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Title

Evaluation of anti-inflammatory and antioxidant properties of *Citrus Sinensis* peels and *Morus Alba* extracts in vitro

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Abstract

In recent years, the antioxidant and anti-inflammatory properties of natural compounds have gained significant attention in the field of health and nutrition.

When an organism experiences stress, it responds by generating free radicals. In normal conditions, the antioxidant system of the organism will keep free radicals in dynamic equilibrium. However, once the balance is disrupted, the free radicals that have been accumulated in excess will attack the normal cells of the body, resulting in oxidative stress. Oxidative stress has been strongly associated with the process of aging and the development of diverse chronic diseases. Studies have shown that antioxidants can attenuate the harm caused by oxidative stress. Natural, harmless antioxidants have been proven to have good antioxidant effects [1].

On the other hand, inflammation commonly occurs as a result of the invasion of infectious microorganisms such as bacteria, viruses, or fungi into the organism. These microorganisms can either reside in specific tissues or circulate within the bloodstream. Additionally, inflammation can be triggered by various processes, including tissue injury, cell death, cancer, ischemia, and degeneration. Numerous inflammatory mediators are synthetized and released during inflammatory responses. Among the various biological activities documented in the literature regarding natural plant products, anti-inflammation is one of the most reported.[2].

Starting from this, this study aims to evaluate the antioxidant and anti-inflammatory properties of extracts from the peel of three varieties of oranges (*Citrus Sinensis*), and two types of white mulberry (*Morus Alba*).

Extracts were obtained via Microwave-assisted distillation, a method that uses microwave energy to accelerate the distillation process. A preliminary evaluation of the potential antioxidant activity of volatile and polar fractions of the extracts was made using DPPH and β-carotene bleaching assays [3]. The extracts were then tested for their potential anti-

inflammatory activity using the NF-kB anti-inflammatory assay and for potential antioxidant activity using CAA (cellular antioxidant activity) assay at the “Masaryk University” Faculty of Pharmacy in Brno (CZ). Each compound was testes in vitro at the concentration of 20 µg/mL, 10 µg/mL and 5 µg/mL, using THP1-Blue™ NF-kB cells purchased from Invivogen. The study revealed that almost none of the tested compounds express a significant anti-inflammatory activity, while some others, like the *Citrus* varieties at the concentration of 10 µg/mL, showed a minimal activity which can be a good starting point for further investigations. On the other hand, antioxidant activity tested promising for all the compounds, especially the *Morus Alba* extracts, at all concentrations.

1.0 Introduction

In the last years there has been an increasing interest over antioxidant and anti-inflammatory properties of natural compounds. In this study we are going to evaluate these properties in three different varieties of orange peels (*Citrus sinensis*) denominated Vanilla, Washington Navel and Navelina from Ribera (Sicily, Italy), and two types of white mulberry (*Morus alba*), from here on referred to as M3 and M4.

These extracts were prepared by prof. Gregorio Peron, prof. Sara Marchizzo, prof. Elisabetta Schievano, prof. Mirella Zancanato and prof. Stefano Dall'Acqua of the University of Padua, Italy and Ca' Foscari University of Venice using the Microwave-assisted distillation.

To extract the natural compounds from these sources, the team used Microwave-assisted distillation, a method that uses microwave energy to accelerate the distillation process. Microwave allows to obtain extraction of essential oil with lower amount of water compared to traditional hydro-distillations. This method has been shown to be effective in extracting essential oils and other volatile compounds from plant materials [3].

Once the extracts were obtained, a preliminary evaluation of the antioxidant potential of both volatile and polar fractions from peels of Vanilla, Washington Navel and Navelina varieties was evaluated using DPPH and β-carotene bleaching assays. DPPH is a stable free radical commonly used to assess the radical scavenging activity of plant extracts. This free radical has a strong purple color, which makes it easy to detect and quantify. Antioxidant molecules have the ability to quench DPPH radicals by providing either a hydrogen atom or electron donation. This process converts the DPPH radicals into a colorless product, indicating the effectiveness of the antioxidant in scavenging free radicals. The β-carotene bleaching assay evaluates the ability of the antioxidant to inhibit lipid peroxidation in both the initiation and propagation phases [4] .

The results of the DPPH assay showed no antioxidant potential of the essential oils from the peels of the Navelina, Washington Navel and Vanilla varieties, which was an expect result based on earlier studies. This is due to the fact that the orange peel is rich of monoterpenes, which do not have the ability to neutralize the DPPH radical by donating a hydrogen [3].

Regarding the carotene bleaching assay, essential oils from the three orange varieties showed a mild and comparable antioxidant potential, with IC_{50} = 23.10 ± 1.32%, 27.22 ± 2.00%, and 20.11 ± 1.74% for Navelina, Washington Navel and Vanilla varieties, respectively. Results of carotene bleaching assay using essential oils from Citrus species available on literature are heterogeneous, and they highlight that the chemical composition of essential oils is determinant [3].

2.0 Orange *(Citrus sinensis)*

2.1 Origins and cultivation

Navel oranges, scientifically known as *Citrus sinensis*, are a unique group of sweet oranges distinguished by the presence of a secondary fruit, the Navel, which develops inside the primary fruit. The occurrence of secondary, tertiary, or quaternary fruit is rare in other plant species. The size and number of extra numerary fruit in Navel oranges vary depending on the cultivar and growing conditions. The distinctive morphology of the fruit was first described by the Italian botanist Ferrarius in 1646 [5].

Despite being one of the most popular citrus fruits in the world, the exact origins of the Navel orange remain a mystery. While it is believed to have originated from a branch mutation in a Selecta orange tree in Bahia, Brazil, in the early 1800s [3], there is also evidence to suggest that oranges with navels were being grown in Spain and Portugal prior to the 1820s. The mutation may have occurred even earlier, possibly in China, making it difficult to pinpoint its true origin.

Regardless of its mysterious beginnings, the Navel orange has played a significant role in the history of citrus fruit cultivation. The name "Washington Navel" and the worldwide commercialization of the Navel orange can be traced back to the year 1870, when 12 budded trees were imported from Bahia by Mr. and Mrs. Schneider, Presbyterian missionaries, to Mr. Saunders, who was the superintendent of gardens and grounds for the USDA in Washington, DC [5].

The fruit quickly gained popularity among local citrus growers, who were drawn to its delicious taste, seedless flesh, and easy-to-peel skin. Within a decade, the "Washington Navel" orange became the most widely planted variety in California, which was the largest producer of citrus fruits in the United States. From there, the Navel orange rapidly spread to other parts of the world, and it soon became a major variety, second only to Valencia orange in popularity [6] *(fig. 1)* [7].

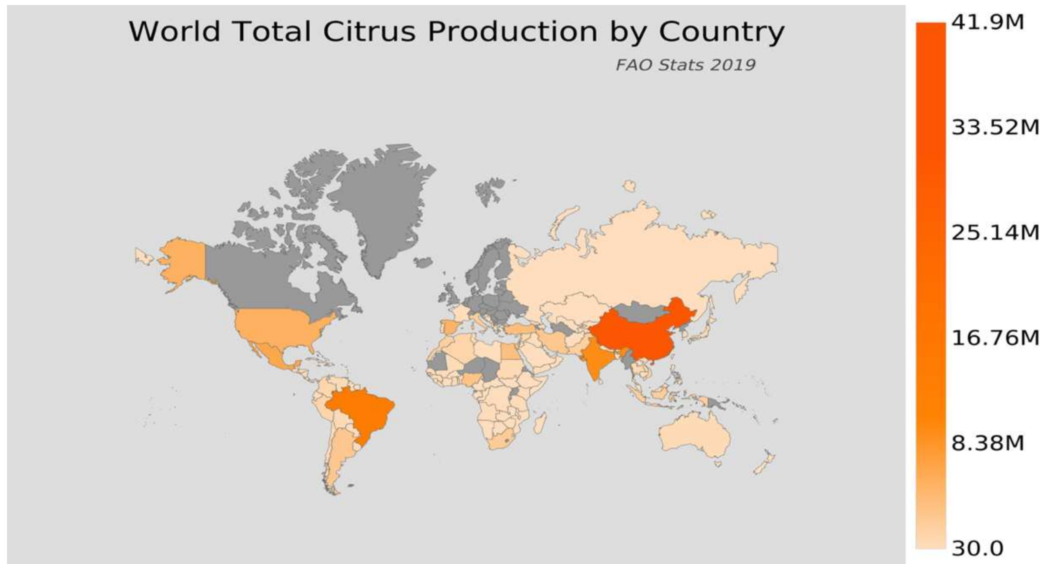


Fig. 1 The map, provided by the Food and Agriculture Organization, displays the global production of citrus fruits by country. We can see China, followed by Brazil and India, is the world largest producer with 44 tons per year while Italy, despite its small size is ranked 12th with a production of approximatively 2.9 tons/year.

Navel oranges are mainly grown for the fresh market and are typically early maturing and seedless due to nonviable pollen and defective embryo sac development. These oranges are generally larger in size than other sweet orange cultivars, with an average of one to two sizes larger. This makes them popular in the gift fruit market due to their impressive size. The highest quality Navels are grown in Mediterranean-type climates, where they develop a deep orange peel color, have moderately high Brix (indicates the sugar content of a solution, typically fruit juice) and acid, and a favorable Brix:acid balance. In contrast, Navel trees grown in tropical and subtropical regions tend to produce poorly colored, low-acid, and high-juice content fruit, and may suffer from more insect and disease pressures. Navel oranges are generally more susceptible to environmental stresses and physiological disorders than other sweet oranges, which may be attributed to the presence of secondary fruit. To attain full production, they require more precise cultural practices than other sweet orange cultivars, particularly in marginal areas.

Sweet orange flowers develop differently depending on the hemisphere. In the northern hemisphere, they usually appear between November and February, while in the southern hemisphere they appear from April to August [5].

In this study we are going to evaluate anti-inflammatory and antioxidant properties of the peel of *Citrus Sinensis* fruits coming from the Ribera region in the Italian island of Sicily, well known for its tradition in the cultivation of the Orange and one of the major contributor of the Italian total orange production, thanks to its typically Mediterranean climate.

2.2 Botanical aspects

As previously stated, citrus trees are distinguished by the presence of a secondary fruit, called Navel, which develops inside the primary fruit (*Fig. 2*) [8].

During the transition from the vegetative meristem to the floral meristem in plants, a series of developmental events takes place, starting with the emergence of the axillary shoot, which breaks through the protective bud scales. It is noteworthy that the floral architecture differs between terminal flowers and axillary flowers. Terminal flowers, located at the tip of the inflorescence, possess a single abscission zone, a specialized region where the flower will eventually detach from the plant. In contrast, axillary flowers, found along the sides of the inflorescence, exhibit two distinct abscission zones. This structural difference between the two types of flowers suggests variations in their development and maturation processes. Terminal flowers generally tend to bloom earlier compared to axillary flowers. This discrepancy in timing could be attributed to the influence of apical dominance, where the terminal bud

Fig. 2 This artwork, extracted from Antoine Risso's "Histoire naturelle des Orangers" (1818-1820), depicts the various components of the Citrus Sinensis plant. It showcases detailed illustrations of the plant's leaves, flowers, fruits, and a cross-section of the fruit.

suppresses the growth of lateral buds. As a result, terminal flowers are released from this inhibitory effect earlier, leading to their earlier blooming. Moreover, within the same inflorescence, flowers on leafy shoots have a tendency to bloom earlier than those on leafless shoots. Leaves play a vital role in providing resources and energy for flower development. Thus, the presence of leaves on the shoot provides favorable conditions for earlier flower initiation and maturation. It is also worth noting that late-blooming flowers often exhibit distinctive characteristics. These flowers tend to exhibit faster growth rates, potentially due to the accumulation of resources over an extended period. Additionally, late-blooming flowers often display an extended blooming duration on the tree, allowing for a prolonged period of pollination and reproductive success. The intricate interplay between meristem development, bud scales, abscission zones, apical dominance, leaf presence, and blooming timing contributes to the complex dynamics of flowering in plants. The rate of flower development is positively correlated with the number of degree days above 12.8 degrees Celsius. Navel orange flowers have perfect, complete flowers with a calyx made up of four or five sepals and a corolla made up of four or five petals. They also have 20 to 40 stamens with bilobed anthers and a primary gynoecium consisting of 10 to 13 fused carpels with a single style and stigma. They also have additional sets of secondary carpels, stigmas, and styles, which are not found on other sweet orange cultivars. Finally, a floral disc, which is carpellary in origin, is located between the stamens and carpels [9].

2.3 Phytochemical profile

The analysis of the volatile profile is a crucial aspect in the study of essential oils and allows us to identify more then 98% of the compounds in the oil. The study revealed that limonene, a common terpene found in citrus fruits, contributed to 85% of the total composition of the essential oils in all three varieties. However, significant differences were observed among the three oranges, particularly in the case of sesquiterpenes. These compounds were found to be more abundant in Washington Navel and Navelina varieties, while the other volatiles had similar profiles for all three varieties.

The study also identified specific marker compounds for each variety. For instance, α-thujene was detected only in the essential oil from Vanilla variety, while 3-carene was found to be higher in Washington Navel variety. Similarly, α-copaene and 3-carene were markers for Washington Navel, while valencene and germacrene D were the markers for Navelina variety.

In addition to analyzing the volatile fraction, an evaluation of the polar component of the orange peels was made through the microwave-assisted extraction method to obtain the essential oil. By employing the optimal conditions of 900 W for 15 minutes, the study attained a polar extract with an average yield of 8.2%. The water that remained in the loading vessel was lyophilized and transformed into powder for chemical characterization.

Results show only slight variations among the three orange varieties in matter of secondary metabolites composition. The analysis revealed that flavones represented the biggest part of these compounds, with an average amount of 4 mg/g of fresh peels. Eriocitrin/neoeriocitrin were the most common compounds in peels from Vanilla variety. Flavenes were the second biggest class of metabolites after flavones, with an average of 1.4 mg/g. Within this class significant variations were observed for narirutin and naringenin-7-O-glucoside-6”-O-HMG, which was the most abundant in Washington Navel and Navelina varieties, but the latter in Vanilla variety; 5,6,7,3′,4′-pentamethoxyflavanone was found in Washington Navel and Navelina; obacunoic acid in Vanilla variety. The

remaining identified metabolites were limonoids (0,4 mg/g) and phenolic acids, with roseoside being the less significant in Vanilla var. (0,5 mg/g) [3].

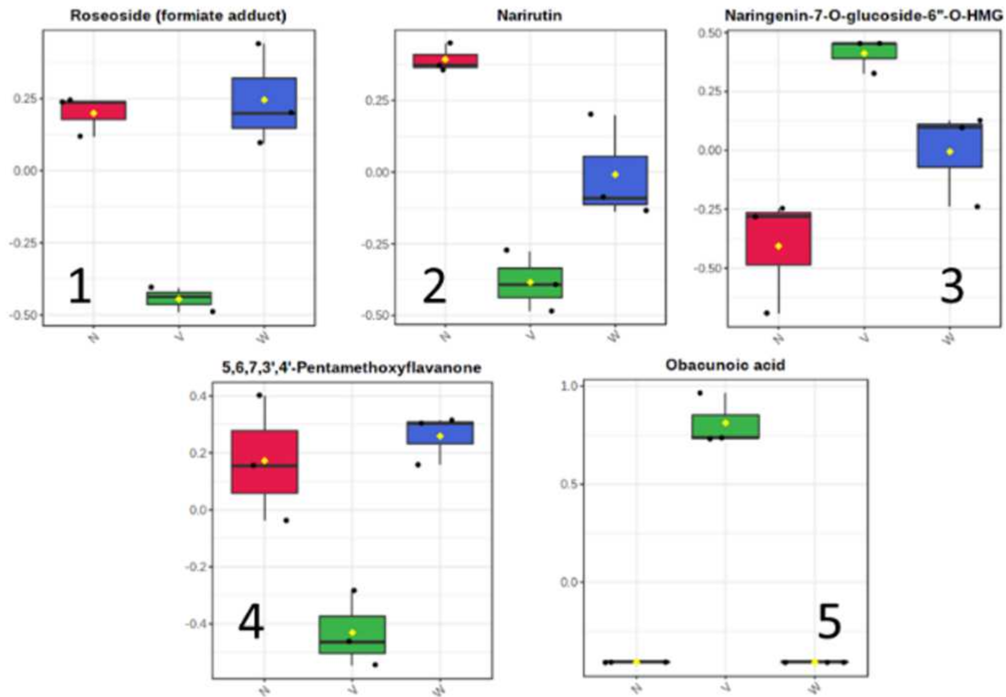


Fig. 3 Range of concentration of different compounds found in the three varieties of Citrus. Red is Navelina, green Vanilla and blue is Washington.

It is important to note that coumarins and furanocoumarins were also evaluated in this study due to their potential inhibitory effect on the intestinal cytochrome P450-3A4. This could cause interference with the metabolism of several drugs. However, the concentration of these compounds was found to be low (0,1 mg/g) [3].

3.0 Mulberry (*Morus Alba)*

3.1 Origins and cultivation

Mulberry is a plant that belongs to the *Morus genus*, which is part of the *Moraceae* family. This genus contains 24 species and one subspecies, with *Morus alba* being the most dominant among them. The plant is widely distributed throughout the world and can be found in a range of climatic conditions, from tropical to temperate regions [10].

Most cultivated mulberry varieties originated from the region of China/Japan and the Himalaya foothills, with China having the largest area of mulberry cultivation, followed by India [11] *(Fig. 4*) [12]. The fruit of *Morus alba*, which is commonly referred to as mulberry in the English-speaking world, is known as Sang Shen in China and Oddi in Korea. Some cultivars of the plant can yield more than 10 kg of fruit per tree in one fruiting season.

Fig. 4 The map shows countries where the species is planted, but it doesn't imply suitability for all ecological zones within them. Green areas represent countries of origin of the tree, while meshed areas are the countries where the plant is planted the most, outside their native regions.

Mulberry fruit is commonly consumed fresh, dried, or processed into various products, such as wine, fruit juice, and jam, in most mulberry-growing countries. This is because the fruit has a delicious taste, pleasing color, low calorie content, and high nutrient content.

In addition to its culinary uses, mulberry fruit is also used in traditional medicine in China, Korea, and Japan, where it is believed to have various pharmacological effects, such as fever reduction, sore throat treatment, liver and kidney protection, eyesight improvement, and blood pressure reduction.

The mulberry fruit was designated one of the first medicinal-and-edible plants by the Ministry of Health of China in 1985, and its medical use was recorded in the Chinese pharmacopoeia. This traditional knowledge has been supported by numerous scientific studies that have identified various chemical constituents of the plant, including amino acids, fatty acids, minerals, polyphenolics, and polysaccharides [10].

3.2 Botanical aspects

The Morus Alba is a rapidly growing deciduous shrub or small to moderate-sized tree that can reach up to 35 meters in height and 1.8 meters in girth. It has a cylindrical, straight bole without buttresses and its bark is rough, dark greyish-brown with vertical fissures. When injured, the plant produces white or yellowish-white latex that can be used for medicinal or industrial purposes.

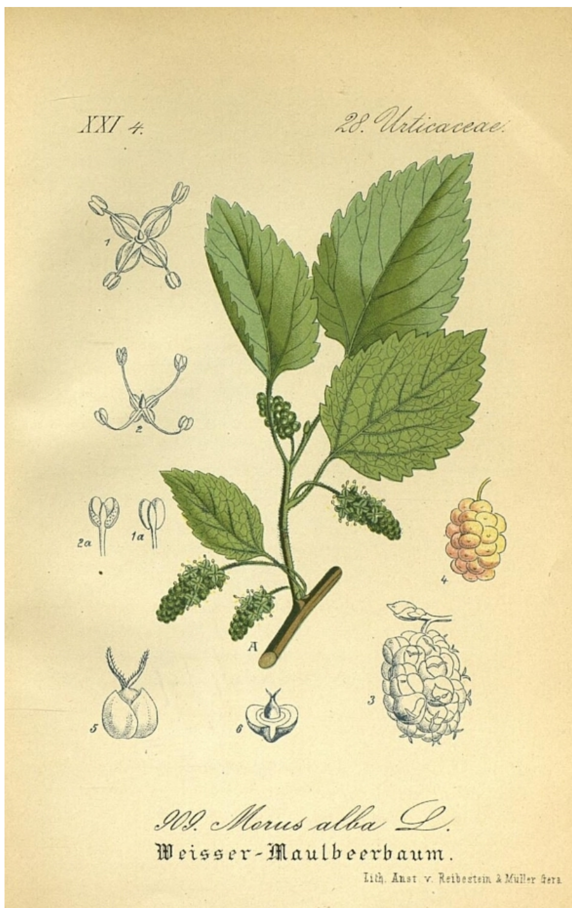


Fig. 5 Illustration of Morus Alba leaves, including venation patterns, flowers, and various fruit stages, sourced from the "Real Jardin Botanico, Madrid" (1892-1898).

The leaves of the *Morus alba* are highly variable, ranging from ovate to broadly ovate in shape, distichous in arrangement, and either simple or 3-lobed. The leaves have dentate margins and are palmately 3-veined at the base. The stipules are lateral and caducous, meaning they are shed early in the plant's life cycle, and are coriaceous, or leathery in texture (*fig. 5*).

The plant produces axillary inflorescences that are pendulous, consisting of small, greenish, and inconspicuous flowers with four free, imbricate petals. Male flowers are arranged in a catkin-like raceme with lax flowers and have four stamens and a top-shaped pistillode. Female flowers are arranged in a long or short spike, with the ovary enclosed and 1-2 locular, containing a single ovule, and a bipartite style.

The fruit of the *Morus alba* is a syncarp, or multiple fruit, consisting of many drupes enclosed in a fleshy perianth that can reach up to 5 centimeters in length. The color of the

fruit can vary from white, pinkish-white, purple, to black, depending on the species and cultivar. The fruit is a valuable food source for wildlife and humans alike and has been used for culinary purposes in various cultures [12].

3.4 Phytochemical profile

As mentioned above, *Morus alba* has a long history of use as an edible fruit and traditional medicine, especially in Asian countries. The fruit is known to contain a diverse assortment of nutritive compounds such as fatty acids, amino acids, vitamins, minerals and bioactive compounds [10]. The type and amount of these compounds can vary depending on the cultivars and maturity stages of the fruit [13]. Among the bioactive compounds found in mulberry fruit, anthocyanins, rutin, quercetin, chlorogenic acid, and polysaccharides have been identified. Recent studies have shown that mulberry fruit extracts and their active components exhibit various biological activities. These activities include antioxidant, neuroprotective, antiatherosclerosis, immuno-modulative, antitumor, antihyperglycemic and hypolipidemic effects. Although we know that mulberry fruit has health benefits, there is still a need to learn more about the specific ways that its compounds work in traditional medicine and diet. Conducting additional research in this field, can lead to the creation of new therapies and treatments that could address a variety of health conditions[10].

This study started taking in consideration nineteen different varieties of mulberry and, after a thorough analysis of the chemical properties of each variety, only two of them were selected for the evaluation of their anti-inflammatory and antioxidant properties. These varieties are the Rosou variety and the Goshoerani variety, from here on named M3 and M4 respectively.

The study started with the quantification of the total anthocyanin and total phenolic content using HPLC-MS analysis. The results showed a great variability between the varieties. We can divide the mulberry into two subgroups: the dark-coloured varieties and the light-coloured ones. In the dark-coloured subgroup total anthocyanins amount ranged from 52.00 ± 10.06 mg/100 g to 372.19 ± 28.21 mg/100 g, while in the light-coloured subgroup it ranged from 0.32 ± 0.02 mg/100 g to 24.13 ± 1.79 mg/100 g. The fruits showing the highest anthocyanins amounts were those from var. 3 (52.00 ± 10.06 mg/100 g), var. 4 (357.32 ± 11.46 mg/100 g) and var. 8 (341.09 ± 13.52 mg/100 g). The greatest

contribution to total anthocyanin content was afforded by two compounds for all the varieties, namely cyanidin glucoside and cyanidin rutinoside (*fig. 6*) [3].

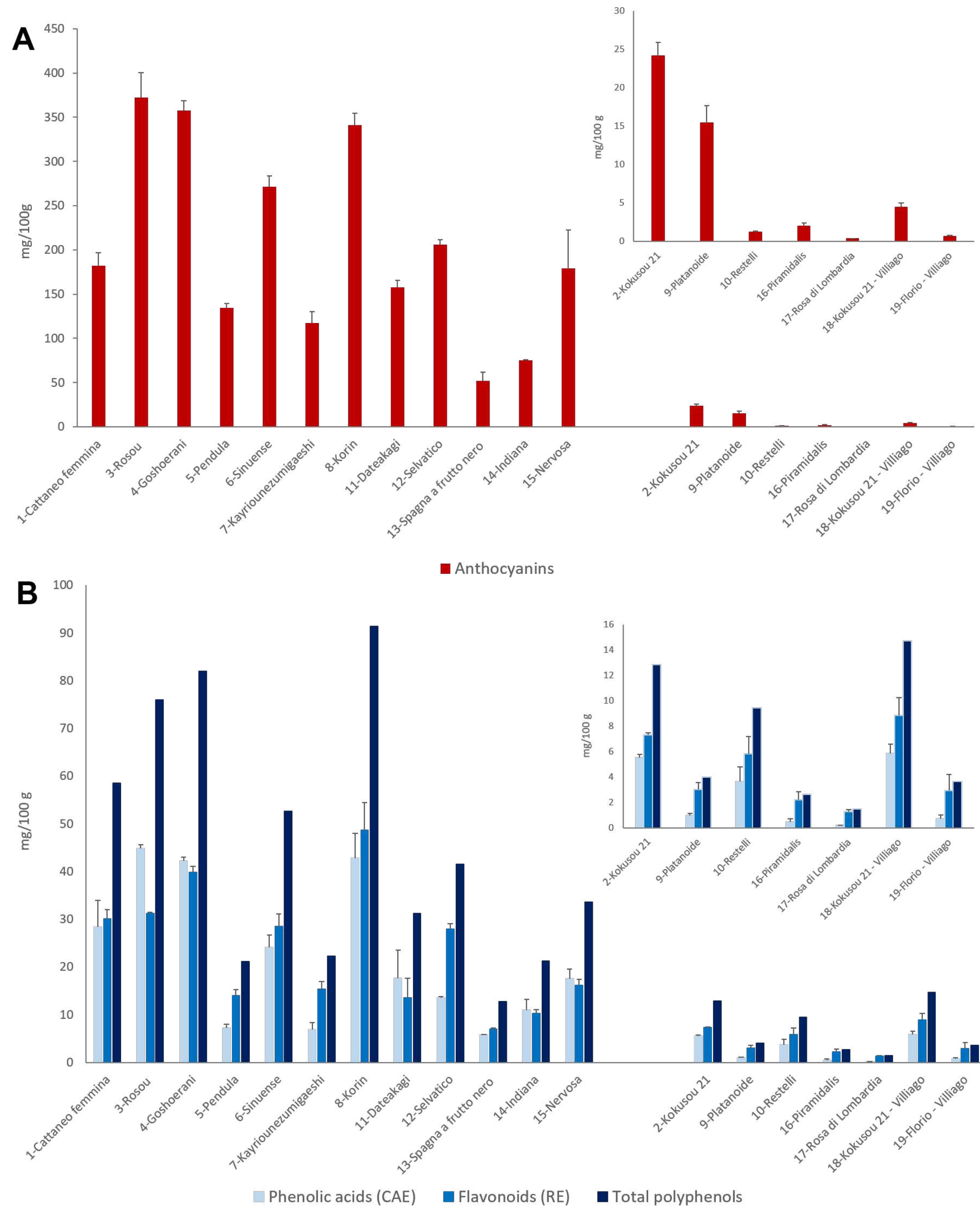


Fig. 6 Histograms showing the amounts of total anthocyanins (panel A) and total phenolic acids (expressed as chlorogenic acid equivalents, CAE), total flavonoids (expressed as rutin equivalents, RE) and total polyphenols (panel B) in fresh Morus alba fruits.

Similarly for the anthocyanin content, the phenolic content results showed a diversity between the two subgroups. The light-coloured species total phenolic content ranged from 1.46 mg/100 g to 14.73 mg/100 g, while in the dark-coloured ones the range was from 12.74 mg/100 g to 91.38 mg/100 g. Variety 8 had the highest phenolic content at 91.38 mg/100 g, with varieties 4 and 3 following at 82.01 mg/100 g and 75.97 mg/100 g, respectively. Flavonoids contributed the most to total phenolic content in almost all the fruits analyzed, ranging from 1.28 to 48.63 mg/100 g, while phenolic acids were less prevalent at 0.18 to 44.79 mg/100 g. However, in varieties 3, 4, 11, 14, and 15, the phenolic acid content slightly higher than the total flavonoid content [3].

4.0 Inflammation and oxidation

Inflammation is a complex physiological response that plays a critical role in mediating the innate and adaptive immune systems. Its primary purpose is to protect the organism against a variety of foreign harmful stimuli like pathogens, particles, and viruses. Depending on such mechanisms and processes, inflammation is classified in two categories: acute and chronic. Chronic inflammation's cellular and molecular processes are diverse and strongly correlated with the manifestation and aggravation of several chronic illnesses including cardiovascular, neurological, pulmonary, metabolic, endocrine, autoimmune disorders, and cancer. Immune system cells release pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which induce the generation of reactive oxygen species (ROS) following the initiation of inflammatory responses. If the inflammation persists, it can cause cellular injury or hyperplasia due to the overproduction of ROS by inflammatory cells. To counteract these damaging effects, cellular antioxidant systems activate genes involved in DNA repair in response to ROS-induced DNA damage. However, excessive oxidative stress increases the levels of inflammatory cytokines and related molecules, which intensifies the inflammatory response [14].

An essential factor in the host defense system are macrophages, which are involved in several immunologic functions, including inflammatory modulation and removal of apoptotic cells. Macrophages are activated by exogenous mediators such as lipopolysaccharide (LPS), an endotoxin expressed in the cell walls of gram-negative bacteria. This phenomenon is considered the first step in the inflammatory process. Researchers have conducted numerous studies investigating the protective effects of anti-inflammatory, immune-modulating, and antioxidant activities mediated by LPS-treated macrophage cells. By understanding the mechanisms involved in inflammation and the role of key players like macrophages, we can develop new therapies and treatments to combat the many chronic diseases associated with inflammation [14].

On the other hand, oxidation is mainly caused by Reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are naturally produced by the body as by-products of cellular metabolic activities. These highly reactive, free radical intermediates play important roles in protecting the body against pathogens, promoting wound healing and tissue repair, and serving as essential signaling molecules. However, overproduction of ROS and RNS can lead to an imbalance known as oxidative stress (OS), which is associated with accelerated aging and the onset of numerous diseases. Oxidative stress has been extensively studied for over three decades and is both a cause and a consequence of disease. It is associated with all diseases and can be caused by a variety of factors, including chemical exposures, age, lifestyle choices, environmental exposures, pre-existing chronic disease, and chronic psychological stress. It is not only a direct cause of non-communicative diseases, but it also indirectly affects the immune system and contributes to communicative diseases. For disease to occur, critical levels of oxidative stress must be reached, which can result from single or multiple sources. Total oxidative stress is the determining factor for disease onset. Therefore, it is crucial to maintain a balance between ROS/RNS production and antioxidant defenses to prevent the negative effects of oxidative stress [15].

Plants, like all living organisms in the presence of oxygen, produce reactive oxygen species as byproducts of their cellular metabolism. ROS are generated in different cellular organelles of plants, such as mitochondria, chloroplasts, peroxisomes, apoplast, glyoxysomes, the plasma membrane, and even the cell wall [16]. At low concentrations, ROS play a vital role as intracellular signaling molecules in maintaining cell homeostasis. They actively participate in various cellular processes such as proliferation, differentiation, growth, metabolic regulation, and programmed cell death. At the tissue level, ROS also contribute to root gravitropism, stoma closure, seed germination, lignin biosynthesis, osmotic stress regulation, and defense against pathogens. At high concentrations, they contribute to oxidative cell injury. The imbalance of ROS levels leads to harmful effects, causing damage at both the cellular and tissue levels. At the molecular level, ROS induce processes such as lipid peroxidation, changes in cell membrane permeability and fluidity,

leakage of ions, oxidation of amino acids, inactivation of enzymes through cofactor oxidation, DNA/RNA damage, and reduced photosynthesis [17].

Plants face various environmental challenges such as drought, salinity, metal exposure, temperature variations, flooding, ozone, soil pH, UV radiation, and high light exposure [16]. All these adverse conditions are known to induce massive production of ROS with harmful consequences, and maintaining cell homeostasis requires a rapid and efficient antioxidant mechanism [18]. Antioxidants found in plants can be categorized into enzymatic and nonenzymatic types. The major enzymatic antioxidants include superoxide dismutase (SOD), catalases, ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione-S-transferase (GST), glutathione peroxidase (GPX), and other peroxidases (POX) [16]. Common nonenzymatic antioxidants found in plants include ascorbate, glutathione, α-tocopherol, carotenoids, flavonoids, cysteine, methionine, polyamines, as well as the more recently discovered dehydrins and annexins [19].

4.1 NF-κB and its role in inflammation

Nuclear factor-κB (NF-κB) represents a family of inducible transcription factors, involved in the regulation of a large array of genes related to different processes of the immune and inflammatory responses [20]. NF-κB is activated through two main signaling pathways,

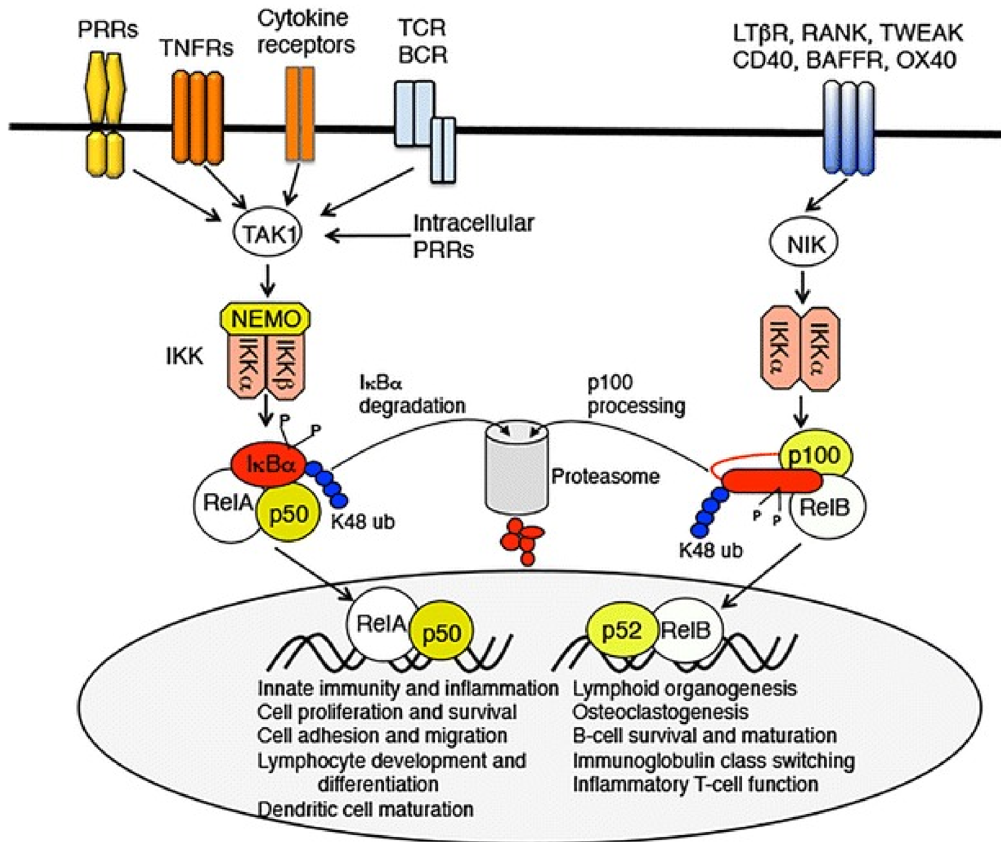


Fig. 7 Illustration of NF-κB canonical (right) and noncanonical pathways (left)

respectively canonical and noncanonical. These pathways, although different in their signaling mechanisms, play crucial roles in regulating immune and inflammatory responses [21]. Canonical NF-κB pathway is activated by various stimuli, such as cytokine receptor ligands, pattern-recognition receptors (PRRs), TNF receptor (TNFR) superfamily members, T-cell receptor (TCR) and B-cell receptor signaling [22].

The canonical NF-κB pathway is primarily activated by phosphorylation and subsequent degradation of IκBα, facilitated by a complex called the IκB kinase (IKK) complex. The IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit called NEMO or IKKγ (*fig. 7*) [22]. Multiple and various stimuli, (e.g. cytokines, growth factors, mitogens, microbial components, and stress agents) can activate the IKK complex. Once activated, the IKK complex phosphorylates IκBα at specific sites, leading to its degradation via ubiquitination in the proteasome. As a result, canonical NF-κB dimers, predominantly p50/RelA and p50/c-Rel, undergo rapid and transient nuclear translocation [21].

Unlike the canonical NF-κB pathway, the noncanonical NF-κB pathway is activated in response to a specific set of stimuli, including ligands of select TNFR superfamily members such as LTβR, BAFFR, CD40, and RANK [23]. In addition, the noncanonical NF-κB activation operates differently. It does not involve the degradation of IκBα, but relies on the processing of the NF-κB2 precursor protein, p100. The key signaling molecule in this pathway is NF-κB-inducing kinase (NIK), which activates and cooperates with IKKα to phosphorylate p100. This phosphorylation leads to ubiquitination and subsequent processing of p100. This results in the generation of mature NF-κB2 p52 and the noncanonical NF-κB complex (p52/RelB), translocates to the nucleus [21]. Functionally, the canonical NF-κB pathway plays a role in various aspects of immune responses, covering a wide range of functions. On the other hand, the noncanonical NF-κB pathway has evolved as a supplementary signaling axis that works in cooperation with the canonical pathway, specifically in the regulation of certain functions within the adaptive immune system [22].

NF-κB is widely recognized for its role in regulating inflammatory responses. It not only induces the expression of various pro-inflammatory genes in innate immune cells but also controls the activation, differentiation, and effector function of inflammatory T cells [24]. Recent studies have also implicated NF-κB in regulating the activation of inflammasomes[25]. Inflammasomes are recognized as one of the culprits of the oxidative stress damage. In general, they can mediate host immune responses to bacterial and cellular damage as forms of cytoplasmic protein complexes. However, in pathological condition, inflammasome activation is a severe complication of oxidative stress and could

cause cell death and tissue damage [26]. Consequently, dysregulated NF-κB activation is a characteristic feature of chronic inflammatory diseases. Therefore, gaining a better understanding of the mechanisms underlying NF-κB activation and its pro-inflammatory functions holds great significance for developing therapeutic strategies to treat inflammatory diseases [21].

Innate immune cells are important players of innate immunity and inflammation. These cells express PRRs that detect various microbial components and molecules released by necrotic cells and damaged tissues. Mammalian cells express five different families of PRRs which receive various stimuli but have many similarities in the signal transduction pathway. Activation of the canonical NF-κB pathway is a typical signaling event triggered by PRRs. It plays a critical role in inducing the transcription of pro-inflammatory cytokines, chemokines, and other inflammatory mediators in various innate immune cells. These inflammatory mediators can directly contribute to the initiation and progression of inflammation. Moreover, they can indirectly influence the differentiation of inflammatory T cells, amplifying the immune response during inflammation [21].

NF-κB is now known to be a crucial factor in the pathogenesis of numerous inflammatory conditions. This includes a range of diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), multiple sclerosis, atherosclerosis, systemic lupus erythematosus, type I diabetes, chronic obstructive pulmonary disease and asthma [27]. NF-κB plays a central role as an inflammatory mediator, responding to a wide range of immune receptors. As a result, therapeutically targeting the NF-κB signaling pathway has gained significant interest as a potential approach for anti-inflammatory treatments. Several classes of inhibitors have been developed to specifically block distinct steps within the NF-κB signaling cascade, offering promising opportunities for therapeutic interventions [21]. To block the catalytic activity of IKK and prevent IκBα phosphorylation, an increasing number of selective IKK inhibitors have been designed [28]. NSAIDs, including aspirin, ibuprofen, indomethacin, and COX-2 inhibitors, are potential NF-κB blockers. They function by either suppressing the inflammatory cell response to indirectly suppress NF-κB, or by directly suppressing NF-κB at key points along the NF-κB activation pathway [28].

Although considerable advancements in developing strategies to inhibit NF-κB have been made, challenges persist in the translation of these approaches into clinically viable NF-κB-based drugs. While the potential therapeutic benefits of NF-κB inhibition in treating inflammatory diseases are recognized, concerns arise regarding the delicate balance between efficacy and safety. This is because NF-κB plays crucial roles in maintaining normal immune responses and cell survival. Emerging evidence indicates that broad inhibition of NF-κB signaling may give rise to significant side effects [21].

5.0 Active compounds

5.1 Anthocyanins

Anthocyanins are a class of molecules that have been widely recognized as the most significant and largest group of water-soluble pigments present in nature. They are responsible of giving coloration to several fruits and vegetables, such as blue, purple, red and orange. The word anthocyanin originated from the Greek words "anthos" meaning flowers, and "kyanos" meaning dark blue, indicating the color range of these pigments [29].

Anthocyanins can be found in abundance in various food sources such as blueberries, cherries, raspberries, strawberries, black currants, purple grapes, red wine and, of course, mulberries. These pigments belong to the flavonoid family, but their unique characteristic is their ability to form flavylium cations (*fig. 8*), which sets them apart from other flavonoids [30].

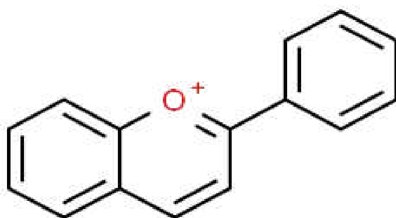


Fig. 8 Flavylium cation

These pigments are mainly present as glycosides of their respective aglycone anthocyanidin chromophores. Typically, the sugar moiety is attached to the 3-position on the C-ring or the 5-position on the A-ring. Although there have been identified approximately 17 types of anthocyanidins in nature, only six pigments, namely cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin, are commonly found in human diet. Among these six, cyanidin is the most prevalent [29].

The variety of colors given by anthocyanins is mainly influenced by the number and position of hydroxyl and methoxy groups on the anthocyanidin skeleton, while the identity, number, and positions at which sugars are attached also play a role, as does the extent of sugar acylation and the identity of the acylating agent. The presence of hydroxyl

and methoxy groups affects the intensity and type of coloration observed, with hydroxyl groups tending to create a bluish shade and methoxyl groups increasing redness.

Anthocyanins' coloration can also vary depending on the pH of the surrounding environment. Under acidic conditions, the presence of eight conjugated double bonds carrying a positive charge causes the pigment to appear intensely red or orange [29]. In contrast, at pH values between 2 and 4, a quinoidal blue species tends to dominate. Between pH values of 5 and 6, only two colorless species, carbinol pseudobase and chalcone, are present. At pH values above 7, anthocyanins degrade depending on the specific substituent groups they contain [31]. This degradation causes a decrease in color stability toward neutrality, although some anthocyanins display an increase in stability at local maxima around pH values of 8-9. Overall, these various factors combine to create the diverse range of anthocyanin coloration observed in nature [29].

Anthocyanins found in various foods and beverages, have been shown to play a crucial role in preventing a wide range of diseases such as cancer and cardiovascular disease. The mechanisms through which anthocyanins exert their preventive effects include antioxidant activity, detoxification activity, anti-proliferation, induction of apoptosis, and anti-angiogenic activity. Additionally, anthocyanins have been found to possess anti-inflammatory activity and inhibit digestive enzymes such as α-glucosidase, α-amylase, protease, and lipase. The inhibition of these enzymes is a clinical therapeutic target for controlling type II diabetes and obesity. Furthermore, anthocyanins are known to improve the immune system, enhance night vision, and even retard the aging process [29].

5.1.1 Bioavailability and metabolism

Despite their potential therapeutic effects, only up to 35% of anthocyanins can be absorbed after undergoing digestion in the stomach and small intestine [32]. However, recent studies have suggested that anthocyanins might still exhibit biological activity along their transit through the gastrointestinal tract, and that they are transformed by the colonic microbiota upon reaching the large intestine. After food consumption, anthocyanins are partially degraded by saliva before entering the stomach, where they are rapidly absorbed and identified in blood and urine as various anthocyanin derivatives. Their stability is reduced in the small intestine due to neutral or mildly alkaline conditions, and absorption occurs mostly in the jejunum as anthocyanidin. However, a small amount of anthocyanin is absorbed in the duodenum, while there is no absorption in the ileum and colon [33]. They can be hydrolyzed to anthocyanidins by enzymes present in the epithelial cells of the gastrointestinal tract. Once absorbed, anthocyanins are transported to the liver where they are further metabolized by various enzymes, while the amount that was not absorbed is metabolized by the intestinal microbiota, producing diverse compounds such as phenolic acids including protocatechuic and gallic acid. Interestingly, the permeability of anthocyanins across the gastrointestinal mucosa is considered quite high, with the former found in high concentrations in intestinal tissues compared to their reduced circulating concentration. This suggests that they could reach biologically active concentrations in gastrointestinal tissues, exhibiting their protective effects before reaching circulation [34].

5.1.2 Role of anthocyanins in the prevention of oxidative stress

The intake of dietary antioxidants, such as anthocyanins, is essential to control an equilibrium between oxidants and antioxidants. The antioxidant properties of anthocyanins have been evaluated both in vitro and in vivo. These compounds can scavenge free radicals and interrupt the chain reaction that initiates oxidative damage. The antioxidant activity of anthocyanins is attributed to their phenolic structure. Anthocyanins are effective at quenching reactive oxygen species (ROS), including superoxide O2·, singlet oxygen (1O2), hydroxyl radical (OH·), peroxide (ROO·), and hydrogen peroxide (H2O2) [34]. The antioxidant capacity of anthocyanins found in various fruits has been demonstrated using a range of assays based on different chemical mechanisms, such as oxygen-radical absorbance capacity (ORAC), hydrogen transfer-based (HAT), ferric-reducing antioxidant potential (FRAP), trolox equivalent antioxidant capacity (TEAC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity.

Anthocyanins and anthocyanidins are known for their high reactivity toward reactive oxygen species (ROS) due to their electron deficiency. However, their antioxidant activity is influenced by their chemical substitutions and the presence of different functional groups on their flavylium B-ring, resulting in varied activity for scavenging different types of ROS and RNS [35]. The antioxidant potential of anthocyanins is also linked to their structural orientation, where the orientation of hydroxyl groups determines their efficacy in donating electrons to stabilize free radicals. Generally, the antioxidant capacity of anthocyanins increases with the number of free hydroxyl groups around the pyrone ring. Moreover, glycosylation affects the antioxidant capacity of anthocyanins, with anthocyanidins exhibiting higher radical scavenger capacity than their corresponding anthocyanins due to the anthocyanin radical's reduced ability to delocalize electrons [36]. Finally, the effectiveness of the functional groups on the flavylium ring of anthocyanins for antioxidant activity follows the order: –OH > –OCH3 ≫ –H. Therefore, anthocyanidins' antioxidant capacity varies in the order: delphinidin > petunidin > malvidin > cyanidin >

peonidin > pelargonidin. (Gonzales). The mechanism described is referred to as "Direct chemical mechanism" [34].

There's also another mechanism called "Indirect chemical mechanism", in which anthocyanins enhance or restore the activity of two antioxidant enzymes (SOD and GPX), increasing glutathione content. In addition, anthocyanins have been found to decrease the formation of oxidative adducts in DNA and endogenous ROS. This is achieved through inhibiting NADPH oxidase and xanthine oxidase, or by modifying mitochondrial respiration. Furthermore, anthocyanins indirectly exhibit antioxidant effects by activating the NF-E2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway, leading to the transcription of genes coding for these enzymes. The enhancement of ARE-regulated phase II enzyme expression by anthocyanins is crucial for cellular defense against oxidative stress. Even if oxidative stress is sufficient to activate Nrf2-ARE signaling pathway and induce the transcription of Nrf2 target genes, anthocyanins can promote oxidative stress-mediated activation because of their specific redox cycling properties [34].

5.2 Quercetin and Rutin

According to the previously observed phytochemical profile, quercetin and rutin are among the major compounds present in mulberries. Studies have shown that through the use of cell-based assays, Quercetin and Rutin, when present in the crude extract of plants, exhibit a noteworthy reduction in the levels of inflammatory mediators such as TNF-α, IL-1β, and IL-6. Some evidence indicates that the combination of Quercetin and Rutin may have a more potent effect than either flavonoid on its own. Rutin has

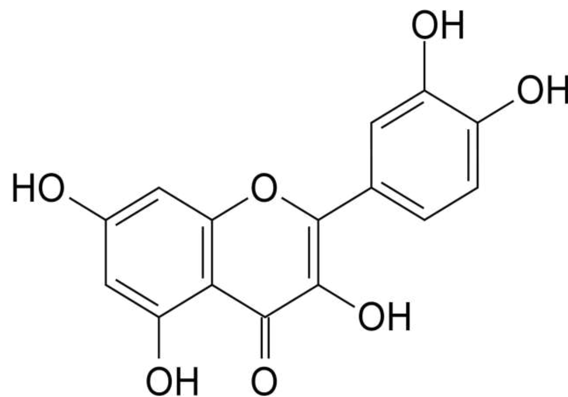


Fig. 9 Quercetin

been found to be particularly effective against nitrosative stress and hepatic damage, whereas Quercetin has greater antioxidant and anti-inflammatory activity.

A study made by D. Shanmugasundaram and J.M. Roza combined quercetin and rutin in a preparation called SophorOx™ to further confirm their antioxidant and anti-inflammatory properties in vitro. The anti-inflammatory activity of a SophorOx™ was investigated in LPS-stimulated RAW 264.7 macrophages. When used at a concentration of 10 µM, SophorOx™ showed a notable reduction in the production of TNF-α and IL-6 induced by LPS, with around 30% inhibition. Pre-treatment of Raw 264.7 cells with SophorOx™ also resulted in a significant reduction of NO/nitrite and ROS generated by LPS stimulation, even at low concentrations (<0.3 µM). These findings suggest that SophorOx™ possesses potent antioxidant and anti-inflammatory properties, effectively blocking mediators of oxidative stress and inflammation [37].

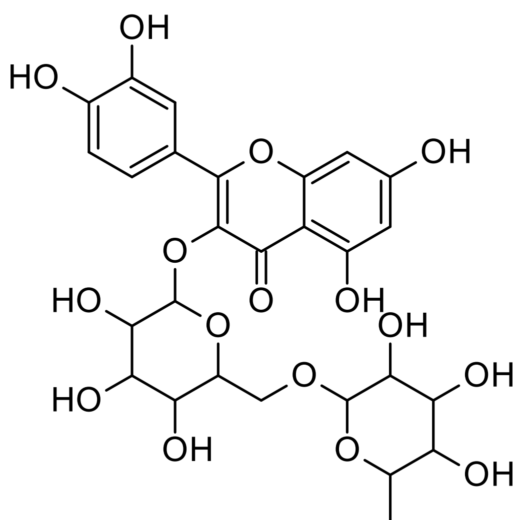


Fig. 10 Rutin

5.3 Eriocitrin

Eriocitrin ((+/−) -5,7,3′,4-tetrahydroxyflavanone 7-o-ruinoside) is a chiral flavanone-7-o-glycoside present in lemons, tamarinds, and other citrus fruits, like the citrus sinensis, as well as in mint, oregano, fennel thyme, and rose hip [38].

Flavone glycosides are one of the largest class of flavonoids. They are characterized by a 2-phenyl-chrome-4-one backbone featuring a unique three-ring skeleton structure (C6-C3-C6). This structure comprises of two benzene rings (A- and B- ring) and an oxygen-containing ring containing a C2-C3 double bond (C-ring) [39].

Eriocitrin found in lemon juice and peppermint leaves, has been shown to possess potent antioxidant activity as a radical scavenger. It was able to scavenge DPPH and superoxide, as well as display a strong anti-H2O2 activity. Eriocitrin has also demonstrated anti-inflammatory effects by significantly reducing ICAM-1 expression in human umbilical vein endothelial cells induced by TNF-α. In addition, eriocitrin extracted from different Mentha species, varieties, hybrids, and cultivars was identified as the dominant radical scavenger in these extracts in an online HPLC-DPPH method [38].

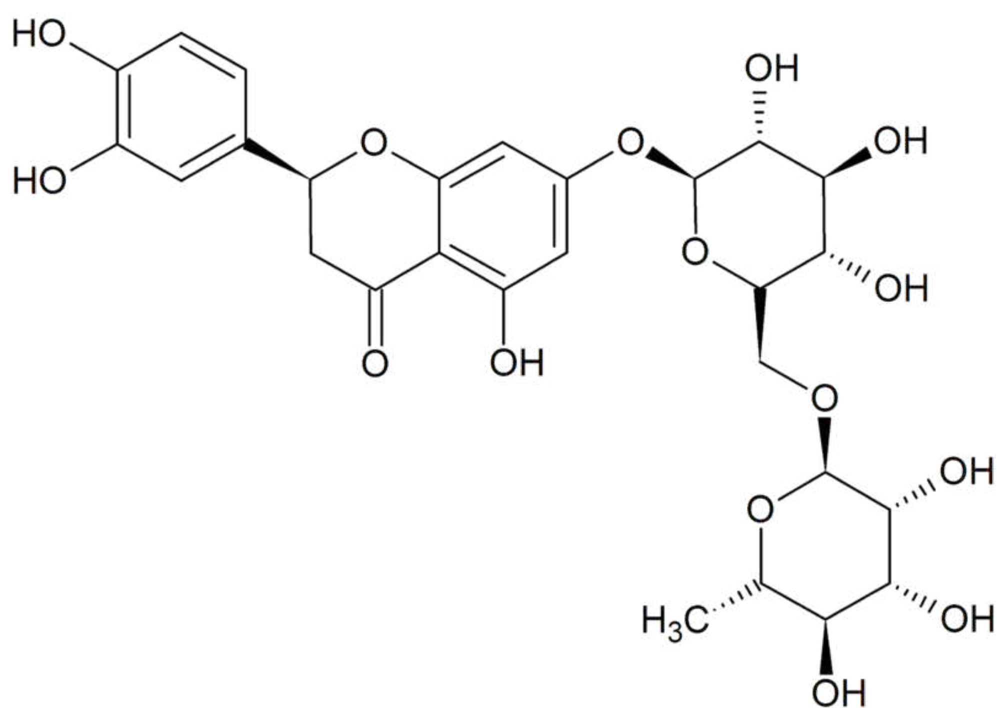


Fig. 11 Eriocitrin

5.5 Narirutin

Narirutin is a flavanone-7-O-glycoside that is present in orange juice and is made up of the flavanone naringenin bound to the disaccharide rutinose [40]. It can be found in Rutaceae citrus plants, including oranges, mandarins, and grapefruits. Fruit and juices from these plants contain flavonoids, including narirutin and naringin in grapefruits [41].

Numerous experimental studies highlight narirutin's wide range of potential pharmacological properties. These studies suggest that narirutin may exhibit anticancer activity, neuroprotective effects, stress relief, hepatoprotection, anti-allergic properties, antidiabetic activity, anti-adipogenic effects, anti-obesity action, and immunomodulatory effects. Narirutin also has antioxidant, and anti-inflammatory activities [40].

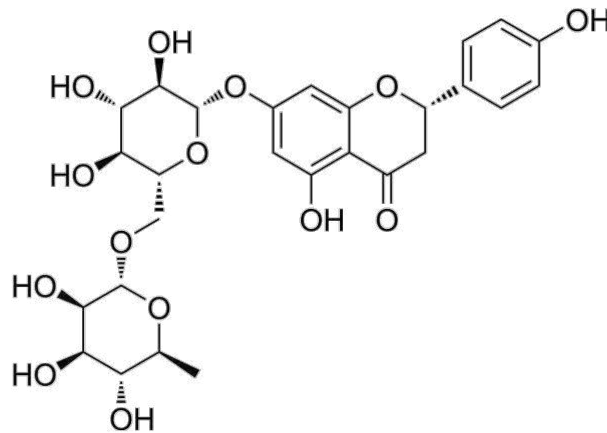


Fig. 12 Narirutin

In a Korean study of 2012, researchers investigated the effects of narirutin fraction on the production of nitric oxide (NO) and the expression of inducible nitric oxide synthase (iNOS) in RAW 264.7 cells stimulated with lipopolysaccharide (LPS). They found that narirutin fraction dose-dependently inhibited LPS-induced NO production and suppressed the expression of iNOS. Furthermore, they examined the impact of narirutin fraction on the production of prostaglandin E2 (PGE2) and the expression of cyclooxygenase-2 (COX-2), which is involved in PGE2 synthesis. The results indicated that narirutin fraction had the potential to reduce PGE2 production and downregulate COX-2 expression in LPS-treated RAW 264.7 cells [42]. Moreover, cells stimulated with LPS increased the activity of NF-kB in the nucleus. However, when the cells were treated with narirutin fraction, this increase in NF-kB activity was inhibited. Additionally, the narirutin fraction prevented the degradation of a

protein called IkB-a and the translocation of NF-kB into the nucleus, proving its activity in the reduction of inflammation [42].

In conclusion, narirutin exhibits promise as a drug candidate for dyslipidemia, tuberculosis, obesity, and Alzheimer's disease, with potential inhibitory effects on HMG CoA reductase, Shikimate kinase, and pancreatic lipase. However, further clinical studies are necessary to validate its efficacy and safety. Additionally, narirutin's properties suggest it could be explored for nano-technology-based treatments targeting various diseases, including cancers and tumors [40].

6.0 Materials and methods

6.1 THP1-Blue™ NF-kB cells

THP1-Blue™ NF-κB cells are a type of cell that are used to monitor the NF-κB signal transduction pathway. These cells were created by introducing a reporter gene into the THP-1 monocyte cell line, which allows us to easily detect NF-kB activity in response to various stimuli. The reporter gene expresses a protein called SEAP (secreted embryonic alkaline phosphatase) in response to NF-κB activation, which can be detected using a reagent called QUANTI-Blue™ Solution. THP1-Blue™ NF-κB cells are highly responsive to certain types of pathogen recognition receptor (PRR) agonists that activate the NF-kB pathway. The strength of their response to different Toll-like receptor (TLR) agonists varies, with some TLR agonists producing strong responses while others produce hardly detectable responses. THP1-Blue™ NF-κB cells are also resistant to the selectable marker blasticidin, which allows for their selection and maintenance in culture [43].

Cells will undergo towards a physiological reduction in responsiveness, which is normal for all stably transfected cells. Hence, it is recommended by the producer to have a frozen stock of the cell line, and not to passage them more then 20 times to maintain their full efficiency.

To culture THP-1 derived cells, a specific cell culture medium is needed. The growth medium should contain RPMI 1640, 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated fetal bovine serum (which has been heated for 30 minutes at 56°C), 100 µg/ml Normocin™, and Pen-Strep (100 U/ml-100 µg/ml). It's important to use Normocin™ together with Pen-Strep to prevent microbial contamination, which could activate TLRs and lead to differentiation of the monocytes and activation of the reporter gene. Initial culture of all THP-1 derived cells should be performed in growth medium with 20% heat-inactivated FBS.

For freezing THP-1 derived cells, it is needed a freezing medium that contains 90% fetal bovine serum (FBS) and 10% DMSO. Finally, for testing the cells, test medium consisting of RPMI 1640, 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, and Pen-Strep (100 U/ml-100 µg/ml) is required [43].

Initial Culture Procedure: The first step in propagating cells is to generate stocks for future use to ensure stability and consistent performance of the cells in subsequent experiments. The following steps should be performed under aseptic conditions:

1. Thaw the vial in a 37°C water bath with gentle agitation, taking care to keep the O-ring and cap out of the water to reduce the possibility of contamination. Thawing should be done rapidly.

2. As soon as the contents are thawed, remove the vial from the water bath and decontaminate by dipping in or spraying with 70% ethanol.

3. Transfer the cells to a vial containing 15 ml of pre-warmed growth medium with 20% heat-inactivated FBS. Do not add selective antibiotics until the cells have been passaged twice.

4. Centrifuge the cells at 150 x g (RCF) for 10 minutes.

5. Remove the supernatant containing the cryoprotective agent and resuspend the cells in 1 ml of growth medium with 20% heat-inactivated FBS.

6. Transfer the contents of the vial to a 25 cm2 tissue culture flask containing 5 ml of growth medium with 20% heat-inactivated FBS.

7. Place the culture at 37°C with 5% CO2.

6.2 Evaluation of anti-inflammatory activity

The evaluation of the anti-inflammatory activity was conducted by measuring the NF-kB activity with QUANTI-Blue. QUANTI-Blue is a colorimetric enzyme assay specifically designed for the detection and quantification of alkaline phosphatase activity (AP) in biological samples, including cell culture supernatants. In the presence of AP, QUANTI-Blue medium changes from a pinkish to a purple-blue color. Secreted embryonic alkaline phosphatase (SEAP), a commonly employed reporter gene, is a truncated variant of placental alkaline phosphatase, a GPI-anchored protein. Unlike intracellular reporters, SEAP is secreted into the cell culture supernatant, presenting numerous advantages [43].

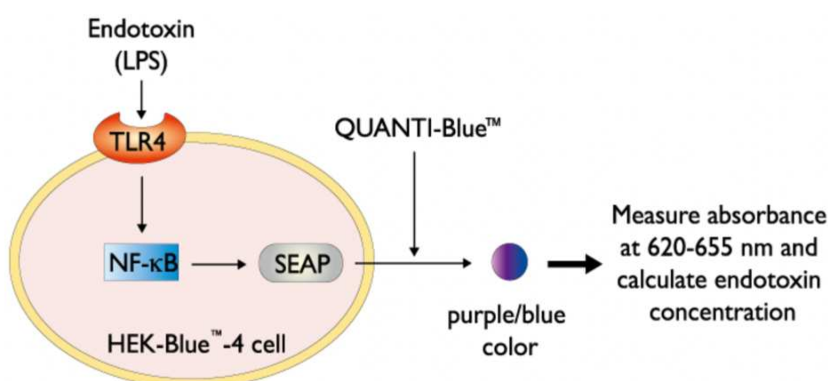


Fig. 13 Mechanism of the NF-kB anti-inflammatory assay with a different cell line

The cell line used in this study, THP1-Blue™ NF-κB cells, express an NF-κB inducible SEAP reporter gene. Lipopolysaccharide (LPS) stimulation of TLR4 receptors in the cells induce signaling cascades leading to the activation of NF-κB and the subsequent production of SEAP. Nuclear factor-κB (NF-κB) consists of a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival. Inflammatory processes are controlled through NF-κB-dependent transcription of cytokines, chemokines, cell adhesion molecules, factors of the complement cascade, and acute phase proteins [20].

Once SEAP is produced, it can easily be detected and measured using QUANTI-Blue which, in the presence of alkaline phosphatase, changes color going from pink to purple/blue

(*fig. 13)* [44]. The intensity of the blue hue reflects the activity of AP. The activity of NF-κB is then determined spectrophotometrically at 630 nm [43].

6.2.1 Procedure

1. Cells are counted to check the viability. If it is appropriate, the test can proceed.
2. Based on the number of cells per mL, a right amount of cells is collected in order to have the concentration of $50 \cdot 10^5$ cells/well.
3. Cells are then centrifuged for 5 minutes at the temperature of 29°C and 300 rcf.
4. The exceeding media is removed under the laminar flow hood and the cells are added with 2 mL of PBS for a further round at the centrifuge.
5. PBS is removed and the cells are finally added with the serum free media (SFM).
6. After dispensing 90 µL of prepared cells into each well, the plate is placed in the incubator for two hours.
7. Meanwhile, 2 µg of each compound are added to 198 µL of SFM. 10 µL of this preparation will be added to the cells. Wells marked as DMSO will be added of 10 µL of a solution prepared using 198 µL of SFM and 2 µL of DMSO.
8. The compounds are then added. For this study each extract has been studied at the concentration of 20 µg/mL, 10 µg/mL, and 5 µg/mL.
9. The plate will undergo an additional one-hour incubation period in the incubator.
10. A quantity of 1 µg/mL of LPS is then added in each well (except for CTRL).
11. After incubating for 24 hours, a mixture of 20 µL of cultivation medium and 180 µL of QUANTI-Blue medium is prepared and subjected to incubation at 37°C for a period of 30-40 minutes, allowing the enzymatic reaction to occur.
12. NF-κB activity is determined with the spectrophotometer at the wavelength of 630 nm.
13. Once results are out, Blank must be subtracted from the values obtained for each compound. To calculate the percentage of NF-kB activation, the mean value of

LPS must be divided from every value obtained by the spectrophotometer. Once this values are calculated, the mean of the values given by the reader for each compound can be calculated. All the mean values will be then collected to have an overall view of the activity of each compound.

6.4 Evaluation of antioxidant activity

The evaluation of antioxidant activity was conducted using the Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. This assay allows the measurement of intracellular ROSs via the use of a 2′,7′-dichlorofluorescin diacetate (DCFH-DA) probe. DCFH-DA possesses a nonpolar and nonionic structure, allowing it to easily cross the cell membrane. Once inside the cell, the ester bond of DCFH-DA is broken down by natural enzymes called esterases. This process converts the probe into its reduced form, which is more susceptible to oxidation by ROS. This converted form of the probe stays inside the cell and cannot escape into the extracellular environment. This characteristic enables the detection and measurement of ROS specifically within the cellular compartment. As ROS interact with the reduced form of the probe, it undergoes oxidation and transforms into a fluorescent molecule known as 2′,7′-dichlorofluorescein (DCF) [45] .

The antioxidant activity of the extracts was measured using the method of Wolfe and Liu (2007) with some modifications, as reported by Malanik et al. [46]. Cells were pre-incubated for 1 h in a serum-free RPMI 1640 medium containing 25 µM 2′,7′-dichlorodihydrofluorescein-diacetate ($DCFH_2$-DA; Sigma-Aldrich, St. Louis, MO, USA) dissolved in DMSO [final concentration of DMSO in the medium was 0.1% (*v*/*v*)] at 37 °C. Cells were then centrifuged and washed with phosphate-buffered saline (PBS), re-suspended in serum-free RPMI 1640 medium, and placed into 96-well black plates in triplicates at the concentration of 60,000 cells/well. Cells were then incubated with the extracts for 1 h and after that, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH; Sigma-Aldrich, St. Louis, MO, USA) was added at a final concentration of 600 µM [47]. AAPH serves as a potent radical initiator that undergoes spontaneous decomposition, resulting in the formation of carbon-centered radicals. These radicals, in the presence of oxygen, instigate a process called lipid peroxidation by targeting the polyunsaturated fatty acids present in the cell membrane (*fig. 14*) [45]. By combining DCFH-DA with AAPH, we can not only detect ROS levels within the cytosolic compartment but also examine the impact of antioxidants on ROS-induced damage [45]. The plate was then immediately placed into a

FLUOstar Omega microplate reader (BMG Labtech) at the temperature of 37 °C. The level of oxidized fluorescent 2',7'-dichlorfluorescein (DCF) was measured for 13 cycles every 5 minutes (excitation wavelength at 485 nm; emission at 538 nm). Each plate included triplicate control and blank wells: control wells contained cells treated with DCFH-DA and AAPH; blank wells contained cells treated with the dye and serum-free RPMI 1640 medium without AAPH. Quercetin (Koch-Light Laboratories, Haverhill, UK) was used as a positive control at the same concentration as the test compounds (20, 10 and 5 µg/mL). After the blank was subtracted from the fluorescence readings, the area under the curve of fluorescence versus time was integrated to calculate the CAA values of the compounds: CAA unit = 100 − (∫SA/∫CA) × 100, where ∫SA is the integrated area under the sample fluorescence versus time curve and ∫CA is the integrated area obtained from the control curve [47].

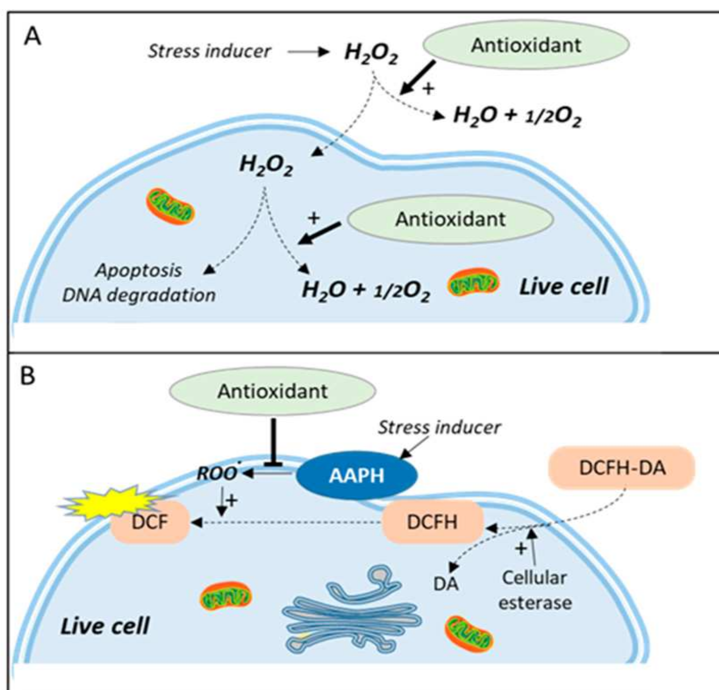


Fig. 14 Oxidation in a live cell (A) and mechanism of the DCFH-DA inside the cell (B)

6.3.1 Procedure

1. Cells are counted to check the viability. If it is appropriate, the test can proceed.
2. Based on the number of cells per mL, a right amount of cells is collected in order to have the concentration of $60 \cdot 10^5$ cells/well.
3. Cells are then centrifuged for 5 minutes at the temperature of 29°C and 300 rcf.
4. The exceeding media is removed under the laminar flow hood and the cells are added with 2 mL of PBS for a further round at the centrifuge.
5. PBS is removed, and the cells are finally added with 1,5 mL of serum free media (SFM) and 1,5 µL of DCF-DA.
6. The prepared sample is placed into the incubator to allow the DCFH-DA to enter the cells, ensuring a proper distribution and a cellular uptake of the compound.
7. After one hour of incubation, cells are centrifuged, suspended in 10 mL of SFM, and dispensed into the wells (100 µL each).
8. Compounds to be tested are prepared adding 2,5 µL of each in 47,5 µL of SFM. DMSO is also prepared (2,5 µL in 47,5 µL of SFM).
9. The diluted compounds are distributed in the amount of 2 µL each in their respective wells (one compound for each line of the plate). In addition, a line of the plate is dedicated to the inclusion of Quercetin, which will serve as a reference being that Quercetin has a strong antioxidant activity. By including Quercetin as a reference, we can assess the relative antioxidant effectiveness of the other compounds in comparison to a known antioxidant agent.
10. The plate is then placed into the incubator for an additional hour of incubation.

11. AAPH is prepared, adding 8,1 mg in 1 mL of water. 10 µL of AAPH solution will be added in column 1,2,3 and 7,8,9 of the plate (*tab. 1*), while the remaining columns will be added with 10 µL of purified water.

12. The sterile black plate is then inserted into the fluorescence reader, which will detect fluorescence emitted by the DCF (2',7'-dichlorofluorescein). The reading process involves 13 cycles, each lasting 300 seconds, with a consistent temperature of 37°C maintained throughout the entire process. During each cycle, the reader captures the intensity of the DCF fluorescence, providing information about the presence and levels of reactive oxygen species (ROS) for each well. This repeated process ensures comprehensive data collection and analysis, permitting a comprehensive evaluation of the antioxidant activity and potential oxidative stress encountered by the samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	AAPH (CTRL)			Blank (-AAPH)			AAPH (DMSO)			Blank - DMSO		
B	AAPH (CTRL)			Blank (-AAPH)			AAPH (DMSO)			Blan - DMSO		
C	WASHINGTON			Blank (W)			WASHINGTON			Blank (W)		
D	VANILLA			Blank (V)			VANILLA			Blank (V)		
E	NAVELINA			Blank (N)			NAVELINA			Blank (N)		
F	M3			Blank (M3)			M3			Blank (M3)		
G	M4			Blank (M4)			M4			Blank (M4)		
H	QUERCETIN			Blank (QUE)			QUERCETIN			Blank (QUE)		

Tab. 1 Representation of the DCFH-DA assay plate. AAPH will be added in coluns 1,2,3 and 7,8,9.

7.0 Results

7.1 NF-κB activity

The anti-inflammatory assay was conducted by evaluating each compound at the concentration of 20 µg/mL, 10 µg/mL and 5 µg/mL, following this order. Compound have been sampled as “W” for Washington Navel variety, “N” for Navelina, “V” for Vanilla and, regarding the Morus, M3 and M4.

7.1.1 Concentration of 20 µg/mL

The assay was performed multiple times to gather a sufficient amount of data. All the data were then combined to obtain a more complete and comprehensive understanding of the overall findings. The following table (*tab. 2*) represents the results of the spectrophotometer at 630 nm after calculations. Only valid results have been selected for the study.

20 µg/mL	M3-20	M4-20	W-20	V-20	N-20	PRED	LPS	CTRL
	102,21%	108,34%	62,87%	74,70%	79,83%	42,28%	102,90%	13,75%
	116,04%	91,52%	63,01%	65,43%	73,13%	45,15%	90,30%	19,48%
	105,35%	76,41%	65,29%	73,84%	65,86%	34,07%	98,00%	5,87%
	117,14%	112,82%	134,22%	97,30%	105,06%	30,08%	98,00%	9,14%
	114,03%	94,71%	107,13%	109,55%	97,47%	32,79%	90,40%	11,39%
	124,38%	102,65%	116,10%	128,35%	112,82%	76,52%	94,71%	21,76%
	125,92%	117,36%	119,68%	134,30%	104,70%	78,48%	114,89%	19,08%
	118,61%	130,38%	101,66%	102,91%	106,12%	74,20%	105,05%	17,48%
	130,02%	114,15%	117,72%	99,35%	94,71%	54,87%	93,28%	24,96%
	84,53%	92,19%	76,64%	70,29%	91,09%	57,89%	101,66%	26,50%
	82,77%	89,12%	74,23%	88,25%	95,47%	64,82%	103,80%	26,72%
	84,64%	90,22%	73,58%	81,90%	90,66%	63,94%	100,95%	23,30%
	93,18%	108,46%	88,78%	85,16%	96,29%	64,38%	95,26%	24,33%
	93,44%	122,69%	91,89%	95,51%	107,94%	71,61%	118,03%	27,18%
	69,17%	98,36%	88,01%	89,56%	114,41%	60,83%	115,44%	
	63,98%	91,32%	88,26%	74,82%	87,55%	58,24%	66,52%	
	68,81%	82,84%	78,00%	84,13%	82,72%	62,38%		
		79,30%	77,53%	80,24%	73,64%			
MEAN	99,66%	100,16%	90,26%	90,87%	93,30%	57,21%	99,32%	19,35%
SD	21,47%	15,31%	21,42%	18,91%	14,12%	15,33%	12,22%	6,95%

Tab. 2 NF-kB activity at 20 µg/mL

LPS is added because of its ability to strongly activate NF-κB and induce a robust inflammatory response. It exhibits maximal inflammatory activity (almost 100%). Prednisolone (PRED) is added because of its strong anti-inflammatory properties. It serves as a positive control to compare the relative efficiency of the tested compounds in inhibiting NF-κB activation and reducing inflammation. Prednisolone values are expected to be relatively low, showing an anti-inflammatory effect.

Standard deviation is relatively high for each compound, indicating a lack of precision in the testing process. Despite considering standard deviation, results show almost no activity at all for any of the tested compounds at the concentration of 20 μg/mL (*fig.15*).

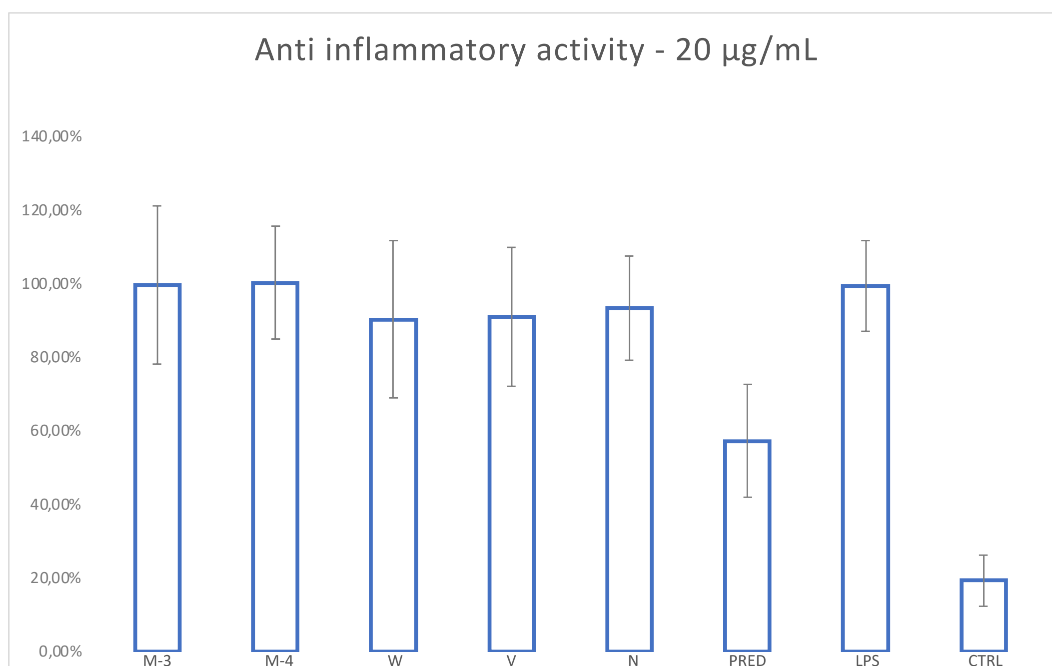


Fig. 15

7.1.2 Concentration of 10 µg/mL

In this range of concentrations, a slightly larger amount of data was collected compared to the previous one. LPS is close to 100%, while PRED, the positive control, demonstrated an average activity of 56%, indicating a favorable level of efficacy (*tab. 3*). Navelina variety seems to be slightly more efficient in the inhibition of NF-kB activity, but a value of 86,92% ± 10,58% is not good enough to be taken into consideration and standard deviation is too high, so it surely could need a further investigation.

10 ug/mL	M3-10	M4-10	W-10	V-10	N-10	PRED	LPS	CTRL
	82,85%	62,80%	83,77%	80,56%	69,90%	42,28%	102,90%	13,75%
	73,57%	84,57%	81,25%	82,28%	75,86%	45,15%	90,30%	19,48%
	112,65%	71,85%	104,82%	65,15%	71,13%	34,07%	98,00%	5,87%
	108,34%	74,27%	63,72%	58,59%	66,00%	30,08%	98,00%	9,14%
	126,80%	73,84%	72,13%	69,00%	73,56%	32,79%	90,40%	11,39%
	103,45%	129,21%	92,64%	95,40%	103,68%	76,52%	94,71%	21,76%
	102,56%	105,23%	96,78%	96,95%	91,09%	78,48%	114,89%	19,08%
	73,48%	77,98%	94,88%	93,50%	95,57%	74,20%	105,05%	17,48%
	92,41%	92,03%	98,10%	99,17%	92,75%	54,87%	93,28%	24,96%
	74,32%	112,37%	97,74%	97,21%	96,67%	57,89%	101,66%	26,50%
	88,79%	97,38%	105,05%	99,70%	90,25%	64,82%	103,80%	26,72%
	89,56%	94,22%	93,77%	88,34%	87,44%	63,94%	100,95%	23,30%
	82,55%	100,55%	105,83%	90,60%	102,21%	64,38%	95,26%	24,33%
	85,40%	95,28%	105,98%	106,28%	96,63%	71,61%	118,03%	27,18%
	95,51%	86,72%	82,12%	84,74%	99,42%	60,83%	115,44%	8,76%
	104,06%	85,62%	82,99%	92,19%	97,66%	58,24%	66,52%	7,76%
	97,58%	84,53%	85,40%	99,42%	84,31%	62,38%	101,36%	9,09%
	87,20%	82,05%	95,51%	91,89%	86,45%	44,93%	102,81%	
	89,55%	92,92%	78,17%	93,44%	98,10%	49,30%	95,83%	
	84,37%	74,81%	74,55%	98,36%	90,85%	55,55%	97,14%	
	80,45%	84,72%	84,13%	93,68%	86,96%		96,31%	
	82,93%	88,61%	84,01%	89,08%	79,89%		106,55%	
	86,56%	82,48%	79,77%	66,10%	75,77%			
		75,00%	82,43%	99,45%	85,57%			
		84,58%	90,20%	105,40%	84,09%			
		83,43%	93,50%	95,32%	78,14%			
MEAN	91,52%	87,58%	88,82%	89,68%	86,92%	56,12%	99,51%	17,44%
SD	13,30%	13,90%	11,03%	12,53%	10,58%	14,42%	10,54%	7,58%

Tab. 3 NF-kB activity at 10 µg/mL

The histogram representation of the results shows a slightly major anti-inflammatory activity at the concentration of 10 µg/mL for all the compounds, but we observe still no significant activity (*fig. 16*). Compounds seems to have more or less the same activity, with Navelina variety and M4 being a little more active than the other tested compounds. Standard deviation is still high.

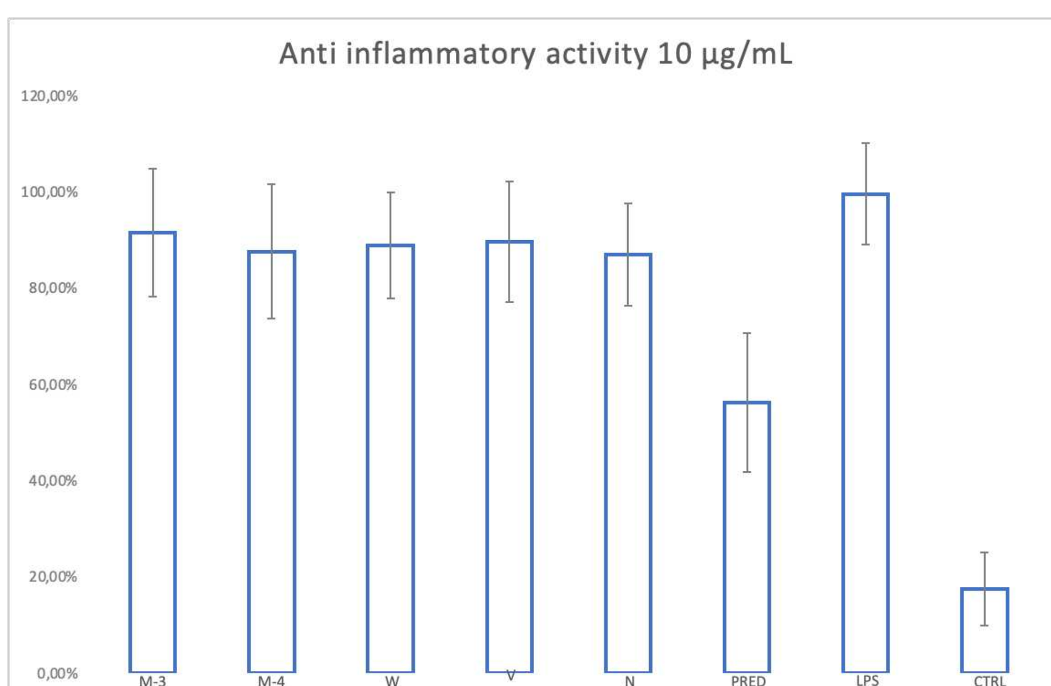


Fig. 16

7.1.3 Concentration of 5 μg/mL

At the concentration of 5 μg/mL, the data collection process encountered some challenges associated with cell cultivation, resulting in a relatively limited amount of data available for analysis. However, despite this limitation, the data collected is adequate to provide a general overview of the activity observed at this particular concentration. LPS shows maximum NF-kB activity, while Prednisolone, with a mean of 57,21% shows a good inhibition of inflammatory activity, meaning the analysis process was conducted properly (*tab. 4*). Navelina variety (82,51% ± 5,90%) once again shows a little higher activity compared to the other tested compounds, followed by Vanilla variety (84,84% ± 4,68%).

5 μg/mL	M3-5	M4-5	W-5	V-5	N-5	PRED	LPS	CTRL
	77,35%	93,33%	89,84%	81,43%	82,29%	42,28%	102,90%	13,75%
	96,83%	91,95%	95,23%	89,19%	86,26%	45,15%	90,30%	19,48%
	106,10%	93,16%	113,34%	82,46%	89,88%	34,07%	98,00%	5,87%
	83,32%	90,38%	97,99%	81,66%	80,60%	30,08%	98,00%	9,14%
	84,01%	93,79%	102,99%	82,01%	72,47%	32,79%	90,40%	11,39%
	99,50%	93,79%	85,55%	92,26%	83,54%	76,52%	94,71%	21,76%
	108,39%		82,36%			78,48%	114,89%	19,08%
	104,32%		81,30%			74,20%	105,05%	17,48%
	91,20%					54,87%	93,28%	24,96%
	85,90%					57,89%	101,66%	26,50%
	86,14%					64,82%	103,80%	26,72%
						63,94%	100,95%	23,30%
						64,38%	95,26%	24,33%
						71,61%	118,03%	27,18%
						60,83%	115,44%	
						58,24%	66,52%	
						62,38%		
MEAN	93,01%	92,73%	93,58%	84,84%	82,51%	57,21%	99,32%	19,35%
SD	10,55%	1,34%	11,06%	4,68%	5,90%	15,33%	12,22%	6,95%

Tab. 4

The following histogram gives us a visual aid in visualizing the activity of the tested compounds (*fig. 17*). We can clearly see Prednisolone being the most active compound, which is normal in these conditions being that it serves as a positive control. Navelina is the most active compound, closely followed by Vanilla, but the activity should not be taken in consideration because it is relatively weak. M4 amongst the other compounds shows to be the most accurate tested compound with a standard deviation of 1,34%.

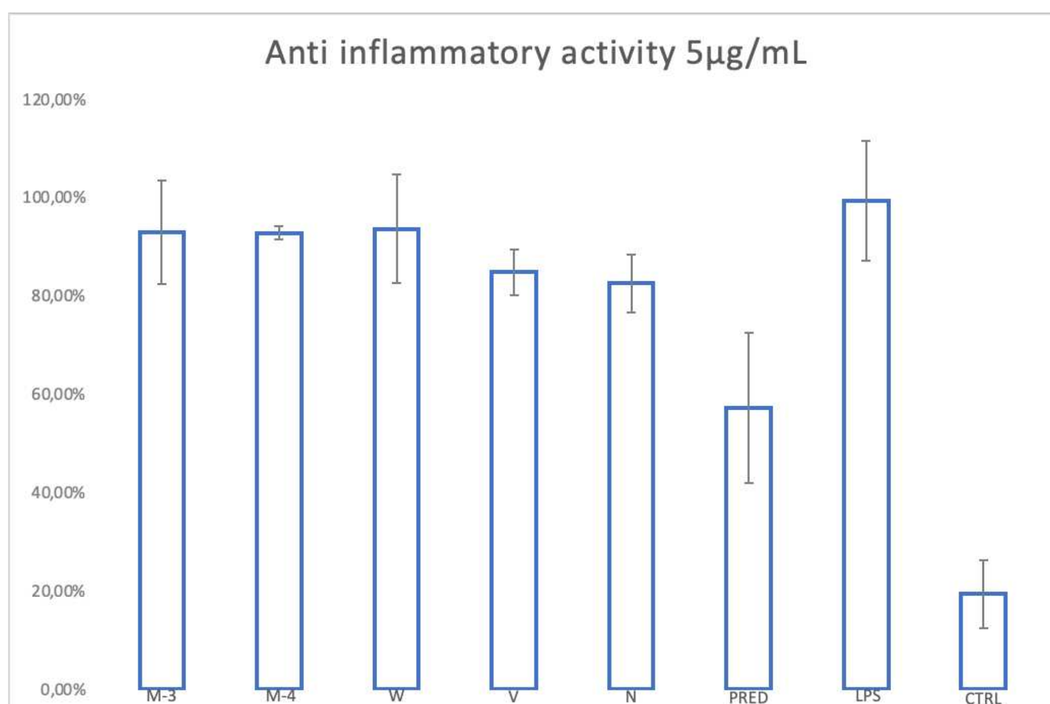


Fig. 17

7.2 CAA

The CAA, like the antioxidant assay, was conducted at concentrations of 20 µg/mL, 10 µg/mL, and 5 µg/mL. For a better comprehension of the results of this assay, Pairwise Comparisons were conducted for each tested compound, both among themselves and in comparison to the negative and positive control (Quercetin), in order to determine their relative effectiveness. Significance values are those with a value of 0,005 or below. Significance values have been also adjusted by the Bonferroni correction for multiple tests. The Bonferroni correction adjusts probability (p) values because of the increased risk of a type I error when making multiple statistical tests [48].

7.2.1 Concentration of 20 µg/mL

From the values in *tab. 5* we can see that, compared to DMSO, only the Washington Navel variety does not exhibit a relative significant value (Significance 0,027 – Adjusted Significance 0,57), while Navelina, Vanilla, M3 and M4 show a relatively good antioxidant activity. Morus 3 and Morus 4 are the only ones to have a significance value below 0,05 compared to Washington Navel, showing a good antioxidant activity, especially M4 with an Adjusted Significance value of 0,002. No compound showed antioxidant activity compared to

Pairwise Comparisons of VAR00001

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.^{a}
DMSO-Washing	-26,889	12,172	-2,209	,027	,571
DMSO-Navel	-49,667	12,172	-4,080	,000	,001
DMSO-Vanilla	-57,333	12,172	-4,710	,000	,000
DMSO-Morus 3	-66,222	12,172	-5,440	,000	,000
DMSO-Morus 4	-74,167	12,172	-6,093	,000	,000
DMSO-QUE	-103,722	12,172	-8,521	,000	,000
Washing-Navel	22,778	12,172	1,871	,061	1,000
Washing-Vanilla	30,444	12,172	2,501	,012	,260
Washing-Morus 3	39,333	12,172	3,231	,001	,026
Washing-Morus 4	47,278	12,172	3,884	,000	,002
Washing-QUE	76,833	12,172	6,312	,000	,000
Navel-Vanilla	-7,667	12,172	-,630	,529	1,000
Navel-Morus 3	16,556	12,172	1,360	,174	1,000
Navel-Morus 4	24,500	12,172	2,013	,044	,927
Navel-QUE	-54,056	12,172	-4,441	,000	,000
Vanilla-Morus 3	8,889	12,172	,730	,465	1,000
Vanilla-Morus 4	16,833	12,172	1,383	,167	1,000
Vanilla-QUE	46,389	12,172	3,811	,000	,003
Morus 3-Morus 4	-7,944	12,172	-,653	,514	1,000
Morus 3-QUE	-37,500	12,172	-3,081	,002	,043
Morus 4-QUE	-29,556	12,172	-2,428	,015	,319

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Tab. 5

Navelina nor Vanilla varieties. M3 and M4 resulted in significance values under 0,05 compared to Quercetin, which serves as positive control, but M4 oddly shows a non-significant value with Bonferroni correction.

Tab. 5 shows the results of each experiment at the concentration of 20 µg/mL. Standard deviation for M3, M4 and Vanilla is a little high, showing a lack of precision in the testing process. M4 seems to be the more active compound, followed by M3. The three variety of *Citrus Sinensis* don't seem to have a good activity, with Vanilla variety being the most active among the three species.

20 µg/mL	Quercetin	Morus 3	Morus 4	Washington	Vanilla	Navelina	DMSO
1st exp	80,517	79,585	80,619	45,273	65,604	52,778	-3,826
	90,345	76,279	80,941	44,643	62,019	52,602	-5,867
	91,118	75,313	81,377	44,974	60,549	49,552	0,140
	89,878	67,198	84,485	42,711	56,735	44,586	-4,178
	90,309	64,359	85,567	44,996	53,867	42,563	-3,513
	90,059	68,506	84,885	44,679	56,831	43,817	-6,130
2nd exp	90,390	35,914	49,320	31,534	32,746	39,492	3,261
	87,341	43,288	55,440	29,636	34,508	39,815	-4,286
	81,067	46,494	41,420	17,121	59,611	41,486	0,459
	82,007	36,835	45,965	30,555	66,290	49,137	-1,971
	79,220	57,190	53,228	33,411	20,186	43,753	-3,473
	82,684	39,752	46,926	33,882	31,818	48,404	-6,528
3rd exp	74,273	49,711	55,890	31,766	44,473	50,218	0,369
	75,071	53,848	53,363	43,675	51,667	54,959	-14,066
	70,929	61,834	51,328	41,694	52,977	52,764	2,909
	75,124	59,094	52,578	43,768	51,062	54,566	-8,295
	72,498	50,943	53,237	40,377	49,859	55,052	6,201
	72,770	55,005	54,420	39,347	50,517	56,362	-6,260
MEAN	81,978	56,731	61,722	38,002	50,073	48,439	-3,058
SD	7,325	13,471	15,907	7,776	12,752	5,645	4,782

Tab. 5

The histogram in *Fig. 18* gives us a visual aid in the comparison of the antioxidant activity of each compound compared to each other and to Quercetin, which is known for being a strong antioxidant.

M4 seems to have the highest activity, followed by M3, Vanilla variety, Navelina variety and finally Washington Navel variety. The *Citrus* fruits don't seem to have a good activity at this concentration.

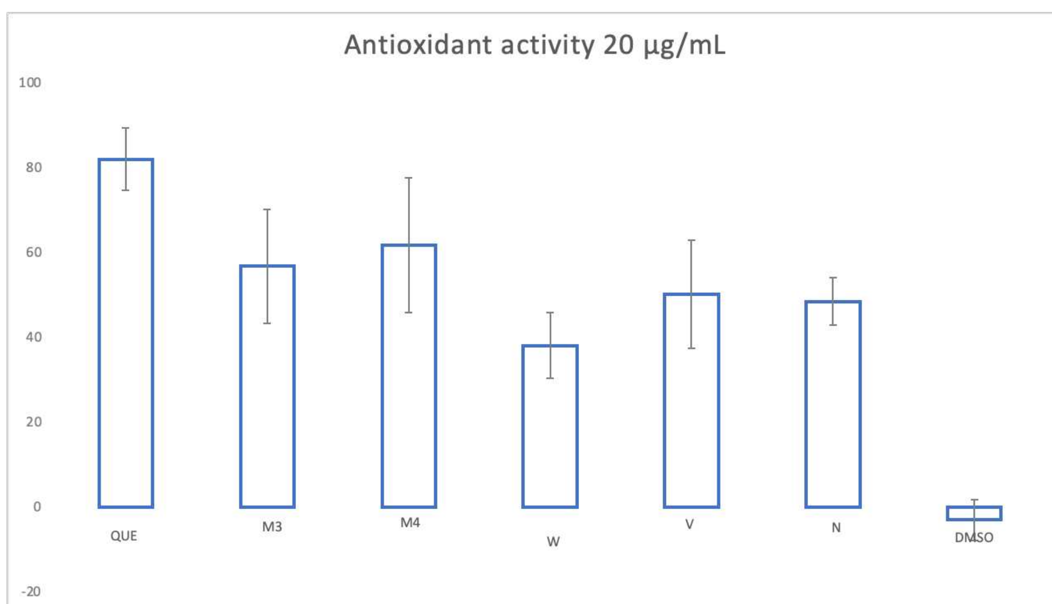


Fig. 18

7.2.2 Concentration of 10 µg/mL

Pairwise comparisons for this concentration shows us there is significance values for each compound compared to DMSO, making them relevant compared to DMSO.

M3, Navelina and Vanilla have an Adjusted Significance value of 1,000 compared to Washington Navel, which cannot be taken in consideration for the study as they result to be non relevant. M4 though is the only one relevant compared to Washington Navel having an adjusted value of 0,049**.**

Pairwise Comparisons of VAR00001

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
DMSO-Washing	-54,250	14,042	-3,864	,000	,002
DMSO-Morus 3	-67,083	14,042	-4,777	,000	,000
DMSO-Navel	-69,292	14,042	-4,935	,000	,000
DMSO-Vanilla	-70,875	14,042	-5,048	,000	,000
DMSO-Morus 4	-97,000	14,042	-6,908	,000	,000
DMSO-QUE	-142,583	14,042	-10,154	,000	,000
Washing-Morus 3	12,833	14,042	,914	,361	1,000
Washing-Navel	15,042	14,042	1,071	,284	1,000
Washing-Vanilla	16,625	14,042	1,184	,236	1,000
Washing-Morus 4	42,750	14,042	3,045	,002	,049
Washing-QUE	88,333	14,042	6,291	,000	,000
Morus 3-Navel	-2,208	14,042	-,157	,875	1,000
Morus 3-Vanilla	-3,792	14,042	-,270	,787	1,000
Morus 3-Morus 4	-29,917	14,042	-2,131	,033	,696
Morus 3-QUE	-75,500	14,042	-5,377	,000	,000
Navel-Vanilla	-1,583	14,042	-,113	,910	1,000
Navel-Morus 4	27,708	14,042	1,973	,048	1,000
Navel-QUE	-73,292	14,042	-5,220	,000	,000
Vanilla-Morus 4	26,125	14,042	1,861	,063	1,000
Vanilla-QUE	71,708	14,042	5,107	,000	,000
Morus 4-QUE	-45,583	14,042	-3,246	,001	,025

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Tab. 6

Moreover, none of the tested compounds have a significance value compared to M3. It is relevant to note though that without the Bonferroni Adjustment, M4 has a value of 0,033 which makes it significant. Adjusted value is 0,696 which exludes M4 from the significant compounds compared to M3.

The following table contains the results of the experiments. Standard deviation is a little high for M3 and M4 and also Navelina and DMSO.

10 µg/mL	QUE	Morus 3	Morus 4	Washingtor	Vanilla	Navel	DMSO
1st exp	87,547	48,860	63,736	57,008	51,030	44,946	2,367
	86,865	95,913	72,626	55,795	46,782	52,802	3,546
	86,506	53,734	60,525	53,513	43,744	51,420	-2,837
	87,976	50,664	64,492	51,547	39,474	59,164	1,907
	88,862	45,101	63,359	53,031	50,369	50,397	0,803
	87,652	47,313	61,063	55,324	47,134	50,299	-5,506
2nd exp	82,043	49,777	65,505	44,415	46,983	56,419	0,012
	83,073	59,546	71,124	44,572	58,529	57,368	8,318
	82,106	58,391	71,156	40,189	53,657	59,584	40,817
	83,926	52,006	74,264	45,915	53,895	58,077	-3,900
	81,861	60,093	75,356	44,038	60,519	60,733	-2,374
	81,961	59,622	72,870	46,606	57,719	61,380	-0,340
3rd exp	84,220	53,270	70,085	54,380	56,209	67,331	-0,734
	85,813	54,673	66,002	50,728	56,744	60,614	1,348
	82,288	53,794	59,021	43,299	54,972	49,135	6,339
	86,538	56,726	66,048	47,991	55,823	60,556	-0,734
	85,813	59,354	63,892	43,782	58,578	58,929	1,348
	82,288	59,929	66,548	47,663	57,106	59,073	6,339
4th exp	78,373	27,854	52,837	52,729	65,480	32,645	-6,803
	91,189	50,450	46,950	59,033	46,527	39,520	6,428
	91,200	46,972	42,267	48,309	60,244	41,713	5,806
	90,589	43,095	37,864	49,599	57,034	41,102	13,469
	90,178	45,122	41,844	53,659	53,528	41,502	-9,955
	87,917	38,344	31,606	53,757	51,809	45,756	32,622
MEAN	85,699	52,942	60,877	49,870	53,495	52,519	4,095
SD	3,480	11,965	12,277	5,054	6,064	8,849	11,355

Tab. 7

From *Fig.19* we can visually see the activity of each compound compared to Quercetin (positive control) and DMSO (negative control). M4 is once again the compound with the major activity amongst the tested ones, followed by Vanilla, M3 and Navelina which have a very similar activity. The least active, once again, is the Washington Navel.

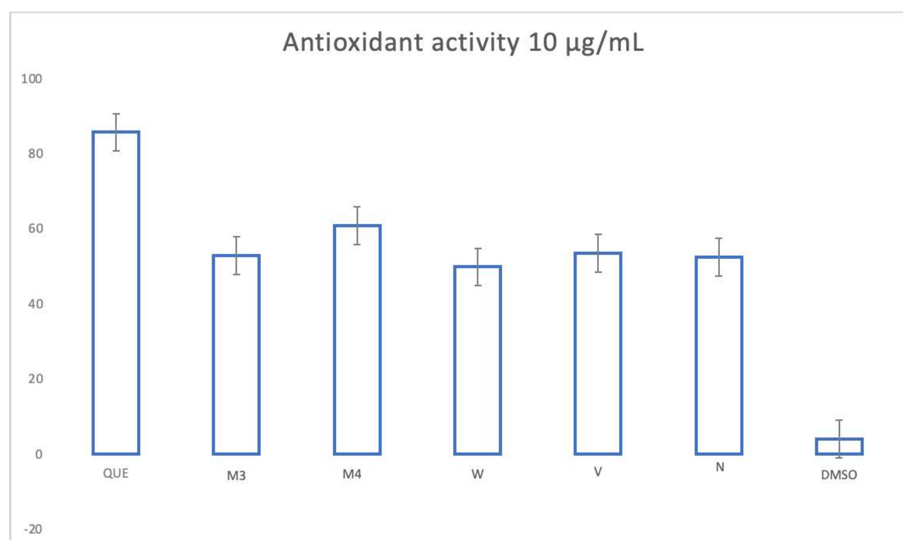


Fig. 19

7.2.3 Concentration of 5 µg/mL

At the lowest concentration at which the compounds were tested we see significance between all the compounds compared to DMSO with values close to 0,000, except for the Washington Navel with an adjusted value of 0,031.

Once again, Washington Navel has shown non-significant values compared to the other tested compounds, with the exception of the positive and negative control.

Pairwise Comparisons of VAR00001

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
DMSO-Washing	-37,528	11,787	-3,184	,001	,031
DMSO-Mor	-47,833	11,787	-4,058	,000	,001
DMSO-Vanilla	-51,333	12,363	-4,152	,000	,001
DMSO-Morus 4	-54,889	11,787	-4,657	,000	,000
DMSO-Navel	-55,324	11,959	-4,626	,000	,000
DMSO-QUE	-100,222	11,787	-8,503	,000	,000
Washing-Mor	10,306	11,787	,874	,382	1,000
Washing-Vanilla	13,806	12,363	1,117	,264	1,000
Washing-Morus 4	17,361	11,787	1,473	,141	1,000
Washing-Navel	17,796	11,959	1,488	,137	1,000
Washing-QUE	62,694	11,787	5,319	,000	,000
Mor-Vanilla	-3,500	12,363	-,283	,777	1,000
Mor-Morus 4	-7,056	11,787	-,599	,549	1,000
Mor-Navel	-7,490	11,959	-,626	,531	1,000
Mor-QUE	-52,389	11,787	-4,445	,000	,000
Vanilla-Morus 4	3,556	12,363	,288	,774	1,000
Vanilla-Navel	3,990	12,527	,319	,750	1,000
Vanilla-QUE	48,889	12,363	3,955	,000	,002
Morus 4-Navel	-,435	11,959	-,036	,971	1,000
Morus 4-QUE	-45,333	11,787	-3,846	,000	,003
Navel-QUE	-44,899	11,959	-3,754	,000	,004

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Tab. 8

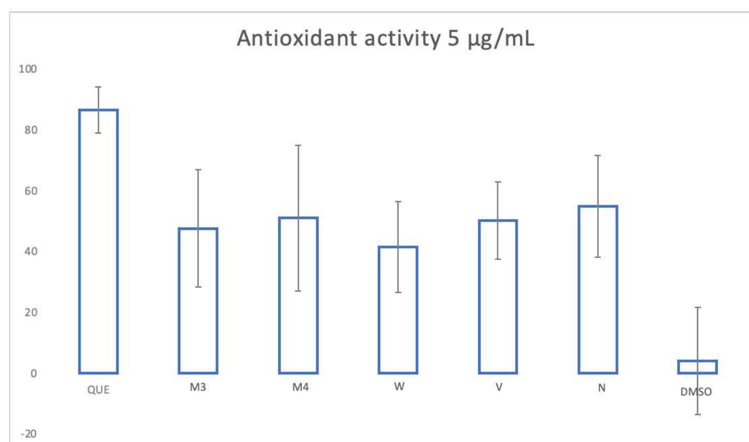
Comparisons between Vanilla variety and the other compounds have shown also non-significant values. To have a better understanding of the values, results should be placed into a histogram.

The following table collects all the data given by the experiments after calculations were made (*tab. 8*). Navelina seems to be the most active extract (54,94% ± 16,83%), closely followed by M4 (51,06 ± 23,91%) and Vanilla variety (50,25% ± 16,83%). The following table collects all the data given by the experiments after calculations were made (*tab. 9*). Navelina seems to be the most active extract (54,94% ± 16,83%), closely followed by M4 (51,06 ± 23,91%) and Vanilla variety (50,25% ± 16,83%). M3 and Washington Navel are the least active. It Is important to notice that the standard deviation is relatively high for all the compounds, which makes it difficult to establish the extracts' activity.

5 µg/mL	QUE	Morus 3	Morus 4	Washingtc	Vanilla	Navel	DMSO
1st exp	89,517	57,981	54,168	63,230	58,859	54,168	1,600
	92,091	67,931	65,076	54,673	57,130	65,076	1,493
	92,559	70,442	71,351	51,674	58,407	71,351	1,828
	93,134	69,367	70,622	52,626	52,195	70,622	-9,420
	94,501	81,790	72,771	50,302	52,998	72,771	-7,712
	94,995	69,415	75,414	48,542	51,334	75,414	-2,272
2nd exp	87,269	55,162	66,608	43,040	49,260	44,646	18,910
	89,669	46,057	68,294	33,617	52,077	42,620	3,934
	88,862	43,860	61,573	50,671	51,924	49,634	-49,098
	88,144	44,701	66,918	64,976	52,175	49,923	14,080
	87,324	43,461	66,200	40,886	67,258	50,237	-8,226
	88,004	53,534	66,773	51,758	53,539	50,671	14,296
3rd exp	74,531	33,197	21,811	30,158	32,419	41,612	31,350
	77,152	23,686	13,404	21,236	51,766	91,145	13,091
	73,397	20,322	22,265	27,769	12,428	38,187	27,411
	94,658	30,506	22,076	19,883		39,533	12,683
	78,505	17,728	17,849	20,170		26,332	8,222
	74,168	28,895	15,937	22,492			-0,095
MEAN	86,582	47,669	51,062	41,539	50,251	54,938	4,004
SD	7,530	19,332	23,913	14,984	12,711	16,830	17,591

Tab. 9 9

As usual, a histogram is necessary to have a better view at the results (*fig. 20*)



8.0 Conclusions

Regarding the NF-kB assay for the evaluation of the anti-inflammatory activity, results revealed that at the concentration of 20 µg/mL the two varieties of *Morus alba*, M3 and M4, exhibited minimal to no activity. The inhibition of NF-kB activity for both varieties was close to 0%. Even when considering the standard deviation, which was 21,47% for M3 and 15,31% for M4, the anti-inflammatory activity did not reach a significant level that could be considered for further experimentation.

On the other hand, the three varieties of *Citrus sinensis* showed a weak activity, with each one of the extracts being close to a 10% inhibition activity of NF-kB (Washington Navel 90,26% ± 21,42%, Vanilla 90,87% ± 18,91%, and Navelina 93,30% ± 14,12%). However, it is important to note that the standard deviation was quite high for these results, so it is difficult to draw a definite conclusion regarding their activity. Further investigations on these extracts should be made to obtain more conclusive and precise results.

At the concentration of 10 µg/mL results seem to be somewhat more promising. The extract that showed a slightly higher activity than the others was Navelina variety (86,92% ± 10,58%). The remaining compounds, anyway, showed similar levels of activity (M4 87,58% ± 13,90% activation of NF-kB, Washington Navel 88,82% ± 11,03%, Vanilla 89,68% ± 12,53% and M3, the least active one, 91,52% ± 13,30%).

The slightly higher levels of activity observed at this concentration could potentially be attributed to improved bioavailability of the active molecules, along with a reduced presence of compounds that reduce the absorption of these active components. However, it is important to note that the results obtained at this concentration should require further investigation, considering the high standard deviation observed in all of the measurements.

At a concentration of 5 µg/mL, the activation of NF-kB is comparable to that observed at a concentration of 10 µg/mL. The results show similar levels of activity, with relatively lower standard deviations, except for Washington Navel (11,06%) and M3 (10,55%). This can be related to the fact that the number of the measurements was lower at this concentration. As previously mentioned, further investigations are necessary to obtain more conclusive findings.

The antioxidant assay at the concentration of 20 µg/mL produced favorable results. Among the tested varieties, M4 demonstrated the highest antioxidant activity (61,72% ± 15,91%), closely followed by M3 (56,73% ± 13,47%). The Vanilla and Navelina varieties also exhibited good antioxidant activity, with percentages of 50,07% ± 12,75% and 48,43% ± 5,65% respectively. The extract from the Washington Navel variety displayed the lowest level of activity compared to the others, yet it can still be considered to possess moderate antioxidant activity. Results are further confirmed by pairwise comparisons, which shows a p value < 0,005 compared to Quercetin (positive control).

At a concentration of 10 µg/mL, M4 once again demonstrated the highest level of activity (60,88% ± 12,28%). The remaining extracts exhibited similar activity compared to the concentration of 20 µg/mL. Interestingly, the Washington Navel extract showed a noticeable increase in its antioxidant activity, rising from 38,00% ± 7,78% to 49,87% ± 5,05%.

Finally, at a concentration of 5 µg/mL, a slight decrease in antioxidant activity was observed, with the highest activity showed by Navelina (54,94% ± 16,83%), closely followed by Vanilla, M4 and M3. The Washington Navel extract displayed the lowest level of activity, with a value of 41,54% ± 14,99%. Standard deviation is quite high, but the activity of all the compounds is to be considered good.

Both antioxidant and anti-inflammatory assays revealed the lowest activity among the tested extracts at the concentration of 20 µg/mL. While the NF-kB assay showed almost no activity at all for any of the extracts, the CAA demonstrated a good activity, especially for the Morus extracts. It is worth noting that the concentration of 10 µg/mL seems to be the most appropriate concentration for the extracts to display their activity. At this concentration the antioxidant assay exhibited the highest activity for all the compounds, while the anti-inflammatory assay showed promising results for further investigations. Finally, at 5 µg/mL, there were minimal differences compared to the 10 µg/mL concentration, with some extracts having a slightly higher activity and some a slightly lower one, both for antioxidant and anti-inflammatory activity.

Overall, *Morus alba* extracts showed a good antioxidant activity across all tested concentrations. This can be explained with the high quantity of anthocyanins which have been proven to have a good antioxidant activity. On the other hand, the anti-inflammatory activity could not be proven by this study.

Citrus sinensis extracts showed a good antioxidant activity for all the concentration, especially at 10 µg/mL and 5 µg/mL. This can be explained by the significant presence of flavones such as Eriocitrin, as well as other established antioxidant compounds like Narirutin. While the study did not establish definitive evidence of anti-inflammatory activity, the results at 10 µg/mL and 5 µg/mL provide a promising basis for further investigations in this area.

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