POLYPHENOL LOADED NANOPARTICLES: A COLON-TARGETED DELIVERY SYSTEM TO ENHANCE STABILITY AND ANTIMICROBIAL PROPERTIES OF BARBERRY (*BERBERIS VULGARIS)* FRUIT EXTRACT

A Dissertation

by

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ABSTRACT

The antibacterial activity against pathogenic bacteria and stimulatory effect of barberry polyphenols nanoparticle (NP) with Pluronic® F127 copolymer on the survival and growth rate of probiotic bacteria through an *in vitro* gastrointestinal tract (GIT) and colonic fermentation models were investigated. Antibacterial activity of free polyphenolic extract showed greater antibacterial activity against Gram-positive bacteria of *Listeria monocytogenes*, *Enterococcus faecium*, and *Staphylococcus aureus* compared to that of Gram-negative bacteria of *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa* ranging from 0.3125 mg/mL to 10 mg/mL. The antibacterial activity of the treatments was dose-dependent. The susceptibility of Gram-positive microorganisms was the order of *S. aureus* > *E. faecium* > *L. monocytogenes* and Gram-negative bacteria in order of *P. aeruginosa* > *S.* Typhimurium > *E. coli* O157:H7.

The stability of barberry extracts was also evaluated in a simulated gastrointestinal digestion model that mimicked oral, gastric, small intestinal, and colon conditions through manipulation of digestive enzymes and pH values at 6.8, 2.5, 5.8, and 6.8, respectively at 3 min, 2 h, and 2 h at 37 °C. The effects of the free treatments on the growth of probiotic bacteria including *Lactobacillus* spp, *Bifidobacterium* spp., co-culture, and *E. coli* O9:H4 strain HS were then investigated at concentrations ranging of 240 mg/L, 480 mg/L, 720 m/L, 960 mg/L, and 1200 mg/L at 37 ºC for 48 h. The concentration of 240 mg/L was found to optimally promote bacterial growth by 88% when co-culture bacteria were treated with free extract, whereas the growth values were up to 82% for *Lactobacillus* spp. and *Bifidobacterium* spp. However, the concentration of 720 mg/L was the most effective concentration among other concentrations that stimulated the growth of *E. coli* HS by 43%.

DEDICATION

A special thanks to my husband, Mehdi Hashemi, for his support, patience, and love during my journey in graduate school.

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Contributors

This work was supervised by a dissertation committee consisting of Professors Stephen Talcott (Advisor), Gary Acuff (Home Department), Mustafa Akbulut (Outside Department), and Susanne Talcott (Home Department).

All the work conducted for the dissertation was completed by Helen Hashemi (Student) independently.

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CHAPTER I

INTRODUCTION

Barberry (*Berberis vulgaris*) belongs to *Berberidaceae* family and is a native shrub that has been cultivated in the Middle East and Europe for up to two centuries. Barberry is grown as a food and food ingredient, and all parts of the plant have been shown bioactive properties including antifungal, antibacterial, antiemetic, antipyretic, and anticancer activities (Hosseini et al., 2016; Motalleb et al., 2005; Rahimi-Madiseh et al. 2015). An increasingly ethnically diverse population in the United States is a potential driving trend toward greater consumption of barberry, ingredients made from barberry, and dried fruits can be found in most international stores and in the Asian section of national food retailers across the country. Barberry is especially popular in Europe and the Middle East but is less known in other parts of the world despite its abundance and diversity of phytochemicals and its sour-sweet and pleasing taste.

The characterization and quantification of polyphenolics in food products is highly relevant as they may serve as a fingerprint for botanical authentication and provide indications of stability, functional properties, and potential health benefits. Polyphenolic compounds are secondary plant and algal metabolites, responsible for flavor, color, and consumer perception of a food product [1], and may provide a variety of health-related and functional properties as antibacterial, antioxidant, anti-inflammatory, and anticancer properties. Specially, CGA was shown to possess antibacterial properties due to its ability to increase plasma membrane permeability and cause leakage of cytoplasmic content and nucleotides of pathogenic bacteria [2-5].

Pluronic® F127, known as poloxamer 407, is the tradename of an ionic copolymer with polar compatibility with compounds. It can form into micelles with hydrophobic Poly propylene oxide (PPO) center core and hydrophilic Polyethylene oxide (PEO) outer shells that interact with water [6]. Nanoparticle (NP) barberry polyphenols rich in chlorogenic acid with Pluronic® F127 polymer may promote the delivery of polyphenols to the bacteria cell due to an optimal size and charge of the molecule that has penetrated through bacteria cell walls resulting in reactive oxygen species (ROS) production to finally damage DNA, protein, and lipid within bacteria cell structures.

Polyphenols (PCs) can act as substrates to increase the survival and functional properties of intestinal microbiota [7], change their composition through an inhibitory effect on pathogenic bacteria and enrichment of beneficial bacteria. However, there is insufficient knowledge concerning the effect of polyphenols loaded NPs on probiotic bacteria in an *in vitro* environment to promote cell viability and growth rate. It is possible that polymeric encapsulating could improve the antibacterial properties against pathogenic bacteria and promote the growth rate of probiotic bacteria *in vitro* GIT and colonic fermentation.

Studies have investigated antibacterial effect of barberry polyphenols NP with Pluronic® F127 against pathogenic bacteria and the possible stimulatory effects on survival and growth rate of probiotic bacteria through *in vitro* GIT and colonic fermentation models. To understand the mode of action of NP barberry polyphenols will give insight into the potential antibacterial properties of NPs on the bacterial cell wall leading to altering the bacterial cell wall integrity and morphology. An increased growth and survival rate of gut probiotic bacteria is expected since it has been hypothesized that encapsulation methods may improve the stability of polyphenols in different pH conditions of a food and also through the oral, gastric, intestinal, and colonic conditions of digestion. It may be concluded that these benefits will result increased availability

of polyphenolic for probiotic bacteria in the colon resulting in a higher concentration of metabolites compared to that of free compounds.

The objectives of this study were to:

1. Assess the efficacy of free barberry polyphenolic extracts using different solvents on viability of Gram-positive and Gram-negative pathogenic bacteria.
2. Evaluate barberry polyphenolic extract loaded Pluronic® F127 in an *in vitro* antibacterial model tested against pathogenic bacteria strains.
3. Assess the stability and dissolution of free and nanoparticle polyphenolic extracts in an *in vitro* gastrointestinal digestive model and evaluate the bio-efficacy of the phytochemicals to promote the growth of probiotic strains in a colonic fermentation model.

CHAPTER II

LITERATURE REVIEW

Barberry

Barberry is a member of *Berberidaceae,* includes up to 500 species and is supplied by Middle Eastern countries, especially Iran and Turkey. The barberry plant can tolerate harsh growing environment conditions including poor soil quality [8]. Barberry fruits are red in color with a sour taste and can be used in the production of jam, jellies, marmalade, tea and juices [2-5]. Fruit of the barberry plant has gained attention as a natural food additive and is grown for medicinal purposes, since all parts of the plant (roots, barks, and fruits) contain polyphenolics such as phenolic acids, flavonoids, anthocyanins, and alkaloids that have been shown to have antifungal, antibacterial, and anticancer properties.

The Global History of Barberry

In the early 1600s, it was brought to North America by early settlers from Europe, where was known for its medicinal purposes. Over time, it adapted well climates in the Midwestern US in 1700s, and was then moved west by farmers in the early 1800s. However, barberry was an alternative host for a fungi causing stem rust in grain cereals, laws were passed in 1916 to protect wheat-producing states especially in the Midwest area of the US. The barberry eradication programs that followed where not phased out until the late 1970's. In today's markets, barberry production is almost exclusively in the eastern part of United States in addition to the Middle East and southern Europe. In Minnesota and Wisconsin, barberry is cultivated in wooden area and hard wood stands. In Pennsylvania, population occurred in stream bank thickets, along roadsides [9].

The Potential Role of Fruits and Vegetables in Health

Fruits and vegetables are widely recognized for their potential health benefit. Regions such as the Middle East and Mediterranean countries generally follow a diet that is rich in fruits and vegetables as part of their traditional cuisine [10]. The Dietary Guidelines for American 2010 recommended that amount of fruits in the Healthy U.S. Style Pattern is 2 cup-equivalents per day at the 2000 calorie level [11].

A diet rich in fruits and vegetables may provide about 1 g of total polyphenols/day [12]. By this association, increasing the daily consumption of fruit and vegetables might lower, the risk of many chronic diseases [13] that are associated with the benefits of polyphenolic consumption such as cancer [14]. The consumption of certain polyphenolics is associated with beneficial changes in the intestinal microbiota such as link between quercetin and rutin that increased the number of *Eubacterium ramulus* along with *Bifidobacterium* spp. and *Lactobacillus* spp. populations [15].

Polyphenolics: Synthesis Nomenclature and Properties

Plant Metabolism, Chemical Structure, and Physical Properties

Polyphenol compounds are a group of small molecules having at least one phenol group which are commonly found in all fruits, herbs, vegetables, seaweed, and some algae. They also are a class of phytochemicals and are known for their potential roles associated with human health [16]. Phenolic compounds can also be classified into different subgroups and functioned based on number of phenol rings such as phenolic acids (hydroxycinnamic and hydroxybenzoic acids), tannins, lignans, flavonoids, stilbenes (resveratrol), coumarins, curcuminoids [17] (**Fig. 1**). On the

other hand, flavonoids are subclassified as flavones, isoflavones, flavonols, flavan-3-ols, flavanones, and anthocyanins. **Fig. 2** also illustrates the parent structure of polyphenols.

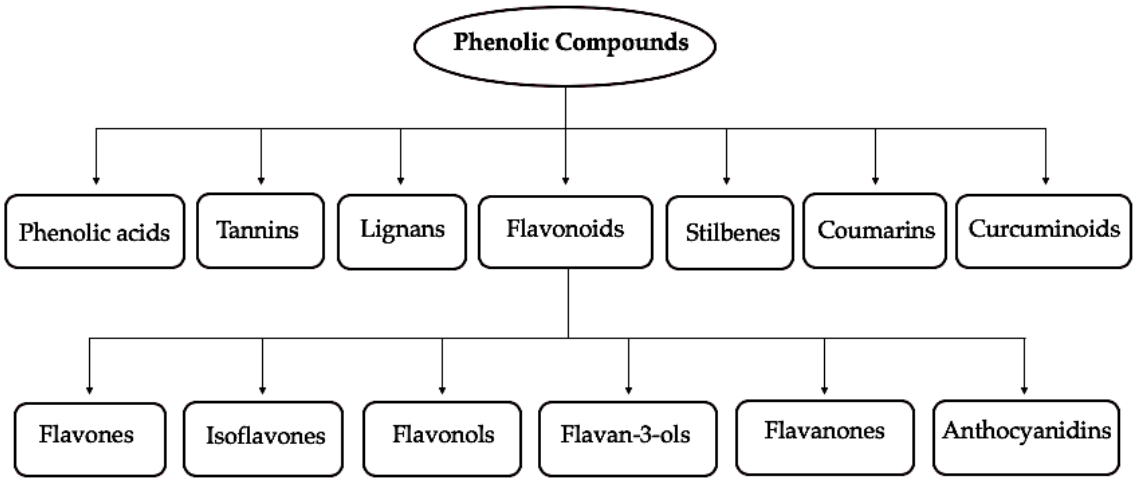


Figure 1. Polyphenols family tree.

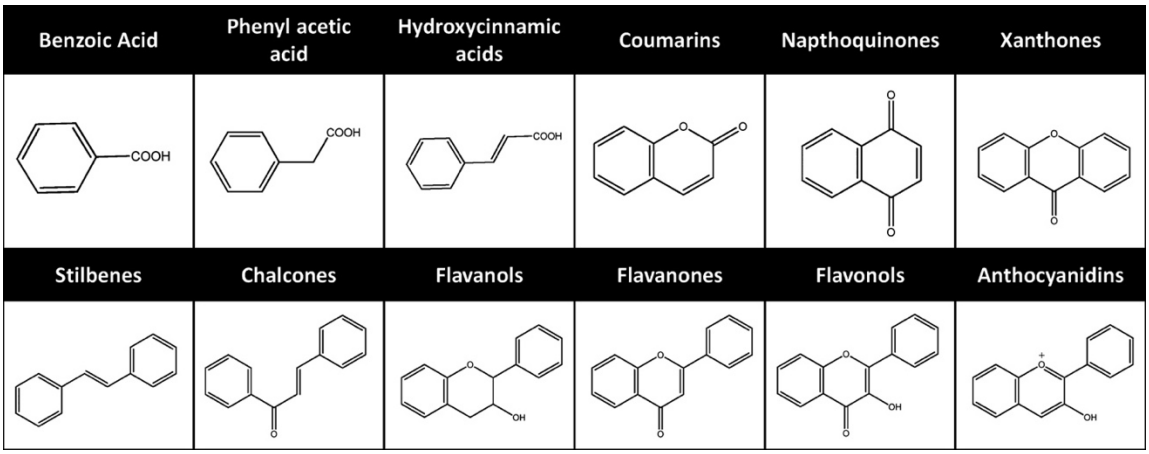


Figure 2. Polyphenols parent structure- Adopted from Soto-Vaca et al., 2012 [18].

Phenolic Acids

Phenolic acids including benzoic and cinnamic acid are two of the most important representatives of non-flavonoid compounds. Gallic acid (3, 4, 5-trihydroxybenzoic acid, caffeic acid (3,4-dihydroxy-cinnamic acid), chlorogenic acid (ester formed from caffeic acid and quinic), *p*-coumaric acid (4-hydroxycinnamic acid), cinnamic acid (trans-3-phenylacrylic acid) are the predominate phenolic acids in most of the fruits especially berries [19-20]. Among fruits, dark plum, cherry, citrus fruits, red grape, and some apple varieties have the highest phenolic acid content [21]. The amount of phenolic acids in barberry extract has been reported as 94% of the total polyphenolic compounds with chlorogenic acid being identified as predominant phenolic acid with a value of 78% [22].

Flavonoids

A large group of polyphenols classified as flavonoids are synthesized by phenyl-propanoid pathway to function as antioxidant, antibacterial, anticancer agents. Flavonoids are well known for their antioxidant activity by scavenging free radical due to having functional hydroxyl groups. Flavonoids usually occur naturally as glycosides and are composed of a fifteen-carbon skeleton with two aromatic rings (A and B) linked by a heterocyclic pyrane ring (C) have a higher antioxidant activity on the number and free OH group position [23-24]. Flavonoids can be subdivided into flavonols, flavones, isoflavones, anthocyanins, flavanols, and flavanones (**Fig. 3**). Almost all of the flavonoids have a capability to act as an antioxidant, but flavones and catechins protect the body more against Reactive Oxygen Species (ROS). ROS may lead to lipid peroxidation and interfere with cellular membrane which further damages the cells by shifting the net charge of the cell, resulting in changing the osmotic pressure, and leading to cell death eventually [25-26]. Nijuveldt

et al. (2001) also indicated that mechanism of antioxidant action of flavonoid can be through suppression of ROS formation, scavenging ROS, and upregulation of antioxidant defense. Flavonoids are able to suppress ROS formation by inhibiting the enzymes that generate ROS including microsomal monooxygenase, glutathione, S-transferase, mitochondrial succinoxidase, and NADH.

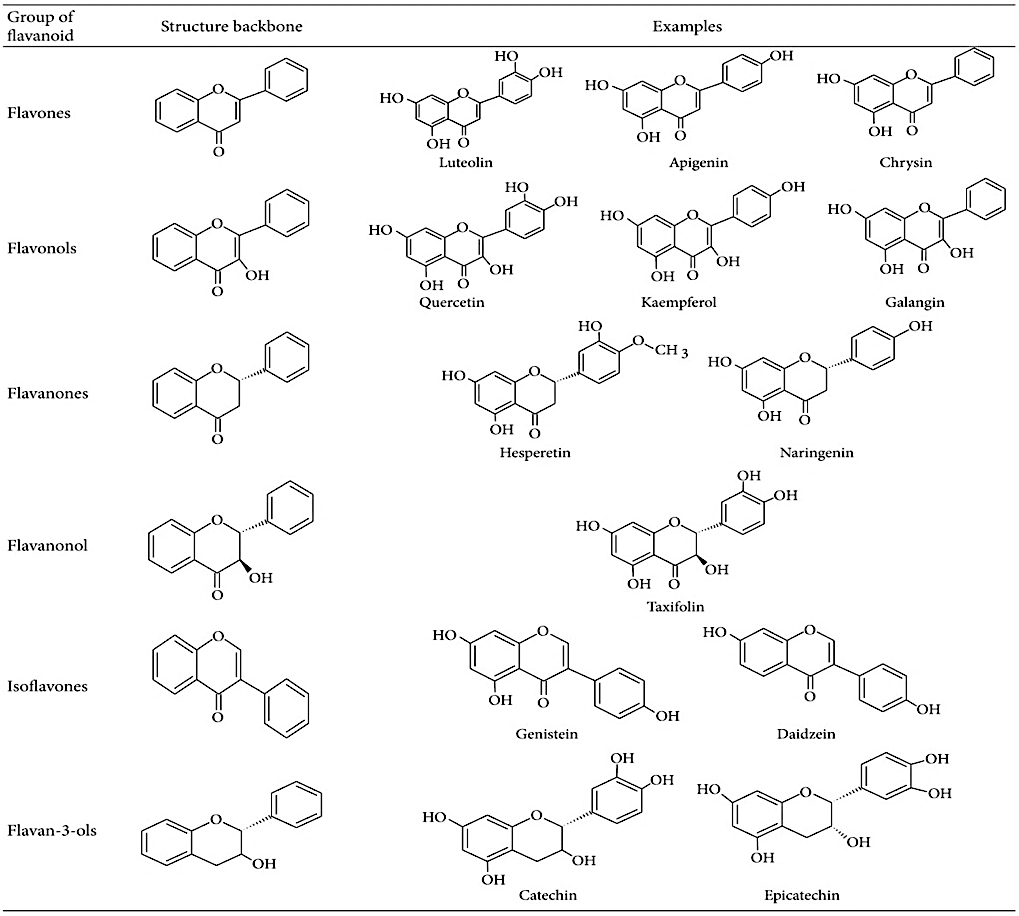


Figure 3. Flavonoid structures- Adopted from S. Kumar et al., 2013 [23].

Anthocyanins (ANCs)

Anthocyanin are described as a bioactive compound within fruits and vegetables with a structure of C_6-C_3-C_6 that may function as an important factor in preventing cardiovascular disease, inflammatory, cancer, and diabetes [27]. Anthocyanins are also colored pigments and can be found in fruits and flowers and are derived from flavonols which has a flavylium structure ion due to a shortage of its ketone oxygen at the 4-position [28]. The 6 major plant anthocyanins have shown in **Fig. 4** including cyanidin (reddish-purple), delphinidin (blue-reddish), pelargonidin (reddish), peonidin (reddish-purple), malvidin (bluish-purple), and petunidin (reddish) pigments are the most common anthocyanidin in edible plants [28]. However, the stability of anthocyanins is related to pigment type, light, temperature, pH, metal ion, and antioxidant. B-ring in anthocyanins and presence of hydroxyl or methyl groups can also decrease the stability of anthocyanidin [28]. Since the structure of anthocyanins has an ionic nature, anthocyanins color depends on the pH of the solution in which they are appeared. They are red color in an acidic solution and turn to blue in a higher pH, due to presence of predominantly flavylium cation [29].

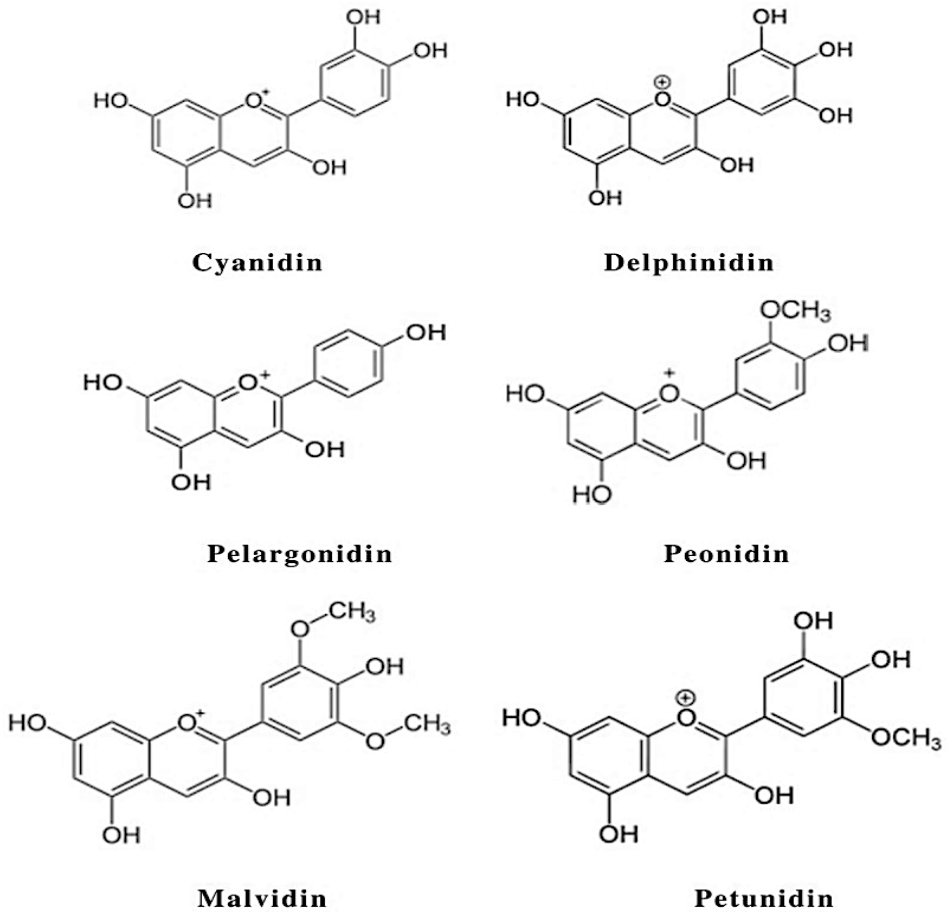


Figure 4. Major plant anthocyanidins- Adopted from H. E. Khoo et al., 2017 [28].

Barberry Compositions

The Physicochemical Characterization of Barberry Fruits

The barberry plant is a shrub that grows to a height of 1-3 m and nearly 2 m in width with fragile, fruit-bearing branches. Yellow flowers appear in May and June with up to 1.30 cm long and develop small fruits of 7-10 mm long and 5-9 mm in diameter that are ripen in 170 days from blossom. The reddish colored fruits have a sour taste and are rich source of vitamin C [30-31] and develop in cluster with a single plant reducing up to 10 kg of fruit per year. The fruits may be

consumed fresh, processing into juice or puree, but are often dried whole, along with their seeds. Barberry fruits are later used in rice dishes or processed into jam and juice, although its major products are tea and drinks made from brewed fruits [32]. Polyphenolic compounds present in barberry, particularly anthocyanins and flavonoids, are secondary metabolites that contribute to the color and consumer perception of the food product [1].

Barberry fruits are reported to contain 70.1% water, and the fruit dimensions was reported on average as 11.3 mm long, 4.24 mm wide, and 4.22 mm thick [33]. Average fruit weight was also determined between 0.051-0.348 g. In moisture content of 89.2%, the length of dried fruit has been reported in 12.01 mm, 5.81 mm in width, with a thickness of 5.80 mm and a volume of 212 mm^3 [34]. The physical properties of barberry fruits have reported by Akbulut et al. (2007) as following a thickness of 3.5 ± 0.07 mm, width of 3.3 ± 0.05 mm, a length of 7.7 ± 0.06 mm, with a volume of 71.0 ± 8.0 mm^3. Nutrient content reported by Ardestani et al. (2015), is as follows 0.73 ± 0.00% ash, 0.60 ± 0.06% fat, 2.6 ± 0.14% fiber, 0.12 ± 0.02% protein, 6.7 ± 0.28% reducing sugar, 9.5 ± 0.07% total sugar, 3.1 ± 0.00 pH, and 75 ± 0.78% moisture content. Study has also reported the energy of 69 kcal/g, reducing sugar 6.5%, protein 10%, cellulose 9.5%, oil 0.84%, ash 1.1%, acidity 3.1%, ascorbic acid 256 mg/kg, total phenolic 789 mg/100g, total anthocyanin 931 mg/kg, and soluble solid 19.4% respectively [35].

Barberry Polyphenols

Phenolic acids, flavonoids, anthocyanin, and alkaloid are predominant bioactive compounds in barberry fruits [36]. The presence of alkaloids, tannins, carotenoid, oleoresin, vitamin C, protein, lipid, anthocyanin and phenolic compounds in barberry have also been reported [37]. Barberry extract contains flavonoids including quercetin glycoside, chrysanthamine, hyperoside,

dolphinidin-3-*O*-beta-D-glucoside, pelargonidin, petunidin-3-*O*-beta-D-glucoside, alpha-tocopherol, and beta-carotene [30]. Barberry consists of monomeric anthocyanins due to lower intensity of red pigments. The red and edible berries of barberry contain Pelargonidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, and petunidin-3-*O*-glucoside [38]. Also, the phenolic compounds within barberry fruit extract are as follows:

Table 1. Concentrations of barberry phenolic compounds- Adopted from N. Gholizadeh-Moghadam et al., 2019 [20] and A. Eroğlu et al., 2020 [36].

Compound name	**Type of phenolic compounds**	**Concentration**	**References**
Gallic acid	Hydroxybenzoic acid	335 mg/L	[20]
p-coumaric acid	Hydroxycinnamic acid	257 mg/L	[20]
Chlorogenic acid	Hydroxycinnamic acid	120-1990 mg/L	[20, 36]
Caffeic acid	Hydroxycinnamic acid	52-152mg/L	[20, 36]
Quercetin	Flavonol	37.2 mg/L	[20]
Cinnamic acid	Hydroxycinnamic acid	0.57 mg/L	[20]
Rutin	Flavonoid	7.6 mg/L	[20]
Apigenin	Flavone	4.4 mg/L	[20]
Vanillic acid	Hydroxybenzoic acid	3-68 mg/mL	[36]
Syringic acid	Hydroxybenzoic acid	13-867 mg/mL	[36]
Ferulic acid	Hydroxycinnamic acid	0.14-39 mg/mL	[36]
Sinapic acid	Hydroxycinnamic acid	136-207 mg/mL	[36]

Chlorogenic (CGA) and caffeic acids (CA) are the predominant phenolic acids and quercetin glycosides are the predominant flavonols. The barberry fruits also contain organic acids including chelidonic acid, malic acid, citric acid, tartaric acid, succinic acid [19, 39]. Malic acid has been reported as the dominant organic acid in barberry fruit [40]. The phenolic compounds of barberry are identified as following: chlorogenic acid, gallic acid, caffeic acid, syringic acid, ferulic acid, *p*-coumaric, *o*-coumaric, protocatechuic acid, vanillic acid, catechin, rutin, and quercetin [19].

Barberry Alkaloids

Alkaloids are a class of naturally occurring nitrogen containing compounds which contain at least one nitrogen atom [41]. All barberry organs such as bark, root, leaf, and fruit contain alkaloids including berberine (BBR), berbamine, palmatine, etc. [37]. Berberine hydrochloride, the more commonly available salt form of berberine, is a quaternary ammonium isoquinoline alkaloid with the chemical formula $C_{20}H_{18}C_{L}NO_4$, belongs to the class of protoberberine alkaloids [42] . The genus *berberis* belongs to Berberidaceae with more than 500 species, including *Berberis vulgaris*, *Berberis asiatica*, *Berberis aristata*, *B. chitria*, *B. lyceum*, and *Coptidis rhizome* [43]. Berberine also presents in plants of Papaveraceae and Ranunculaceae [44]. Plants containing berberine have been used in many prescriptions to treat hypertension, diarrhea, stomatitis, and hepatitis as an antioxidant, antibacterial, anti-inflammatory, hypoglycemic, hypotensive, and hypolipidemic [45]. The antibacterial property of BBR may be due to its potential inhibitory effect on enzymatic and endotoxic activities of bacterial.

Barberry and Chlorogenic Acid

CGA is the predominant polyphenolic in barberry fruits with a value of 120-1990 mg/L [20, 36]. Berries, apples, plums, pears and vegetables such as sweet potatoes, potatoes, blueberries, carrot, spinach, lettuce, coffee beans, tea are a good source of CGA [46]. CGA composed of *trans*-cinnamic acids such as caffeic or ferulic linked to quinic acid via an ester bond. CGA can be divided to caffeoylquinic acids (1-CQA, 3-CQA, 4-CQA, and 5-CQA), feruloylquinic acids (1-FQA, 3-FQA, 4-FQA, and 5-FQA), diacylquinic acids (1,3-diCQA, 1,5-diCQA, 3,5- diCQA, and CFQA), other acylquinic acids (*p*-coumaroylquinic acid (*p*-CoQA), *m*-coumaroylquinic acid (*m*-CoQA), and 3′,4′-dimethoxycinnamic acid (DQA), and lactones (feruloylquinic lactone (FQL), caffeoylquinic lactone (CQL), and *p*-coumaroylquinic lactone (*p*-CoQL)) [47]. There are three most common isomers of CGA including mono-caffeoylquinic acid also known as 5-*O*- caffeoylquinic (5-CQA), neochlorogenic acid also reported as 3-*O*-caffeoylquinic acid (3-CQA), and cryptochlorogenic acid also suggested as 4-*O*- caffeoylquinic acid (4-CQA). The most abundant of CGA isomers is 5-caffeoylquinic acid (5-CQA) [48].

CGAs are synthesized by esterification of a C6-C3 trans- hydroxycinnamic acids with 1_L-(—)-quinic acid, *p*-coumaric, caffeic, ferulic, and sinapic acids (**Fig. 5**). Most common natural form of quinic acid is described as 1_L-1(OH), 3,4/5-tetrahydroxycyclohexanecarboxylic acid named (—)-quinic acid or $_L$-quinic acid [49]. Glucose initiates the biosynthesis of CGA compound by shikimic acid, *p*-coumaroyl shikimic acid is then hydroxylated to caffeoyl shikimic acid, and finally caffeoyl shikimic acid is converted to caffeic acid or to caffeoyl-CoA. Caffeic acid can then be formed to CGA after trans-esterification with quinic acid. Therefore, CGA is an ester form of either caffeic or ferulic acids with quinic acid [50].

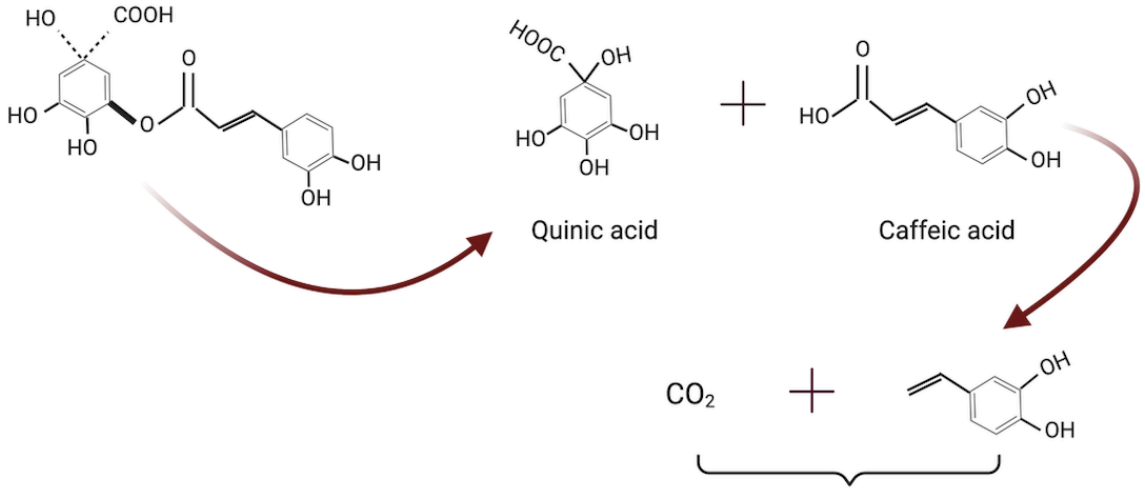


Figure 5. Structure of chlorogenic acid- Adopted from R. Bel-Rhlid et al., 2013 [51].

Polyphenols and Human Food Pathogenic Prevention

Several disease-causing bacteria can contaminate foods, they also called foodborne or food poisoning. More than 250 foodborne disease have been identified by researchers [52]. Foodborne illnesses always caused by pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, and *Salmonella enterica*. Nausea, vomiting, fever, diarrhea, and sometimes blood in the stools are common symptoms of foodborne illnesses [53]. The food poisoning and it happens when individuals consume a contaminated food which has already been contaminated with either microbial pathogen or their toxin, parasites, and chemicals [54]. While American food supplies may be among the safest the world, 48 million foodborne illnesses are reported every year [54].

Antibacterial Activity of Polyphenols

In many developing countries, different parts of medicinal plants including fruits, roots, and leaves have been used in traditional medicine [55] and evidence showed compounds synthesized as part of the secondary metabolism of the plants such as polyphenols play a crucial role as antimicrobial agents against foodborne pathogens [56]. Phenolic acids are non-flavonoid polyphenolic compounds such as CGA, gallic acid (GA), ferulic acid, and caffeic acid. Gallic and caffeic acids were demonstrated to have antimicrobial activity against *S. aureus*, both compounds inhibited the production of α-hemolysin. The antimicrobial mechanism of effect of GA was reported to be through interfering the adhesion properties of bacteria such as *S. aureus* while caffeic acid influences the stability of the bacterial membrane [57]. However, most studies on the mechanism of action of plant-based antimicrobial such as fruits, spices, and essential oils have indicated the effect of those compounds on the cytoplasmic membrane of targeted microorganism [58-59].

Chemical preservatives are approved food antibacterial compounds that are naturally present in food or directly added to inhibit growth of bacteria [60]. Various synthetic food additives including organic acids, benzoate, propionate, sorbate, nitrate, nitrite, and sulfate have also been used to control pathogenic and spoilage bacteria [61]. It has been hypothesized that long term consumption of synthetic additives may cause many allergic reactions, liver damage, and even cancer [62]. For example, vitamin B1 (thiamine) in food can be degraded using sulfites [63]. Due to consumer demand for safe, high-quality foods but free of artificial ingredients, naturally occurring antimicrobials like flavonoids, tannins, sesquiterpenes, lactones, terpenoids, and alkaloids may be the good substitutes [64-65]. The benefits associated with the consumption of natural antibacterial compounds have been suggested to be inexpensive, non-toxic, and active at low concentration in

a natural form [66]. The antimicrobial effect of plant extracts can be varied by plant species, polyphenol chemical composition, the type of solvent, and extraction method. For example, cinnamon containing 3.6% epicatechin, 23.2% proanthocyanidins, and 64.1% cinnamaldehydes can inhibit the growth of *B. cereus* at a concentration of 625 µg/mL [67]. Also, red grape juice rich in resveratrol, ellagic acid, quercetin, catechin, and epicatechin demonstrated an inhibitory effect on *E. coli* by increasing the membrane permeability of bacteria cell wall [68].

Gram-positive bacteria have a thick layer of peptidoglycan in their cell wall, whereas Gram-negative bacteria have a thin peptidoglycan layer with an additional outer membrane (OM) of lipopolysaccharide (LPS) and lipoprotein (LP) molecules with negative charges **(Fig. 6)**. Silva et al. (2017) reported that Gram-positive bacteria are more susceptible to phenolic compounds due to their cell wall structure. Generally, the mechanism of action for polyphenols such as phenolic acids and flavonoids is to degrade bacteria cell walls and the cytoplasmic membrane due to the presence of –OH groups that incorporate into microorganism cell wall membrane, leading to a change in permeability. Polyphenols can inactivate the intracellular enzyme, which is vital for metabolic processes, and inhibit the synthesis of DNA, RNA, polysaccharide, enzyme, and protein. [69]. The inhibitory effect of phenolic acids may be related to the position and number of hydroxyl groups on the phenol ring, resulting in disruption of membrane integrity and leading to a leakage of intracellular constituents [70]. Thus, the inhibitory effect of flavonoids on foodborne bacteria can be attributed to inhibiting energy and DNA synthesis further interfering with protein and RNA synthesis due to hydroxylation at position 2 that is important for antibacterial activity in open-chain flavonoids (chalcones) [71]. However, antibacterial activity of flavonoids depends on the number and position of –OH groups, their backbone structure, and the presence of glycosidic linkages [72].

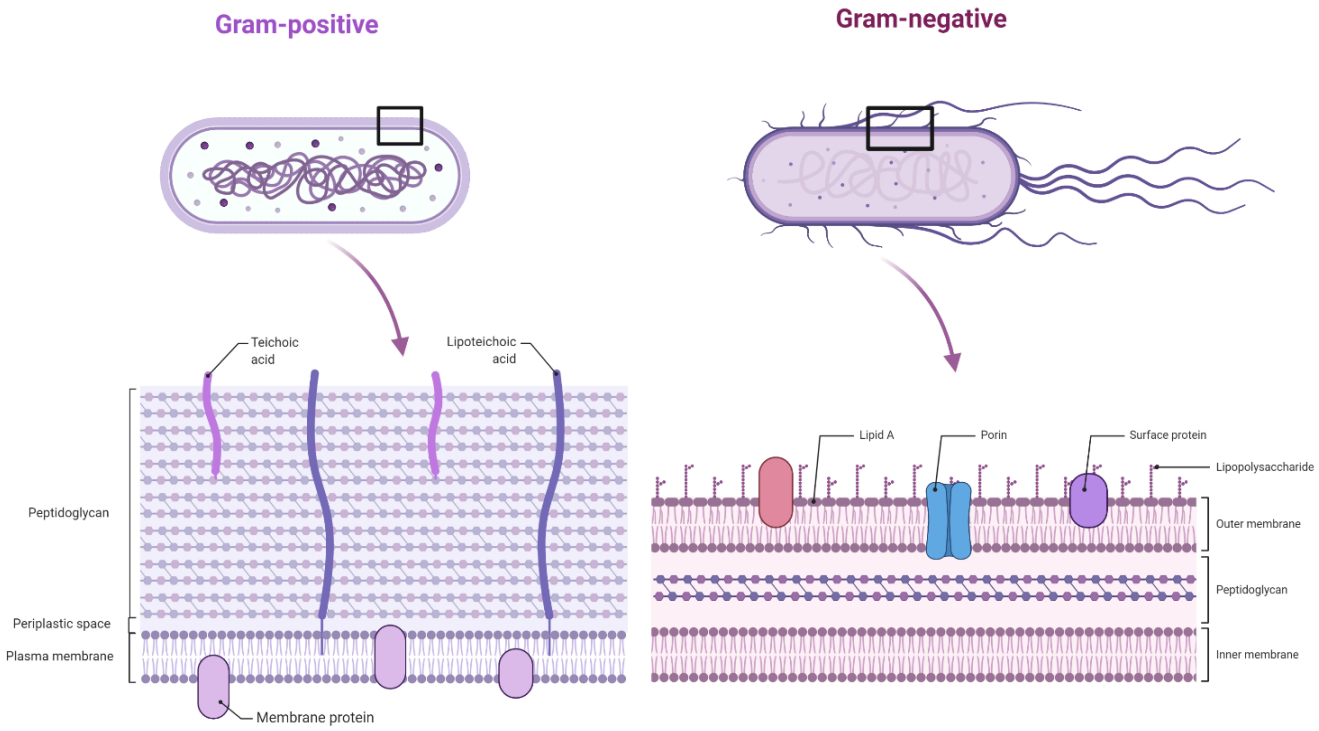


Figure 6. The mechanism of action of natural compound in a bacterium, created with BioRender.com (2021).

Antibacterial Activity of Barberry Extract Rich In CGA

Chlorogenic acid is among the most common cinnamic acid-derived polyphenol in the higher plants, and its use as an antimicrobial agent is widely reported in the literature. CGA was shown to have antibacterial properties at concentrations ranging from 5 mg/mL to 10 mg/mL against *Staphylococcus epidermidis*, *S. aureus*, *S.* Typhimurium, *E. coli*, and *Klebsiella pneumonia* [73]. CGA extracted from blueberry fruits inhibited the growth of *S. epidermidis* and *P. aeruginosa* by 40% and 42%, respectively, at a concentration of 4 mg/mL [74]. Phenolic acids such as cinnamic acid , *p*-coumaric, ferulic, and caffeic acids also inhibited *L. monocytogenes* at the concentrations

of 0.20% w/v, 0.22% w/v, 0.27% w/v, and 0.29% w/v, respectively, but GCA at a 1.0 % w/v concentration was ineffective [75].

A possible mechanism of action for polyphenolic's antimicrobial activity is when they underdo oxidation reactions that alter their size, polarity, and interactions with their environment. For example, hydroxycinnamic acids have exhibited greater microbial inhibitory effects that were attributed to the presence of hydroxyl (OH) groups and higher hydrogen donating capacity within their chemical structures [76]. The mechanism of action of CGA against *S. dysenteriae*, and *S.* Typhimurium was due to an adverse effect on the bacterial barrier function by inducing massive changes in plasma membrane permeability and causing an efflux of K^+, along with leakage of cytoplasmic content and nucleotides [70]. Therefore, the potential role of CGA as an antibacterial agent compound to control bacteria such as *S.* Typhimurium and *P. aeruginosa* is through damaging the outer membrane (OM) and the release of intracellular materials [77] due to compromise the molecules of lipopolysaccharides (LPS) and lipoprotein (LP) that are connected by electrostatic interaction. The CGA as an anionic molecule might disrupt the OM resulting in the loss of the cell barrier function, a decrease in extracellular pH and modifying the regulation of genes causing cell death [78].

The observation of cell morphology changes in treated bacteria using Scanning Electron Microscopy (SEM) is a proper method of determining if treatments are effective. The morphological change in *Alicyclobacillus acidoterrestris* cells involved in the spoilage of fruit juices was observed by SEM method and showed that the cells treated with CGA became wrinkled and irregular at MIC value of 2.0 mg/mL [79]. Scanning Electron Microscopy of *K. pneumoniae* rod-shape cells treated by 10 g *S. polyanthum* extract mixed with water as food sanitizer at different

interval time of 5, 10, and 15 min appeared to be damaged and deflated while untreated *K. pneumoniae* showed normal rod-shape cells [64].

Drug Delivery System of Polyphenols

Encapsulation (EN) of Polyphenolic

Pluronic® F127 is the trade name for an amphiphilic polymer used in biomedical and drug applications and consists of a triblock PEO-PPO-PEO copolymers of hydrophilic polyethylene oxide (PEO), and hydrophobic polypropylene oxide (PPO). Linear and branched PEO-PPOs chain are known as Pluronic and Tetronic respectively [80]. It is a nontoxic surfactant that can be found less expensive and has a polarity compatibility with the most compounds. The PF127 is an ionic polymer and is able to form into micelles with hydrophobic PPO center core and hydrophilic PEO outer shell as it interacts with water. The hydrophobic core is a storage for hydrophobic compounds and outer shell provides an integrity of nanoparticles (NPs) in an aqueous environment [6].

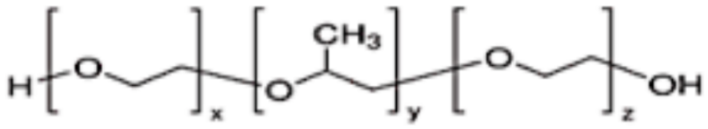


Figure 7. Pluronic® F127- Adopted from Spectrum Chemical Manufacturing Corp (SCMC), 2020 [81].

The amphiphilic micelles with a capability of self-assembly with a size range of 10-200 nm may be used as chemical carriers by delivering compounds into the subcellular compartment and slowly release encapsulated compounds into physiological aqueous solutions [82]. Encapsulation is a process where core (the targeted compounds) is surrounded by wall materials as an outer shell [83]. Encapsulation techniques have been successfully applied as drug delivery systems in the pharmaceutical industry [84] and the coating materials protect polyphenols and drugs in a way that they enable target delivery and control their release during digestion. The coating materials are often made from macromolecules and other encapsulating agents such as carbohydrates (i.e. starch, maltodextrin), cellulose (i.e. carboxymethyl cellulose), natural gum (i.e. sodium alginate and carrageenan), protein (i.e. casein, gelatin), and polymers (i.e. DL- lactode-co-glycolide (PLGA), polyvinyl acetate) [85]. Encapsulation of polyphenolic extracts using encapsulating agents may promote the dispersion of nanoparticles and protect them from oxidation, enzymatic reactions, light exposure, temperature, precipitation, and other degrading factors especially for sensitive compounds such as anthocyanins [86].

Encapsulating hydrophilic compounds such as some naturally occurring compounds (phenolic acids and flavonoids) into hydrophobic polymeric core may improve their effectiveness, the low solubility, dissolution, and poor stability in the GIT due to the hydrophilic layer of PF127 micelles contains polar groups within the polymer which increase the dissolution of the target compounds [87]. The release of polyphenols from polymer depends on different factors including particle size and surface charge, dispersity of the compounds, polymer composition, and type of solvent used. These factors may directly affect nanoparticle physicochemical properties [88].

Antibacterial Activity of Barberry Nanoparticle Polyphenols

Besides the antioxidant activity of polyphenols, many compounds may exhibit antibacterial properties. The mechanism of action of polyphenols on the bacteria could be due to permeability of those polyphenols through the bacterial cell wall, followed by an increase in the lipophilic characteristic of those compounds which results an enhancement in antibacterial activity [89]. It has been suggested that Gram-positive bacteria are more resistant to nanoparticles (NPs) treatments due to their thicker peptidoglycan layer compared to Gram-negative bacteria [90-91]. The presence of OM with negatively charged molecules such as LPS and LP and their interactions with bacterial cell wall structures as well as NPs will also make Gram-negative bacteria more susceptible to NPs compared to that of Gram-positive bacteria. This is due to the higher affinity to the ions among negatively charge molecules within Gram-negative OM that leads to an increase uptake of ion through cell wall membrane, and eventually damage the cell wall [92].

The mechanisms involved in the interaction of NPs and bacteria either through an accumulation in cytoplasm and interference with cellular functionality or production of ROS, mostly from singlet oxygen and hydroxyl radicals. Free radicals are able to break down the membrane lipids resulting in disruption of cell membranes through and finally an alteration of cell membrane integrity [93]. Antibacterial activity of encapsulated *Sesbania grandiflora* extract (SGE) in PF68 and PF127 has indicated that both NPs inhibited the growth of *S. aureus* at MICs value of 0.5 and 1.0 mg/mL respectively compared to that of micelles of PF68 and PF127 without SGE [94]. The mechanism of the nanoparticle could be due to an enhancement of bacterial cellular uptake of polymeric micelles [95] and release the polyphenols inside the bacteria at the target site [96]. CGA loaded silica nanoparticles (CSC NPs) at concentration of 45 μg/mL exhibited that nanoparticles

could disrupt cell membrane, breach the bacteria cell walls of *E. coli* and *B. subtilis* by accumulation of high level of reactive oxygen species (ROS) resulting in cell death [97].

Size Distribution and Polydispersity Index (PDI) of Nanoparticles

The size distribution of nanoparticle is a critical factor that influences the absorption or penetration of a targeted compound through the cell wall and to aid in disruption the growth and replication cycles of the bacteria [98]. Using some polymers such polyvinyl alcohol, Pluronic® F68 and Pluronic® F127 as stabilizers may result in homogeneity in size distribution with an optimal range from 10 nm to 100 nm [88]. Particles must be sufficiently small within the optimal range to pass through transmembrane and interfere with cells constituent and metabolism [99]. Thus, the size of nanoparticle has also been important to help penetrating the compound through the cell walls and it may be responsible for bacteria structure to be disrupted [98].

Polydispersity index (PDI) is a parameter that defines the size range of polymer or lipid nanocarrier system. The term of polydispersity is recommended by IUPAC and describes the degree of non-uniformity of a size distribution of particles [100]. The values of polydispersity index (PDI) exhibit a homogeneous dispersion when the value is close to zero, whereas the sample higher than 0.3 indicates more heterogeneity [101]. It has been hypothesized that homogeneous dispersion of nanoparticle may play major role in penetrating the compound through the cell wall and inhibiting bacteria proliferation due to interaction of nanoparticle with lipopolysaccharide molecule present in Gram-negative bacteria resulting in oxidation of biomolecules and accumulation of reactive oxygen species (ROS). The ROS production can cause the enhancement of carbonyl content and rupture the protein structure present in bacterial membrane resulting in the disruption of the cell wall integrity by losing the small ion such as K^+ and PO_4^{3-} followed by DNA,

and RNA molecules [102]. A cation nanoparticle of silver (Ag^{+}) as small as 5 nm can easily transport across the cell membrane and become more toxic to bacteria cells due to the higher surface volume ratio compared to that of large NPs, resulting in (ROS) production and finally damage DNA, protein, and lipid [99]. Therefore, entrapment of antimicrobial agents in a nanosized core-shell micelle may promote the compounds' transportation into bacterial membrane and increase the efficiency of the compounds.

Polyphenol Absorption, Distribution, Metabolism, and Excretion (ADME)

When polyphenols within a food matrix are consumed, they will undergo ADME to exert any biological effects, after ingested by grinding and mixing with saliva juice in the mouth, then transferred to the stomach and effected by the gastric juice as well as enzymes. The digestion is defined as breakdown the large and insoluble food molecules into smaller soluble compounds by various digestive enzymes in different pH conditions. First, polyphenols must be released from the food matrix (bioaccessible) via digestion to be absorbed into the body cells. Metabolism and distribution will then take place. Most of the compounds that are not absorbed in small intestine, will be transferred to the colon and bio-transformed by microbiota in the duodenum, jejunum, and ileum [103]. The parent compounds are converted to metabolites that can be absorbed more easily. Absorption of polyphenols is a complex process due to dose of their availability (bioaccessibility), molecular size, the degree of polymerization, solubility, interaction with other compounds, gut microbiota populations, and transfer through the GIT [104]. The route of polyphenol absorption can also be either through the stomach, small intestine or if absorption did not happen in these sites, gut microbiota may metabolize them by producing enzymes such as α-rhamnosidase, β-galactosidase, β-glucuronidase, sulfatase, and esterase [105].

Absorption initiatives in the stomach where the digestive enzyme can effect on intact polyphenols. An *in vitro* study reported that a complete recovery of 30 mg CGA and 15 mg of caffeic acid after incubation in gastric juice for 0.5 and 2 h respectively [106]. After reaching the small intestinal, the hydrolysis of CGA and dimethoxycinnamyl quinic acid will occur which is slow and a small portion of CGA is hydrolyzed. Then, some intact CGA as well as free caffeic acid cross the small intestinal epithelium to get metabolized and reduced their double bounds. These compounds are further absorbed and sulfated to form of caffeic acid-3-*O*-sulfate and following methylation to form ferulic acid-4-*O*-sulfate. However, only one third of CGA are absorb in small intestine with a large variability among individuals, mostly in form of free caffeic acid by passive diffusion and monocarboxylic acid transporter (MCT) [107]. The remainder two third of the initial CGA remain unabsorbed and passed into the large intestine [73]. The production of enzymes such as α-rhamnosidase, β-glucuronidase, sulfatase, and esterase by human colon microbiome convert the CGA to dihydroferulic acid and dihydrocaffeic acid in colonic microbiota-mediated conversion step. Finally, in the further step, metabolites of dihydroferulic acid-4-*O*-sulfate and dihydrocaffeic acid-3-*O*-sulfate have been identified in the blood circulation. Theses enzymes are also able to hydrolyze phenolic-quinic acid linkage [73, 108]. CGA that has already reached the colon may undergo microbial metabolism which converts this acid to 3-(3-hydroxyphenol) propionic acid and benzoic acid [109]. Also, the formation of caffeic acid and its esters including 3-(3,4-dihydroxyphenyl) propionic acid, followed by the 3-(3-hydroxyphenol) propionic acid, 3-(3-hydroxyphenol)propanoic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-Dihydroxybenzoic acid, 3-phenyllactic acid, and 3-hydroxyphenylpropionic acid were observed [110] (**Fig. 8**).

Caffeic acid, its esters, and chlorogenic acids used in a gastrointestinal model were transformed into 3-(3´- hydroxyphenyl)propionic acid and benzoic acid [111]. Metabolites then are absorbed through the colonic epithelial cell where caffeic and quinic acids are transported across the epithelial colon cells. The most ingested amount of CGA is degraded during digestion, biotransformation by gut microbiota, or excreted via feces. However, after consumption 5-CQA, only small amount of caffeic acid, a hydrolysis product of CQA has been identified in human plasma and urine samples [112].

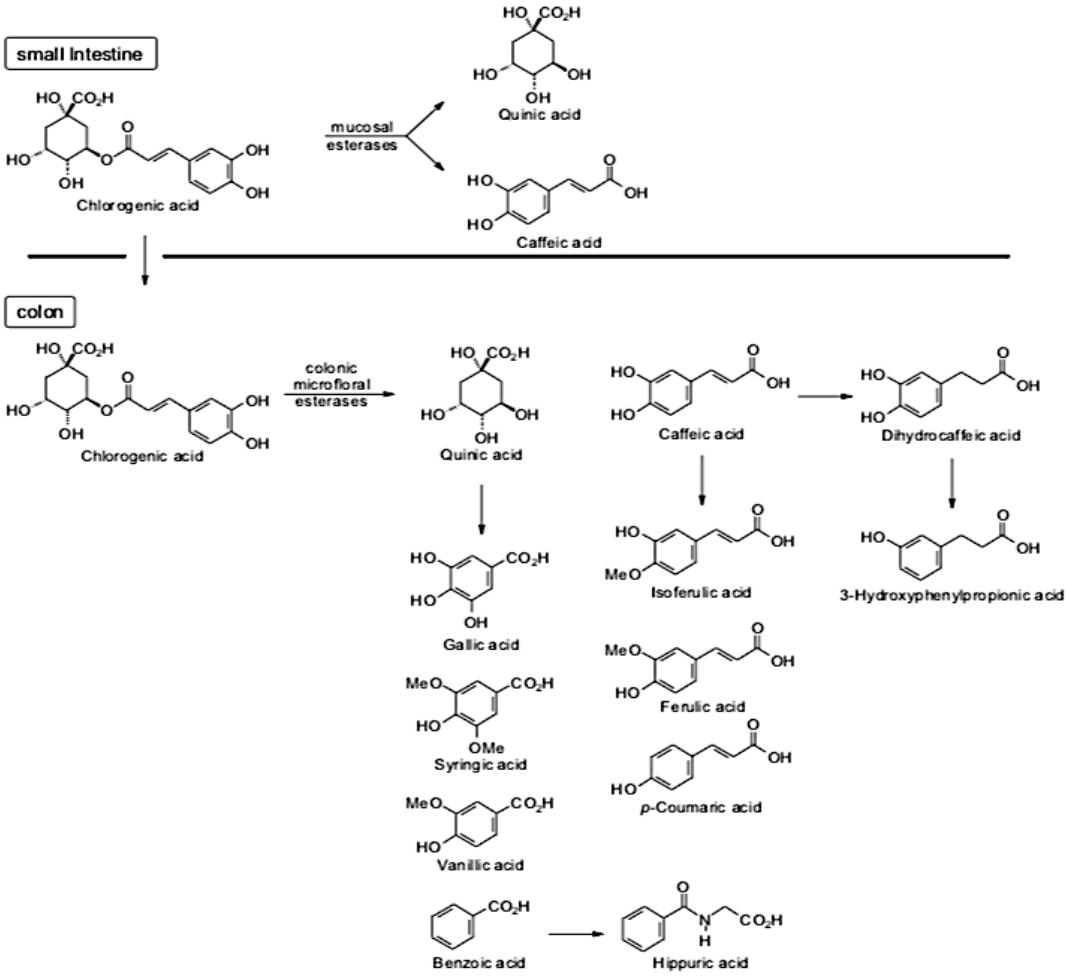


Figure 8. Metabolic route of CGA- Adopted from S. F. Nabavi et al., 2017 [113].

Human Gut Microbiome and Health

The human colon microbiome is the most populated microbial ecosystem on the planet having over 10^{14} bacteria cells which is 10 times more than human cells [114]. It is estimated 500-1000 different microbial species in gastrointestinal tract with a concentration of 10^{14} colony per gram of feces. However, gut microbiota populations are not stable throughout the entire human life-span and is also highly variable between individuals, and their bacterial colonization could also be influenced by many factors including the breast-feeding period, diet, lifestyle, environment, inflammation, and disease [115]. The dominant gut microbial phyla are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicobi [116]. Up to 90 % of gut microbiota are categorized as either Firmicutes or Bacteroidetes [117]. Some bacteria are beneficial to the host while it has been hypothesized that a change in the composition of the microbiome may be led to chronic disease and stimulate inflammation [118].

The FDA and WHO have defined probiotic bacteria as the live microorganisms that may have health benefit to the host. *Lactobacillus*, *Bifidobacterium*, and *faecalibacterium* are considered as probiotic specially *L. casei*, *L. plantarum*, and *F. prausnitzii* that have shown potential role on promoting anti-inflammatory cytokine interleukin-10 biomarker by downregulating the expression of key proinflammatory cytokines and neutralizing the proinflammatory effect of pathogenic bacteria such as *Escherichia coli* [119]. For einstance, *E. coli* is associated with IBD and colon cancer and another strain of this bacteria functions as a commensal bacterium such as *E. coli* HS [118]. *Escherichia coli*, *Bifidobacterium, Lactobacillus*, *Bacteroides*, *Eubacterium* species are involved in catabolic pathways in the human body [120]. So that, gut microbiota metabolic transformation is known through three main reactions, hydrolysis (O-deglycosylation, hydrolysis of esters), cleavage (C-ring cleavage, demethylation), reduction

(dehydroxylation, hydrogenation, carbon-carbon double bond), and aromatic hydroxylation [121]. However, dominant colonic microbiota especially short chain fatty acid (SCFA) producers are responsible for breaking down the parent polyphenols structures into lower molecular weight phenolic metabolites which may affect on human health more than parent compounds [118, 120].

Synbiotic Interaction of Polyphenol and The Gut Microbiota

The colon microbiome and their host also have a synbiotic beneficial where the host could benefit by the microbiome when the gut immune system is regulated [7]. The concept of "three P" such as Probiotic, Prebiotic, and Polyphenols are related to gut health, specially polyphenols may enhance the survival and growth rate of probiotic bacteria [7]. There is also reciprocal interaction between polyphenols and gut microbiota, where they may modulate the composition of the host gut microbiota improving a variety of biomarkers and risk factors for chronic disease [122]. For example, C6 phenols derived from CGA and most flavonoids are active biologically and may promote the growth of human gut microbiota population [50].

The polyphenols may promote the growth of beneficial microbiota while pathogenic strains are inhibited. From an *in-vitro* study of 1000 mg/L gallic acid treatments added to a fecal slurry, the number of *Clostridium histolyticum*, a harmful bacterium decreased without impacting other beneficial bacteria present [123]. Fruit extracts from different tissues such as pulp, seed, and peel have shown prebiotic activity related to their polyphenolic profiles, for instance, the potential role of catechin, GA, vanillic, and ferulic, protocatechunic acid within mango extract on the growth of *Lactobacillus rhamnosus* and *Lactobacillus acidopholus* individually and in combination exhibited a growth of Lactobocilli when treated with gallic, protocatechuic, vanillic acids and catechin while ferulic acid did not promote probiotic growth [124]. An increased in number of

probiotic bacteria (i.e. *Bifidobacterium*) can improve the gut barrier function and reduce the effect of pathogenic microorganism and inflammatory substances such as lipopolysaccharide (LPS)[125]Lee et al. (2006) reported that microbiota from fecal source treated with tea polyphenols in aerobic and anaerobic conditions showed a growth inhibition on pathogen and regulating probiotic bacteria (i.e. *Lactobacillus* and *Bifidobacterium*) resulting in decreased in formation of harmful amine procarcinogens in the large intestine, and reduce acid production that raises fecal pH [126]. The ability of some gut bacteria to produce α-rhamnosidase, β-galactosidase, and β-glucuronidase enzymes which can break down the parent polyphenol structure has been studied [124]. The growth of the *Lactobacillus* and *Bifidobacterium* have also been induced when treated with purple sweet potato anthocyanin at concentration of 7.06 mg/g dry weight for tested samples. The ANCs content of the treatments were further fragmented to phenolic acid metabolites with an increase in the production of formic, acetic, propionic, butyric, and lactic acid [127]. However, the acidic environment and the effect of the pepsin enzyme in the gastric may effect on the survival rate of gut microbiota [128].

Stability of Polyphenols in the GIT

Evaluate the stability of polyphenols during their passage through the GIT, a simulation of gastric intestinal has been suggested [129-131]. Due to the effect of the pancreatic enzyme and basic pH on the polyphenolic profile of chokeberry juice, a reduction of 43% of ANCs, 26% of flavonols, 19% of flavan-3-ols, and 28% of neochlorogenic acid in the small intestinal phase was observed, while CGA increased by 24% due to isomerization of neochlorogenic acid to chlorogenic acid under basic condition [132]. Chlorogenic acid was also observed to be stable in gastric and small

intestine conditions. After colonic fermentation, a major qualitative change happened in the colonic fermentation.

ANCs can be divided into monomeric, acylated anthocyanins, pyranoanthocyanins, and polymeric anthocyanins. The predominant ANCs in barberry were identified as pelargonidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, and petunidin-3-*O*-glucoside. Monomeric ANCs had an increase of 8.22 % in ANCs concentration after 6 h exposure time in stomach condition whereas 49% total anthocyanin concentration in small intestinal phase was decreased. Dominant structures for monomeric ANCs are flavylium cation under the acidic conditions. Basic pH may facilitate the C-ring cleavage when ANCs move from acidic to basic pH. An instability of methoxy groups present in the B-ring could also be responsible for ANCs structure stability whereas hydroxyl groups lead to a decrease in their stability [133]. The concentration of blueberry ANCs in a crude extract was decreased by 10-15% after exposure to intestinal digestion [129].

Nanoencapsulation of barberry polyphenols rich in chlorogenic acid and simulating in oral, gastric, intestinal, and colonic phases with a pH of ~6.8, ~2.5, ~5.8-6.8 in GIT conditions may be an approach to evaluate the stability of phenolic compounds. Despite the acidic environment of the stomach as well as the presence of proteolytic pepsin enzyme and bactericidal activity of bile salts, survival rate and population of gut microbial may change [134]. A *vivo* study on rats reported high stability for nanoparticles modified with PF127-Lip in simulated GI fluids when compared to other formulations of liposome (Lip) and chitosan-coated liposome (CS-Lip) in a simulated gastric fluid (SGF) for 1 h and CS-Lip incubated in simulated intestinal fluid (SIF) for 2 h [135]. It could represent a protective role of PF127 against pH changes in GIT. Nanoencapsulated in polymeric micelles could protect the compounds of interest against enzymatic degradation in the stomach and promote the transport of compounds of interest to the small intestinal and colon [136].

EN of polyphenols using nanotechnology could be an approach to improve the delivery system, food stability, increase antioxidant, antibacterial efficacy, and bioavailability by protecting the botanical compounds from degradation [137]. There are some obstacles regarding phytochemical administration due to their low water solubility in large polyphenol molecules and degradation over time as well as their instability *in vivo* GI tract, including the influence of different pH conditions and presence of enzymes [138-139]. In addition, the advantages of EN are numerous; polyphenols loaded polymers (nanoparticles) will play a potential role in preserving polyphenols from degradation, improving stability, particle size uniformity, drug loading capacity, and controlled release of encapsulated compounds. The fabrication procedures to create polymeric nanoparticles depend on many factors such as the EAs, the physicochemical nature of the compounds, and the desired properties of the nano-polymer [140].

However, there is still a gap in knowledge regarding the stability of NP barberry polyphenols with Pluronic® F127 in an *in vitro* simulated gastric (SGF) and intestinal fluid (SIF) as well as the effect of gastric and intestinal fluids on the growth of healthy gut bacteria. Therefore, these studies investigated the mechanism of action of polymeric nanoparticles made from barberry fruit extracts nanoparticle with Pluronic® F127 against pathogenic food bacteria and the mechanism and stimulatory effect of barberry NPs on promoting the growth of healthy gut microbial.

CHAPTER III

PHYTOCHEMICAL CHARACTERIZATION AND EFFICACY OF BARBERRY (*BERBERIS VULGARIS*) POLYPHENOLIC EXTRACTS USING DIFFERENT SOLVENTS ON VIABILITY OF BACTERIA

Introduction

Barberry fruits contain various phytochemical substances that are known for human health, including carbohydrates, organic acids, vitamins, and polyphenols. The barberry plant is also well known as a medicinal plant in Asia and Europe especially Iran and Turkey as a medicinal shrub against various diseases such as malaria, gall stones, and hypertension (Tan et al., 2018). The characterization and quantification of polyphenolics in food products such as barberry is such as barberry is highly relevant as they may serve as a fingerprint for botanical authentication and provide indications of stability, functional properties, and potential health benefits. Polyphenolic compounds are secondary plant metabolites that are responsible for flavor, color, and consumer perception of the food product [1]. The polyphenols are contained in such products made from food and botanicals which can also be used as food additives to enhance nutritional attributes, and improve food quality, and serve as antimicrobial agents to enhance food safety (Genskowsky et al., 2016).

The consumption of natural antimicrobial has become valuable since the use of synthetic preservatives is increasing concern among consumers. Plants used in traditional medicine have been investigated to search for possible compounds that can be used to develop natural drugs [141]. Compounds synthesized as part of the secondary metabolism of the plants such as tannins, essential oils, and phenolic compounds exhibited antimicrobial activity and other clinical benefits [56]; for

instance, phenolic acids are known to possess antibacterial properties due to their ability to increase cell membrane permeability and cause leakage of cytoplasmic content and nucleotides of pathogenic bacteria [76]. However, the effectiveness of plant extracts as antibacterial agents may vary due to plant species, chemical composition and structure of phenolic compounds, the type of solvent in extracting polyphenols, and extraction method.

Materials and Methods

Fruit

Barberry (Berberis vulgaris) dried fruits with 19% moisture content were purchased from Cyrus Saffron company, WA, USA. The fruits were shipped in polyethylene bags (500 gr) to the Department of Food Science and Technology at Texas A&M University and stored at 4 °C for further analysis.

Barberry Extraction

Dried barberry fruits (5 g) were grounded using a Miracle Mill grinder (MC-17B, 150 W, Yugoslavia) and transferred into 50 mL Falcon tubes containing methanol:water, ethanol:water, acetone:water and acidified with glacial acetic acid at a fixed ratio of 70:30 (solvent:water) to maintain a pH of 2.9-3.0. Extraction was carried out three times by homogenizing the fruits using a Fischer Scientific tissumizer (PowerGen500S1, 500W) for 10 min followed by a 15 min sonication (Branson sonicator_3510R-MT,130W). Samples were then centrifuged for 10 min at 3000 x g in Eppendorf centrifuge (5810R,1300W, Germany) and the supernatants were filtered through a 9.0 cm filter paper (Qualitative P8, Fischer Scientific). Solvents were removed under reduced pressure using a Büchi rotary evaporator (R-200) at 45°C. All the extracts were

normalized to a volume of 4 mL. The samples were evaluated for Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and monomeric and polymeric anthocyanins content. Free extracts were stored at -20°C until polyphenolic analysis.

Determination of Total Reducing Capacity (TRC)

The total reducing capacity was determined using the Folin-Ciocalteu method [142]. According to this method, 1 mL of 0.25 N Folin-Ciocalteu reagent was mixed with 0.1 mL of the fruit extract. After 3 minutes, 1 mL of 1 N sodium carbonate was added to the solution and let it stand for 7 minutes. Then, 5 mL of water was added to the test tubes. After an hour, its absorbance was read at 726 nm using a spectrophotometer. The TRC was calculated against the standard curve of gallic acid according to gallic acid equivalents (GAE) per g of the dry weight of fruit.

Determination of Total Anthocyanin Content (MAC)

The monomeric anthocyanin content (MAC) was determined by a pH differential method (Giusti and Wrolstad, 2001), using a UV-VIS spectrophotometer. Absorbance was measured at 520 nm and 700 nm in buffers at pH 1.0 and 4.5 using the equation $A = (A_{520}-A_{700})$ pH 1.0 - $(A_{520}-A_{700})$ pH 4.5 with a molar extinction coefficient of 15,600. The absorbance at pH 1.0 and 4.5 was measured at 700 nm which allowed for haze correction. Results were expressed as mg of pelargonidin-3- glucoside equivalents per g of dried barberry fruits.

Determination of Monomeric and Polymeric Anthocyanin Content (PAC)

Polymeric anthocyanin content (PAC) was measured using a UV-VIS spectrophotometer (Ferrari et al., 2010). For the assay, 0.4 mL of 5% sodium sulfite (Na_2SO_3) was added to 4 mL of

the free extract. A blank was prepared using 0.4 mL of water (pH 3.0) added to 4 mL of extract. The absorbance was recorded after subtracting the absorbance for the blank at 520 nm. The polymeric anthocyanin content was determined according to the following equation:

% Monomeric Anthocyninns: $(A_{blank} - A_{SO2}) / A_{blank} * 100$

% Polymeric Anthocyaninns: $(A_{SO2} / A_{blank}) * 100$

Determination of Total Flavonoid Content (TFC)

The total flavonoid content was determined using the methods previously described [143]. A volume of 0.5 mL of free barberry extract and 1.5 mL of methanol were mixed. Then, 100 µL of 10% aluminum chloride ($AlCl_3$), 100 µL of 1 M sodium acetate ($NaCH_3CO_2$), and 2.8 mL of distilled water were added to test tubes. After 40 min incubation, absorbance was measured using a spectrophotometer at a wavelength of 415 nm. Total flavonoid content was determined using the standard curve of rutin equivalents in mg per liter. All assays were be carried out in triplicate.

HPLC-PDA-ESI-MS^n Analysis

Free barberry polyphenolic extract was characterized and analyzed using a Thermo-Finnigan Surveyor HPLC-PDA in tandem with an LCQ Deca XP Max ion trap spectrometer with an ESI source equipped with a C18 column (Sunfire C18 Reversed-phase, 4.6 x 250 mm, 5 µm particle size (Waters Company, Milford, Massachusetts, USA) at room temperature. The sample loop was 50 µL. Mobile phase A was consisted of water and mobile phase B consisted of, methanol, both containing 0.1% formic acid at a flow rate of 0.45 mL/min. A gradient separation was run with 100% mobile phase A and maintained for 2 min. Phase A then decreased to 90% over 10 min, 60% for 25 min, 35% at 35 min, and 15 at 41 min and maintained for an additional

15 min. A capillary temperature at 350 °C was set with a ESI source voltage of 4000 V. The electrospray interface ionization worked in negative ion mode for phenolic acids and flavonoids and positive ESI ionization for anthocyanins each run under the same chromatographic conditions [144].

Preparation and Inoculation of Microorganism

The Gram-positive bacteria such as *L. monocytogenes* (Scott A Ser 4b)*, E. faecium* (NRRL B-2354), and *S. aureus* (ATCC 6538) along with the Gram-negative bacteria *E. coli* O157:H7 (43895™), *S. enterica* serovar Typhimurium (ATCC 700720), and *P. aeruginosa* (ATCC 10145) were obtained from food Microbiology lab (Department of Food Science, Texas A&M University). Culture preparation of the microorganisms was accomplished using the method of (Perez et al., 2012). For each targeted bacteria, working cultures were prepared on tryptic soy agar (TSA, Becton, Dickinson and CO Sparks, MD, USA). A loop (10 µL) of the culture was transferred into 10 mL sterile Brain Heart Infusion broth (BHI; Becton, Dickinson, and Co.), followed by incubation for 24 h at 37 °C. The bacterial concentration was determined by spread plating decimal dilutions of 0.1 % (w/v) peptone water and TSA plates and incubated at 37 °C for 24 h.

In Vitro Screening Using Hole Diffusion Method

The antimicrobial activity of barberry extracts was determined using the hole diffusion technique on Tryptic Soy Agar (TSA) growth medium according to [145]. A standardized bacterial suspension of 10^6-10^7 CFU/mL was prepared in Brain Heart Infusion broth (BHI). A bacterial suspension volume (120 µL) was mixed with 120 mL of Tryptic Soy Agar (TSA), 20 mL of prepared bacteria suspension then poured into Petri dishes (100 mm x 25 mm). After 10 min of

rest at ambient temperature, holes were punched into the agar using a sterile 5 mm diameter cork borer and the filled with 50 µL of free extract. Ethanol (100%), methanol (100%), acetone (100%), and gentamicin (10 mg/mL) were used as positive controls and free extract was as a negative control all added at 50 µL. The antimicrobial assessment was evaluated by measuring the diameter of a clear inhibition zone of growth of each of six bacteria strains after 24 h at 37 °C under aerobic conditions. The average diameters of the inhibition zones were used as a measure of antimicrobial activity and each assay was repeated in triplicate.

In Vitro Screening Using Broth Microdilution Method

The antibacterial activities of barberry fruit extracts were assessed based on broth microdilution assay previously reported [146] (**Fig. 9**). Briefly, a 96-well microplate, two-fold (2x) Brain Heart Infusion (BHI) medium, and 6.0-7.0 $\log_{10}$ CFU/mL inoculum bacteria were used to measure the growth of bacteria treated with free barberry extracts. The free extracts were diluted with two-fold BHI (2x BHI) to obtain final concentrations of 0.312, 0.625, 1.25, 2.5, 5, 6, 7, 8, 9, and 10 mg/mL. In the 96 well plates, 100 µL of free extract was transferred to the wells. Column 1 contained the highest concentration of 10 mg/mL and column 10 contained the lowest concentration of each extract (0.312 mg/mL). To each well, 100 µL of each bacterial suspension was added for a total volume of 200 µL. Each plate had a control set of 100 µL of 2x BHI medium as a negative control in while 100 µL of 2x BHI mixed with 100 µL of microbial suspensions was considered as a positive control. The microplate was loaded into an Epoch UV/Visible scanning spectrophotometer (Bio-Tek® Cytation Hybrid Multi-Mode Reader, Cytation 5 imaging, USA) at 37 °C for 24 h and readings were conducted at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h.

Changes in optical density at 630 nm represented the growth (increase) or death (decrease) of each bacterium in suspensions.

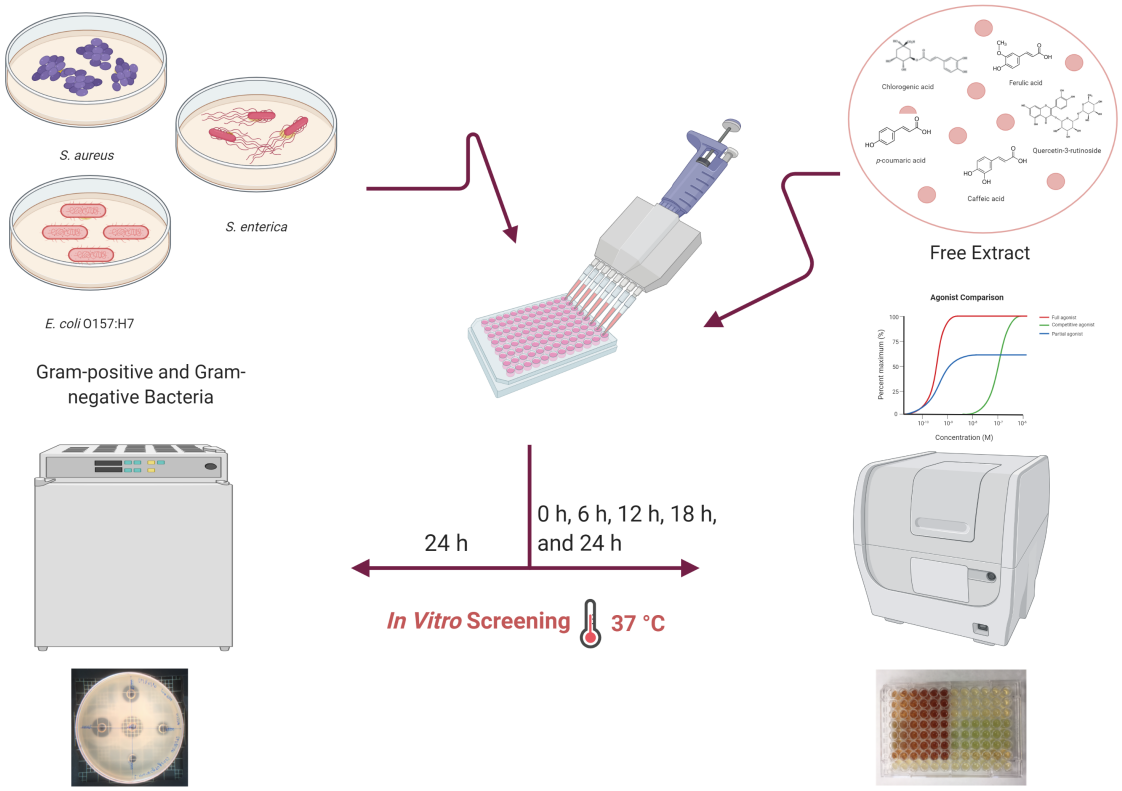


Figure 9. A schematic illustration of the antibacterial methodology, created with BioRender.com (2021).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC of barberry polyphenolic extract were determined using a microbroth dilution assay and evaluating the visible growth of microorganisms in the media [147]. A stock solution of the free extract was prepared in a 15-mL Falcon tube by diluting the free methanolic

extract with BHI media to a concentration range of 0 mg/mL to 18 mg/mL using 100 µL of BHI media in each well of 96-well microplate with adjusted bacterial concentration (1×10^8 CFU/ml, 0.5 McFarland's standard). The positive control contained inoculated broth and the negative control contained BHI media. The volume of each well was made up to 200 µL using 100 µL of the tested bacteria suspension. The 96-well plate was then incubated for 24 h at 37 °C and read to confirm MIC value.

For the determination of MBC, 100 µL of culture suspension from each well in the MIC plate was transferred into a new 96-well microplate. The volume of each well was then raised to 200 µL by adding 100 µL BHI broth to each well. The 96-well plate was incubated at 37 °C for 24 h and read to determine the changes in OD values.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to determine potential morphological changes caused by exposure to free barberry fruit extract. Cells were prepared as previously described method [148] whereby, cells were treated with 10 mg/mL of the free extract, and the culture was incubated for 24 h at 37 °C. After the incubation, bacteria pellets were collected by centrifugation at 12,000 x g for 5 min at 25 °C, then washed with Milli-Q water 3 times to remove the residual. A volume of 100 µL of isolated cells were fixed on dry poly-lysine coated glass coverslips using glutaraldehyde (2.5 w/v%) for 2 hours under a fume hood. The pellets were washed with 3 mL of 0.1 M cacodylate buffer gently for 10 min three times. The post fixed-step was accomplished by applying 1 mL of 1 wt% osmium tetroxide on the poly-lysine coated samples at ambient temperature for 1 h. The cells were then washed with 3 mL of cacodylate buffer and dehydrated first with 50% ethanol for 30 seconds and secondarily with absolute ethanol to complete the dehydration process for 15 min.

Lastly, cells were coated with ~10 nm carbon and scanning electron micrographs were imaged using Scanning Electron Microscope (SEM) at Materials Characterization Facility (MCF)-Division of Research at Texas A&M University.

Statistical Analyses

All results were expressed as mean ± standard error (SE) using GraphPad Prism version 5. Data of growth inhibition of the bacteria were evaluated in triplicates using the calculated means from ANOVA (analysis of variance) followed by comparing each pair with a control.

Results and Discussion

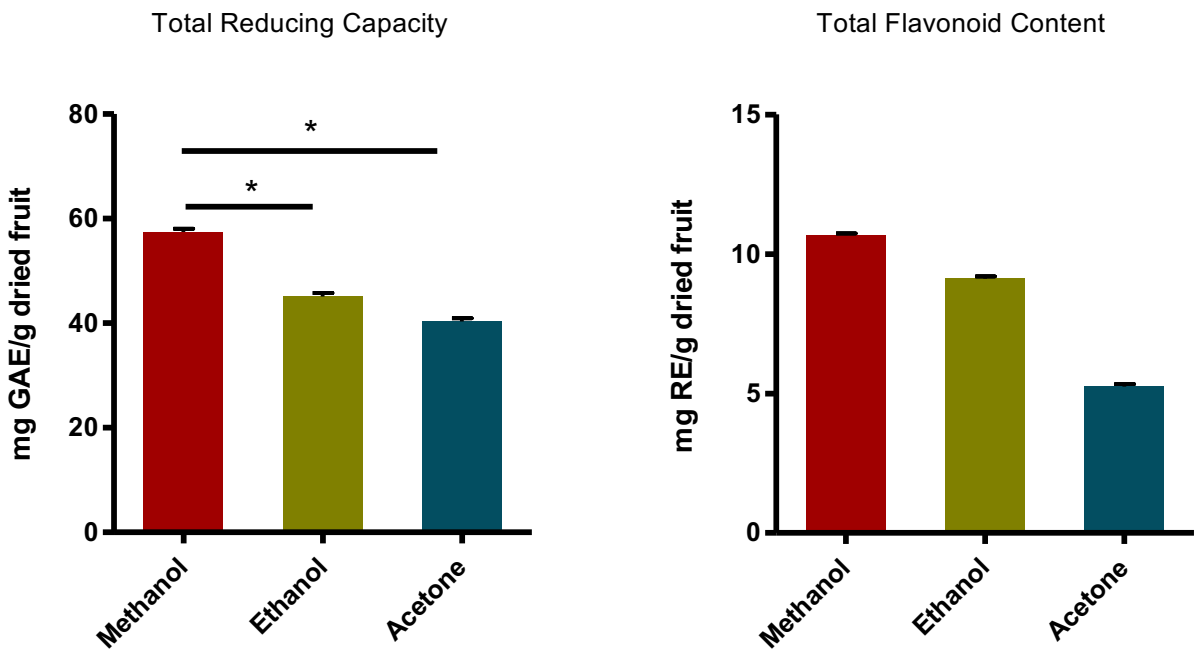
Total Reducing Capacity, Monomeric and Polymeric Anthocyanin, and Flavonoid Contents of Barberry Fruits

Total reducing capacity of barberry extraction using different solvents analyzed in this study was measured in methanol, ethanol, and acetone extracts at values of 57.4, 45.1, and 40.3 mg gallic acid (GAE) per g of dried weight, respectively (**Fig. 10a**). 70% methanol was a superior extraction solvent for polyphenolic recovered in three extractions at 57.4 mg GAE/g dried fruit compared to 45.1 and 40.3 mg/g dried fruit for 70% ethanol and 70% acetone, respectively. This result contrasts with other studies reporting TPC of barberry where extraction with absolute acetone had a higher concentration than methanol and ethanol at 92.8 mg GAE/g fruit [32]. The concentration of total phenolic content of 80% methanol was observed to be at a value of 280 mg GAE/g extract and for 100% aqueous 100 mg GAE/g extract, respectively [149]. The lowest concentration of total phenolic content for methanol extraction of fresh barberry was 12.2 mg GAE per 10 g of fresh fruit and the highest concentration of total phenolic content was obtained from aqueous extraction of dried

barberry for 59.6 mg GAE per 10 g of sample [150]. The amount of total phenolic concentration obtained from barberry fruit varied from 25.9 mg GAE/100 mL extract in *Berberis integerima* to 94.0 mg GAE/100 mL extract in *Berberis vulgaris*.

The mechanism behind the total reducing assay using Folin-Ciocalteu reagent is based on transferring the electrons and oxidation of phenolic compounds in alkaline medium to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that can be detected spectrophotometry [151]. Some non-phenolic compounds, including ferrous ion and ascorbic acid, can also react with the Folin-Ciocalteu reagent. However, the type of solvent can be important in extracting polyphenols. Methanol and ethanol are the most commonly used solvents with the relative polarity of 0.762 and 0.654, respectively. In this current work, the polarity of solvents increased by adding 30% water, since higher polarity enables the breakage of hydrogen bonds in polyphenols structure and improves their solubility in organic solvents [152]. However, the total reducing capacity, anthocyanins, and flavonoids of the fruit differ based on soil type, phenotype, environment, climate condition, storage, and processing condition.

10a. **10b.**



The total flavonoid content results were derived from rutin calibration curve (Y=0.0018-0.0099, R^2=0.9996) at a concentration of 0-0.5 mg/mL (**Fig. 10b**). The highest TFC amount of methanolic extract was 10.7 mg rutin per g of dried fruit weight and ethanolic and acetone extracts were with values of 9.1 and 5.2 mg rutin per g dried fruit weight, respectively. The concentration of total flavonoids was 157.8 mg (+)(-) catechin equivalent per 100 g fresh fruit weight. A value of 12.2 mg per g fruit extract of total flavonoid content was also reported in barberry fruits (Rahimi et al., 2017). Hassanpour et al., (2015) reported a range of 133-280 mg/100 g fruit weight in various barberry genotypes reported. However, the differences in the composition of barberry fruits could be due to growing conditions and cultivars, geographic conditions, and degree of fruits maturity at harvest time, and drying process [153].

10c. **10d.**

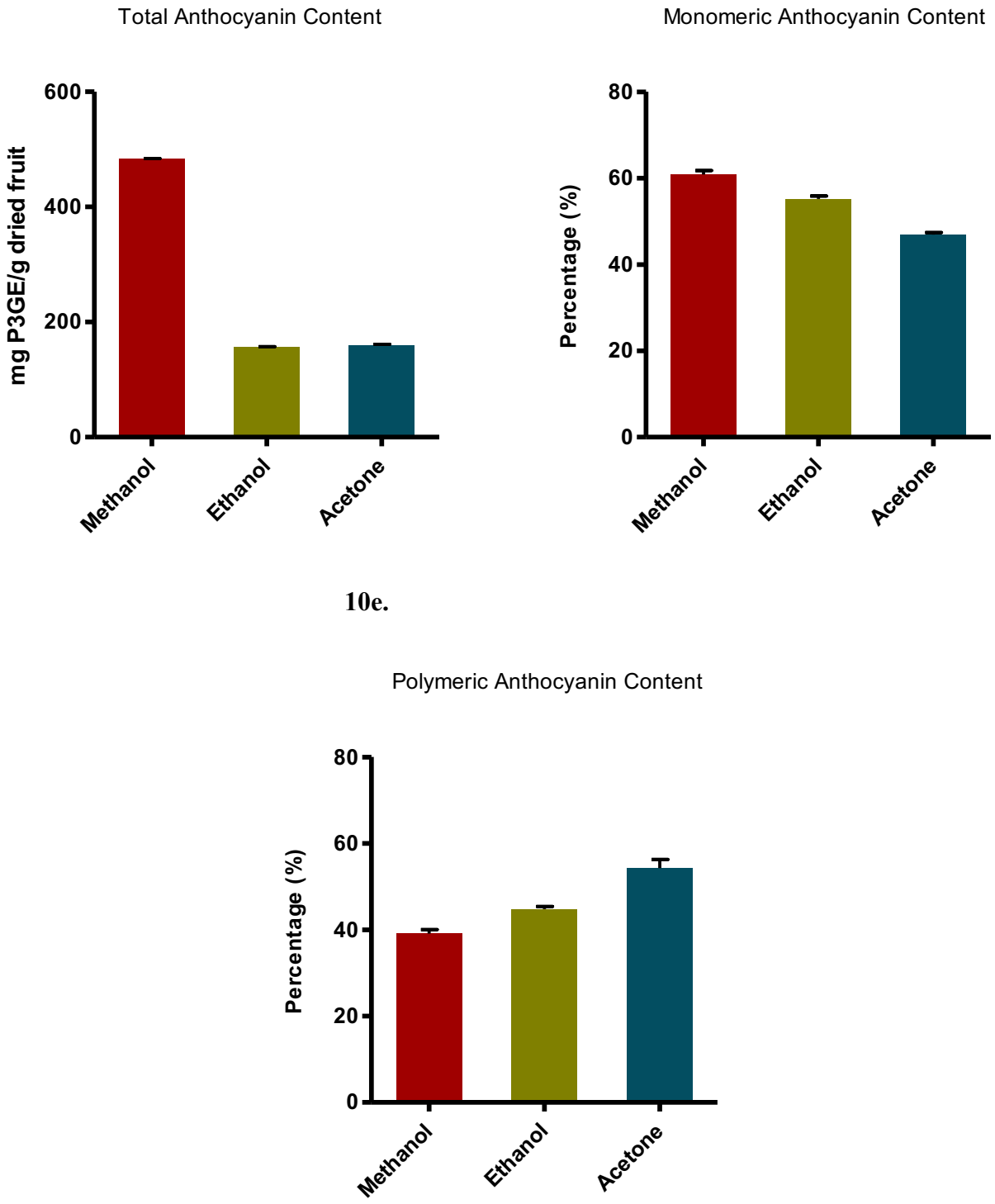


Figure 10. Total reducing capacity (a), Total flavonoid content (b), Total anthocyanin content (c), Monomeric anthocyanin content (d), and Polymeric anthocyanin content (e) in methanol, ethanol, and acetone barberry polyphenolic extracts.

Pelargonidin-3-*O*-glucoside was found to be the most predominant anthocyanin in barberry fruit along with cyanidin-3-*O*-glucoside and petunidin-3-*O*-glucoside in this study. Total anthocyanin evaluation based on mgs of pelargonidin-3-*O*-glucoside equivalents per g dried weight is shown in **Fig. 10c**. The results indicated that methanolic extract recovered in three extractions was also efficient by extracting anthocyanins at 483 mg pelargonidin-3-*O*-glucoside equivalents per g dried weight and for acetone and ethanol were 159 and 157 mg pelargonidin-3-*O*-glucoside equivalents per g dried weight, respectively. The total anthocyanin content of barberry fruit was 14.8 mg pelargonidin-3-*O*-glucoside (P-3-G) /100 g of fresh fruit (Ardestani et al., 2015). The TAC was 0.70 mg/ g of ethanolic extract and 153 mg/ g in barberry juice as equivalent cyanidin-3-*O*-glucoside per g of ethanolic extract and g in barberry juice respectively [154]. A study on fourteen different barberry selections grown in Turkey reported the total anthocyanin content range from 360 to 874 mg cyanidin-3-glucoside per liter fruit juice [40]. Anthocyanins aglycon has higher solubility in alcoholic solvents than its glucoside, whereas glycosylated anthocyanin is highly soluble in water. This is because anthocyanin structure induces hydrophobic characteristics to its structure which would be more soluble in organic solvents such as methanol and ethanol. In contrast, the presence of 3-hydroxyl group in the C-ring of anthocyanin makes them more soluble in water [28].

The results of monomeric and polymeric anthocyanin content are shown in **Figs. 10d** and **e**. The highest monomeric anthocyanin content was for methanolic extraction at 60.9% followed by ethanol at 55.2%, and acetone at 46.9%. The polymeric anthocyanins pigment was also obtained at a value of 39.1%, 44.8%, and 53.1 for methanol, ethanol, and acetone extracts. The hydroxylation of the anthocyanins in the B ring can affect the color stability due to the effect on the delocalized electrons. Degree of the methylation in the B ring contributes more redness [155].

Anthocyanins are water-soluble compounds, therefore, polar solvents are good choices in extracting anthocyanins from fruits matrices [156]. A study on MAC and PAC values of barberry indicated that varieties of the barberry fruits, extracting solvent, presence or absence of light, temperature, and pH may affect on concentration of individual anthocyanin [8]. In general, acidified organic solvents such as acidified methanol are good choices for extracting anthocyanins from plant tissues, due to their potential destructive role on the cell membrane and dissolve and stabilize the anthocyanins [157].

Identification and Quantification of Barberry polyphenolic Extract

The polyphenol content of fruit can vary based on the variety growing condition, and post-harvest environment, processing variables, and shelf-life conditions. In these studies, commercially available, dehydrated barberries were evaluated with no indication of the variety, growing conditions, processing environment, storage duration and conditions that were likely factors impacting the polyphenolic concentration at the time of sampling. Previous studies have all reported the polyphenol composition of barberry fruits [20, 158]. The major phenolic acids in barberry fruits as gallic acid, caffeic acid, chlorogenic acid, p-coumaric, and cinnamic acid and as well as flavonoids rutin, apigenin, and quercetin among 18 barberry genotypes were characterized [20]. In this work, CGA, ferulic acid, protocatechuic acid, *p*-coumaric, caffeic acid, caffeic acid, and rutin were present in methanolic barberry fruit extract. Characterization of the barberry phenolic acid and flavonoid were performed using HPLC-MS.

The characterization of free barberry extract is shown in **Fig. 11**. The concentration of CGA, ferulic acid, protocatechuic acid, *p*-coumaric, caffeic acid, gallic acid, and quercetin-3-rutinoside were found to be at 1475 ± 31 μM, 16.6 ± 1.5 μM, 43.6 ± 2.8 μM, 10.2 ± 0.02 μM, 118

± 4.8 µM, 686 ± 35 µM, and 154 ± 15 µM for free methanolic extract, respectively. The concentration of CGA (70.24 mg/kg) and rutin (27.09 mg/kg) were previously reported in barberry fruits [158]. Phenolic compounds in ethanol extracts in which chlorogenic and syringic acid were found to be the most abundant polyphenols in barberry fruit extracts. The presence of gallic acid, sinapic, caffeic acid, vanillic acid, and *trans*-ferulic acid was observed [36]. However, the presence of 4-hydroxy benzoic acid and *p*-coumaric was not observed, whereas, in this work *p*-coumaric was identified and quantified at 16.6 ± 1.5 µM.

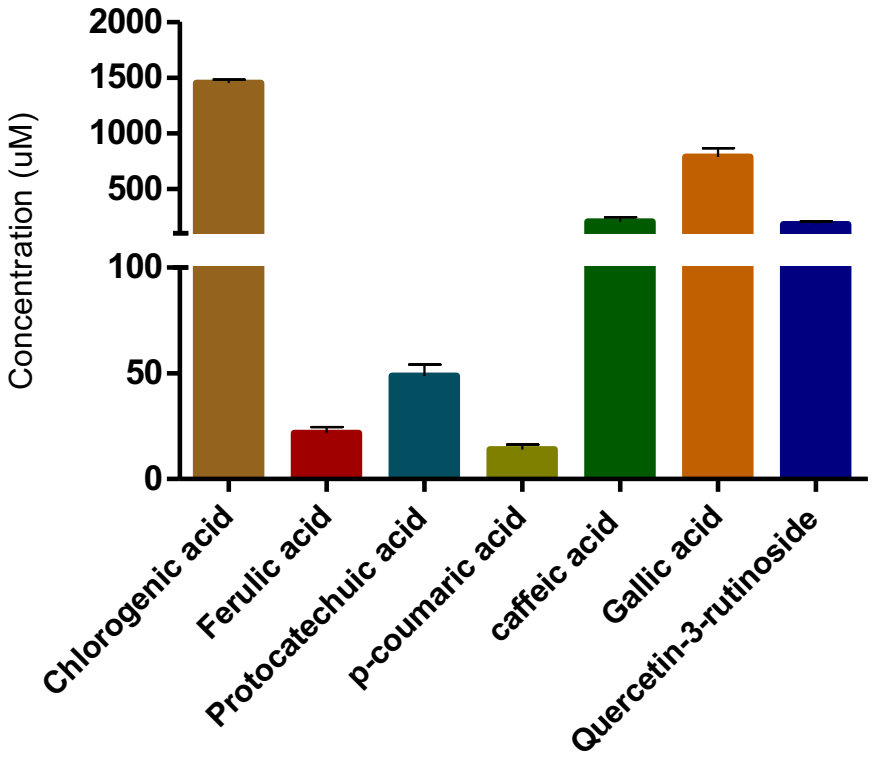
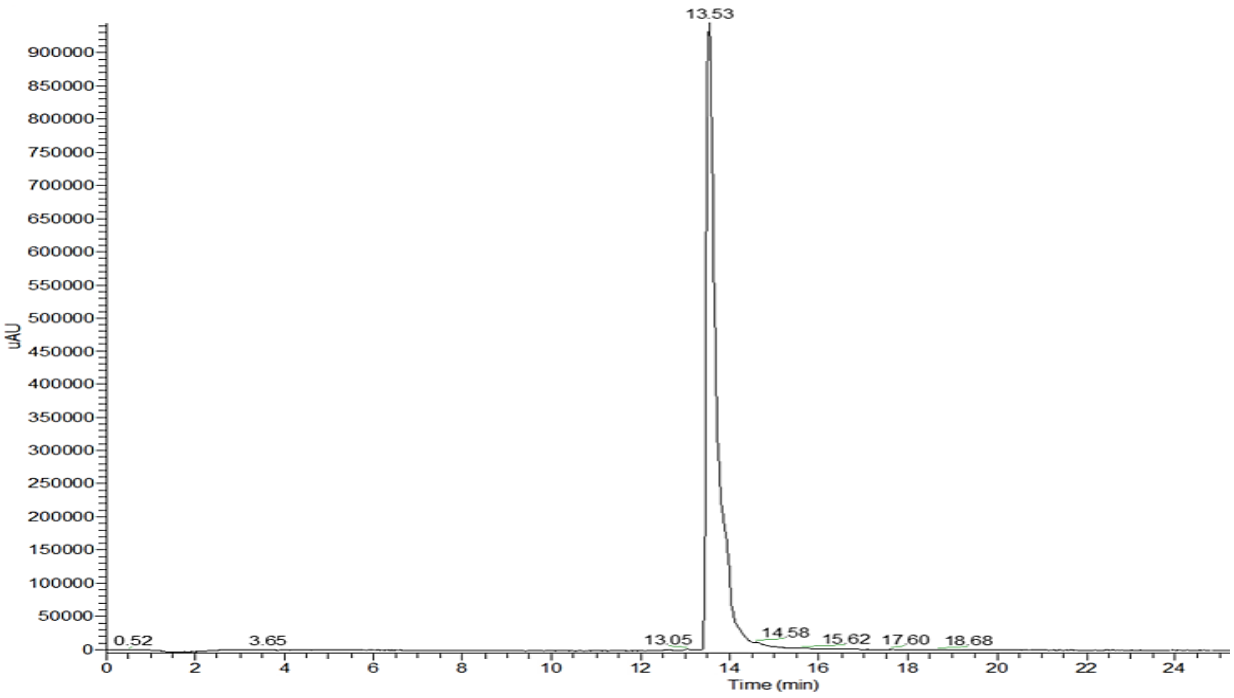


Figure 11. Concentrations of chlorogenic acid, ferulic acid, protocatechuic acid, p-coumaric, caffeic acid, gallic acid, and quercetin-3-rutinoside identified in methanolic extract.

Barberry polyphenolic profiles can differ based on different types, varieties of fruits, and maturity. In the current work, CGA was characterized from a *m/z* 353 and λ_{max} of 280 nm (**Fig. 12**). It was clear from the chromatogram, there were three peaks, one as the main peak and two peaks as confirmation peaks. From ESI-MS analysis of mono-acyl CGA, the following fragmentation patterns were yielded (**Table 3**). The *p*-coumaroylquinic acid (*p*CoQA) was characterized at a m/z of 337 $[M-H]^-$ and product ions at m/z 129 (decarboxylated coumaric acid); m/z 173 (dehydrated quinic acid) and m/z 191(quinic acid) ions. The ion representing quinic acid at m/z 191 was fragmented and product ion at m/z 129 was yielded by losing a water molecule and carbon oxide as part of the parent's molecular structure.

12a.



12b.

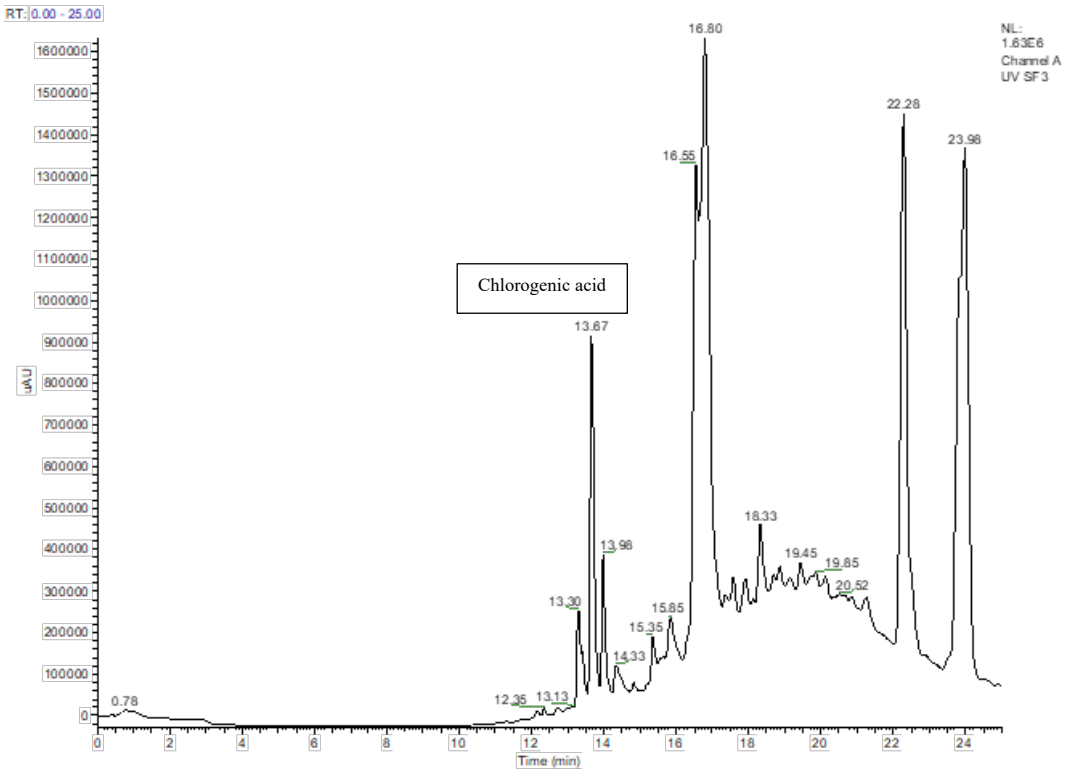


Figure 12. Chromatogram of CGA at 280 nm represents chlorogenic acid standard (a) compared to presence of this compound in methanolic extract of barberry (b).

The caffeoylquinic acid (CQA) was also characterized with a precursor ion at a m/z 353 $[M-H]^-$ and product ions were determined at a m/z of 85, 173, and 191. The product ions at m/z 85 were identified as decarboxylated caffeic acid, at m/z 191 was quinic acid which is formed by cleavage of the ester bond between the caffeic and quinic acid moieties. Further, quinic acid lost a water molecule to form dehydrated quinic acid at m/z 173, the product ion of quinic acid is explained by the presence of carboxylic acid as the functional group that can be easily ionized in the negative ion mode [48]. The feruloylquinic acid (FQA) was also determined at a m/z of 367 [M-

H]⁻ and with product ions at m/z 179, 191, and 231. Interestingly, a peak of dicaffeoylquinic acid (diCQA) was identified at a m/z of 515 [M-H]⁻. The MS fragmentation of this peak exhibited three products at m/z of 425, 353, and 191, CQA, and quinic acid, respectively. The fragment ion at m/z 353 could be as glycoside of CQA and at m/z 191 was quinic acid. The result was in agreement with a study that peaks were detected with a precursor ion at m/z 515 [M-H]⁻. Two ions were yielded fragment ions at m/z 353 and m/z 173 that identified as glycoside of 4-CQA [159].

Table 2. Product ions of CGA acid isomers were observed in the negative ion mode during CID-MS/MS analysis.

Compound (nm)	[M-H] (m/z)	First generation product ions (MS/MS)	Second generation product ions (MS^2)
p-CoQA	337	191, 173	129
CQA	353	191, 173	85
FQA	367	231, 191	179
diCQA	515	425, 353	191

In Vitro Screening Using Hole Diffusion Method

An *in vitro* antibacterial activity was performed on six types of bacteria with three different extracts of methanolic, ethanolic, and acetone. All six bacteria were inhibited after exposure to normalized barberry extracts at 5, 10, 15, 20, and 25 mg/mL using the hole diffusion test (**Table 4**). Antibacterial efficacy against tested bacteria was greater in the methanolic extract, likely due to its superior extraction of polyphenolics. Methanolic extracts impacted Gram-positive bacteria with inhibition zones for *L. monocytogenes* (10.3-20.7 mm), *E. faecium* (11.1-21.6 mm), *S. aureus*

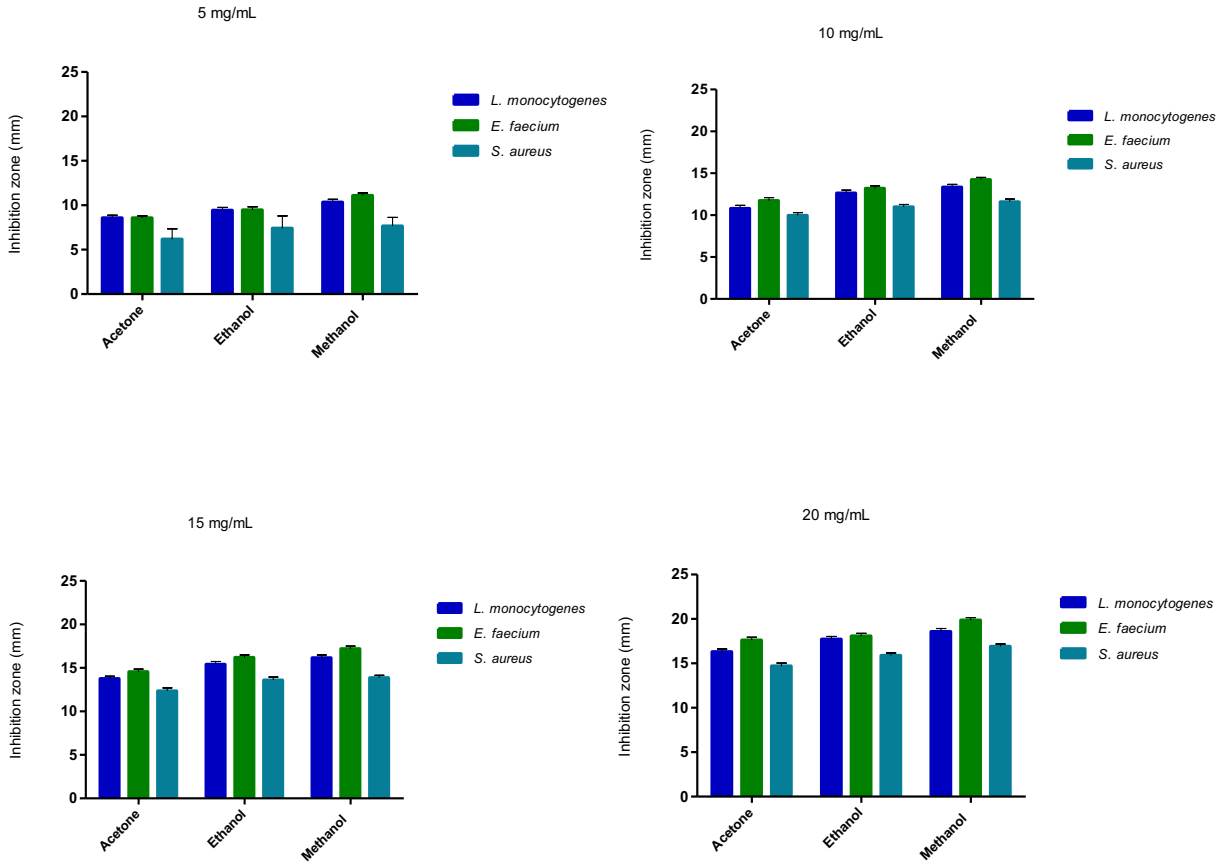
(7.6-19.5 mm). Methanolic extracts were found to be more effective against *E. faecium* at 5 mg/mL and 20 mg/mL compared to ethanol and acetone of free extracts while the effect of methanolic extract was significant at 25 mg/mL against *S. aureus*. However, no statistical difference observed for *L. monocytogenes* when compared to ethanolic extract. The methanolic extract at a concentration of 10 mg/mL inhibited *L. monocytogenes* at 13.3 mm, *E. faecium* at 14.2 mm, and *S. aureus* at 11.6 mm.

Table 3. Barberry extracts growth inhibition zone (mm) against Gram-positive bacteria.

Concentration (mg/mL)	Type of solvent	Gram-positive		
		L. monocytogenes	*E. faecium*	*S. aureus*
5	Methanol	10.3±0.33A	11.1±0.28A	7.6±0.98A
	Ethanol	9.4±0.33A	9.5±0.36B	7.4±1.40A
	Acetone	8.6±0.31B	8.6±0.24B	6.2±1.17A
10	Methanol	13.3±0.33A	14.2±0.26A	11.6±0.32A
	Ethanol	12.6±0.36A	13.2±0.28A	11.0±0.30A
	Acetone	10.8±0.36B	11.7±0.36B	10.0±0.33B
15	Methanol	16.2±0.32A	17.2±0.31A	13.9±0.27A
	Ethanol	15.4±0.33A	16.2±0.29A	13.6±0.34A
	Acetone	13.8±0.29B	14.5±0.32B	12.4±0.36B
20	Methanol	18.6±0.35A	19.9±0.28A	16.9±0.28A
	Ethanol	17.7±0.32A	18.1±0.32B	15.9±0.30A
	Acetone	16.3±0.31B	17.6±0.34B	14.7±0.32B
25	Methanol	20.7±0.32A	21.6±0.28A	19.5±0.36A
	Ethanol	20.2±0.30A	21.2±0.28A	18.4±0.31B
	Acetone	17.9±0.32B	19.4±0.33B	17.9±0.32B

Results are presented as means of the three observations.
Mean values ± standard error sharing the big letters in the columns are significant at $P<0.05$.

Fig. 13 shows the effect of methanol, ethanol and acetone of free barberry extracts on the growth inhibition zones against Gram-positive bacteria. The best inhibition property observed against *E. faecium* ranged from 11.09 to 21.59 mm. Mojaddar Langroodi et al. (2018) reported that the inhibitory effect of ethanolic extract of barberry was higher than aqueous extract [160]; however, in this work the effect of aqueous extract was not tested compared to the other alcoholic extracts.



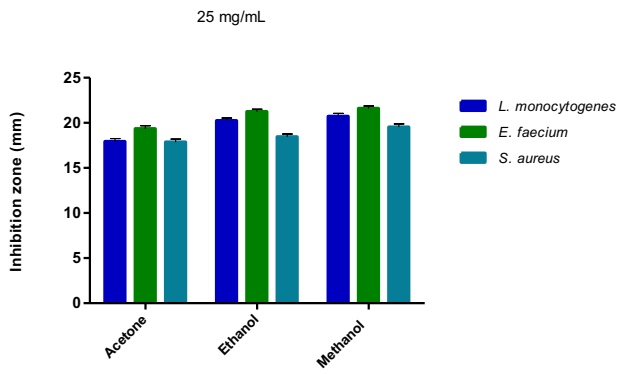


Figure 13. Effect of methanol, ethanol and acetone of free barberry extracts on the growth inhibition zones against Gram-positive bacteria at an exposure time of 24 h at 37 °C.

The results of exposure to the methanolic extract of free barberry samples against Gram-negative bacteria including *E. coli* O157:H7 (6.000-19.99 mm), *S.* Typhimurium (5.593-19.61 mm), and *P. aeruginosa* (5.070-17.08 mm) is shown in **Table 5**. The effect of methanol treatment was found to be more effective against *E. coli* O157:H7 at the concentrations of 10 mg/mL, 15 mg/mL, 20 mg/mL, and 25 mg/mL while it was not statistically different at 5 mg/mL. A comparison between methanol and ethanol of free barberry extracts indicated that there is no difference among these two extracts against *S.* Typhimurium at the concentrations of 5 mg/mL, 15 mg/mL, 20 mg/mL, and 25 mg/mL while the inhibitory effect was observed at the concentration of 10 mg/mL. So that, 10 mg/mL of methanolic extract inhibited *E. coli* O157:H7 at 10.3 mm whereas the zone inhibitions were 8.6 mm and 8.0 mm for ethanol and acetone, respectively. Also, the methanolic extract exhibited the zone inhibitions at 12.1 mm against *S.* Typhimurium and 9.6 mm against *P. aeruginosa*. The observed inhibitory effect of methanol extract was significant compared to ethanol only at a concentration of 20 mg/mL against *P. aeruginosa*. The highest and lowest antimicrobial activities of methanolic extract among other samples were found to be against *E. coli* O157:H7 and *P. aeruginosa*, respectively (**Fig. 14**). However, the hole diffusion test

sometimes has a limitation such as the ability of compounds, especially hydrophobic molecules to penetrate through media agar.

Table 4. Barberry extracts growth inhibition zone (mm) against Gram-negative bacteria.

Concentration (mg/mL)	Type of solvent	Gram-negative		
		E. coli O157:H7	*S.* Typhimurium	*P. aeruginosa*
5	Methanol	6.0±1.15A	5.6±1.40A	5.1±1.27A
	Ethanol	5.6±1.07A	5.4±1.35A	4.7±1.18A
	Acetone	0.0±0.00B	0.0±0.00B	0.0±0.00B
10	Methanol	10.3±0.32A	12.1±0.31A	9.6±0.30A
	Ethanol	8.6±0.30B	10.8±0.31B	8.7±0.28A
	Acetone	8.0±0.23B	10.3±0.33B	8.5±0.22B
15	Methanol	14.5±0.32A	15.0±0.35A	12.1±0.29A
	Ethanol	11.8±0.32B	14.0±0.33A	11.2±0.30A
	Acetone	10.8±0.35B	13.8±0.32A	10.6±0.33B
20	Methanol	16.5±0.30A	16.9±0.35A	14.6±0.33A
	Ethanol	14.5±0.35B	16.9±0.26A	13.0±0.34B
	Acetone	13.4±0.30B	16.7±0.30A	13.0±0.26B
25	Methanol	20.0±0.31A	19.6±0.32A	17.1±0.31A
	Ethanol	18.16±0.26B	18.98±0.31A	16.00±0.30A
	Acetone	17.02±0.33C	18.49±0.33A	15.59±0.32B

Results are presented as means of the three observations.
Mean values ± standard error sharing the big letters in the columns are significant at $P<0.05$.

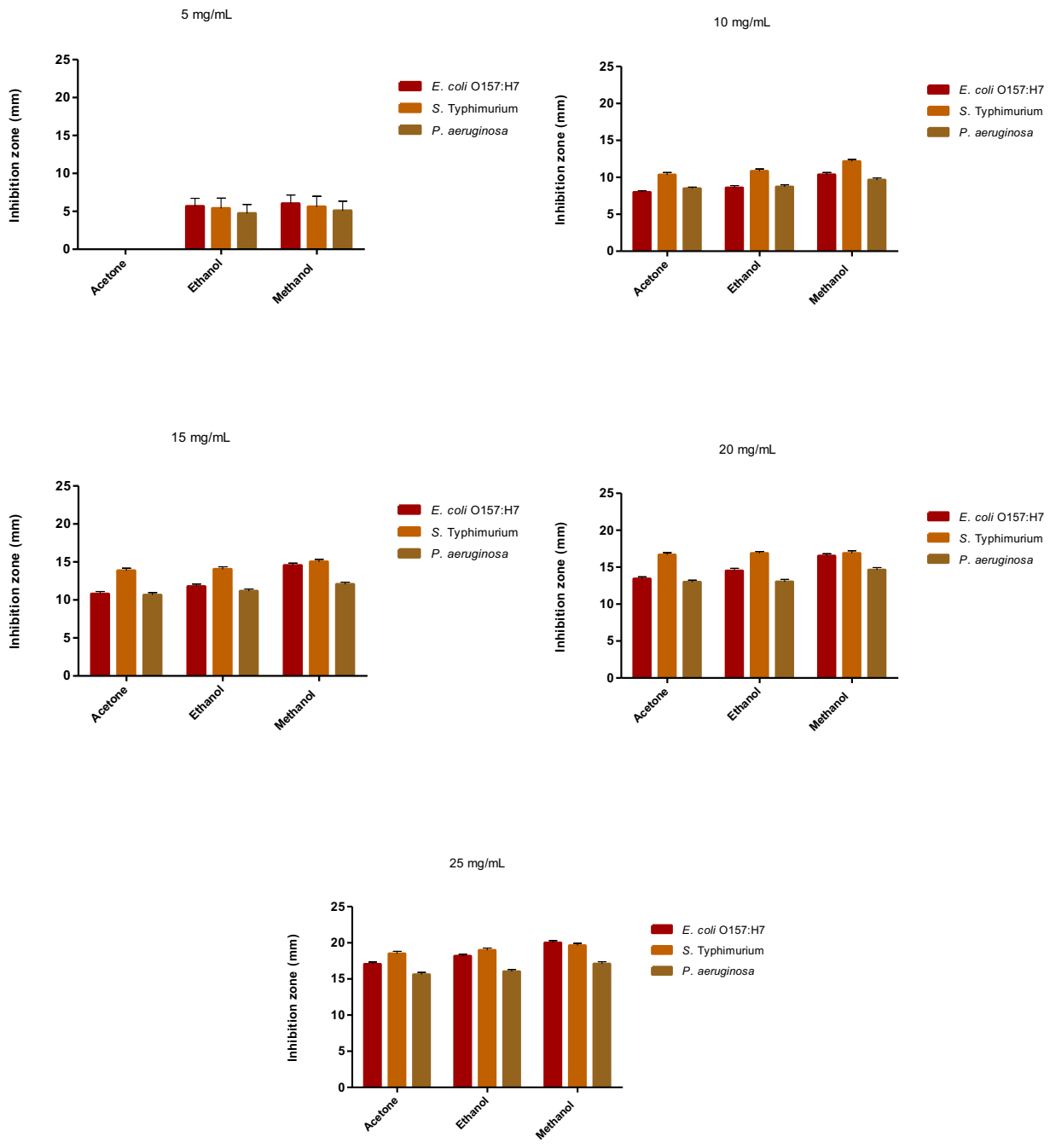


Figure 14. Effect of methanol, ethanol and acetone of free barberry extracts on the growth inhibition zones against Gram-negative bacteria at an exposure time of 24 h at 37 °C.

The effect of the free barberry extract can be explained by the differences in the cell wall of bacteria between Gram-positive and Gram-negative bacteria. Gram-positive have a thick layer of peptidoglycan (50%) in their cell wall and a long anionic polymer called teichoic acid (40%), threading through the peptidoglycan layer. The teichoic acids are covalently bound to peptidoglycan in the bacterial cell wall. Another molecule is lipoteichoic acid which is attached to the head group of membrane lipid. Therefore, there is a negative charge on the surface of bacteria due to glycerol phosphate and glucosyl phosphate [161]. Gram-negative bacteria have three layers of a thin peptidoglycan layer, a cytoplasmic inner membrane, and an additional outer lipid membrane (OM) of lipopolysaccharide (LPS). LPS is considered as glycolipid, lipoprotein (LP), and ß-barrel protein molecules which are maintained together by electrostatic interaction with divalent cation.

Antibacterial Activity of Free Barberry Extract In Vitro Screening Model

After confirmation of the antibacterial properties of free barberry extracts, the antibacterial effect was confirmed in a broth microdilution method (**Table 6**). The inhibitory effect of free extract was tested at the concentrations of 0.312, 0.625, 1.25, 2.5, 5, 6, 7, 8, 9, and 10 mg/mL. The free extract was found to be more effective against Gram-positive bacteria overall.

Table 5. Effect of free extract on the growth of bacteria strains at concentrations of 0.312, 0.625, 1.25, 2.5, 5, 6, 7, 8, 9, and 10 mg/mL for 24 h exposure time at 37 °C.

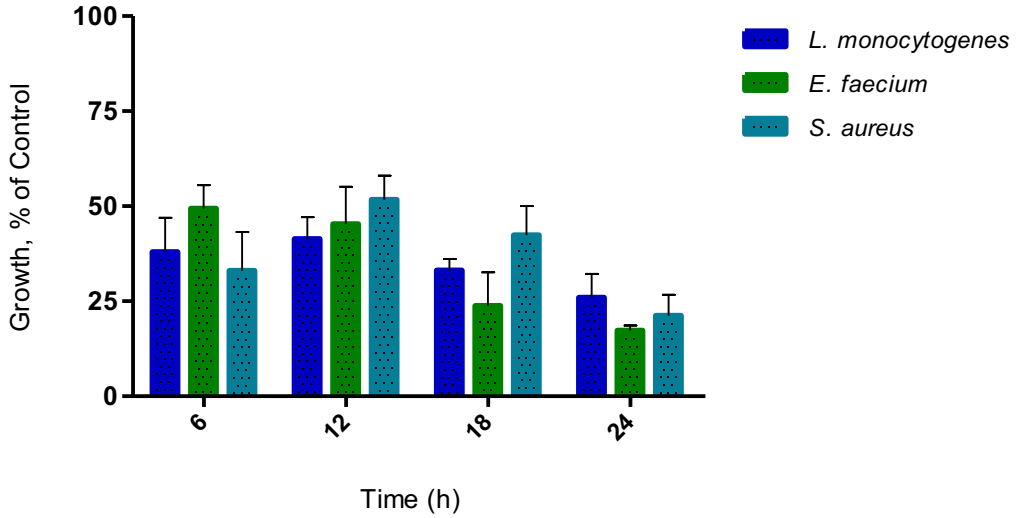
Type of Bacteria	Concentration (mg/mL)										
	Control	0.312	0.625	1.25	2.5	5	6	7	8	9	10
L. monocytogenes	0.9±0.27	0.8±0.32	0.8±0.28	0.7±0.26	0.7±0.24	0.6±0.22	0.6±0.22	0.5±0.20	0.5±20	0.4±0.17	0.5±0.18
E. faecium	0.2±0.49	1.0±0.46	0.9±0.39	0.8±0.35	0.7±0.26	0.6±0.23	0.7±0.25	0.6±0.25	0.7±0.26	0.6±0.23	0.6±0.26
S. aureus	0.9±0.34	0.7±0.36	0.7±0.37	0.8±0.38	0.6±0.31	0.7±0.35	0.7±0.32	0.6±0.32	0.5±0.34	0.5±0.33	0.5±0.28
E. coli O157:H7	0.8±0.35	0.5±0.18	0.5±0.17	0.5±0.17	0.5±0.14	0.4±0.14	0.4±0.13	0.4±0.14	0.4±0.13	0.4±0.13	0.4±0.12
S. Typhimurium	0.7±0.35	0.5±0.18	0.5±0.16	0.5±0.15	0.4±0.14	0.4±0.15	0.4±0.13	0.4±0.13	0.4±0.12	0.3±0.13	0.3±0.10
P. aeruginosa	0.8±0.36	0.7±0.37	0.7±0.32	0.7±0.35	0.7±0.33	0.7±0.35	0.7±0.34	0.7±0.35	0.6±0.34	0.6±0.33	0.5±0.29

Values represent the means and standard errors of growth curve of bacteria strains from triplicate at 37 °C for 24 h incubation time.

The growth of Gram-positive and Gram-negative bacteria was analyzed after 6 h, 12 h, 18 h, and 24 h exposure to a concentration of 10 mg/mL of free extract (**Fig. 15**). The results indicated that *L. monocytogenes* had a growth of 38%, 41%, 33% and 30% at 6 h, 12 h, 18 h, and 24 h after incubation time whereas, *E. faecium* exhibited a growth of 49% at 6 h, 45% at 12 h, 24% at 18 h, and 17% at 24 h. Also, *S. aureus* demonstrated similar growth to *L. monocytogenes* and *E. faecium*, so that, the growth was continued by 33% and 52% at 6 h, and 12 h, respectively. The growth of *S. aureus* was decreased by 42% at 18 h, and 21% at 24 h. According to the result, *L. monocytogenes* was the strongest microorganism toward the treatments, following were *S. aureus*, and *E. faecium*.

Gram-negative bacteria were more resistant toward free barberry extract. *E. coli* O157:H7 exhibited a growth of 58%, 59%, 42%, and 21% at 6 h, 12 h, 18 h, 24 h, whereas *S.* Typhimurium had a growth of 64% at first 6 h and 49% at 12 h. However, the growth of this microorganism reduced to 37% at 24 h. *P. aeruginosa* had a growth of 43% at 6 h, an additional growth by 63% at 12 h, but the growth of this microorganism was decreased to 38% at 24 h. *E. coli* O157:H7 demonstrated more resistant toward the free treatments, following were *S.* Typhimurium and *P. aeruginosa*.

15a.



15b.

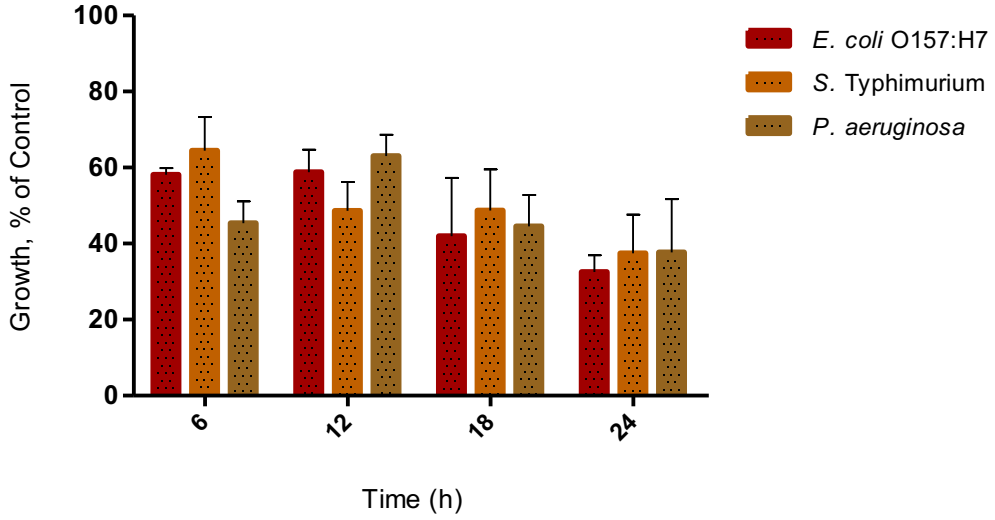


Figure 15. Effect of free barberry extracts on the growth of Gram-positive (a) and Gram-negative (b) bacteria over 24 h at 37 °C.

Barberry fruits contain a diversity of polyphenolics, mostly hydroxycinnamic acids with the potential to have synergic effects when present in whole food extracts. A study was evaluated the antibacterial activity of a mixture of cinnamic, *p*-coumaric, caffeic, and ferulic acid to establish whether the interaction between phenolic acids can influence *L. monocytogenes*. The combined effect of *p*-coumaric acid and caffeic acid were greater or equal to the sum of the effects observed with tested acids alone at the tested concentrations between 0.0% and 0.5% (w/v) [75]. The mechanism of phenolic acids such as CGA can be compared to common organic acids used in the food industry as surface and food-contact sanitizers due to their ability to penetrate bacteria cells walls and dissociate, resulting in acidification of the cytoplasm and eventual cell death [162]. In Gram-negative bacteria, the electrostatic interaction maintains the integrity of OM. Generally, in this type of bacteria, the outer membrane is partially permissive for hydrophobic compounds and functions as a barrier that allows only small hydrophilic compounds to pass [64]. The effect of CGA on *S. pneumoniae* membrane integrity was previously characterized and was found to first disrupt the cell membrane permeability and then depolarize the cell membrane leading to an ability for cells to maintain metabolites [76]. In another study on the effect of 25% CGA treatments against *P. aeruginosa* P1 and revealed that CGA likely caused leakage of intracellular proteins and ATP and also disrupted intracellular membrane permeability resulting in the release of cell components [77].

MIC and MBC of the Treatments

The MIC and MBC of free extract were assessed using a microdilution assay with a concentration of 0-18 mg/mL. **Table 7** shows the MIC and MBC value presented the differences OD values as a function of time at 0 h and 24 h. The MIC was the lowest concentration of antibacterial agent that inhibited the growth of bacterial pathogens, determined for methanolic free

barberry extract [163]. Minimum bacterial concentration (MBC) assay was completed following the completion of MIC assay and determined as the lowest concentration of the free extract that killed 99.99 % of the targeted bacterial pathogen. The MICs of *L. monocytogenes*, *E. faecium*, and *S. aureus* were lower at the tested concentration compared to that of *E. coli* O157:H7, *S.* Typhimurium, and *P. aeruginosa* at the same tested concentrations. However, the results showed that there was no significant difference between *S. aureus* ~ *E. faecium* samples, whereas *L. monocytogenes* was exhibited a greater MIC and MBC with values of 15 mg/mL and 17 mg/mL, respectively.

The concentration higher than 18 mg/mL was required to achieve a minimum inhibitory and bactericidal effect for mentioned *S.* Typhimurium and *P. aeruginosa*, whereas *E. coli* O157:H7 was inhibited at a MIC value of 17 mg/mL and MBC value of 18 mg/mL. A study previously suggesting that *S. aureus* and *S.* Typhimurium were more sensitive to grape stem polyphenolic extract (rich in GA and CGA) at a MIC value of 16 g/L, while *L. monocytogenes* and *E. coli* O157:H7 exhibited a higher MIC value at a concentration of 18 g/L [164].

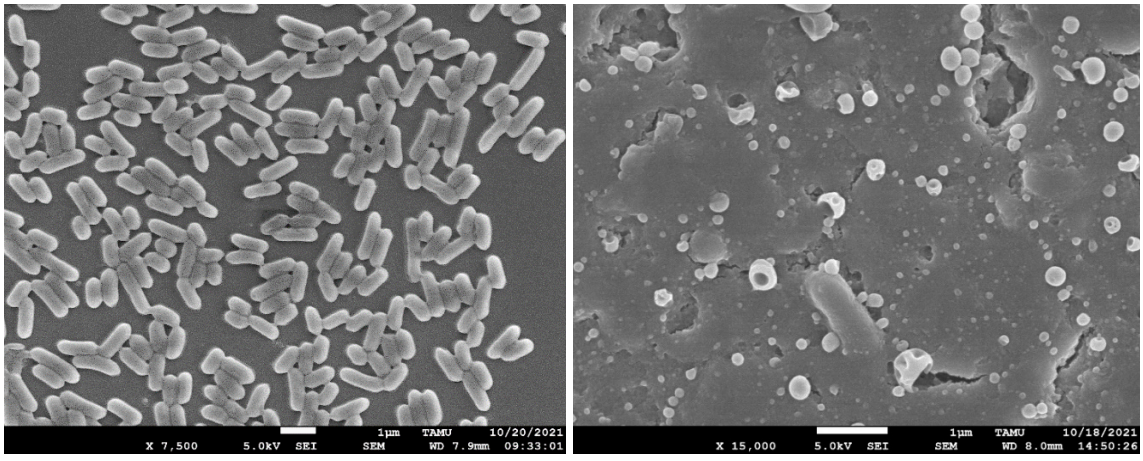
Table 6. The MIC and MBC of free extract against tested bacteria after treatments for 24 h at 37 °C.

Type of Bacteria	MIC (mg/mL)	MBC (mg/mL)
L. monocytogenes	15	17
E. faecium	> 11	13
S. aureus	> 11	13
E. coli O157:H7	17	18
S. Typhimurium	> 18	>18
P. aeruginosa	> 18	>18

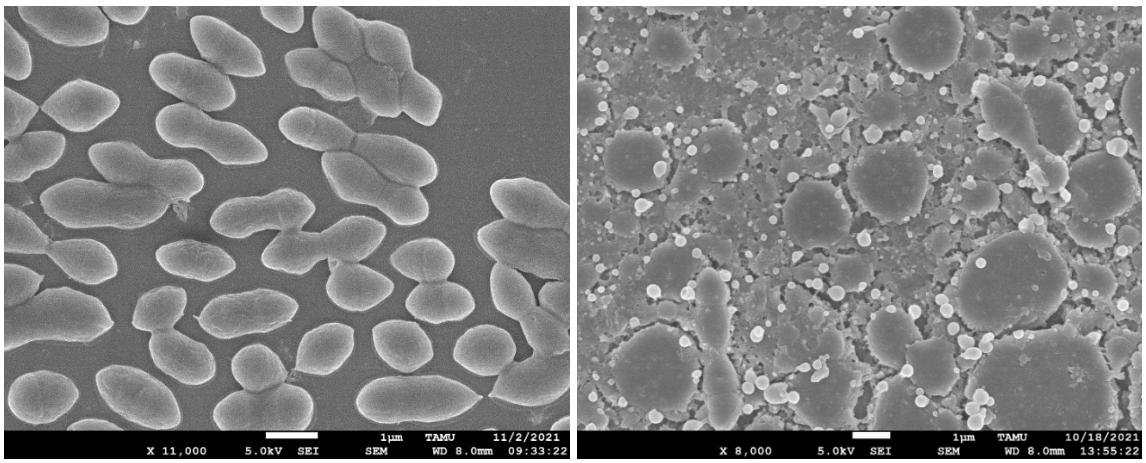
Influence of Barberry Polyphenolic Extract on the Bacterial Cell Morphology

The effects of the free extract on the cell morphology were visualized after 24 h of exposure at a concentration of 10 mg/mL and examined by SEM (**Fig. 16**). The untreated *L. monocytogenes* showed a normal cell morphology with a rod shape, smooth surface, and intact cell structure (**Fig. 16a**). Treated *L. monocytogenes* cells displayed morphological damage after treatment at 37 °C for 24 h. As seen (**Fig. 16b**) the collapse and deformation of cells, as well as the leakage of intracellular components is evident. As seen in the control group (**Fig. 16e**), the cells of *S. aureus* are smooth, but after free barberry treatment for 24 h, there were vesicular and irregular changes on the surface and what appears to be intracellular materials around the cells (**Fig. 16f**). These results are in agreement with a study [165] that evaluated the effect of vine tea extract (VTE) on the morphology of *S. aureus*. After a 12 h exposure a large amount of intracellular materials around the cells was observed.

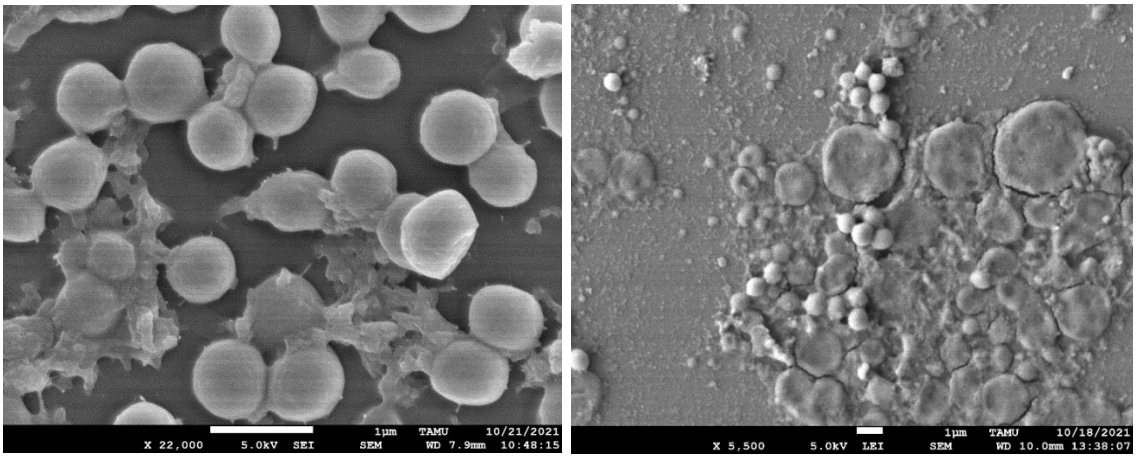
a. **b.**



c. **d.**



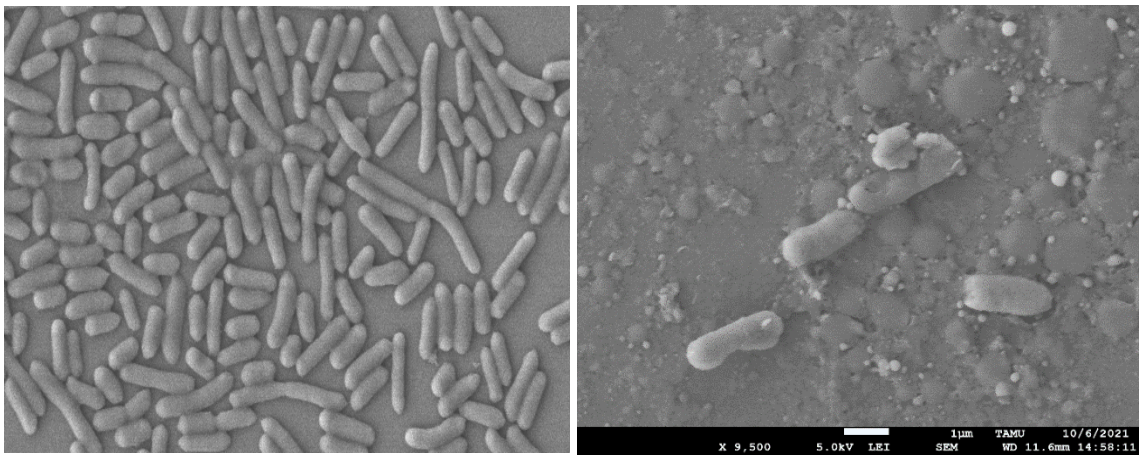
e. **f.**



In **Fig. 16h** as compared to the untreated control *E. coli* O157:H7 cells, free barberry extract revealed damage to the cell (**Fig. 16g**). Untreated cells exhibited rod-shaped and intact morphology, while the bacterial cell treated with the free extract showed damaged cell walls, shrunken cells, and deformed cell morphology. The result of a study indicated an irregular and striated wall for *E. coli* O157:H7 untreated cells, while treated cells with Spanish oregano at the MICs of 0.025, 0.05, and 0.05 % (vol/vol) showed holes or white spots on the cellular wall [166].

A possible mechanism of the antibacterial activity of weak acid may be due to the impact of pH on the proportion of non-dissociated acid molecules in solution and lipophilicity parameter (logK) [167]. The higher the proportion of non-dissociated forms of phenolic acids can occur in the higher pKa. A decrease in pH of media due to the presence of phenolic acids treatments may be associated with the effect of those compounds on bacterial growth through the penetration of their non-dissociated forms into the cells, causing an acidification of the cytoplasm and leading to K^+ efflux and cell death gradually[168]. The dissociation constant (p*K*) of CGA is 3.4 compared to other phenolic acids, including *p*-coumaric acid, 4.57; ferulic acid, 4.45; caffeic acid, 4.45, and chlorogenic acid, 3.4. Therefore, the effectiveness of this compound can be lower than hydroxycinnamic acids such as *p*-coumaric, ferulic acid, and caffeic acid [167].

g. **h.**



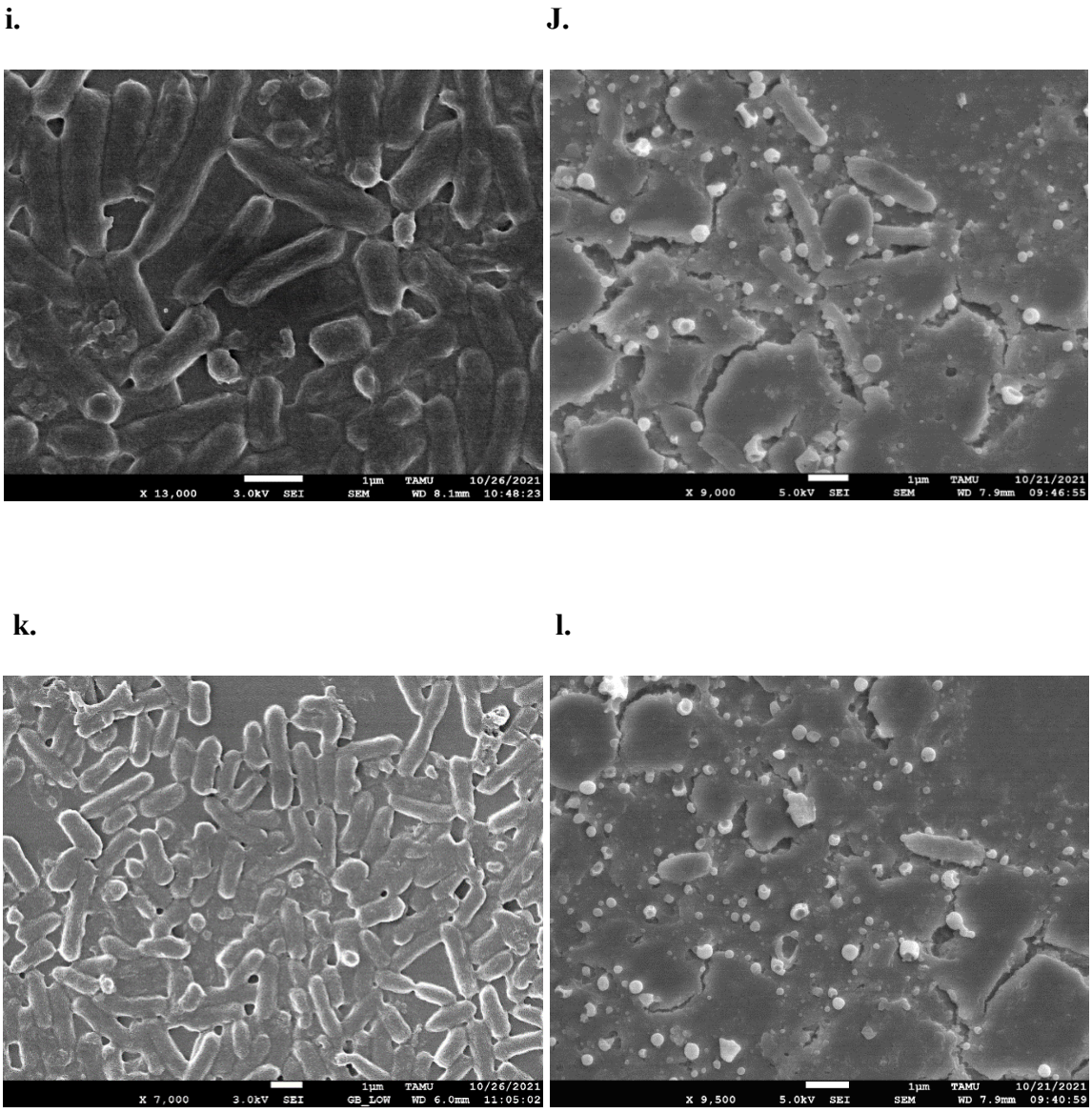


Figure 16. Scanning electron micrograph of untreated *L. monocytogenes* (a), *E. faecium* (c), *S. aureus* (e), *E. coli* O157:H7 (g), *S.* Typhimurium (i), and *P. aeruginosa* (k) and treated bacteria of *L. monocytogenes* (b), *E. faecium* (d), *S. aureus* (f), *E. coli* O157:H7 (h), *S.* Typhimurium (j), and *P. aeruginosa* (l) with free extract at the concentration of 10 mg/mL for 24 h at 37 °C.

Conclusions

The polyphenol compositions of methanolic, ethanolic, and acetone barberry fruit extracts were studied for their total reducing capacity, total flavonoid, total anthocyanins, monomeric and polymeric anthocyanins contents in the current work. 70% methanol had the highest total reducing capacity for polyphenolic recovered in three extractions at 57.4 mg GAE/g dried fruit compared to 45.1 and 40.3 mg/g dried fruit for 70% ethanol and 70% acetone, respectively. The highest TFC amount of methanolic extract was 10.7 mg rutin per g of dried fruit weight. The results indicated that methanolic extract was also an efficient solvent by extracting anthocyanins at 483.4 mg pelargonidin-3-*O*-glucoside equivalents per g dried weight. In the present work, the concentration of CGA, ferulic acid, protocatechuic acid, *p*-coumaric, caffeic acid, gallic acid, and quercetin-3-rutinoside were found to be at 1475 ± 31 μM, 16.6 ± 1.5 μM, 43.6 ± 2.8 μM, 10.2 ± 0.02 μM, 118 ± 4.8 μM, 686 ± 35 μM, and 154 ± 15 μM for free methanolic extract, respectively. Additionally, barberry fruit extract exhibited a rich source content of CGA compound and its geometric isomers of *p*CoQA, CQA, FQA, and diCQA with molecular weights of 337, 353, 367, and 515 Da. However, the comparison of identified compounds with external pure standards could be a proper way to accurately identify and quantify the targeted compounds.

The methanolic extract showed the highest antibacterial efficacy against tested bacteria among other free extract, likely due to its superior extraction of polyphenolics. From the hole diffusion result, methanolic extracts impacted Gram-positive bacteria with inhibition zones for *L. monocytogenes* (10.3-20.7 mm), *E. faecium* (11.1-21.6 mm), *S. aureus* (7.6-19.5 mm). The result of the inhibitory effect of methanolic extract of free barberry samples against Gram-negative bacteria including *E. coli* O157:H7 (6.0-20.0 mm), *S.* Typhimurium (5.6-19.6 mm), and *P. aeruginosa* (5.1-17.1 mm). Also, the inhibitory effect of methanol extract was significant only at

a concentration of 20 mg/mL against *P. aeruginosa* compared to ethanol. For the first time, the SEM morphology of six untreated and treated with methanolic free polyphenolic extract rich in CGA were shown in this work. The untreated *L. monocytogenes* showed a normal cell morphology with a rod shape, smooth surface, and intact cell structure while morphological damage, collapse, and deformation of cells were observed after 24 h of incubation time. Untreated cells of *E. coli* O157:H7 exhibited rod-shaped and intact morphology, while the bacterial cell treated with the free barberry extract showed a damaged cell wall, shrunken cells, and deformed cell morphology.

CHAPTER IV

IN VITRO ANTIBACTERIAL EVALUATION OF NANOPARTICLE BARBERRY POLYPHENOLICS

Introduction

Pluronic® F127 is an ionic polymer and has a polar compatibility with most compounds. They can form into micelles with hydrophobic PPO center core and hydrophilic PEO outer shells that interact with water [6]. The nanoparticle of barberry polyphenols with Pluronic® F127 polymer may improve the delivery of these bioactive compounds to the bacteria cell due to an improvement in size and a charge of the molecule which has penetrated through bacteria cell walls resulting in reactive oxygen species (ROS) production, and finally damage DNA, protein, and lipid within bacteria cell structures. Nanoparticles may enhance cellular uptake with a mechanism involved in the interaction of NPs and bacteria cell walls either through an accumulation in cytoplasm and interference with cellular functionality or potential disruption of membrane integrity leading to the leakage of intracellular constituents.

Barberries are rich in plant-derived polyphenols such as phenolic acid and anthocyanin that small compounds that are known to possess a health benefit to the host. The bioactivity of the phenolic acids including CGA found in barberry has been widely investigated and received much attention due to potential antimicrobial, antidiabetics, and anticarcinogenic properties. Regarding phenolic acid biological activities in the human body, they need to withstand the different conditions of the digestive system. Stability is the ability to keep the structure and content once they are subjected to various circumstances such as (pH, light, temperature, oxygen, the presence

of enzyme proteins, and metallic ions) (Patras et al., 2010). Despite the health promising effects, the application of polyphenols with barberry extract has been restricted to their high rate of metabolism, and excretion from the body makes them less bioavailable to reach the systemic circulation. Recently, nanoencapsulation has been an effective approach to enhance the stability of the susceptible compounds in *in vitro* simulated gastrointestinal digestion and colonic fermentation [169]. In addition, the usage of polymeric coating material may improve the stability, uniformity of particle size, drug loading capacity, and controlled release of nanoparticle polyphenols in the gastrointestinal (GI) digestive system.

There is limited research work regarding the identification and synthesis of a stable nanoparticle of barberry extract that can function as an antimicrobial agent and withstand the low pH of gastric fluid, enzymes, and basic condition of the intestinal during the digestion system. Therefore, the aim of this research chapter was to synthesize a nanoparticle of barberry extract to evaluate the antibacterial property of free and NP barberry polyphenolics in *in vitro* model against pathogenic Gram-positive and Gram-negative bacteria strains. Also, the stability of free and NP barberry will be studied in the next chapter.

Material and Methods

Nanoparticle Preparation

free barberry polyphenolic-loaded polymeric NPs were prepared using a nano-precipitation method to determine the best quality of nanoparticles as previously reported (Akbulut et al., 2009). The method for the preparation of polymeric nanoparticles was developed using Pluronic® F127, tetrahydrofuran (THF) solvent. Pluronic® F127 as amphiphilic triblock copolymer along with barberry polyphenol samples was dissolved in THF solvent (**Fig. 17**). The solution was then

impinged rapidly against milli-Q water to produce NP barberry. The flow rates of water and THF solution were 50.0 mL/min and 5.0 mL/min, respectively. Following the encapsulation process, the NP-contained solution was dialyzed for 6 h to remove tetrahydrofuran solvent from the samples.

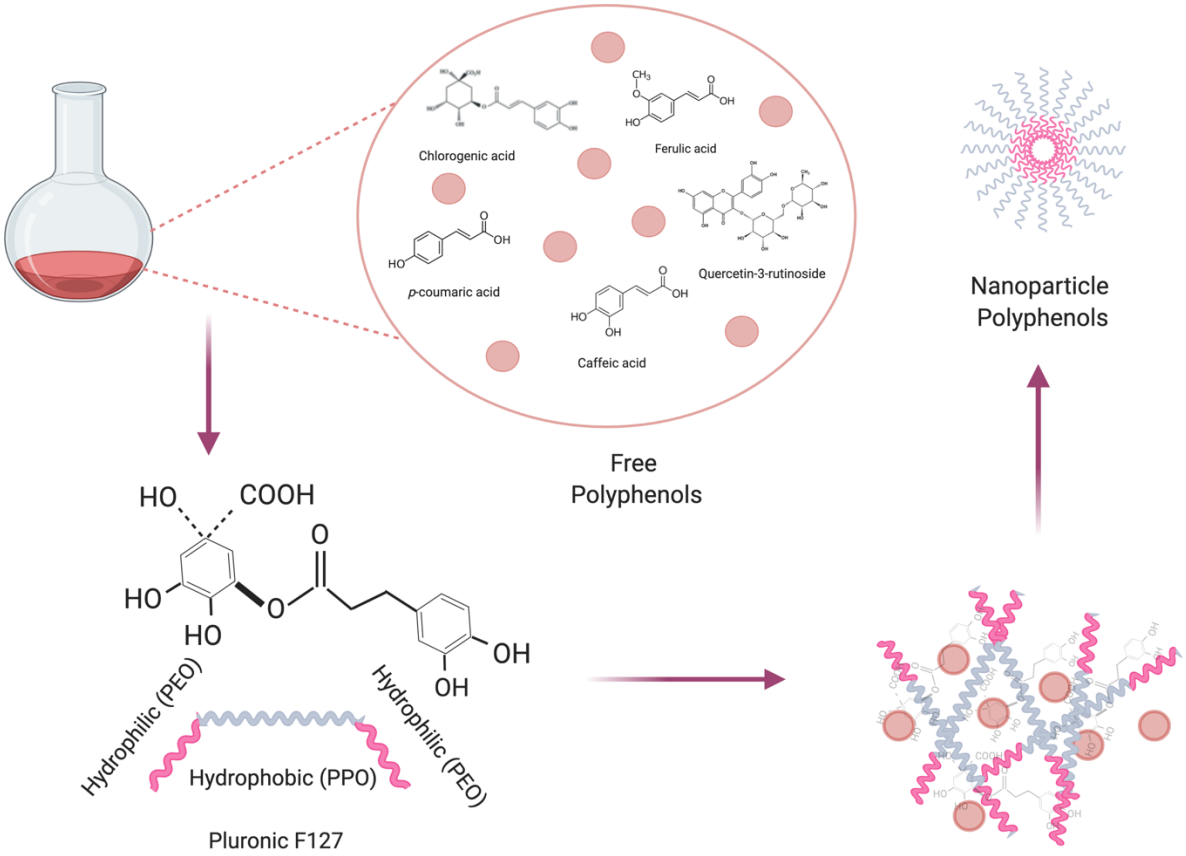


Figure 17. A schematic illustrating the preparation of nanoparticle barberry polyphenolic extract, created with BioRender.com (2021).

Characterization of nanoparticles and Surface Charge of Nanoparticles

Dynamic light scattering (DLS) and Ζ-potential were performed to determine the particle size distribution and surface charge of nanoparticle barberry using Zetasizer ZS90 analyzer

(Malvern Instrument, L.t.d., Westborough, MA) by adjusting specimens in 1 cm path length at 95° scattering angle, with a pinhole set to 20 μm [170].

Encapsulation Efficiency

Encapsulation efficiency (EE%) was measured by using a UV-1800 UV/Visible scanning spectrophotometer (Shimadzu Corp., Columbia, MD) at 310 nm according to following method previously reported [171]. Briefly, the nanoparticles of barberry polyphenols with PF127 were sonicated for 5 min and then vortexed for a few seconds. The compounds were separated using a centrifuge at 14,000 × g for 30 min. The resulting supernatant was collected and quantified at 520 nm. To determine the EE of barberry polymeric NPs, a calibration curve was obtained by linear regression and its standard equation. The corresponding results were then plotted in the standard curve equation to determine the encapsulation efficiency. The EE% was calculated by the following equation:

$$\textbf{EE (\%)} = {}^{Amount\ of\ extract\ in\ NPs}/_{Total\ amount\ of\ extract} \times 100\%$$

In Vitro Dissolution Release from Pluronic Formulation

The *in vitro* release behavior study of NPs barberry polyphenolic compounds was carried out by the membrane equilibrium method [172]. Briefly, a 10 mg/mL suspension of NPs was prepared Milli-Q water (pH 7.4). About 10 mL of prepared NPs suspension was placed in equilibrium membrane pouches with 12-14 kDa molecular weight cut-off (MWCO) (Thermo Scientific Inc., Rockford, IL, USA) and with a pore diameter of 2.0 nm. The THF solvent was released by

submerging each pouch into a beaker containing 2000 mL Milli-Q water at 25 ºC for 5 h. To mimic the physiological condition of body systems, the bags were tied and then immersed in 200 mL of Milli-Q water (pH 7.4) using screw-capped glass bottles. The bags were stirred gently with an orbital shaker under 90 rpm and at 25 ºC. At specific time intervals of 0, 1, 2, 4, 8, 16, 24, 48, 72, 96, 120, 144, and 168 h, 3 mL of release medium were taken and analyzed by UV-1800 UV/Visible scanning spectrophotometer (Shimadzu Corp., Columbia, MD) at the absorbance range of 200-800 nm. The kinetic analyses of the release data were performed according to the highest correlation coefficient (R^2).

Scanning Electron Microscopy (SEM)

Characterization of the size and shape of nanoparticles barberry extract was determined using a scanning electron micrograph (SEM). And followed by the method previously reported [148]. Briefly, the targeted bacteria cells were treated with 5 mg/mL of NP extract, and the culture was incubated for 24 h at 37 °C. The pellets were collected and washed with Milli-Q water to remove the media residuals. The cells samples were then fixed on dry poly-lysine coated glass coverslips using glutaraldehyde (2.5 w/v%) for 2 hours under the fume hood, then were washed using 0.1 molar cacodylate buffer gently for 10 min three times. The Post fixed-step was followed by applying 1 wt% osmium tetroxide on the poly-lysine coated samples at ambient temperature for 1 h. The cacodylate buffer and dehydrated first with 50% ethanol solvent for 30 seconds were applied to the cells. The dried specimens were coated with ~10 nm carbon in an evaporator and scanning electron micrographs were imaged using Scanning Electron Microscope (SEM) at Material Characteristic Facility (MCF) in Texas A&M University.

Statistical Analysis

All experiments were replicated three times. All results were expressed as mean ± standard error (SE) using GraphPad Prism version5. Significant differences between means of the treatments were identified by ANOVA (analysis of variance) followed by Tukey's honesty significant differences (HSD) and Bonferroni test comparing each pair with the control.

Results and Discussion

Size Distribution and Zeta-Potential of Nanoparticle Extract

The shape and size of NP barberry extract were determined using a scanning electron micrograph (SEM) (**Fig. 18**). The average size of free barberry extract was determined using dynamic light scattering (DLS) (Malvern Instrument, Ltd., Westborough, MA) for particles made from 1:1.6 ratio barberry extract:PF127.

a. **b.**

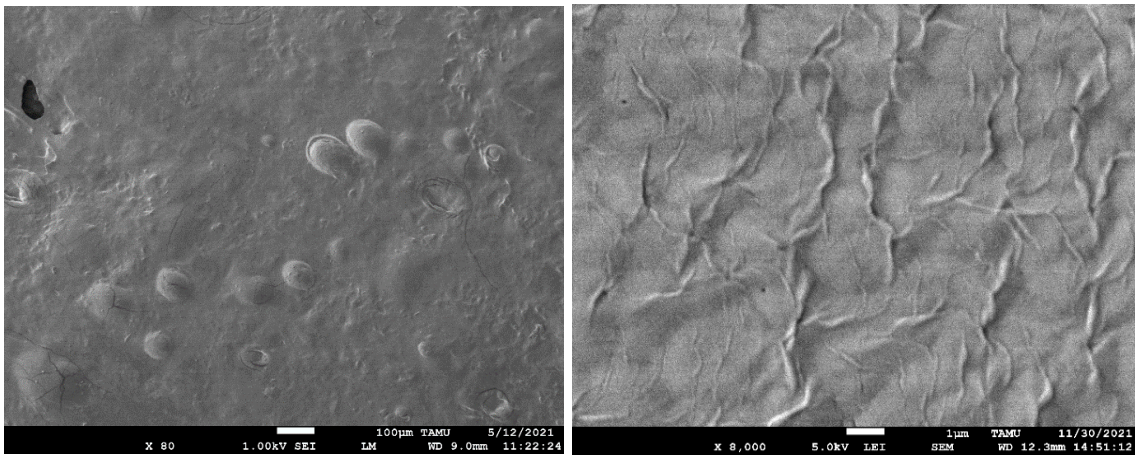


Figure 18. Scanning electron micrographs (SEM) represents free barberry (a) and PF127 polymer (b).

The result showed that the hydrodynamic size of NP extract ranged from 10 to 100 nm (0.01-0.1 μm) with a polydispersity index (PDI) of 0.39 at ambient temperature (**Fig. 19**). The result was in agreement with a study that previously reported a particle size of 66 nm when berberine was loaded in shellac using P407 as a stabilizer [173]. The silver NP of aqueous barberry from leaves and fruits extracts were showed an average size of 30-60 nm with a polydispersity value of 0.281[174], which was consistence with the result in this work. The study of the electrophoretic movement in the nanoparticle was expressed as zeta-potential (ZP). free barberry extract showed the ZP of -0.63 ± 1.5 mV, whereas NP exhibited the ZP at 0.16 ± 1.6 mV.

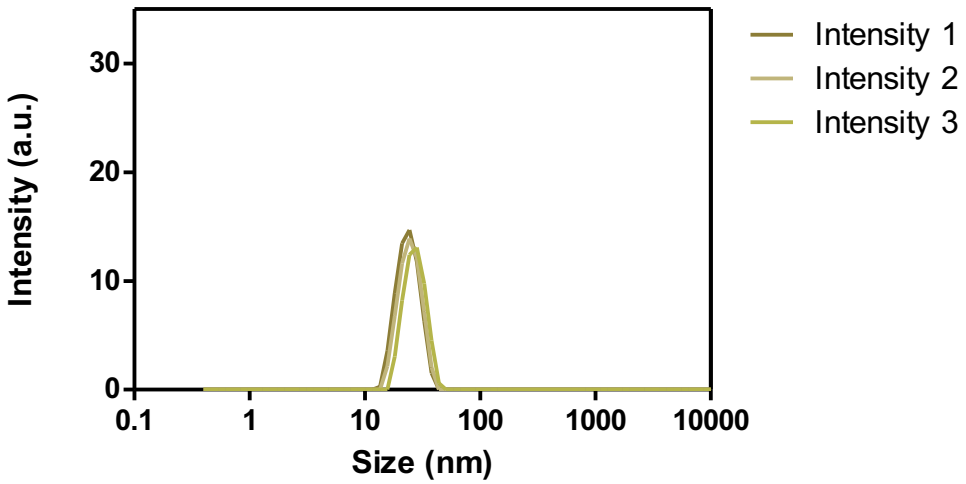


Figure 19. Particle size obtained at 1:1.6 ratio of barberry extract:PF127 polymer.

Size Distribution and Zeta-Potential of Treated Bacteria with Extract

Sizes of tested bacterial treated of (*L. monocytogenes* (Scott A Ser 4b), *E. faecium* (NRRL B-2354), *S. aureus* (ATCC 6538) as well as Gram-negative including *E. coli* O157:H7 (43895™), *S. enterica* serovar Typhimurium (ATCC 700720), *P. aeruginosa* (ATCC 10145) when treated with free barberry extract are shown in **Table. 8**. Untreated (control) of *L. monocytogenes*

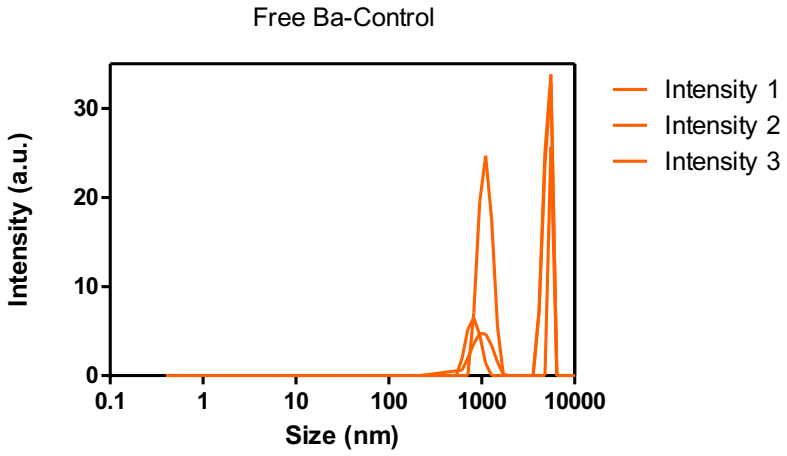
exhibited an average mean size of 1.2 ± 0.01 μm and PDI value at 0.08 ±0.03. The *L. monocytogenes* showed a relatively narrow distribution of size when treated with free treatment and its size distribution was slightly reduced by 4%. The size distribution for control of *E. faecium* was 2.6 ± 0.12 μm, and a size reduction was observed by 58% at a size value of 1.1 ± 0.06 μm with a PDI at 0.90 ± 0.32. *S. aureus* was also displayed a size distribution of 1.7 ± 0.02 μm and a PDI of 0.21± 0.04 before treating with free extract. However, the size of this bacteria reduced by 35% after exposure to the treatment with a value of 1.1 ± 0.06 μm and PDI of 0.79 ± 0.36.

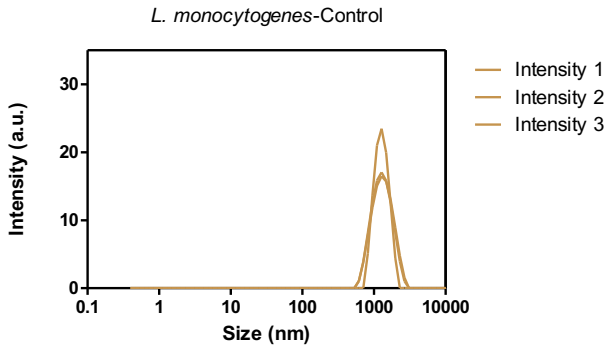
Table 7. Average particle size and polydispersity index of bacteria control without treatments along with treated bacteria with free and nanoparticle barberry extract.

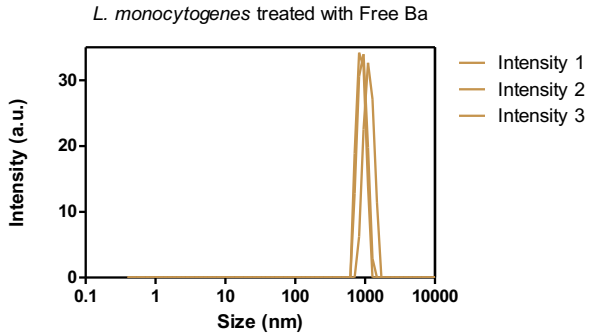
Type of Bacteria	Average Particle Size (μm)			Polydispersity Index (PDI)		
	Bacteria Control	Treated with free Ba	Treated with NPBa	Bacteria Control	Treated with free Ba	Treated with NPBa
L. monocytogenes	1.2 ± 0.01	1.2 ± 0.06	3.6 ± 0.16	0.08 ± 0.03	0.82 ± 0.32	0.71 ± 0.05
E. faecium	2.6 ± 0.12	1.1 ± 0.06	6.3 ± 0.41	0.29 ± 0.21	0.90 ± 0.18	0.69 ± 0.09
S. aureus	1.7 ± 0.02	1.1 ± 0.06	5.9 ± 0.38	0.21 ± 0.04	0.79 ± 0.36	0.79 ± 0.03
E. coli O157:H7	2.0 ± 0.04	1.2 ± 0.13	4.2 ± 0.13	0.18 ± 0.16	0.48 ± 0.49	0.74 ± 0.08
S. Typhimurium	2.2 ± 0.13	1.2 ± 0.14	2.5 ± 0.30	0.44 ± 0.50	0.40 ± 0.53	0.76 ± 0.21
P. aeruginosa	1.3 ± 0.03	1.3 ± 0.02	5.4 ± 1.1	0.24 ± 0.02	0.67 ± 0.57	0.26 ± 0.02

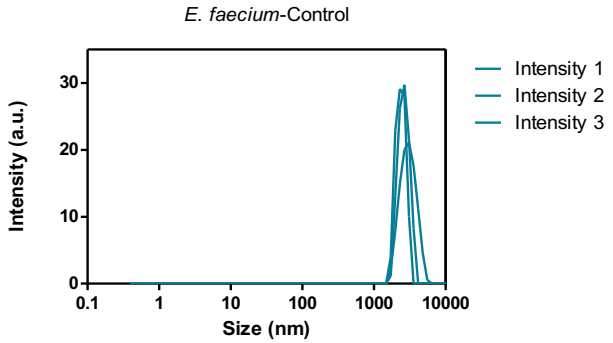
Values are the mean and standard deviation of average particle size and polydispersity index.

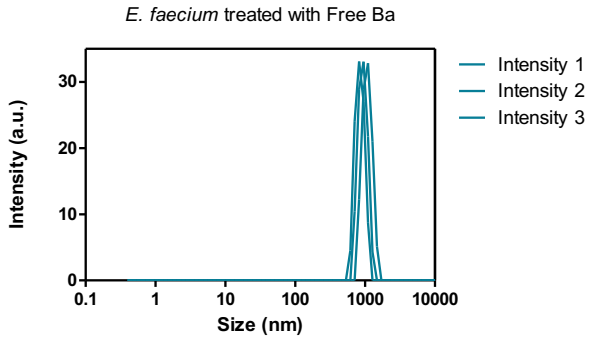
Moreover, Gram-negative bacteria exhibited the same reduction trend compared to Gram-positive bacteria. The size of *E. coli* O157:H7 was 2.0 ± 0.04 µm and a value of 0.18 ± 0.16 for PDI. A size reduction was observed by 40 % in treated cells. Also, the size of untreated *S.* Typhimurium was at a value of 2.2 ± 0.13 µm which was decreased by 46 percent after exposure to the free treatment. The size of treated *P. aeruginosa* exhibited a similar value as the control (1.4% reduction) at the value of 1.3 ± 0.02 µm and a PDI value of 0.67 ± 0.57. Interestingly, all bacteria sizes were decreased after exposure to free extract except *L. monocytogenes* and *P. aeruginosa* which showed almost similar value size as their controls. **Fig. 20** shows the sizes intensity for free barberry before combination with PF127 as well as the particle size distribution of untreated and treated bacteria strains with free barberry for 24 h at 37 °C. The free barberry extract showed an average size distribution of 2.0 ± 0.13 µm and a PDI value of 0.33 ± 0.06.

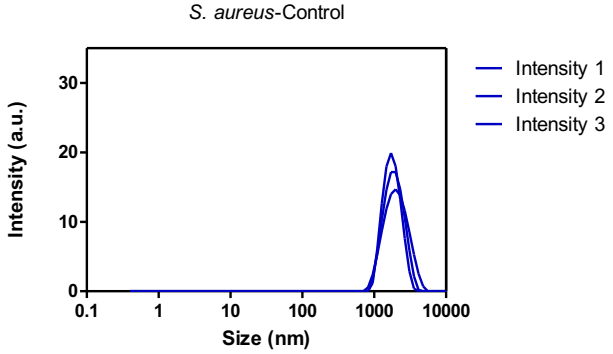


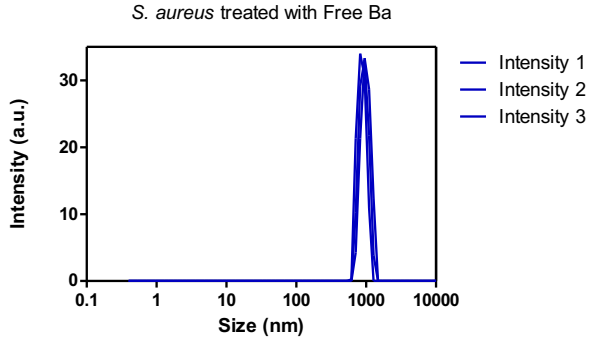

L. monocytogenes-Control
Intensity (a.u.)
Size (nm)
Intensity 1
Intensity 2
Intensity 3


L. monocytogenes treated with Free Ba
Intensity (a.u.)
Size (nm)
Intensity 1
Intensity 2
Intensity 3


E. faecium-Control
Intensity (a.u.)
Size (nm)
Intensity 1
Intensity 2
Intensity 3


E. faecium treated with Free Ba
Intensity (a.u.)
Size (nm)
Intensity 1
Intensity 2
Intensity 3


S. aureus-Control
Intensity (a.u.)
Size (nm)
Intensity 1
Intensity 2
Intensity 3


S. aureus treated with Free Ba
Intensity (a.u.)
Size (nm)
Intensity 1
Intensity 2
Intensity 3

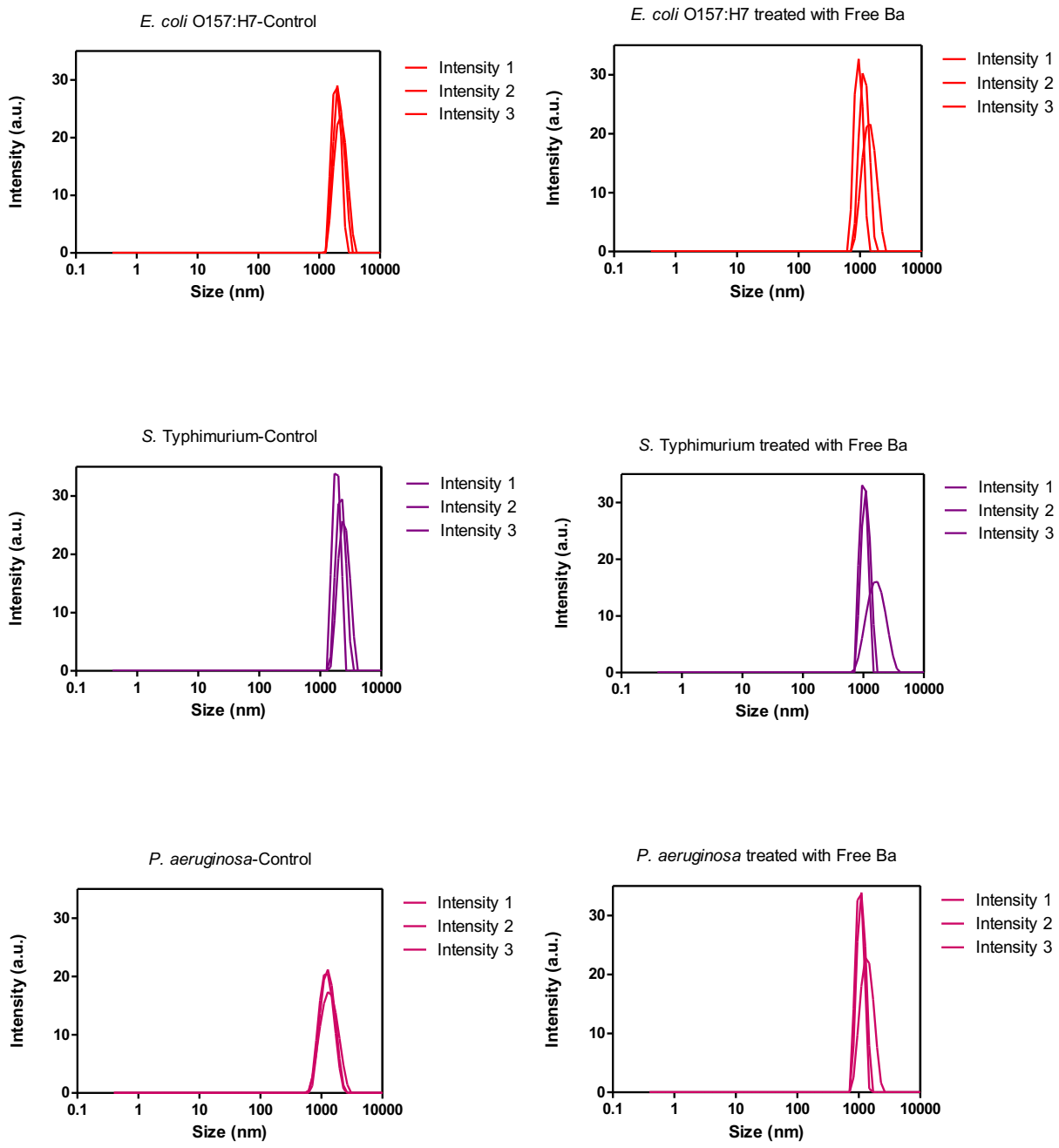
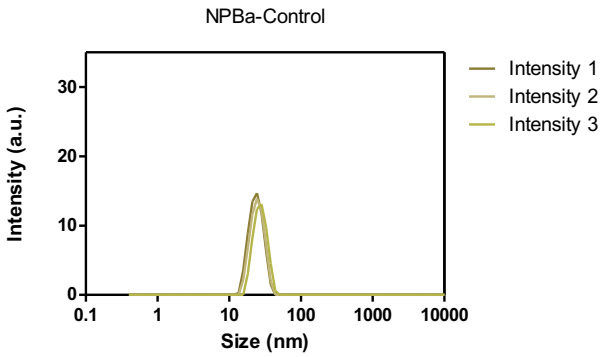
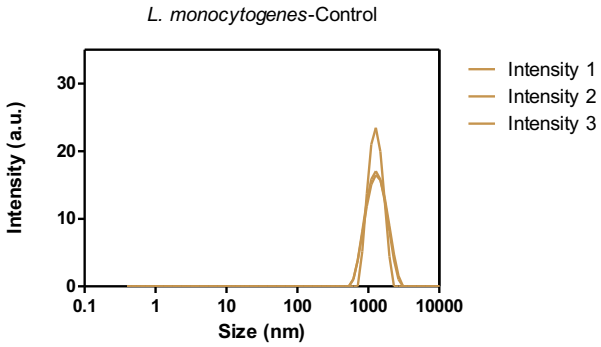


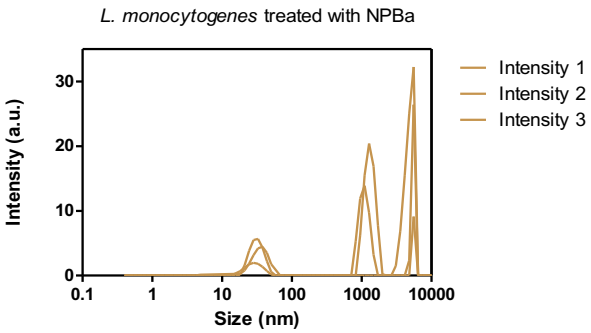
Figure 20. Particle size distribution of bacteria controls and treated strains with free barberry extract at the concentration of 10 mg/mL over 24 h at 37 °C.

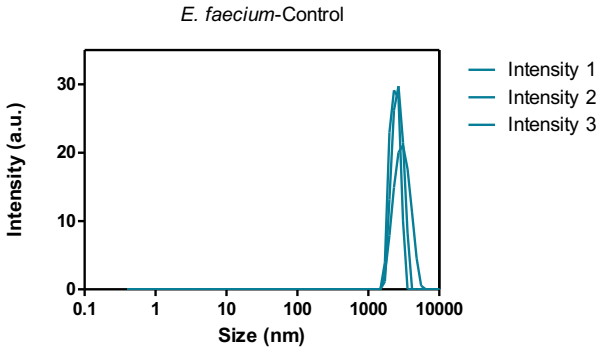
As seen in **Fig. 21**, the size of NP barberry extract showed a size distribution of 14 ± 0.01 μm and a PDI value of 0.40 ± 0.05 which can exhibit a homogeneous system at where the particle sizes present in the suspension are more uniform. The effect of the NP treatment on the tested bacteria indicated that the average size distributions of all bacteria strains including *L. monocytogenes, E. faecium*, *S. aureus*, *E. coli* O157:H7, *S.* Typhimurium, and *P. aeruginosa* were increased by 197%, 136%, 250%, 104%, 13%, and 310%, respectively, when these microorganisms were treated with NP barberry extract. The *S.* Typhimurium was the only bacteria that exhibited the least increase in their size of their cells by 13% reduction in size at a value of 2.5 ± 0.30 μm when treated with NP extract compared to other bacteria. Contrary, the size distribution of treated *S.* Typhimurium with Cetylpyridinium chloride (sanitizer) and lecithin was increased from 1.3 ± 0.07 μm in untreated to 4.0 ± 1.1 μm, due to the effect of the treatment on the aggregation of bacteria cells.

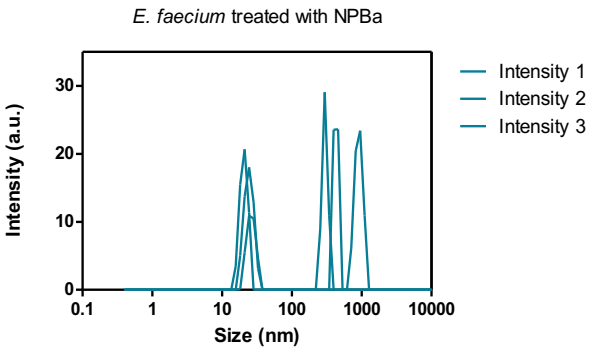
The possible mechanism involved in cell aggregation can be due to compromising the bacteria cell membrane by the NP treatment, resulting in the release of the intracellular components from cells and causing aggregation of lipids and protein following cell death [175]. Interestingly, increases in cells size were observed for *L. monocytogenes* and *P. aeruginosa* when treated with NP treatment with a mean size distribution of 3.6 ± 0.16 μm and 5.4 ± 1.07 μm, respectively. These microorganisms had exhibited almost the same cells size when exposed to the free treatments.

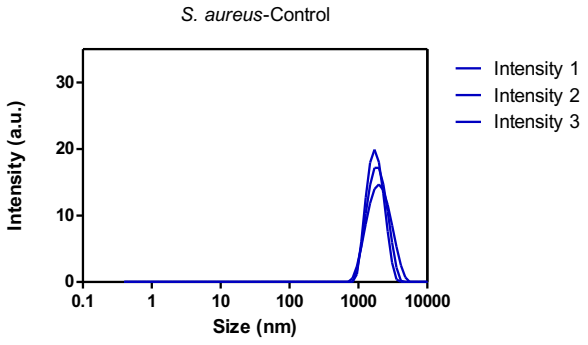

NPBa-Control
Intensity 1
Intensity 2
Intensity 3
Intensity (a.u.)
Size (nm)

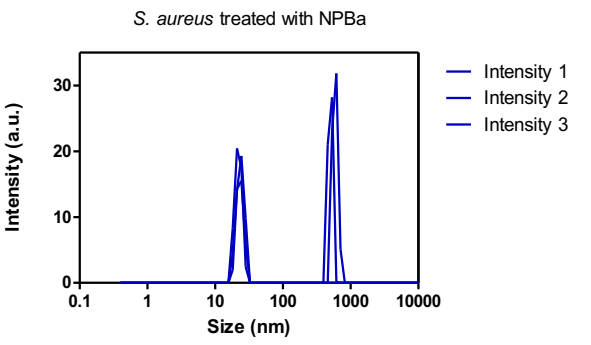

L. monocytogenes-Control
Intensity 1
Intensity 2
Intensity 3
Intensity (a.u.)
Size (nm)


L. monocytogenes treated with NPBa
Intensity 1
Intensity 2
Intensity 3
Intensity (a.u.)
Size (nm)


E. faecium-Control
Intensity 1
Intensity 2
Intensity 3
Intensity (a.u.)
Size (nm)


E. faecium treated with NPBa
Intensity 1
Intensity 2
Intensity 3
Intensity (a.u.)
Size (nm)


S. aureus-Control
Intensity 1
Intensity 2
Intensity 3
Intensity (a.u.)
Size (nm)


S. aureus treated with NPBa
Intensity 1
Intensity 2
Intensity 3
Intensity (a.u.)
Size (nm)

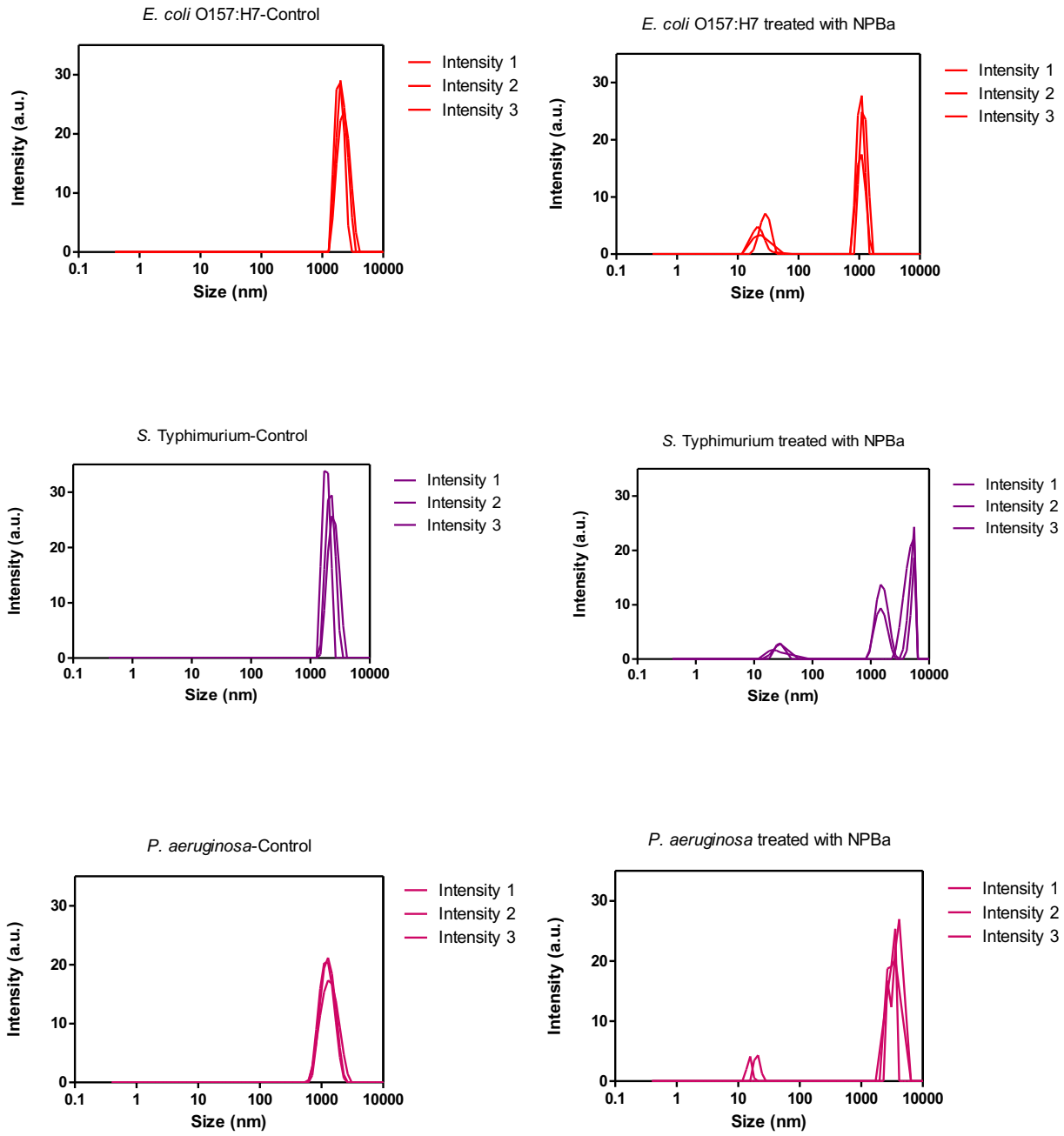
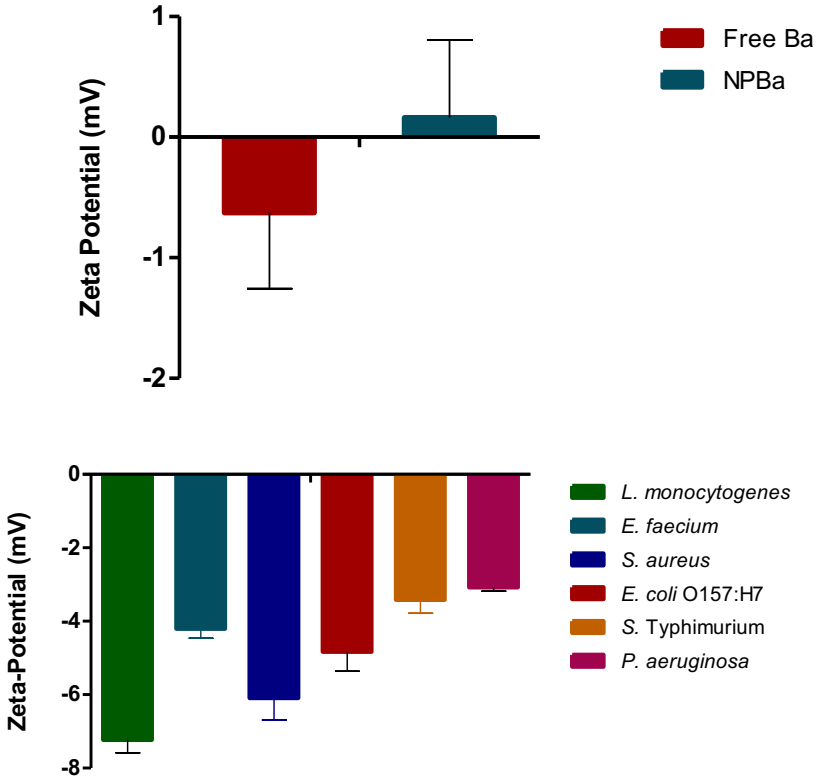


Figure 21. Particle size distribution of bacteria controls and treated strains with nanoparticle barberry extract at the concentration of 10 mg/mL over 24 h at 37 °C.

The zeta-potential (ZP) values of free, NP barberry extracts, and untreated (control) and treated bacteria strains are shown in **Fig. 22**. The ZP values were at -0.63 ± 1.5 mV and 0.16 ± 1.6 mV for free and NP barberry extracts, respectively. *L. monocytogenes* had the highest negative value of -7.2 ± 0.6 mV and the *P. aeruginosa* had the lowest ZP value at -3.1 ± 0.18 mV. Zeta potential is a net superficial charge on the nanoparticle, which determines the degree of repulsion between particles and suspension interaction [176], and depends on a combination of size and surface properties. The surface charge of tested bacteria exhibited a shift toward a less negative value when treated with free and nanoparticles of barberry extracts encapsulated with PF127. The treated *L. monocytogenes* with free showed a decrease in the ZP at the values of -3.6 ± 0.27 mV. However, the negativity of treated cells with NP was slightly less than that of treated cells with free extract at a value -3.8 ± 0.24 mV. Responses of Gram-negative bacteria of *E. coli* O157:H7, *S.* Typhimurium, and *P. aeruginosa* to the NP extract were monitored, so that, the surface charge of *E. coli* O157:H7 was decreased from -4.8 ± 0.92 mV to -4.3 ± 0.66 mV and -1.9 ± 0.59 mV when treated with free and NP extract, respectively. Untreated *S.* Typhimurium had a surface charge of -3.4 ± 0.18 mV. This value exhibited a slight increase at a value of -4.0 ± 0.26 mV when treated with free extract, whereas NP extract was able to decrease the negative charge bacteria cells to a value of -1.6 ± 0.73 mV. As mentioned, the size of treated cells of *P. aeruginosa* did not change when treated with free extract, however, the size of treated *P. aeruginosa* with NP extract increased the size of this bacteria overall. The ZP result from untreated and treated *P. aeruginosa* supports an increase in size value of this bacteria when exposed to different treatments. While the ZP of untreated cells exhibited a value at -3.1 ± 0.18 mV, this value was increased to -3.9 ± 0.24 mV when treated with free extract. However, this value had a reduction to -1.1 ± 0.28 mV with NP treatment. Therefore, a higher affinity of *P. aeruginosa* toward the positively charged NP treatment

caused a higher penetration and consumption of the compounds by *P. aeruginosa* resulting in aggregated bacteria cells.

The Gram-positive and Gram-negative bacteria carry a negative surface charge which the charge magnitude is different from strain to strain. In Gram-positive bacteria phosphate group of teichoic acid that is present in the peptidoglycan layer of these bacteria are responsible for the negative charge [161]. Molecules of lipopolysaccharide (LPS) and lipoprotein (LP) present in OM provide the Gram-negative bacteria with a negative charge [92].



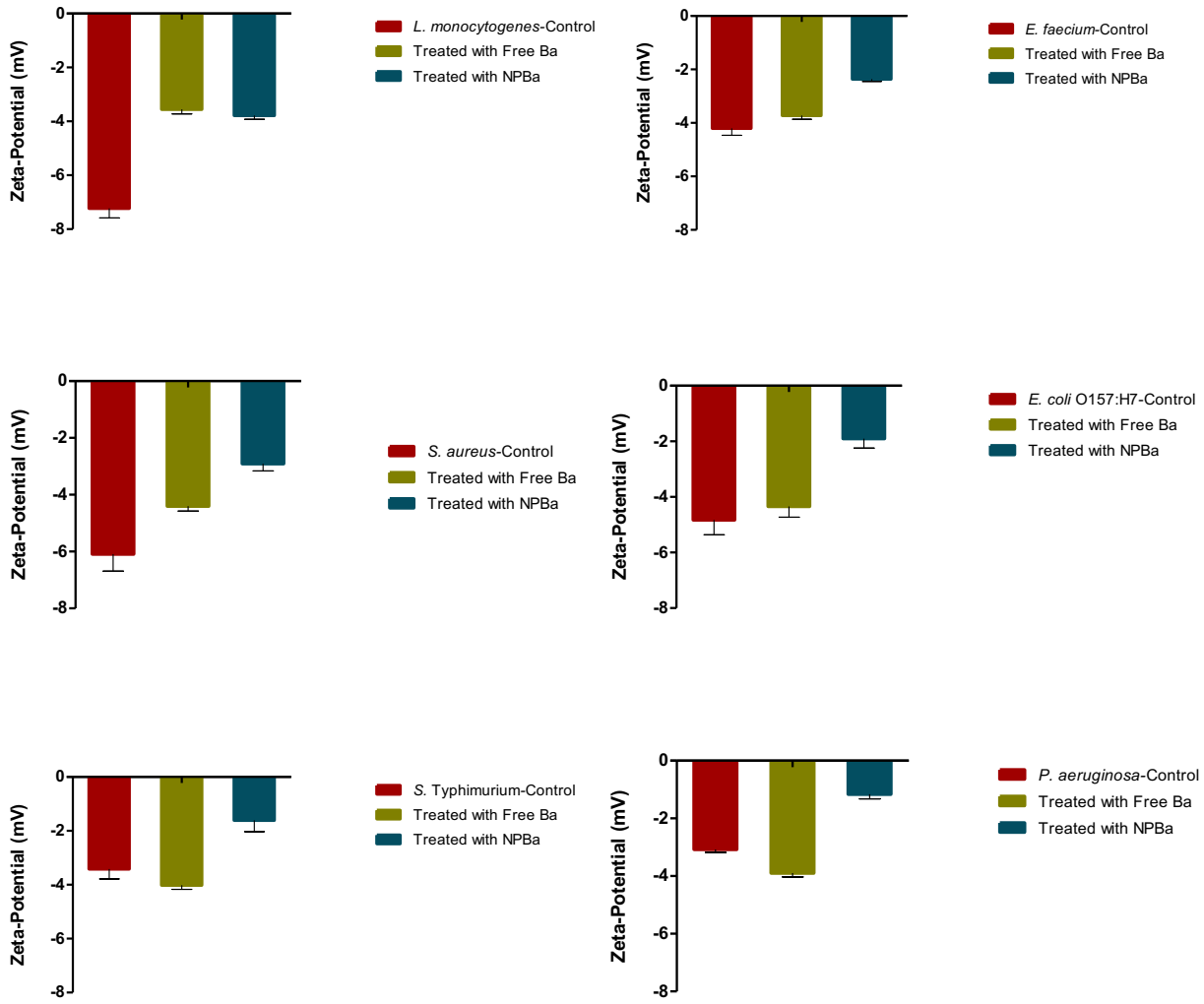


Figure 22. A comparison of zeta-potential distribution of tested bacteria (a), free and nanoparticle barberry extract (b), *L. monocytogenes* (c), *E. faecium* (d), *S. aureus* (e), *E. coli* O157:H7 (f), *S.* Typhimurium (g), and *P. aeruginosa*.

Encapsulation Efficiency in Percent (EE%) of Nanoparticle Extract

The EE of barberry polymeric NPs was determined by using a calibration curve obtained by linear regression and its standard equation (**Fig. 23**). The corresponding absorbances were then plotted in the standard curve equation and EE% was calculated. It was found that the highest

encapsulated amount of free barberry extract with nanoparticle encapsulated with PF 127 was 76% ± 0.33 at the composition of 1:1.6 ratio of barberry extract:PF127 polymer, respectively.

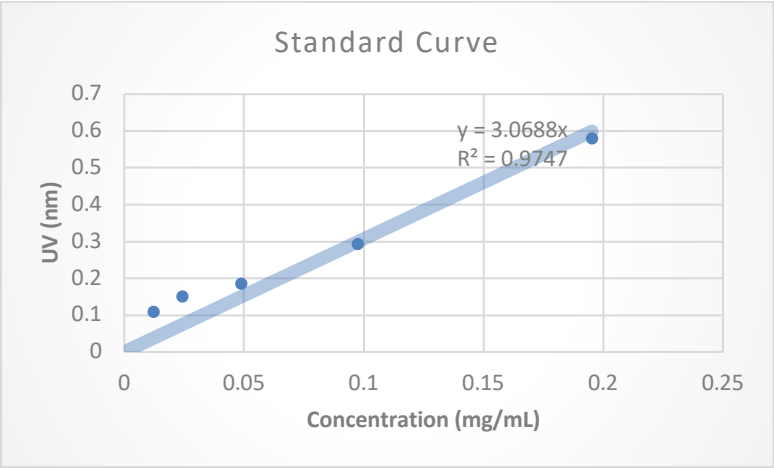


Figure 23. Standard curve for encapsulation efficiency of nanoparticle barberry polyphenolic extract.

Release Kinetic of Nanoparticle barberry extract

The *in vitro* release profile measurement of barberry polyphenolic extract from PF127 was carried out in Milli-Q water at pH 7.4 over 168 h. **Fig. 24** shows the process of instantaneous polyphenol release from the NP extract. The release rate of polyphenols was 50 % for the first 2 h, and a burst in the release at 8 h was observed. The release was continued over the course of 168 h. A possible explanation of burst release observed in 8 h can be due to release of untrapped compounds to the solution.

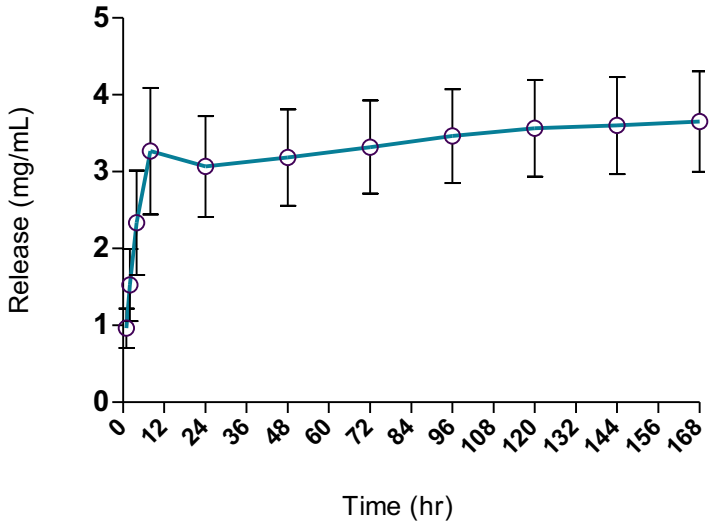


Figure 24. *In vitro* barberry polyphenolic release from PF127 as a function of time at pH 7.4 obtained at 310 nm.

The release rate can vary based on the different locations of the compounds that can be in the core, core-corona interface, and corona. The burst release is triggered if the drug located are the core-corona interface or hydrophobic-hydrophilic interface. The thymoquinone encapsulated with PF68 and PF127 exhibited a different release profile, where thymoquinone NPs encapsulated with PF68 was no longer to release the compound after 24 h upon exposure to the aqueous solution. However, NPs made with PF 127 release the thymoquinone by 62% after 48 h [171]. Moreover, the burst effect observed in the released of NP cinnamon bark extract (CBE) encapsulated with PLGA was due to the location of the extract compounds that were close to or attached to the surface of nanoparticles [170].

Impact of Nanoparticle Barberry Polyphenolic as an Antibacterial Agent

The antibacterial properties of barberry polyphenolic extract loaded in PF127 micelle were determined against pathogenic bacteria with the same microbial cultures as free extract was cultured and tested in various concentrations of 0.312 mg/mL, 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, and 10 mg/mL at 37 °C for 24 h of incubation time. The aim was to evaluate the efficacy of nanoparticles on the tested bacteria of *L. monocytogenes, E. faecium*, *S. aureus, E. coli* O157:H7*, S.* Typhimurium, and *P. aeruginosa* based on the types of bacteria are involved. The results of the growth inhibition are shown in **Table 9**. The most effective concentration against microorganisms was the concentration of 10 mg/mL compared to other tested bacteria, which exhibited the antibacterial activity of the treatments to be dose-dependent. *S. aureus* exhibited more susceptibility compared to the other two Gram-positive bacteria, the following were *L. monocytogenes* and *E. faecium*. The NP treatment exhibited a more inhibitory effect against *S.* Typhimurium among the other tested Gram-negative bacteria, whereas the following bacteria were *P. aeruginosa* and *E. coli* O157:H7, respectively.

Table 8. Effect of nanoparticle extract on the growth of bacteria strains at concentrations of 0.312, 0.625, 1.25, 2.5, 5, 6, 7, 8, 9, and 10 mg/mL for 24 h exposure time at 37 °C.

Type of Bacteria	Concentrations of Nanoparticles [a] (mg/mL)										
	Control	0.312	0.625	1.25	2.5	5	6	7	8	9	10
L. monocytogenes	0.8±0.09	0.8±0.06	0.7±0.06	0.5±0.06	0.3±0.03	0.2±0.06	0.2±0.08	0.2±0.08	0.1±0.06	0.2±0.06	0.2±0.09
E. faecium	0.7±0.10	0.9±0.09	0.90±0.10	0.8±0.10	0.4±0.05	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.2±0.01
S. aureus	0.8±0.10	0.8±0.10	0.8±0.14	0.8±0.12	0.3±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01
E. coli O157:H7	0.9±0.08	0.7±0.06	0.6±0.05	0.4±0.04	0.2±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01
S. Typhimurium	1.2±0.14	0.6±0.05	0.51±0.05	0.3±0.03	0.2±0.01	0.2±0.01	0.2±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01
P. aeruginosa	0.9±0.14	0.8±0.10	0.8±0.11	0.8±0.14	0.3±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01

[a]Values represent the mean and standard errors from triplicate of antibacterial properties of nanoparticle barberry treatments on tested bacteria after an exposure time of 24

The growth behavior of the treated bacteria was also studied based of the specific time intervals of 6, 12, 18, and 24 h (**Fig. 25**). Each bacteria strain exhibited a different growth trend. The growth of *L. monocytogenes* was 15% at 6 h, followed by 22% at 12 h, 19% at 18 h, and finally, 13% growth at 24 h incubation time. The *E. faecium* exhibited a higher growth rate by 30% at 6 h compared to *L. monocytogenes* and *S. aureus*. However, the growth of this microorganism was decreased by 29% at 12 to 13% at the end of the experiment. *S. aureus* also exhibited a growth rate of 23% at 6 h and 23% at 12 h, whereas its growth was inhibited by 11% and 10% at 18 h and 24 h, respectively. The study on the Gram-negative bacteria in defined time intervals resulted in lower growth of rate for *P. aeruginosa* and *S.* Typhimurium by 7.1% and 6.7% after 24 h incubation time. The *E. coli* O157:H7 was still the most resistant bacteria to the treatments by with 9.0% growth at the end of the incubation.

A possible explanation for the observed growth result can be due to different lag, log or exponential, stationary, and death phases of the microorganisms. Depending on the bacteria growth rate and their responses to the treatments, various results were obtained not only between Gram-positive and Gram-negative bacteria but also between each individual bacteria among the same Gram stains in this current work. Moreover, the treatment characteristic can also be significant when it comes to stimulatory or inhibitory effects of the treatments. For instance, the polyphenols with a lower molecular weight with the existence of hydroxyl groups as reactive sites (such as phenolic acids) were found to be more effective compared to the higher molecular weight molecules. Nevertheless, this aspect can be related to hydrophobicity/hydrophilicity of the polyphenols tested as well as the unique composition of each Gram stains bacteria cell wall [177].

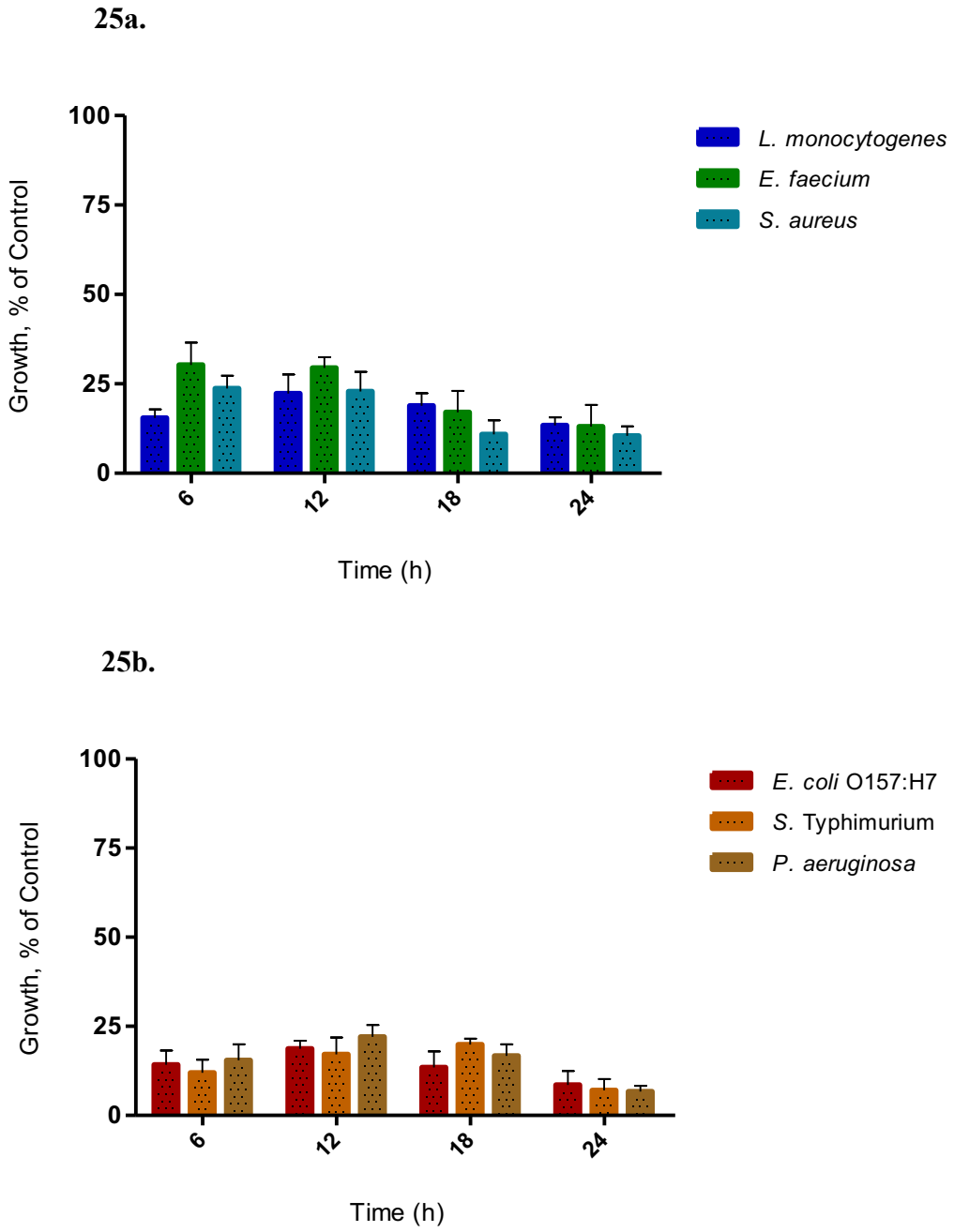


Figure 25. Effect of NP barberry extracts on the growth of Gram-positive (a) and Gram-negative (b) bacteria over 24 h at 37 °C.

A possible mechanism of antibacterial property of the NP treatment can be found through the electrostatic binding force between the nanoparticles and negatively charged bacterial cell

membrane, where they may bind to lipoteichoic acid and LPS in Gram-positive and Gram-negative bacteria, respectively, and disrupt the cell membrane. It was previously reported that the mechanism of action of the polyphenols such as CGA against Gram-negative bacteria is to remove divalent cations from their binding sites in LPS and disrupt the cell integrity. LPS and LP in OM are maintained together by electrostatic interactions with divalent cations that stabilize the OM. The CGA carries a negative charge and its OH^- functional groups act as reducing agents that cause this compound to be bonded to OM resulting in disrupting and causing a loss of barrier function [78]. Contrary, CGA (an ester form of quinic acid and caffeic acid) exhibited a lower antibacterial activity against *Salmonella enteritidis* compared to free phenolic acid, due to steric hindrance that may decrease the interaction of this compound and bacterial cell wall [89].

MIC and MBC of the Nanoparticle Barberry Extract

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of NPs extract were determined over 24 h incubation time at 37 °C. **Table 10** represents the MIC and MBC values that inhibited The MIC and MBC values for *L. monocytogenes* were 17 mg/mL and 18 mg/mL. The *E. faecium* and *S. aureus* were responded to the treatment at MIC values of 17 mg/mL and >11 mg/mL. The MIC and MBC values for *E. coli* O157:H7 were >18 mg/mL. The *S.* Typhimurium and *P. aeruginosa* needed the same concentrations to be inhibited with MIC values at 17 mg/mL and MBCs at 18 mg/mL. The MIC and MBCs results were consistent with the growth inhibitory study over 6, 12, 18, and 24 h for NP barberry extract that exhibited the susceptibility of Gram-positive microorganism in order of *S. aureus* > *E. faecium* > *L. monocytogenes* and Gram-negative bacteria in order of *P. aeruginosa* > *S.* Typhimurium > *E. coli* O157:H7.

Table 9. The MIC and MBC of nanoparticle extract against tested bacteria after treatments over 24 h at 37 °C.

Type of Bacteria	MIC (mg/mL)	MBC (mg/mL)
L. monocytogenes	> 18	> 18
E. faecium	17	> 18
S. aureus	> 11	13
E. coli O157:H7	> 18	> 18
S. Typhimurium	17	18
P. aeruginosa	17	18

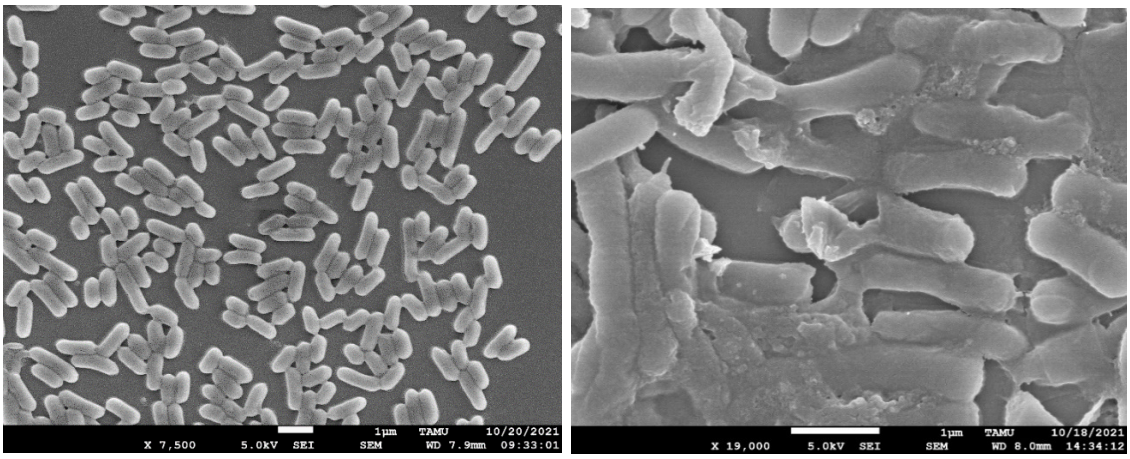
Antibacterial property of Nanoparticle Extract on the teste bacteria by SEM

The cell morphological changes of *L. monocytogenes, E. faecium*, *S. aureus, E. coli* O157:H7*, S.* Typhimurium, *P. aeruginosa* were studied in presence of NP barberry extract over 24 h at 37 °C. Untreated *L. monocytogenes* cells (the one on the left side) were intact with a typical rod-shape, whereas an injury in the cell's membrane of the treated cell was occurred, and cytoplasmic substances were released (the one on the right). Some of the cells were collapsed and severe damage was happened (**Figs. 26a** and **b**). In the control samples of *S. aureus*, the cells look around and undamaged. Some bacteria had holes in their cell wall as well as the surface of a couple of cells was slightly coarse following incubation with NP barberry extract for 24 h at 37 °C. The untreated *E. coli* O157:H7 cells displayed a smooth and intact surface. The morphology of *E. coli* O157:H7 cells was changed, so that, the holes were observed in their cell wall after incubation with the NP treatment (**Figs. 26g** and **h**). In the surface of *S.* Typhimurium (**Figs. 26i** and **j**) and *P. aeruginosa* cells (**Figs. 26k** and **l**), wrinkles and deformities were observed, especially an

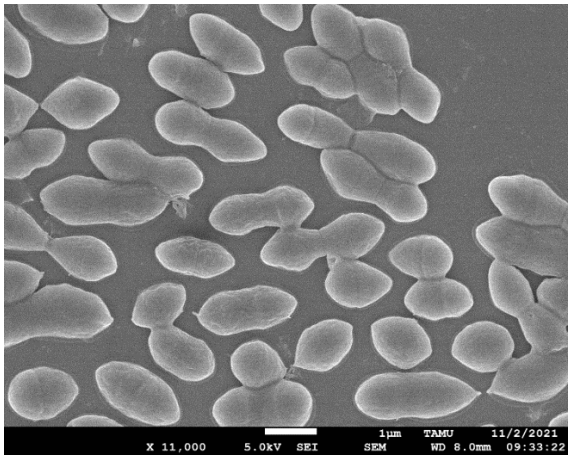
accumulation of the treatment compounds on the surface of the *P. aeruginosa* cells was observed that can be due to interaction of polyphenols within the treatment with the bacteria cell wall.

The effect of polyphenols on the bacteria can vary ranging from bacterial growth stimulation to antimicrobial property at which bacterial strains, electronic, and charge of the polyphenols play an important role. The mechanism of action for polyphenols against bacteria strains suggested that the hydrogen binding capacity of polyphenols to enzymes may induce changes in various intracellular functions. For instance, tannins can bind to cell's enzymes, damage the cell membrane, and even inactivate the cell metabolism, whereas phenolic acids can disrupt the cell integrity and cause leakage of essential intracellular constituents [89].

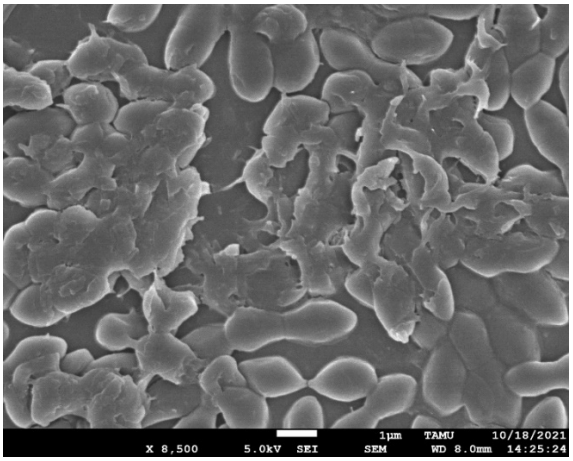
a. **b.**



c.


X 11,000 5.0kV SEI 1µm SEM TAMU WD 8.0mm 11/2/2021 09:33:22

d.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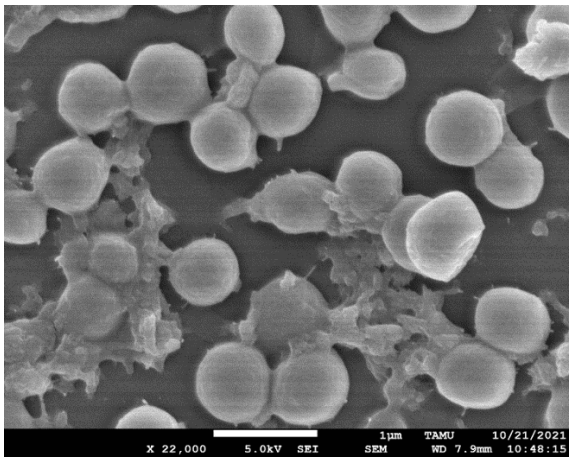
e.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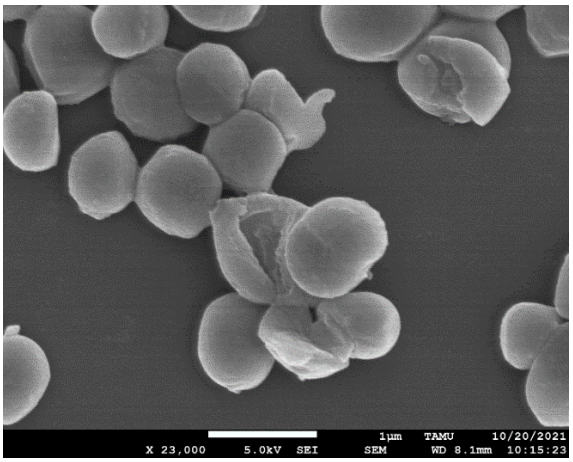
f.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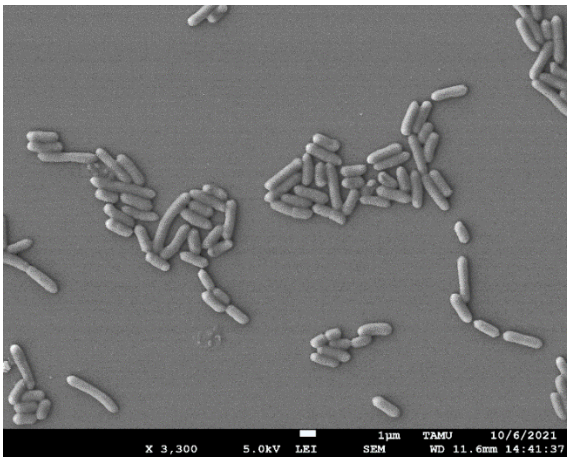
g.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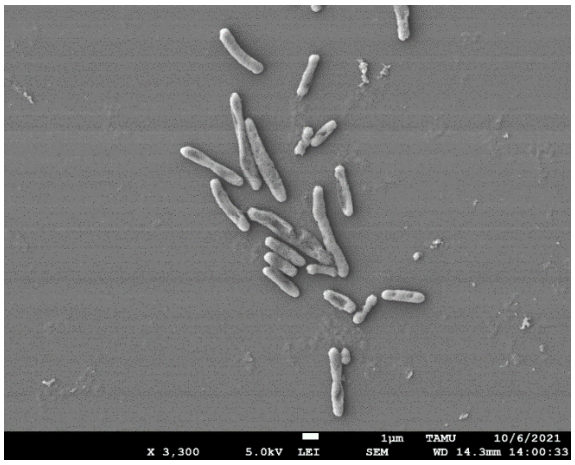
h.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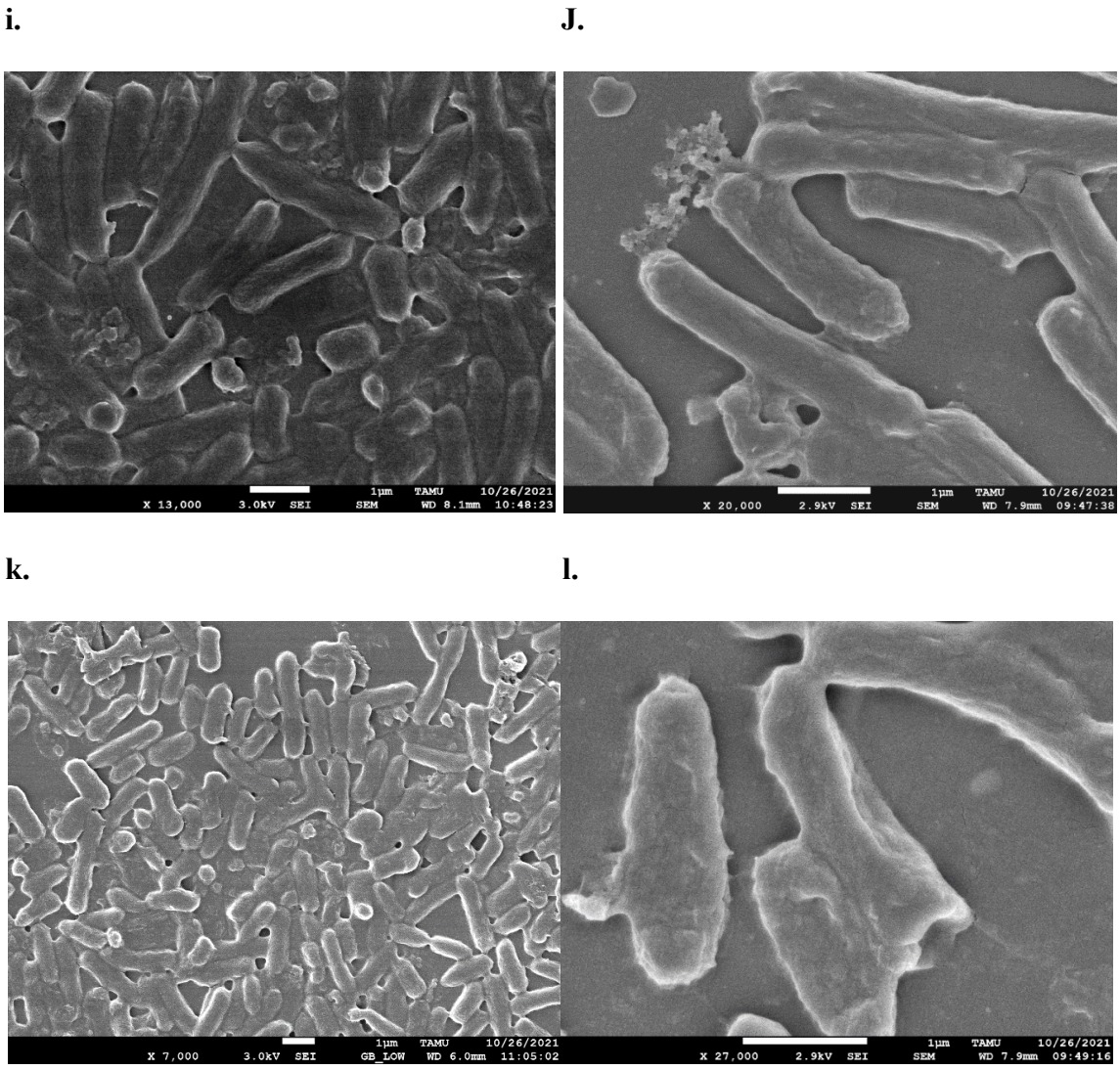


Figure 26. SEM of bacteria control of *L. monocytogenes* (a), *E. faecium* (c), *S. aureus* (E), *E. coli* O157:H7 (g), *S.* Typhimurium (j), and *P. aeruginosa* (k), and treated bacteria of *L. monocytogenes* (b), *E. faecium* (d), *S. aureus* (f), *E. coli* O157:H7 (h), *S.* Typhimurium (j), and *P. aeruginosa* (l) with nanoparticle extract (10 mg/mL) after 24 h exposure time at 37 °C.

Conclusions

The result showed that the hydrodynamic size of nanoparticle (NP) barberry extract ranged from 10 to 100 nm (0.01-0.1 μm) with a polydispersity index (PDI) of 0.39 at ambient temperature. The ZP values were at -0.63 ± 1.5 mV and 0.16 ± 1.6 mV for free and NP barberry extracts, respectively. Also, *L. monocytogenes* had the highest negative value of -7.2 ± 0.6 mV and *P. aeruginosa* had the lowest ZP value at -3.1 ± 0.18 mV. Nevertheless, the surface charge of tested bacteria exhibited a shift toward a less negative value when treated with free and NP barberry extracts encapsulated with PF127. A higher affinity of *P. aeruginosa* toward the positively charged NP treatment caused a higher penetration and consumption of the compounds by *P. aeruginosa* cells resulting in aggregated bacteria cells. The antibacterial activity of the treatments was dose-dependent, due to a greater antibacterial property of the treatments at the higher concentration. The *S. aureus* exhibited more susceptibility compared to the other two Gram-positive bacteria, the susceptibility of Gram-positive microorganisms was in order of *S. aureus* > *E. faecium* > *L. monocytogenes* and Gram-negative bacteria in order of *P. aeruginosa* > *S.* Typhimurium > *E. coli* O157:H7.

CHAPTER V

IN VITRO GASTROINTESTINAL SUMIULATION MODEL, CHEMICAL STABILITY, AND METABOLITES FORMATION OF FREE AND NANOPARTICLE BARBERRY POLYPHENOLICS

Introduction

Polyphenols are secondary plant metabolites that include chemical class variation such as phenolic acid and flavonoids, are the predominant antioxidant class present in barberry fruit. The phytochemicals present in fruit, vegetables, and spices were reviewed and shown to possess a diverse array of biological activities, including antimicrobial, antioxidant, anticancer, and anti-inflammatory [178]. Up to 5-10% of ingested polyphenols are absorbed in the small intestinal, and about 90-95% reach the large intestinal (colon) where they continue to be absorbed or are biotransformed into microbial metabolites in a complex system of substrate-microbiome interaction that varies by individual's gut microbiota. Although, polyphenolics are not known to be essential nutrients, they are generally regarded as beneficial to human health. Host-substrate-microbiome interaction can therefore produce novel metabolites, not present in the original food, and produce drug-like small molecules that are not only more bioavailable but are often more bioactive than their parent compounds. Polyphenol compounds can act as substrates or prebiotics for select probiotic bacteria to aid in their survival and functionality while simultaneously inhibiting pathogenic bacteria [7], Therefore, the polyphenols may possess positive health outcomes by inhibiting the pathogenic bacteria and enhancing the growth of probiotic bacteria in the production of novel bioactive polyphenolic metabolites.

The International Scientific Association for Probiotic and Prebiotic (ISAPP) defines probiotic bacteria as "*live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host*" [179]. Probiotic bacteria, mostly classified as Gram-positive bacteria, live all over the human body (gut, skin, hair, membrane, etc). Beneficial probiotic species belonging to *Lactobacillus* spp. and *Bifidobacterium* spp. can be influenced by plant polyphenols. The combination of probiotic bacteria with prebiotic provides synbiotics environment that may affect the gut microbiota population and posse health benefits to the host. However, there is not enough knowledge on the influence of barberry polyphenols in forms of free and NP on the growth and proliferation of probiotic bacteria an *in vitro* model. Therefore, the aim of this work was to evaluate the bio-efficacy of barberry polyphenolic extract on promoting the growth of probiotic strains including *Lactobacillu*s spp., *Bifidobacterium* spp., and a mixed *co-culture* of Gram-positive *Lactobacillus* and *Bifidobacterium* with a Gram-negative and pathogenic strain of *E. coli* HS in a colonic fermentation model. It was hypothesized that the growth of gut probiotic bacteria may be promoted when exposed to barberry polyphenol treatments, resulting in a higher concentration of microbial metabolites production. The stability of barberry polyphenols that can withstand the gastrointestinal digestive pH in an *in vitro* gastrointestinal digestive model was also studied.

Materials and Methods

Standards

Standards of phenolic acids (CGA, caffeic, ferulic, *p*-coumaric, protocatechuic, gallic acid), 3,4-dihydroxy-phenylacetic acid, and 3-(3-hydroxyphenyl) propionic acid along with chemical solvents such as methanol, ethanol, formic acid as well as UPLC grade of methanol, and water for chromatography were purchased from Sigma Aldrich, Co. (St. Louis, MO, USA).

In Vitro Gastrointestinal (GI) Digestion

Three steps of the human digestion system were simulated including oral, gastric, and small intestinal to assess the stability and release of barberry polyphenols nanoparticles compared to the stability of free polyphenolics. The aliquots were then characterized by UPLC-MS-MS at the end of each incubation stage (**Fig. 27**).

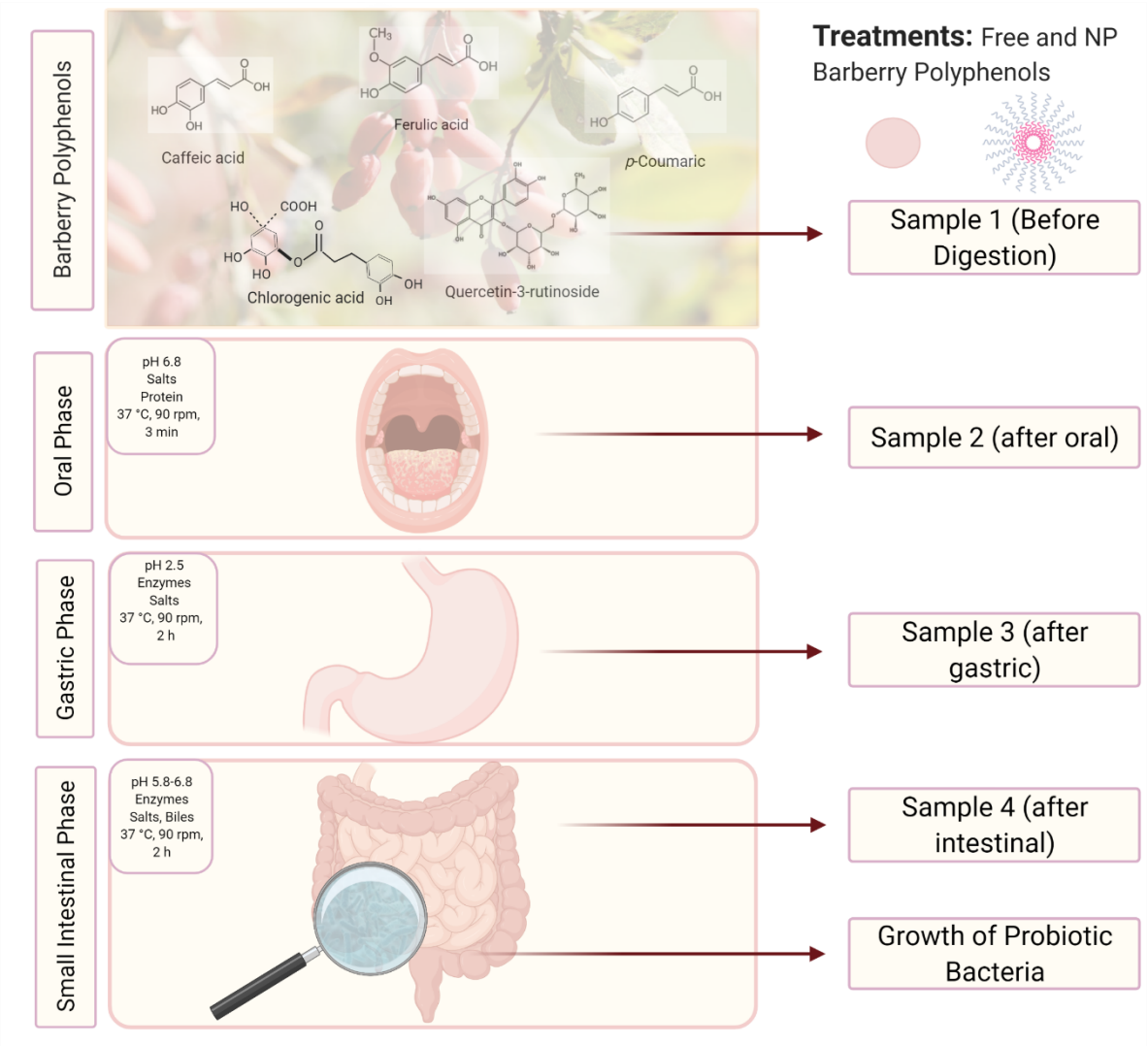


Figure 27. A schematic illustration of gastrointestinal digestive model, created with BioRender.com (2021).

Oral Phase

A simulated saliva fluid containing 3% mucin was prepared with a previously method described [180]. This stage consisted of 1.6 g/L Sodium chloride (NaCl), Ammonium nitrate (NH_4NO_3), Potassium phosphate (KH_2PO_4), Potassium chloride (KCl), Potassium citrate $K_3C_6H_5O_7.H_2O$), Uric acid sodium salt ($C_5H_3N_4O_3.Na$), Lactic acid sodium salt ($C_3H_5O_3Na$) were added to Urea (H_2NCONH_2), Porcine gastric mucin (Type II), and water (Fisher Scientific, San Jose, CA, USA). Briefly, 1 g of free and 2.5 g of NP were digested with 40 mL containing artificial saliva. The initial concentration of free and NP after subjecting to artificial saliva was 25 mg/mL. The fluid pH was adjusted to 6.8 with 1 M NaOH and placed at 37 °C with continuous shaking at 90 rpm for 3 min in a shaking water incubator to mimic the oral condition. The aliquots of free and NP were then taken for characterization by Ultra Performance Liquid Chromatography (UPLC-PDA-ESI) mass spectrometry at the end of the incubation time.

Gastric Phase

The simulation of gastric phase (SGF, pH 2.5) in an *in vitro* digestion model was followed using a method previously described [180-181]. The gastric digestion experiment was simulated by adding 2 g sodium chloride (NaCl), 7 mL of SGF hydrochloric acid solution (0.05 M, pH 1.2) containing 5 g pepsin to a flask and diluting the flask content with distilled water to a volume of 1 liter. The samples obtained from the oral phase were mixed with 200 mL of SGF (ratio 1:5 v/v). The pH of the mixture was then adjusted to 2.5 using an adequate volume of 2 M HCl, incubated at 37 ºC and shielded from the light in a shaking water bath for 2 h to mimic gastric digestion. The aliquots were taken at the end of incubation time and mixed with methanol acidified with 0.1%

formic acid (1:5 ratio) for inactivation of pepsin, then cooled down in an ice bath. The samples were then centrifuged at 4 ºC, 12,000 rpm, for 20 min and filtered using a 0.22 µm Polyethersulfone (PES) filter for analysis by UPLC-PDA-ESI mass spectrometry.

Small Intestine Phase

The simulated intestinal fluid (SIF, pH 6.8) was maintained as previously described method with a slight modification [181]. After gastric digestion, the pH of digesta was raised to 5.8 by adding approximately 2.0 mL of 1 M $NaHCO_3$. The diluted fluid was incubated for 10 min at 37 ºC to mimic the duodenum. Next, 10 mL of simulated small intestinal fluid (SIF) containing 24 mg/mL porcine pancreatin, 50 mg/mL of bile salts, and saline solution (0.5 M $CaCl_2$ and 5.5 M NaCl) were added to the digestion model. The final pH of digestion samples was adjusted to 6.8, adding 0.5 M NaOH, and then incubated at 37 ºC for 2 h under 90 rpm in a shaking incubator. Once the pancreatic digestion ended, the aliquots were collected and inactivated at a ratio of 1:5 with methanol acidified with 0.1% formic acid, then placed in an ice bath for 10 min to cool down for enzyme deactivation. All the collected aliquots at the end of each phase (oral, gastric, and intestinal) were centrifuged at 4 ºC, 12,000 rpm, for 20 min and filtered using a 0.22 µm PES filter. The samples were kept in -80 until UPLC characterization.

Size Stability as Function of Gastric and Intestinal Environments

The particle size stability of free and NP barberry was studied as a function of different pH of the gastrointestinal environment [182]. The free (0.625 g) and NP (1.625 g) specimens were mixed with a volume of 150 mL of Simulated Gastric Fluid (SGF) with a pH value of 2.5 and kept at 37 ºC on a shaking incubator at 90 rpm for 2 h. Then, 12.5 mL of Simulated Intestinal Fluid (SIF)

(pH 5.8) was added to the mixture and kept on the shaking incubator for 10 min to simulate the upper intestinal condition. The pH of the mixture was adjusted to 6.8 and held for 32 h at the same above-mentioned condition. At the time intervals of 0, 2, 4, 8, 16, and 32 h, then 1 mL of aliquots were pulled and cooled down for 10 min. Dynamic light scattering (DLS) was used to measure the particle size distribution of the samples using the Zetasizer ZS90 analyzer (Malvern Instrument, L.t.d., Westborough, MA) at a scattering angle of 90^{o} at 25 ^{o}C.

Viability of Probiotic Bacteria

Bacteria Culture Preparation

One capsule of adult probiotic bacteria commercially available containing 50 billion live cultures including *Lactobacillus* (*L. casei, L. rhamnosus, L. reuteri, L. acidophilus, L. paracasei, L. bulgaricus, L. salivarius,* and *L. plantarum*) and *Bifidobacterium* (*B. bifidum*, *B. longum, B. breve,* and *B. lactis*) from Renew Life dietary supplements Co. (Durham, NY, USA) was used. *E. coli* HS was donated by the *E. coli* Reference Center from the Department of Food Science at Penn State University. *Faecalibacterium prausnitzii* was also purchased from American Type Culture Collection (ATCC, Manassas, VA). *Lactobacillus* sp. and *Bifidobacterium* sp. were cultured in deMann-Rogosa Sharpe (MRS) broth at 37 ^{o}C for 36 to 48 h in anaerobic conditions. *E. coli* HS was also precultured in BHI broth at 37 ^{o}C for 36 to 48 h. *F. prausnitzii* did not grow under anaerobic conditions provided by the anaerobic chamber and it was not considered for further experiment. The suspensions of *Lactobacillus*, *Bifidobacterium*, and *E. coli* HS were prepared and grown until the early exponential phase (Optical Density at 630 (OD630= 0.6)). The absorbance was measured at OD630 using a microplate reader (CLARIOstar^{Plus}) to determine log-phase of 0.6 [183]. The bacteria cells were centrifuged at 4 ^{o}C, 4000 rpm, for 15 min), then washed twice with a

phosphate-buffered saline (PBS) (pH 5.8). The bacterial pellets were then resuspended in a 10 mL of media solution prior to inoculation and used for further experiments.

Standard Growth Curve

All bacteria suspension were cultured in MRS broth in 96 well microplate and incubated at 37 ºC for 48 h of incubation. The absorbances were measured at OD630 after 0, 2, 4, 8, 16, 32, and 48 h.

Impact of Free and Nanoparticle Barberry Polyphenols on the Growth and Survival of Probiotic Bacteria

The growth of the probiotic bacteria assay was evaluated based on a previously reported method [183]. A volume of 100 µL of *Lactobacillus*, *Bifidobacterium*, co-culture, and *E. coli* HS bacteria suspensions in MRS broth were treated with 100 µL of free and NP barberry extract at the concentrations of1200 mg/L, 960 mg/L, 720 mg/L, 480 mg/L, and 240 mg/L. The 96 well microplate was incubated at 37 ºC for 48 h. During the incubation, the absorbance was measured at OD630 nm in a microplate reader after 0, 2, 4, 8, 16, 24, 32, and 48 h. The positive control was determined as bacterial suspension mixed with MRS without treatments, and the MRS medium as a negative control. Obtained results were expressed as a percentage of growth of tested probiotic bacteria at each concentration compared to positive control.

Assessment of Metabolite Production from Free and Nanoparticle Extract Upon Microbial Fermentation

The measurement of chlorogenic acid metabolites production such as caffeic acid and its esters 3-(3-hydroxyphenyl) propionic acid and 3,4-dihydroxyphenylacetic acid from free and NP barberry extract by probiotic fermentation were determined using LC-MS instrument. For quantification, *Lactobacillus*, *Bifidobacterium*, co-culture, and *E. coli* HS were treated with 100 µL of 1320, 1200, 960, 840, 720, 600, 480, 360, 240, 120 mg/L samples and incubated at 37 ºC for up to 48 h. The optical density of bacteria suspension was measured at different time intervals of 0, 2, 4, 8, 16, 24, 32, and 48 h. The bacteria's growth was ended by adding methanol acidified with 0.1% formic acid at a ratio of 1:6 (v/v) and were filtered using 0.22 µm PES filter and stored at -80 ºC for further analysis.

Statistical Analysis

All the experimental results were expressed as mean standard deviation of three replicates. Significant differences between means of treatments were identified by ANOVA (analysis of variance) followed by Tukey's Honestly Significant Differences (HSD) and Bonferroni tests comparing each pair with control.

Results and Discussion

The Impact of pH of Simulated Gastrointestinal Digestion on the Individual Polyphenols from Free and Nanoparticle Barberry

An *in vitro* digestion model was investigated as a simulation of oral, gastric, and intestinal conditions of the human body. To evaluate the stability of barberry polyphenolic rich in CGA

during their passage through the GIT, free and NP barberry samples were prepared and subjected to a simulated oral solution and incubated for 3 min at pH 6.8. The samples were also incubated with gastric fluids (pepsin enzyme, NaCl, and HCl) at pH of 2.5 for 2 h to simulate digestion in the stomach. To mimic a small intestinal environment, free and NP specimens were mixed with porcine pancreatin, bile salts, and saline solution (0.5 M $CaCl_2$ and 5.5 M NaCl). The initial pH of this stage was adjusted to 5.8 for 10 min for a simulation of the intestinal duodenum, and then pH raised to 6.8 for 2 h. After each step of *in vitro* digestion, aliquots were collected and inactivated with 5-fold methanol acidified with 0.1% formic acid, then placed in an ice bath for 10 min to cool down for enzyme deactivation. The results expressed as a percentage variation between the initial concentration submitted to the digestion and the amount that recovered after digestion. Although various digestion models have been proposed using different enzymes, different pH, types of minerals, and the time of digestion, the simulated gastrointestinal digestion in this work was designed to be close to the Infogest method [184].

Plants may contain one of three predominant isomers of Acyl-quinic acids (chlorogenic acids) and commonly exist as chlorogenic acid (5-caffeoylquinic acid; CQA), cryptochlorogenic acid (4-CQA), and neochlorogenic acid (3-CQA), all derived from the Shikimic acid pathway and are generally classified as hydroxycinnamic acids derivatives [185]. CGA consists of water-soluble esters between quinic acid and hydroxycinnamic acids, including caffeic acid (3,4-dihydroxycinnamic acid), *p*-coumaric (4-hydroxycinnamic acid), and ferulic acid (3-methoxy-4-hydroxycinnamic acid) [47]. Barberry (*Berberris vulgaris)* is a native fruit of the Middle East, which contains high levels of polyphenols such as phenolic acids, anthocyanins, and flavonols. Previous identification of phenolic acids of 18 different barberry genotypes shows the fruits of this plant contain 2.01 mg/L to 119.5 mg/L of chlorogenic acid, 3.67 mg/L to 51.78 mg/L of caffeic acid, 4.10 mg/L to

171.1 mg/L of *p*-coumaric, 16.13 mg/L to 334.8 mg/L of gallic acid, and 2.73 mg/L 7.61 mg/L of rutin [20].

The concentrations (μM) of hydroxycinnamic acids of free and NP barberry such as chlorogenic acid, ferulic acid, *p*-coumaric, and caffeic acid as well as hydroxybenzoic acids (protocatechuic acid and gallic acid) and a flavonol of quercetin-3-rutinoside before and after *in vitro* oral, gastric, and intestinal digestive phases were measured. **Figs. 28a** and **b** show the hydroxycinnamic acids in oral, gastric, and intestinal phases at where the oral digestive phase had a significant effect on the CGA with up to 66% decrease compared to its control in free extract ($p < 0.05$), while CGA in NP decreased slightly at 4.6%. The impact of *in vitro* gastrointestinal digestion on expresso coffee from a single-dose coffee indicated a loss of higher than 10% for CGA where oral digestion had a significant impact on the CGA concentration [186].

CA and FA concentrations had a decrease of 64 % and 36% compared to their controls in free, but the concentration of these compounds increased for 14% and 108% in NP extract, respectively, and *p*-coumaric showed resistance to changes in the oral condition with a slight decrease of 0.72% in free and an increase of 0.1% in NP barberry extract. However, salivary albumin, mucins, and proline-rich proteins may affect the digestibility of specific polyphenols in the oral phase. For instance, tannins were precipitated by such proteins through hydrogen bonding and hydrophobic interactions [132]. The only difference observed between the control, and the oral digestive phase was for CGA among of the other hydroxycinnamic acids ($p < 0.05$).

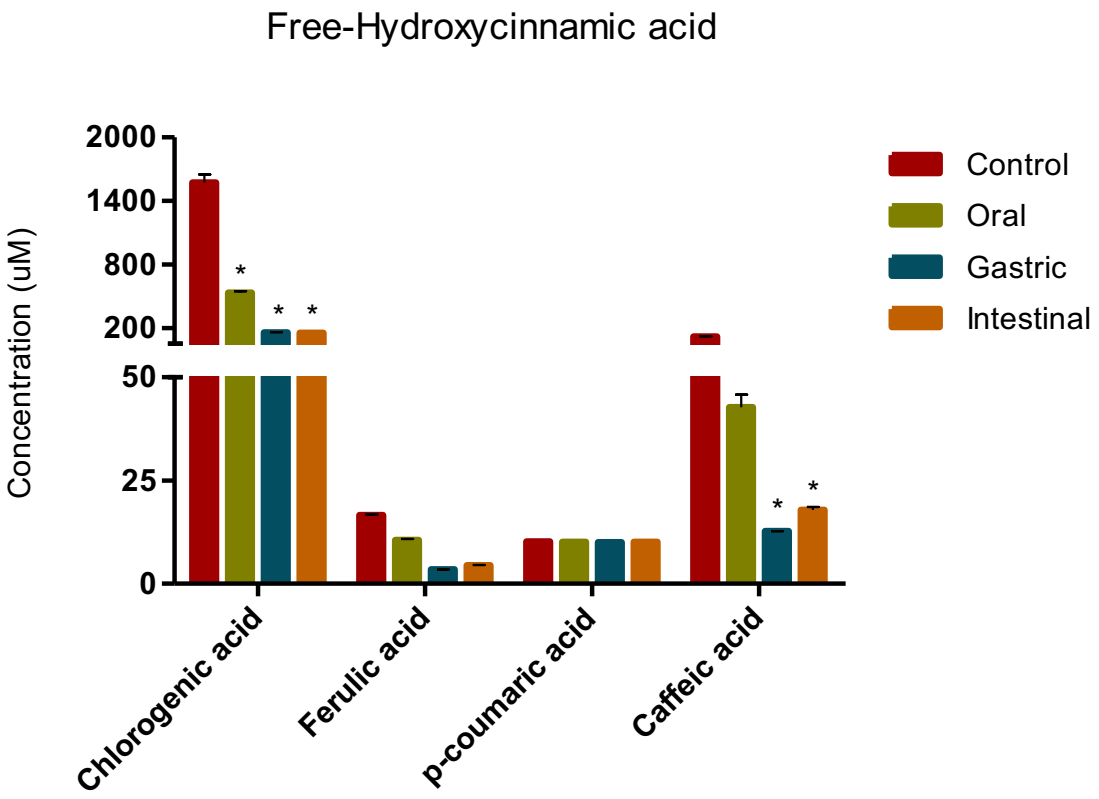
The CGA decreased by 70% the during gastric phase and an additional 2% in 2 hours at pH 6.8 in the intestinal phase in the free extract. In contrast, the neochlorogenic acid (3-caffeoylquinic acid) and chlorogenic acid (5-caffeoylquinic acid) were not altered during gastric treatments at a pH of 2.0 for 2 h [187]. An acidic pH of 2.5 in the gastric phase could alter the CGA concentration

in NP, so that 72% decrease and an additional 7.9% was observed. The CA showed a loss of 70% in the gastric phase, while the amount of this compound increased up to 41% in the intestinal digestive phase. The reason for an increase in CA concentration can possibly be due to the degradation of CGA in the gastric phase to CA and quinic acid. A study found that the content of CA and quinic acid in intestinal digestive phase was significantly higher than those in the oral and gastric phases, due to the effect of the SIF on the degradation of chlorogenic acid dimers and convert them into CA and quinic acid [188]. In a previous study, the degradation of CGA was pH dependent. When 1.2 mM CGA solution was incubated at 37 ºC at pH 6.0-9.0, 4-CQA and 3-CQA were produced [185]. Due to various conditions of GIT, including the presence of digestive chemicals, enzymes, and pH, 5-CQA showed to be more affected by GID. During the degradation, the positional isomerization of 5-CQA was occurred and isomers of 3-CQA (pH values of 6.0, 6.5, 7.0) and 4-CQA (pH values of 5.0, 5.5, 6.0, 6.5, 7.0) were produced. A possible proposed mechanism for this phenomenon could be that the 5-CQA was first isomerized to 4-CQA, then to 3-CQA. The isomerization, which is a major degradation pathway of 5-CQA were observed after 8 h exposure to intestinal fluid with a range pH value of 6.3-7.7, and formation of 3-CQA, 4-CQA, caffeic acid occurred [189-190].

The ferulic acid also exhibited a similar decrease trend compared to caffeic acid with a decrease of 67% in the gastric digestive phase and an increase of 30% at the intestinal phase in free extract, while this compound was decreased 73% in the gastric phase in NP extract. However, a 17% increase in the concentration of FA was observed in the intestinal phase in the same sample. The *p*-coumaric was the strongest hydroxycinnamic acids in an acidic pH of 2.5 in the gastric condition with only 0.4% decrease and a slightly increase (0.09%) in the intestinal phase in free extract compared to 0.19% decrease in gastric phase for NP. The possible explanation for increased

phenolic acid could be attributed to the action of pancreatin enzyme and bile salts on the extract, leading to the release of bound phenolics into the digestive model. The Meghalayan cherry (*Prunus nepalenis*) pomace extracted with microwave-assisted extraction (MAE) showed a slight increase in TPC from gastric to intestinal digestion compared to conventional solvent extraction (CSE). In the transition from the gastric to intestinal digestive phase, a higher decrease of 49.79% was observed in the TPC of CSE compared to the MAE with a value of 44.87% [169].

28a.



28b.

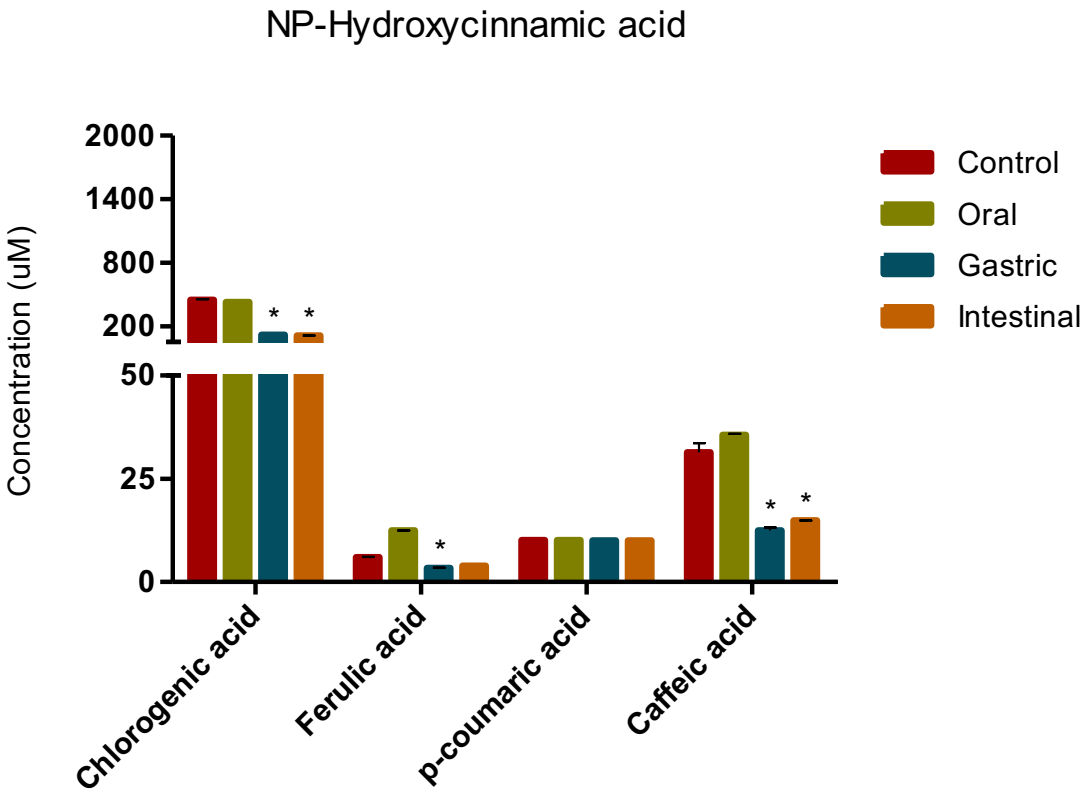


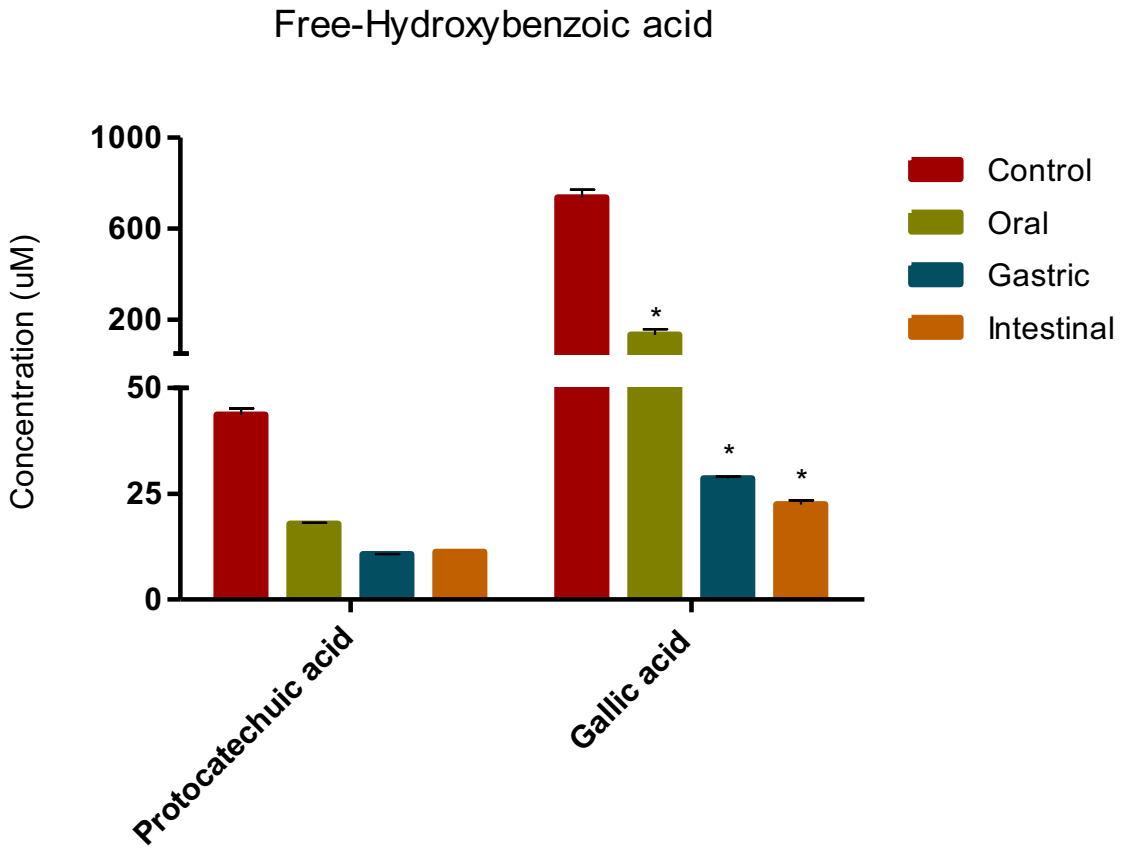
Figure 28. Digestion of hydroxycinnamic acids present in free and NP barberry extract in oral (PH 6.8), gastric (pH 2.5), and intestinal (pH 5.8 & 6.8) digestive phases at 37 ºC after 3 min, 2 h, and an additional 2 h incubation times. Asterisks represents a significant differences ($p < 0.05$) in ANOVA and Bonferroni post-tests.

In comparison to hydroxycinnamic acids, the hydroxybenzoic acids also showed susceptibility to simulated digestive phases (**Fig. 29a)**. An appreciable decrease was observed with values of 59% and 82% for protocatechuic acid and GA between the control and oral digestive phase in free extract ($p < 0.05$). The gallic acid was also more susceptible to the gastric digestive phase with a loss of 78% an additional 21% in the intestinal phase. However, protocatechuic acid showed up to 40% decrease at pH of 2.5 and a slightly increase in the intestinal phase (4.6%). This finding was

in contrast with previous studies that suggested phenolic compounds were stable in pH 2.0 of gastric condition [191].

A possible explanation could be that some key factors play significant roles in the stability of the polyphenols in the gastrointestinal digestive phases, including their chemical characteristics, solubility, hydrophobicity, molecular weight, and isomer configuration. The stability of the phenolic compounds in various pH was directly related to polyphenol structures [192]. Thus, the phenolic compound with antioxidant property in low pH of 2.0 or gastric phase, is not necessarily to have the same antioxidant capacity at the alkaline pH of 7.4 (intestinal phase) [133]. For instance, the pH condition of GIT may promote producing quinone intermediate compounds, which are unstable and alter the chemical properties of antioxidant compounds.

29a.



The concentration of protocatechuic acid was not impacted under the oral digestive phase in NP polyphenols extract. A slight decrease of 0.48% was observed in its concentration, whereas gallic acid concentration decreased by 46% in the oral phase for NP. This reduction was up to 35% less than the GA concentration at the oral stage in the free extract. The GA showed a decrease of 75% in gastric in NP, which was almost similar to the reduction of this compound in free extract. In NP extract, the protocatechuic acid was also reduced by 36% in the gastric phase, whereas a 5.5% increase was observed for this compound in the intestinal digestive phase. Some non-extractable polyphenols such as (protocatechuic acid and vanillic acid in purple carrots) could conjugate to cell walls of polymers (pectin, cellulose) during ingestion. A value of 35.3% increase

in the recovery of polyphenols was observed due to the interaction of neutral cellulose or pectin containing hydrophobic cavities, which could potentially encapsulate phenolic acids [193].

29b.

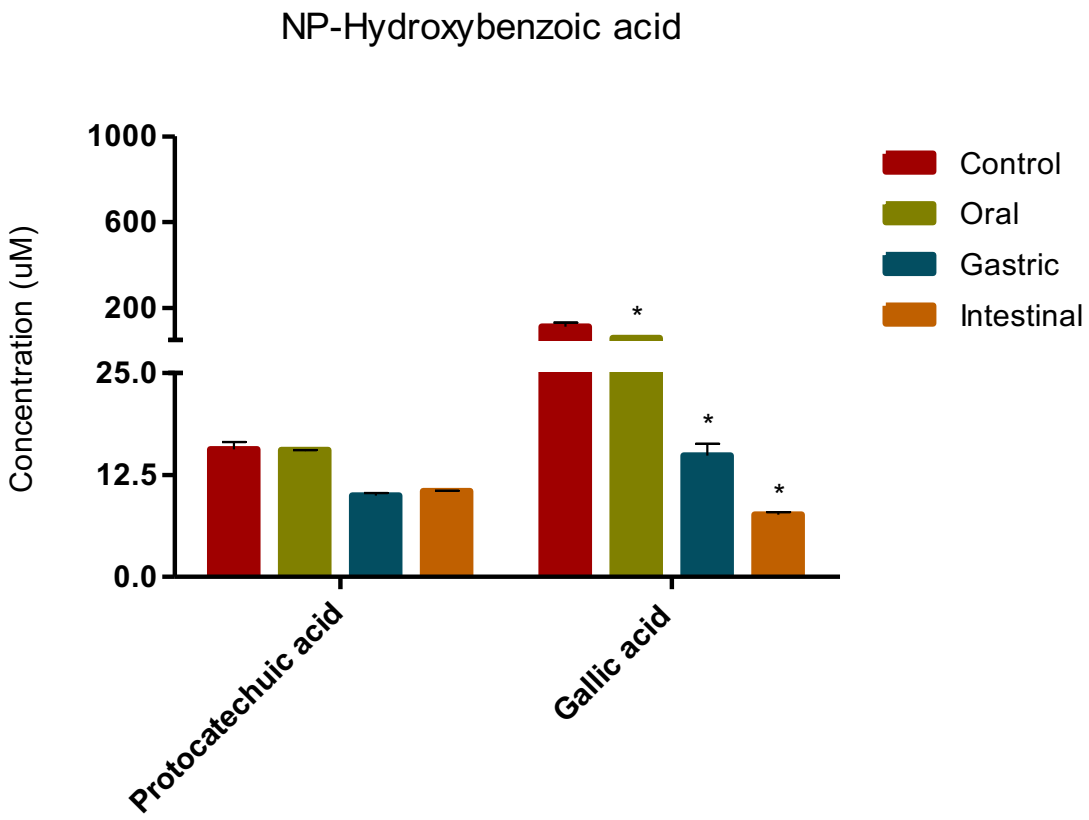
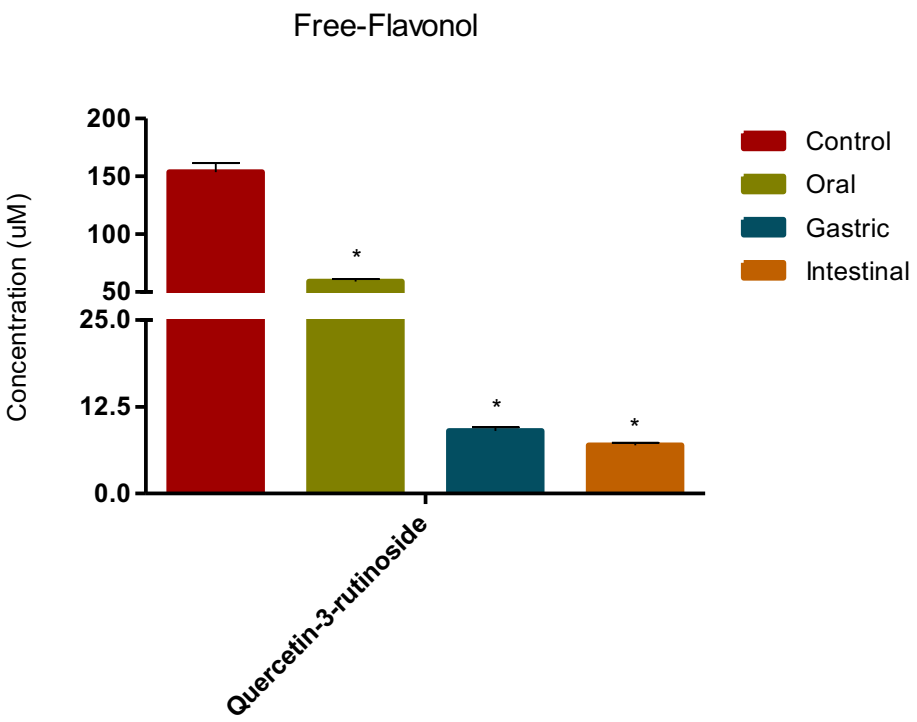


Figure 29. Digestion of hydroxybenzoic acids present in free (a) and nanoparticle (b) barberry extract in oral (PH 6.8), gastric (pH 2.5), and intestinal (pH 5.8 & 6.8) digestive phases at 37 °C after 3 min, 2 h, and an additional 2 h incubation times. Asterisks represents a significant differences ($p < 0.05$) in ANOVA and Bonferroni post-tests.

Figs. 30a and **b** show the concentration of quercetin-3-rutinoside (rutin) in free and NP barberry extract. The concentration of quercetin-3-rutinoside in the free specimen was decreased by 62% in oral that was significant compared to the control and an additional 85% and 23 %

decreases were also observed in gastric and intestinal digestive phases, respectively ($p < 0.05$). The dominant phenolic compounds, such as rutin detected in original samples, were not stable under gastrointestinal conditions with an 88% loss in the standard and a 1.7% loss in Caper (*Capparis spinosa L.*) was observed at the end of the simulated digestion [194]. In the low pH of the stomach, the flavonoids exist as aglycones and enter the upper small intestine. In the high pH of intestinal, deglycosylation, glucuronidation, methylation, sulfonating, and hydroxylation can occur [132]. In a comparison of free with NP barberry, quercetin-3-rutinoside was decreased by 1.5% after oral phase regarding to initial concentration followed by 79% in gastric and 17% in intestinal digestive phases.

30a.



30b.

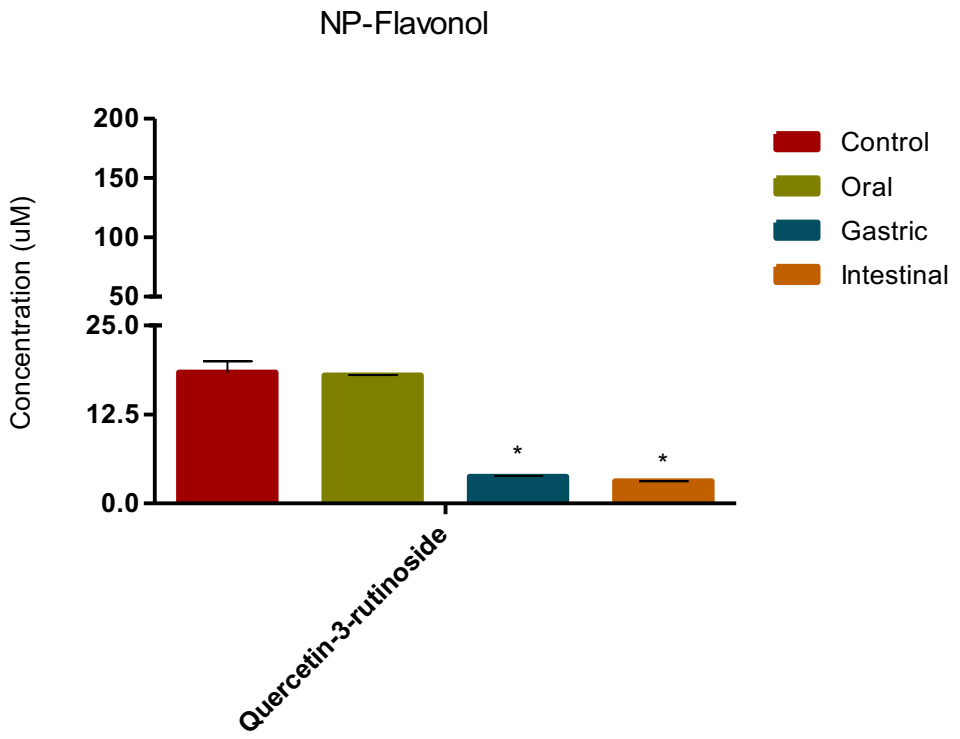


Figure 30. Digestion of quercetin-3-rutinoside present in free (a) and nanoparticle (b) barberry extract in oral (PH 6.8), gastric (pH 2.5), and intestinal (pH 5.8 & 6.8) digestive phases at 37 ºC after 3 min, 2h, and an additional 2 h incubation times. Asterisks represents a significant differences ($p < 0.05$) in ANOVA and Bonferroni post-tests.

Size Stability of Barberry Free and Nanoparticle in Simulated Gastric and Intestinal Conditions

In order to investigate the fate of size distribution of free and nanoparticle barberry polyphenols under a gastrointestinal digestive pattern and evaluate the influence of differential acidity levels on the particle size, the specimens were subjected to SGF (pH 2.5) and SIF (pH 6.8) over 32 h. Particle size distribution of samples collected at different digested stages of gastric and

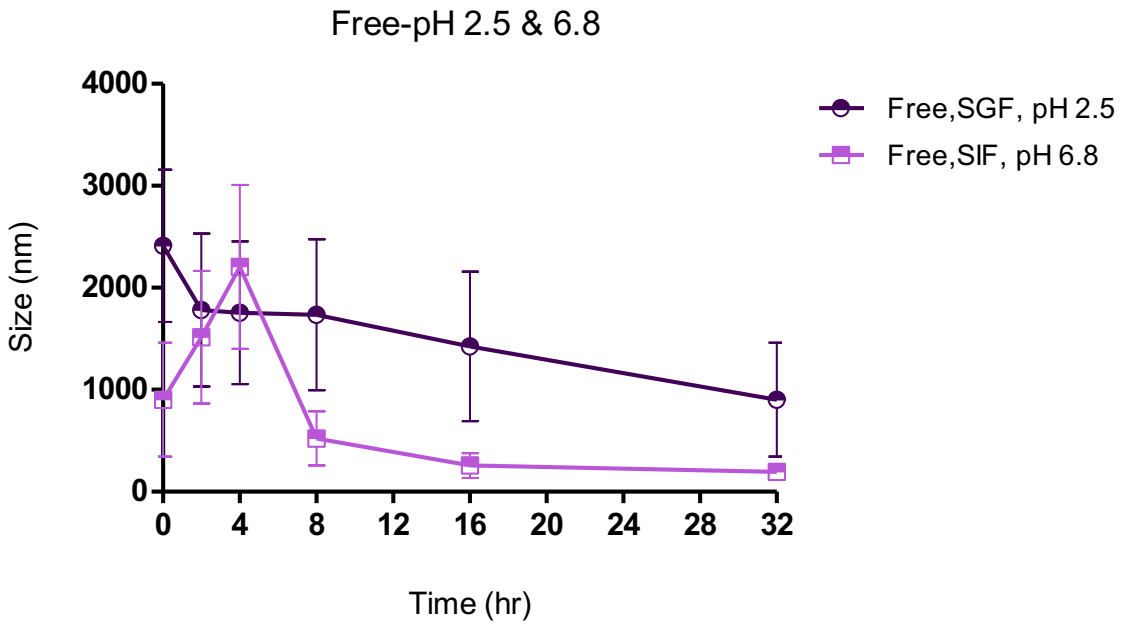
intestinal were measured through a laser light scattering instrument using a Zetasizer ZS90 analyzer (Malvern Instrument, L.t.d., Westborough, MA). **Figs. 31a** and **b** show the size stability in PBS with different pH conditions. The size distribution of free in SGF was 2411 nm and dropped after 2 h by 26%. At 27%, 28%, 41, and 62%, reduction was observed at 4 h, 8 h, 16 h, and 32h after incubation with SGF. The free barberry extract was exposed to SIF for an additional 32 h. The size of the free extract was increased by 67% and 144% in first 2 h and 4 h after incubation with SIF, respectively. However, the size distribution was decreased at 8 h, 16 h, and 32 h by 42%, 71%, and 78%, respectively.

In contrast, the size of nanoparticles was found to be increased by 26%, 40%, and 15% in the first 2 h, 4 h, and 16 h to values of 2087 nm, 2312 nm, and 1890 nm at pH 2.5 of SGF, respectively. In comparison, the size distribution of nanoparticles was reduced to 877 and 755 nm after 8 h and 32 h exposure time to SGF. In agreement with this work, the mean particle diameter of tannic acid-loaded nanoparticle (zein/pectin nanoparticles) increased in a shifted pH from pH 8.0 to pH 2.0 resulting in the nanoparticle coming into contact with each other [195]. However, upon exposure of samples to an acidic environment of the gastric phase, a size increase was observed and the compounds within nanoparticle extract were aggregated. Factors including temperature, pH, and salts can alter the nanoparticle characteristic due to changing the size distribution through aggregation [196]. Typically, aggregated nanoparticles are held together by physical forces, such as electrostatic interaction, hydrogen bonding, and hydrophobic forces [197].

Barberry nanoparticles also encountered salt (NaCl) and pepsin protein in simulated gastric fluid (SGF). The presence of salt may reduce the repulsive force between nanoparticles, causing particle surfaces to contact each other and more aggregation and particle attachment to occur in SGF (pH 2.0). A previous study indicated that the aggregation of PLA nanoparticles occurred by

increasing in size from 158 nm to 501 nm upon incubation of 10 mg/mL of nanoparticle treatment at SGF (pH 1.2) for 24 h [198]. However, when nanoparticle treatments are exposed to various environmental conditions, they may undergo changes of size distribution along with changes in surface chemistry due to an ionic strength of the solution [199].

31a.



31b.

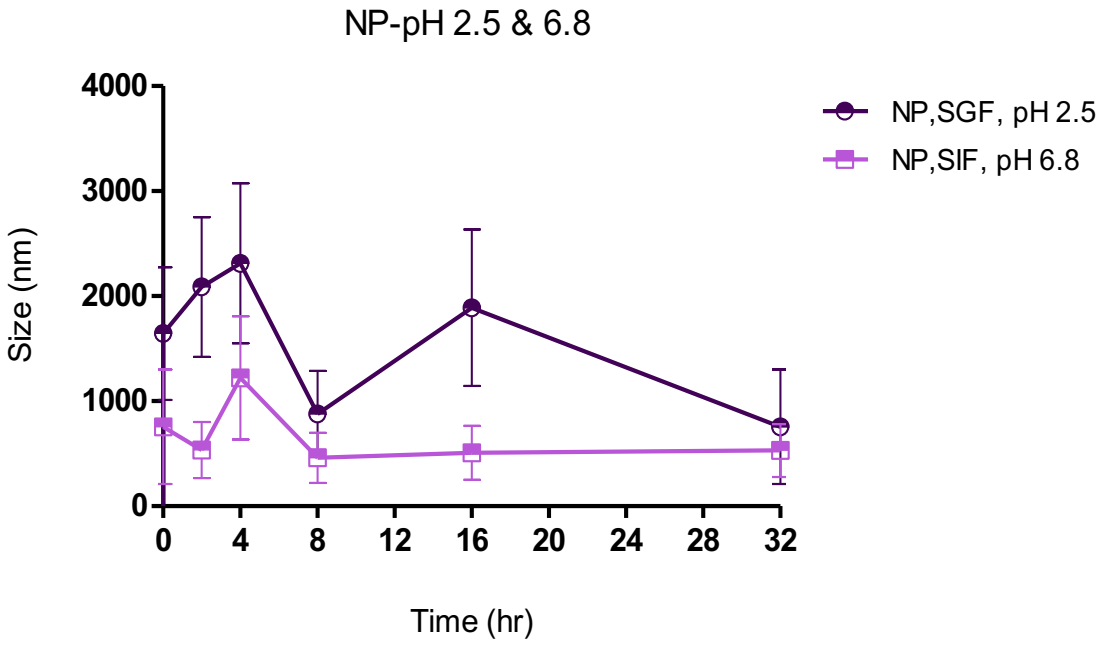
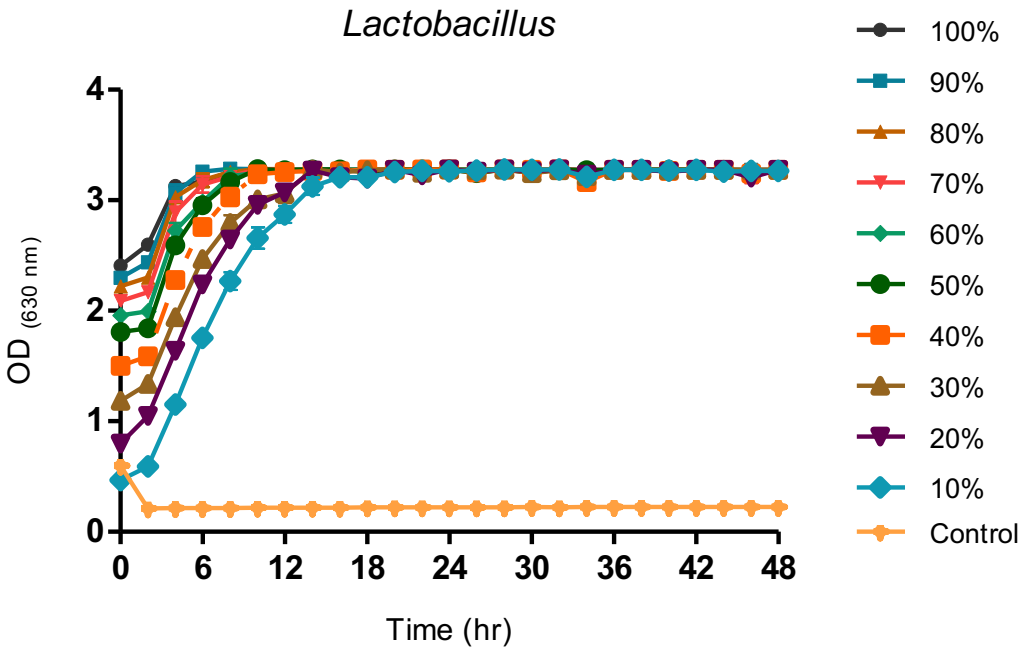


Figure 31. The effect of gastrointestinal stage on the particle size distribution of free (a) and nanoparticle (b) barberry polyphenolic extract at pH of 2.5 and 6.8 at 37 ºC.

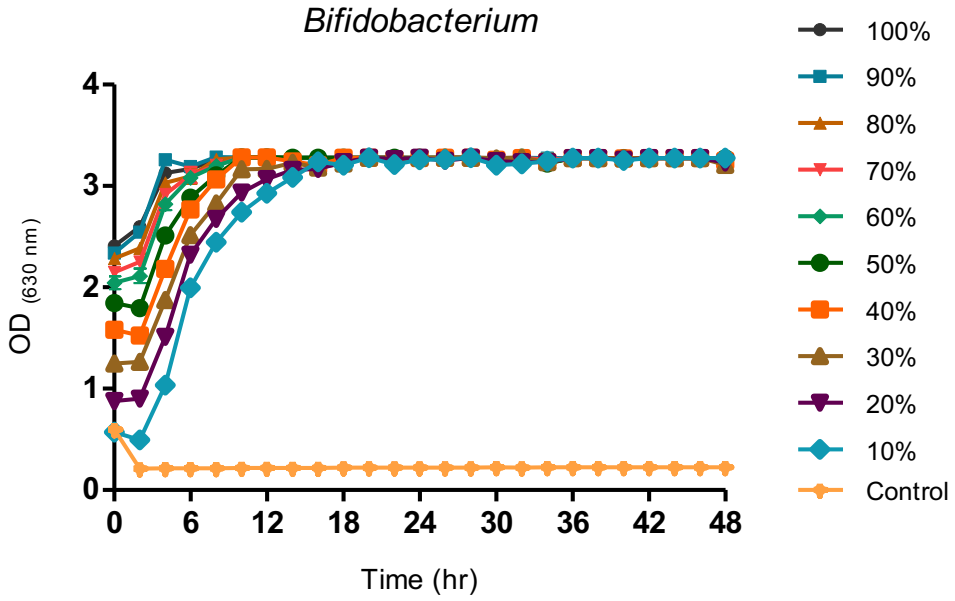
Stimulatory Effect of Barberry Polyphenolics on Probiotic Bacteria Growth

A commercial probiotic supplement was used to isolate *Lactobacillus* spp. (*L. casei, L. rhamnosus, L. reuteri, L. acidophilus, L. paracasei, L. bulgaricus, L. salivarius,* and *L. plantarum*) and *Bifidobacterium* spp. (*B. bifidum*, *B. longum, B. breve,* and *B. lactis*) for use alone and in a co-culture as Gram-positive compared to a Gram-negative of *E. coli* HS were cultured for 36-48 h within two consecutive days. *Lactobacillus* spp., *Bifidobacterium* sp., and a co-culture were incubated in MRS media and *E. coli* HS in BHI at the concentrations of control 0 (MRS medium as control), 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% probiotic bacteria for 48 h at 37 ºC. **Fig. 34** shows the growth curve of tested probiotic bacteria in MRS to set up a consistent model and evaluate the microbial metabolism.

34a.


Lactobacillus
OD (630 nm)
Time (hr)
0
1
2
3
4
0
6
12
18
24
30
36
42
48
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
Control

34b.


Bifidobacterium
OD (630 nm)
Time (hr)
0
1
2
3
4
0
6
12
18
24
30
36
42
48
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
Control

34c.

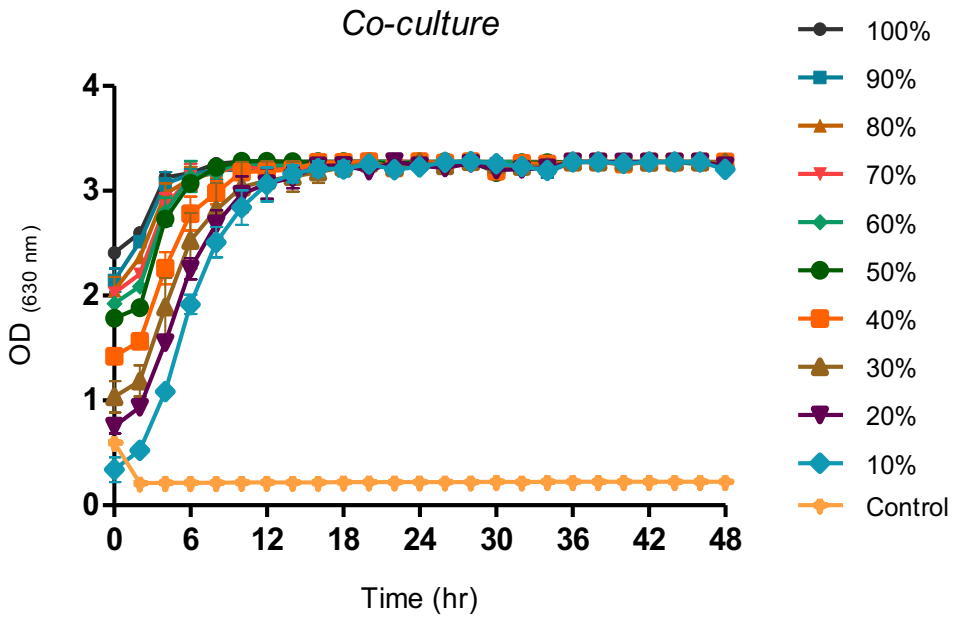


Figure 32. Growth curve of *Lactobacillus* (a), *Bifidobacterium* (b), and Co-culture (c) in MRS media over 48 h at 37 ºC.

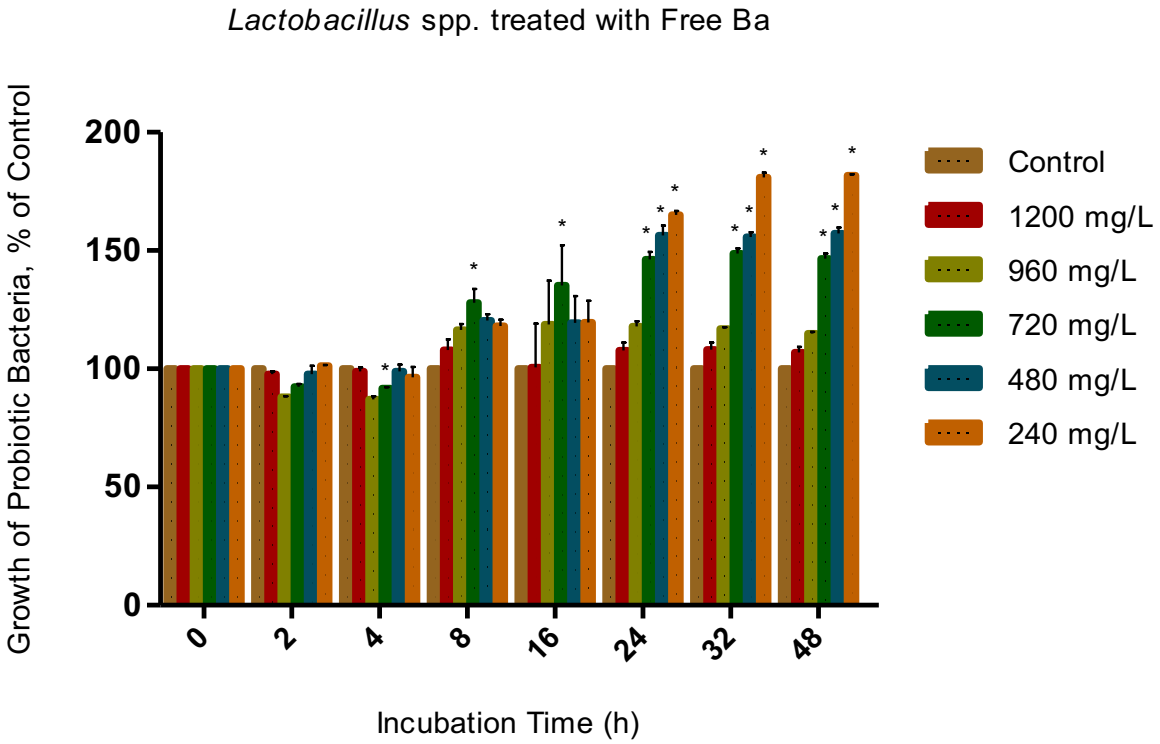
In a further step, each treatment of free and NP barberry extracts was added to bacterial cultures in concentration ranges of 240 mg/L-1200 mg/L. The effect of the treatments on the growth of probiotic bacteria including *Lactobacillus* sp. was then investigated (**Fig. 35a**). The results indicated that free barberry extract stimulated the growth of *Lactobacillus* spp. after 48 h of the incubation time, whereas a slight growth was observed by 1.32% at 2 h of incubation (exponential phase) at 240 mg/L. The growth was also reached maximums for concentrations of 1200 mg/L, 960 mg/L, 720 mg/L, and 480 mg/L by 7%. 15%, 47% and 57% at 48 h of incubation time, respectively. Moreover, stimulatory effect of 720 mg/L concentration was significant 8 h by 28% and 16 h by 35% after incubation times. However, a slow growth was observed for next 24

h, 32 h, and 48 h, whereas the growth was boosted at the concentration of 480 mg/L and 240 mg/L by 56% and 81%, respectively.

The growth of L*actobacillus* spp. was decreased by 2.3% and 1% after 2 h and 4 h incubation time at the concertation of 1200 mg/L, stayed on 7% growth until end of the incubation. During 10 days supplementation of polyphenols derived from Sea buckthorn, an initial 20% inhibition was observed, however, after a 48 h incubation, *Lactobacillus* adapted to environment condition [200]. This finding was in agreement with the current study especially at the concentrations of 720 mg/L and 240 mg/L.

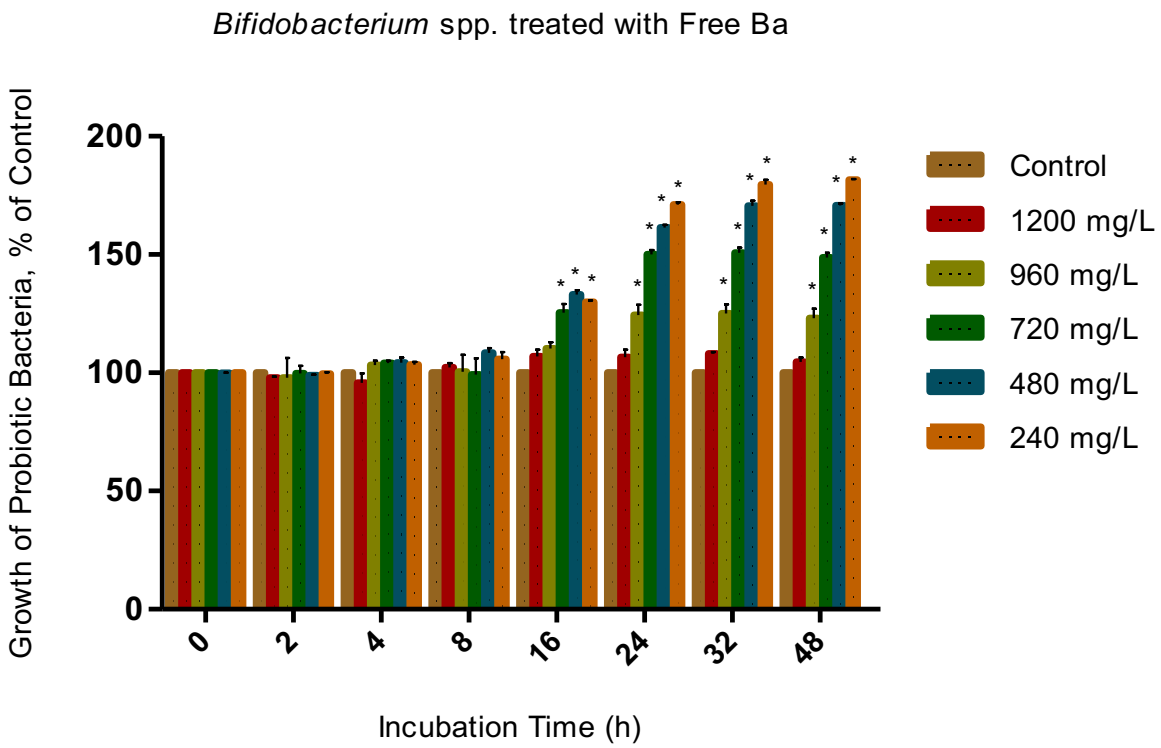
It can be a possible of autoxidizing of polyphenols that leads producing hydrogen peroxide and may act as bactericidal substances. Also, the basic chemical structure of polyphenols can be responsible for their impact on the bacteria, depending on their concentration, the polyphenolic extract can act as stimulators and inhibitors. The phenolic acids such as hydroxycinnamic acids (caffeic acid, *p*-coumaric acid, and ferulic acid) affect the growth of *Lactobacillus* spp. depends on the type of nutrient matrix they are present. First, the phenolic acids may interfere with their metabolic process such as lactic fermentation. For instance, gallic acid stimulated the growth and metabolic activity of *Lactobacillus* spp. bacteria at the concentration of below 3 mM, whereas a concentration above 3 mM disrupted the integrity of bacteria cell walls, resulting in a disturbed the pH gradient [201].

35a.



The potential stimulatory effect of free barberry extract on *Bifidobacterium* spp. was also studied (**Fig. 35b**). The *Bifidobacterium* spp. showed a slight increase in their growth at 2 h of incubation by up to 2% at the concentration of 960 mg/L. Similar to *Lactobacillus* spp, the highest growth rate was observed at 48 h after exposure time to the treatments. As the time of incubation reach 48 h, *Bifidobacterium* spp. exhibited the highest stimulatory effect by 82% and 71% at the concentrations of 240 mg/L and 480 mg/L ($p < 0.05$). The values of 48% and 23% and 4.5% increase in the proliferation of *Bifidobacterium* spp. were observed at the concentrations of 720 mg/L, 960 mg/L, and 1200 mg/L.

35b.



Bifidobacterium spp. Are heterofermentative which promote the production of organic acids such as lactic acid and acetic acid, resulting in a pH decrease [202]. This result was in agreement with the study that indicated the stimulatory and inhibitory effects of *Bifidobacterium* spp. at the same time. The strongest stimulatory effect was observed for the addition of 2, 20, 100 mg/L of coumaric acid, at which 20%, 36%, 50% growth increased, respectively [183]. A possible explanation for the inhibitory influence of polyphenols against *Bifidobacterium* at a higher concentration may be due to the stimulatory effect of the treatment on the growth of lactic acid bacteria (LAB) at 24 h which cause a significant drop in the pH value of the environment resulting a cell death afterward [128]. This trend of inhibitory effect was observed in the current study, although, slight increases was

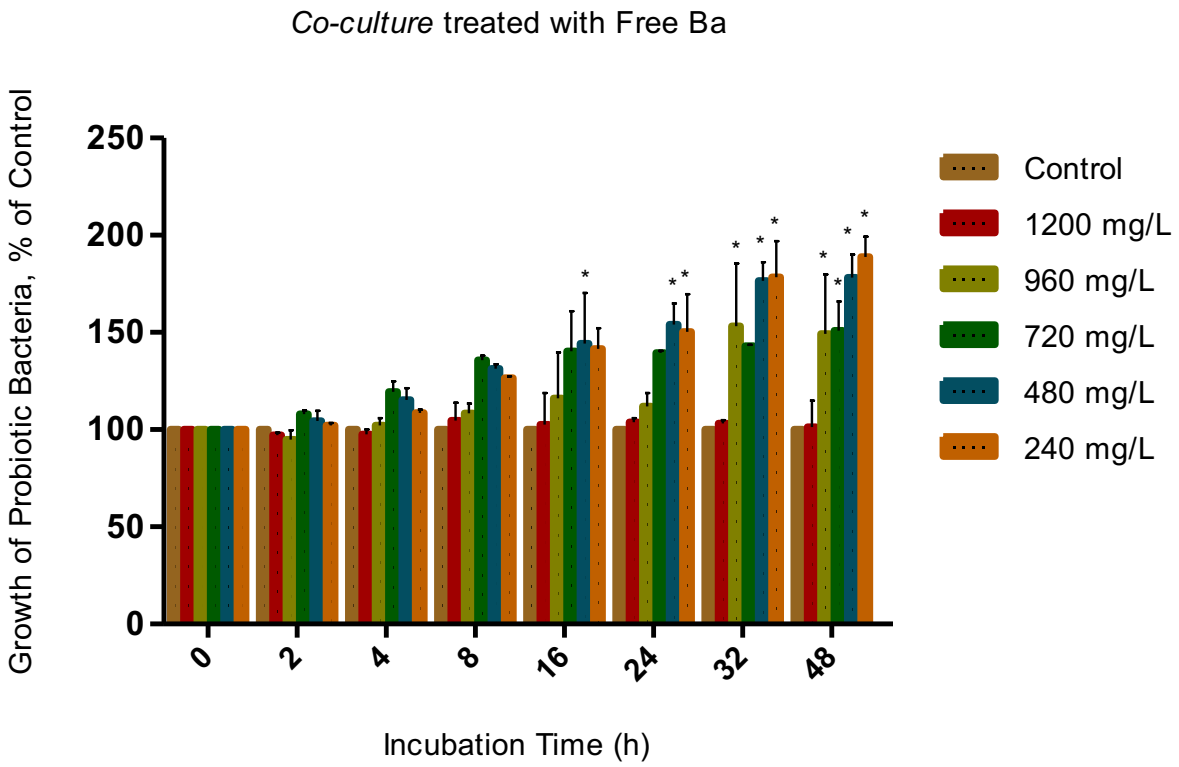
occurred at 24 h to 32 h after incubation times for 720 mg/L, 960 mg/L, 1200 mg/L, the growth levels were decreased by 23%, 3%, and 4%, respectively.

The stimulatory effects of polyphenolic compounds are dose, structural, and strain-dependent [183]. For instance, the effect of hydroxycinnamic acids and their quinic esters including ferulic, *p*-coumaric, and caffeic aid on the growth of wine-spoilage LAB was observed at 100 mg/L, while LAB was inhibited at concentrations of 500 and 1000 mg/L. Also, the stimulatory effect of catechin on the *Bifidobacterium sp*. growth, while epicatechin showed less proliferation effect [200]. The 500 mg/L of caffeic, coumaric, and ferulic acid inhibited the development of *L. brevis*, whereas chlorogenic and caffeic acid had a reduction on *L. rhamnosus* at the concentration of 250 mg/L [201].

Fig. 35c shows the impact of free barberry polyphenolic extract on the multiplication of co-culture, a mixture of *Lactobacillus* spp. and *Bifidobacterium* spp. The result indicated that a combination of 8 strains of *Lactobacillus* spp. (*L. casei, L. rhamnosus, L. reuteri, L. acidophilus, L. paracasei, L. bulgaricus, L. salivarius,* and *L. plantarum*) and 4 strains of *Bifidobacterium* spp. (*B. bifidum*, *B. longum, B. breve,* and *B. lactis*) increased the proliferation of those bacteria by 21% and 7% at the concentration of 480 mg/L compared to that of *Lactobacillus* and *Bifidobacterium* bacteria. At the end of the 24 h using 240 mg/L significantly affected the bacterial growth, whereas the growth of co-culture bacteria was significant at the concentration of 480 mg/L after 16 h. The concentration level of 240 mg/L stimulated the greatest percentage increases by 88% when co-culture bacteria were treated with, whereas the growth values were at up to 82% for *Lactobacillus* spp. and *Bifidobacterium* spp. The contrary, the concentration of 1200 mg/L stimulated the co-culture bacteria by 1.2%, while values of 7% and 4.5 were observed when *Lactobacillus* spp. and *Bifidobacterium* spp. were treated with this concentration.

The microorganisms from the same species or genus exhibited substantial variation in sensitivity once exposed to phenolic acids which were different from strain to strain. Nevertheless, the strain that showed the highest metabolic activity towards phenolic acid, also were more tolerant strains. For instance, *L. reuteri* which had an esterase enzyme activity only toward conversion of CGA to CA, was the most sensitive strain among *L. plantarum*, *L. fermentum*, and *L. hammesii*. A possible explanation can be due to the effect of hydroxyl and methoxy groups as well as the contribution of carboxyl groups and the double bond in hydroxycinnamic acids [203].

35c.



The effect of free barberry extract was studied on commensal *E. coli* HS as Gram-negative bacteria (**Fig. 35d**). The *E. coli* HS bacteria was exposed to different concentrations of the treatments at 1200 mg/L, 960 mg/L, 720 mg/L, 480 mg/L, and 240 mg/L. The growth trend for all

concentrations was similar to *Lactobacillus* spp., *Bifidobacterium* spp., and a mixture of co-culture at 0 h, 2 h, 4 h, 8 h, 16 h, 24 h, 32 h, and 48 h after incubation. However, the treatments showed a different growth trend from 24 h to 48 h. So that, the growth of 720 mg/L was significant at 24 h by 42% increased, whereas 240 mg/L showed a reduction by 4% after 24 h. The growth of *E. coli* HS was reduced from 24 h to 48 h upon fermentation by 14% at 1200, 19% at 960 mg/L, 15% at 720 mg/L, 14% at 480 mg/L, and 11% at 240 mg/L. The concentration of 720 mg/L was the most effective concentration among other concentrations that stimulated the growth of *E. coli* HS by 43%, following were the concentrations of 480 mg/L and 240 mg/L by 36% and 32%, respectively.

35d.

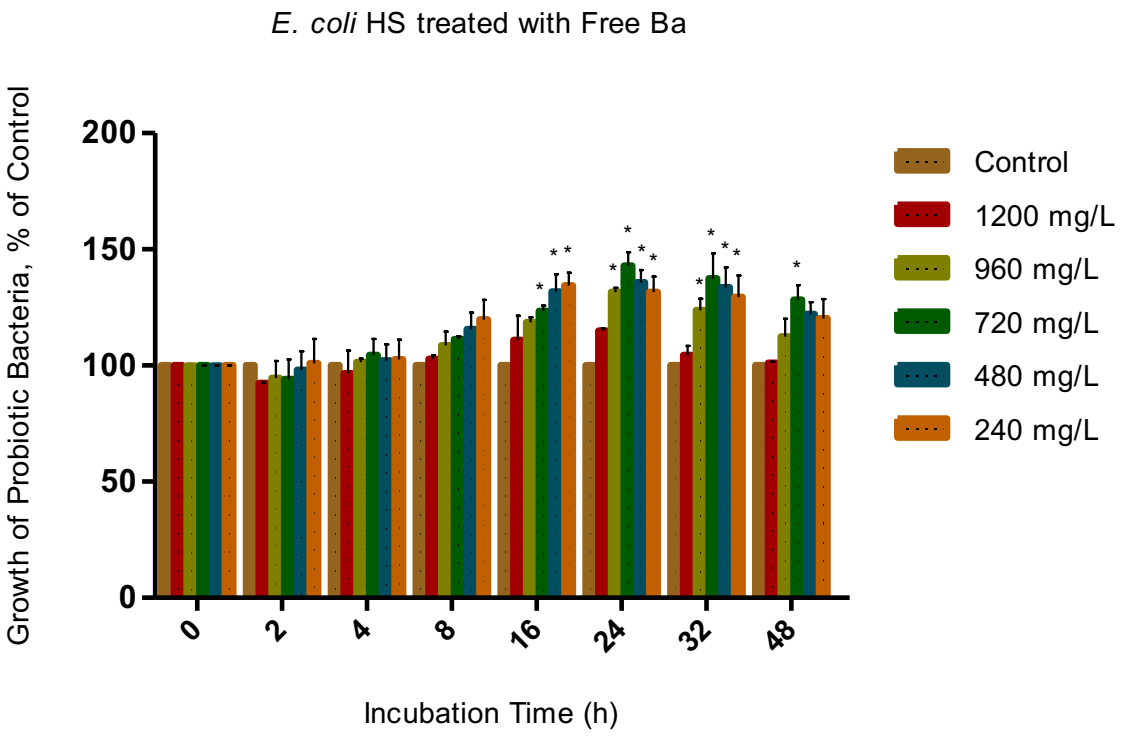


Figure 33. The modulatory/inhibitory effects of free barberry polyphenolic extract on the growth of *Lactobacillus*, *Bifidobacterium*, Co-culture, and *E. coli* HS treated at the concentrations of 240-1200 mg/L at 37 ºC during 48 h incubation time anaerobically ($p < 0.05$).

The result of the current study indicated that the probiotic strains such as *Lactobacillus* spp. and *Bifidobacterium* spp. were more resistant toward polyphenols, whereas commensal bacteria were more susceptible to polyphenolic compounds compared to probiotic bacteria. *E. coli* K-12 MG1655 *recA::gfpmut2* strain treated with cinnamic, caffeic, ferulic, and chlorogenic acid was incubated for 24 h and 48 h. The result indicated a significant increase in growth inhibition values for CGA and FA by 45% and 36% especially at the concentrations of 28.2 μM/L, 2.82 μM/L, and 2.82×10-1 μM/L for CGA and 51.5 μM/L, 5.15 μM/L, 5.15×10-1 1 μM/L for FA [204]. The microbial metabolites production of tea catechins were able to inhibit the growth of several pathogenic and non-beneficial intestinal without affecting the growth of probiotic bacteria significantly. For instance, *Bifidobacterium* spp. are resistant to flavan-3-ols, which are iron-chelating compounds. Since these bacteria do not use heme-containing enzymes, they are less susceptible to this class of compound than pathogenic bacteria such as *E. coli*, *S. aureus*, and *Salmonella* spp., *Clostridium perfringens*, and *Clostridium difficile* [205].

Another possible explanation regarding the resistance of probiotic to polyphenolic compounds can be due to their ability to produce lactic acid which makes them stronger to withstand the acidic environment. Some other factors can also affect the polyphenols to being effective when treated with beneficial bacteria, such as interaction with medium matrix and precipitate such as tannins as well as the interaction with protein present in the bacteria cell wall. Also, the hydrophilicity of the phenolic compound can influence their stimulatory effect, lipophilic phenolics such as quercetin and kaempferol could not inhibit lactic acid bacteria, whereas hydrophilic compounds such as myricetin were exhibited inhibitory effect [206].

Metabolism of Free and NP Barberry Polyphenolics by Probiotic Bacteria

Treated probiotic cultures with free and NP polyphenolic extract were collected at specific intervals for 48 h of incubation and deactivated with 5-fold methanol acidified (0.1 % formic acid), LC-MS/MS was used for the metabolite quantification. **Fig. 36** shows a schematic illustration of methodology and metabolites production by probiotic bacteria.

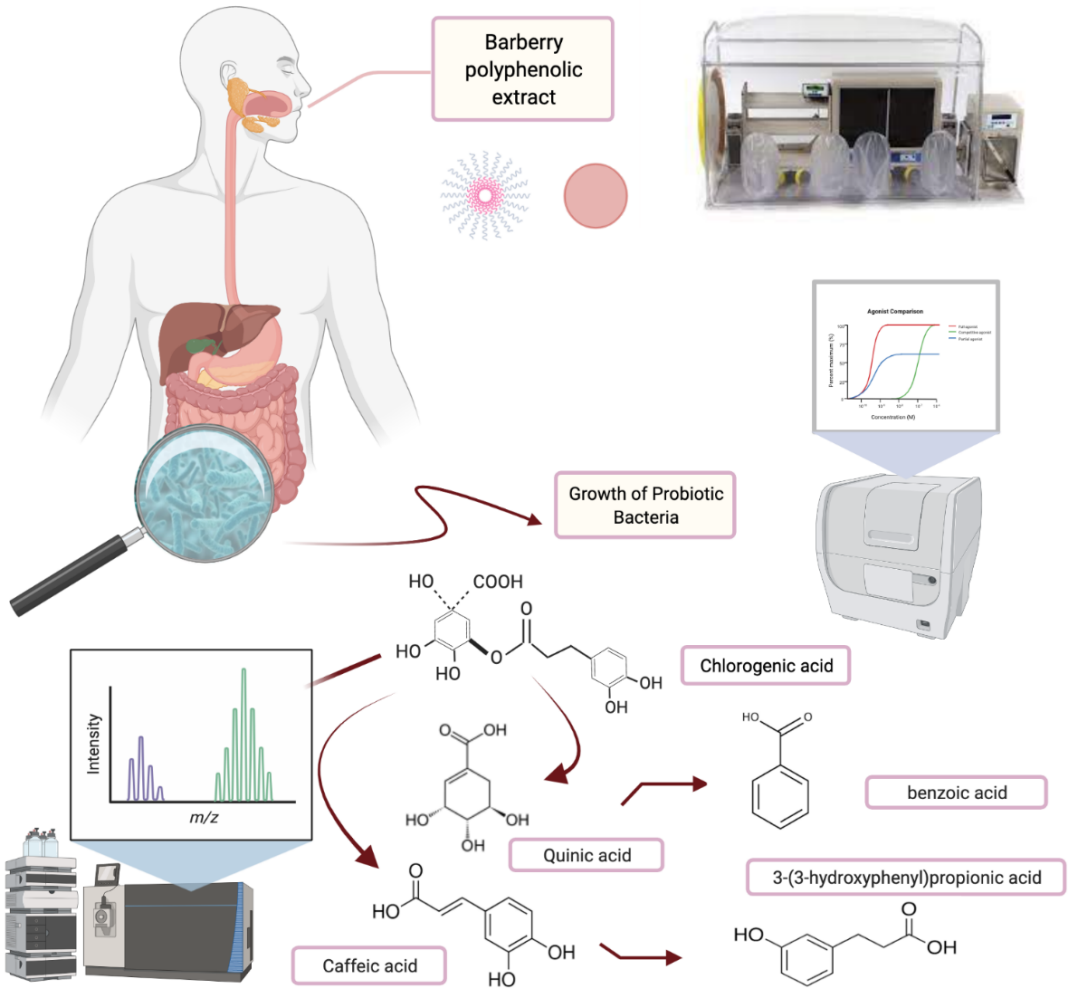


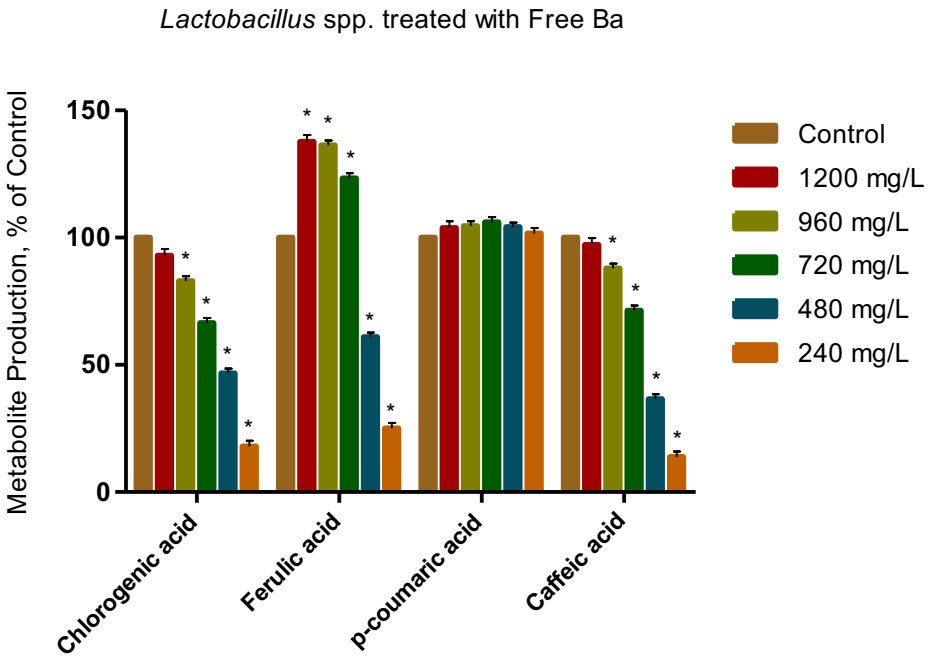
Figure 34. A schematic illustration of methodology and metabolite production of free and NP polyphenolic extract, created with BioRender.com (2021).

The final step of catabolism by probiotic bacteria *in vitro* were studied which yields the molecules with lower molecular weight. These polyphenols metabolites can further be absorbed in the body easily. This study was aimed to investigate the changes in the content of barberry extract by probiotic bacteria (*Lactobacillus* sp., *Bifidobacterium* sp., co-culture of *Lactobacillus* sp. and *Bifidobacterium* sp.) as well as *E. coli* HS after 48 exposure times with free and NP polyphenolic extract. The concentration of targeted compounds of CGA (5-CQA), FA (4-hydroxy-3-methoxycinnamic acid), *p*-Coumaric acid (4-hydroxycinnamic acid), CA (3,4-dihydroxycinnamic acid), protocatechuic acid (3,4-hydroxybenzoic acid), GA, rutin (quercetin-3-*O*-rutinoside), 3-(3-hydroxyphenyl) propionic acid, and 3,4-dihydroxyphenylacetic acid in µMolar were evaluated. Results of impact of *Lactobacillus* spp. treated with free extract was indicated that a decrease in the amount of CGA was observed as the concentration of polyphenols decreased (**Fig. 37a**). Up to 10% reduction was observed in CGA concentration at 1200 mg/L, with a value of 1324 µM whereas the concentration of this compound decreased by 85% at 240 mg/L with a value of 232 µM. The FA was the only hydroxycinnamic acid among the others that showed an increase in its concentration by 35%, 34%, and 21% at the free extract at concentrations of 1200 mg/L, 960 mg/L, and 720 mg/L with values of 25 µM, 24.5 µM, and 22 µM, respectively. The caffeic acid also showed a similar reduction trend to CGA in its concentration, whereas by decreasing the concertation of treated free extract from 1200 mg/L to 240 mg/L, the concentration of caffeic acid was decreased from 118 µM to 10 µM.

A decrease in CGA and an increase in the concentrations CA and FA by *E. coli*, *B. lactis*, and *L. gasseri* was reported. These bacteria can hydrolyze CGA into the CA, FA, and other aromatic acid substances [207], whereas *L. plantarum* was not able to metabolize CGA. However, the metabolisms of CA, FA, GA, *p*-coumaric, *p*-hydroxybenzoic, and protocatechuic acid by *L.*

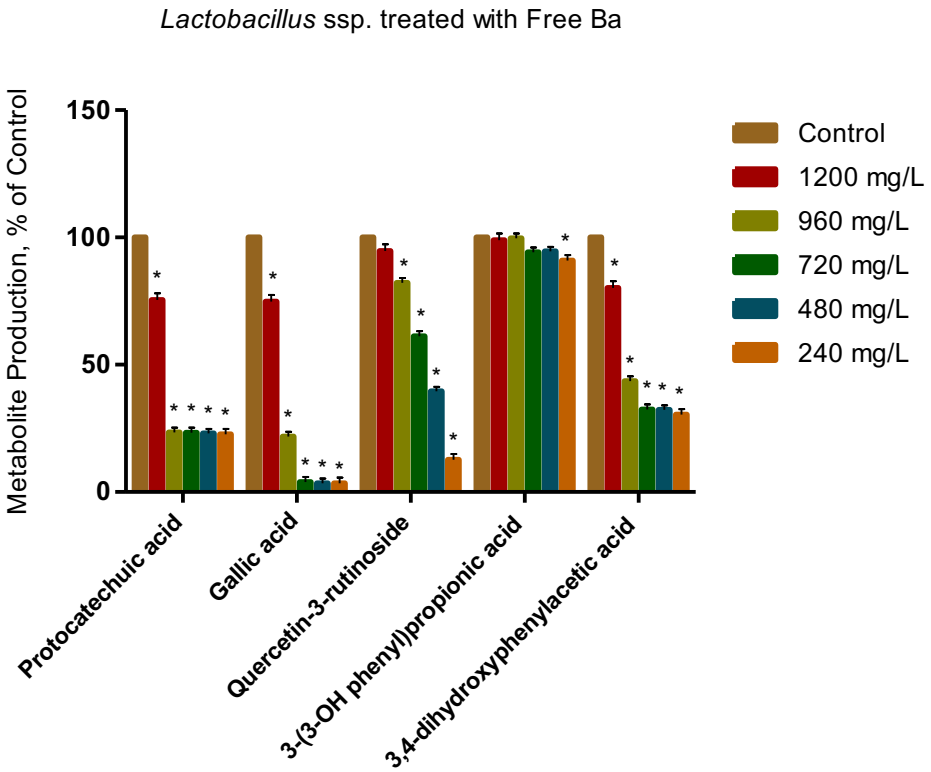
plantarum have been reported. *L. fermentum* metabolized *p*-coumaric and FA, whereas CGA was the only compound that was metabolized by *L. reuteri* [203]. An increase in the concentration of phenolic acid after exposure to probiotic bacteria can be explained that, first, some hydroxycinnamic acids such as ferulic and *p*-coumaric acids are ester-linked form to plant cell wall polymers and esterase enzymes released by probiotic bacteria can hydrolyze and break the ester bonds resulting in releasing of free phenolic acids [208]. Second, some probiotic bacteria such as *B. lactis*, *L. gasseri*, and *E. coli* produce cinnamoyl esterase which catalysis the cleavage of quinic acid releasing the cinnamic acid and moiety from the CGA molecules produce caffeic and ferulic acids. The free acid is then released from phenolic acid in ester forms by bacterial esterase. These compounds undergo further analysis to reduce the double bond and produce metabolites by demethylation, dehydroxylation, decarboxylation, and isomerization [207].

37a.



The result of the current study indicated that 3-(3hydroxyphenyl) propionic acid concentration showed an increase by 78% at the concentration when *Lactobacillus* strains were treated with 240 mg/L of free extract (**Fig. 37b**). The concentration of 3,4-dihydroxyphenylacetic also had a slight increase by 27% at the concentration of 240 mg/L compared to the control. By decreasing the concentration of free extract from 1200 mg/L to 240 mg/L (1 to 6-fold dilution), the concentration of 3,4-dihydroxyphenyacetic acid was increased with a value of 15 µM.

37b.

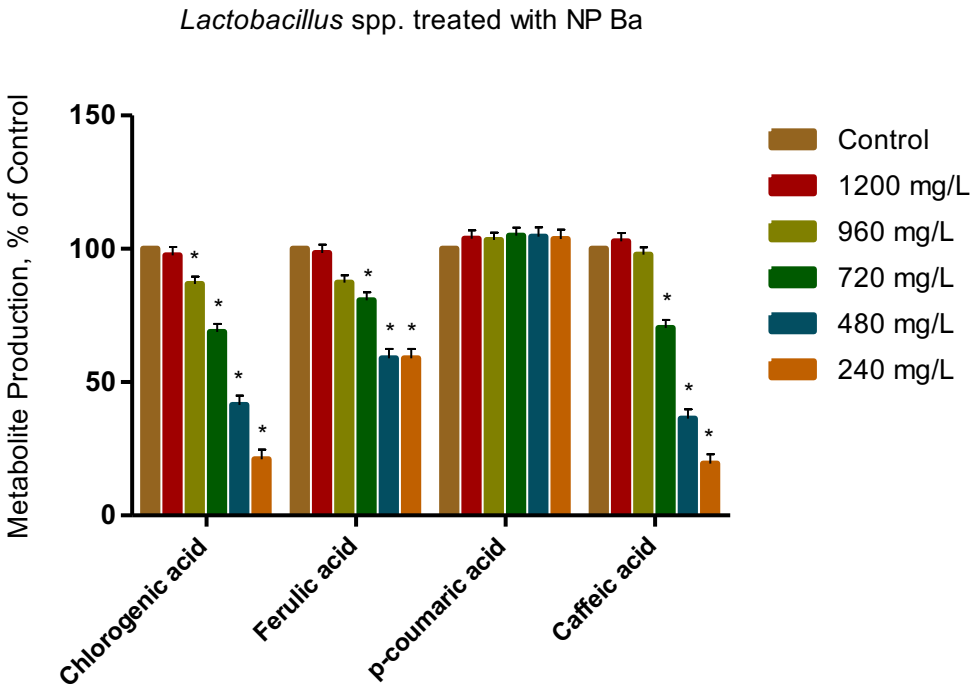


Two metabolism pathways were suggested for hydrolyzing CGA to its metabolites: first, a formation of 4-vinyl derivatives which further can be reduced to 4-ethyl derivatives. Second, formation of phenylpropionic and then decarboxylation [209]. Microbial metabolisms of CQA are produced by hydrolyzing CGA to CA and quinic acid. The free CA may be further transformed into m-coumaric, hydroxylated derivatives of phenylpropionic, benzoic acid, and hippuric acid [210]. The hydrogenation or dehyroxylation of CQA can also be happened by colonic microbiota, before even hydrolyzing the ester bond and breaking the QA moiety. The degradation of polyphenolic acid by *lactobacillus* spp. including *L. plantarum* 299v, *L. rhamnosus* GG, and *L. reuteri* DSM17938 isolated from three different supplement was previously studied. The *L. plantarum* 299v was able to metabolize the hydroxycinnamic acids (caffeic acid, ferulic acid, *p*-coumaric) by decarboxylation and reduction reactions. The contrary, *L. plantarum* 299v was found to be resistant to CGA (5-CQA). *L. reuteri* DSM17938 decomposed 5-CQA by 88% by producing esterase enzyme, whereas *L. rhamnosus* GG did not display any substrate enzymatic (decarboxylase, reductase, or esterase) activity on caffeic acid, ferulic, and CQA [209]. However, other strains of *L. plantarum* TMW 1.46 exhibited an ability on hydrolyzing the 5-CQA [211]. Thus, the expression of certain enzymes depends on the bacteria strains and their affinity to CGA substrate among other substrates.

The CGA concentration was reduced by 14% at the treatment concentration of 240 mg/L with a value of 227 µM. The concentrations *p*-Coumaric acid and 3-(3-hydroxyphenyl)propionic acid were found to be increased by 100% for NP extract when exposed to *Lactobacillus* strains **(Fig. 37c**). The concentration of FA and 3, 4-dihydroxyphenylacetic acid were also increased by 30% and 39% compared to their controls at the concentration of 240 mg/L with a value of 3.24

μM and 15.5 μM. The concentration of quercetin-3-*O*-rutinoside showed a decrease by 41% at the concentration of 240 mg/L with a value of 2.64 μM.

37c.



37d.

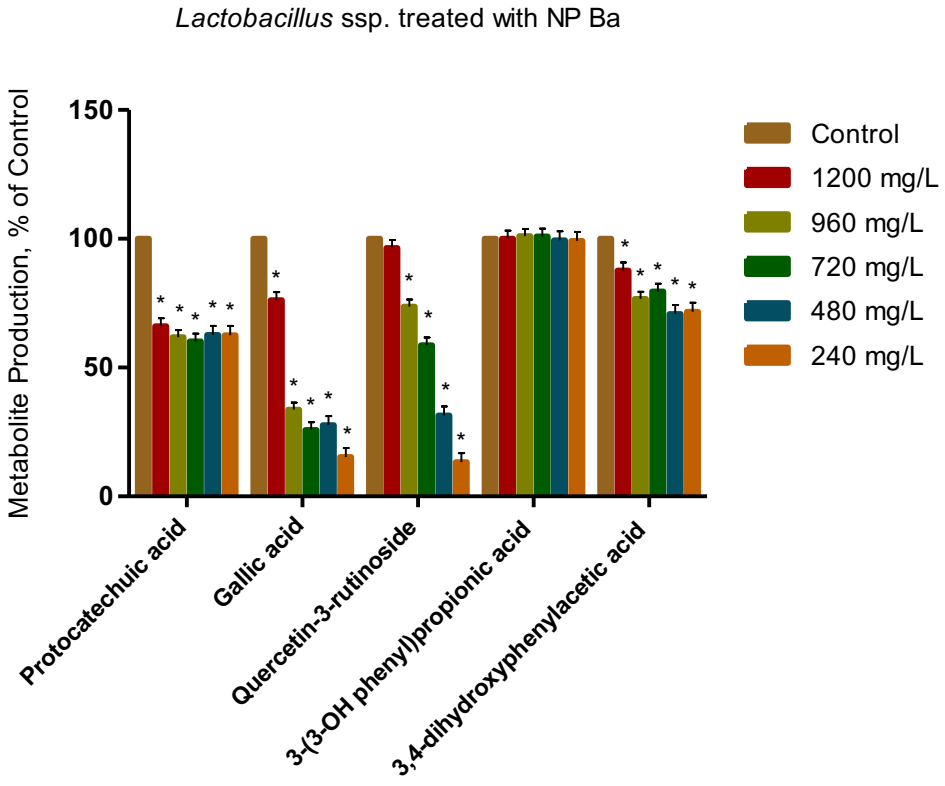


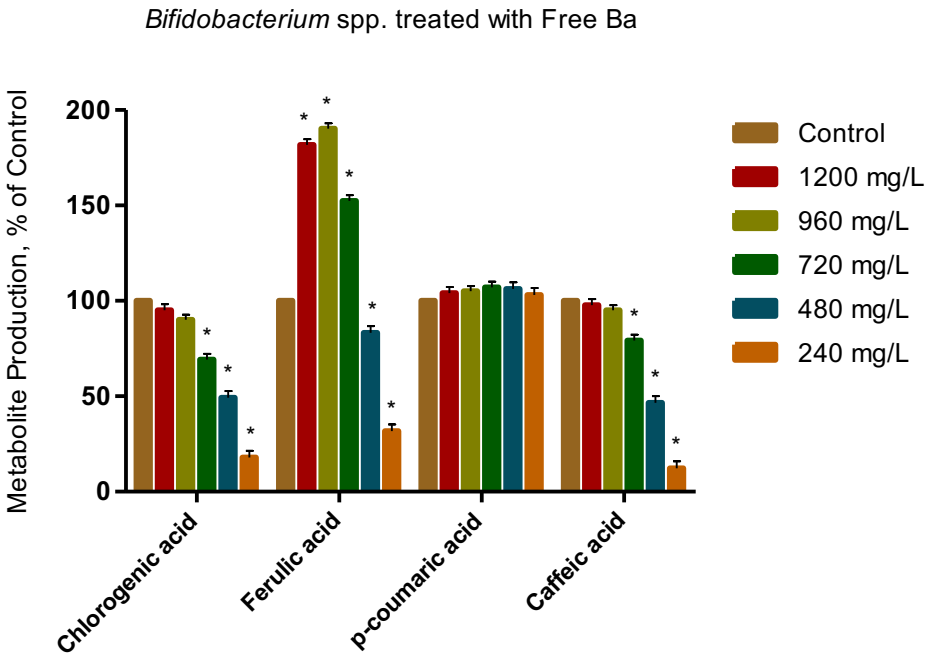
Figure 35. Metabolites production of hydroxycinnamic acids, hydroxybenzoic acid, and flavonoid of free and nanoparticle barberry polyphenolic extract upon fermentation by *Lactobacillus* spp. at the concentrations of 1200, 960, 720, 480, and 240 mg/L at 37 ºC for 48 h anaerobically ($p < 0.05$).

Bifidobacterium spp. (*B. bifidum*, *B. longum, B. breve,* and *B. lactis*) were treated with free and EN barberry extracts (**Fig. 38**). The result indicated that the FA was significantly increased at the concentrations level of 1200 mg/L by 95%, at 960 mg/L by 121%, at 720 mg/L by 101%, at 480 mg/L by 49%, and at 240 mg/L by 15% ($p < 0.05$). The concentrations of *p*-coumaric in free extract treated by *Bifidobacterium* spp. exhibited increases at the concentration levels of 1200 mg/L, 960 mg/L, 720 mg/L, 480 mg/L, and 240 mg/L. It can be explained that by diluting the

treatment to 1 to 6-fold (from control to 240 mg/L), the concentrations of *p*-coumaric did not only change, but also increased by 18%, 37%, 56%, 72%, and 89%.

It was expected for *Bifidobacterium* spp. to metabolize the phenolic compounds at a higher rate at the concentrations of 480 mg/L and 240 mg/L since the growth of *Bifidobacterium* spp. was stimulated at those concentration levels. For another hydroxycinnamic acid (caffeic acid), 12%, 26%, 28%, and 12% increases were observed at the concentration levels of 1200 mg/L, 960 mg/L, 720 mg/L, 480 mg/L. However, caffeic acid concentration was decreased by 4.8% at 240 mg/L.

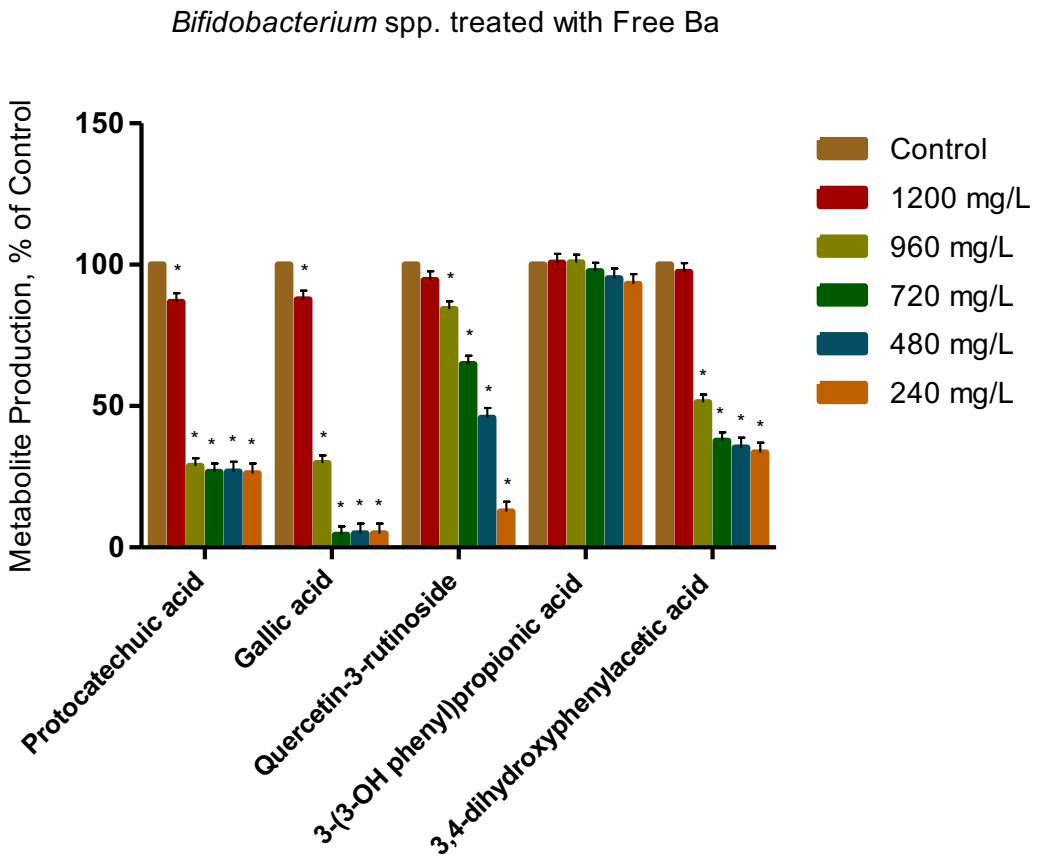
38a.



The 3-(3-hydroxyphenyl) propionic acid and 3,4-dihydroxyphenylacetic acid, colon metabolites of caffeic acid or dihydrocaffeic acid (3,4-Dihydroxyphenylpropionic acid) were identified in this study. Besides, the formation of hydroxyphenyl propionic acids from C-C double bond reduction in ferulic acid by *Lactobacillus* spp. such as *L. plantarum* was previously reported

[212]. The 3-(3-hydroxyphenyl) propionic acid which is a metabolite of caffeic acid by gut bacteria, was also increased at all the tested concentrations by 15% at 1200 mg/L, 32% at 960 mg/L, 46% at 720 mg/L, 61% at 480 mg/L, and 76% at 240 mg/L. The 3,4-dihydroxyphenylacetic acid had an increase at 1200 mg/L by 12% and an additional 17% at 960 mg/L. The concentration of this compound had a reduction by 14% at 720 mg/L, however, the 3,4-dihydroxyphenylacetic acid concentration was increased by 1% at 480 mg/L and an additional 16% at 240 mg/L.

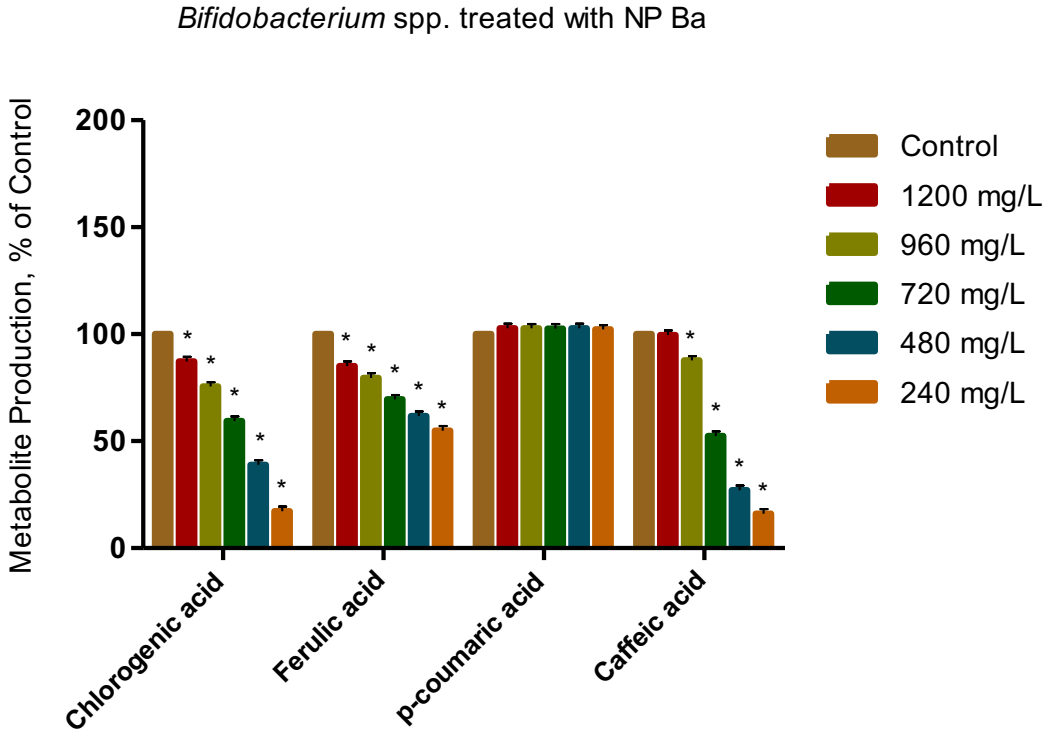
38b.



In NP extract, CGA showed slight increases at all concentrations being tested. The amount of this compound increased by 2% to 414 µM at the concentration of 1200 mg/L, so that, the

highest increases were observed at the 960 mg/L by 7% and at 720 mg/ L by 8%. The concentrations of *p*-coumaric were increased to 10.1 µM by 17% at 1200 mg/L, 34% at 960 mg/L, 51% at 720 mg/L, 68% at 480 mg/L, and 85% at the concentration of 240 mg/L compared to the control NP. An increase was also observed for caffeic acid concentrations by 14% at 1200 mg/L with a value of 35.9 µM and by 19% at 960 mg/L with a value of 28.9 µM compared to the control. The ferulic acid concentration was increased significantly in NP extract especially at the lower concentrations of 720 mg/L, 480 mg/L, and 240 mg/L by 18%, 27%, and 35%, respectively.

37c.



The colonic microbiota can hydrogenate or dehydroxylate CGA first, before hydrolyzing and breaking the ester bond that results in producing QA moiety. Also, the free CA produced by

hydrolyzation of CGA can be metabolized in further reaction to hydroxylated derivatives of phenylpropionic and phenylacetate. Moreover, the CGA metabolism is a strain-dependet, so that, *Bifidobacterium* strains especially *B. animalis* subsp. *lactis* was able to hydrolyze CQA to CA by 50%, whereas all other strains including the *B. bifidum*, *B. breve*, *B. longum* were incapable of transforming CQA [213].

38d.

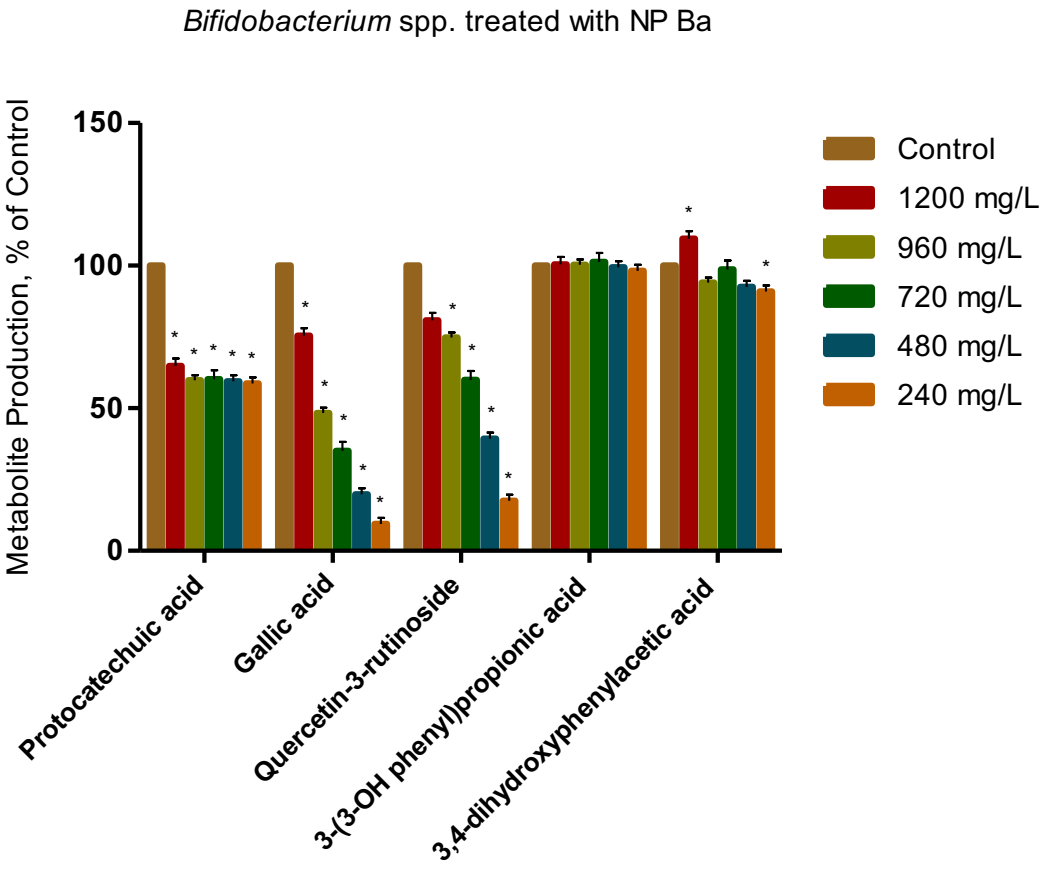


Figure 36. Metabolites production of hydroxycinnamic acids, hydroxybenzoic acid, and flavonoid of free and nanoparticle barberry polyphenolic extract upon fermentation by *Bifidobacterium* spp. at the concentrations of 1200, 960, 720, 480, and 240 mg/L at 37 °C for 48 h anaerobically ($p < 0.05$).

Fig. 39 shows possible metabolic pathways of hydroxycinnamic acids by gut bacteria enzyme. However, an *in vitro* study was conducted to determine the free and NP polyphenolic metabolites upon fermentation by probiotic bacteria. Gut microbiota metabolic transformation can be done into three main reactions, hydrolysis (O-deglycosylation, hydrolysis of esters), cleavage (C-ring cleavage, demethylation), reduction (dehydroxylation, hydrogenation, carbon-carbon double bond), and aromatic hydroxylation [121]. Therefore, the CGA (5-CQA), an ester form CA and QA, can be hydrolyzed by chlorogenate esterase to quinic and caffeic acids compared to 3-CQA and 4-CQA. Since CA is a metabolite product of hydrolyzing the CGA by bacteria enzyme upon fermentation, the concentration of CA was expected to be high in this study. However, a decrease in its concentration can be due to the autoxidizing of polyphenols during the fermentation process which may be a reason for decreasing the amount of phenolic acid metabolites.

A possible explanation regarding an increase in the concentration of *p*-coumaric can be due conversion of FA to *p*-coumaric by probiotic bacteria through a reduction reaction. The probiotic bacteria can demethylate FA and add a hydrogen bond to convert FA to CA by reduction reaction. Further step, the CA can be dehydroxylated by a probiotic enzyme and produced the *p*-coumaric. It can also be concluded that the ferulic source of hydroxycinnamic acid in barberry extract can be through 5-O-Feruoylquinic acid beside 5-O-Caffeoylquinic acid.

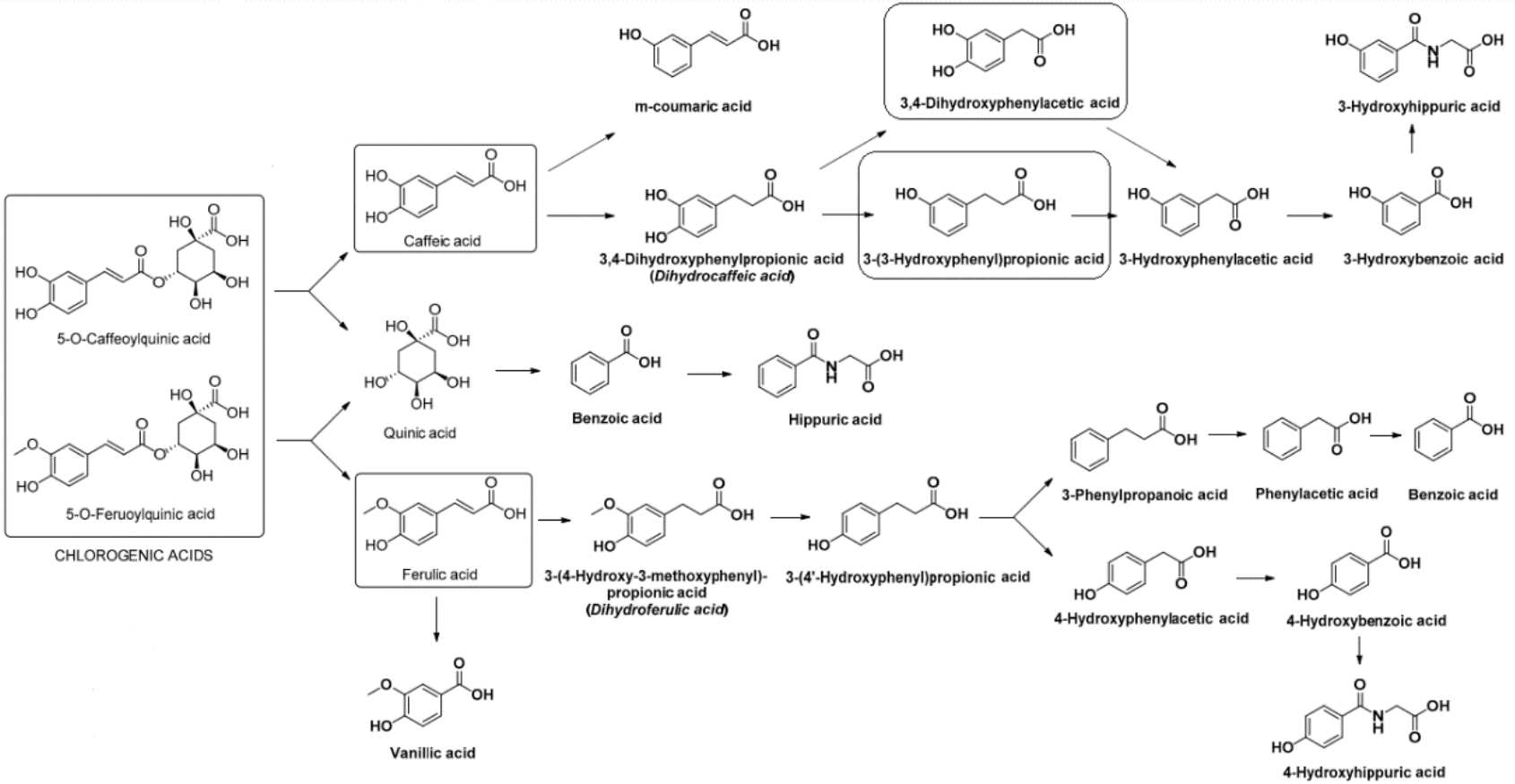


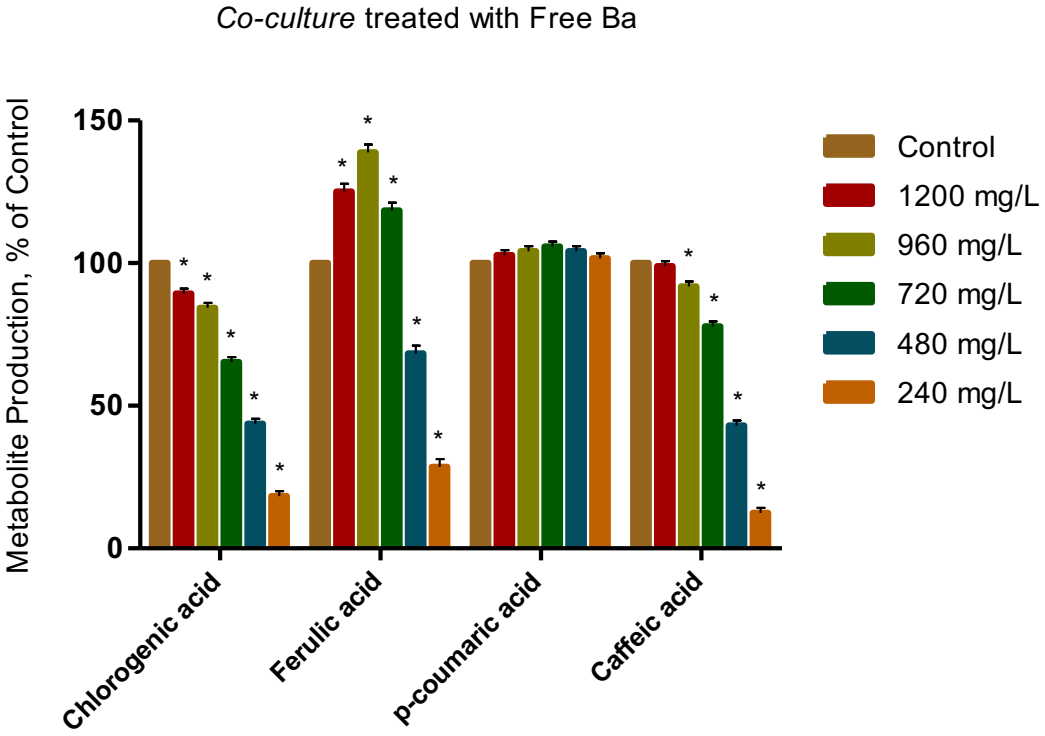
Figure 37. Metabolism pathways of hydroxycinnamic acids by gut bacteria- Adopted from M. Sova et al., 2020 [214].

In co-culture, a mixture of *Lactobacillus* spp. and *Bifidobacterium* spp. treated with free polyphenolic extract, FA showed an increase in its concentration compared to initial which was expected to be at a range of 1 to 6 reduction as the treatments were diluted. An increase by 39% at 1200 mg/L, 70% at 960, 67% at 720 mg/L, 34% at 480 mg/L, and 11% at 240 mg/L were observed (**Fig. 40**). During the fermentation process, the *p*-coumaric had a consistent increase in its concentration level when barberry extract was exposed to probiotic bacteria, an increase by 17% at 1200 mg/L, 36% at 960 mg/L, 54% at 720 mg/L, 70% at 480 mg/L, and 85% at 240 mg/L.

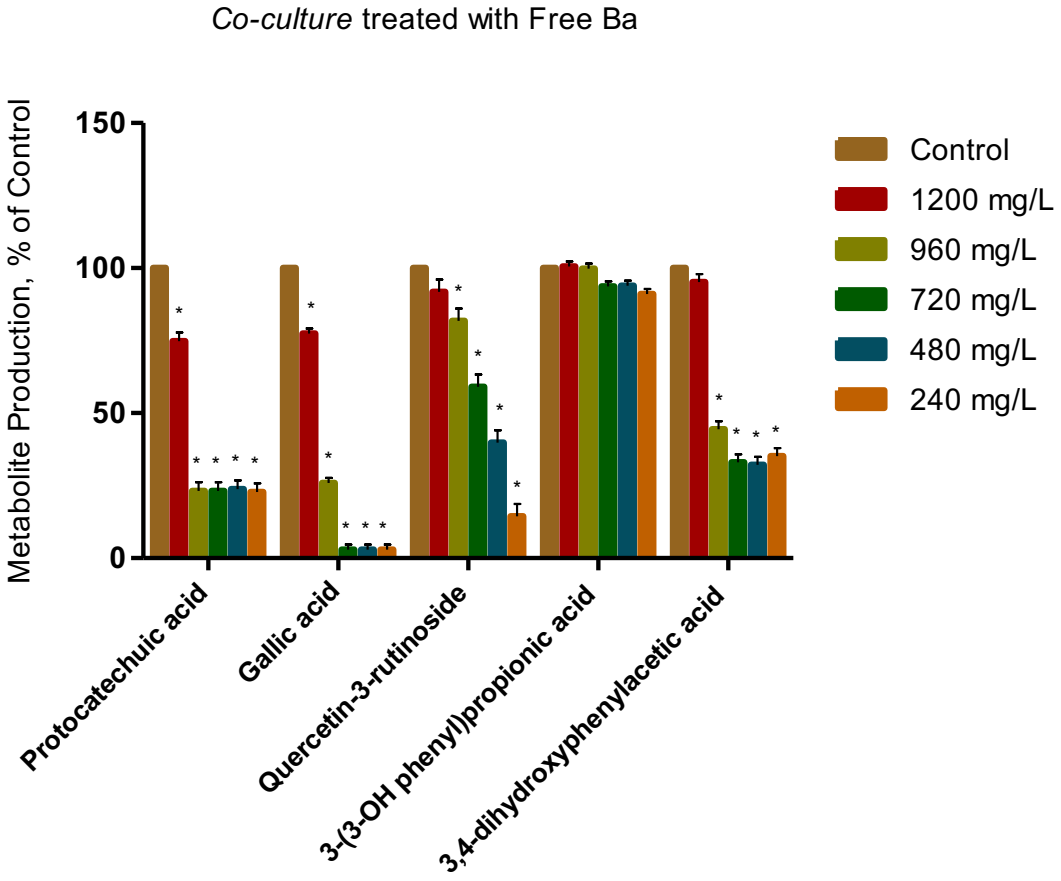
As mentioned, the hydrogenation or dehydroxylation of CQA can occur by colonic microbiota, before even hydrolyzing the ester bond and breaking the QA moiety, for instance, *L. plantarum* can cause degradation in caffeic acid and ferulic acid by decarboxylation and reduction reactions [209]. A study on the enzymatic activity of probiotic bacteria toward phenolic acid was indicated that *L. plantarum* can also produce *p*-coumaric decarboxylase enzyme (Filannino et al., 2015). Thus, an increase in the concentration of *p*-coumaric can be also due to the conversion of CA produced by hydrolyzing CGA to *p*-coumaric acid by probiotic bacteria since they can reduce CA through dehydroxylation and produce *p*-coumaric.

The concentrations of 3-(3-hydroxyphenyl)propionic acid and 3,4 dihydroxyphenylacetic acid was evaluated in this current work. As mentioned, the metabolism of CGA by GIT microbiota result in production of diverse aromatic acid metabolites including *m*-coumaric, benzoic, and phenylpropionic derivatives [215]. The 3-(3-hydroxyphenyl)propionic acid was increased by 15% at 1200 mg/L, 31% at 960 mg/L, 42% at 720 mg/L, 60% at 480 mg/L, and 74% at 240 mg/L. Moreover, 3,4 dihydroxyphenylacetic acid concentration showed a reduction at 960 mg/L, 720 mg/L, and 480 mg/L by 24%, 18%, and 2%, respectively. However, the concentration of this compound increased by 18% that reached 17.1 μM at 240 mg/L of the treatment.

40a.


Co-culture treated with Free Ba
Metabolite Production, % of Control
150
100
50
0
Chlorogenic acid
Ferulic acid
p-coumaric acid
Caffeic acid
Control
1200 mg/L
960 mg/L
720 mg/L
480 mg/L
240 mg/L

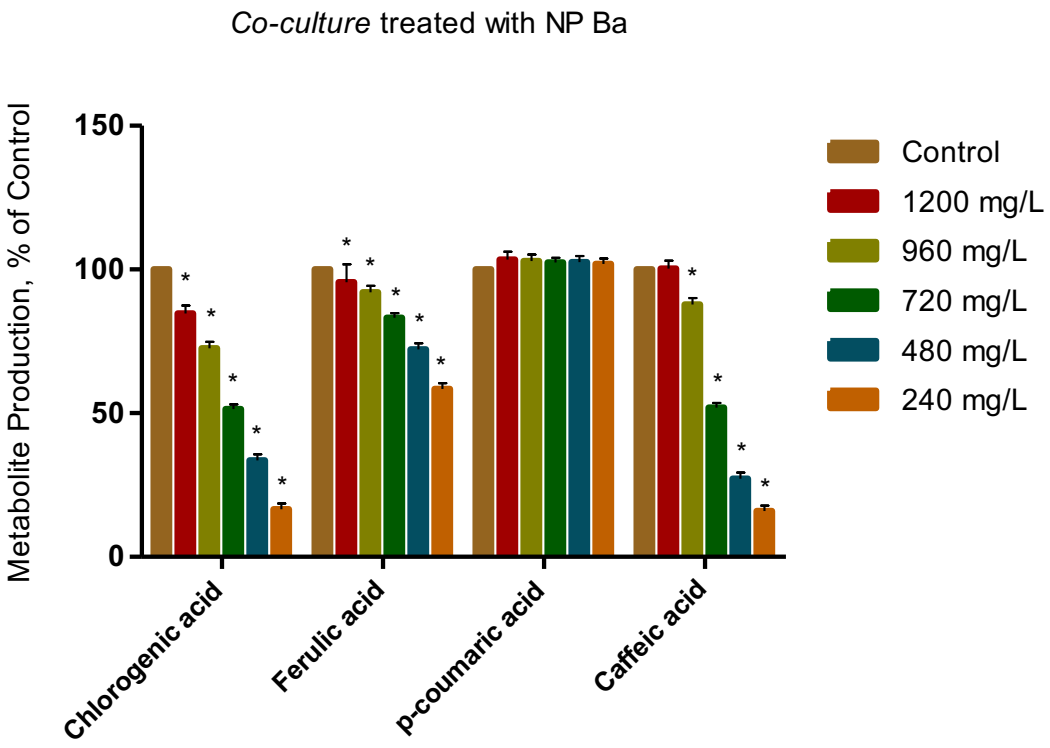
40b.



Once co-culture bacteria treated with NP extract, the trend on the metabolites production for hydroxycinnamic acids (chlorogenic acid, Ferulic acid, Caffeic acid, and *p*-coumaric acid) was similar to that of free extract. However, a different trend of metabolite production was observed for hydroxybenzoic acids and phenylpropionic derivatives. So that, the concentration of protocatechuic acid was increased significantly by 12%, 21%, 38%, and 47% at the concentration levels of 960 mg/L, 720 mg/L, 480 mg/L, and 240 mg/L. A slight decrease of 2% at 1200 mg/L was the only reduction in the concentration of protocatechuic acid in NP extract, whereas this compound was decreased by 45% when co-culture treated with free extract. The 3-(3-

hydroxyphenyl) propionic acid concentration levels were increased by 17% at 1200 mg/L and additional of 33%, 48%, 64%, and 81% at the concentrations of 960 mg/L, 720 mg/L, 480 mg/L, and 240 mg/L, respectively. The 3,4 dihydroxyphenylacetic acid was exhibited greater concentration values compared to the free extract. An increase of 20% was observed at the concentration of 1200 mg/L, following additional increase by 33% at the concentration of 960 mg/L, 30% at 720 mg/L, 46% at 480 mg/L, and 64% at 240 mg/L.

40c.



40d.

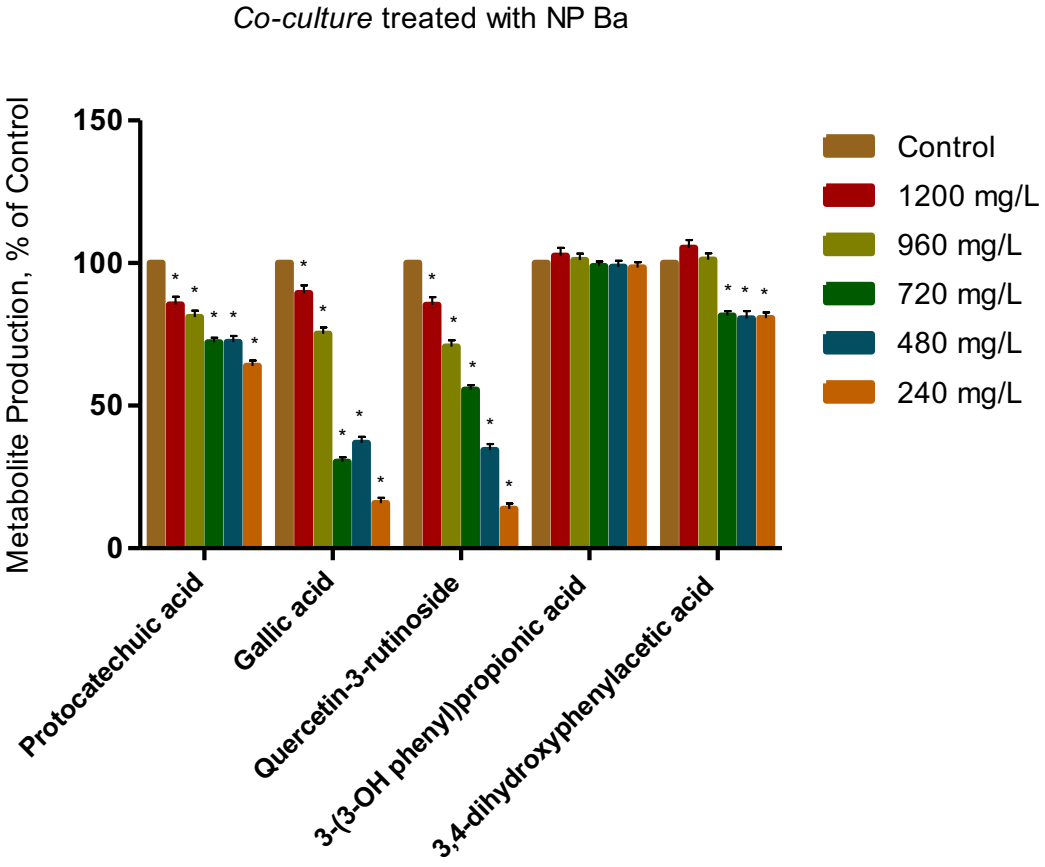


Figure 38. Metabolites production of hydroxycinnamic acids, hydroxybenzoic acid, and flavonoid of free and nanoparticle barberry polyphenolic extract upon fermentation by Co-culture at the concentrations of 1200, 960, 720, 480, and 240 mg/L at 37 ºC over 48 h anaerobically ($p < 0.05$).

The probiotic supplement used in the current study was contained a mixture of *Lactobacillus* strarins including *L. casei, L. rhamnosus, L. reuteri, L. acidophilus, L. paracasei, L. bulgaricus, L. salivarius,* and *L. plantarum* as well as *B. bifidum*, *B. longum, B. breve,* and *B. lactis,* therefore, there is a possibility that *B. lactis* was only *Bifidobacterium* spp. that involved in hydrolyzing of CQA in this work. To understand the mechanism of hydrolyzing fate of CQA by

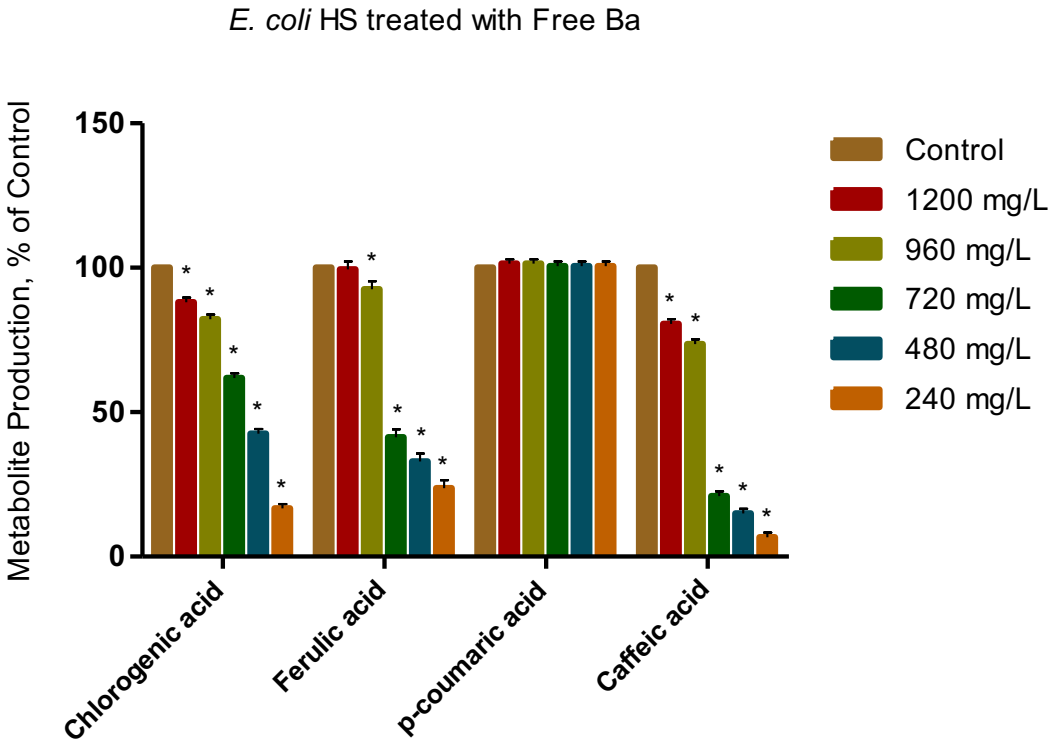
Lactobacillus spp. and *Bifidobacterium* spp., it may be better to investigate the enzyme involved in the reaction. The production of cinnamoyl esterase capable to hydrolyze chlorogenic acid (5-CQA) and ethyl ferulic acid was previously studied. Strains of *E. coli*, *B. lactis*, and strains of *L. gasseri* isolated from the adult human feces were capable of producing cinnamoyl esterase enzyme [216]. Fritsch et al., (2017) also investigated cinnamoyl esterase activity of *L. acidophilus*, *L. plantarum*, *L. helveticus*, *L. gasseri*, *L. reuteri*, *L. fermentum*, *B. animalis* subsp *lactis* to be able to hydrolyze esterified hydroxycinnamic acid. During fermentation, *L. helveticus* and *L. acidophilus* were able to decrease the CGA concentration by 5.3% among these tested probiotic bacteria, whereas > 99% reduction in CGA concentration was observed by *L. plantarum*, *L. gasseri*, *L. reuteri*, *L. fermentum*, *B. animalis* subsp *lactis* [211].

The free and NP barberry extract were fermented by commensal *E. coli* HS to quantify the concentration produced metabolite by this bacteria strain. The result indicated that the overall metabolite production by *E. coli* HS resulting in less concentration of metabolites compared to *Lactobacillus* spp, *Bifidobacterium* spp. as well as a co-culture mixture of those bacteria. The commensal *E. coli* HS was able to influence the CGA concentrations at all tested treatments. The amount of this compound was measured by 2%, 13%, 10%, 8% increases at the concentration levels of 1200 mg/L, 960 mg/L, 720 mg/L, and 480 mg, respectively. The concentration of 240 mg/L was the only treatment that showed a reduction by 0.5% in CGA concentrations (**Fig. 41**).

The FA concentration was also increased by 13% and 24% to a value of 16.5 µM at the concentration levels of 1200 mg/L and 960 mg/L, whereas this compound was decreased by 10% and 1.3% at the 720 mg/L and 480 mg/L, respectively. Additional production of FA was observed by 7% at 240 mg/L. The FA in NP extract showed a different trend of metabolite production, so that, the concentration of this compound decreased by 19% and 11% at 1200 mg/L and 960 mg/L,

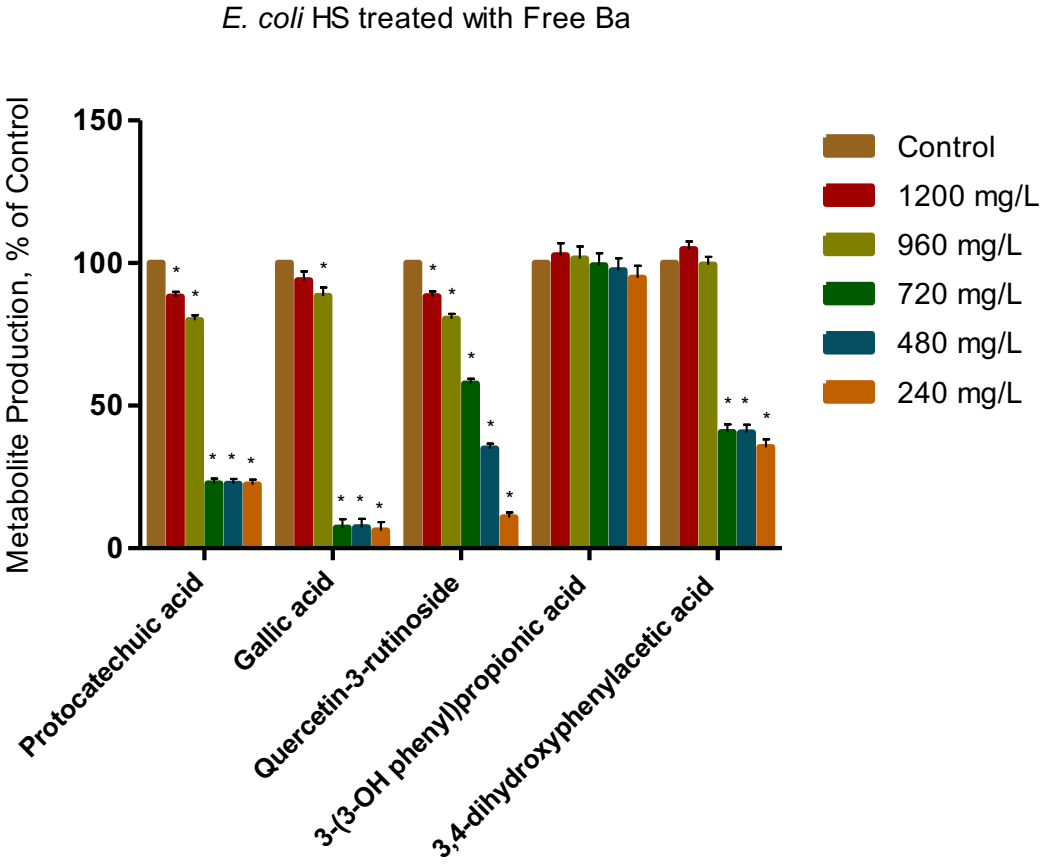
whereas 6%, 28%, and 34% increase was observed at the concentration levels of 720 mg/L, 480 mg/L, and 240 mg/L. Unlike other tested concentrations, the effective concentration that stimulated the growth of commensal *E. coli* HS was 720 mg/L, whereas the treatments of 480 mg/L and 240 mg/L conferred more proliferation effects on *Lactobacillus* spp, *Bifidobacterium* spp., and co-culture bacteria. The 10% reduction in the FA concentration at the concentration of 720 mg/L can be due to metabolize of the ferulic acid compound by the higher amount of commensal *E. coli* HS cell at the tested concentration (720 mg/L). Moreover, a possible mechanism for a decrease in the concentration of FA and increase in *p*-coumaric can be also due to hydrogenation of FA and conversion of this compound to CA. Further, *E. coli* HS was able to dehydroxylate the CA and produced *p*-coumaric acid.

41a.



An increase in *p*-coumaric concentrations was observed by 16%, 33%, 49%, 66%, 83% at where the concentrations of the treated free extract were 1200 mg/L, 960 mg/L, 720 mg/L, 480 mg/L, and 240 mg/L. In NP extract, the concentration of *p*-coumaric was slightly increased by up to 1% at 1200 mg/L, 960 mg/L, 480 mg/L, and 240 mg/L, whereas 35 increase at the concentration of 720 mg/L was observed. In comparison free extract data with co-culture, even though the concentration of was significantly high, however, *p*-coumaric concentration showed a slight decrease of production by *E. coli* HS in all tested treatments. The concentration of caffeic acid was also had the most reduction by 31% to 10.1 µM when commensal *E. coli* HS was treated by 720 mg/L free extract. It is noticeable that the extract polyphenols matrices and the concentration to which bacteria cells are exposed, may be associated with uptake of the targeted compounds by different probiotic strains. Thus, Acyl-quinic acid esterase and cinnamoyl esterase activities have been found in *Lactobacillus* spp., *Bifidobacterium* spp., and *Escherichia coli* may contribute to various pathways based on the type of microorganism. For instance, *E. coli* can remove the double bound from CA and produce dihydrocaffeic acid (DHCA) which can be converted to 3-(3-hydroxyphenyl)propionic acid by dehydroxylation reaction and removing 4-hydroxyl group [47, 216].

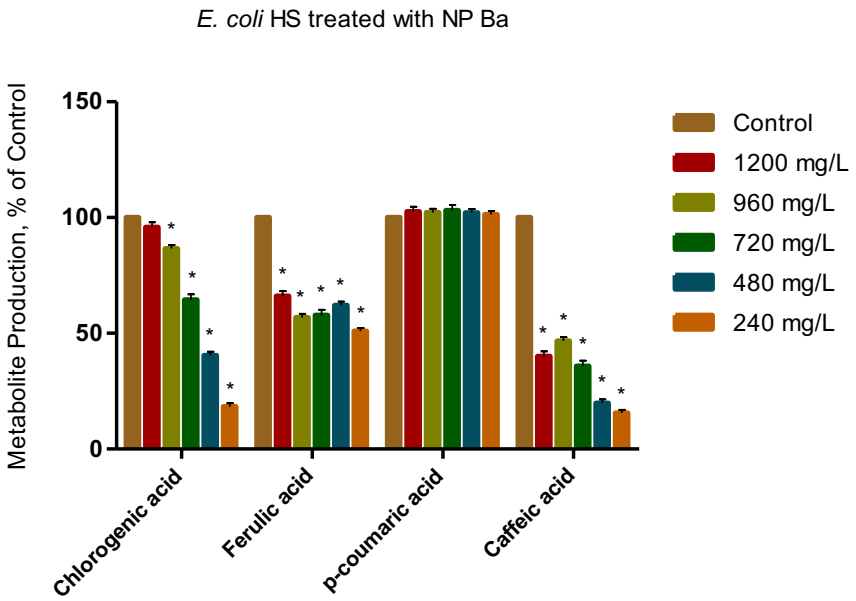
41b.



An increase in the concentration of 3-(3-hydroxyphenyl)propionic acid was observed by 17% at the concentration of 1200 mg/L, an additional increase of 33% at 960 mg/L, 47% at 720 mg/L, 63% at 480 mg/L, and 78% at 240 mg/L when bacteria strain treated with free extract. The concentration of 3-(3-hydroxyphenyl)propionic acid was increased by 17% at the concentration of 1200 mg/L, an additional increase of 33% at 960 mg/L, 47% at 720 mg/L, 63% at 480 mg/L, and 78% at 240 mg/L in NP extract. The result indicated that the amount of 3-(3-hydroxyphenyl)propionic acid produced by commensal *E. coli* HS was greater than 3,4-dihyroxyacetic acid in all tested probiotic strains. It could be due to the ability of probiotic bacteria

to metabolism CGA into aromatic metabolites. The 3-(3-hydroxyphenyl)propionic acid was also considered as the preferred route of caffeic acid metabolism in rats [205].

41c.



41d.

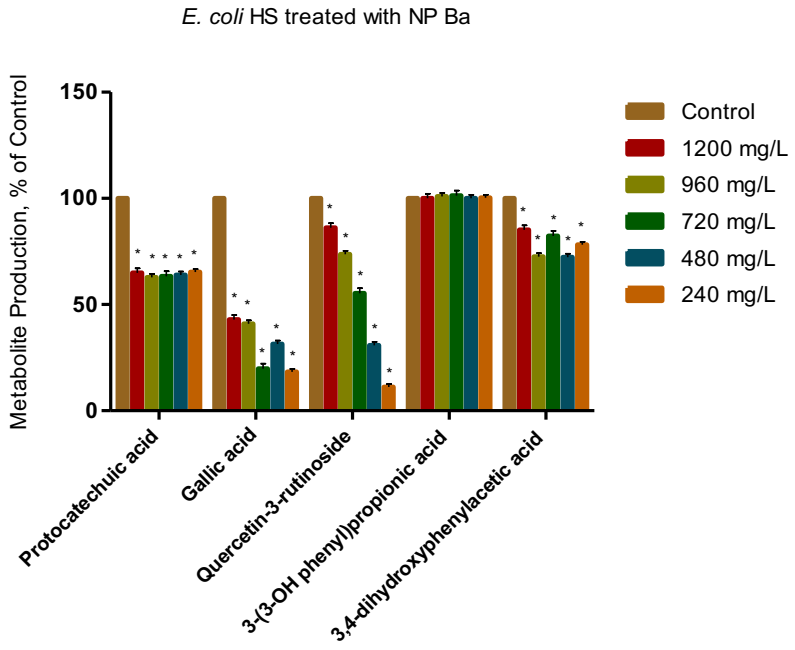


Figure 39. Metabolites production of hydroxycinnamic acids, hydroxybenzoic acid, and flavonoid of free and nanoparticle barberry polyphenolic extract upon fermentation by *E. coli* HS at the concentrations of 1200, 960, 720, 480, and 240 mg/L at 37 ºC over 48 h anaerobically ($p < 0.05$).

Conclusions

The hydroxycinnamic acids in oral, gastric, and intestinal phases at where the oral digestive phase had a significant effect on the CGA with up to 66% decrease compared to its control in free extract ($p < 0.05$), while CGA in NP decreased slightly at 4.6%. CA and FA concentrations had a decrease of 64 % and 36% compared to their controls in free, but the concentration of these compounds increased for 14% and 108% in NP extract, respectively, and *p*-coumaric showed resistance to changes in the oral condition with a slight decrease of 0.72% in free and an increase of 0.1% in NP barberry extract. The influence of different pH of PBS solution (2.5, 5.8, 6.8, and 7.4) of free and NP barberry polyphenols was indicted an increase in release as the pH, shifted from acidic to basic.

The effect of the free treatments on the growth of probiotic bacteria including *Lactobacillus* spp, *Bifidobacterium* spp., co-culture, and *E. coli* HS was then investigated at the concentrations ranging of 240 mg/L, 480 mg/L, 720 m/L, 960 mg/L, and 1200 mg/L at 37 ^{o}C for 48 h. The concentration level of 240 mg/L stimulated the greatest percentage increases by 88% when co-culture bacteria were treated with, whereas the growth values were at up to 82% for *Lactobacillus* spp. and *Bifidobacterium* spp., whereas the concentration of 720 mg/L was the most effective concentration among other tested concentrations that stimulated the growth of *E. coli* HS by 43%.

CHAPTER VI

CONCLUSIONS

In conclusion, it was demonstrated that polyphenolic in barberry fruits were obtained at higher concentrations in total reducing capacity, total anthocyanins, total flavonoid contents when extracted with methanol solvent. It can be due to its greater relative polarity of 0.762 compared to other solvents, which improve polyphenols solubility in organic solvents. It was also observed that methanol extract exhibited greater antimicrobial properties due to higher efficiency in extracting polyphenolics from fruits matrices.

Nanoparticle barberry exhibited a smaller size and slightly positive surface charge than the free extract that promoted the penetration and consumption of the compounds by bacteria, especially Gram-negative bacteria cells resulting in aggregated bacteria cells. It was observed that polyphenols characteristics could also be significant when it comes to stimulatory or inhibitory effects of the treatments, and the polyphenols with a lower molecular weight with the existence of hydroxyl groups as reactive sites (such as phenolic acids) were found to be more effective compared to the higher molecular weight molecules.

Polyphenols loaded nanoparticles could withstand different pH of the simulated gastrointestinal conditions, resulting in lower degradation of polyphenols in oral, gastric, and intestinal digestive phases when encapsulated with Pluronic F127. It was also concluded that barberry extract promoted the growth of *Lactobacillus* spp., *Bifidobacterium* spp., co-culture, and *E. coli* HS, resulting in producing a higher concentration of metabolites upon fermentation by probiotic and commensal bacteria. Overall, this current work targeted investigating the stability of the polyphenolic compounds in unfavorable pH conditions that can likely be available during

digestion and colonic fermentation and be capable of health benefits in the body due to metabolite production of barberry polyphenols which need to be better characterized and quantified for health benefits.

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