	19	NC	19
Dog 8	93	64	7	32	-	1

5.3.6 Hematology

The clinical laboratory values for the blood panel were within normal limits during the study period of 8 weeks for most dogs (Table 12) (Willard and Tvedten, 2012). Values that were higher or lower than reference values are indicated in the table. There was no observed effect of dietary supplement on the hematological parameters.

Table 12. Routine hematology - complete blood count (CBC) and differential blood counts

Test Name	Unit	Dog 1		Dog 2		Dog 3	
		Baseline	Follow up	Baseline	Follow up	Baseline	Follow up
WBC	10^3/μl	7.1	6.5	8.1	11.3	8.7	10.4
Red Blood	10^6/μl	7.68	7.89	7.35	7.23	7.59	7.05
Hemoglobin	g/dl	18.2	18.1	18.5	11.2	18.7	17.5
Hematocrit (automated)	%	51.6	52.5	52.1	50.9	53.6	50.2
Packed Cell Volume (Spun)	%	50	50	50	49	53	49
Mean Corpuscular Volume	fl.	67.2	65.6	70.8	70.4	70.6	71.2
Mean Corpuscular Hemoglobin Concentration	g/dl	35.2	34.5	35.5	35.7	34.9	34.9
Plasma Protein	TS-g/dl	7.2	6.8	7	7.1	7.4	7
Platelet Count (automated)	/μl	258000	241000	273000	296000	248000	225000
Segmented Neutrophils	%	67	56 L	77	82 H	53 L	58 L
Absolute Neutrophil		4757	3640	6237	9266	4611	60.32
Lymphocytes	%	22	30	15	10	33	26
Absolute Lymphocyte		1562	1950	1215	1130	2871	27.04
Monocytes	%	4	8	6	6	3	3
Absolute Monocyte		284	520	486	6.78	261	312
Eosinophil	%	7	6	2	2	11	13 H
Absolute Eosinophil		497	390	162	226	957	1352 H

*H = high; L= low

Table 12. Continued

Test Name	Unit	Dog 4		Dog 6		Dog 7	
		Baseline	**Follow up**	**Baseline**	**Follow up**	**Baseline**	**Follow up**
WBC	10^3/µl	7.3	7.2	10.1	9.7	8	6.9
Red Blood	10^6/µl	6.08	6.19	6	6.06	6.39	6.26
Hemoglobin	g/dl	16	15.8	14.9	14.8	14.9	14.5
Hematocrit (automated)	%	44.9	45.4	42.1	43.3	43.2	43.5
Packed Cell Volume (Spun)	%	43	43	40	40	43	41
Mean Corpuscular Volume	fl.	73.9	73.3	70.2	71.4	67.5	69.5
Mean Corpuscular Hemoglobin Concentration	g/dl	35.5	34.7	35.3	34.1	34.6	33.3
Plasma Protein	TS-g/dl	7.1	7.4	7.6	7.4	6.3	6.1
Platelet Count (automated)	/µl	233000	275000	411000	283000	224000	226000
Segmented Neutrophils	%	72	78 H*	85 H	87 H	75	75
Absolute Neutrophil		52.56	5616	8585	8439	6000	5175
Lymphocytes	%	17	14	7 L	6 L	15	17
Absolute Lymphocyte		1241	1008	707 L	582 L	1200	1173
Monocytes	%	6	2 L*	7	4	5	5
Absolute Monocyte		438	144 L	707	388	400	345
Eosinophil	%	5	6	1 L	3	5	3
Absolute Eosinophil		365	432	101	291	400	207

*H = high; L= low

5.3.7 Force Plate Analysis

Force plate gait analysis is a valuable method to obtain objective data on limb loading in dogs (McLaughlin, 2001). In this study, dogs were trotted across the force plates by a single handler and five acceptable trials were obtained from each limb of each dog. Peak vertical force (PVF) is the largest of the orthogonal forces and is the one most commonly evaluated for gait analysis in animals. Vertical impulse (VI) is also commonly reported and is represented by the area under the curve of a force over time graphical representation (McLaughlin, 2001). The mean values of PVF and VI of each dog were derived from the average PVF and VI of the first five valid trials on the most affected limb (specified in Table 9). Due to the fact that enrolled patient's most affected limb could be either a thoracic or pelvic limb, force plate data was converted into unitless values and reported as percent change from baseline (for each dog) to account for differences in thoracic vs. pelvic limb (Figure 39A).
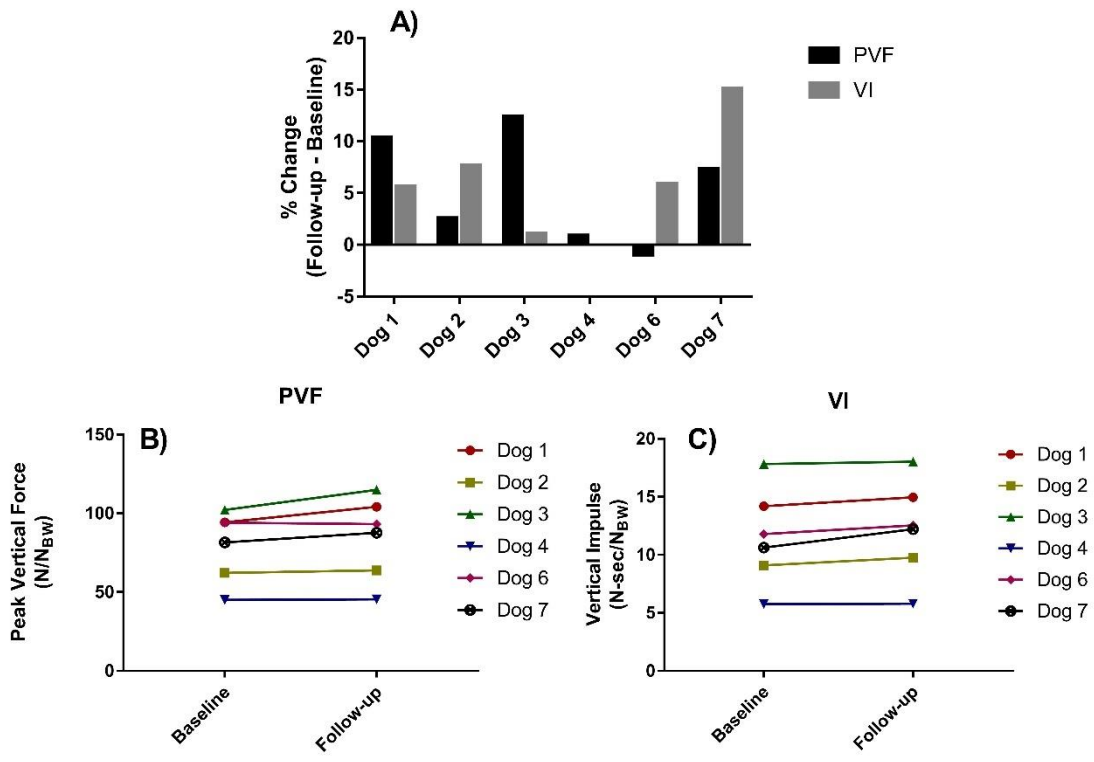
Figure 39. Force plate gait analysis of dogs with osteoarthritis (OA) before and after dietary supplementation with the PCQ formulation for 8 weeks. A) represents the percent (%) change of peak vertical force (PFV) and vertical impulse (VI) of the most affected limb of each dog compared to baseline. B) and C) represents the baseline and follow-up values obtained for PVF and VI in 6 enrolled dogs.

When analyzing each dog individually, different levels of change in PVF and VI measurement were observed and the number of positive responders (positive % difference to baseline) was greater than non-responders, as shown in Figure 39A. There was a positive percent change in 4 out of 6 of dogs for PVF and 5 out of 6 dogs for VI, after 8 weeks taking the supplement. Dog 4 was considered a non-responder, as the percent difference for PVF was close to 0 and there was no difference for VI. Dog 6

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showed a negative response in PVF, although VI increased after 8-week supplementation. Dogs affected by OA and experiencing pain tend to bear less weight on painful limbs, therefore an increase in PFV is an indication of improvement in pain and lameness. Similarly, an increase in VI indicates improvement in the force applied over time. Improvements in raw PVF and VI values can be seen in Figure 39B and C.

Moreau et al., 2014 detected significant increase in PVF in dogs (n = 13) taking a medicinal herb-based natural health product after 8 weeks, although no differences could be seen between treated and placebo-group at the 8-week time-point. On the other hand, Comblain et al., (2017) did not detect differences over time in PVF or VI in dogs (n = 21) taking a curcuminoid-collagen-green tea extract for 3 months. In these studies, even though it was not possible to always identify significant increase in PVF and VI specifically, positive effects observed in other outcome measurements (owner questionnaires and orthopedic evaluations) indicated that supplementation improved clinical signs of osteoarthritis. Based on overall favorable results obtained from this n=6 case study, it is believed that a larger sample size would allow more confidence about the overall outcome.

A limitation of the force plate analysis is that it only evaluates the animals outside their normal environment and at one specific time point. Therefore, it is recommended that researchers use other outcome measures in addition to the force plate. The "Canine Brief Pain Inventory" for example, is a validated questionnaire that quantifies owners' assessment of clinically relevant pain–related behaviors with the dog at its environment and over a larger period of time. Taken together, these different

assessment tools are able to better clarify the benefits of supplements and nutraceuticals in pain, lameness and overall quality of life of dogs with OA.

5.3.8 Canine Brief Pain Inventory (CBPI)

The Canine Brief Pain Inventory (CBPI) was developed as an owner-completed questionnaire designed to quantify the owner's assessment of the severity and impact of chronic pain in their dogs with osteoarthritis (Brown *et al.*, 2010). Owners were asked to complete the Canine Brief Pain Inventory (CBPI) prior to starting the supplementation and at the follow up visit. This tool consists of four questions pertaining the pain severity (PSS – pain severity score) and six questions pertaining pain interference (PIS – pain interference score), i.e., how much the perceived pain interferes with the dog's regular activities (Brown *et al.*, 2010). A score of 1 represents no pain or no interference and a score of 10 represents extreme pain/interference. The CBPI also has a qualitative question about the animals' overall quality of life. This question is scored in a 5-point scale, from poor, fair, good, very good and excellent. The scores for each group of questions were averaged and reported as median and range, and a difference between baseline – follow-up was also calculated for each individual dog. According to the CBPI user guidelines, individual treatment success was defined as a reduction equal or greater than 2 for PI and equal or lesser than 1 for pain severity (Brown *et al.*, 2013a).

When analyzing each dog individually, all dogs except #2 showed reduction in pain scores (Figure 40A and B). According to the CBPI guidelines, treatment was

successful in reducing PSS in 3 (42%) after 8 weeks (Dogs 1, 3 and 7) and for PIS, 3

dogs (42%) had a reduction ≥ 2 at the follow-up visit (Dogs 1, 4 and 7) (Figure 40C and

D). Taken together, two dogs responded positively to both PSS and PIS (Dog 1 and 7),

two dogs responded positively for one or the other score (dogs 3 and 4) and two dogs

did not respond according to these two scores (Dogs 2 and 6).
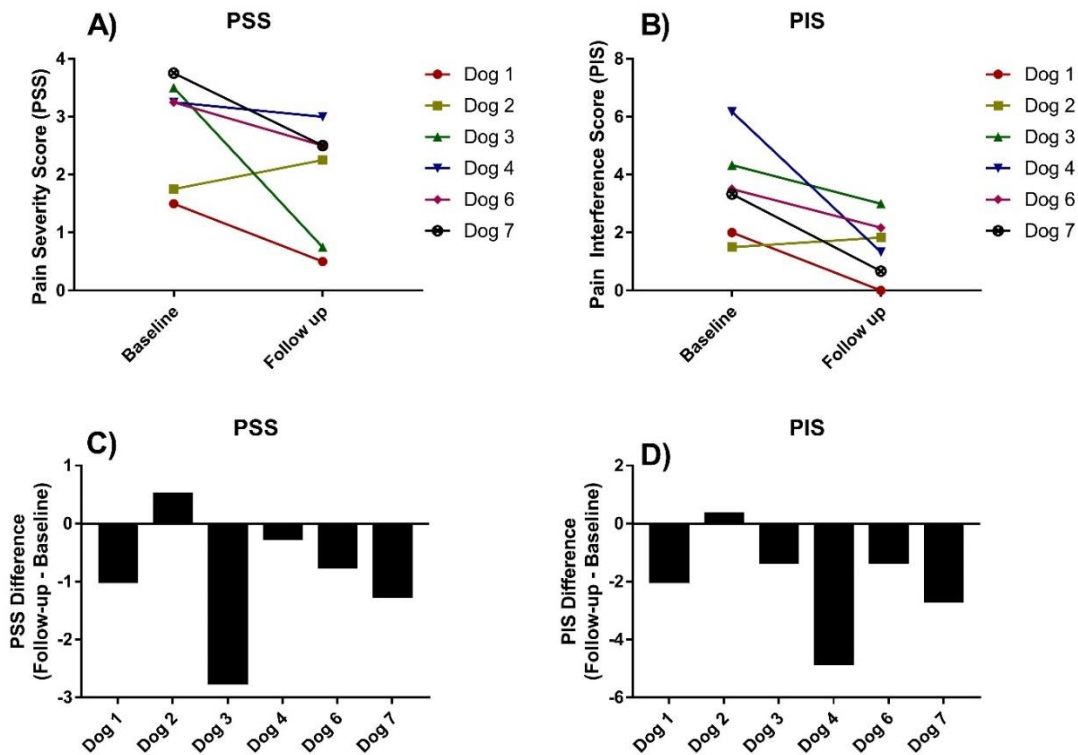
Figure 40. Canine brief pain inventory (CBPI) scores in dogs with osteoarthritis (OA) before and after dietary supplementation with the PCQ formulation for 8 weeks. A and B represents the scores given before and after the 8-week intervention for each dog. C and D represents the difference between follow-up and baseline pain scores (PSS and PIS) for each individual dog. A reduction ≥ 2 in PIS and ≥ 1 for PSS represent a successful treatment response.

When comparing CBPI median scores from baseline and follow-up visits, there was an overall reduction in both pain severity scores (PSS) and pain interference score (PIS) after the 8-week supplementation period (Figure 41A and B). For pain severity (PS), median scores were 3.25 and 2.38 at baseline and follow-up, respectively. For pain interference (PI), median scores were 3.42 and 1.58. A large range on values is expected, as baseline scores for each animal's pain is different and subjectively evaluated by each owner.
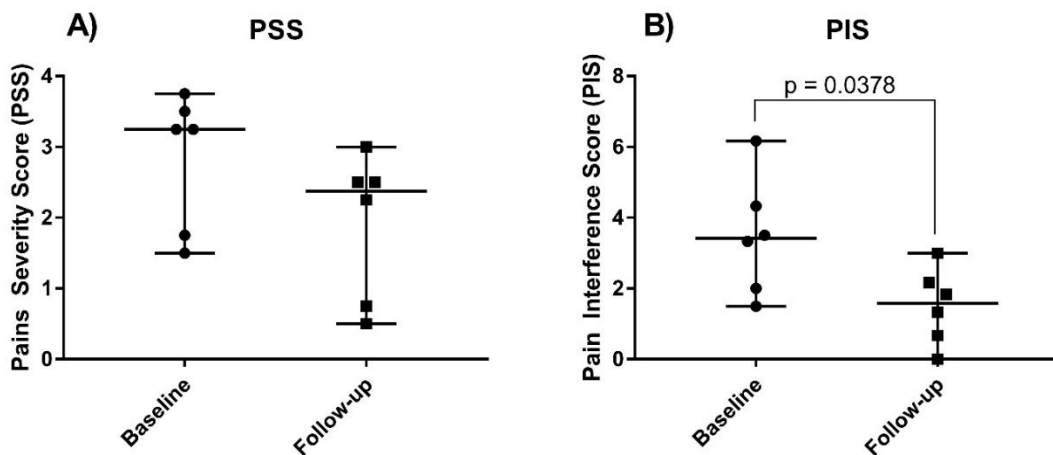
Figure 41. Median and range for CBPI Pain Severity Score (PSS) and Pain Interference Score (PIS) in 6 enrolled dogs. Indicated p value indicate statistical significance between baseline and follow-up visits.

Owners were also asked to rate the animals' overall quality of life (QOL) in a qualitative scale, that comprised 5 levels: poor, fair, good, very good and excellent. Even though the baseline score for each dog was different, overall medians were significantly higher (p = 0.025) at the follow up visit, indicating improvement in dogs'

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activities and perception of pain (Figure 42). Figure 42B shows changes in QOL scores

for each dog individually.
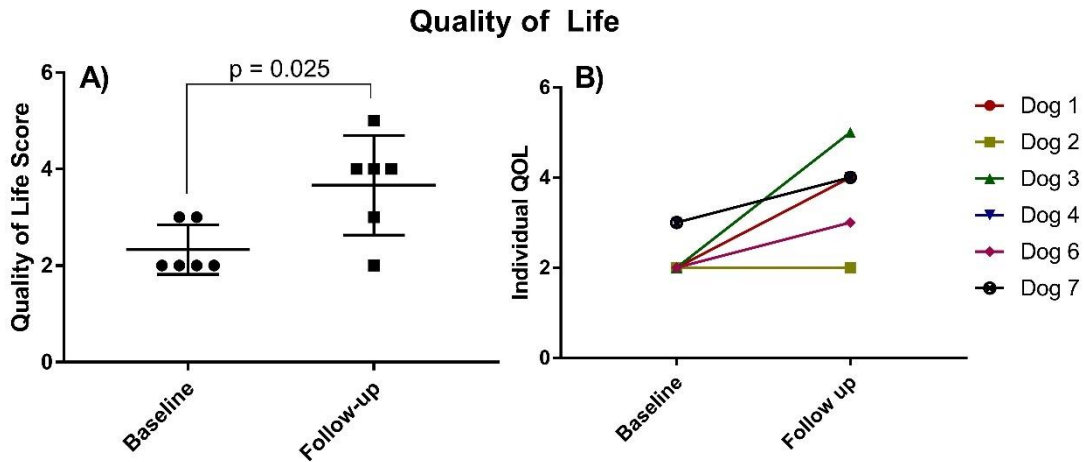
Figure 42. Canine brief pain inventory (CBPI) quality of life (QOL) scores for 6 enrolled dogs. Scale goes from 1 (poor) to 5 (excellent). A) Boxplots represent the median and range for baseline and follow-up visits (n = 6). B) Lines represent scores at baseline and follow-up visits. Indicated p-value indicate statistical significance (Paired t-test, $p < 0.05$).

It is common to see the CBPI being used as an outcome measurement of pain in

studies assessing efficacy of nutraceuticals and dietary supplements. In a placebo-

controlled clinical trial investigating the effects of a diet supplemented with

curcuminoids extract, hydrolyzed collagen and green tea extract, researchers were able

to detect differences between the control and the supplemented group, although no

differences were seen within the supplement group after 3 months (Comblain *et al.*,

2017). When evaluating the effects of a commercially available joint supplement

compared with carprofen in working dogs with OA, Alves et al. (2017) did not detect a clinical response based on CPBI scores.

Even though the CBPI is a validated tool to access pain is osteoarthritic dogs and widely used in several OA intervention studies (Brown *et al.*, 2013a,b; Alves *et al.*, 2017), it is important to consider other assessment tools and interpret outcomes accordingly. It is known that CBPI scores are not always correlated with force plate analysis results (Brown *et al.*, 2013b). When analyzing individual results for force plate and CBPI before and after supplementation, 4 dogs showed improvement in both PVF and VI, while only 1 dog improved PS and PI scores. It is possible that the force plate may be detecting levels of lameness that were not detectable by the human eye. Therefore, it is important to consider multiple outcome measurements when studying the effect of interventions in osteoarthritic dogs.

5.3.9 Effect of 8-week supplementation with PCQ formulation on serum inflammatory markers of osteoarthritic dogs

With OA increasingly being considered an inflammatory disease, cytokines are being assessed as possible candidates for biochemical markers that could aid in determining severity of the disease, monitoring efficacy and safety of interventions as well as having the potential to act as a diagnostic and prognostic tool (Mabey and Honsawek, 2015). The synovial fluid is an important potential source of biomarkers in osteoarthritis (OA), as it reflects the biological environment of the joint, offering a

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direct measure of joint pathophysiology (Jayadev *et al.*, 2012). However, often OA joints do not offer sufficient SF volume to be collected for analysis. This study protocol included a joint fluid collection for detection of local inflammatory cytokines. Nevertheless, it was only possible to collect joint fluid from 2 dogs at the follow-up visit and no baseline sample. Therefore, concentrations of inflammatory markers in joint fluid could not be reported in this study. Alternatively, quantification of cytokines in serum was performed and can provide useful information about systemic inflammation associated with OA (Stannus *et al.*, 2010; Paquet *et al.*, 2012).

Cytokine concentrations found in this study are reported in Table 13. Compared to tissue-specific assessments, minimal data evaluating systemic biomarkers in dogs with OA are available and comparison of quantified levels is challenging due to substantial variation across different methods and heterogeneity of the studied dogs (Hegemann *et al.*, 2005; Hillström *et al.*, 2016; Muller *et al.*, 2019). Therefore, analyzing biomarker profile before and after the intervention may provide a better insight about the systemic inflammatory response and contribute to research and clinical applications.

Table 13. Serum cytokine concentrations for enrolled dogs on Day 0 (baseline) and after 8 weeks (follow-up)

Biomarker (pg/mL)	Baseline Median (min - max)	Follow-up Median (min - max)
GM-CSF	44.52 (0.36 -881.1)	106.2 (4.70 - 849.2)
IFN-γ	17.34 (7.308 - 33.22) *	11.12 (4.043 - 18.1)
IL-6	40.39 (5.348 - 47.87)	49.72 (5.348 - 124.1)
IL-10	60.14 (3.703 - 92.87)	45.38 (3.703 - 111.9)
MCP-1	206.9 (148.5 - 270.8)	192.8 (148.5 - 353.3)
TNF-a	< LD**	< LD

*Different from follow-up (p = 0.0078, Wilcoxon matched-pairs signed rank test)
**LD: limit of detection

Baseline levels of the different cytokines varied greatly among enrolled dogs. Therefore, the data range was large and significant effects were not observed for GM-CSF, IL-10, IL-6 and MCP-1 levels after the 8-week supplementation. IFN-γ levels exhibited a significant decrease (p = 0.0078) compared to baseline (Figure 43). The overall reduction on median levels of three cytokines (IFN-γ, IL-10 and MCP-1) observation might suggest a reduction in systemic inflammation associated with OA. However, the reductions were small and only IFN-γ reached statistical significance.
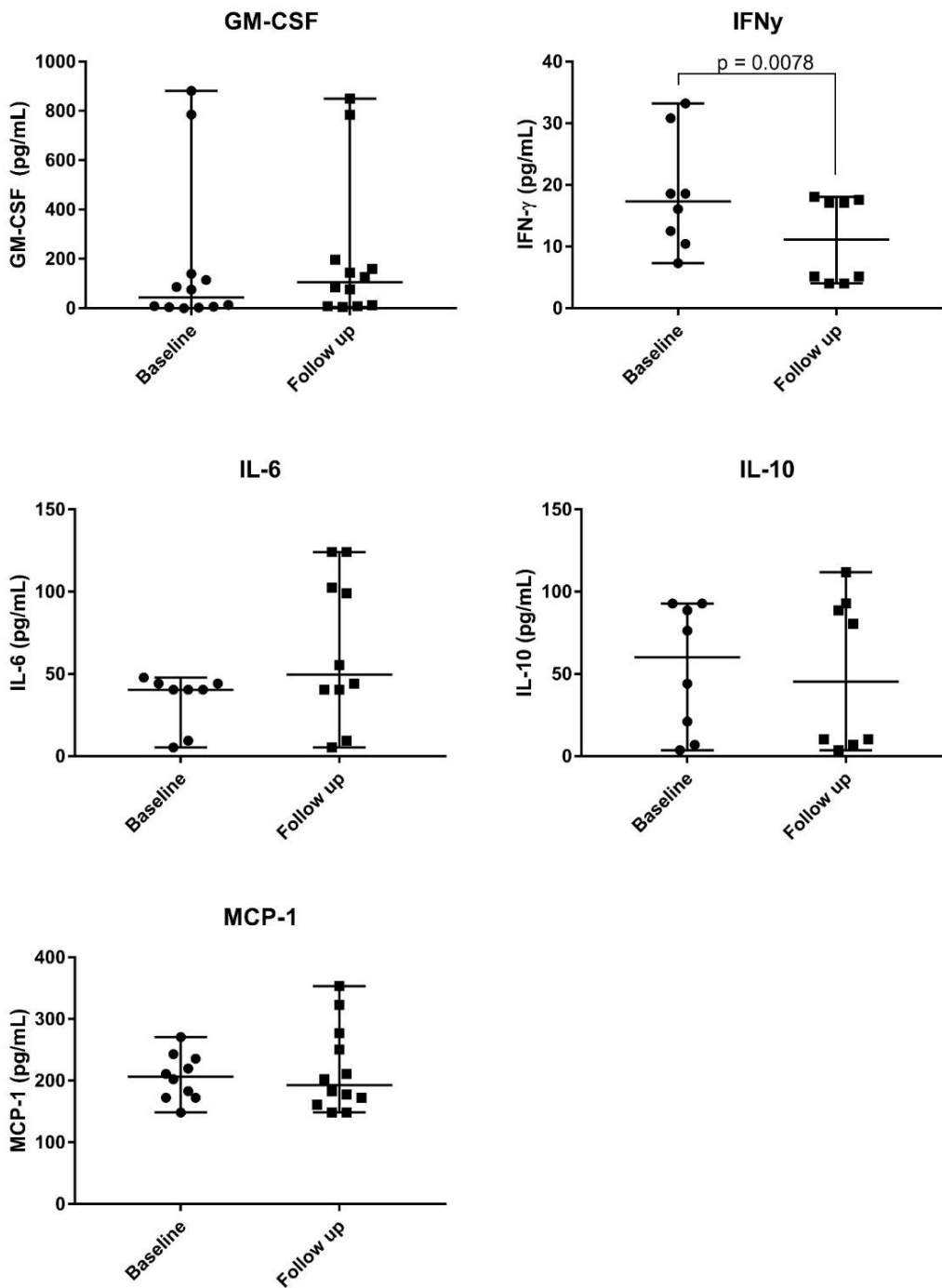
Figure 43. Cytokine concentrations (median and range) measured in OA dogs at baseline and follow-up visits. Indicated p-values indicate statistical significance (Wilcoxon matched-pairs signed rank test, $p < 0.05$)

There was little to no variation in granulocyte-macrophage colony-stimulating factor (GM-CSF) levels in the participant dogs (Figure 44). GM-CSF is a growth factor that can also act as a proinflammatory cytokine and in dendritic cell function. It has been shown to be important in the development of inflammatory models of rheumatoid arthritis (RA) (Conaghan *et al.*, 2019). Cook et al., (2012) proposed that GM-CSF is key to the development of experimental osteoarthritis and its associated pain in a collagenase-induced arthritis model in mice. In this pilot study, no differences were observed in GM-CSF levels after supplementation with PCQ formulation.

When analyzing individual levels of IL-6, Dogs 3 and 6 showed a marked increase, while levels of all other dogs did not change (Figure 44). Serum levels of IL-6 are reportedly higher in human OA patients and also associated with cartilage loss (Stannus *et al.*, 2010). In dogs, IL-6 levels in synovial fluid of OA joints was 35x higher than normal joints (Allen *et al.*, 2019) Additionaly, together with other cytokines, IL-6 is believed to stimulate the production matrix-metalloproteinases (MMPs) and inhibit synthesis of proteoglycan and collagen type II synthesis, and therefore can be related to OA progression (Wojdasiewicz *et al.*, 2014). However, we found no evidence of a positive effect from the PCQ supplement on IL-6 concentrations in serum. Similar result was reported in a pilot study investigating effects of a commercially available dietary supplement in OA dogs (Muller *et al.*, 2019).

Individual IFNγ levels were mostly reduced after 8-week supplementation, with Dogs 3, 4, and 6 showing decrease compared to baseline (Figure 44). The role of IFNγ in the pathogenesis and progression of osteoarthrtitis is poorly defined. However, this

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cytokine is frequently used as a biomarker in chronic inflammation and a sustained release of IFN-γ in the inflamed tissues of patients with rheumatoid arthritis is also reported (Ivashkiv, 2018). The trend observed in IFNγ in this pilot study might indicate an overall improvement and downregulation of the inflammatory response in most dogs after suplementation with PCQ formulation.

Interleukin-10 (IL-10) is a cytokine reported to mediate chondroprotective effects in OA (Waly *et al.*, 2017). The observed trend for IL-10 median levels was a reduction at the follow-up visit, although this was not statistically significant (Figure 43). Levels of IL-10 in Dog 3 increased, while Dog 6 exhibited a marked decrease (Figure 44). IL-10 levels were not detected in Dogs 1 and 7. Contrary to the tendency observed in this study, a trial testing a multicomponent dietary supplement in OA dogs reported reduction in IL-6 and increase in IL-10 levels, which confirmed the beneficial effects of the tested supplementation (Musco *et al.*, 2019).

There were no significant changes in MCP-1 levels in this study (Figure 44). Monocyte chemoattractant protein 1 (MCP-1) is a chemokine that plays a role in inflammation by recruiting mononuclear leukocytes and lymphocytes in inflamed knee joints (Paquet *et al.*, 2012). Sohn *et al.*, (2012) found increased concentration of inflammatory proteins, including MCP-1, in sera of osteoarthritis human patients compared to controls. It is also proposed that elevated MCP-1 levels promote the pathogenesis of OA by enhancing the apoptosis of chondrocytes while inhibiting their proliferation, consequently facilitating cartilage damage (Xu *et al.*, 2015). Although limited data is available on MCP-1 serum concentration in dogs and its association with

OA, MCP-1 levels in synovial fluid in dogs was identified as the most accurate marker to distinguish OA from normal joints (Allen *et al.*, 2019).

In this study, TNF-α levels were below the assay minimal detectable concentration (6.1 pg/mL) and could not be reported. TNF-α concentration in canine serum is frequently reported to be very low and below the limit of detection of several tests. In healthy dogs, TNF-α levels ranged from "below limit of detection", to 11.9 pg/mL in a review from (Richter *et al.*, 2018). More recently, Muller et al. (2019) also found extremely low levels of TNF-α in serum of OA dogs (median 1.6 pg/mL).
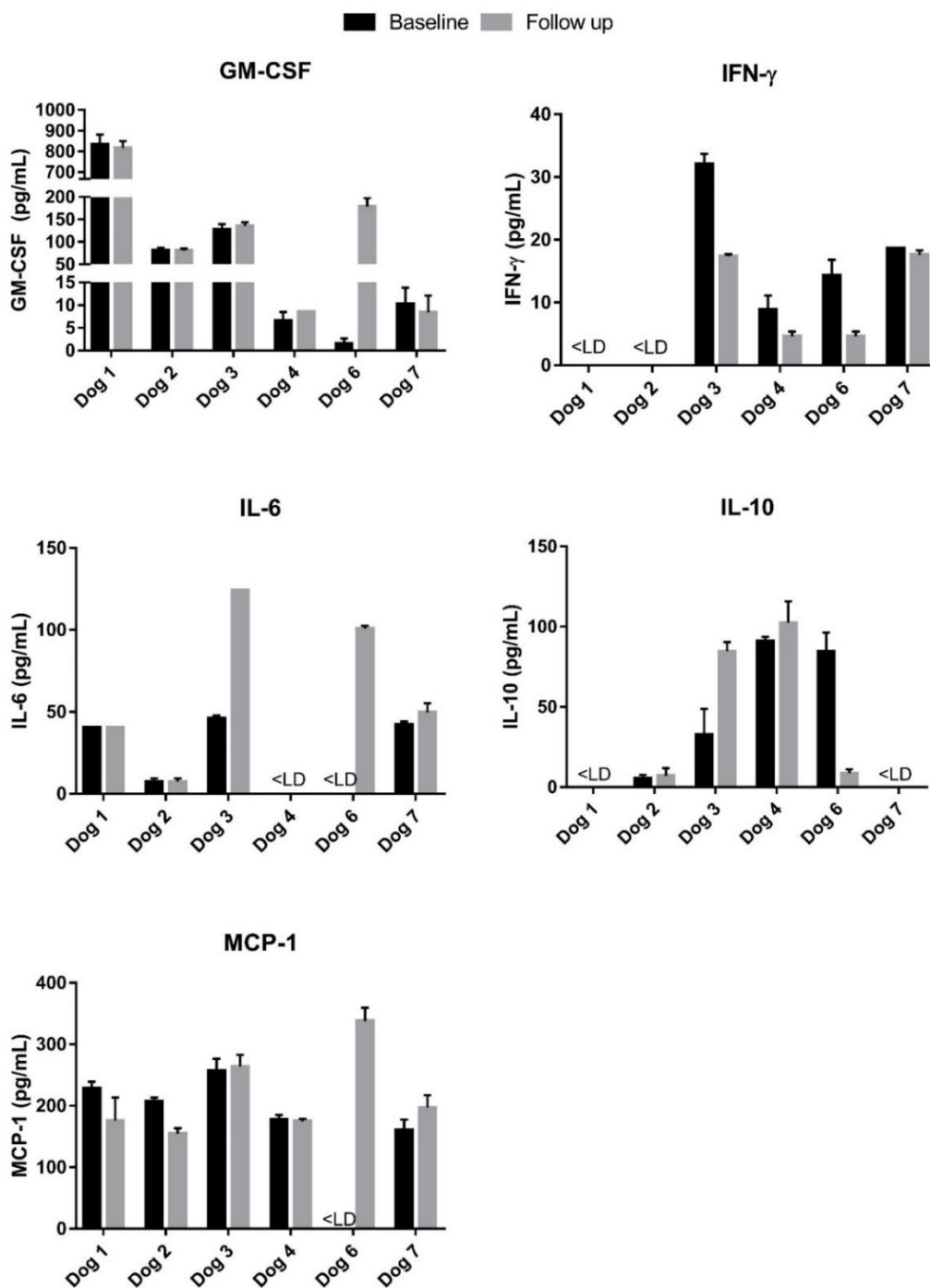
Figure 44. Individual levels of inflammatory cytokines (pg/mL) in six OA dogs at baseline and follow-up visits.

Quantification of IL-1b in dog serum was performed with a commercially available canine-specific ELISA kit. IL-1b concentrations fell below the limit of detection of the assay (10 pg/mL) for most dogs and could not be determined (Table 14) IL-1b level for dog 4 could not be detected at baseline, and at follow-up visit it was 8.99 pg/mL (± 2.35 SD), which might indicate that there was an increase after the 8-weeks supplementation. For dog 7, levels were 21.7 pg/mL (± 8.78 SD) and 18.91 pg/mL (± 0.37 SD) at baseline and follow-up visits, respectively.

Table 14. Mean IL-1b concentrations measured in dog serum at baseline and follow-up visits (average ± SD)

	Baseline (average ± SD)	**Follow-up** (average ± SD)
Dog 1	< LD	< LD
Dog 2	< LD	< LD
Dog 3	< LD	< LD
Dog 4	-	8.989 ± 2.35
Dog 6	< LD	< LD
Dog 7	21.7 ± 8.78	18.91 ± 0.37

LD: Limit of detection

In several studies, TNF-α and Il-1b are reported to be relevant biomarkers in the pathogenesis of OA, and able to control the degeneration of articular cartilage matrix, which makes them prime targets for therapeutic strategies (Kapoor *et al.*, 2011). In human cultured osteoarthritic (OA) articular cartilage, inhibition of either or both of these cytokines promoted reduction in gene expression of enzymes involved in cartilage matrix degradation (MMPs), as well as favoring its repair (Kobayashi *et al.*, 2005). In this study, however, it was not possible to draw conclusions about the role of systemic

levels of TNF-α and Il-1b in OA. Fox & Cook (2011) point out to effects of dilution and degradation on serum marker concentrations as a challenge when trying to correlate systemic levels of OA inflammatory biomarkers to local (synovial fluid) levels. It is important to consider that even though cytokine profiling in OA patients is an essential starting point, different inflammatory subtypes arise from affected joints (Ren *et al.*, 2018). Ideally, patients with a more similar OA pathophysiology and affected joint should be assessed in a study for a more comprehensive understanding of the role of circulating cytokines.

There are currently no published studies investigating the use of PEA in dogs suffering from osteoarthritis. Considering that several in vitro and animal trials present strong evidence of efficacy of PEA in the pathophysiology and clinical signs of the disease, its investigation for use as a nutraceutical in dogs with OA becomes of interest. PEA has been shown to be present in the synovial fluid of osteoarthritic dogs (Valastro *et al.*, 2017), although it is not clear how these levels would compare to healthy joints. Because PEA is synthesized and act locally, it can be hypothesized that PEA is produced by immune cells in the OA joint in response to inflammatory stimuli caused by the disease. Furthermore, PEA has been safely used in studies involving dogs and it appears in trials targeting skin conditions (Abramo *et al.*, 2014; Noli *et al.*, 2015) and it was also tested in a small pharmacokinetic experiment in beagle dogs (Petrosino *et al.*, 2016). In a human study comparing the effects of PEA versus ibuprofen for pain relief in temporomandibular joint (TMJ) osteoarthritis, a higher pain reduction was seen for subjects taking PEA (Marini *et al.*, 2012). Recently, the safety, tolerability and efficacy

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of PEA on symptoms of knee osteoarthritis was evaluated in adults with mild to moderate knee osteoarthritis (Steels *et al.*, 2019). Results demonstrated a significant reduction in several pain, stiffness and function scores compared to placebo. The exact mechanism through which PEA can counteract inflammation and pain in OA patients is yet to be elucidated, although Marini et al. (2012) highlight the ability of PEA to control mast cell degranulation as a potential mechanism.

Turmeric and standardized curcuminoid extracts have been used as a nutritional supplement for osteoarthritis, both alone or as part of herbal formulations for humans (Panahi *et al.*, 2014) and companion animals (Sgorlon *et al.*, 2016; Comblain *et al.*, 2017; Musco *et al.*, 2019). Downregulation of pro-inflammatory cytokines and inhibition of cartilage degrading enzymes by inhibition of the NF-kB and AP-1 pathways, as well as exhibiting and radical scavenging properties are some of the proposed mechanism through which curcuminoids can assist in pain relief and reduction of inflammation in OA (Bello *et al.*, 2017). Similarly, quercetin have supportive effect in OA, by downregulating MMP-13, upregulating SOD and TIMP-1, as well as weakening the oxidative stress responses and inhibiting the degradation of cartilage extracellular matrix (Wei *et al.*, 2019). An indication of the potential of combining PEA with polyphenols to mitigate OA-related inflammation and pain is a study that investigated a co-ultramicronized formulation containing PEA and quercetin in a carrageenan-induced paw edema model in rats. Improvement in pain scores and inflammatory markers were reported with this formulation, as well as superior effects compared to meloxicam, which is the standard NSAID for treatment of arthritis (Britti

et al., 2017). To date, this present study is the first to investigate the effects of a formulation containing PEA in dogs with osteoarthritis and moreover, the first to propose its combination with polyphenols for this purpose.

5.3.10 Study limitations

As an initial screening of the potential effects of the PCQ formulation in OA dogs, this pilot study was conducted over 8 weeks. Given that osteoarthritis is a chronic progressive disease that causes cartilage damage in the long run, a longer supplementation time might be indicated for following studies. This might allow a better ability to detect improvement or a slowdown in disease progression.

Although adequate inclusion/exclusion criteria were used, this study enrolled animals having OA diagnosed in different joints and at different disease progression levels. It is known that different inflammatory response patterns can arise from different affected joints in OA (Ren *et al.*, 2018), and therefore clinical observations, especially biochemical marker analysis can be very heterogeneous. In order to have a more homogeneous response, narrower inclusion criteria may be used, enrolling only dogs with a specific affected joint or even using experimental models of induced-OA.

This is the first time this formulation is being tested in vivo and dosage was based on published literature using the individual compounds as nutraceuticals and dietary supplement. Upon the conclusion of this pilot trial, it might be determined that a

different dosage of the formulation might be needed in order to detect a clearer improvement in OA pain and inflammation.

5.4 Conclusions

Here we demonstrate for the first time that a new formulation containing palmitoylethanolamide (PEA), and the natural polyphenols quercetin and curcuminoids extract exert beneficial effects in osteoarthritis (OA) in dogs. Results demonstrated improvement of pain scores from CBPI in 3 dogs and increase in vertical forces (VF) and impact (VI) in 4 out of 6 dogs, indicating a potential benefit of supplementation with the PCQ formulation in the lameness in OA dogs. Furthermore, reduction of several biomarkers in some dogs after supplementation also indicates a potential mitigation of the inflammatory cascade related to the disease. Given that OA is a degenerative disease and worsening is expected, these preliminary observations indicate favorable outcomes from the use of a PCQ formulation as a dietary supplement in OA dogs. The study is currently ongoing and as the number or participating dogs increases the statistical significance of results is expected to increase. The overall goal is to translate the use of the PCQ formulation into future human clinical trials to ultimately support the treatment of human OA.

CHAPTER VI

SUMMARY AND FUTURE RESEARCH

For the first time, the anti-inflammatory effect of palmitoyethanolamide (PEA) was investigated in combination with the biologically active polyphenols quercetin and curcuminoids. We have demonstrated that these compounds are able to mitigate oxidative stress by reducing ROS and NO production, as well as to downregulate expression of inflammatory markers such as Cox-2, iNOS and TNF-α. Most importantly, a synergistic interaction was demonstrated between PEA and quercetin and PEA and curcuminoids in downregulation of IL-1b mRNA expression. Synergy parameters such as the combination index (CI), dose reduction index (DRI) and isobolograms for two different experimental designs were presented and indicated stronger synergy for the PEA and quercetin combination. These results provide evidence and rationale for the use of these ingredients in association in nutraceutical formulations targeting prevention and treatment of chronic inflammation and pain. Initial mechanistic investigations allowed us to determine that downregulation of IL-1b by PEA in RAW264.7 cells occurred via PPAR-α activation, while the effects of quercetin and curcuminoids appear to be mediated by different mechanisms. They are known to be highly pleiotropic molecules, exhibiting action towards multiple targets. Furthermore, quercetin and curcuminoids did not affect the expression of PEA-degrading enzymes in macrophages, demonstrating that the synergistic mechanism is not arising from transcriptional inhibition of these enzymes by the polyphenols. They

may, however, have an inhibitory effect on enzymatic activity, leading to higher intracellular PEA concentrations. Therefore, mechanistic studies targeting usual receptors that can be activated by quercetin and curcuminoids (PPAR-γ for example), as well as focus on antioxidant enzyme profiles upon treatment with combined compounds may assist in elucidating the underlying molecular mechanisms of this synergistic interaction, to expand application and facilitate translation into in vivo investigations.

By initially assessing the stability and cellular uptake of PEA by RAW264.7 cells, we have demonstrated that PEA has good stability under cell culture conditions, as 82% and 78% of added PEA and d4-PEA, respectively, can still be found in supernatants after 12h. The cellular uptake of PEA by macrophages was demonstrated and the presence of quercetin and curcuminoids did not change the chemical stability or the cellular uptake after 3h incubation. It was not possible to determine if there was an effect of the polyphenols on the actual transport into the cells or on the hydrolysis by endogenous enzymes based on the brief experiments that were conducted in this pilot investigation. Thus, additional exploration using enzyme inhibitors and enzymatic assays measuring PEA hydrolysis in the presence of quercetin and curcuminoids are recommended to determine if there are any effects of polyphenols on the uptake and pharmacokinetic parameters of PEA absorption by macrophages. Monitoring the enzymatic hydrolysis products palmitic acid and ethanolamine might also provide useful information on the kinetics of breakdown of the active compound and possible accumulation of metabolites.

A combined formulation using PEA, quercetin and curcuminoids (PCQ) was developed and tested in a pilot study with family-owned osteoarthritic dogs. For the first time, PEA in combination with polyphenols was investigated as a potential treatment for osteoarthritis (OA) in dogs. Results indicated that an 8-week supplementation with the PCQ formulation exerted beneficial effects, demonstrated by improvement in pain scores and gait, overall quality of life and also positively affecting pro-inflammatory markers in some dogs. As an initial pilot study, the current work provided useful information to guide future in vivo research using the developed formulation. Next steps should focus on testing the PCQ formulation in a controlled (standard of care) or placebo-controlled study design, including a larger number of enrolled dogs and potentially longer intervention time. Considering the positive results of the use of PEA for human OA and joint pain reported on the literature, and the beneficial outcomes of this pilot study in dogs, human clinical trials testing the use of the PCQ formulation to support the treatment of human OA are also encouraged.

Overall, this research focused on demonstrating the anti-inflammatory effect of PEA, quercetin and curcuminoids and the synergistic interaction that arises when these compounds are used in association. Upon positive response in vitro, a combined formulation was developed and tested in a pilot animal trial, demonstrating beneficial effects. Further studies are still needed and can contribute to better understand the interaction between PEA and polyphenols, potential mechanisms of action and the overall disease modifying properties that these synergistic combinations might have on osteoarthritis (OA).

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APPENDIX A

DOSE REDUCTION INDEX (DRI) TABLES FOR FIXED RATIO EXPERIMENTS

Table A-1. Dose Reduction Index (DRI) for from tested combinations between PEA and quercetin in the fixed ratio design

Effect (compared to LPS = 1)	PEA (µg/mL)	QUER (µg/mL)	DRI PEA	DRI QUER
0.81	0.49	0.69	1.97	5.50
0.56	1.97	1.01	3.94	4.04
0.59	1.68	0.97	1.68	1.93
0.38	4.41	1.26	2.94	1.69
0.17	15.30	1.79	7.65	1.79
*0.8	0.52	0.70	2.02	5.40
*0.5	2.55	1.08	3.11	2.65

* DRI for simulated effect levels

Table A-2. Dose Reduction Index (DRI) for from tested combinations between PEA and curcuminoids extract in the fixed ratio design

Effect (compared to LPS = 1)	PEA (µg/mL)	CUR (µg/mL)	DRI PEA	DRI CUR
0.53	2.18	1.00	8.73	2.01
0.67	1.14	0.78	2.28	0.78
0.34	5.59	1.44	7.45	0.96
0.46	3.11	1.15	3.11	0.58
*0.8	0.52	0.58	20.48	11.30
*0.5	2.55	1.07	4.64	0.97

* DRI for simulated effect levels