ANTI-INFLAMMATORY PROPERTIES OF ACEROLA (*MALPIGHIA EMARGINATA*) LEAF AND RIPE FRUIT GENOTYPES FOR PROTECTION AGAINST LPS-INDUCED INFLAMMATION IN MACROPHAGE CELLS AND THEIR SELECTIVITY TO CYCLOOXYGENASE-2 (COX-2) ACTIVITY

A Thesis

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ABSTRACT

Conventionally, NSAID has been proposed to have inhibitory action against COX and therefore has traditionally been used for treatment of acute and chronic inflammation. This study aims at exploring putative anti-inflammation mechanism of acerola. Previous studies have illustrated that phytochemicals like alkaloids, terpenoids, flavonoids, curcumin and phenolics have COX inhibitory activities as well. However, a natural occurring selective inhibitor of COX-2 that can modulate inflammation and can overcome the limitations of drugs like aspirin is still a priority. Aspirin is known to form an irreversible and non-competitive binding to COX which proves to be a potent cardiovascular protective agent. On the other hand, irreversible binding has implications by initiating inhibition of blood platelet aggregation.

Acerola has been earlier studied for its antioxidant, antimicrobial, anti-inflammatory, anticancer, antigenotoxic and antihyperglycemic properties. In the present study, anti-inflammatory properties of acerola have been established where different genotypes of acerola fruit and leaf fractions were studied for their biological properties. A comparative study using TLC, LC-MS and bioassays using macrophages is employed to identify which groups of phytochemicals are responsible for scavenging and inflammation inhibitory effect of acerola. Initially, phytochemicals were extracted using methanolic and methanolic/acetone/water solvents which isolated different groups of compounds in two fractions, including polyphenols and a mixture of polyphenols/terpenoids, respectively. The two fractions were explored to elucidate mode of action for different acerola genotypes. Results indicated that the methanolic fractions

of acerola showed higher activity exhibited suppression of ROS and partial decrease of nitric oxide levels in LPS-stimulated RAW264.7 macrophage cell line. This fraction also demonstrated inhibition of enzyme expression of COX-1/2. Moreover, BRS-238, a ripe fruit genotypes of acerola had a selective action against COX-2 - confirming the hypothesis that acerola's mode of anti-inflammatory action is through selective inhibition of COX-2.

DEDICATION

To my parents and brother

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CHAPTER I

INTRODUCTION

Phytochemicals are naturally occurring plant derived compounds that account for bitterness, astringency, color, flavor, odor and oxidative stability in foods. They include carotenoids, lycopenes, flavonoids, anthocyanins, terpenoids, polyphenols etc.

Epidemiological studies have illustrated that intake of these phytochemicals have an inverse correlation with obesity, diabetes, hepatitis B, cardiovascular diseases, neurodegenerative diseases and HIV [1-10]. They are known to possess antioxidant properties and have been identified to scavenge singlet oxygen ions, peroxide decomposer, quencher of singlet oxygen radicals and electron donors. Therefore plants are called bio-factories of polyphenols [1]. Phytochemicals are absorbed in the small intestine where they undergo series of hydrolysis before they are finally metabolized. After consumption of the foods polyphenols circulate in the blood plasma in their glucurinated or sulfated forms [9, 10]. These oxidative stresses have been coupled with inflammatory studies.

Inflammation is a central feature of metabolic syndrome. Inflammation is defined as a physiological response that triggers a defense mechanism against various stimuli. They may be categorized as systemic or localized according to the gravity of inflammatory reaction. Neutrophils play a pivitol role during the course of inflammation. They circulate within the blood stream and extravasates from the endothelial cell barrier in response to a mediator of inflammation [11-14].

MACROPHAGES AND INFLAMMATION

Macrophages were first described by Noble laureate Élie Metchnikoff [15]. As depicted from their name, they were previously thought to be involved in phagocytic functions; and were expected to possess an ability to stimulate phagocytosis at the port of inflammation. However, decades of research has demonstrated that these phagocytic cells have more functions than just defense mechanisms [15, 16].

Several reports have amplified the role of macrophages in inflammation. Macrophages are essential in homeostasis [15, 17]. They are omnipresent and circulate in the blood stream; either in response to stress in activated mode or to maintain the homeostasis in unstimulated state. They are involved in removal of the cell debris of the apoptotic cells through scavenger receptors like phosphotydl serine, thromobospondin, complementary receptors and integrin. These responses are independent and do not require signaling pathways to initiate cytokines or transcription of different genes [16, 18].

Macrophages are integral in identifying the endogenous danger signals through intracellular pattern recognition receptors and interleukin-1 receptors. Activated macrophages are further classified as M1 and M2 [16, 18]. M1 are the ones that are classically activated and induce proinflammatory cytokines, inducible nitric oxide and reactive nitrogen/oxygen intermediates. M1 promote tumorcidal and microbicidal activities [17, 19]. Whereas M2 are alternately activated cells and helps in eradication of parasites by inducing IL-4 and IL-13 cytokines.

Interaction between metabolic cells and macrophages is cardinal for inflammation pathogenesis. During dysfunctions, recruitment process is intervened by pro-inflammatory cytokines and chemokines. Macrophages have specific roles that can be utilized for therapeutic purposes [19]. Cytokines like IFN-γ are induced by innate or adaptive immune cells during endogenous stress. For example through LPS which transduces macrophages to secrete pro-inflammatory markers. Toll-like receptors (TLR) bind to the pathogen which induces transcription of various genes [19-21].

MACROPHAGES INFILTRATION AND ACTIVATION

Recruitment

At the port of inflammation, a well-coordinated signaling pathway is initiated. Activation of macrophages is an essential step in pathogenesis of inflammation. Chemokines like monocyte chemoattractant protein-1 (MCP-1), also called as CC chemokine ligand-2, are produced at the site of dysfunction and they recruit macrophages. Studies have demonstrated that mice with knockout MCP-1 have higher insulin sensitivity and are protected from atherosclerosis [17, 19].

Activation

In the presence of external stimuli, the recruited macrophages begin host dependent signaling pathways to eradicate the cause of stress in the host. Therefore, they trigger an intrinsic or an extrinsic regulatory function. Overexpression of TNF-α, IFN-γ and iNOS traverses induction M1 macrophages which promotes pro-inflammatory markers. Whereas M2 phenotype of macrophages induce anti-inflammatory responses,

leading to production of IL-10, IL-13 and IL-4 causing wound healing, tissue repair and homeostasis [17, 19].

Deactivation

This is a critical step in restoring balance in the host; else pro-inflammatory markers will continue inducing an inflammatory response even after suppression of inflammation in the host tissues. The anti-inflammatory cytokines like IL-10 are produced to abort pro-inflammatory signals. Studies have established polymorphic nature of IL10 and its close association with metabolic syndrome [22]. Anti-inflammatory cytokines mediate phagocytosis of the apoptotic cells and prevent systemic inflammation and necrosis. IL-10 is expressed in both M1 and M2 cytokines [19].

Regulatory macrophages

Hypothalamic pituitary-adrenal (HPA) axis releases glucocorticoids to inhibit the action of macrophages in the host. They interrupt the transcription of pro-inflammatory cytokines. TLR ligand signals production of IL-10 and downregulates production of IL-12. This ratio of the two interleukins is an essential step in regulation. Other mechanisms to generate regulatory macrophages involve activation of MAPK and ERK [16, 17].

EXTRAVASATION IN CASE OF ENDOTHELIAL INFLAMMATION

In case of endothelial dysfunction, macrophages circulating in the blood extravasate from the endothelial cell barrier in response to the stimulus [11-13]. The endothelial cells increase the production of E and P-selectins as well as pro-

inflammatory cytokines like IL-1 or TNF-α which signals the macrophages to the port of inflammation [11-14, 23].

ROLE OF REDOX MOLECULES IN INFLAMMATION

Redox molecules include nitric oxide (NO)/ reactive nitric oxide synthase (RNS) and Reactive oxygen species (ROS). They serve as immune-toxins as well as immune-modulator; they are capable of eradication of the pathogen and initiate an immunosuppression by restoring the tissue balance. They mediate a series of downstream signals pathways triggering diverse action against the pathogen [21, 24, 25].

Reactive oxygen species comprise of singlet oxygen ions, hydrogen peroxide, hydroxyl ions and superoxide anions, generated by partial reduction of dioxygen in the mitochondrial oxidation pathway. These ROS are capable of oxidizing biological macromolecules [25, 26]. The inducible nitric oxide synthase mediates the production of NO. There are three types on nitric oxide synthases- eNOS, nNOS and iNOS. iNOS is the one which is associated with the immune system and is produced from nM levels to uM levels. NO and NO_2 are lyophilic in nature, then permitting it to migrate across. Despite of being crucial for, homeostasis ROS/NO are capable of causing oxidative stresses, lipid peroxidation, oxidize nucleic acids, protein modification and increased levels of free radicals in the host tissue [26].

The presence of reactive oxygen species (ROS) in tissues was first proposed by Gerschman, Gilbert and co-workers as early as 1954. They discovered the toxic mechanism of oxygen in presence of increased partial pressure [27]. Subsequent discovery

of superoxide dismutase lead to the postulation that ROS has a phagocytic action justifying their action to prevent oxidative stresses [25, 26].

ROS is produced via two mechanisms in an intact cell- enzymatically and non-enzymatically. Non-enzymatic reactions occur when foreign substances or endogenous metabolites like by-products of mitochondrial respiratory chains are auto-oxidized. On the other hand, enzymatic reactions are controlled and are known to promote signaling pathways within the cells [24]. Primary sources of ROS include mitochondria and NADPH oxidase. NADPH oxidase (NOX) is a multi-protein present in the plasma membrane. A small G-protein initiates activation of NOX in presence of external stimuli like bacterial lipopolysaccharide (LPS) or various proinflammatory cytokines [24]. After stimulation LPS, LPS binds to a Toll Like Receptor-4 (TLR-4), specific for LPS and induces ROS via mitochondria and NOX which leads to expression of IFN- β which promote iNOS and further induce NO production and also mediate iNOS via MAPK pathway [21].

APPROACHES UTILIZED TO QUANTIFY ANTIOXIDANT CONTENTS OF POLYPHENOLS

Numerous approaches have been employed to quantify the antioxidant content of fruits and vegetables. Frequently, utilized techniques for measuring the total phenolic compounds are DPPH, ABTS free radical decolorization assay, FRAP (ferric reducing antioxidant power assay) and Folin-Ciocalteau reaction.

Different motley tests have illustrated results for total phenolic content [28] The following table is a summary of acerola content reported in the literature.

Table 1. Phenolic content in acerola reported

Test employed	Fresh acerola sample	Lyophilized sample
Folin ciocalteau (mg GAE/100 g)	1055.9 ±46.5	900 ±54.6
ABTS (mg/100 g)	1315.0±25.3	1783.4±15.0
DPPH (EC_{50} mg/100 g)	838.8±0.4	795.0±6.8
FRAP (mg/100 g)	495.1±12.3	491.3±7.2

(Adopted from[28])

Concentrate of acerola are endorsed as potent anti-tumor which attenuates NKK formation which leads to suppression of development of lung tumor. Extracts can be used as new candidate for multidrug resistance by inhibiting the action of p-glycoprotein and antibacterial agent [29]. It can be used as dietary supplements to increase the dermal density of skin and treating cutaneous aging [30]. Apparently in a study juice from acerola was recommended as substitute for orange juice to infants [31].

ACEROLA AS A SOURCE OF ANTI-INFLAMMATORY COMPOUNDS

Acerola, is associated to genus *Malpighigia*, and in scientific taxonomy is called *Malpighia emarginata* D.C. It's belongs to an evergreen small tree or a shrub. It is a drupaceous round fruit with thin epicarp which turns green to yellow to bright red after ripening. It is habituated to tropical or subtropical conditions, and requires fertile soil composed of clay plus sand which can hold humidity longer. It is endemic to Southern Texas, Mexico, Central and South America including Brazil and Caribbean. It is even cultivated in few areas of Asia like India, but currently Brazil is the largest grower of acerola [32, 33]. It was conventionally called as West Indian Cherry, Haiti or Barbados cherry and is commercially utilized as juice, marmalade, gelatin, ice creams, frozen concentrate, jelly, gums, nutraceutical, liquor and yoghurt [32, 34].

Mature acerolas are extremely fragile and perishable with a shelf life of 2-3 days. After cultivation the cherries are hand-picked, early morning to avoid transpiration and maintain its quality attributes. Optimal storage temperature lies between 7-8^{o}C [33]. Phenolic compounds in acerola vary according to its maturity level and growth conditions. It has been exhibited that with changing conditions of maturity, phenolic content also varied [35]. Composition of the fruit depends on various factors including climatic, culture treatment, location of the farms, utilization of pesticides, level of maturity, processing and storage [33, 35]. The amount of ascorbic acid decreases during the ripening process, for example, from initial ranges of 2.15 - 3.20 g/100 g to1.45 - 1.83 g/100 g [36].

Acerola is a natural source of vitamin C with abundance of polyphenols and terpenoids. Previous reports have identified radical scavenging properties of acerola due to presence of anthocyanin and quercetins [37]. Flavanoid- aceronidin has been isolated from green acerola has exhibited higher DPPH activity as compared to α-tocopherol [38].

OBJECTIVES

In this study, genotypes of acerola are screened for anti-inflammatory effects and to elucidate its putative mechanism using a mammalian cell model. The aim is to identify which genotype has a selective inhibitory action against COX-1 and COX-2.

HYPOTHESIS

Aspirin is a potent NSAID target which has an inhibitory action against COX-2 and COX-1. We hypothesized that the extracts of different genotypes used for this

investigation have a COX inhibitory action like aspirin or a selective action like that of ibuprofen. Selective COX-2 inhibitors are a subject of interest in strategies to mitigate diseases such chronic inflammation and cancer and overcome limitations of NSAIDs. The aim is to endorse acerola as a natural NSAID.

Specific objective 1: To determine an extraction procedure and identify the compounds present in each genotype of acerola.

Chapter II deals with evaluation of chemical compounds present in acerola and which solvent should be employed to extract phytochemicals from the lyophilized acerola samples. Extracts were dissolved in methanol and were analyzed using TLC and LCMS. Different peaks depicting different masses at different retention time were identified using literature survey.

Specific objective 2: To differentiate between different extraction processes using biological assays and determine selectivity of genotypes in COX assay kit.

In chapter III, deals with comparing the effects on ROS, NO and mitochondrial activity on the basis of the different compounds present within each genotype. Furthermore acerola fractions were tested for their inhibitory action against COX-2 and some genotypes were selected for their selective action against COX-1.

CHAPTER II

PHYTOCHEMICAL ANALYSIS OF ACEROLA LEAVES AND FRUIT GENOTYPES

SYNOPSIS

For this investigation two approaches were used. First, a qualitative analysis was performed using thin layer chromatography (TLC) and second, the identification of the various phytochemicals was performed using liquid chromatography-mass spectrometry (LC-MS). Isolation and identification of different components existent in a plant is an essential step in elucidating the mechanism of action in biological systems.

TLC is mainly a qualitative analysis that determines the type of compounds present in an extract and gives insight of the possible amounts present. This test was executed to select the extraction procedure for biological assays. TLC results showed presence of high levels of terpenoids in the methanolic samples as compared to methanol/acetone/water. Isolation using methanol/acetone/water had negligible amounts of terpenoids. Polyphenols subsisting in each genotype was identified using LCMS.

Nine out of thirteen peaks in distinct leaf genotypes were identified in the mass spectra at negative ionization mode at specific retention time. Likewise nine out of eleven peaks were identified in fruit fractions using positive ionization mode. These different flavanoids have hitherto ascertained their antioxidant activities in literature.

INTRODUCTION

Acerola has been studied for the presence of high amounts of vitamin C content [31, 34] while other studies have reported presence of other phytochemicals including flavonoids [28, 31, 34].

Anthocyanins are the most ubiquitously present polyphenol imparting red, violet color to the fruit belongs to class of flavonoids which are composed of six membered rings. The bioactivity of anthocyanin is based on the structure and function relationships and on the side chain [39]. According to USDA database (2011) acerola has 15.71 mg/100 g edible portion and 6.84 mg/100 g edible portion of cyanidin and pelargonidin respectively; and has 1.05 mg/100g of kaempferol with 4.74 mg/100g of quercetins [40].

Functional characterization of green and ripe acerola have revealed them to be an abundant source of anthocyanin, carotenoids, flavanol, benzopyrone, polyphenolic acid and aceronidin, whereas leaves have been reported to have high flavanol, phytosterol, triterpenes and sesquiterpenes [38, 41, 42].

In a human intervention, comparing a synthetic ascorbic acid juice versus acerola juice, bioavailability of ascorbic acid from acerola was higher than the synthetic one. The investigation suggested that prominence of cyanidin-3-aO-rhamnoside and pelargonidin-3-a-O-rhamnoside present in the acerola juice enhanced the absorption of ascorbic acid into plasma and reduced its excretion through urine [43].

In another study, analyzing the effects of acerola juice on body fat mass., mice were fed with cafeterian diet (Mortadella, marshmallow, cheese chips, chocolate wafer,

water and Guaraná soft drink etc.) supplemented with water (for control), acerola juice (produced industrially or from ripe fruit, or immature acerola) and synthetic vitamin C. The results confirmed that a diet supplemented with acerola juice reduced the inflammatory proteins (TNF-α) and increased lipolysis in mice fed a cafeteria diet [44].

The study of therapeutics from antioxidant derived from phytochemicals has been an area of interest for decades. These antioxidants participate in pathogenesis of coronary heart diseases, diabetes and cancer [8]. These secondary metabolites are non-essential metabolites utilized in the defense mechanism against the ultraviolet radiation and pathogenic attacks on the plants. Among the range of antioxidants present in plants, phenolic compounds are the largest group including flavonoids, anthocyanins, hydroxycinnamic acids, hydroxybenzoic acids and catechins [8].

In the present study, for detection of the polyphenols present in a range of genotypes of acerola we used the LC-MS technique as well a different solvents for the extraction procedure. Liquid chromatography mass spectroscopy (LCMS) is a reliable approach utilized in detection and separation of chemicals on the basis of the molecular mass from a mixture of compounds. It is a powerful system used for identification and isolation in pharmaceutical, drug and food industries. LCMS separates components on the basis of their molecular masses. Being highly sensitive this technique has been used for identification of natural compounds from a plant derived mixture [45].

This study begins by employing three different solvents for extraction of phytochemicals including methanol, methanol/acetone and methanol/acetone/water under the hypothesis that different solvent mixtures will extract different group of

compounds based on polarity. Methanol is able to extract maximum amount of polar/non polar phytochemicals while a mixture of methanol and acetone (1:1) could favor the extraction of more non polar compounds compared to only methanol. The third solvent mixture methanol/acetone/water potentially could extract high content of polar compounds compared to methanol alone. In the present study we evaluated the use of different solvents for the extraction of bioactive compounds from acerola, determined the presence of terpenoids using TLC and identified the presence of polyphenols by LCMS. The selection of the appropriate solvents and compound identification in this chapter 2 would be critical for the studies of anti-inflammatory properties in chapter 3.

MATERIAL AND METHODS

Materials

Genotypes of Acerola leaf and ripe fruits (BRS-186, BRS-235, BRS-236, BRS-237, BRS-238, BRS-366) were brought from EMBRAPA Brazil. The chemicals used were purchased from VWR.

Extraction of the acerola samples

Extraction was performed using two methods: 100% methanol and extractions with methanol/acetone/ water (5:4:1). Around 100 mg of the raw lyophilized (leaf and fruit) samples were dissolved in 1000 µl of one of the above solvents. The mixture was vortexed followed by sonication for 40 mins. Then the mixture was stirred for overnight at a 4°C. After that the mixture was centrifuged for 10 min, at >3000 rpm (The Drucker Company, Port Matilda, PA) and the supernatant was filtered using 0.2 µm syringe

filters (VWR, Radnor, PA, USA) in pre-weighed tubes. Leaves extracts were additionally washed with 1000 µl hexane and the supernatant of the biphasic layer formed was separated using a separating funnel. Then the supernatants were concentrated in a vacuum centrifuge (LABCONCO) at 45°C to dryness. Then the extracts were re-suspended in methanol prior to LC-MS analysis or DMSO for biological assays.

Evaluation of terpenoids using Thin Layer Chromatography

100 mg of raw lyophilized BRS-238 leaf sample was dissolved in 1000 µl methanol, methanol/ acetone (1:1) and methanol/acetone/water (5:4:1) separately. They were vortexed and centrifuged for 10 min and later stirred in the cold room for overnight. After incubation, they were centrifuged and the supernatant was filtered using 0.22um filter; subsequently collected in a pre-weighed tubes. The supernatants were washed with 1000 µl of hexane and the biphasic layers formed was separated using a separating funnel and both the layers were collected in separate pre-weighed tubes. The residues from each solvent were washed using 1000 µl chloroform and the supernatant was collected in the pre-weighed tubes after being stirred and centrifuged for 30 min. Small amount of all the various supernatants were spotted on the silica plate. The TLC plate was run using all the various supernatants in a mobile phase of 10% methanol and DCM (dichloromethane). The run was then visualized using UV and anisaldehyde/sulfuric acid spray reagent as shown in Figure 2.1.

HPLC/MS methodology

Identification of phenolics from the acerola leaf genotypes by LC-MS analysis

The system used was a Surveyor (Thermo Scientific, USA) coupled to Surveyor DAD. The eluents were composed of acetonitrile/methanol (1:1), formic acid (0.5:99.5, v/v) (phase A) and formic acid–water (0.5:99.5, v/v) (phase B). 10 µl of the sample were injected applying an elution conditions of: 0-2 min, 2% A, 98% B; 3-5 min, 5%A, 95% B, 5-30 min, 20% A, 80% B; 30-72min, 35% A, 65% B; 72-83 min, 100% A, 0% B; 83-85 min was held isocratic, 100% A; 87-90min 2% A, 98% at the starting condition. The chromatograms were monitored at 330, 280, 210 nm; and complete spectral data were recorded between the range of 200–600 nm. A reversed-phase Phenomenex (Torrance, USA) Luna C_{18} column (150mm×4.6mm i.d. and particle size 3µm) with a Waters Nova-Pack C_{18} guard column (10mm×3.9mm i.d, 4 µm) was used and a flow of 200 µl/min from the DAD eluent was directed to the ESI interface using a flow-splitter. Nitrogen was used as desolvation gas, at 275°C and a flow rate of 60 L/h, and no cone gas was used. Mass spectra were obtained on a MS Finnigan LCQ Deca XP Max, Ion trap mass spectrometer coupled at the exit of the diode array detector and equipped with a *Z*-spray ESI source, and run by Xcalibur version 1.3 software (Thermofinnigan-Surveyor, San José, USA). A potential of 1.5 kV was used on the capillary for negative ion mode. The source block temperature was held at 250 °C.

Identification of phenolics from the acerola fruits genotypes by LC-MS analysis

100mg each of lyophilized ripe fruits genotypes was dissolved in a 1000 µl of MeOH separately and stirred for 24 h at 4°C. The extracts were centrifuged and then filtered. The filtrate was concentrated until all the volatile solvent was evaporated to dryness. The above samples were re-dissolved in MeOH this extract was used to analyze the profiling of the compounds present in the ripe fruits genotypes.

Individual compounds were identified on the basis of retention time, UV spectra, and their mass-to-charge ratio using LC-MS/MS. Chromatographic separations were performed on a LCQ Deca XP Max MS^n system (Thermo Finnigan, San Jose, CA, USA) equipped with an autosampler, a Surveyor 2000 quaternary pump, and a Surveyor UV 2000 PDA detector using a Hydro-RP18 Phase (150 mm x 4.6 mm x 3 mm, Phenomenex, Torrance, CA, USA, particle Sizes (4 µm) and pore size of 100 Å) and a guard column of the same chemistry. 10 µl of the sample was injected and elution gradients were performed with solvent A consisting of acetonitrile/methanol (1:1 containing 0.5% formic acid) and solvent B: water containing 0.5% formic acid. The elution gradients were performed with acetonitrile/methanol (1:1), formic acid (0.5:99.5, v/v) (phase A) and formic acid–water (0.5:99.5, v/v) (phase B). The applied elution conditions were: 0-2 min, 2% A, 98% B; 3-5 min, 5%A, 95% B, 5-30 min, 20% A, 80% B; 30-72min, 35% A, 65% B; 72-83 min, 100% A, 0% B; 83-85 min was held isocratic, 100% A; 87-90min 2% A, 98% to the starting condition. The chromatograms were monitored at 330, 280, 210 nm; and complete spectral data were recorded in the range 200–600 nm. Nitrogen was used as desolvation gas, at 275◦C and a flow rate of 60 l/h,

and He gas was used as damping gas. Mass spectra were obtained on a MS Finnigan LCQ Deca XP Max, Ion trap mass spectrometer coupled at the exit of the diode array detector and equipped with a Z-spray ESI source, and run by Xcalibur version 1.3 software (Thermofinnigan-Surveyor, San José, USA). A potential of 6.8 V was used on the capillary for positive ion mode. Spray voltage of 4.57 kV and the source block temperature was held at 255°C.

RESULTS AND DISCUSSION

Evaluation of terpenoids using Thin Layer Chromatography

In thin layer chromatography the mobile phase (generally liquid or gas) traverses over the stationary phase (solid or liquid) displacing different component present in it. The chemicals present in the stationary phase travels according to its speed on the mobile phase. Basically this test estimates the quantity of different components present in the mixture. In this study, strong intensity steroid band were detected in the supernatants of MeOH and MeOH/Ac extracted fractions. However, very weak intensity band was observed in extracts of MeOH/Acetone/H_2O. Hexane did not remove any steroids. The residues were treated with chloroform to detect presence of any terpenoids, however no bands were observed. Terpenoids and polyphenols were isolated using the different solvents. MeOH/Acetone/H_2O had negligible amounts of terpenoids assuming it to potentially have higher polyphenols whereas MeOH extracts had terpenenes as well (Table 2.1).

During the extraction procedure the pH of acerola was measured using a pH indicator strip giving pH values between 3 – 4 which matches with previous studies of reported pH values of 3.7 [46]. This confirms that the stability of anthocyanins were not affected during the extraction procedure in the present.

The fractions from the two extracts were used to quantify the biological activities, discussed in chapter III.

Identification of phenolics from the acerola leaf genotypes by LC-MS analysis

The HPLC-DAD chromatogram of the acerola leaves at 330, 280, 210 nm was carried out using liquid-chromatography-mass-spectrometry (LC-MS) as shown in Figure 1. The fragmentation patterns of MS and MS^2, along with potential compounds identified are shown in Table 2.2.

The derivatives of flavonoids were detected and identification was achieved by comparison of MS^2 fragmentation pattern, UV spectra (nm) and retention time (RT) with data reported in the literature. The MS^2 data mining process begins by identifying the fragments of aglycone flavonoid is recognized to determine the backbone of each compound; thus quercetin ([M-H] m/z 301 Da), isorhamnetin ([M-H] m/z 315 Da) and kaempferol ([M-H] m/z 285 Da) were identified. Further step involves determining the number and type of sugar units attached to each flavonoid skeleton.

The retention times, molecular weight (MS) and MS^2 data of the phenolic compounds detected in the extracts are listed in Table 2.2. The compounds were

identified according to their fragmentation data and UV absorption and their structures are shown in Figure 2.2.

The MS^2 analysis of the precursor ions from peaks **1**, **2**, **4, 5** and **7** yielded major MS^2 fragment at m/z 285, thus, these compounds were tentatively identified as kaempferol *O*-glycosides (Table 2.2). Compound **1** gave $[M-H]^-$ m/z 711; yielding fragments at m/z 579 $[M-H-132]^-$ and m/z 285 $[M-H-132-146-132]^-$. According to these fragments, two sugar units, such a hexose (162 Da), and two pentose (132 Da) are attached to a kaempferol molecule. In order to confirm the structure, the compound **1** was isolated for NMR studies. Hence the structure was confirmed on the basis of 2D NMR and the new molecule was assigned as kaempferol 3-*O*-β-D-[- arabinopyranosyl-(1'''→2'')-*O*-α-L-apiose-(1''''→5'')-*O*-β-Dglucopyranoside [47].

The compound **2**, precursor ion at m/z 725 $[(M-H)-]^-$, yielded fragments at m/z 579 $[(M-H)-146]^-$, *m/z* 598 $[(M-H)-132]^-$ and *m/z* 285 $[(M-H)-146-132-162]^-$. According to these fragments, three different sugar units, such a hexose (162 Da), a deoxyhexose (146 Da) and a pentose (132 Da) are attached to a kaempferol molecule. Thus compound **2** was identified as kaempferol 3-O-robinobioside-7-alpha-L-arabinofuranoside. The fragmentation pattern of compound **3**, at *m/z* 755 $[(M-H)]^-$, yielded ions at *m/z* 623 $[(M-H)-132]^-$, *m/z* 315 $[(M-H)-146-162]^-$. The tri-glycoside **3** was identified as quercetin 3'-methoxyquercetin-7-O-α-L-rhamnopyranosyl-3-O-α-arabinofuranosyl-(1→6)-β-D-glucopyranoside (**3**) and fragmentation of this compound ions at *m/z* 623 $[(M-H)-132]^-$ suggests that pentose is directly attached to the ring and the similar compound from the aerial parts of *Atriplex halimus L.* collected from Sardinia has pentose at position 7 and

rutinoside at position 3, precursor ion at *m/z* 593 $[(M-H)]^-$, generated fragments at *m/z* 490 $[(M-H)-146+41]^-$ and m/z 301 $[(M-H)-146-162]^-$. Hence compound **3** was identified as 3'-methoxyquercetin-7-O-α-L-rhamnopyranosyl-3-O-α-arabinofuranosyl-(1→6)-β-D-glucopyranoside [48]. Compound **4** and **5** gave $[M-H]^-$ *m/z* at 579 and the MS^2 analysis of precursor ion at *m/z* 579, yielded the fragments at *m/z* 285 $[(M-H)-162]^-$. The compound **4** and **5** was identified as kaempferol *O*-glycosides isomers, according to the fragmentation pattern in the literature [49].

The compound **7** with the precursor ion at m/z 593 $[(M-H]^-$, showed similar fragments at *m/z* 285 $[(M-H)-146-162]^-$ and *m/z* 257.18 $[(M-H)-146-162-28]^-$, then these di-glycosides are isomers. Hence the compound **7** was identified as kaempferol 7-*O*-neohesperidoside, previously characterized by LC-ESI-MS analysis in cocoa (*Theobroma cacao*) [50]. This is the first report of kaempferol 7-*O*-neohesperidoside in *Malpighia emarginata* samples.

Compound **8**, precursor ion at *m/z* 623 $[(M-H)]^-$, yielded fragments detected at *m/z* 461 $[(M-H)-162]^-$and at *m/z* 315 $[(M-H)-146-162]^-$, showing that this compound is a di-glycoside. The fragmentation pattern of compound **8** allowed identification of isorhamnetin 3-O-rutinoside (narcissin), according to the data obtained by [51]. Compound **9, 10, 12 and 13** gave precursor ion m/z $[M-H]^-$ 917, 931, 857 and 871 respectively and yielded MS^2 fragments 711, 693 for *m/z* 917 and 739, 725, 605, 298 for *m/z* 931 and 693 for *m/z* 857 and 751, 725, 707, 441 for *m/z* 871. These fragments were not reported in the literature and their structures need to be confirmed by NMR. The precursor ion at *m/z* 785 gave MS^2 fragments at m/z 593 and m/z 313, which is

characteristic of hexose isomer and hence the compound was tentatively identified as digalloyl-HHDP-glucose [52].

Identification of phenolics from the acerola fruit genotypes by LC-MS analysis

Peaks at R.T 26.5 and 27.3 had the molecular ion ($[M+H]^+$) at m/z 434 and 418 respectively, and by MS/MS experiment a major fragment at *m/z* 270 and *m/z* 287 respectively and was assigned to cyanidin-3-rhamnoside and pelargonidin 3-rhamnoside respectively[53]. The exact mass of the Peak at retention time (R.T.) of 28.4 could not be identified clearly but it gave a major MS/MS fragment ion at 301 which suggested a peonidin derivative, peaks at R.T 31.0, 31.7 and 44.5 gave molecular ions at *m/z* 596, 451 and 464 respectively, on MS fragmentation *m/z* 596 gave fragments at *m/z* 449 and 287 and was assigned to cyanidin 3-rutinoside, *m/z* 451 gave mass fragments at *m/z* 303 and *m/z* 316 and was assigned to peonidin-3-xylopyranoside, *m/z* 464 gave a major fragment ion at *m/z* 301 suggesting again a peonidin derivative and was assigned to peonidin-3-rutinoside[54]. Peaks at R.T 53.2 and 58.8 also gave fragmentation ion at *m/z* 301 and *m/z* 316 suggesting again a peonidin derivative and hence the peaks were identified as peonidin-3-rutinoside and peonidin-3-O-diglucoside respectively. Peaks at R.T 50.88 and 58.8 gave fragment ions at *m/z* 303 and *m/z* 339, *m/z* 423 and *m/z* 365 respectively; however they could not be identified as shown in Table 2.3 and figure 2.3.

CONCLUSIONS

From the above experiments LCMS has revealed that leaves and fruits comprise of derivatives from flavonoids and anthocyanins respectively. It was for the first time

these genotypes were examined. Ripe fruits are red making them ample in anthocyanin. Moreover the derivatives identified were cross checked from the literature. TLC results validate presence of terpenes in methanol rather than methanol/acetone/water. It was hypothesized that MeOH/Acetone/H_2O had negligible amounts of terpenoids concluding it to be rich in polyphenols whereas MeOH extracts had presence of both phenolic as well as terpenoids

Individual compounds are known to contribute to various biological activities and have been linked with protection as antiallergic, antiinflammatory, antiviral, antiproliferative, and anticarcinogenic activities [39, 55].Generally flavonoids consumed are flavonols, flavanones, and isoflavones. A large spectrum of beneficial pharmacological properties have been studied for flavones [39, 55]. Anthocyanins, are recognized as a natural colorant and a substitute for synthetic colorants which toxic for human [56].

Nine out of thirteen peaks identified in acerola leaf were quercetin and kaempferol derivatives which have been reported previously in acerola. In case of fruits peonidin-3-xylopyranoside and peonidin 3-rutinoside has been recorded for the first time in acerola. Derivatives of peonidin have been analyzed to be down-regulating metastasis [57]. We conclude that there is a broad range of polyphenols and terpenoids existent in leaf and fruit samples give acerola the potential to exert anti-inflammatory properties which is further explored in chapter 3.

CHAPTER III

ANTI-INFLAMMATORY PROPERTIES OF ACEROLA LEAVES AND FRUIT GENOTYPES

SYNOPSIS

This chapter comprises of the biological aspect of acerola in their anti-inflammatory properties. Extracts of different leaf and ripe fruit samples were selected on the basis of the TLC work from chapter 2. It was hypothesized that due to presence of a terpenoids/polyphenols mixture, methanolic extracts have more biological activity as compared to Me/Acetone/water which only contains polyphenols. A decrease in ROS levels and partial decrease in NO production were observed in methanolic extracts compared to Me/Acetone/water extracts. This effect was evident for leaf methanolic extracts which contained a mixture of terpenoids/polyphenols very likely responsible for the effect.

Methanolic extracts were further analyzed to study the action on COX enzymatic expression. Almost all genotypes exerted inhibition against COX-2 and COX-1 activity. From all samples studied, BRS-238 fruit had a selective action against COX-2 and COX-1. Consequently, this genotype is likely to have a putative mechanism similar to that of known drugs like celecoxib and rofecoxib. NSAIDs are known to exhibit analgesic, antipyretic, anticarcinogenic, anti-inflammatory properties. The ultimate goal is to propose acerola as a functional fruit with a potent NSAID like property, with the

advantage of being a natural occurring selective inhibitor of COX-2 which can overcome the limitations of commercial drugs like aspirin.

INTRODUCTION

Humans differ in their response to drugs for multiple reasons but particularly as a result of inter-individual genetic differences in drug absorption, distribution, metabolism and excretion. All of these are potential sources of pharmacokinetic variability and can have significant effects on treatment outcomes. For example, the chemo-preventive potential of NSAIDs (Non-Steroidal Anti-inflammatory Drug) could be enhanced, and more selectively targeted, by taking into account of such relevant pharmacogenetic differences.

During the succession of inflammation, transcription factors like JNK (c-Jun N-terminal kinase), NF-κB (Nuclear factor κB) and MAPK (Mitogen-activated protein kinase) are activated and they eventually transcribe pro-inflammatory and anti-inflammatory cytokines which are indispensable for signaling pathway [16, 18]. NF-κB is an omnipresent redox transcription factor involved in regulation of various genes associated with immune responses against pathogens. It is localized in the cytoplasm and is translocated in the nucleus in presence of stimuli after being phosphorylated [16, 18, 26].

Redox mechanism of NF-κB and inflammation

The pathway is mediated via phosphorylation and subsequent ubiquitination IκB. IκB and NF- κB are present in the cytoplasm in basal levels in an unstressed cell. In macrophage, the LPS, binds to TLR-4 (Toll like receptor-4), a pathogen-associated

molecular pattern (PAMP) and induce degradation of IκB leading it to translocation of NF-κB to the nucleus and initiating a transcription and production of gene expressions of TNFα, COX-2 and iNOS, which are mediated by LPS/TLR4 signaling pathway [58]. The TLR4 signaling is also mediated by mitogen-activated protein kinase (MAPK) signaling cascades [59]. Briefly, TGFβ-activated kinase 1 (TAK1) induces the extracellular signal-regulated kinase (ERK) pathways via IκB kinase (IKK) [60, 61]. The MAPKs triggers an interferon β signaling pathway and induces iNOS expression via activating the STAT-1. iNOS expressions amplifies production of NO after 6 h via IFN-β [62].

Antioxidant compounds are compounds that quench free radicals by donating electrons. There are two types of antioxidants, first which exist endogenously like glutathione peroxidase, catalases and superoxide dismutase; and second which needs to be supplemented from outside sources are exogenous [63]. Plethora of reports have elucidated that plant derived phytochemicals have antioxidant capacity. There are several mechanisms targeted by phytochemicals in *in-vivo* systems. For example: flavanoids, commonly consumed polyphenolic compound are known to exhibit anti-inflammatory effects in LPS stimulated macrophages by inhibiting NF-κB and further expression of iNOS in a dose dependent manner. Quercetin inhibits STAT-1 transcription factor along with iNOS expression [64, 65]. Curcumin, commonly found in rhizhomes have inhibited proliferation and ROS in TPA induced mouse epidermal cells [66].

Different array of prostaglandins (PG) are produced by each cell type in response to inflammation, wound healing, nerve growth and development, blood clotting and

ovulation. The production of PG in response to external stimuli is mediated by the biosynthesis of eicosanoids. Briefly, diacyglycerol from the membrane undergoes hydrolysis to archiadonic acid which is a substrate for cyclooxygenases. Subsequent peroxidation of archiadonic acid promotes production of prostaglandins by cyclooxygenases [67, 68]. NSAID drugs targets the cyclooxygenases which are the precursors of prostaglandins, consequently inhibiting inflammation. There are two isozymes of cyclooxygenases-COX-1 and COX-2. COX-1 is a precursor for thromboxane synthesis. NSAIDs like aspirin are known to bind to COX-1 irreversibly preventing platelet aggregation [67, 69, 70]. This binding has its pros and cons. In terms of cardiovascular disease aspirin binding to COX-1 it is an important mediator to prevent thrombosis; however inhibition of COX-1 affects homeostasis and even disrupts renal functions. On the other hand COX-2 is induced in presence of inflammation or during cases of stresses. It is reported to be produced in high quantities in presence of fever and synovial tissues during osteoarthritis. COX-2 specific inhibitors like Celecoxib and ibuprofen have demonstrated to exert suppression of inflammation and overcome innumerous adverse effects associated with non-selective drugs like aspirin [67-71].

This chapter aims at elucidating a putative acerola phytochemical responsible for the scavenging effect of ROS and partially suppression of NO levels and COX inhibitory activity. Thereby two solvent based extraction procedures were selected for this investigation. One set of extracts (MeOH/Acetone/H_2O) were abundant in polyphenols whereas another set (MeOH) possessed a combination of terpenes and phenolics. A comparative analysis indicates that methanolic fruit and leaf fractions attest greater

biological effect, probably due to presence of both terpenes and phenolics. In addition, this chapter deals with COX inhibitory activity of acerola extracts where MeOH extracts inhibited COX-1/2 enzyme activity indicating it to have similar actions as commercial NSAIDs.

MATERIALS AND METHODS

Materials

The following chemicals were used in the experiments: Lipopolysaccharide (LPS), 2′,7′-Dichlorofluorescin diacetate (DCFA), Griess reagent, Sodium nitrite solution, Dulbecco's Modified Eagle's Medium (DMEM)/low glucose, phenol red-free DMEM/low glucose, penicillin/streptomycin mixture, DMSO and Fetal Bovine Serum (FBS) were purchased from Sigma (St. Louis, MO). Glucose and sodium bicarbonate were purchased from Acros Organics (Fair Lawn, NJ) and sodium bicarbonate from Mallinckrodt Chemicals (Phillipsburg, NJ) respectively. The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Madison, WI). The Macrophages RAW 264.7 (cell line TIB-71™) was acquired from the American Type Culture Collection (ATCC) (Manassas, VA). COX (human) Inhibitor Screen Assay kit was purchased from Cayman Chemical.

Cell culture and drug treatment

RAW 264.7 mouse macrophages were cultured in the DMEM-low glucose (pH 7.2 – 7.4) supplemented with 4 g/l glucose, 3.7 g/l sodium bicarbonate, 10% fetal bovine

serum (FBS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere with 5% CO2 at 37°C. They were passaged in every 2-3 days.

Macrophages were plated at 0.5 $\times 10^5$ cells/well in a 96-well black and clear bottom plates (Costar, Cambridge, MA). The cells were incubated with the acerola extracts for 5 h and then cells were treated with 1 µg/ml LPS for 4 h or 19 h in this study. The extracts of genotypes were dissolved in 100% DMSO. For treatment of cells 0.5% of DMSO dissolved samples were exposed in all experiments.

Cell viability test

The proliferation of viable cells was evaluated using 3-(4,5-dimethylthiazol-1)-5-(3-carboxymeth-oxyphenyl)-2H-tetrazolium, CellTiter 96®AQueous One Solution according to the manufacturer's manual (Promega, Madison, WI). Briefly, 0.5×10^5 cells were seeded in a 96-well plate (BD Biosciences, Franklin Lakes, NJ). Then cells were incubated for 24 h with growth media containing 400 µg/ml of acerola fruits and leaves extracts. Afterwards, cells were re-suspended with 100 µl of growth media supplemented with 20 µl CellTiter 96®AQueous One Solution and the plate was incubated for 2 h. The assay is known to detect the absorbance of formazan produced which could be measured at 490 nm.

Cell viability was calculated using the following equation:

$$A_{\text{treatment 490 nm}}/A_{\text{control 490 nm}} * 100 = \% \text{ cell viability}$$

Detection of extracellular nitric oxide and intracellular reactive oxygen species production

Macrophages were plated 0.5×10^5 cells/well in a 96-well black and clear bottom plates and cultured overnight. The cells were stimulated by LPS for 4 h and 19 h and treated with or without the 5 h-pre-treatment with 400 µg/ml acerola fractions. Finally, cells and medium was used for ROS and NO detections respectively. The extracts of genotypes were dissolved in 100% DMSO. For treatment of cells 0.5% of DMSO dissolved samples were exposed in all experiments.

First, nitric oxide production was assessed after 19 h LPS treatment. The nitrite (NO_2^-) levels accumulate in the medium and can be determined using a colorimetric reaction with the Griess reagent. 50 µl of cell culture supernatants were mixed with an equal volume (50 µl) of Griess reagent. The readings were calculated against the standards between 0 to 100 µM sodium nitrite solutions. Subsequently, the absorbance was measured at 540 nm using a 96-well microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT).

Second, the intracellular ROS production was evaluated by measuring the fluorescence of 2′,7′-Dichlorofluorescin diacetate (DCFA). This non fluorescent probe permeates inside the cell membrane; ROS oxidizes the probe into fluorescent DCF. Briefly, after LPS stimulation for 4 h and 19 h, the cell culture medium was removed and subsequently cells were exposed to phenol red/FBS-free DMEM media containing 10 µM DCFA for 30 min. Then the cells were washed twice with the phenol red/FBS-free DMEM. Finally, fluorescence was read immediately at wavelengths of 485 nm for

excitation and 528 nm for emission using a 96-well microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT).

LPS-induced mitochondrial activity using MTS

Mitochondrial activity was evaluated in macrophages using the MTS assay kit (Promega, Madison, WI). Cells were seeded 0.5×10^5 cells/well after a pretreated with/ without 400 µg/ml of acerola fruits and leaves extracts acerola fractions for 5 h, Raw 264.7 were challenged with/without LPS for 19 h. The absorbance was measured using the manufacturer's protocol as explained above.

Evaluation of COX-1 and 2 binding nature of acerola fractions

The experiment was performed using a 'COX (human) Inhibitor Screen Assay' kit (Cayman Chemical), which measures $PGF_{2\alpha}$. PGH_2 is reduced by $SnCl_2$ into $PGF_{2\alpha}$ which is measured according to the manufacturer's protocol. Briefly, 10 µl of 200 µg/ml of acerola fractions were diluted with 90 µl of reaction buffer (4 mg/ml mixture provided from the manufacturers which was dissolved in 10% DMSO). From their 10 µl of the samples, DMSO (negative control), DuP-697 (positive control) were mixed with heme, COX-1/2 (human recombinant) enzyme and reaction buffer. The tubes were incubated for 10 min at 37^OC. Afterwards the reaction was initiated by addition of arachidonic acid for 2 min and later was terminated by 1 M HCl followed by addition of stannous chloride ($SnCl_2$). The reaction tubes were diluted, 2000X using EIA buffer and plated into the 96 well plate according to the protocol. Then level of PGF2α produced was

measured using microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT) 410 nm and the calculations were made against the standards.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) and t-test, using the software JMP pro v11.0. Results are expressed as means ± standard errors (SE). Different letters show significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

TLC results demonstrate that methanolic extracts are rich in terpenes and phenolics whereas MeOH/Acetone/H_2O extracts lacked steroids so have greater phenolic content. For this study, a comparison is executed to illustrate the putative action of acerola. That is, which compounds are responsible for scavenging ROS and NO as well as their inhibitory effect against COX activity in RAW 264.7.

Cytotoxicity of leaf and fruit extracts

MTS assay results indicate that 400 µg/mL acerola leaf and fruit extracts did not show any cytotoxic effects on macrophage cells (Figure 3.1). An increase in mitochondrial activity is observed in acerola treated cells as compared to the non-treated cells which did not affect the overall results of the present study.

Protection of acerola extracts against LPS-induced inflammation in Macrophages

The measurement of ROS in the present study was done at two time intervals of 4 and 19 h. According to a previous study the levels of LPS induced ROS are maintained

until 6 h, afterwards the levels of ROS induction in macrophages are partially mediated through nitric oxide. Therefore this investigation was divided into an early (before 6 h) ROS stage before NO induction and a late stage (after 6 h) after NO induction [21, 72].

LPS challenged cells stimulates TLR mediated pathway. LPS binds to TLR4 and activates a ROS- NO mediated redox signaling [16, 18]. ROS is induced from NADPH oxidase and mitochondria which initiates translocation of NF-κB into the nucleus after being phosphorylated. It then further leads to expression of iNOS and COX-2.

In this investigation we illustrate the effects of various genotypes of acerola leaf and fruit fractions extracted from two different solvents in LPS-stimulated RAW 264.7 macrophage cell lines to determine which group of phytochemical would most likely be involved in the ROS signaling.

For methanol extracts all of the leaf samples exhibited scavenging effect after 4 h (Figure 3.2 A.1) stimulation of LPS and the scavenging effect continued even after 19 h (Figure 3.3 A.1). However there was a partial scavenging after 19 h which could be due to the extracts being depleted after the long term LPS stimulation. Accordingly, acerola methanolic leaf extracts had early ROS scavenging properties. On the other hand, forfruit samples, other than BRS 238 and 366 none of them showed scavenging effect after stimulation of LPS for 4 h (Figure 3.2 A.2), however, a remarkable decrease was exhibited by all genotypes after 19 h (Figure 3.3 A.2) of the LPS challenge. This would suggest different scavenging kinetics between leaf and fruit acerola phytochemicals mainly due to terpenoid/polyphenol mixtures.

For methanol/acetone/water leaf extracts, these could not suppress ROS after 19 h stimulation (Figure 3.3 B), however, BRS-236 and BRS 366 leaf samples scavenged ROS levels at 4 h (Figure 3.2 B). On the other hand, despite only BRS- 366 fruit extract reduced LPS challenged cells after 4 h (Figure 3.2B), all genotypes exhibited partial decrement after 19 h stimulation of LPS (Figure 3.3B). Once again, these results would suggest different scavenging kinetics between leaf and fruit acerola phytochemicals, which in this case is associated solely to the polyphenols present in the samples.

In case of *methanol extracts* all genotypes of leaf samples displayed partial suppression of NO levels after 19, however, no suppression was observed as far is fruits are concerned (Figure 3.4 A).

A weak suppression effect was observed in *methanol/acetone/water* fruit extract-BRS 236 and BRS238 (Figure 3.4 B)

LPS stimulates iNOS gene which promotes production of nitric oxide (NO) after 6 h and it reaches its maximal levels in around 8 h [21, 64]. Enzymatic reaction by nitric oxide synthases on L-arginine produces nitric oxide [24]. Generally iNOS is responsible for prolonged production of nitric oxide levels. LPS stimulates ROS from various sources which activate transcription of NF-κB, AP-1 and subsequently other cytokines like TNF-α. For methanolic as well as MeOH/Acetone/water extracts nitric oxide levels were partially suppressed or mostly unaffected. In the case of acerola leaf extracts to the partial suppression of levels of ROS at 4 h, was responsible for the partially suppression in the production of nitric oxide levels. For all other samples the levels of ROS

suppression at 4 h was not enough to affect transcription factors and iNOS gene expression so as to affect NO production. .

Terpenes have been known to exhibit immunomodulatory, antiallergic and immunosuppressant actions [73-76]. Phenolic compounds also have scavenged ROS and nitric oxide [1, 35, 37, 39]. It is clear that the extent to which each phytochemical may display an effect determines the response. In this investigation two solvents were used assuming that each extraction procedure potentially isolated and enriched extracts into mixtures of terpenes/polyphenols and solely polyphenols. For instance methanolic compounds demonstrated to have high quantity of terpenes as compared to methanol/acetone/ water as shown in the TLC work in Chapter 2. One hypothesis could be that terpenes displayed higher activity or a synergistic effect with polyphenols when mixed in the methanolic extracts as compared to methanolic/acetone/water which contained only polyphenols.

LPS-induced mitochondrial activity using MTS

Tetrazolium salt in MTS is bioreduced to a blue colored compound formazan by NADH or NADPH. It is a reflection of the glycolysis pathway and the Kreb's cycle activity inside the cells which are main sources of NADH or NADPH. In presence of LPS macrophages undergo increased aerobic glycolysis where glucose is rapidly consumed and is converted into lactate while mitochondrial respiration activity decreases with a simultaneous increase in ROS production due to an increase in electron leaks from the mitochondrial electron transport system which react with water to produce free radicals. Such effect has been termed as Warburg effect [77-79]. This phenomenon is commonly witnessed in tumor cells, however inflammated cells also

switch to aerobic glycolysis in presence of oxygen leading to upregulation of hypoxia factor-1α (HIF-1α) and PKM-2. Both (HIF-1α) and PKM-2 seem to be upregulated by transcription factor NF-κb and thus are ROS dependent. Protein kinase (PKM-2) is an essential enzyme in glycolysis which catalyzes phosphophenol pyruvate to pyruvate [78]. It is thought that the Warburg effect is irreversible in cancer cells, while in inflammation, the effect seems to be reversible [77]. Here, in this study cells were challenged with or without LPS for 19 h after 5 h pre-treatment of 400 µg/ml acerola fractions. Macrophages were stimulated with LPS that increased the Warburg effect however acerola samples were unable to decrease these levels, so the levels were maintained. One plausible explanation is that acerola leaf or fruit extracts (for both solvents) were unable to scavenge ROS completely after 19 h, nor were they able to affect the glycolysis pathway or the Krebs cycle, thereby they were unable to bioreduce tetrazolium salt into formazan and thus acerola could not reverse the Warburg effect (Figure 3.5).

Selective inhibition of Cyclooxygenase-2 enzyme activity

NSAID drugs are traditionally used by targeting cyclooxygenases. Cyclooxygenase exists in two isoforms COX-1 and COX-2 and are responsible for production of prostaglandins, prostacyclin's and thromboxane's. COX-1 is expressed constitutively and is responsible for the maintenance of the homeostasis whereas COX-2 is induced during inflammation and proliferative diseases. Stimulus like LPS and overexpression of cytokines mediates expression of COX-2 for biosynthesis of prostaglandins, prostacyclin and thromboxane [68, 80, 81].

Arachidonic acid undergoes peroxidase and cyclooxygenase action which lead to production of prostaglandins H_2. Cayman COX inhibitor screening assay directly measures $PGF_{2\alpha}$ produced by $SnCl_2$ reduction of COX-derived PGH_2. The results are quantified by a plate reader assay.

Acerola leaf extracts showed strong inhibition of COX-2 enzyme expression (Figure 3.6 A). On the other hand, the action of COX-2 enzyme was attenuated by fruits as well, but BRS-238 had a stronger inhibition as compared to other extracts (Figure 3.6 B). BRS-238 ripe fruit and other leaf samples suggest that a possible mechanism of action is through inhibition of COX-2 activity in a mechanism similar to that of an NSAIDs [82, 83]. So further, to pinpoint the genotype with a selective mode of action COX-1 enzyme activity was measured for all leaf samples (BRS 186, BRS 235, BRS 236, BRS 237, BRS 238, BRS 366) and BRS 238 sample of fruit species. The leaf extracts from acerola demonstrated suppression in the COX-1 activity making them potent NSAID drugs with non-selective cyclooxygenase inhibition, like aspirin [82, 83]. However, in BRS-238 fruit a selective inhibition was observed (Figure 3.7 A). BRS-238 does not trigger the COX-1 enzyme activity but inhibited the action of COX-2 enzyme only (Figure3.7 B) , proposing that consumption of acerola genotype BRS-238 ripe fruit not only is a source of antioxidants but can also substitute NSAID commercial drugs like Diclofenac, Celecoibs and ibuprofen [70]. COX inhibitors bind to both isozyme- COX-1 and COX-2.Their kinetics reckons on the bond inhibitors develops with cyclooxygenases depending upon time. Supposing a bond between the isozyme and the inhibitor is

irreversible then the inhibitors are termed as non-selective. However, in case of selectivity the bond dissociates from with one of the isozyme quicker than the other. [71].

CONCLUSION

Results exhibited an increase in ROS due to induction by LPS which was further reduced by methanolic extracts of leaf and few ripe fruit genotypes after 4 h or 19 h stimulation of LPS; however, in the case of MeOH/Acetone/water extracts, the levels of ROS were partially suppressed mainly at 19 h. Since only leaf methanolic extracts showed a reduction of ROS levels at 4h, there was a partial reduction in NO production for these samples as well. For all other acerola extracts, the NO production was not altered. It is likely that the mixture of terpenoids/polyphenols present in the methanolic leaf samples played a role in the response of ROS and NO reduction.

During inflammation, LPS binds to the TLR4 receptor and triggers ROS dependent NF-κB activation. NF-κB is translocated via IκB and Ref-1 into the nucleus where it mediates the gene expression of COX-2 and iNOS [21, 72]. iNOS mediates production of nitric oxide and cyclooxygenases (prostaglandin endoperoxide H synthase-1 and 2) are known to catalyze the synthesis of prostaglanoids. Prostaglandin E-2 and D-2 are produced during inflammation for resolution of inflammation [68]. In a previous study with macrophages, acerola leaves and ripe fruit genotype BRS-238 exhibited suppression in the production of PGE2 in cell lysates [84]. In the present study we observed a drastic decrease in COX-2 (enzyme upstream of prostaglandins) enzymatic activity confirming that the suppressed level of PGH2α by acerola genotypes takes place in in-vitro cell assays as well as in biochemical enzymatic assays (Figure 3.8).

Non-steroidal anti-inflammatory drugs (NSAID) are involved in a range of pathologies for inflammation. Inhibitory effect of COX-2 and COX-1 by acerola leaf and fruit extracts for a range of genotypes in this project indicates its potential to be used as a natural source of NSAID.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Evidences have established a pivotal role of macrophages in metabolic syndrome [18]. Being the key effector cells they are known to maintain homoeostasis by efferocytosis that is eradicating the necrotic cells [15-17].

They have been used as a model cell line for understanding pathogenesis of chronic inflammation and preventing them utilizing various metabolites from plants, ceramide and different drugs. They elicit inflammatory responses by increasing the metabolic stress during maladaptation through a feed forward signaling pathway leading to activation of various transcription factors. They induce various cytokines like TNF-α, IL-1β, IL-10, IFNγ and IL-4 [16, 19].

In the presence of external stress LPS, pathogen is recognized by a pattern recognition receptor TLR-4 resulting in its upregulation. This ligation initiates production of copious ROS and NO [26]. ROS is known to be produced from mitochondria and NADPH oxidase. Complex I and III are known to be involved in production superoxide radicals. NADPH oxidase in presence of endogenous stimuli converts O_2 to $O_2^{\cdot-}$. These superoxide radicals are further converted into hydrogen peroxide (H2O2) by action of superoxide dismutase. H_2O_2 is well known to impact the gene expression and proliferation in stressed macrophages [25, 85]. iNOS (inducible isoform of nitric oxide synthase) gene responsible for high throughput production nitric oxide in presence of LPS. Nitric oxide levels are also stimulated by cytokines TNF-α, IL-1β and IFN-γ when

macrophages are activated and NO levels increase exponentially after 6 h. IFN-β is an autocrine and paracrine activator for iNOS gene which induces JAK/STAT and IRF signaling pathway [21].

The crosstalk between ROS/NO and NF-κB has been studied to understand insight mechanism of inflammation. Activation of NF- κB is mediated by Ref-1 (redox factor-1) which induces gene expression of various pro-inflammatory markers [26].

This investigation explores to measure at two different time intervals and analyzes the effect of acerola on the basis of ROS and NO in presence of external stimuli. Results from early ROS indicate the action of acerola on ROS produced from NADPH. Whereas late ROS are mediated by action of TNF-α and other cytokines. Interestingly, acerola showcases suppression of ROS howbeit there is no or partial suppression for the levels of nitric oxide. As explained above, ROS mediates transcription of NF-κB which advocates iNOS gene which further fabricates nitric oxide. This connotes that ROS and NO, two redox factors are linked. Acerola's putative mechanism may involve suppression of ROS from NADPH oxidase whereas it was unable to suppress the ROS produced from mitochondria. Exhorting the fact that ROS was still being produced which promoted NO levels.

The extraction process by two different solvents bestows a comparative analysis on the basis of the phytochemicals that are being involved in scavenging ROS in case of acerola. Phenolics with or without presence of terpenes are known to scavenge ROS. Yet in this study phenolics alone could not scavenge the ROS however phenolics along with terpenoids scavenged ROS at early and late stimulation of LPS. This confirmed the

hypothesis that either terpene alone or synergistically is involved in imparting the antioxidant activity.

Further analysis on COX enzyme inhibition activity was measured using the fractions including terpenes and phenolic both. It was observed that all acerola fractions could suppress COX-2 enzyme expression; however the effect seen by acerola leaf were more poignant as compared with fruits. COX-1 enzyme activity was further measured for leaf fraction and one genotype of fruit sample. All leaf samples demonstrated a mechanism similar to traditional NSAID drugs where they block both COX levels by binding to the active site in the C-terminal of cyclooxygenases and block the production of prostaglandins making them not selective. Howbeit fruit sample (BRS-238) manifests a selective COX-2 inhibitor which means that probably it does not bind to the active site of cyclooxygenase C-terminal and bind to some other side pocket enhancing its activity over traditional NSAIDs [69].

FUTURE RECOMMENDATIONS

Acerola's mechanism associated with inhibition COX enzyme expression needs to be elucidated. Acerola could be both binding to enzyme itself and further inhibiting the expression of prostaglandins or it could be suppressing the gene associated with COX. According to this study, acerola methanolic leaf fractions are capable of inhibiting the enzymatic activity of COX-1 and 2. One possibility is that acerola binds at the catalytic site of the cyclooxygenase enzyme, in a similar mechanism that of aspirin. Or another possibility could be that acerola suppresses the cyclooxygenases expression.

Chemical analysis of acerola fraction is required to determine the underlying role of individual phytochemical responsible for the biological analysis.

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APPENDIX

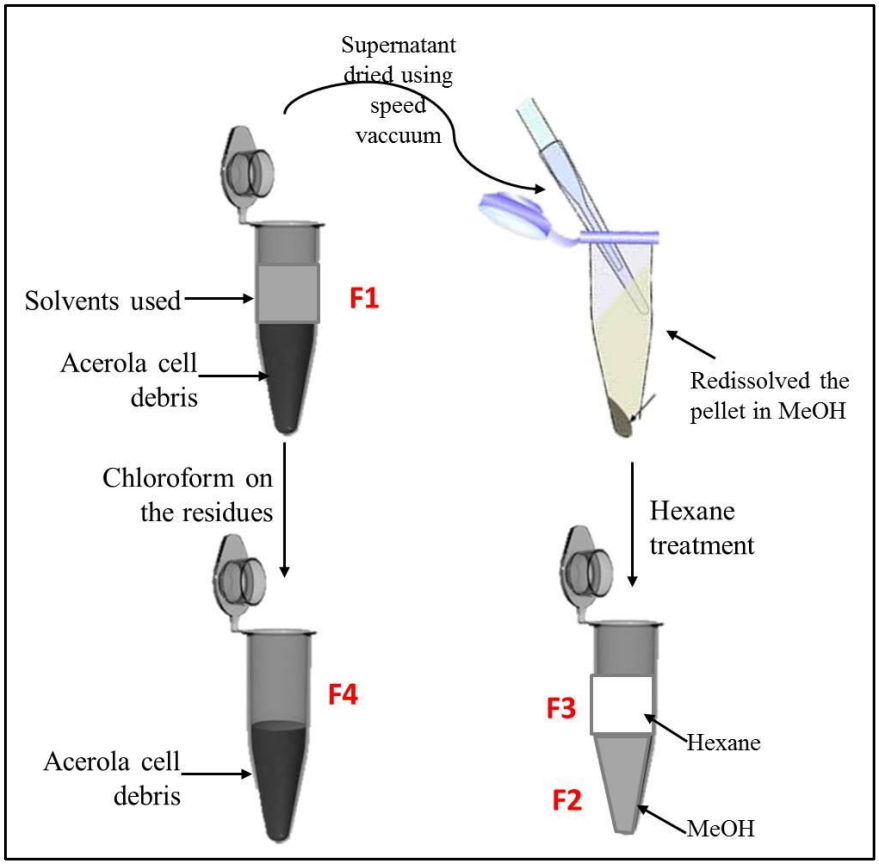


Figure 2.1. Systematic flowchart for TLC

Table 2.1. Terpenoid and steroid screening using TLC in 10% DCM.

	Secondary Metabolite	**Solvent combination for extraction**		
F1		Methanol	MeOH: Acetone (1:1)	MeOH:Acetone:water (5:4:1)
	Terpenoids/Steroids	+++	+++	+
	Secondary Metabolite	**Solvent combination for extraction**		
F2		After hexane extraction		
		Methanol	MeOH: Acetone (1:1)	MeOH:Acetone:water (5:4:1)
	Terpenoids/Steroids	+	+	-
	Secondary Metabolite	**Solvent combination for extraction**		
F3		chloroform treatment on the residues		
		Methanol	MeOH: Acetone (1:1)	MeOH:Acetone:water (5:4:1)
	Terpenoids/Steroids	+	+	-
	Secondary Metabolite	**Solvent combination for extraction**		
F4		Hexane fractions		
		Methanol	MeOH: Acetone (1:1)	MeOH:Acetone:water (5:4:1)
	Terpenoids/Steroids	-	-	-

+++ Strong intensity reaction, ++ Moderate intensity reaction; + Weak intensity reaction; - Non-detected

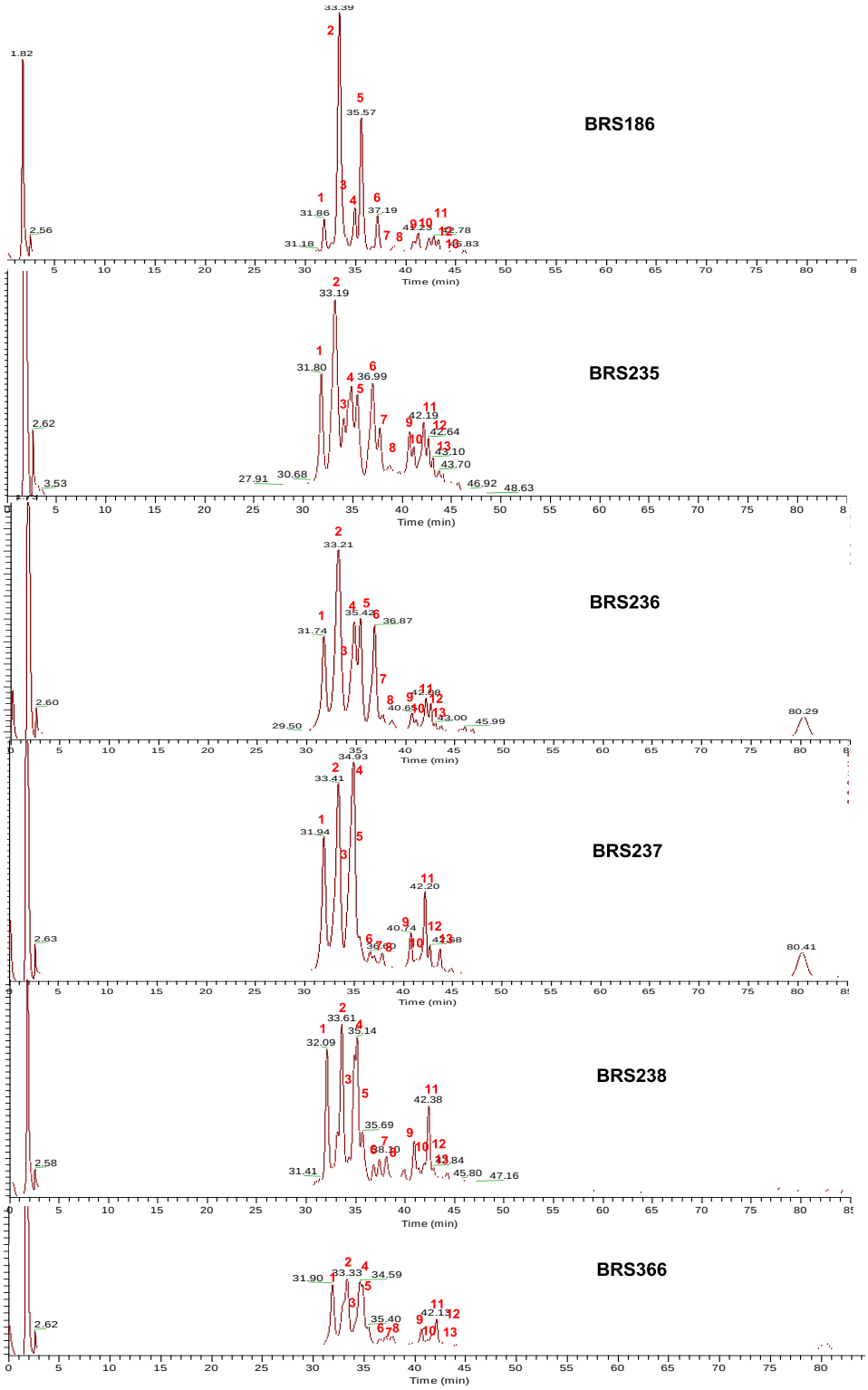
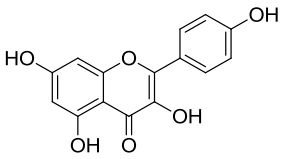


Figure 2.2. HPLC/MS profile of Acerola Leaves genotypes

Table 2.2.Identified components from the Acerola leaves extract

Peak no	Retention time	UV	MS $[M-H]^-$	MS^2	Identification
1	31.8	253,328, 352	711	579,285	kaempferol 3-*O*-β-D-[-arabinopyranosyl-(1'''→2'')-*O*-α-L-apiose-(1''''→5'')-*O*-β-Dglucopyranoside
2	33.2	242,271, 382	725	593,392, 285,	Kaempferol 3-*O*-robinobioside-7-*O*-arabinofuranoside
3	33.43	242,265, 299,353	755	623,605, 490,357, 315	3'-methoxyquercetin-7-O-α-L-rhamnopyranosyl-3-O-α-arabinofuranosyl-(1→6)-β-D-glucopyranoside
4	34.58	242,266, 307,348	579	285	kaempferol *O*-glycosides
5	34.84	242,266, 345	579	285	kaempferol *O*-glycosides
6	35.48	242,266, 347	609	301	Rutin
7	36.99	242,266, 345	593	447,285	Kaempferol 7-*O*-neohesperidoside
8	37.74	253,328, 354	623	315,242	isorhamnetin 3-O-rutinoside
9	40.73	230,242, 270,312	917/459	711,693	-
10	41.16	253,328, 354	931	739,725, 707,605, 298	-
11	42.19	253,328, 354	785	593,313	digalloyl-HHDP-glucose
12	42.64	230,242, 270, 315	857	693	-
13	43.10	230,242, 270,315	871	751,725, 707, 441	-

Structure of Kaempferol



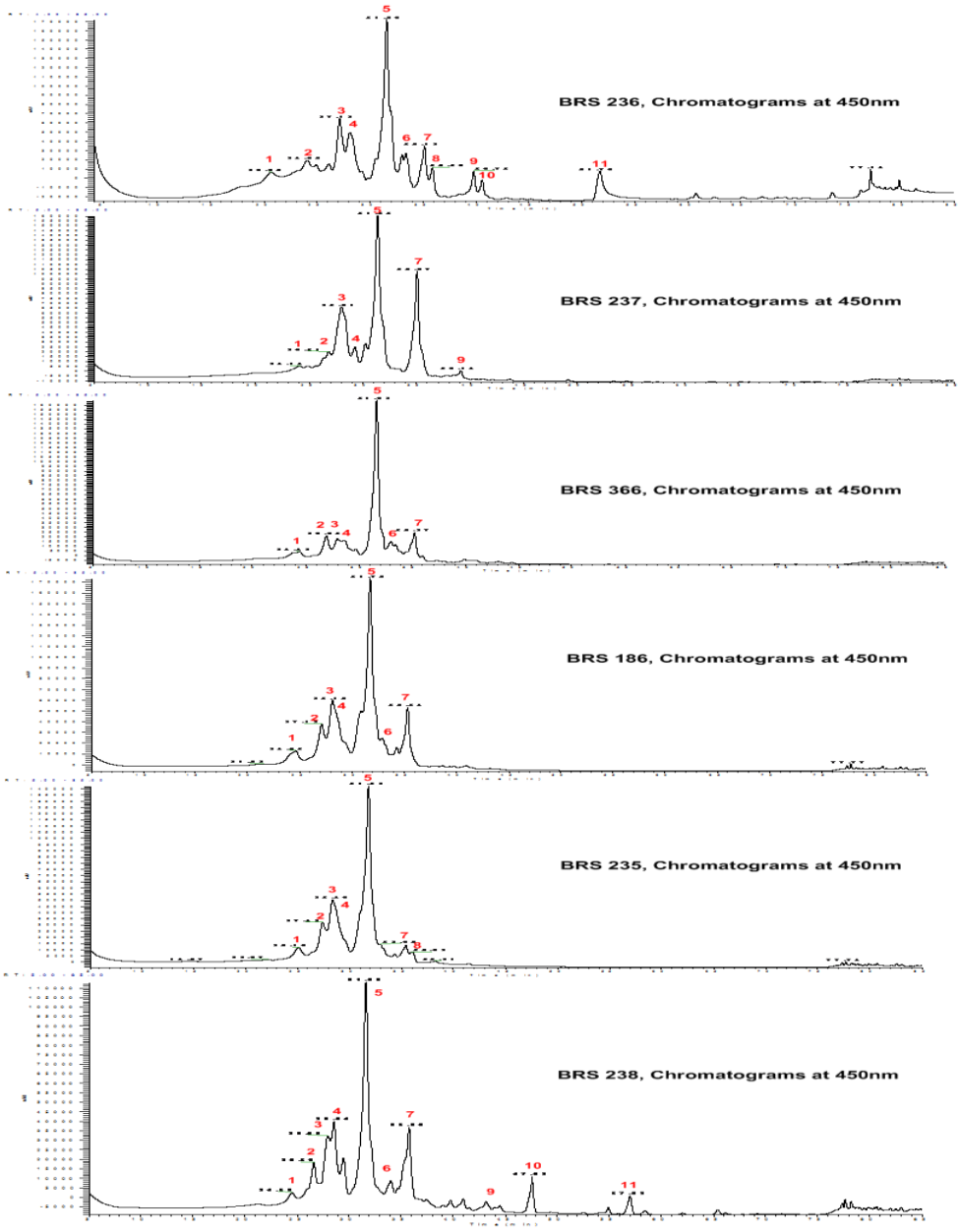
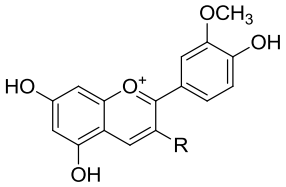


Figure 2.3. HPLC/MS profile of Acerola fruits genotypes

Table 2.3. Identification of components from acerola fruits genotypes

Peak no	Retention time	UV	MS [M+H]	MS/MS	Identification	BRS 186	BRS 235	BRS 236	BRS 237	BRS 238	BRS 366
1	26.5	208, 510	433	270	Cyanidin 3-rhamnoside	+	+	+	+	+	+
2	27.3	208, 510	417	287	Pelargonidin 3-rhamnoside	+	+	+	+	+	+
3	28.4	208, 510	327	301	Unknown Peonidin derivative	+	+	+	+	+	+
4	31.0	208, 510	596	449, 287	Cyanidin 3-rutinoside	+	+	+	+	+	+
5	31.7	208, 510	451	303, 316	Peonidin-3-xylopyranoside	+	+	+	+	+	+
6	44.5	208, 510	464	301	Peonidin 3-glucoside	+	-	+	+	-	+
7	46.6	348	448	285	Kaempferol 3-*O*-glucoside	+	+	+	+	+	+
8	50.8	-	383	303	-	-	+	+	-	+	-
9	53.2	266, 349, 478	610	464, 301	Peonidin 3-rutinoside	-	-	+	+	+	-
10	56.4	241, 310	424	339, 423, 365	-	-	-	+	-	+	-
11	62.1	241, 264, 322	641	627, 464, 316	Peonidin-3-O-diglucoside	-	-	+	-	+	-

Structure of Peonidin



R	Glucoside
	Rutinoside
	Diglucoside

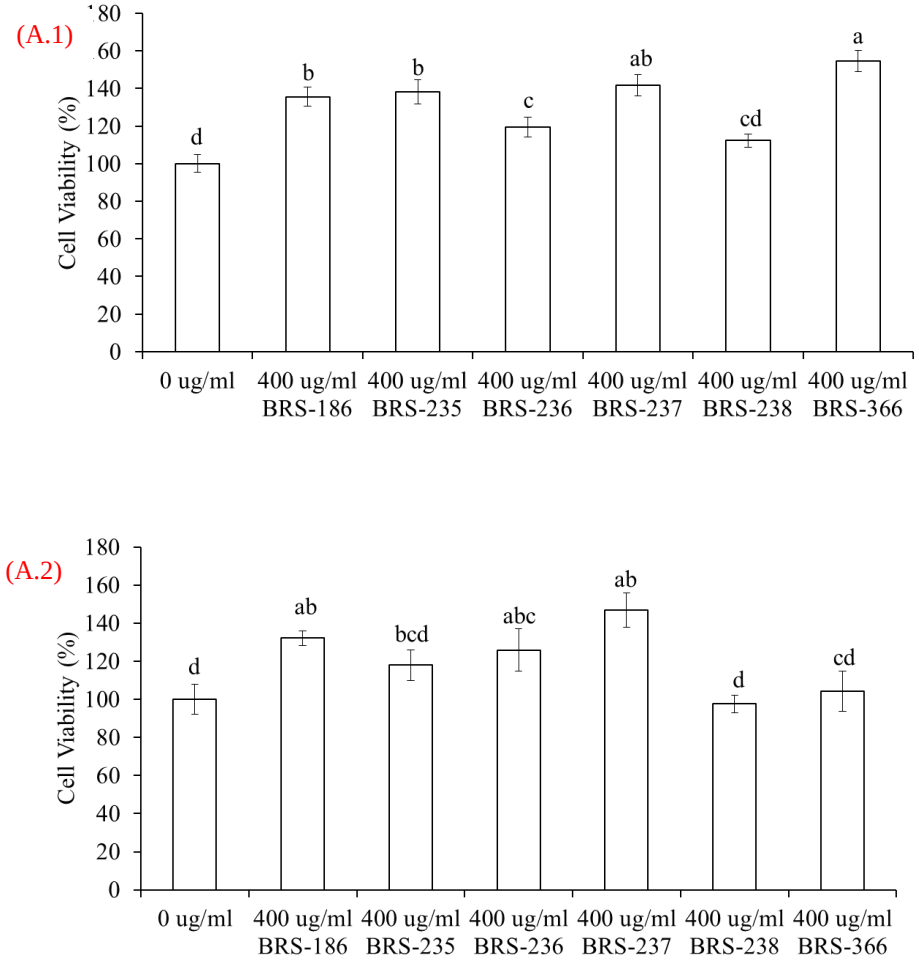
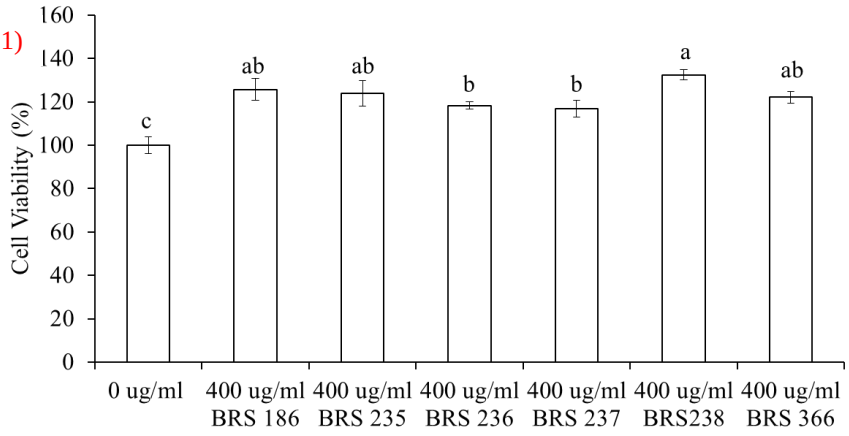


Figure 3.1. Effect of Acerola on cell viability in macrophage cells

RAW 264.7 cells (0.5×10^5 cells/well in 96-well culture plates) were pretreated with 400 µg/ml Acerola samples for 5 h and then stimulated with LPS (1 µg/ml) for 19 h. The cell viability was measured using microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT) with the MTS CellTiter 96®AQueous One Solution. The 0 µg/ml is a control, indicating the pretreatment of 0.5 % DMSO in DMEM medium without any LPS stimulation. Data, obtained from triplicate repeats at least, are shown as mean ± SE. Different letters indicate significant differences by the ANOVA/Student's t-test ($p < 0.05$).

(A) Methanolic extracts (A.1) leaf and (A.2) fruits; (B) methanol/acetone/water (B.1) leaf and (B.2) fruits.

(B.1)



(B.2)

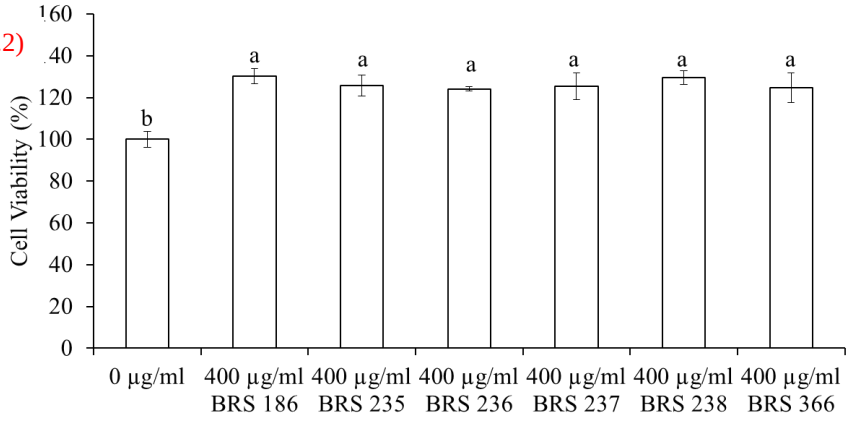
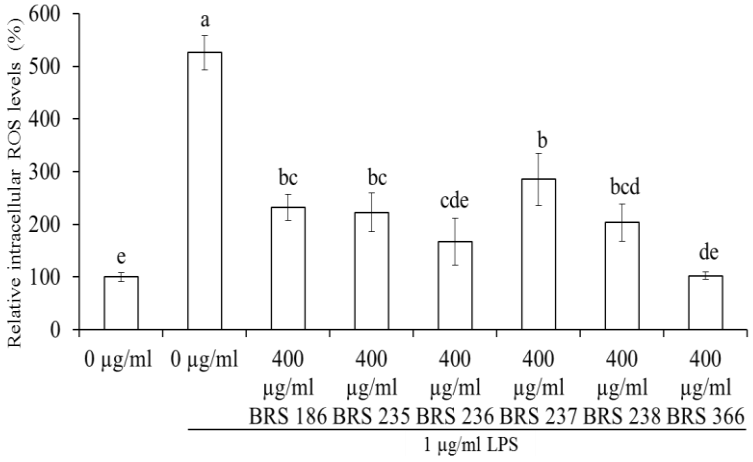


Figure 3.1. Continued

(A.1)



(A.2)

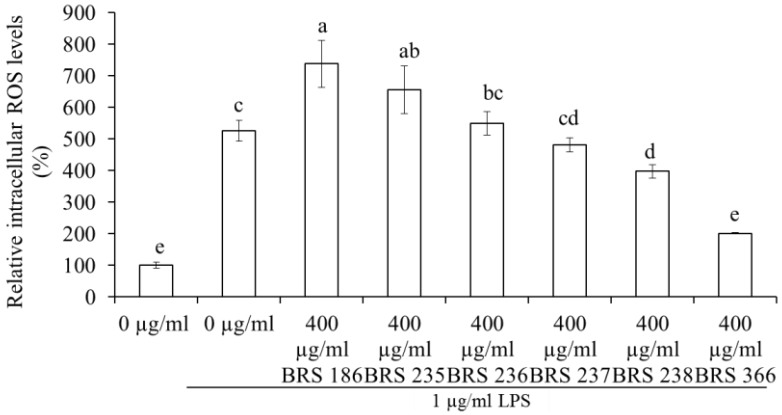
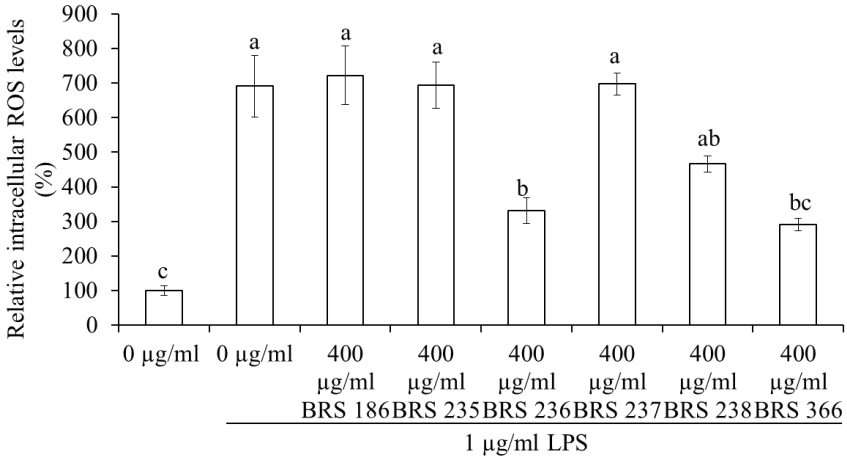


Figure 3.2. Effect of Acerola on LPS-induced ROS production for 4 h.

RAW264.7 cells (0.5×10^5 cells/well in 96-well culture plates) were pretreated with 400 µg/ml Acerola samples for 5 h and then stimulated with LPS (1 µg/ml) for 4 h. DCFA was used to determine the generation of intracellular ROS. The 0 µg/ml is a control, indicating the pretreatment of 0.5 % DMSO in DMEM medium without any LPS stimulation. Data, obtained from triplicate repeats at least, are shown as mean ± SE. Different letters indicate significant differences by the ANOVA/Student's t-test ($p < 0.05$).

(A) Methanolic extracts (A.1) leaf and (A.2) fruits; (B) methanol/acetone/water (B.1) leaf and (B.2) fruits.

(B.1)



(B.2)

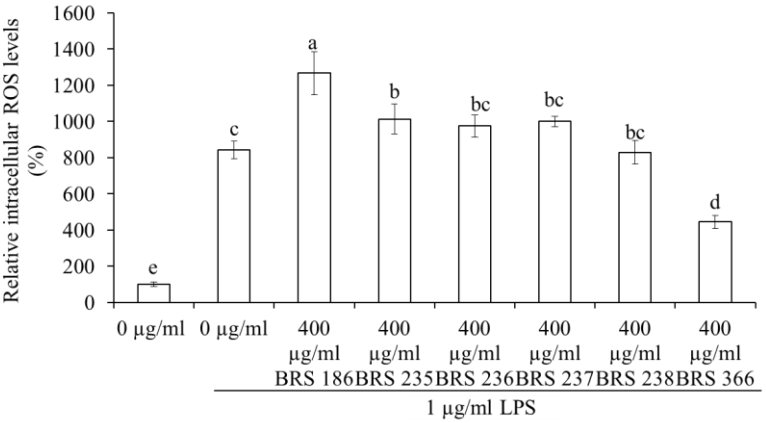
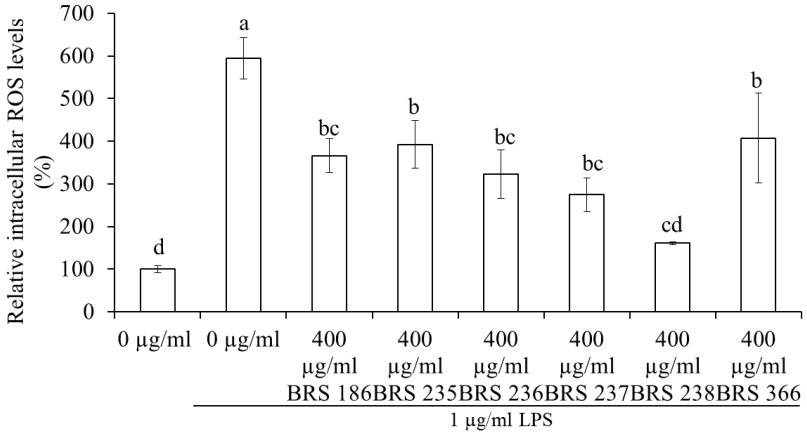


Figure 3.2. Continued

(A.1)



(A.2)

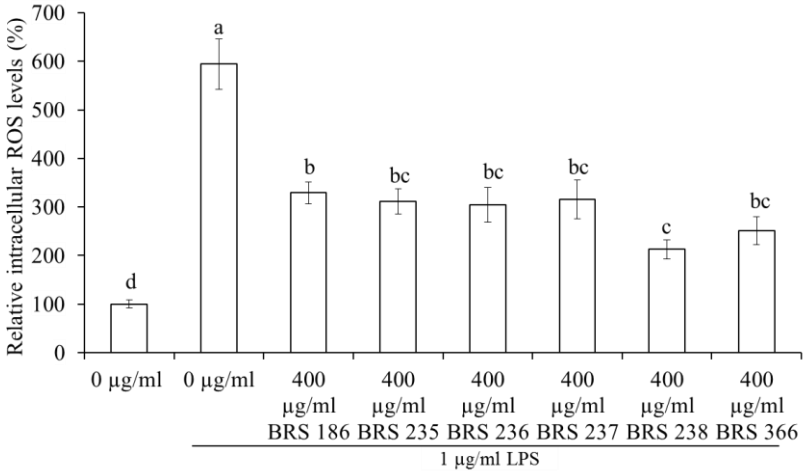
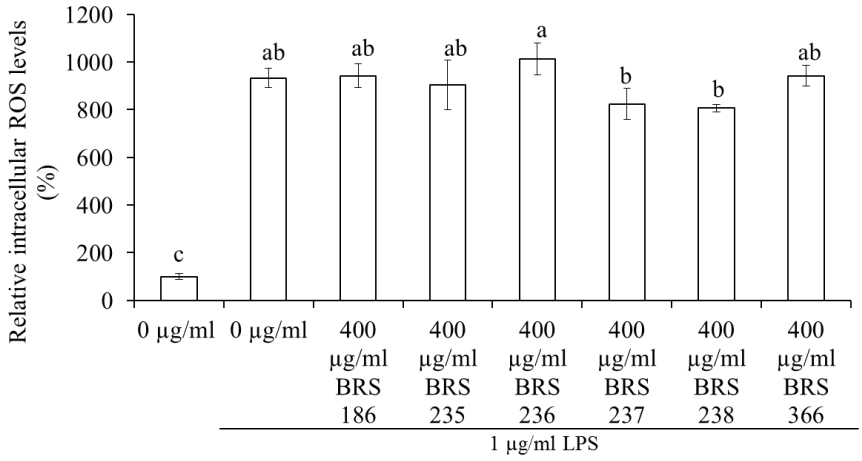


Figure 3.3. Effect of Acerola on LPS-induced ROS production for 19 h.

RAW264.7 cells (0.5×10^5 cells/well in 96-well culture plates) were pretreated with 400 µg/ml Acerola samples for 5 h and then stimulated with LPS (1 µg/ml) for 19 h. DCFA was used to determine the generation of intracellular ROS. The 0 µg/ml is a control, indicating the pretreatment of 0.5 % DMSO in DMEM medium without any LPS stimulation. Data, obtained from triplicate repeats at least, are shown as mean ± SE. Different letters indicate significant differences by the ANOVA/Student's t-test ($p < 0.05$).

(A) Methanolic extracts (A.1) leaf and (A.2) fruits; (B) methanol/acetone/water (B.1) leaf and (B.2) fruits.

(B.1)



(B.2)

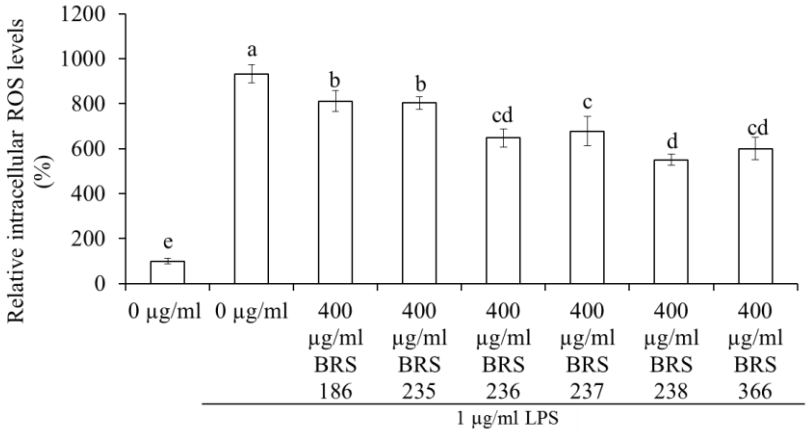
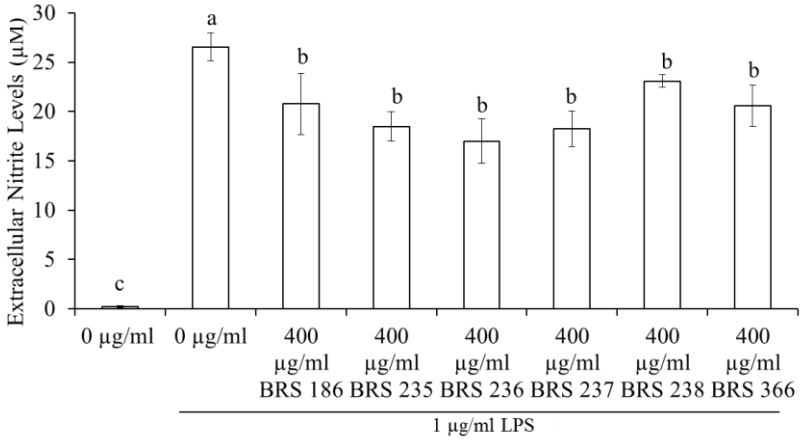


Figure 3.3. Continued

(A.1)



(A.2)

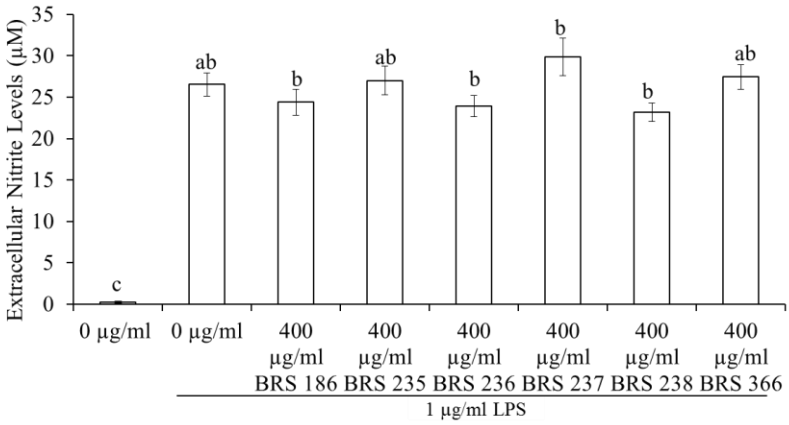
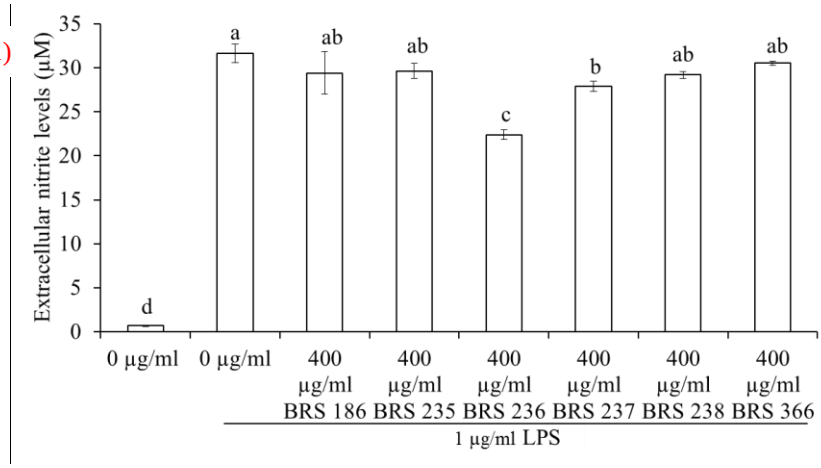


Figure 3.4. Effect of Acerola on LPS-induced NO production.

RAW264.7 cells (0.5×10^5 cells/well in 96-well culture plates) were pretreated with 400 µg/ml Acerola samples for 5 h and then stimulated with LPS (1 µg/ml) for 19 h. Griess reagent was used to detect the generation of extracellular nitrite 19 h after the LPS challengeThe 0 µg/ml is a control, indicating the pretreatment of 0.5 % DMSO in DMEM medium without any LPS stimulation. Data, obtained from triplicate repeats at least, are shown as mean ± SE. Different letters indicate significant differences by the ANOVA/Student's t-test ($p < 0.05$).

(A) Methanolic extracts (A.1) leaf and (A.2) fruits; (B) methanol/acetone/water (B.1) leaf and (B.2) fruits.

(B.1)



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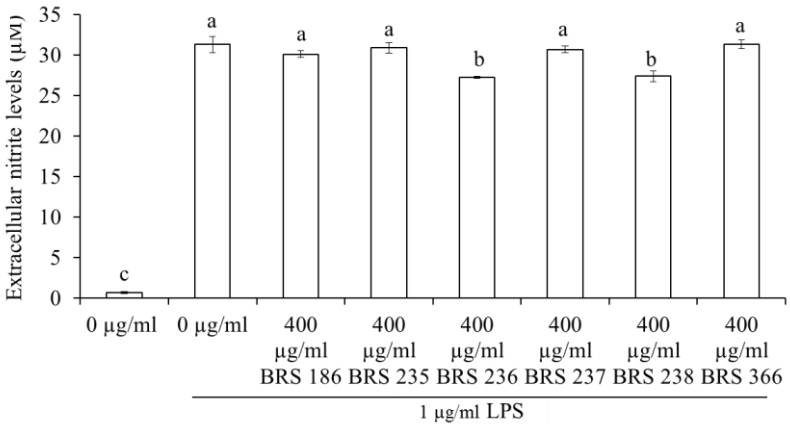
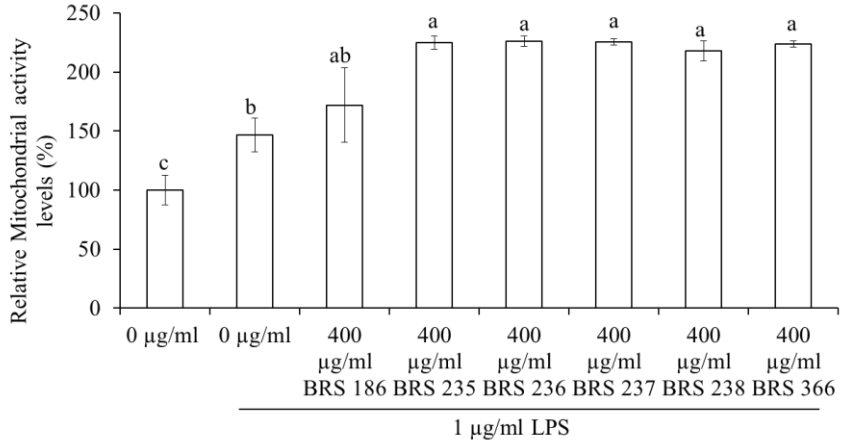


Figure 3.4. Continued

(A.1)



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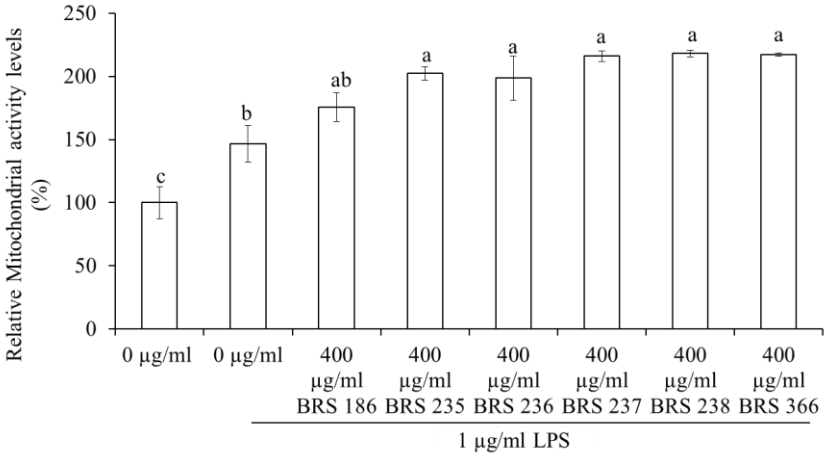
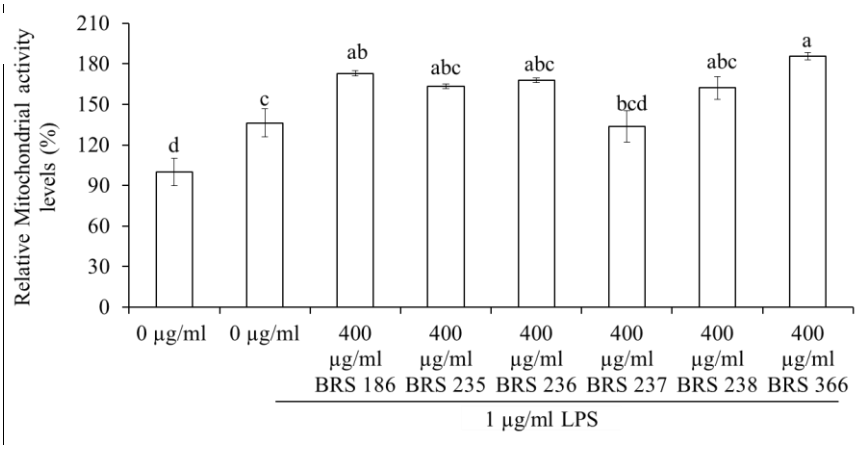


Figure 3.5. Effect of Acerola on mitochondrial activity after LPS stimulation.

RAW 264.7 cells (0.5×10^5 cells/well in 96-well culture plates) were pretreated with 400 µg/ml Acerola samples 5 h and then stimulated with LPS (1 µg/ml) for 19 h. MTS assay was performed to determine the mitochondrial activity as described in materials and methods. The 0 µg/ml is a control, indicating the pretreatment of 0.5 % DMSO in DMEM medium without any LPS stimulation. Data, obtained from triplicate repeats at least, are shown as mean ± SE. Different letters indicate significant differences by the ANOVA/Student's t-test ($p < 0.05$).

(A) Methanolic extracts (A.1) leaf and (A.2) fruits; (B) methanol/acetone/water (B.1) leaf and (B.2) fruits.

(B.1)



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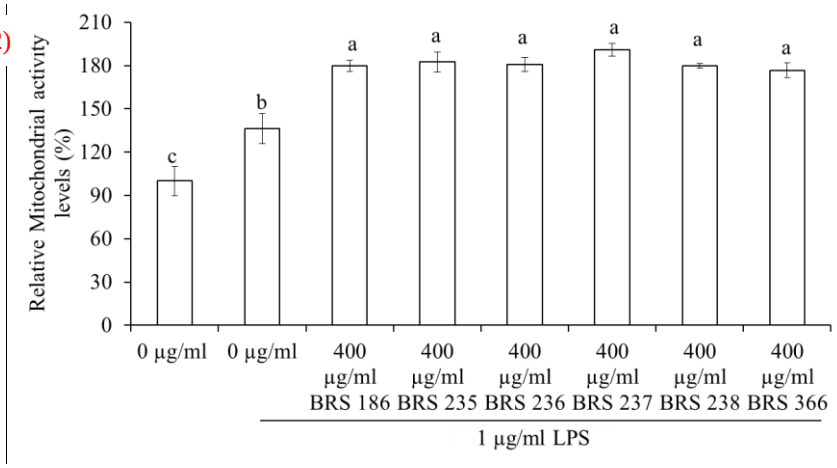

Figure 3.5. Continued

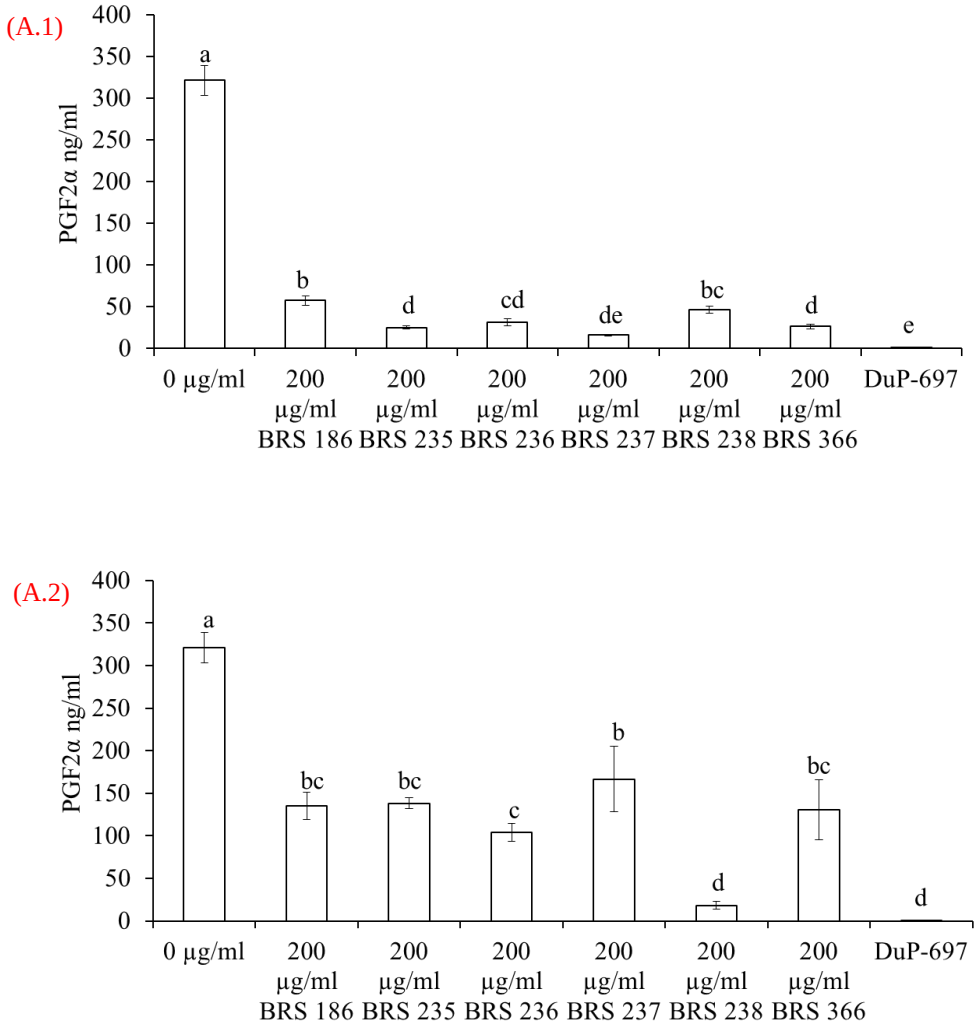

Figure 3.6. Effect of Acerola on COX-2 enzyme activity.

According to the instruction of COX Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI), COX-2 enzyme was incubated with 200 µg/ml Acerola leaf and fruit methanolic extracts and then its activity was examined by measuring the level of PGE2α. DuP-607 was used as a positive control for COX-2 reaction. Data, obtained from three biological repeats at least, are shown as mean ± SE values. Different letters indicate significant differences by the ANOVA/Student's t-test ($p < 0.05$). Methanolic extracts (A.1) leaf and (A.2) fruits

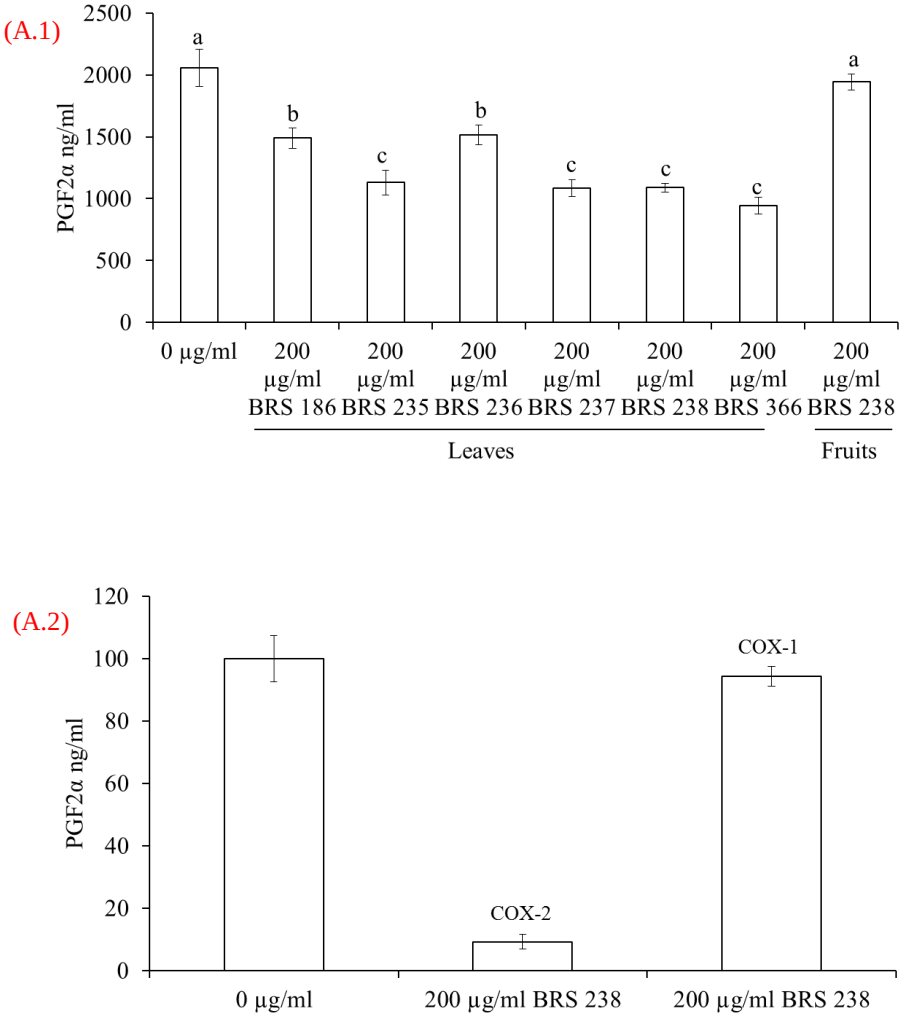

Figure 3.7. Effect of Acerola on COX-1 enzyme activity.

According to the instruction of COX Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI), COX-1 enzyme was incubated with 200 µg/ml Acerola leaf and fruit extracts and then its activity was examined by measuring the level of PGE2α. Data, obtained from three biological repeats at least, are shown as mean ± SE values. Different letters indicate significant differences by the ANOVA/Student's t-test ($p < 0.05$).

(A.1) Leaf (BRS-186, BRS-235, BRS-236, BRS-237, BRS-238, BRS-366) and Fruit (BRS-238)
(A.2) COX 1 and 2 combined.

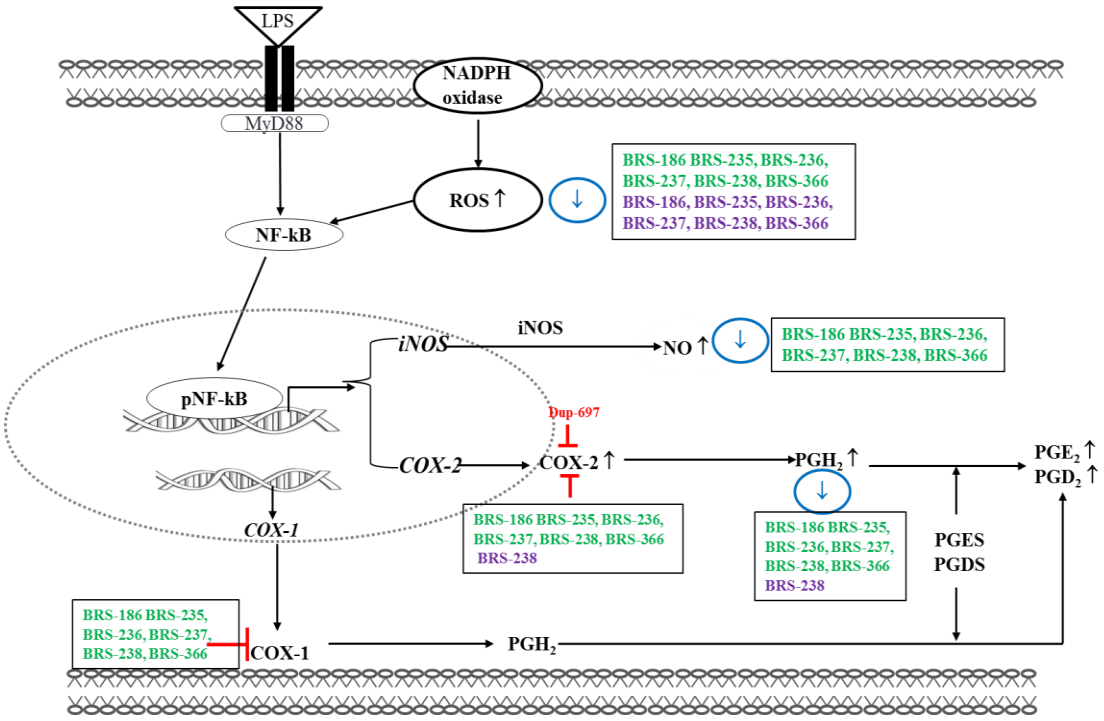

Figure 3.8. Proposed model of action of methanolic genotypes of acerola

LPS binds to the TLR-4 receptor, fostering ROS from NADPH oxidases and mitochondria. ROS initiates activation of NF-κB and induces COX-2 and amplification of the iNOS expressions. Methanolic extracts of acerola genotypes partially scavenged ROS at 19 h LPS treatment and the leaf fractions partially attenuated nitric oxide levels. Acerola genotypes displayed inhibitory action of COX-1 and COX-2 enzyme activity. However, BRS-238 fruit fraction displayed selectivity and suppressed enzyme activity of only COX-2. Here, green and purple color signifies the leaf and fruit genotypes respectively.