Original Article

Anti-Inflammatory Effect Of Quercetin-Rich Fractions On Lps Stimulated Raw 264.7 Macrophage Cells

Arya S[1] and Usha R[2*]

1. Department of Microbiology, PMS College of Dental Science & Research, TVPM, Kerala 695028
2. Department of Microbiology, Karpagam Academy of Higher Education, Coimbatore, TN 641021
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Corresponding author*:**ushaanbu09@gmail.com**

Abstract

Quercetin, a natural flavanol found in vegetables and plants, possesses unique biological properties that enhance physical and mental well-being and minimize infection risk. The study suggested the possible benefits of this plant-based flavonoid quercetin to be exploited as an effective anti-bacterial and anti-inflammatory agent. Isolated quercetin-rich fraction of Psidium guajava rom leaf extract following standard protocols of column chromatography. The present study investigated the anti-bacterial effect of the quercetin-rich fraction of Psidium guajava leaf extracts by agar well diffusion method with test organisms Enterococcus faecalis and streptococcus mutans. The composition analysis was done by FTIR spectrometer. The anti-inflammatory effect of quercetin-rich fraction of Psidium guajava leaf extracts on Lipopolysaccharide stimulated RAW 264.7macrophage cells were evaluated. The diameter of zone of inhibition was 24 mm and 14 mm, respectively., and functional groups were characterized by FTIR spectroscopy. Myeloperoxidase, Cellular nitrite level, inducible Nitric oxide synthase, Cycloxxygenase, and Lipoxygenase assay were done, and percentage inhibition concerning quercetin rich fraction of Psidium guajava leaf extract was studied. The current study suggests that a quercetin-rich fraction of Psidium guajava decreased the production of different pro-inflammatory mediators on LPS-treated macrophages and is a potent anti-bacterial agent. The present study proposed the medicinal value of Quercetin rich fractions in treating Periodontal Disease.

Key words: Antibacterial, Antiinflammatory ,Periodontitis, Phytochemical ,Inflammatory mediators, Flavanoid

INTRODUCTION

Quercetin, a natural flavanol found in vegetables, plants, possesses unique biological properties that enhance physical and mental well-being and minimize infection risk. Various studies proved quercetin's anti-inflammatory, ant-carcinogenic, and anti-oxidant properties. The plant flavonoid quercetin is found worldwide in plants and possesses several biological properties of medical importance [(1).] As stated by S. Chen et al., 2016, quercetin was found to inhibit various biological functions like lipid peroxidation, platelet aggregation, and capillary permeability and promote mitochondrial biogenesis. Hence, a detailed study is necessary to evaluate the novel therapeutic potential of the phytochemical quercetin [(2).]

Traditional medicines exploit various plants and their products in treating infections due to their ability to combat several clinical conditions. Psidium guajava is a popular conventional medicinal plant generally used in traditional medicine. We isolated quercetin-rich fractions from *Psidium guajava* leaf extract (QRF-PG) and investigated their anti-bacterial and anti-inflammatory activities in the present study. Anti-bacterial activity was evaluated by agar- well diffusion method. The current research focused mainly on anti-inflammatory property of quercetin rich fractions of *Psidium Guajava* leaf extract. To analyse the anti-inflammatory activity of the QRF_PG leaf extract, a study was done to evaluate its ability to down regulate the amount of inflammatory mediators increased by Lipopolysaccharide stimulated macrophage cell line (RAW 264.7). Analysing previous studies, the

molecular mechanism in which QRF-PG stops the expression of inflammatory mediators is due to the refrainment of NF-κB activation through the decrease of LPS-induced IκB-degradation. Choi et al., 2012 investigated the anti-inflammatory activity of quercetin. In a study conducted by Choi et al., 2012, highlighted the inhibitory potential of Quercetin in Lipopolysaccharide stimulated macrophage cell lines, and identified the elevated amounts of NO production, iNOS expression, and (NF)-κB stimulation [3]. Jang et al , confirmed the anti-inflammatory potential of Psidium guajava leaf extracts by demonstrating the percentage inhibition of LPS-induced NO, as 52.58% [4].

The anti-inflammatory effect of QRF-PG on Lipopolysaccharide stimulated raw 264.7 macrophage cell lines established the ability of QRF PG to down-regulate LPS -induced Nitric Oxide , Cycloxygenase 2 , and myeloperoxidase level, through the down-regulation of NF-κB [5]. Similarly, Kaileh et al. proposed that the down-regulation of NF-κB could be at the transcription level due to the decreased binding between nuclear factor-κB and DNA[6] [7]. At the same time, Jang et al. investigated that LPS-induced production of NO and specific inflammatory mediators may result from down-regulation of protein expression by Psidium guajava leaf extract [8]. Simultaneously, Sen et al. also established the down-regulation of NF-κB by the flavonoid content of Psidium guajava leaf extract [9]. Furthermore, the anti-inflammatory property of quercetin by the reduced NF-κB expression may be due to the equilibration of the NF-κB/IκB complex and IκB degradation and pro-inflammatory mediators & iNOS expression [1]. In the meantime, another study by Laily et al. suggested using Psidium guajava leaves as immune modulators as it aids lymphocyte proliferation response [10]. All these studies bring out the efficacy of (QRF-PG) as an excellent anti-inflammatory agent and a potent immuno stimulator.

The process by which our body defends the intruding pathogens and tissue response linking phagocytic cells, such as macrophages, mast cells, dendritic cells, and finally reconstructing the typical cell structure and restoring the biological function is known as inflammation. On the other hand, the unpredictability of immune homeostasis and a persistent inflammatory condition may progress to acute inflammatory diseases like periodontitis, cancer, etc. [11].

Macrophages, a specialised immune cell type evolving as blood monocytes, have a precarious role in the primary immune response, i.e., instigating and spreading inflammatory reactions by secreting inflammatory mediators, like NO, interleukin-6, TNF-α, & prostaglandins by inducible cyclooxygenase (COX-2). The bacterial cell wall is a highly compact structure with varied chemical compositions. The most potent initiator of inflammation is the LPS in the cell wall of GNB. On exposure to bacterial Lipopolysaccharide, monocytes and macrophages are activated to release various pro-inflammatory cytokines. Lee et al., 2018 explained the relevance of Nuclear factor-κB, as it controls the transcription of multiple genes involved in immunity, inflammation, and protection from apoptosis [11] .

The current study analyzes the anti inflammatory effects of QRF_PG on LPS roused macrophages. Since bioactive products can inhibit inflammatory mediators, they can suppress the inflammatory response, which helps treat inflammatory conditions like periodontal disease. QRF_PG down regulated the production of cyclooxygenase (COX) and pro-inflammatory mediators on LPS treated macrophages [12] in various tissues. From the relevant previous studies, QRF PG can be considered as an effective anti-bacterial agent and anti-inflammatory restricting inflammation

MATERIALS AND METHODS

The fresh leaves of *Psidium guajava* were collected and shade dried. Using mortar and pestle shade dried Psidium guajava leaves were powdered and stored in tight container. 100g of shade dried leaf powder was extracted with 250ml ethanol for 24 hours and filtered. The extract obtained was dried under controlled condition in a temperature range of 40°C ± 5°C and refrigerated for future studies [13]. Isolated QRF-PG from leaf extract following standard protocols of coloumn chromatography.

The anti-bacterial assay was done by agar well diffusion method with Streptomycin as the standard anti-bacterial agent (concentration 10mg/ml). The test organisms used were *Enterococcus faecalis* (ATCC 29212) and *Streptococcus mutans* (MTCC 890) [14]. The composition analysis was performed by FT-IR spectrophotometer.

The macrophage cell line was purchased from the NCCS Pune, India. The cells were grown in DME Medium (Sigma Aldrich, USA) supplemented with 10% heat-inactivated Foetal Bovine Serum and antibiotics. The cultured cells were maintained in a CO2 incubator at 37^0C (NBS Eppendorf, Germany). Macrophages cultivated in the Lipopolysaccharide added medium (1 mg/ml) are the positive control for macrophage activation. The sample solution in varying concentrations was added to Lipopolysaccharide stimulated RAW 246.7 cells and incubated for twenty four hours. After incubation, the anti inflammatory assays were carried out [15].

Anti-bacterial analysis

Test organisms (Streptococcus mutans and Enterococcus faecalis) were seeded into petri plates containing MHA. Growth of culture adjusted according to McFarland Standard, 0.5%. Using a well cutter wells of approximately 10mm were bored and different concentrations of the sample such as 250µg, 500µg and 1000µg were added. The plates were then incubated at 37°C for 24 hours. The anti-bacterial activity was assayed by measuring the diameter of the zone of inhibition formed around the well [16]. Streptomycin was used as a positive control.

Spectrum analysis

The composition analysis was performed by the FTIR method. The Fourier transform infrared spectroscopy (FTIR) spectra of quercetin were recorded on Nicolet iS50 spectrometer. The FTIR spectra were recorded at 1 cm^{-1} resolution, ranging from 500 to 4000 cm^{-1}. The FTIR spectra obtained for the compound are compared with the standard FTIR chart.

Anti-inflammatory assay

Myeloperoxidase Assay

Release of myeloperoxidase from activated neutrophils leads to inflammatory tissue injury, as it catalyzes the formation of hypochlorous acid, a powerful anti-oxidant [17]. Hence inhibiting myeloperoxidase release may be crucial in minimizing the risk of inflammation. We evaluated the effect of quercetin rich fraction of Psidium Gujava leaf extract on LPS-activated macrophage RAW 246.7 cells. Potassium phosphate buffer 50 milli molar concentration and 0·57% Cetyltrimethyl ammonium bromide was used to prepare the cell lysate and maintained in liquid Nitrogen. After thawing, supernatant collected after centrifugation of the sample & MPO activity was assayed by adding PO4 buffer containing catechol monomethyl ether and H_2O_2. A change in absorbance was noted at 460nm. One part of Myeloperoxidas**e** activity is calculated as that degrading 1 µmol of Perroxide per minute at twenty five degree Celsius. Based on previously standardised protocol Myeloperoxidase effect was evaluated as units /milli litre of lysate[18].

Nitrite assay

Nitric oxide is a potent inflammatory marker due to cytokine-activated macrophages. Nitric oxide is involved in many clinical conditions like vascular, neurological, and autoimmune diseases [19]. Hence, nitric oxide inhibitors should be considered in developing therapeutic aids for managing inflammatory diseases. Cellular nitrite levels were assayed in LPS activated Macrophage cells treated with quercetin rich fractions of Psidium gujava leaf extract. Protein-free supernatant was prepared by centrifuging 3% Sulfosalicilate (0.1ml) treated with 0.5 ml cell lysate. The supernatant (20mico liter) is mixed with thirty microlitres of 10% NaOH and 300 microlitres THA. 530 microlitre o Griess reagent was added to the mixture and incubated in the dark for 15 minutes. The concentration of cellular nitrite was observed colorimetrically at 540 nm against a Griess reagent blank. The standard used here was Sodium nitrite (100–500 mg) [20].

Inducible Nitric Oxide Synthase assay

Inducible nitric oxide synthase is the critical enzyme in forming pro-inflammatory mediators like Nitric oxide. Inducible nitric oxide synthase assay was done to evaluate the efficacy of quercetin-rich fractions of Psidium gujava leaf extract in controlling pro-inflammatory mediators. To the cell lysate loaded HEPES buffer, NADPH, tetrahydropterin MgCl(0.1ml), 30 microgram Cleland's reagent & oxygenated hemoglobin was added. The absorbance was read colorimetrically at 401 nm [21].

Cyclooxygenase (COX) activity

Cycloxygenase is a vital enzyme involved in various inflammatory conditions and tumor progression. Inhibition of cyclooxygenase 2 may be advantageous in treating different physiologic conditions [22].100µl cell lysate was added to Tris HCL buffer at pH 8 and incubated with glutathione, hemoglobin at 25℃ for 1 minute. The process began with adding 200 mM/L arachidonic acid and was completed after 20 minutes of incubation at 37^0C. Then, 200µL of 10% TCA was added in 1 N HCL and incubated at 37°C. The mixture was then centrifuged, and added with 200micro liter of 1% thiobarbiturate, followed by boiling the tubes for twenty minutes. After centrifugation, COX activity was estimated colorimetrically by noting absorbance at 632 nm [23].

Lipoxygenase (LOX) activity

50 µL Cell lysate added to Tris HCL buffer at pH 7.4 with 200 µl Sodium Linoleate. Reaction mixture made up to 2ml as required. The formation of 5-HCT acid indicates the LOX activity [24].

RESULT AND DISCUSSION

The present study was formulated to analyze the anti-inflammatory and anti-bacterial effects of QRF-PG. We assayed the anti-bacterial activity of the quercetin-rich fraction of Psidium Gujava leaf extract against the oral pathogens Streptococcus mutans and Enterococcus faecalis. Percentage of inhibition of various inflammatory mediators like Myeloperoxidase(MPO), Cellular nitrite levels, inducible nitric oxide synthase, Cyclooxygenase (COX), Lipoxygenase (LOX) on Lipopolysaccharide stimulated macrophage cells were evaluated.

The anti-bacterial assay was carried out by agar well diffusion method with streptomycin as standard anti-bacterial agent.. The anti-bacterial assay revealed the potential of Quercetin-rich fraction of Psidium guajava leaf extract to control the oral pathogens. The test organisms Enterococcus faecalis and Streptococcus mutans showed a zone of inhibition of 21 mm and 14 mm, respectively, with 1000 µg of QRF-PG, as depicted in Tables I and Table II. The zone of inhibition observed with both test organisms is shown in Figures 1 and 2. Periodontal pathogenesis is a combined outcome of both bacterial action and immune reaction. Understanding a compound that can act as an anti-bacterial and anti inflammatory agent will surely improve and enhance the treatment regimen for oro dental infection.

Sample	**Concentration(µg)**	**Zone of inhibition (mm)**
Quercetin rich fractions of Psidium gujava	Streptomycin (100 µg)	20
	250	12
	500	14
	1000	21

Table I: **Agar well diffusion method** - Diameter of Zone of inhibition of Enterococcus faecalis in various concentration of *Psidium guajava* leaf extract.

Sample	**Concentration(µg)**	**Zone of inhibition (mm)**
Quercetin rich fractions of Psidium gujava	Streptomycin (100µg)	26
	250	Nil
	500	12
	1000	14

Table II: **Agar well diffusion method**- Diameter of Zone of inhibition of Streptococcus mutans in various concentration of *Psidium guajava* leaf extract.

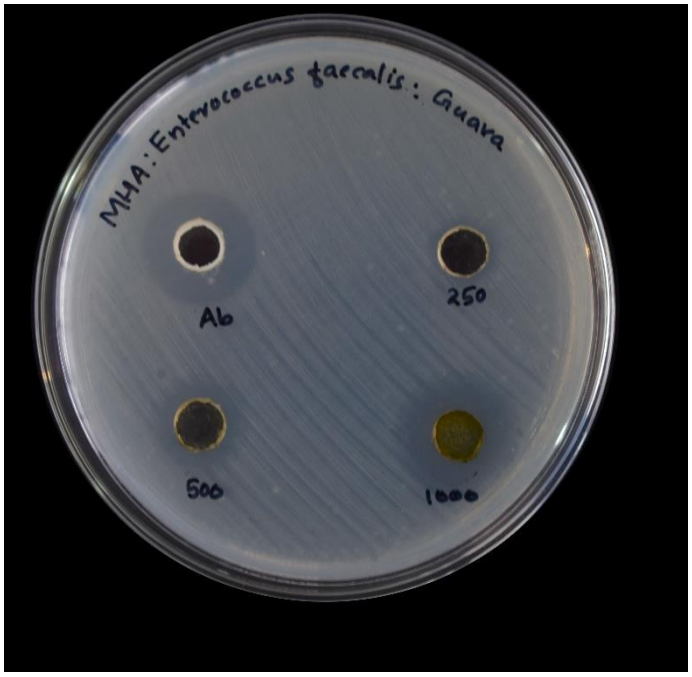

Figure 1: Anti-bacterial assay-. MHA agar plate showing anti-bacterial assay with test organism Enterococcus facecalis at various concentration of quercetin rich fraction of *Psidium guajava* leaf extract.

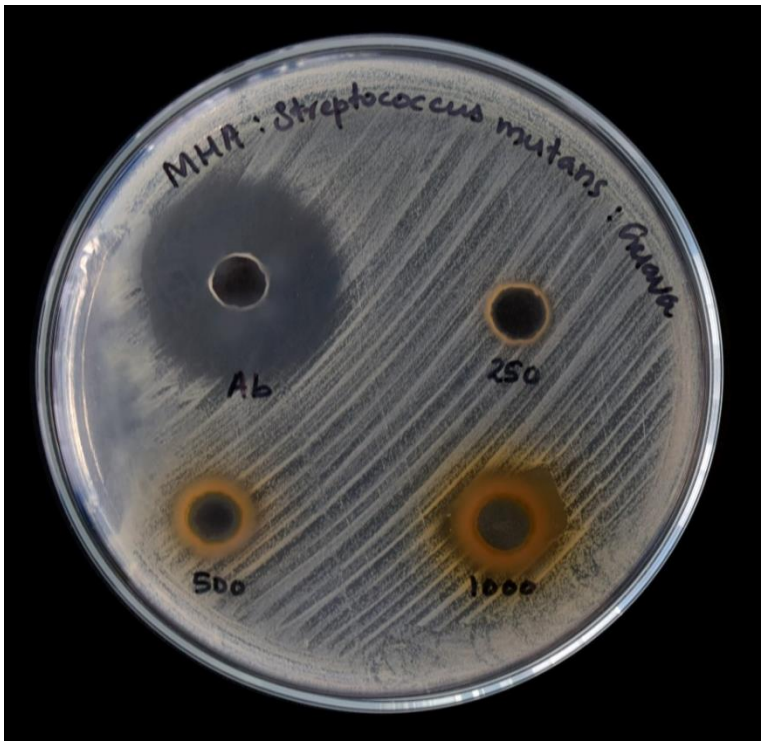

Figure 2: Anti-bacterial assay- MHA agar plate showing anti-bacterial assay with test organism Streptococcus mutans, in various concentrations of quercetin rich fractions of *Psidium guajava* leaf extract.

FTIR spectrum of quercetin is shown in Figure 3, in which quercetin showed several characteristic bands representing O–H stretching (3700 to 3100 cm^{-1}), C=O stretching (1700 to 1800 cm^{-1}), C–C stretching (1600 to 1700 cm^{-1}), C–H bending (1500-1600 cm^{-1}), C–O stretching in the ring structure (1270 cm^{-1}), and C–O stretching (1000 to 1200cm^{-1}) [25]. The functional groups obtained for quercetin are represented in Table III.

Sl no	Functional group
1	-OH stretching
2	C=O stretching
3	C–C stretching
4	C–H bending
5	C–O stretching
6	C–O stretching

Table III: Functional groups from FTIR analysis

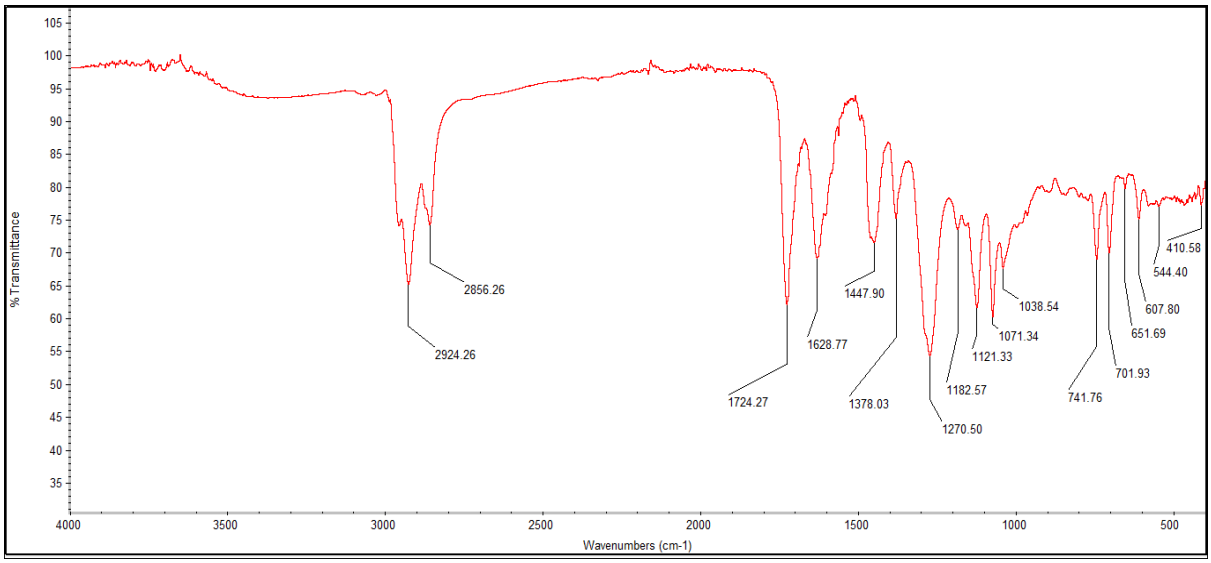

Figure 3: FTIR spectrum of Quercetin-rich fraction of Psidium guajava leaf extract

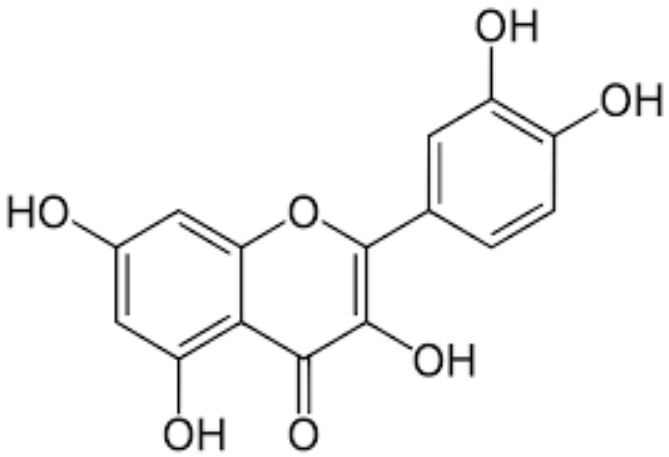

Figure 4: Structure of Quercetin

The accelerated immune reaction is a harmful condition that can be life-threatening due to an unbalanced host response to infection. Global researchers investigated flavonoids' biological effects, like free radical scavenging and anti-oxidant properties, anti-inflammatory potential, anticarcinogenic effects, etc. Among the multiple flavonoids studied, quercetin is vital in alleviating inflammation by inhibiting inflammatory mediators in a controlled manner. Quercetin is rich in many edible fruits and vegetables and is widely distributed in nature. Quercetin is an exciting area of research due to its varied biological activities like neuroprotective, anticariogenic,anti-inflammatory, antistress, modulation of oxidative stress, etc.[26]. Multiple studies prove the immune-modulatory action of quercetin.

Cytokines are the pivotal mediators which manage the process of inflammation and repair. Oishi & Manabe, 2018 stated that pro-inflammatory cytokines exhibit an immediate initiation of inflammation to defend the pathogens, simultaneously initiating the repair process, highlighting the close connectivity between inflammation and regeneration mechanisms. Poorly regulated cytokine signals hinder this synchronized mechanism and can result in various pathology [27].

Macrophages are the immune cells initially activated in fighting the extraneous attack and thus are an accepted in-vitro model for evaluating immune responses [28]. Hyperactivation of macrophages can increase pro-inflammatory cytokines and impair tissue repair and fibrotic tissue formation [29]. On exposure to Lipopolysaccharide, xenobiotic, and interferon-gamma, macrophages are classically activated. These activated macrophages, i.e., M1 macrophages, are efficient in up-regulating cytokines such as TNF-alpha, IL 1 beta, iNOS, RO, and N, etc.[30]. The recruitment of various immune cells to the targeted tissue is mediated by pro-inflammatory mediators [31].

Morton & Dongari-Bagtzoglou, 2001, stated that elevated levels of prostaglandins within the periodontal tissue are pathognomic for periodontitis, and its progression. MPO is a pro-inflammatory mediator expressed during foreign body invasion. MPO is an iron-containing enzyme primarily found in neutrophils and monocytes. Furthermore, MPO is proven to cause necrosis and subsequent immune reactions in various clinical conditions. Recent studies investigated the role of MPO to be targeted as an essential therapeutic mediator in the treatment of chronic inflammatory conditions [33]. A substantial elevation in the MPO level was noted in the Lipopolysaccharide stimulated cell and compared with the control. MPO activities in LPS-treated RAW 246.7 macrophage cells were compared to the result of QRF PG treated cells. The results indicate reduced inflammation with increasing concentration of QRF-PG, shown in figure 5. The activity of MPO is determined as that degrading 1 µM of peroxide per minute at twenty-five degree celsius.

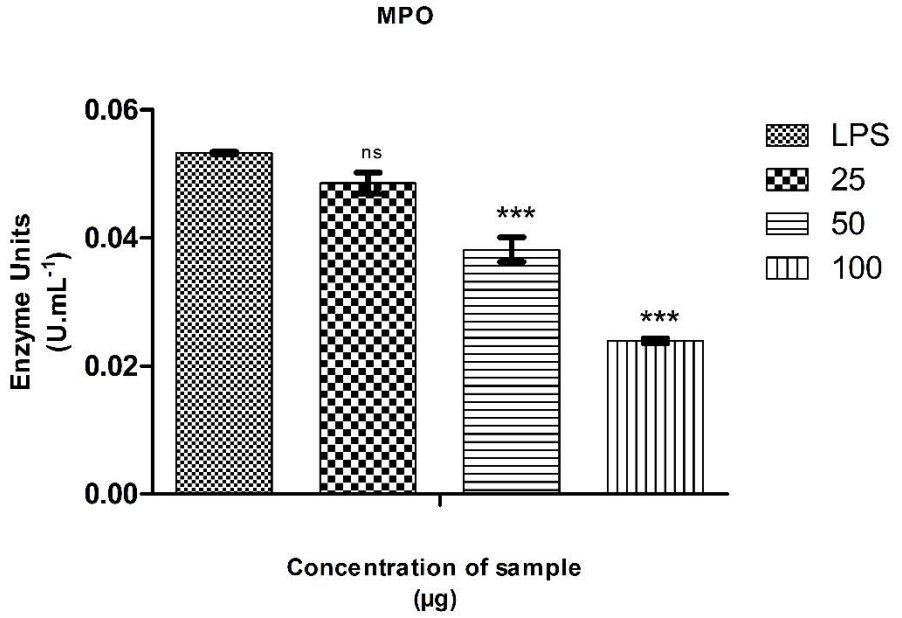

Figure 5:. Percentage inhibition of Myeloperroxide by QRF-PG

NO is the tiniest bioactive substance synthesized by NOS and produced by various cells. NO involves multiple mechanisms like nerve impulse transmission, vasodilator, defense against infection, and immune homeostasis. Inducible nitric oxide synthase is secreted by nuemerous cells in response to diverse stimuli including various cytokines. Being a crucial early -inflammatory mediator, NO plays a vital role in immune modulation [(34)]. NO is a versatile substance having crucial roles in inflammatory response, endothelial cellular activity, and anti-bacterial response. It is indisputable that human activated macrophages produce NO. The increased levels of NO in activated macrophages specifies chronic inflammation [(35).] A study conducted by Carmody & Cotter, 2001 proves the effect of Nitric oxide to bring apoptosis in macrophage cells invitro [(36).] The immunological activity of NO in instigating cell toxicity and inflammation in macrophages marks its importance as an immune stimulatory parameter [(37)]. NOS catalyzes nitric oxide synthesis. Among the different Nitric oxide synthase forms, iNOS is abundantly seen in activated macrophage cells. Numerous physiological functions have been credited to Nitric oxide.

Activated macrophages induce nitric oxide production as the primary anti-bacterial defence mechanism. Lipopolysaccharide, a dreadful agent of sepsis, is an essential activator of macrophages and the induction of iNOS [(38).] The iNOS in the macrophage cell lines were assayed. Results are observed as the percentage of inhibition in cells, 0 percentage inhibition in the LPS system, and increased inhibition percentage in the cells treated with the QRF-PG. The results were compared with those for control cells. Figure 6 shows the percentage inhibition of iNOS in LPS-treated RAW 246.7 cells. . iNOS activity in RAW 246.7 cells incubated with QRF-PG compared with LPS -system, indicating the elevated levels of inhibition percentage with increasing concentration. An elevation in absorbance was recorded at 401nm.

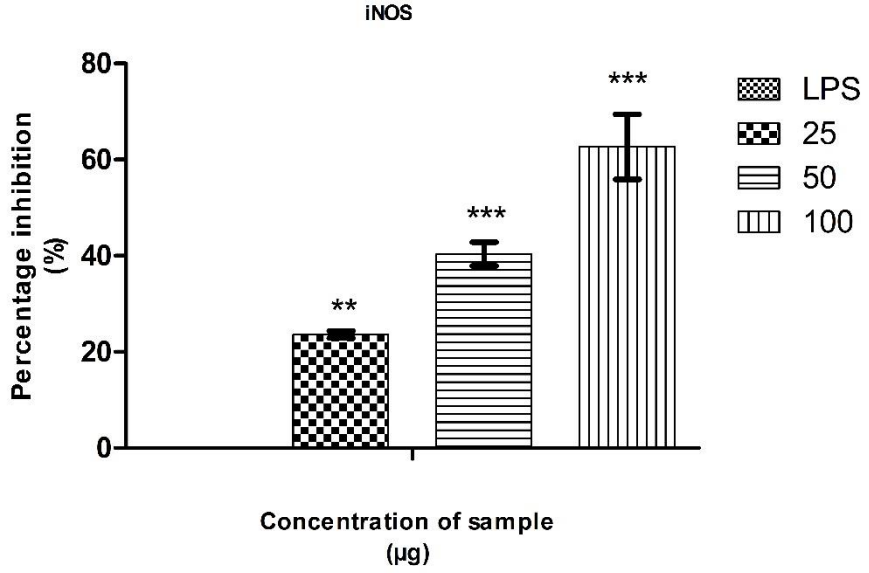

Figure 6: Percentage of inhibition of iNOS

The synthesis of Nitric oxide in QRFPG-treated RAW 246.7 cells is estimated by the amount of nitrate content formed and is depicted in fig 7. Values show the reduced inflammatory stimulation with increasing concentration of QRF-PG to LPS. The percentage of nitrate content is plotted graphically.

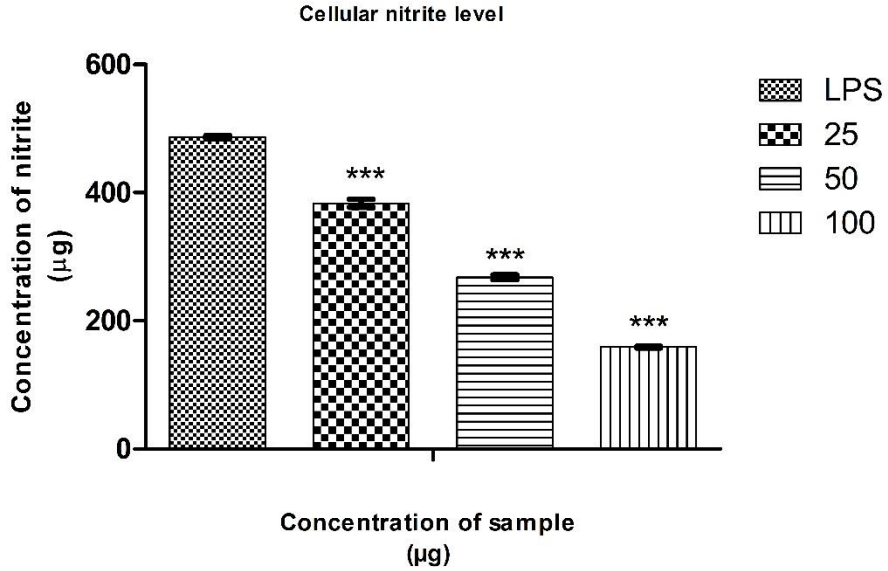

Figure 7: Concentration of nitrite in macrophage cells with QRF PG.

Cyclooxygenase (COX) catalyses the conversion of Prostaglandins from arachidonic acid, which is the rate-limiting step. Currently, two known isoforms of the enzyme COX are studied i.e COX 1 & 2. COX-1 and COX-2 regulate numerous biological activities in tissues , are secreted due to diverse external stimuli, and are induced in different cell types. COX 2 is supposed to synthesis prostaglandins at the site of inflammation hence is considered as an inducible enzyme [(39) (32)]. Current research in different models of inflammation and infection proved that COX 2 induced Prostanoids are the pivotal factors in controlling the secretion of pro inflammatory mediators [(39).] Besides, different chronic inflammatory diseases are associated with COX-2-induced production of

prostaglandins. This study aimed to analyze COX-2 expression in Lipopolysaccharide stimulated RAW cells. QRF PG is supposed to suppress COX-2 expression and prostaglandin production. Previous studies pointed out that, unbalanced expression of COX-2 is an essential mediator in an inflammatory response. A study done by Morton & Dongari-Bagtzoglou, 2001, concluded that in inflamed periodontal tissue COX-2 expression is suggestively up regulated. Pathogenicity of periodontitis is the combined outcome of bacterial constituents and pro inflammatory mediators like interlukin-1β , both of which constitute to the elevated expression of COX 2 and increased synthesis of prostaglandins invivo [32]. To determine whether QRF PG regulated COX-2 expression in immune regulation, we evaluated the effect of QRF PG on COX-2 protein by ELISA. Figure 8 shows the percentage of inhibition of COX 2 by QRF-PG in Lipopolysaccharide stimulated RAW 246.7 cells. Evaluation of Cycloxygenase 2 as a mediator of inflammation in Lipopolysaccharide stimulated macrophages depicts the elevation in the percentage of inhibition with varying concentrations of QRF -PG.

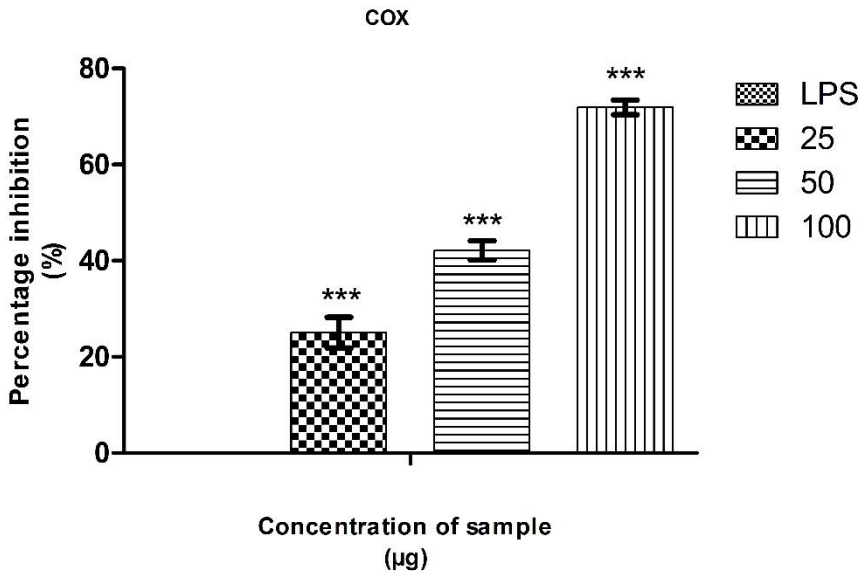

Figure 8: Percentage of inhibition of COX-2 by QRF-PG

Che et al., 2017 proposed the involvement of LOX-1 as a possible drug target in human dental peri-implantitis. LOX 1induces the production of Interleukin-1β and triggers the extracellular matrix breakdown through a unique inflammatory pathway, finally resulting in dental peri-implantitis [40]. The Lipooxygenase assay was determined by the increased absorbance at 234 nm, reflecting the production of arachidonate-5-lipoxygenase (5-hydroxyeicosatetraenoic acid), which is depicted in figure 9. By down regulating the synthesis of inflammatory mediators, inflammation can be reduced to larger extend, thus bioactive natural compounds capable of inhibiting these mediators can be considered as an alternative in treating periodontal disease. QRF-PG stopped the synthesis of inflammatory enzymes COX, and LOX induced by inflammatory reaction in vitro.

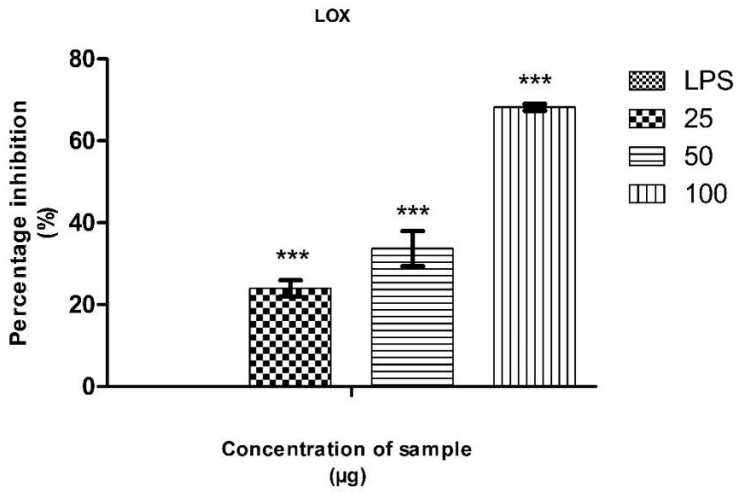

Figure 9: Inhibition of LOX in Lipopolysaccharide activated RAW 246.7 cells.

CONCLUSION

Inflammation is vital in immune pathogenesis and reacts to microbial invasion, stress and tissue damage. Activated macrophages play critical part in acting as a first-line defense in combating infection. On exposure to microbial Lipopolysaccharide (LPS), tumor necrosis factor-α or interferon-γ, macrophages are classically activated and the inflammation process will be initiated. Meanwhile, as the inflammatory response progresses, macrophages produce numerous inflammatory mediators, comprising nitric oxide, prostaglandins, and pro inflammatory mediators. Conversely, the pathology of several inflammatory diseases including arthritis, asthma, cancer, diabetes, periodontitis attributes to the sustained release of pro inflammatory mediators by the macrophage cells. Hence, to treat inflammation-related diseases, anti-inflammatory agents are a therapeutic target [(41).] Inflammation is also associated with infection. So, an anti-bacterial activity is beneficial, and an added advantage when targeting drug design and future treatment strategies. The study suggested the possible benefits of this plant-based flavonoid quercetin to be exploited as an effective anti-bacterial and anti-inflammatory agent. In the current study, we evaluated the anti-inflammatory effect of QRF of *Psidium guajava* leaf extracts on Lipopolysaccharide stimulated RAW 246.7 cells. The use of quercetin would surely provide a unique and promising treatment strategy for clinical treatment in supporting global dental health. Finally, the study showed that QRF–PG decreased the activity of different cytokines on LPS-treated macrophages. More studies focussed on this aspect may reveal the novel therapeutic potential of quercetin in combatting infections.

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CONFLICT OF INTREST

It is hereby declared that there is no conflict of interest.

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