

EVALUATION OF THE FUNCTIONAL PROPERTIES OF PHENOLIC-COMPOUND
RICH EUROPEAN CRANBERRYBUSH (*VIBURNUM OPULUS* L.): BIOLOGICAL
ACTIVITIES, BIOAVAILABILITY, AND SAFETY

by
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Submitted to Graduate of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in
Biotechnology

Yeditepe University

2022

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ACKNOWLEDGEMENTS

First of all, I would like to convey my sincere gratitude to my advisor Prof. F. Yeşim Ekinci who is always show her continuous guidance patience even her busy times due to administrative duties. My advisor is my role model since my under graduation education and I would like to thank for contributing my academic and personal development with her knowledge and experience as well as for enlightening me the glance of research. Besides my advisor, I am deeply appreciative to Prof. Ahmet Aydın for being my thesis committee member and for especially designing and conducting toxicity studies on animal models. I would like to express my appreciation to my thesis committee members Prof. Gülgün Tınaz for her time and valuable advice. Also, I would like to convey my great pleasure to Assist. Prof. Alev Cumbul and Assist. Prof. Özge Seçmeler offering their time and support.

I appreciate for the financial assistance from the TÜBİTAK 2211-C National Graduate Scholarship Program. Many thanks to Yeditepe University for awarding me with Graduate Research and Teaching Assistant scholarship, which has been a financial supports and providing me the opportunity to complete my thesis, whilst refining my research and teaching skills.

I had a great chance to work different esteemed research groups during my thesis experiments in Yeditepe University, so I would like to express my appreciate to all of them for their collaborative works. I would like to thank to Assoc. Prof. Muhammed Hamitoğlu and Sinem Helvacıoğlu, PhD who are in Pharmaceutical Toxicology Department, for mutagenic and antimutagenic activities study. Assist. Prof. Alev Cumbul and her research team who are in Histology and Embryology Department, for the histopathological experiments and also examinations. It gives me great pleasure to thank Engin Sümer, PhD and his team at the Experimental Research Center (YUDETAM) for their supports and encouragements during animal studies.

My sincere thanks to also goes to all academic staff and laboratory technician in Food Engineering Department. I was very lucky to conduct my experiments in the well-established laboratories in this department. I would like to special thanks to my friends, lab mates, undergraduate students and our lab technician for their help and support during my PhD journey.

Special thanks to Tlay Dalgıç, Graduate School of Secretary, and her team in Graduate School of Natural and Applied Sciences for their support.

Last but not the least, I would want to express my sincere gratitude to my gorgeous family for their unwavering love and support over the years.



ABSTRACT

EVALUATION OF THE FUNCTIONAL PROPERTIES OF PHENOLIC-COMPOUND RICH EUROPEAN CRANBERRYBUSH (*VIBURNUM OPULUS* L.): BIOLOGICAL ACTIVITIES, BIOAVAILABILITY, AND SAFETY

The aim of this study was to examine the functional properties of European cranberrybush (ECB) (*Viburnum opulus* L.) fruit containing high phenolic compounds, known for their beneficial health effects. Biological activities (antimicrobial, mutagenic, antimutagenic, antiproliferative activities) and bioavailability of ECB fruit juice extract were firstly assessed by *in vitro* assays. Then, ECB juice was used to evaluate the viability and growth performance of probiotic cultures in the formulated environments for potential use of a new whey-based functional beverage. Additionally, since ECB fruit juice was found to be strongly inhibitory against *Listeria monocytogenes*, antimicrobial effect of ECB fruit juice in milk-based environment with probiotics cultures was investigated by challenging in the presence of this pathogen. Finally, acute and subacute oral toxicities of ECB fruit extracts were evaluated in *in vivo* animal studies to establish a toxicity profile by detecting the LD₅₀ and NOAEL. ECB extracts inhibited the growth of pathogens and spoilage microorganisms but showed a slight inhibitory effect on probiotics only at high concentrations. ECB extracts had no mutagenic activity and showed moderate levels (27.3%) of antimutagenic effect. ECB fruit juice induced antiproliferative effect on AGS cells in dose and time dependent manner. Total phenolic and anthocyanin contents of ECB extract remained after gastric digestion, but values decreased during intestinal digestion due to their low stability in alkaline conditions. Probiotic LAB maintained their viability in the presence of ECB juice added whey-based beverage resulting that utilization of whey powder could be an alternative medium for development of functional beverages. On the other hand, ECB juice was not inhibitory against *L. monocytogenes* when it was added in milk. Toxicity results showed that although ECB extract was not toxic after a single high dose exposure, consumption in high dose for a prolonged period showed toxicity on the liver, kidney, and adipose tissues. The LD₅₀ was higher than 2000 mg/kg, whereas NOAEL was 500 mg/kg/day for long time consumption. Upon all these results, ECB juice shows potential use in functional food and nutraceutical industries.

ÖZET

FENOLİK BİLEŞİKCE ZENGİN EUROPEAN CRANBERRYBUSH'UN (*VIBURNUM OPULUS* L.) FONKSİYONEL ÖZELLİKLERİNİN DEĞERLENDİRİLMESİ: BİYOLOJİK AKTİVİTELER, BİYOPYARARLANIM VE GÜVENLİK

Bu çalışmanın amacı, sağlığa yararlı etkileri ile bilinen yüksek fenolik bileşikler içeren European cranberrybush (ECB) (*Viburnum opulus* L.) meyvesinin fonksiyonel özelliklerini incelemektir. İlk olarak, ECB suyu ekstraktının biyolojik aktiviteleri (antimikrobiyal, antibiyofilm, mutajenik, antimutajenik, antiproliferatif aktiviteler) ve biyopyararlanımı in vitro deneylerle değerlendirildi. Daha sonra, ECB suyu yeni peynir altı suyu bazlı fonksiyonel içecek formülasyonunda probiyotik kültürlerin canlılığını ve büyüme performansını değerlendirmek için kullanım potansiyeli araştırıldı. Ek olarak, ECB meyve suyunun *Listeria monocytogenes*'e karşı güçlü bir inhibitör etkisi olduğundan, ECB meyve suyunun probiyotik kültür içeren süt bazlı ortamda antimikrobiyal etkisi bu patojen varlığında araştırılmıştır. Son olarak, ECB meyve suyu ekstraktlarının toksisite profili için akut ve subakut oral toksisiteleri in vivo hayvan çalışması ile LD₅₀ ve NOAEL değerleri değerlendirilmiştir. ECB ekstraktları, patojenleri ve bozulmaya neden olan mikroorganizmaların büyümelerini engelledi ancak probiyotikler üzerinde sadece yüksek konsantrasyonlarda hafif bir inhibitör etki gösterdi. ECB ekstraktları mutajenik aktivite göstermedi ve orta derecede (%27) antimutajenik etki gösterdi. ECB meyve suyu, doz ve zamana bağlı olarak AGS üzerinde antiproliferatif etki göstermiştir. ECB ekstraktının toplam fenolik ve antosiyanin içeriği mide sindiriminden sonra kalmıştır, ancak alkali koşullarda düşük stabilite nedeniyle bağırsak sindirimi sırasında değerler düşmüştür. Probiyotik LAB, peynir altı suyu bazlı içeceklerde ve süt ortamına eklenen ECB suyu varlığında canlılıklarını korudu ve peynir altı suyu tozunun kullanılmasının fonksiyonel içeceklerin geliştirilmesi için alternatif bir ortam olabileceğini gösterdi. Öte yandan, ECB suyu süte eklendiğinde *L. monocytogenes* büyümesi engellemedi. Toksisite sonuçları, yüksek doz bir kere alındığında toksik olmamasına rağmen uzun süre yüksek doz tüketiminin karaciğer, böbrek ve yağ dokularında toksik etki gösterdiği gözlenmiştir. LD₅₀ değeri 2000 mg/kg üzerindeyken, uzun süreli tüketim için NOAEL 500 mg/kg/gün olarak belirlenmiştir. Tüm bu sonuçlar, ECB suyunun fonksiyonel gıda ve nutrasötik endüstrilerde potansiyel kullanımını göstermektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

ADI	Acceptable daily intake
AGS	Human gastric adenocarcinoma cancer
ALT	Alanine aminotransferase
ANOVA	One-way analysis of variance
AST	Aspartate aminotransferase
ATCC	American type of culture collection
BHI	Brain heart infusion
BW	Body weight
CFU	Colony forming unite
DMSO	Dimethyl sulfoxide
ECB	European cranberrybush
FAO	Food and agriculture organization
GAE	Gallic acid equivalent
GRAN	Granulocyte
H&E	Hematoxyline and eosine
HCT	Haematocrit
HED	Human equivalent dose
HGB	Hemoglobin concentration
HPLC	High performance liquid chromatography
ISAPP	International scientific association for probiotics and prebiotics
LAB	Lactic acid bacteria
LD	Lethal dose
LDH	Lactate dehydrogenase
LYM	Lymphocyte
MBC	Minimum bactericidal concentration
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration

MPV	Mean platelets volume
MRS	Man, rogosa and sharp
Nd	Not detected
NOAEL	No observed adverse effect level
NPD	4-nitro-o-fenilendiamine
OECD	Organization for economic co-operation and development
PA	Phenolic acids
PAS	Periodic acid-schiff
PC	Phenolic compounds
PCT	Platelet counts
PDA	Photodiode array
PDV	Plateletcrit
P-LCR	Platelets larger cell ratio
PLT	Platelet volume distribution width
RBC	Red blood cells
RC	Regenerated cellulose
RDW	Red blood cell distribution width
RI	Refractive index
ROS	Reactive oxygen species
SDA	Sabouraud dextrose agar
SEM	Scanning electron microscopy
TAC	Total anthocyanin content
TCM	Masson's trichrome
TPC	Total phenolic content
TSA	Tryptic soy agar
WBC	White blood cells
WHO	World health organization
ZI	Zone of inhibition

1. INTRODUCTION

Consumer preference toward more natural and healthier products with greater effectiveness is encouraging food, pharmaceutical and cosmetic companies to invest in research and development and look for natural ingredients. Particularly, the change in eating behaviors of consumers, their desire to consume more healthier foods or/and nutraceuticals with natural ingredients that could boost and support their immunity, are growing more crucial including the preservation of health and battling various diseases. Anthocyanins, phenolic acids (PA) and organic acids are only a few of the naturally occurring components called as phenolic compounds (PC) that are abundant in berries [1,2].

European cranberrybush (ECB) (*Viburnum opulus* L.), grown in Europe, Africa, Asia and central zone of western Russia, have potential use in food products and therapeutic purposes [3]. Fruits's polyphenol content (especially flavonoids and anthocyanins), vitamin content, and organic acid content have all been linked to ECB fruit's positive health effects [4–11]. ECB fruits represent an excellent source of PC and contribute both by providing beneficial health effects and by giving sensorial properties of juice of ECB fruit such as color, taste and flavor. Many of them include mostly catechin, chlorogenic acid, epicatechin, and cyanidin-3-glucoside, yet even the PC content of ECB fruits varies depending on several factors such as genotype, geographic region, production procedures, storage conditions and ripeness etc. [3,12].

Fruits of ECB are edible, but they generally are not preferred to consume directly due to their unpleasant smelling, bitter-sour and astringent taste [13–15]. In Turkey, ECB fruit juice produced commercially, is highly consumed as a traditional fermented drink (gilaburu juice). Microflora analyses of gilaburu juice showed that *Lactobacillus plantarum* species was the predominant lactic acid bacteria (LAB) species and some isolated LAB strains found to possess potential probiotic activity [16].

With this knowledge, in our previous study [12], the interaction of PC in ECB fruit juice with different *Lactobacillus species* namely, *L. plantarum*, probiotic *L. plantarum* and *L. brevis* was investigated. This study results revealed that the growth of LAB strains could be stimulated in the presence of freshly squeezed ECB fruit juice at low concentrations (5–10%) without altering the fermentation performance; however, at high concentrations

of the juice, the growth of LAB was inhibited indicating that ECB juice might be used in the production of functional products with probiotics added, to sustain and develop survivability of beneficial microorganisms and products enrichment in terms of PC with strong antioxidant properties.

However, further studies are needed before ECB juice can be considered as nutraceuticals or functional food ingredients. In this study, the biological activities of ECB fruit juice such as antimicrobial, antimutagenic and antiproliferative were evaluated in Section 3. Besides their biological activities, PC present in ECB fruit must be bioavailable to be utilized as a functional compound which means PC should be delivered to body for achieving biological activities in as specific tissue or organs by effectively absorbed from gastrointestinal tract [17–19]. Therefore, in Section 3, *in vitro* bioavailability ECB fruit juice was studied by mimicking biochemical factors of gastrointestinal tract at selected concentration.

Following the assessment of the biological activities of ECB fruit juice, in Section 4, the effect of ECB fruit juice was investigated on viability and growth performance of probiotic microorganisms in the formulated whey-based environments to evaluate the potential development of a new functional beverage. Besides in Section 4, since ECB fruit juice was found to be strongly inhibitory against *Listeria monocytogenes* (Section 3), microbial safety of ECB fruit juice was examined in a food model. Investigation of the effect of ECB fruit juice added probiotic cultures containing milk environment was done by challenging the environment with the presence of pathogenic *L. monocytogenes* ATCC 15313.

Although, the promising evidence of potential use of PC in disease prevention, there is not enough data to recommend optimum intake or dietary suggestions for their consumption [20]. High dose intake of PC might cause adverse health effect, therefore, toxicological analysis should be done to ensure safe levels of intake for consumptions. That's why, in Section 5, acute and subacute oral toxicities of PC-rich ECB extracts were evaluated on an animal model to establish a toxicity profile ECB extract by detecting the lethal dose (LD₅₀) and no observed adverse effect level (NOAEL).

2. LITERATURE REVIEW

2.1. BERRY FRUITS

Berry fruits, especially cranberry, grape, strawberry, blackberry, blueberry, raspberry, black currant, and others, are significant components of health diet due to presence of rich sources of valuable bioactive compounds such as phenolic compounds (PC), vitamins, minerals, organic acids, and fiber [21–23]. Due to their high antioxidant content, berries have been found to have a positive impact on human health. Berries are commonly utilized in a variety of food products, such as juice, jams, jellies and other foods, in addition to being consumed fresh. Besides, fruit extracts are utilized as an ingredient in the development of nutraceuticals and functional foods due to their benefits for maintaining good health [24].

2.1.1. Biological Activities, Bioavailability and Safety Evaluation of Phenolic Compounds Present in Berries

In recent decades, PC have indicated several biological activities such as antioxidant, antimicrobial, antimutagenicity, antiinflammatory properties, they have gained significant attention for their beneficial properties [25–28]. The composition of PC in berries varies depend on their genotype, degree of ripeness, harvesting time, processing, storage conditions, and environmental factors [29]. They are generally classified mainly into four different groups which are phenolic acids, flavonoids, stilbenes and lignans depend on their number of phenolic rings, carbon skeleton and substituting groups [18,30]. They have a one or more aromatic ring and hydroxyl substituents with different functional derivatives such as esters, methyl esters, glycosides [30].

Phenolic acids (PA) and flavonoids are main polyphenols found in berries. Hydroxybenzoic and hydroxycinnamic acids are subclasses of PA. They are mostly monomeric phenols and are generally attached to an organic acid, mostly quinic acid. For instance, the caffeic and quinic acids that are mostly present in foods like blueberries and raspberries combine to make chlorogenic acid. There are several subclasses of flavonoids, such as flavanones, flavones, flavonols, flavan-3-ols, dihydroflavonols, isoflavones and proanthocyanidins containing two phenolic rings and polymerized flavonoids have multiple phenolic rings. The

primary connected sugars to the glycosides that flavanols, flavanones, and anthocyanins exist as are glucose and rhamnose. Flavanols are present mostly in their free forms, but can be galloylated or polymerized to form proanthocyanidins [25,31,32]. Quercetin, myricetin, catechin, epicatechin, hesperetin, cyaniding, malvidin etc are mainly found flavonoids in berries [33]. Their chemical structures have an enormous impact on their bioavailability and biological characteristics. Moreover, conjugation reactions with derivative groups such as methyl, sulfate or glucuronide and the amounts of metabolites formed by gut microbiota may affect their absorption and their bioavailability [30,34,35].

2.1.1.1. Biological Activities

Chemical structure, diversity, and consumption amounts of PC in present of berries affects their biological activities, resulting in their bioavailability and bioactivity in the human body [22,32,36]. Due to their capacity to scavenge reactive oxygen and nitrogen species, PC have antioxidant characteristics and the capacity to control a number of a enzymes and cell receptor, which are the main sources of their biological activities [36]. Ability of PA to increase low-density lipoprotein resistance to lipid peroxidation, protect proteins from oxidation, chelate transition metals that promote oxidative reactions, and inhibit oxidative stress-related enzymes are just a few of the important activities that have been document in the literature [37–40]. Similar to PA, flavonoids have also demonstrated high antioxidants activity.

PC have also been considered as alternative sources for safe and effective natural antimicrobial agents to overcome the problems related with chemical antimicrobial agents against several strains [29,41–43]. Antimicrobial activity of them was correlated with the inactivation of cellular enzymes and disruption of membrane permeability [44]. At cellular state, PC showed different actions as in Figure 2.1. Apart from different actions, their antimicrobial activity also varies depend on types of PC, their concentration, extraction method and tested microbial strains. Ellagitannins, present high amount in cloudberry, raspberry and strawberry, showed powerful antimicrobial activity against *Helicobacter pylori*, *Bacillus cereus*, *Campylobacter jejuni* and *Candida albicans* [45]. Condensed tannins (proanthocyanidins) of cranberry polyphenolics and derived from other berries have exhibited prevention of adhesion of uropathogenic fimbriated *Escherichia coli*, cariogenic *Streptococcus mutans* and oxacillin-resistant *Staphylococcus aureus* [46–48]. Cranberry

phenolics also inhibited the adhesion of *H. pylori* to human gastric mucosa which has an important role for the prevention of gastric ulcer development [49].

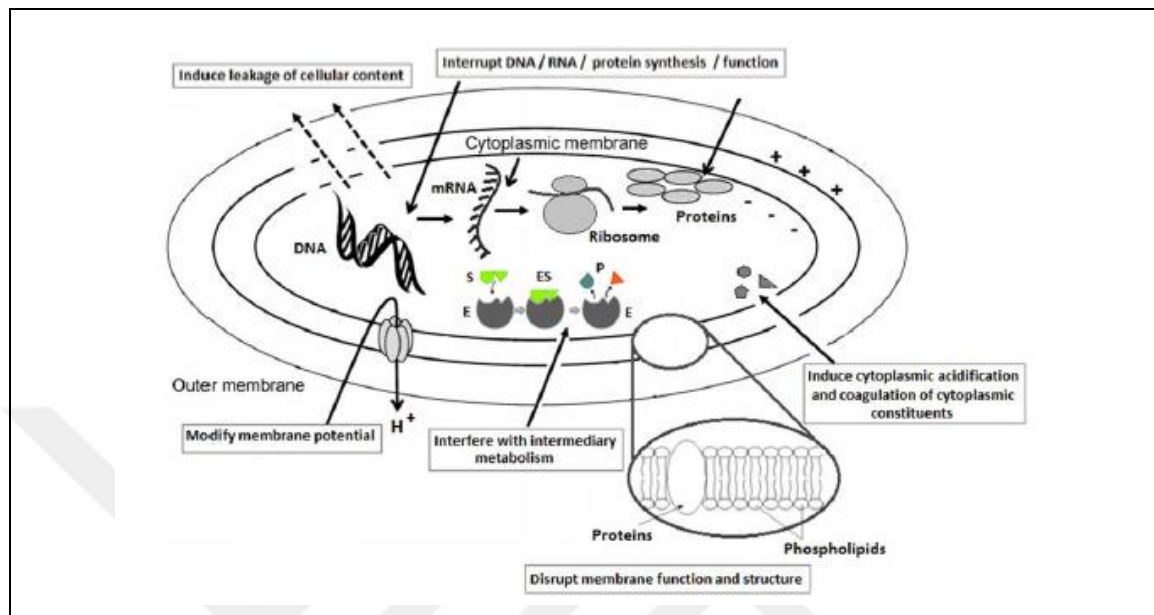


Figure 2.1. Antimicrobial actions of PC at different sites in cellular state [50]

PC is a strong anti-biofilm agent that has the ability to both prevent and eliminate the production of established biofilms [51,52]. Their biofilm suppression mechanism studies revealed that they may either show their antibacterial activity on bacteria via interacting bacterial proteins and cell wall structures, damaging to cytoplasmic membranes, reducing membrane fluidity, inhibiting nucleic acid synthesis, cell wall synthesis or energy metabolism, or may affect regulatory mechanism of bacteria such as quorum sensing or other regulatory systems, without an effect on bacterial growth [41,51,53,54]. The antimutagenic properties of strawberry, blueberry, and raspberry fresh juice and various solvent extracts were shown in literature [55]. The results showed that both fresh polyphenol rich berries and their solvent extracts significantly inhibited mutagenesis. The effect of different types of anthocyanins and anthocyanidins were studied on AGS cell line, malvidin showed more efficiency in anti-proliferation and cytotoxicity against AGS cell with a time and dose-dependent manner [56]. Pomegranate has high concentration of tannins (hydrolysable and condensed tannins) and anthocyanins as polyphenol source. The extract of pomegranate showed apoptosis in different breast cancer cell lines [57]. Similarly, 100% cherry juice demonstrated both cellular protection and death on breast cancer cell line in a dose dependent manner [58]. This activity may come from PC content including chlorogenic acid,

flavonoids, flavonols, flavan-3-ols, anthocyanins such as glycosides of cyanidin and pelargonidin in cherries [59]. Quercetin, is a flavonoid, have found anticancer effects inhibiting cell proliferation and induction of apoptosis [60]. PC have demonstrated cytotoxicity activity against cancer cells, so they are able to induce apoptosis in cancer cells could be used as a new alternative anticancer agents [61].

2.1.1.2. Bioavailability

Bioavailability is defined as the fraction of PC that is metabolized, absorbed and digested via normal mechanisms [18]. There are different PC in berries and their chemical structures affect biological activities, bioavailability and specific interactions between cell receptors and enzymes [62,63]. They must be absorbed in the intestinal tract to be bioavailable in the circulatory system to exert their beneficial health effects on human. Bioavailability seems different among the PC due to their physicochemical properties. Although type of PC, their molecular weight, degree of polymerization or glycosylation, solubility, and conjugation with other phenolics are the main factors, digestibility by gastrointestinal enzymes and absorption into the enterocytes are another critical parameter for bioavailability [34,64,65]. Digestion process affects the biological activity of PC and also their stability in turn, affect their bioavailability and their possible beneficial health effects for human [64]. The *in vitro* digestion models replicate the intricate physicochemical and physiological characteristics of the human gastrointestinal tract in order to better understand structural changes, interactions with PC, digestibility, and metabolite release [66]. Despite the *in vivo* studies provide more accurate results, they are more complex, technically difficult, expensive and ethical considerations are needed. Therefore, *in vitro* models offer an replacement to animal and human models due to relatively simple, affordable, and reproducible properties [17,67].

PC are normally found as different forms in terms of ester, glycosides, or polymeric that will not be absorbed directly. Therefore, these compounds should be hydrolyzed by enzymatic reactions such as methylation, glucuronidation or sulfation, before absorption [68,69]. The small intestine does not absorb PC (only absorb 5-10% of PC) due to their structure and polymerization, therefore most of the them are not found in urine [63]. High molecular weight cannot absorb in small intestine and reach the colon almost unchanged. Unabsorbed PC may build up in the large intestine where they are thoroughly digested by the gut bacteria through interactions with digestive enzymes. Low molecular weight phenolic metabolites which are more absorbable, are produced by colonic microbiota and produced metabolites

may show more bioactive than original compound [34]. Biological activities of PC are exerting through intestinal tract and when they reach colon, they can interact with colonic microbiota. Amount of PC in bloodstream is consist in modification of PC during absorption and metabolism in the gastrointestinal tract [19].

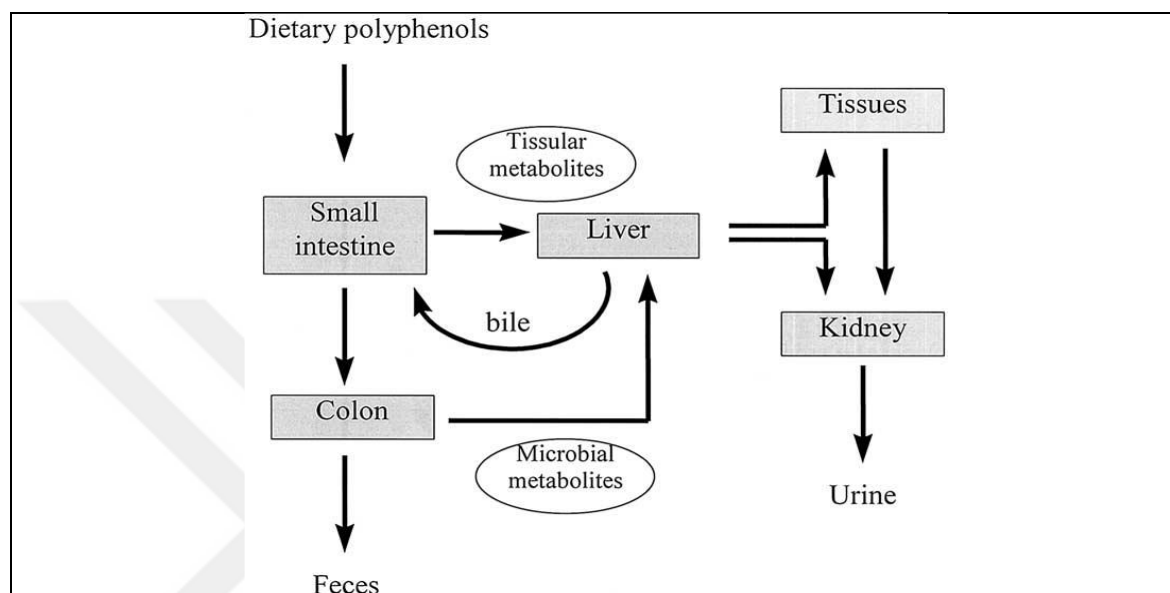


Figure 2.2. The route of PC and their metabolites in humans [34]

Metabolism of PC is extensively done either in tissues or colonic microbiota. They are absorbed through the gut barriers, however, if they are not absorbed and re-excreted in the bile, goes to colon as seen Figure 2.2. [34]. Glycosidic polyphenols are cleaved from sugar moieties (glucose, xylose, and galactose) in small intestine before absorption. Different types of enzymes in small intestine hydrolyzed and then aglycones enter epithelial cells by passive diffusion. Generally, after absorption, all PC are conjugated in gut barrier to form O-glucuronides, sulphate esters and O-methyl ether and then reach liver. These conjugates transported to the bloodstream until they are secreted in urine and unabsorbed metabolites are eliminated via feces [64,70].

2.1.1.3. Safety Assessment

Despite encouraging findings indicating PC's potential function in disease prevention regarding their advantageous biological actions, toxicity and safety evaluation of PC are essential to know their potential adverse effects on human health [22,63,71]. Toxicological tests are needed to report their safety for use in various foods, nutraceuticals and

cosmeceuticals products [72–74]. Bioavailability, dose effects and different forms of PC including in complex food matrix or as pure compound, mixture, or extract, are important parameters affecting safety evaluation during analysis of toxic effects on human health. Therefore, to establish upper safe limits, acceptable daily intake (ADI) and no observed adverse effect level (NOAEL) should be known [75–77]. Therefore, safety evaluation and risk assessment of PC are needed to determine consumption amount by humans. In literature, although most of the PC studies were on their beneficial effects, there are some studies examined possible toxicity of them. Until now, reported adverse effects include carcinogenic/genotoxic effects at high doses and concentrations, thyroid toxicity, estrogenic activity, inhibition of iron absorption, interaction with certain pharmaceutical agents, and immunosuppressive effect [71,78–82].

Toxicological investigations can be done by using *in silico*, *in vitro*, *in vivo* studies and human interventions [83]. These types of studies are the parts of preclinical studies. The *in silico* studies which are based on computer or computer simulation, are widely used due to comparatively less expensive, more efficient and highly effective, however, this method cannot be suitable for absorption, distribution, metabolism and excretion of PC.

Toxicological studies usually begin with *in vitro* experiments, which can be used in a variety of ways, including isolated tissues or organs, cell-based assays, and receptor enzyme binding tests. Mode of action, the fate of PC in absorption, distribution, metabolism and excretion, toxicology, possible biomarkers, dose-response relationship can be obtained from results of *in vitro* studies [84].

For risk assessment, there are four general toxicological approaches including toxicokinetics, toxicity studies subchronic, chronic toxicity, carcinogenicity, genotoxicity, and reproductive toxicity [85]. Toxicokinetics, is essential for determination of risk of health that provides valuable information for toxicity depending on applied doses and species difference [86]. Absorption studies are another important to establish the potential of bioactive compounds, their metabolites, by products, to be absorbed from gastrointestinal tract [87]. Genotoxicity studies can be also done to identify genetic alterations by both *in vitro* (bacterial reverse mutation, mammalian cell micronucleus assays) and *in vivo* (comet assay, *in vivo* micronucleus, transgenic rodent tests) studies that provide enough knowledge on mechanism and mode of genotoxic action about tested compounds. Finally, long term experiments are needed to detect reproductive and development toxicities [83].

Ames test has been widely used for the determination of mutagenic and antimutagenic activities of chemical compounds [88]. Investigation of mutagenic activity of PC such as high doses of quercetin (2% or 4%), caffeic acid (2%) for rats and mice, are gaining importance to ensure their safe use because some PC have mutagenic effect and they may interfere with essential biochemical pathways [71,89–91]. Many mutagens and carcinogens work by producing reactive oxygen species (ROS), which have the potential to disrupt macromolecules including DNA, protein, and lipids. As a result, ROS may be linked to cancer, heart disease and aging [92,93]. The *in vitro* techniques might potentially be carried out in a high-throughput way employing automated devices that boost productivity, but the cost of the research would rise [94]. The last step of preclinical research is *in vivo* animals studies that provide significant information on toxicity. The results of *in vivo* studies provide a basis on safety of PC for clinical studies and also validate proposed biomarkers and dose-response relationships. However, human clinical studies for examination of a PC dose response are definitely needed to ensure final evidence of safety relation with health rather than *in vitro* and animal studies [71].

Acute, subacute, subchronic and chronic studies show the adverse effect of polyphenols and ensure the safe use [95]. Acute toxicity study determines as an adverse effect occur within a short time after administration of a single dose and this test performs principally in mice and rats providing information on its potential toxicity [96,97]. Subacute toxicity is another obligatory step to support the safety of tested substances. This study is also done to provide information on possible health hazard that may arise from repeated exposure of compounds over a limited time periods. Moreover, setting doses for longer-term subchronic research with it is beneficial [98–100].

2.2. EUROPEAN CRANBERRYBUSH FRUIT

Among various berry fruits, European cranberrybush (*Viburnum opulus* L.) (ECB) fruits also have one of the most valuable bioactive compounds. ECB fruits that is cultivated in Europe, Africa, Asia, and the central region of western Russia. It is a member of the *Adoxaceae* family and formerly known as *Caprifoliaceae* family [101–103]. The red berry fruits of ECB contain one seed which ripened in August-September and persisted over the winter. They are not preferred to consume directly due to their unpleasant smelling, bitter and astringent

taste. The unpleasant smelling ECB fruits were attributed to presence of odor compounds which are 2-methyl-butanoic acid, 3-methyl-butanoic acid, linalool and ethyl decanoate [13]. However, the ECB fruits have been generally consumed as a drink [103,104]. In Türkiye, the fruits of ECB are generally grown in Kayseri where the fruits are consumed as a fermented drink. Gilaburu juice is prepared traditionally by allowing washed ECB fruits to stay in water for three to five months at room temperature for fermentation. In microflora analyses of fermented ECB fruit juice (gilaburu juice) demonstrated that *Lactobacillus plantarum* species was the dominant LAB species. Some isolated LAB strains found to possess potential probiotic activity suggesting that fermented ECB fruit juice has as a potential of being functional food [16].

In traditional medicine, ECB fruits are widely used to cure a variety of diseases, including high blood pressure, ulcers, menstruation pain, coughs and colds [3,103,105]. Fruits of *V. opulus* have been analyzed for their composition and have potential use in food and therapeutic purposes [3,103,106]. Most of the studies have been shown that health promoting properties attributed abundant sources of flavonoids and anthocyanins as PC, vitamins and organic acids present in ECB fruit [3,12,104,107]. High concentration of PC contributes not only to provide beneficial human health effects, but also to define sensory properties of juice such as color, taste and flavor. Freshly squeezed ECB fruit juice is primarily composed of chlorogenic acid, quercetin glycosides, catechin, epicatehin, cyanidin-3-glucoside and cyanidin-3-rutinoside even though the phenolic composition of ECB fruits varies depending on genotypes, geographic region, cultivation practices, storage conditions and ripeness [103,106,108]. The total content of phenolic compounds of ECB fruit was in the ranges of 325.4-1460 g/100 g depending on fruit genotype [3]. Total anthocyanins content of different variety of ECB fruit was in the range of 23-45 mg cyanidin 3-glucoside /100 g [108]. Apart from the PC of ECB fruit, other valuable compounds such as sugar and organic acid, were also determined in our previous study and literature. Glucose and fructose found as sugar sources. Malic, acetic, quinic, oxalic, L-ascorbic, maleic, citric and fumaric acids are present in ECB fruit [12,104,109,110].

2.2.1. Health Promoting Effects of ECB Fruit Juice and Extracts

Due to rich PC in ECB fruits, immense amounts of studies have shown the antioxidant activities of juice and various extracts obtained from fruit [12,104–106,109,111–114]. Several different antioxidant methods were applied and results showed difference depending on the amount of PC concentration, extraction solvent or genotypes of fruit. Not only *in vitro* assays showed antioxidant activity of ECB fruit but also various cellular models that are used to evaluate antioxidant activity and mechanisms of action of antioxidants on various oxidative stressors results summarized in the review paper of Kajszczyk et al. (2020) [3].

Antimicrobial activity of ECB fruit juice and extract demonstrated in many different studies [115–119]. The antimicrobial properties of ECB fruit can be utilized in functional food. Moreover, these extracts would be fascinating as natural antimicrobial agents for medicinal treatment or preserving the microorganisms growth in food products [41,50,120]. Antibacterial activity of methanol extract of dried ECB fruit showed inhibitory effect on the growth of various important pathogens such as *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Yersinia enterocolitica* at 10% concentration application but not effective at 2% concentration of extract [115]. The antimicrobial properties of different variety of ECB fruit juice evaluated by using agar well diffusion method [117]. Inhibitory effect of fruit juices varied depending on variety and types of microorganisms, for example strongly inhibited growth of a wide range of pathogenic bacteria (*S. Typhimurium*, *S. agona*, *S. aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*), but did not inhibit spoilage yeasts *Debaryomyces hanseii* and *Torulaspora delbrueckii* and weak inhibitory on *Saccharomyces cerevisiae*, *Candida parapsilosis*, *Trichosporon cutaneum* and *Kluyveromyces marxianus* var. *lactis*. Later, the research of same group investigated antimicrobial activities of several variety of fruit juices and ethanol extracts of varieties by using agar well diffusion method [116]. All varieties of juice and ethanol extracts showed inhibitory effect on microorganism, but fruit juices showed more effective than ethanol extracts. Similarly, growth of yeasts did not affect in the presence of both fruit juice and ethanol extracts. Although the low inhibition zone obtained against some strains of yeast than pathogens, juice and ethanol extracts showed inhibition zone against the *T. cutaneum*, *S. cerevisiae* strains and *K. marxianus*. Antimicrobial activity of water and ethanolic (75%) extracts of ECB fruit investigated on *S. aureus* ATCC25923,

methicillin resistance *S. aureus* ATCC43400, *B. subtilis* ATCC6633, *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *Klebsiella pneumoniae* RSKK574, and *C. albicans* ATCC10231 by using disc diffusion and tube dilution techniques. Although water extracts did not show antimicrobial activity, ethanol extract showed inhibitory effect on *P. aeruginosa*, *E.coli* and *Klebsiella pneumoniae* by disc diffusion assay and minimum inhibitory concentration (MIC) value was 125 µg/ml [118]. Kirazlı and Tunca (2022) studied the inhibitory activity of commercially available ECB fruit (gilaburu) juice against *S. aureus* showing that the MIC value of *S. aureus* was 50% ECB fruit juice [119].

Apart from antioxidant and antimicrobial activity, *in vitro* anti-diabetic activity was investigated in study of Paşayeva et al. (2021). They examined inhibitory effect of different ECB extracts, decoction, infusion, ethanol extract of fruit and fruit juice, on digestive enzymes correlated to diabetes resulted that findings of this study could contribute to advanced studies and beneficial for the development of novel products in both pharmaceutical and food industries [121].

Recently, researchers have investigated the health beneficial effects of ECB fruit in cell-based, *in vivo* and human studies that were summarized in Table 2.1. ECB fruits and their extracts were investigated on various cell lines. ECB fruit extracts showed anticancer activity against human breast and cervical cancer cell lines [122], neuroprotective effect against hydrogen peroxide induced oxidative stress in human SH-SY5Y neuronal cells [123]. DNA damage, apoptosis and cytotoxicity in colon cancer (Lovo) cells in a dose dependent manner due to its pro-oxidant activity [124]. Gilaburu juice, an ECB fruit juice that is marketed, may stop the development of the Caco-2 and HeLa cancer cell lines, but it could not show antiproliferative activities against lung epithelium A549 and normal cell line (MDCK and HUVEC) [125]. Moreover, cytoprotective agent potential to decrease free fatty acids and glucose uptake [107], anti-obesity potential of ECB fruit extracts in mouse differentiated adipocytes [8], cytoprotective potential against chemically induced oxidative stress in mouse insulin-secreting pancreatic MIN6 [126], osteogenic potential on osteosarcoma Saos-2 cell activity to understand the use of juice as a diet component potentiating bone health [5], molecular mechanism in adipogenesis regulation in 3T3-L1 cells and dietary fat lipolysis [127], and protective activity against hepatic lipotoxicity by using human hepatoma HepG2 cell [128] of ECB fruit juice and its extracts were examined.

The *in vivo* rat models of ECB fruit have been showed the ability of decreasing gastrointestinal mucosal damage [129], preventing colon cancer at initiation phase [4], anti-urolithiatic effect [7], ability to use in the treatment of endometriosis [6], prevention or reduction the structural and functional damages in reproductive organs, alleviation of testicle and sperm damages [11], protective effects against oxidative stress during lung transplantation [10], and vasorelaxant activity which is reduction in tension of blood vessel walls [130] of ECB fruits and their extracts.

Apart from *in vivo* studies, the effect of commercially available ECB fruit extracts were tested on patients to observe effect of supplements on kidney stones. There is a dietary supplement produced as film-coated tablets of *V. opulus* extract which is called “Gilaburine” on the market in Turkey. Each tablet is 1000 mg and contain 0.3% chlorogenic acid. The use of tablets recommended to consume as tablets (2x 3 times) for maximum 4 weeks. The usage area of this supplement is kidney stone disease. Kidney stones cause severe pain in both the kidney and the canal. Also, motion of stones can cause tears in the canal and cause bleeding and inflammation. This supplement was given to patients who received 6000 mg dose of *V. opulus* extract per day (2 x 1000 mg tablet x 3 times a day). The results showed that *V. opulus* extract alleviates the passage of ureteral stones smaller than 10 mm , therefore it can be effectively used as an alternative treatment [131,132].

Table 2.1. Health promoting effects of ECB fruit juice and extracts

Cell-based studies			
Cell line	Sample	Health benefits	References
Caco-2 and HeLa cancer cell lines	Commercially available ECB (gilaburu) fruit juice	Antiproliferative activity	[125]
MCF-7 and HeLa cancer cell lines	Fruit juice, polyphenolic fraction, methanol and acetone extracts	Anticancer activity against tested cancer cell lines	[122]
Human SH-SY5Y neuronal cells	Ethanol extract, decoction and fruit juice	neuroprotective effect	[123]
Caco-2 cells	Fresh fruit juice, and different solvent extracts	cytoprotective agent potential	[107]
Murine 3T3-L1 preadipocyte cell line	Acetone extract of ECB fruit and semi-purified phenolic extract	anti-obesity effect by inhibiting adipogenesis process in a dose dependent manner	[8]
Murine 3T3-L1 preadipocyte cell line	Fresh fruit juice and solid-phase extraction	inhibited adipogenesis and pancreatic activity	[127]
Mouse insulin-secreting pancreatic MIN6 cells	Fresh juice and phenolic-rich fraction	cytoprotective potential against chemically induced oxidative stress	[126]
Osteosarcoma Saos-2 cell	Fresh and purified fruit juice	osteogenic potential to understand the usage juice as a diet component potentiating bone health	[5]
Human hepatoma HepG2 cell	Fresh and purified fruit juice, solvent extracts from fruits (methanol and acetone extracts)	protective activity against hepatic lipotoxicity	[128]
in vivo studies			
Animal	Application time and dose of sample	Health benefits	References
Male Wistar rats	Orally administered dose (25-50 and 75 mg/kg) proanthocyanidins of fruit	ability to decrease gastrointestinal mucosal damage	[129]
Male Balb-c mice	Commercially available ECB (gilaburu) juice (65% fruit pulp, pH:3.09) 30 week given with cancer induced chemical 18 week given after applied cancer induced chemical	prevent initiation stage of colon cancer	[4]
Male Wistar rats	100 mg/kg doses of hexane, ethyl acetate and methanol extracts and lyophilized commercial fruit juice	anti-urolithiatic activity	[7]

Female Sprague Dawley rats	100 mg/kg doses of hexane, ethyl acetate and methanol extracts by oral gavage	anti-endometriotic activity	[6]
male Wistar albino rats	10 weeks oral daily 100 mg/kg water extract of fruit	alleviation of structural and functional damages in reproductive organs induced by taxane-based chemotherapeutics	[11]
Female Wistar albino rats	200 mg/kg dose of methanol extract administered intraperitoneally	protective effects against oxidative stress during lung transplantation	[10]
Male Sprague Dawley rats	Acetone-methanol-acetic acid mixture extract of frozen fruits	vasorelaxant activity	[130]
Human study			
Patients with distal ureteral calculi	Commercially available fruit extract tablet taken as peroral 1000 mg consuming (2 x 3 times) per a day for 4 weeks	facilitates passage of ureteral stones smaller than 10 mm	[131,132]

2.2.2. Potential of Functional Ingredients and Dietary Supplements Developed from ECB Fruit

In Turkey, commercially available freshly squeezed ECB fruit juice is bottled and pasteurized. On the other hand, different reseaches have carried out on development of ECB fruit juice added functional food such as a pear juice with the addition of different concentrations of ECB juice [133]. They concluded that the addition of ECB fruit juice into pear juice has positive effect on the enhancement of bioactive compounds (PC, Vitamin C) and antioxidant activities. The addition of 2.5 and 5% of ECB juice was the most attractive to consumer preferences and acceptability after sensory analysis results. In the study of Şeker et al. (2016) examined the valorization of ECB fruit pomace after juice extraction which is a waste product, incorporated into cake formulation at different levels (5-10 and 15%). Based on sensory analysis, the highest overall acceptability score was the 5% pomace added cake sample [134]. Furthermore, some studies indicate the possibility of ECB fruit in cooked minced turkey or homogenized meat products [135,136]. Commercial available ECB fruit juice concentrate was used in the study of Çemtekin et al (2019) as an alternative antioxidant source for nitrites and butylated hyroxytoluene to delay oxidative changes and authors concluded that the use of ECB juice especially at 10% may be effective strategy in delaying the oxidative changes in poultry meat [135]. In the study Mazur et al. (2021) demonstrated the addition of freezed-dried ECB fruit in homogenized meat for improving microbiological

quality as an antimicrobial agent in meat production despite cooking loss due to low pH value of ECB fruit [136]. Moreover, evaluation of traditionally produced vinegar from ECB fruit was done by Erdal et al. (2022) and also showed antimicrobial and anticancer activity [137].

Due to their valuable content, ECB fruits are also employed in Türkiye's pharmaceutical industry as dietary supplements for traditional treatment. PC-rich ECB fruit extracts have been of great interest to obtain extracts with high concentrations from them, hence it is increasing demand for applications in nutraceuticals and functional foods. In Türkiye, there are commercially available various products such as "Gilaburine" which is a film-coated tablets of *V. opulus* extract (Natural Health Products, NHP, Türkiye), "Viburnum plus" liquid and powder extracts (Talex Pharma, Türkiye) and "Gilaburu Extract" (Immu-nat, Türkiye) on the internet under dietary supplement category for the treatment of kidney stone.

2.3. AIM OF THIS STUDY

The purpose of this thesis is to examine functional properties of PC-rich ECB fruit juice to understand its potential offerings to the food and nutraceutical industries.

The specific objectives:

- i. Assessment of biological activities (antimicrobial, antimutagenic, and antiproliferative activities) of ECB fruit juice and extract
- ii. Evaluation of bioavailability of ECB fruit extract *in vitro* gastrointestinal digestion system
- iii. Evaluation of the survival of probiotic cultures in presence of ECB fruit juice added different formulations in whey as growth medium
- iv. Assessment of inhibitory effect of ECB fruit juice against *Listeria monocytogenes* in milk as a model food
- v. Assessment of safety of ECB fruit extracts by determining lethal dose (LD₅₀) and no observed adverse effect level (NOAEL) *in vivo* animal study

3. ASSESSMENT OF *IN VITRO* BIOLOGICAL ACTIVITIES OF ECB FRUIT JUICE

3.1.INTRODUCTION

Demand on phenolic compound (PC) containing products by consumer is boosting the market growth because of the increase in awareness of consumption of healthy foods and benefits of maintaining good health. The usage of PC-rich products, particularly berries, is gradually expanding in the pharmaceutical, cosmetic, and functional food and beverage industries on the international market [138]. Berries, whose consumption is increasing worldwide, have great interest for both nutraceuticals and novel functional food production as raw materials. They are globally recognized for their antioxidant properties that contain diverse and abundant phenolic compounds and also are used natural food coloring agents [21,139,140]. European cranberrybush (ECB) fruits have been reported to be thriving sources of valuable biologically active substances, including flavonoids, phenolic acids, anthocyanins, possessing antioxidants and other beneficial health properties [3,12]. The wide range of biological activities have reported to possess antioxidant, antimicrobial, antibiofilm, antimutagenic, anticancer and anti-inflammatory properties [32,141–143]. The growing concern on preference of consumer to natural safe preservatives instead of chemical preservatives have stimulated the search for valuable alternative agents without any mutagenic effect on people to use in foods, nutraceuticals, food surfaces to fight against harmful microorganisms [50,144,145].

Due to their potential biological actions against pathogenic microorganisms, PC are incorporated into foods, nutraceuticals, and surface cleaning solutions, among others, to treat and prevent different microbial infections [41]. Different studies in literature, antimicrobial activities of ECB fruit juice and its solvent (methanol, ethanol) extracts against various foodborne pathogens and spoilage yeasts have been demonstrated [115–117,146]. Different susceptibilities were observed due to differences in applied concentrations and microorganism strains. In conjunction with antimicrobial activity, most of the bacterial infections result in the formation of biofilms and more resistant to antimicrobial agents, antibiotics, and disinfectants, thus an alarming need to develop new control strategies. In the study of Wójcik-Bojek et al. (2021) investigated the impact of solvent extracts of ECB fruit

and bark on biofilm formation of *Staphylococcus aureus* to understand use of them as an alternative for development of biofilm-associated infections by *S.aureus* showing that ethanol extracts of ECB fruit was effective against different strains of *S.aureus*, decreasing biofilm biomass around 20% [147].

Even though PC has antioxidant, antimutagenic and anticancer properties that are able to protect from damage by ROS and protect cells from mutation on initiating event in carcinogenesis, PC might have pro-oxidant and mutagenic properties at high concentrations [55,71,148,149]. That's why, there is a concern about the consumption of PC-rich fruits or extracts, which can cause toxicity, and thus the biological properties of these extracts should be well investigated to ensure their safe use. Studies have been conducted to look into the mutagenic and genotoxic effect of different ECB varieties' juices and their solvent extracts [124,125,150,151]. Arslan et al (2018), Paulauskas et al. (2015), and Koparal (2019) did not find mutagen and genotoxic potential in tested analysis, however, high dose of methanol extract of ECB caused DNA damage [124]. Many studies have reported the anticancer effect of ECB juice or extracts against various cancer cell lines [122,124,125]. Methanol extracts of ECB fruit showed cytotoxicity in colon cancer (Lovo) cells in concentration-dependent manner [124]. Commercially available ECB fruit juice inhibited the growth of Caco-2 and HeLa cells, but it did not show antiproliferative activities against A549 (human type II lung epithelium) [125]. When the applied concentration of ECB juice, solvent (methanol, acetone) extracts and solid phase extracted PC fraction increased, cell viability of human breast (MCF-7) and cervical (HeLa) cancer cells decreased [122].

The types of PC varies in different berry fruits based on species, harvesting area and conditions [152]. The fruit of ECB has shown that genotype influences the concentration of these compounds and their relative amounts [106,153,154]. This finding suggests that changes in environmental conditions such as light, temperature and relative humidity along the cropping season may also affect the concentration of bioactive compounds at various harvesting times.

Considering these aspects, despite many studies on the biological activities of ECB fruit, in this study, antimicrobial, antibiofilm, mutagenic, antimutagenic, and antiproliferative activities and bioavailability of ECB fruit juice harvested at same cropping season were assessed *in vitro* assays in more holistic approach to comprehend the potential use of ECB fruit juice as a safe food or food supplement in functional food or nutraceutical products.

3.2. MATERIALS AND METHODS

3.2.1. Plant Material and Sample Preparation

The fruits of ECB were freshly harvested from Gürpınar Village, located in Kayseri, Central Anatolia region of Türkiye in harvesting time, September. Fruits were washed and removed from their seeds and stalks. They were freshly squeezed and centrifuged at 10 000 x g for 20 min at 4°C. Both freshly squeezed ECB juice manually and lyophilized form of ECB fruit juice were stored at -80°C until further analysis.

3.2.2. Antimicrobial Activity

3.2.2.1. Microorganisms and Growth Conditions

A total of 24 microorganisms were used including several pathogenic gram-negative and gram-positive bacterial strains, yeasts, probiotic bacteria. *Pseudomonas aeruginosa* ATCC 15442, *P. aeruginosa* PAO1, *P. aeruginosa* ATCC 9027, *Escherichia coli* ATCC 25922, *E. coli* O157:H7, *E. coli* O111, *Salmonella* Typhimurium ATCC 14028, *Yersinia enterocolitica* ATCC 23713, *Shigella flexneri* ATCC 12022, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 43300, *S. epidermis* ATCC 12228, *Listeria monocytogenes* ATCC 15313, *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 19443, *E. faecalis* ATCC 29212, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Lactobacillus plantarum* ATCC 8014, *L. brevis* ATCC 8287, and *L. acidophilus* ATCC 4356 were obtained from the Yeditepe University Food Microbiology Laboratory Collection, Istanbul, Türkiye. Probiotic cultures of *L. plantarum* Lp-115, *L. rhamnosus* Lr-32, and *Streptococcus thermophilus* St-21 were kindly provided by Danisco, USA.

All microorganisms were kept at -80°C in proper growth medium, which contained 30% glycerol for bacteria and 10% glycerol for yeasts. The viable counts were performed on Tryptic Soy Agar (TSA, Merck, Germany) at 37 °C for overnight incubation for pathogenic bacteria, Sabouraud Dextrose Agar (SDA, Merck, Germany) at 30°C for 24 h for the yeasts, Man Rogosa Sharp agar (MRS, Merck, Germany) at 37°C for 48 h under flowing CO₂ (5%)

for *Lactobacillus* strains and M17 Agar (Conda Pronadisa, Spain) at 37°C for 48 h under flowing CO₂ (5%) for and *S. thermophilus*.

3.2.2.2. Antimicrobial Activity of Freshly Squeezed ECB Fruit Juice

Due to growth inhibitory effect on *Lactobacillus* strains at high concentration [12], antimicrobial activity of ECB fruit juice were tested on one of the most important foodborne pathogens and spoilage microorganisms, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, *L. monocytogenes* ATCC 15313, *C. albicans* ATCC 10231 and *S. cerevisiae* ATCC 9763 by monitoring their growth during incubation period.

At the same time, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of ECB juice on microorganisms were performed using a standard protocol based on broth microdilution assay with slight modifications (CLSI, 2012). Briefly, 100 µl of suitable broth medium was first added into a 96-well microplate (Nunc, Thermo Scientific, USA). Then, 100 µl freshly squeezed ECB fruit juice (100%) was added to the first rows of microplates and two-fold dilutions were done until 0.39% of ECB fruit juice by dispensing solutions to the remaining wells. ECB juice added without microorganisms and no ECB juice added medium were use as control groups.

All grown cultures were centrifuged at 10 000 x g for 10 min at 4°C, then supernatants were discarded and pellets were dissolved in saline (0.9% NaCl) to obtain 10⁸ CFU/ml organisms by comparing turbidity of 0.5 McFarland standard solutions. After adjustment, final concentrations of all cultures were 10⁵ and 10⁶ CFU/ml. Adjusted culture (100 µl) was added to the microplate to reach 10⁵ CFU/ml. The microplates were tested with or without the presence of ECB juice and incubated for 24 h under suitable conditions for all cultures. The turbidity was measured at 600 nm absorbance using a microplate reader (MultiScanGo, Thermo Scientific, Finland). After incubation of microplates, five µl of inoculum was taken from wells and put onto agar media, then incubated under suitable conditions to obtain MBC. The MIC was the lowest concentration of extract that hindered a microorganism's ability to grow visibly after 24 h incubation, whereas MBC/MFC was the lowest concentration at least 99.9% of microbial cells in the initial inoculation were destroyed.

3.2.2.3. Antimicrobial Activity of Lyophilized Form of ECB Juice Extracts

In this study, antimicrobial activities of ECB extract was examined at higher concentrations prepared by using the powder form of ECB fruit juice, due to the obtaining valuable results with ECB juice from the previous study (section 3.2.2.2). Agar well diffusion assay was applied to both microorganisms used in section 3.2.2.2 and another different microorganisms at different concentrations of ECB juice extracts (800, 400, 200, and 100 mg/ml). Different concentrations of ECB juice extracts (800, 400, 200, and 100 mg/ml) were firstly tested by using the agar well diffusion assay as a screening method. Lyophilized ECB powder was dissolved in a sterile 1X PBS solution and the maximum soluble ECB concentration was 800 mg/ml. Pathogenic bacteria, yeasts, *Lactobacillus*, and *S. thermophilus* strains were grown as described in section 3.2.2.1. The supernatants of each organism were discarded and the pellet was dissolved in saline solution (0.9% NaCl) to produce 0.5 McFarland standards, approximately 10^8 CFU/ml organisms. Final concentrations of the cultures were adjusted to 10^5 and 10^6 CFU/ml and 100 μ l was streaked onto proper solid media with a sterile cotton swab to generate a lawn of organisms. Wells were made in a sterile glass cylinder with a 5 mm diameter, and 100 μ l of ECB extracts (800-100 mg/ml) were placed inside the wells. Chlorhexidine (drogSan, Türkiye) and diluted with water penicillin-streptomycin (Gibco, UK) were used as positive control for yeast and pathogenic bacteria, respectively. PBS was used as a negative control. The result of antimicrobial activity was determined by measuring the zone of inhibition around the wells and was expressed in millimeters by using a digital calliper.

To eliminate the antimicrobial activity effects of pH and malic acid against microorganisms were also checked since the presence of the high amount of malic acid and low pH value of ECB fruit juice could have contributed to antimicrobial activity. The pH value of ECB extracts at 800, 400, 200, and 100 mg/ml, prepared by dissolving PBS solution, was measured with a pH meter (Hanna Instruments, Italy). Among the four ECB extracts, 800 mg/ml had the lowest pH values contained highest malic acid. Malic acid (Sigma-Aldrich) was added to the PBS solution to adjust the pH values to 2.50, 2.60, 2.72, and 2.90. Agar well diffusion assay was done with the acidified PBS solutions against tested on selected microorganisms with the same inoculation levels and experimental procedure. Results were measured as the diameter of inhibition zone around the wells and expressed as mm.

As described in section 3.2.2.2, after adding a 100 µl of broth medium into a 96-well microplate, then 100 µl ECB extract (800 mg/ml) was added into the first rows of microplates and two-fold dilutions of the extract (400 to 3.13 mg/ml) were made by dispensing solutions to the remaining wells. The same procedure was applied that was explained in detail above for applying broth microdilution method.

3.2.3. Mutagenicity and Antimutagenicity Activities

Standard plate incorporation Ames method was used by using *Salmonella* Typhimurium TA98 and TA100 for the determination of mutagenic and antimutagenic activities of ECB extract [156]. The *S. Typhimurium* TA 98 strain detects frame shift mutation and TA 100 detects base pair mutation. ECB extracts (100 mg/ml stock) were prepared by dissolving in dimethyl sulfoxide (DMSO, Merck, Germany). Various ECB extract concentrations (10000, 5000, 1000, 500 and 100 µg/plate) were used against know mutagens and carginogens compounds.

Plates containing various bacterial strains and a concentration of ECB extracts were incubated at at 37°C for 48 h as part of the mutagenicity test. Revertant colonies were enumeratated after incubation. Additionally, same experiment was applied with liver S9 fraction to evaluate the effect metabolites of ECB extract. Each concentration of ECB extracts was tested to compared with and without S9 fraction.

The antimutagenicity assay was performed by using the same strains and ECB extracts with and without S9 fraction. The 4-Nitro-o-Fenilendiamine (NPD) was used positive mutagen for TA98 strain and sodium azide was used as positive mutagen for TA100 strains in the absence of S9 experiment, whereas 2-aminofluorene was used as positive mutagen agent for both TA98 and TA100 strains in presence of S9 fraction. After 48 hours of incubation at 37°C, the number of revertant colonies on the plates was counted. The number of revertant colonies grown on plates containing the mutagen without ECB extracts was defined as 100% with 0% inhibition. Calculation of the percent of inhibiton was done with the formula: $[1 - T/M]$ where T is the number of revertants per plate in the presence of mutagen and the test sample and the M is the number of revertants per plate in the positive control. According to percentages, more than 40% inhibitory was considered as strong antimutagenic effect,

inhibitory percentage ranges between 25-40% was moderate and less than 25% inhibitory was considered as weak effect [157,158].

3.2.4. Antiproliferative Activity

3.2.4.1. Cell Culture

Previous study [129] indicated gastroprotection of *V. opulus* fruit and also realized the protective activity against ulcerogenesis. Along with this information, in this study we used Human gastric adenocarcinoma cancer (AGS) cells ATCC CRL1739 to search the effect of PC-rich ECB fruit juice on this cell line. Cell line was present in Culture Collection of Food Microbiology at Yeditepe University, Istanbul, Türkiye. Cells are cultured in RPMI-1640 (Roswell Park Memorial Institute) medium (the pH value 7.4, Gibco, USA), supplemented with 10% FBS (Gibco, USA) and incubated at 37°C under flowing CO₂ (5%) (New Brunswick, UK).

3.2.4.2. Cell Proliferation Assay

Freshly squeezed ECB fruit juice diluted with RPMI-1640 medium from 25 to 0.20% (v/v) and filter sterilized through a 0.2-µm pore size of syringe filter. Percentage of ECB fruit juice could be assumed as 25-0.20 mg/ml according to our calculation because it was determined that 1 ml ECB juice contained 100 mg of dry weight. Briefly, AGS cells were diluted to 1×10^5 cells/ml, and 100 µl added to each well of a 96-well plate (TTP, Germany). Cells were treated with different ECB fruit juice concentrations for 24 h and 48 h following overnight incubation at 37°C in 5% CO₂ incubator. Control cells were treated with medium for negative control and 10% DMSO as a positive control. Both treatment and control groups were performed in 6–8 replicate wells. Cell viability was evaluated by using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation MTS Assay (Promega, Madison, WI, USA), based on the manufacturer's instructions. Then, optical values were read by using ELISA plate reader (Bio-Tek ELx800, USA) at 490nm. Cell viability was calculated as dividing average absorbance value of test culture by average absorbance value of control culture.

3.2.4.3. *Observation of Cell Morphology*

Scanning Electron Microscopy (SEM) was used to observe the morphology of control and sample-treated cells as described previously [159]. AGS cells were pre-incubated for 24 h on sterile coverslips, were prefixed in glutaraldehyde solution (2.5%) for 2 h, and then rinsed three times in 0.15 M cacodylate buffer. Different concentrations of ECB fruit juice (6.25%, 3.13% and 1.56% (v/v)) was chosen according to cell proliferation assay results. Medium and 10% DMSO used as positive and negative controls. Samples were then dehydrated by exposing to ascending ethanol series for 10 min each. Samples were then coated with gold for 40 s with a sputter coater (Baltec SCD 005, USA) and were examined using a SEM (Evo-40, Carl Zeiss AG, Germany), at different magnifications ranging from 500× to 5,000× at 10 kV.

3.2.5. *In vitro* Gastrointestinal Digestion Procedure

The *in vitro* gastrointestinal digestion procedure was adapted from published studies [17,160]. The approach involves two procedures that simulate the small intestine and stomach's digestive processes, respectively. The lyophilized ECB fruit juice was dissolved in distilled water at 200 mg/ml. To simulate gastric conditions, pepsin (1.6 g, Sigma-Aldrich, USA) and NaCl (1 g) were added into ECB extract and the pH of the mixture was adjusted to 2.0 with 5 M HCl. For two hours at a speed of 100 rpm, the mixture (20 ml) was incubated at 37 °C to mimic peristaltic action. At the end of this step, sample (2 ml) was taken as a post gastric (PG) digestion and placed into an ice bath to terminate enzymatic process. The remaining solution was placed in a 250 ml glass beaker. A segment of cellulose dialysis tubing (Molecular weight cut-off 12kDa, Sigma-Aldrich, USA) containing 1 M NaHCO₃ (18 ml) to neutralize the titratable acidity was placed inside the beaker, which was sealed with parafilm. Diffusion of NaHCO₃ out of the dialysis tubing imitates the absorption process taking place in the gastrointestinal tract that is the simplest and most convenient to mimic gradual rise in pH from the stomach to the small intestine. When the pH of the solution reached neutrality within the 20 min, 4.5 ml of 4 mg/ml pancreatin (Sigma-Aldrich, USA) and 25 mg/ml bile salts mixture (Sigma-Aldrich, USA) was added into the beaker. The solution was incubated under the same conditions for an additional 2 h to simulate small intestine conditions. At the end of incubation process, the solution outside the dialysis tubing

was taken as OUT sample representing material that remained in the gastrointestinal tract (colon available) and the solution that had diffused into the dialysis tubing was taken as IN sample (serum available) and were cooled by immersing in an ice bath. All samples were maintained at -20°C for further analysis after being centrifuged at 10000 x g for spectrophotometric measurement.

3.2.5.1. Total Phenolic and Anthocyanin Contents

The total phenolic and anthocyanin contents of before and after digestion samples were determined by the Folin-Ciocalteu [161] and pH-differential methods [162], respectively. Gallic acid (Sigma, USA) was used as a standard in the concentration range of 0.02–0.2 mg/ml for the calibration curve for phenolic content analysis. While the results of total phenolic content were expressed as mg gallic acid equivalents per g fruit, total anthocyanin results were expressed as cyanidin 3-glucoside equivalents per g fruit.

3.2.6. Statistical Analysis

SPSS was used to conduct the statistical analysis. The outcomes were the average and range of at least three separate studies, each of which was carried out in triplicate for every treatment. Data were also analyzed using a One-way analysis of variance (ANOVA) with the Tukey test. However, in the statistical evaluation of antimicrobial activity results, General Linear Model-Univariate analysis model with Tukey's post hoc test were used to evaluate effect of different ECB extract concentrations and type of microorganisms. Differences were deemed significant for $p \leq 0.05$.

3.3.RESULTS AND DISCUSSION

3.3.1. Antimicrobial Activity of ECB Fruit Juice and Extracts

The growth inhibitory effect of ECB fruit juice at various concentrations (100-50-25-12.5-6.25-3.13-1.56-0.78-0.39% (v/v)) by using broth microdilution method on foodborne pathogenic and spoilage microorganisms was measured by taking optical density values during incubation and graphs were drawn for each microorganism (Figure 3.1.-3.2.). As seen

from the graphs, the presence of 100-50% ECB fruit juice was inhibitory to all microorganisms. When the percentage of ECB juice decreased from 50% to 25%, *C. albicans* ATCC 10231 and *S. cerevisiae* ATCC 9763 started growing slowly (Figure 3.2.), while 25% ECB juice was still inhibitory to all pathogenic bacteria. Among the foodborne pathogens, only *E. coli* was able to grow in the presence of 12.5% ECB juice, even the growth numbers were still low. When the concentration of ECB juice in growth medium decreased from 12.5 to 6.25% and lower than 6.25%, all pathogenic bacteria started to grow, but the growth was still less compared to control cultures.

The values of MIC, MBC, and MFC of freshly squeezed ECB fruit juice against microorganisms was presented in Table 3.1. The obtained values of MIC (50%) and MFC (100%) on spoilage microorganisms, *S. cerevisiae* and *C. albicans*, were higher which means that more resistant against ECB fruit juice compared to pathogenic bacteria. Among foodborne pathogenic bacteria, MIC (25%) and MBC (50%) values of *E. coli* was higher than other pathogens. The MIC and MBC values of other pathogens were 12.5% and 25%, respectively. Based on these values, *E. coli* was the most resistant bacteria against ECB fruit juice. In the study of Česonienė et al. (2014), *V. opulus* fruit juice showed the highest inhibitory effect against *S. Typhimurium*, *L. monocytogenes* and *S. aureus* based on inhibition zone measurements. Similarly, *E. coli* was more resistant than others and yeasts were much more resistant than pathogens, inhibition zone of *S. cerevisiae* was measured low [116].

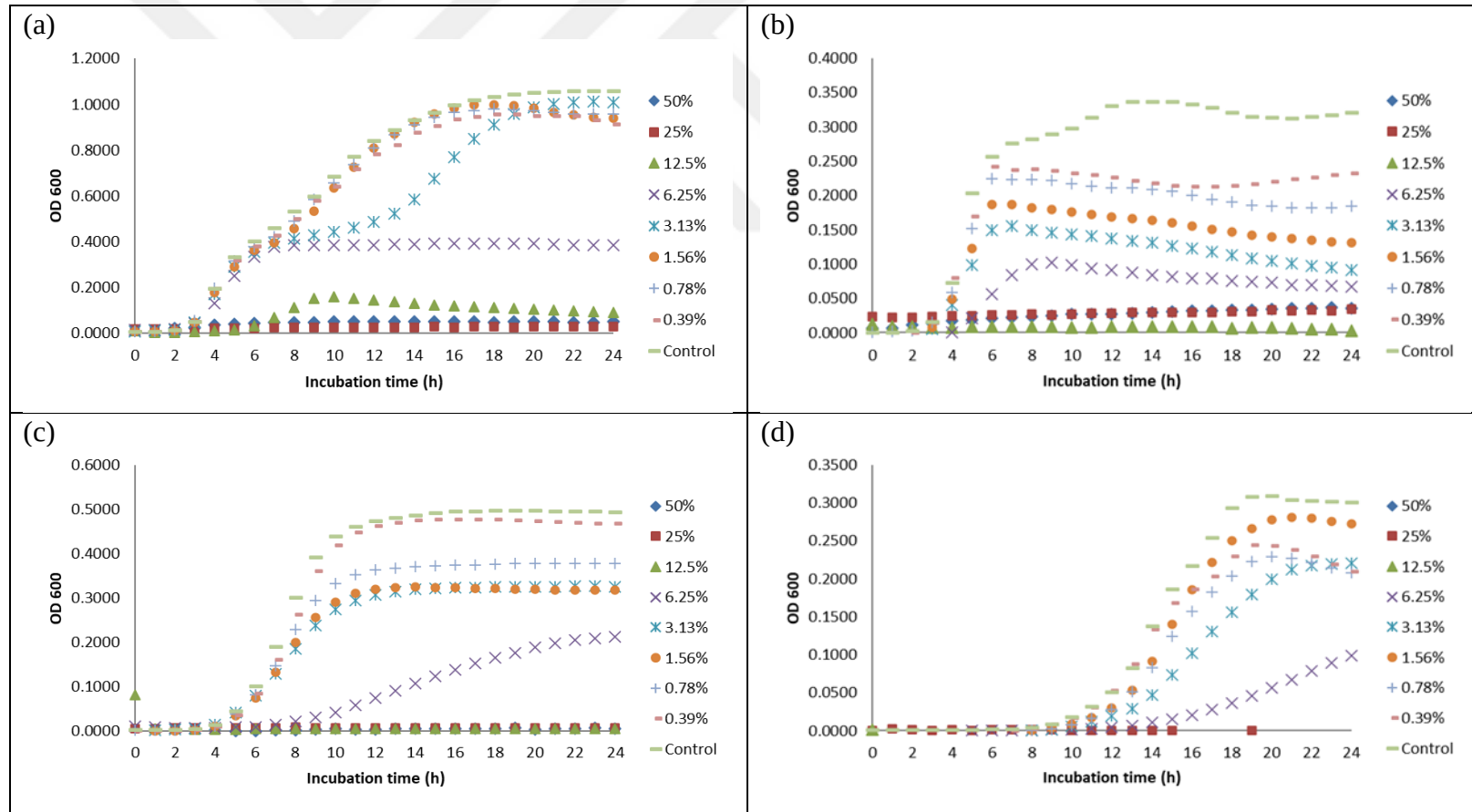


Figure 3.1. Growth curve of *E. coli* ATCC25922 (a), *Salmonella Typhimurium* ATCC14028 (b), *S. aureus* ATCC25923 (c) and *Listeria monocytogenes* ATCC15313 (d) in growth medium different concentrations of ECB fruit juice at 37°C during 24 h of incubation

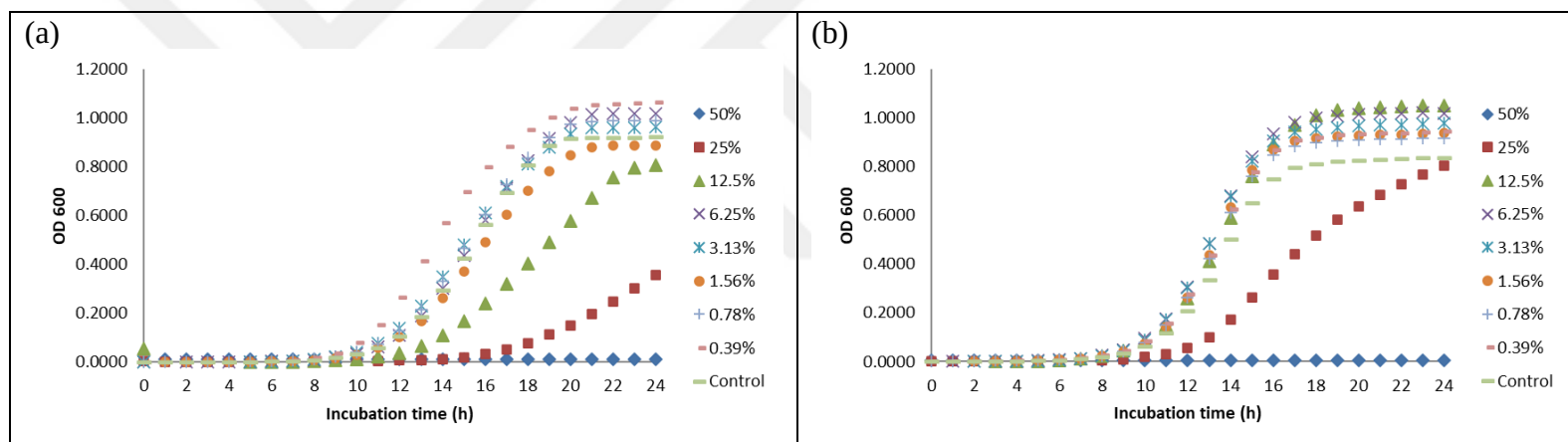


Figure 3.2. Growth curve of *Candida albicans* ATCC10231(a) and *Saccharomyces cerevisiae* ATCC9763 (b) in growth medium different concentrations of ECB fruit juice at 37°C during 24 h of incubation

Table 3.1. Antimicrobial activity of freshly squeezed ECB fruit juice on tested microorganisms

Microorganisms	MIC (%)	MBC /MFC (%)
<i>Escherichia coli</i> ATCC 25922	25	50
<i>Salmonella</i> Typhimurium ATCC 14028	12.5	25
<i>Staphylococcus aureus</i> ATCC 25923	12.5	25
<i>Listeria monocytogenes</i> ATCC 15313	12.5	25
<i>Candida albicans</i> ATCC 10231	50	100
<i>Saccharomyces cerevisiae</i> ATCC 9763	50	100

Table 3.2. Antimicrobial activity of ECB extracts against food pathogens, spoilage yeasts, and probiotic LAB

Microorganisms	Zone of Inhibition (mm)				MIC (mg/ml)	MBC/MFC (mg/ml)		
	ECB Concentrations (mg/ml)							
	800	400	200	100				
Gram-negative pathogens								
<i>Pseudomonas aeruginosa</i> ATCC 15442	6.24±0.1 ^{fg}	4.44±0.2 ^{fg}	2.51±0.2 ^b	*	50	50		
<i>Pseudomonas aeruginosa</i> PAO1	6.61±0.3 ^g	5.04±0.4 ^{gh}	2.65±0.3 ^b	1.45±0.1 ^{ab}	50	50		
<i>Pseudomonas aeruginosa</i> ATCC 9027	4.91±0.2 ^{de}	3.91±0.3 ^{ef}	1.98±0.2 ^{ab}	0.77±0.1 ^a	50	100		
<i>Escherichia coli</i> ATCC 25922	4.91±0.4 ^{de}	2.56±0.2 ^{bc}	1.33±0.1 ^a	*	50	100		
<i>Escherichia coli</i> O157:H7	5.31±0.2 ^{ef}	3.27±0.2 ^{cde}	*	*	50	100		
<i>Escherichia coli</i> O111	4.63±0.3 ^{de}	3.86±0.3 ^{def}	*	*	50	100		
<i>Salmonella</i> Typhimurium ATCC 14028	3.89±0.2 ^{cd}	2.78±0.2 ^{cd}	*	*	50	100		
<i>Yersinia enterocolitica</i> ATCC 23713	9.96±0.5 ^j	9.37±0.4 ^l	5.49±0.3 ^d	*	50	50		
<i>Shigella flexneri</i> ATCC 12022	7.31±0.3 ^g	6.52±0.6 ^{ij}	1.58±0.3 ^a	*	50	100		
Gram-positive pathogens								
<i>Staphylococcus aureus</i> ATCC 43300	7.00±0.4 ^g	5.62±0.2 ^{hi}	2.40±0.2 ^b	*	50	50		
<i>Staphylococcus aureus</i> ATCC 25923	6.54±0.7 ^g	5.20±0.3 ^{gh}	2.60±0.3 ^b	1.21±0.2 ^{ab}	25	50		
<i>Listeria monocytogenes</i> ATCC 15313	12.73±0.3 ^l	11.33±0.5 ^m	6.48±0.5 ^f	3.63±0.5 ^c	25	50		
<i>Bacillus cereus</i> ATCC 11778	8.63±0.3 ^h	8.31±0.3 ^{kl}	5.20±0.3 ^{de}	1.75±0.1 ^{ab}	100	100		
<i>Enterococcus faecalis</i> ATCC 19443	11.19±0.6 ^k	9.11±0.9 ^l	4.39±0.2 ^c	0.91±0.1 ^a	50	100		
<i>Enterococcus faecalis</i> ATCC 29212	9.64±0.5 ^{hj}	8.28±0.4 ^{kl}	4.51±0.1 ^{cd}	*	50	100		

<i>Staphylococcus epidermis</i> ATCC 12228	9.83±0.5 ^j	7.58±0.6 ^{jk}	4.04±0.6 ^c	2.41±0.4 ^{bc}	50	100
Spoilage yeasts						
<i>Candida albicans</i> ATCC 10231	2.32±0.4 ^{ab}	1.32±0.3 ^a	*	*	200	200
<i>Saccharomyces cerevisiae</i> ATCC 9763	2.46±0.1 ^{ab}	1.12±0.1 ^a	*	*	200	200
Probiotic LAB						
<i>Lactobacillus plantarum</i> ATCC 8014	1.58±0.2 ^a	*	*	*	100	100
<i>Lactobacillus plantarum</i> Lp-115	2.16±0.2 ^{ab}	1.14±0.0 ^a	*	*	200	200
<i>Lactobacillus rhamnosus</i> Lr-32	2.85±0.2 ^{bc}	1.09±0.2 ^a	*	*	100	100
<i>Lactobacillus acidophilus</i> ATCC 4356	1.47±0.1 ^a	*	*	*	100	100
<i>Lactobacillus brevis</i> ATCC 8287	*	*	*	*	200	200
<i>Streptococcus thermophilus</i> St-21	2.48±0.2 ^{ab}	1.63±0.2 ^{ab}	*	*	400	400

* Inhibition not detected

^{a-m} Different letters in the same column mean statistically significant difference among the values of same parameter, according to Tukey test ($p < 0.05$)

Subsequently, antimicrobial activity of lyophilized ECB juice extracts against various pathogenic, spoilage, LAB and probiotic bacteria at various concentrations were presented in (Table 3.2.). The obtained results indicate that, zone of inhibition (ZI) values of ECB extracts against all strains were in the ranging from 0.77 ± 0.1 to 12.73 ± 0.3 mm. Inhibitory effect of penicillin-streptomycin used as a positive control was measured as 5.03 ± 0.25 , 2.08 ± 0.37 , 5.88 ± 0.49 and 4.61 ± 1.16 mm against *S. aureus*, *Salmonella* Typhimurium, *E. coli* and *L. monocytogenes*, respectively. Following pathogens, the ZI results of chlorhexidine used as a positive control for yeast, was 8.9 ± 0.1 and 9.3 ± 0.2 mm against *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763 (data not shown).

The highest ZI was measured at the two highest concentration of ECB extracts (800 and 400 mg/ml) against all pathogenic and spoilage microorganisms. Contrarily, the effect of these extracts on probiotic lactic acid bacteria (LAB) strains were slight. *Lactobacillus brevis* (800 and 400 mg/ml), *L. plantarum* and *L. acidophilus* (400 mg/ml) did not display ZI values. Although the measurement of ZI decreased as the applied ECB concentration decreased, most of the pathogenic bacteria were still susceptible to low concentrations (200 and 100 mg/ml) of ECB extracts. Among all microorganisms, gram-positive pathogenic bacteria showed the highest ZI at all ECB concentrations according to statistical analysis ($p < 0.05$), especially *L. monocytogenes* was the most sensitive bacteria among all strains and all ECB concentrations. After gram positive pathogenic bacteria, the highest inhibition was on gram negative bacteria. There were no significant differences between the ZI values of spoilage yeasts, LAB and probiotic bacteria that were more resistant strains compared to pathogenic bacteria.

In the present study, gram-positive pathogenic bacteria were more susceptible to ECB extracts than gram-negative pathogenic bacteria. The ZI values ranged between 12.73 ± 0.3 mm and 0.91 ± 0.1 mm among gram-positive pathogenic bacteria, whereas zone of inhibition values of gram-negative pathogenic bacteria varied 9.96 ± 0.5 mm to 0.77 ± 0.1 mm (Table 3.2). The strongest inhibitory effect was observed against *L. monocytogenes* and the highest zone of inhibition values were 12.73, 11.33, 6.48 and 3.63 mm at 800, 400, 200 and 100 mg/ml ECB extracts, respectively ($p < 0.05$). After *L. monocytogenes*, the most susceptible strain was *E. fecalis* ATCC 19443 and ZI were in the range of 11.19 to 0.91 mm at concentration of 800-100 mg/ml ECB extracts.

Among gram-negative pathogenic bacteria, the highest inhibitory effect was seen against *Y. enterocolitica* ATCC 23713 which means that the most susceptible bacteria among them. The measured ZI values were between 9.96-5.49 mm at 800, 400 and 200 mg/ml ECB extracts. Moreover, *S. flexnerii* was the second susceptible microorganism. As seen from Table 3.2, the lowest ECB extract (100 mg/ml) was only effective on *P. aeruginosa* PA01 and *P. aeruginosa* ATCC9027 that the zone of inhibition measured as 1.45 ± 0.1 mm and 0.77 ± 0.1 mm, respectively. The most resistant gram-negative bacteria against ECB extracts were *S. Typhimurium* ATCC 14028 and the zone of inhibition values were 3.89 and 2.78 mm at 800 and 400 mg/ml ECB, respectively ($p<0.05$).

The ZI values of *C. albicans* and *S. cerevisiae* were 2.32 and 2.46 mm at 800 mg/ml ECB and 1.32 and 1.12 mm at 400 mg/ml ECB, respectively. The 200 and 100 mg/ml ECB extracts did not show any inhibition zone on both yeast strains and they were among the resistant microorganisms against ECB extracts ($p<0.05$). All tested yeasts, LAB and probiotic cultures were the most resistant microorganisms against ECB extracts ($p<0.05$). The ZI values of *Lactobacillus* strains and *S. thermophilus* were significantly lower than pathogenic bacteria ($p<0.05$) (Table 3.2). Only 800 and 400 mg/ml of ECB extracts showed inhibitory effect on some of *Lactobacillus* strains and *S. thermophilus*, except *L. brevis* ATCC 8287. The measured ZI values were in the range of 1.47 ± 0.1 to 2.85 ± 0.2 and 1.09 ± 0.2 to 1.63 ± 0.2 mm at 800 and 400 mg/ml ECB, respectively.

Antimicrobial activity of ECB fruit was compared with the literature [116,117,146]. Similar results were seen that *L. monocytogenes* and *E. faecalis* were the most sensitive bacteria against ethanol extract of *V. opulus* [146]. High antibacterial activity of ethanol extract and *V. opulus* juice observed against *S. Typhimurium*, *L. monocytogenes*, *E. faecalis*, and *S. aureus*. In contrast, ethanol extract and *V. opulus* juice showed no or little inhibitory activity against various spoilage yeasts [116,117]. In our study the highest inhibitory effect was seen against *Y. enterocolitica*, however, they found that the most resistant bacteria was *Y. enterocolitica* against methanol extract of *V. opulus* [115]. Although tested microorganisms were same, results of antimicrobial activity were different due to differences in sample preparation and the presence of their PC concentrations.

In our previous study [12], organic acid composition of ECB fruit juice was determined and high amount of malic acid (19.79 mg/ml) was detected about 74% of total organic acid of ECB juice. Low pH and presence of organic acid can be responsible for the antimicrobial

activity against microorganisms. The effect of adjusted pH values of PBS solutions with malic acid (2.50, 2.60, 2.72 and 2.90) according to native pH values of ECB extracts (800, 400, 200 and 100 mg/ml) were also investigated on antimicrobial activity against *P. aeruginosa* ATCC 15442, *E. coli* ATCC 25922, *S. Typhimurium* ATCC 14028, *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 15313. The result showed that the zone of inhibition was observed only against *P. aeruginosa* ATCC 15442 at pH 2.50 (1.24 ± 0.2 mm ZI) and pH 2.60 (0.78 ± 0.1 mm ZI). The pH adjusted PBS solutions did not show any inhibitory effect against other tested microorganisms showing that there were no pH and malic acid effect on antimicrobial activity. Therefore, antimicrobial activity of ECB fruit clearly showed that PC present in ECB were strongly responsible for the inhibitory effects against microorganisms.

Although antimicrobial activity of ECB fruit against various pathogenic bacteria and spoilage yeasts is reported, there is a lack information on inhibitory effect against beneficial probiotic microorganisms. Zakłós-Szyda et al. (2020b) tested the inhibitory activity of fresh *V. opulus* juice and phenolic-rich juice obtained from fresh juice against both beneficial and pathogenic bacteria. *S. aureus*, *E. faecalis*, and *L. monocytogenes* were sensitive to *V. opulus* juice samples and these samples were no show inhibitory effect on tested other pathogenic strains and *C. albicans*. Furthermore, both samples did not show inhibitory effect against various LAB strains such as *L. rhamnosus*.GG, *L. plantarum* LOCK.0981 and 0989, *L. brevis* LOCK.0983, *L. paracasei* LOCK.0985, and *L. delbrueckii* LOCK.0987 [5].

MIC and MBC/MFC values of foodborne pathogenic, spoilage yeast, LAB and probiotic microorganisms were ranged between 50 and 400 mg/ml ECB (Table 3.2). *Streptococcus thermophilus* St-21 had the highest MIC and MBC (400 mg/ml) among probiotic LAB. After that, the highest MIC and MBC values (200 mg/ml) were observed on *C. albicans* and *S. cerevisiae*. Yeast strains were found to be more resistant to ECB extracts than pathogenic bacteria. The reason could be the difference in yeast and bacterial cell wall structure and protein synthesis [163]. Antimicrobial activity of PC was correlated with the inactivation of cellular enzymes and disruption of membrane permeability [44]. The reason of higher ZI values observed in gram-positive pathogenic bacteria in other words more susceptibility against ECB extracts, might be the differences in cell wall structure between gram-positive and negative bacteria. Gram positive bacteria have less complex cell wall structure that

makes it more permeable to PC, however gram-negative bacteria have an outer membrane which can act as a barrier, and thus they are more resistant to PC [164].

The MIC values of all gram-negative pathogenic bacteria had 50 mg/ml ECB and the MBC was 50 mg/ml ECB for *P. aeruginosa* ATCC.15442, *P. aeruginosa* PAO1 and *Y. enterocolitica* ATCC.23713, 100 mg/ml ECB for the remaining gram-negative pathogens. The MIC values of gram-positive bacteria showed different concentrations depend on strains that ranged between 25-100 mg/ml ECB extracts. The MBC value of *S. aureus* ATCC.43300, ATCC 25923, and *L. monocytogenes* ATCC.15313 were 50mg/ml, other gram-positive pathogens had 100 mg/ml MBC value. MIC and MBC values of *L. plantarum* Lp-115 and *L. brevis* ATCC 8287 were 200 mg/ml, whereas 100 mg/ml for the *L. plantarum* ATCC 8014, *L. rhamnosus* Lr-32 and *L. acidophilus* ATCC 4356.

In literature, similar results found that pure PC or PC-rich berries demonstrated antimicrobial effects against pathogens while conserving or promoting growth of LAB and probiotic strains. Puupponen-Pimiä et al. (2001) also reported that the growth of *Lactobacillus* and probiotic strains were not affected by the presence of PC-rich berries [165]. The 100% blueberry juice did not affect growth of probiotic *L. bulgaricus* and *Bifidobacterium bifidis* bacteria while inhibiting pathogens [166]. In another study of blackberry juice (10%) showed that viable number of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* decreased in both skim and whole milk, but the growth of *L. casei*, *L. plantarum* and *L. rhamnosus* significantly stimulated by blackberry juice in both milk and MRS broth media [167]. The study of Pacheco-Ordaz et al. (2018) showed that phenolic compounds (gallic, vanillic, protocatechuic and ferulic acids and catechin) selectively inhibited the pathogenic *E. coli* 0157:H7 ATCC 43890 and *S. Typhimurium* ATCC 14028 bacteria without affecting viability of probiotic *L. rhamnosus* GG ATCC 53103 and *L. acidophilus* NRRLB 4495 bacteria [168]. The reason of *Lactobacillus* strains resistance might be that they can withstand and survive in a low pH environment because they can produce organic acids during fermentation and are more resistant to hyper-acidification. Besides, *Lactobacillus* strains could detoxify the phenolic acid during metabolism and therefore PC could not inhibit the growth of strains [169,170].

There are several studies showed that PC- rich berry fruits had antimicrobial activity against pathogens [43,165,166,171]. At a concentration of 1 mg/ml cloudberry, blackberry, raspberry and strawberry inhibited *S. Typhimurium* [165]. In another study of Puupponen-

Pimiä et al. (2005) reported that *Staphylococcus* and *Salmonella* were the most sensitive strains against cloudberry and raspberry [43]. Biswas et al. (2012) found that 100% blueberry juice inhibited growth and viability of *S. Typhimurium*, *E. coli* O157:H7 and *C. jejuni* strains [166]. Shen et al. (2014) examined antimicrobial activity of blueberry extract on the *L. monocytogenes* and *Salmonella* Enteritidis [171]. Antimicrobial activity results reported that blueberry disrupted metabolism via changing in fatty acid profile of *L. monocytogenes* and affected on efflux pump of *S. enteritidis* by weakening to adaptation and survival the harsh environment. In our experiment, *L. monocytogenes* was the most susceptible bacteria against ECB extracts. However, although Puupponen-Pimiä et al. (2005) reported that *Listeria* stains were not affected by the PC-rich extracts of bilberry, cloudberry, strawberry, raspberry, blackcurrent, lingonberry and buckthorn berry, Wen et al. (2003) found antilisterial activities of selected pure PC, especially hydroxycinnamic acids, exhibited activity against several strains of *L. monocytogenes* [43,172]. These results suggested that high amount of chlorogenic acid, hydroxycinnamic acid, in ECB fruit juice might be responsible for the inhibitory activity against *L. monocytogenes*. In the study of Wen et al. (2003) also investigated the relationship between pH and anti-listeria activity and found that cinnamic, caffeic, p-coumaric and ferulic acids were bactericidal at pH 4.5 and bacteriostatic at higher pH value [172], however, chlorogenic acid inhibited *L. monocytogenes* growth only at pH 6.5. In our study, ECB extracts at 800 mg/ml has the lowest pH value (2.50), but it contained the highest PC content compared to other concentrations, therefore we can conclude that PC content might be more effective than pH values of ECB extract.

As a result, different microorganisms exhibited different sensitivities toward PC-rich ECB fruit juice. ECB extracts selectively allowed the growth of beneficial microorganism, *Lactobacillus* strains and probiotics, and inhibited pathogenic and spoilage microorganisms. This selectivity is significant for developing new functionally enhanced products for both PC and beneficial microorganisms, potentially improving overall health of the consumers. PC-rich ECB fruit juice have also been considered as alternative sources for safe and effective natural antimicrobial agents to overcome the problems related with chemical antimicrobial agents against several strains or it can be used as natural food preservatives and may replace with chemical ones to increase food safety.

3.3.2. Mutagenic and Antimutagen Activity of ECB Extract

The number of revertant colonies per plate was not noticeably higher than the negative control at any tested ECB concentrations against *Salmonella* Typhimurium strains TA98 and TA100, either with or without S9 activation. ECB extracts did not exhibit any mutagenic effect (Table 3.3). The average revertant colony numbers in negative control were 30 ± 8.7 and 165.6 ± 7.0 without S9 fraction, 26.5 ± 0.7 and 122 ± 2.8 with S9 for TA98 and TA100 strains, respectively. The results of revertant colony numbers showed that the various ECB extract concentrations did not influence the viability of bacterial strains.

Table 3.3. Mutagenic activity of ECB extracts against *Salmonella* Typhimurium TA98 and TA100 strains without and with S9 fraction

ECB extracts (ug/plate)	Number of revertant/plate			
	TA 98		TA 100	
	without S9	with S9	without S9	with S9
0	30 ± 8.7	26.5 ± 0.7	165.6 ± 7.0	122 ± 2.8
100	32.3 ± 8.3	25 ± 0.0	151.6 ± 7.1	98.5 ± 2.1
500	29.3 ± 7.57	29 ± 8.5	159.3 ± 4.04	130 ± 2.8
1000	35.7 ± 2.52	33 ± 4.2	160.7 ± 15.2	124 ± 1.4
5000	31.7 ± 6.42	29 ± 5.7	146.3 ± 2.08	128 ± 2.8
10000	33.3 ± 2.08	30.5 ± 6.4	152.3 ± 10.8	97 ± 4.2

The results of antimutagenicity of ECB extracts against *Salmonella* Typhimurium TA98 and TA100 were presented in Table 3.4 and 3.5. The percentage inhibition of ECB extracts ranged from 3.2 to 25.7% and 8.8 to 17.5% in *S. Typhimurium* TA98 without and with S9 fraction, respectively. The percent of inhibition was increased when the concentration of ECB extracts increased based on both with and without S9 fraction results. The highest inhibition ratio (25.7%) was observed in 10000 $\mu\text{g}/\text{plate}$ ECB extract without S9 and 17.5% inhibition was observed in the presence of S9 fraction. In TA100 strain, the inhibition was only seen at high concentrations of 5000 and 10000 $\mu\text{g}/\text{plate}$ ECB extract without S9 as 11.3 and 16.1%, respectively. On the other hand, antimutagenic activity of TA100 varied from

9.6 to 27.3% with S9 fraction. The highest inhibition ratio of TA100 (27.3%) was seen in 10000 µg/plate ECB extract with S9.

Table 3.4. Antimutagenic activity of ECB extracts against *Salmonella Typhimurium* TA98 without and with S9 fraction

ECB extracts (ug/plate)	TA 98			
	without S9		with S9	
	Number of revertant/plate	% Inhibition	Number of revertant/plate	% Inhibition
0	1234±41.58	0.0	1450.5±57.3	0.0
100	1195±43.84	3.2	1323±42.5	8.8
500	981±20.50	20.5	1296±62.2	10.7
1000	964.5±21.80	21.8	1235±77.8	14.9
5000	927.5±24.80	24.8	1271±55.2	12.4
10000	917±25.70	25.7	1196.5±9.2	17.5

In this study, due to the fact that ECB extracts at all investigated concentrations (100-10000 µg/plate) had no mutagenic effect on the *S. Typhimurium* strains TA98 and TA100, either with or without S9 activation, ECB fruit extract can be deemed to be genotoxically safe. The result of this study was also supported by the study of Paulauskas et al. (2015) who reported that different *Viburnum* cultivars were not genotoxic [151]. The study of Arslan et al (2018) reported antimutagenic activity of water added ECB fruit extract with Ames test by using both *S. Typhimurium* strains TA98 and TA100. They found that there were no statistically significant reduction in any dose compared to positive control in TA100 strains, but in TA98 strains, only one dose (40 µl /plate) of extract showed a reduction compared to positive control. Therefore, only TA98 strain at high dose of ECB extract showed antimutagenic activity [150].

Table 3.5. Antimutagenic activity of ECB extracts against *Salmonella* Typhimurium TA100 without and with S9 fraction

ECB extracts (ug/plate)	TA 100			
	without S9		with S9	
	Number of revertant/plate	% Inhibition	Number of revertant/plate	% Inhibition
0	777.3±16.44	0.0	689.5±14.8	0.0
100	895±35.36	-15.1	623±1.40	9.6
500	804.5±6.36	-3.5	607.5±46	11.9
1000	885.5±92.63	-13.9	573±52.4	16.9
5000	689.5±14.85	11.3	562.5±24.7	18.4
10000	652±25.46	16.1	501±21.2	27.3

The antimutagenic activity of ECB extract might be because of the presence of phenolic compounds [173]. In the study of Edenharder and Tang (1997) reported that PC can be classified as potent antimutagens [174]. The metabolically activated carcinogen benzo[*a*]pyrene and the direct-acting mutagen methyl methanesulfonate also prevented mutagenesis in many berry fruits such strawberry, blueberry and raspberry [55]. While safety evaluation of catechin rich green tea and proanthocyanidin rich grape seed extracts have shown no toxicity in mutagenicity test [74,175]. PC extracted from apple extract had slight mutagenic effect at a high concentration of 2500 µg/plate [176]. In our study, the highest ECB concentration was 10000 µg/plate that were 4 fold higher than PC-rich apple extract. Acai, cashew apple, kiwi, and strawberry exhibited mutagenic activity at high fruit concentrations of 5%, 10% and 15% (w/v), whereas coconut, raspberry, red guava, apple, papaya, mango, peach, and red grape frozen fruits displayed a significant antimutagenic effect at all concentrations [177].

Chlorogenic acid is found one main phenolic compound in ECB fruits, did not show mutagenic activity with or without S9 fraction [178,179]. The antimutagenic activity of ECB extract was determined as being dose dependent. Low concentrations of ECB extracts showed weak inhibition effect on both TA98 and TA100 strains because inhibition percent

less than 25%. However, at the highest concentration of ECB extract (10000 µg/plate) exhibited moderate inhibitory effect as 25.7 and 27.3% in TA98 and TA100, respectively.

3.3.3. The Effect of ECB Fruit Juice on AGS Cell Proliferation

PC have been played as a crucial roles in preventing the development of abnormal cell due to cancer. For this reason, the use of PC-rich products in cancer treatment may reduce the risk of gastric cancer [180]. Current gastric cancer treatment includes surgery and chemotherapy, however there are potentially severe side effects of chemotherapy, present a considerable challenge. Therefore, the potential anticancer effect of PC against cancer cells have increased due to their minor side effects and showing the ability for prevention of cancer as an alternative treatment [181,182].

The potential of ECB fruit juice in inhibiting AGS cell was evaluated and obtained results of antiproliferative activity of ECB fruit juice was presented in Figure 3.3 for both dose and time dependent manners against AGS cell line using MTS assay. The percent of AGS cells viability decreased significantly when the ECB juice concentration increased in both 24 and 48 h exposure time ($p < 0.05$). At high concentrations of ECB juice (25 to 3.13% (v/v)), the viability percentage of AGS cells varied from 10.5 to 76.9% for 24 h, from 9.3 to 35.2% for 48 h exposure. However, low concentrations of ECB fruit juice (1.56-0.20%) were no show any efficacy on AGS cells and the viability of cells increased compared to the control. ECB juice was effective of viability of AGS cell at a minimum of 3.13% (v/v) in both two different time points (24 and 48 h). Therefore, two lowest cytotoxic concentrations of ECB juice (3.13 and 6.15%) were selected to observe morphological changes in AGS cell compared to control cell under SEM after 48 h incubation. SEM images were also in agreement with percentage of cell viability results demonstrating high concentrations of juice decreased the number of viable cells and caused the alteration in the morphology (Figure 3.4.). SEM images also confirm the growth of AGS cell was inhibited by ECB juice in a concentration dependent manner.

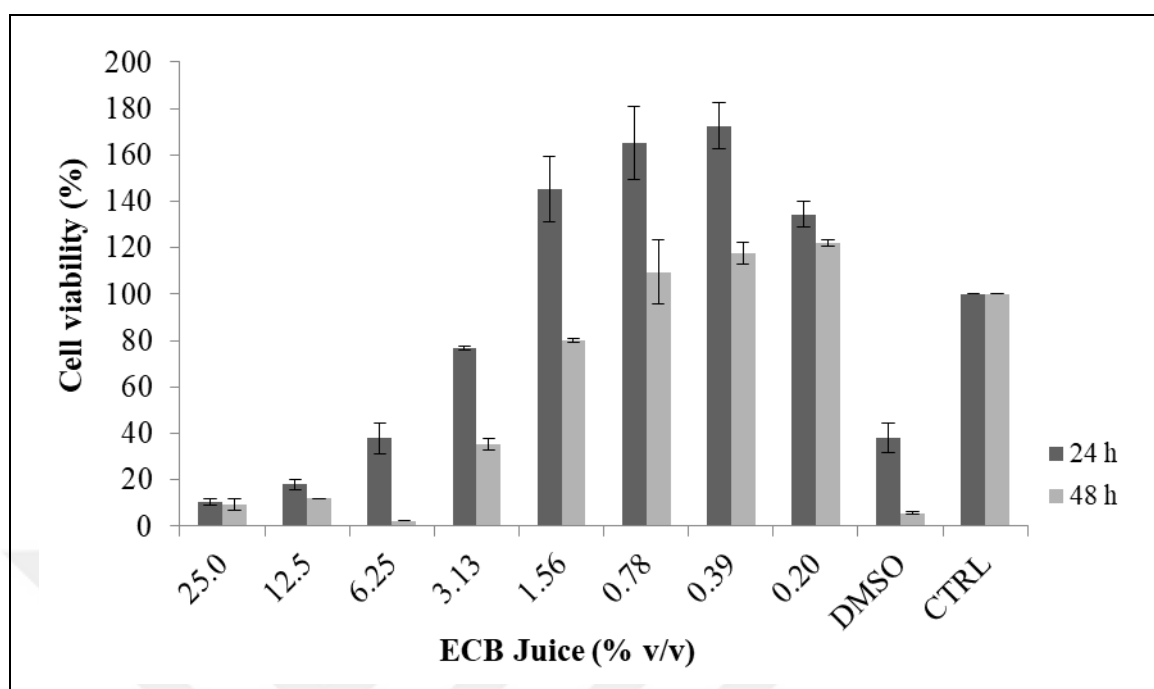


Figure 3.3. Percentage viability of AGS cells in the presence of various concentrations (25-0.20%) of freshly squeezed ECB fruit juice for 24 and 48 h. Values given as mean $\pm$ standard deviation of triplicate independent experiments

Previous studies have focused on the antiproliferative activity of ECB fruit or extracts on different cell lines to evaluate the potential use for alternative treatment and prevention of cancer, but there was no study that showed an antiproliferative activity on the AGS cell line. Commercially available ECB fruit juice demonstrated cytotoxic activities against cancer cell lines of HeLa and Caco-2 [125]. Similarly, same ECB fruit juice was used in the of Ulger et al (2013) that investigate the chemopreventative effects of juice on colon cancer. They found that commercially available ECB fruit juice might be useful in the prevention of colon cancer induced by 1,2-dimethylhydrazine (DMH) in Balb-c mice at the initiation stage [4]. PC-rich fraction obtained from ECB juice has also been chemo-preventive activity against oxidative stress induced by chemically and is able to reduce glucose uptake and free fatty acids in colon cancer cells [107]. Moreover, methanol and acetone extracts of ECB fruit have been displayed anticancer effect against breast and cervical cancer cells line by suppressing cell migration and reduce mitochondrial potential [122]. In addition, commercially available ECB fruit juice has been reported to exhibit anticancer activity against Ehrlich ascites carcinoma, is a murine spontaneous breast cancer, *in vivo* mice tumor model [183].

Collectively, these results show that ECB fruit juice/extracts has antiproliferative activities due to their high content of PC.

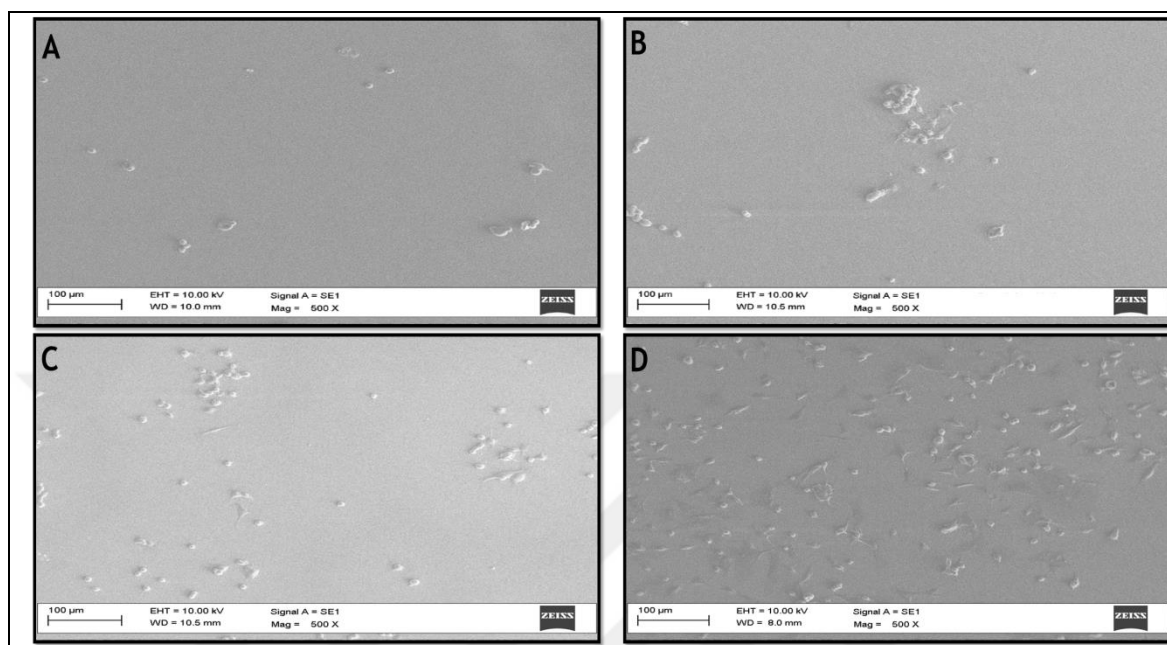


Figure 3.4. SEM images of AGS cells after 48 h treatment with ECB juice (A) 6.25%, (B) 3.13%, (C) 1.56%, (D) Control (only medium) at 500X magnification

PC-rich extracts have gained significance for the treatment anticancer that may reduce cancer risk by suppression of the production of potential carcinogens within the body [55,173,184]. Also, due to severe side effects of conventional cancer medications and evidence of secondary toxicity of these drugs in the body, people who had cancer are increasingly turning to natural alternatives. Therefore, pharmaceutical industry is trying to search for natural products and investigate efficacy of active compounds which contribute to their potent effects on inhibiting carcinogenesis for the anticancer treatment [185,186].

PC have been shown the potential to regulate cell apoptosis and cytotoxic activity against cancer cells therefore they are able to induce apoptosis in cancer cells could be used as new alternative anticancer agents [61]. As the efficacy of PC may changed with dose, phenolic composition, cell type, exposure time and conditions. In *in vitro* study, PC present in berry fruits may modulate cellular processes such as proliferation and apoptosis [187]. For example, in the study of Shih et al. (2005) examined the effect of different anthocyanins on the inhibition of proliferation and induction of apoptosis in AGS cells and resulted that

depending on the structure of anthocyanins, malvidin showed much efficacy than others and inhibited growth inhibition of AGS cells [56]. Likewise the previous biological activity, the antiproliferative ECB fruit juice might be associated with the presence of high content of PC in ECB fruit.

3.3.4. Evaluation of *in vitro* Bioavailability of ECB Fruit Juice

ECB fruit have reported as valuable source of PC that contribute to the beneficial biological properties of this fruit. To produce any biological effect in a particular tissue or organ, PC must be bioavailable, which means that PC must be efficiently absorbed from the gastrointestinal tract into the circulation and transported to proper spot inside the body [17]. In our previous study, antimicrobial activity of ECB fruit juice was investigated at different concentrations on both several strains of pathogenic and probiotic bacteria. The antimicrobial activity results showed that 200 mg/ml ECB fruit juice were effective on pathogenic bacteria, but there was no any inhibition zone on probiotic bacteria. Therefore, this concentration was selected to evaluate bioavailability of ECB fruit juice by mimicking both physiochemical and biochemical factors of the upper gastrointestinal tract with *in vitro* gastrointestinal digestion procedure. Besides, same concentration was used in Section 5 for the evaluation of PC-rich ECB fruit juice safety and then, effect of gut microbiota will investigated at this concentration *in vivo* animal study for further study.

Total phenolic (TPC) and anthocyanin (TAC) contents of ECB extract before and during digestion were exhibited in Figure 3.5. and 3.6. The decrease in the amount of TPC and TAC was low in post gastric compared to initial measurements of ECB fruit juice. After post gastric, the recovery of phenolics and anthocyanins were 94.97% and 91.15%, respectively as shown in Table 3.6 indicating that stable PC under gastric conditions. However, the amount of TPC and TAC were dramatically decreased during intestinal part of digestion. TPC decreased from 130.77 ± 6.19 to 29.77 ± 2.42 mg GA/g fruit and TAC decreased from 0.13 ± 0.01 to 0.009 ± 0.00 mg cyanidin 3-glucoside/g fruit in before digestion of ECB fruit juice and IN sample, respectively. The recovery of TPC and TAC was 45.98% and 11.70% in digested ECB extract in OUT, respectively (Table 3.6.). The reason might be degradation of PC throughout the intestinal digestion due to their low stability at alkaline conditions, pH values increased around 8 after gastric part (around pH 2.0) [188].

Table 3.6. The percent of recovery of TPC, TAC of ECB extract and the changes in pH values during digestion

Samples	% Recovery		pH values
	TPC	TAC	
ECB extract	100	100	2.89±0.07
PG	94.97±5.32	91.15±0.02	2.10±0.04
OUT	45.98±3.46	11.70±0.02	8.24±0.34
IN	22.84±2.20	6.81±0.01	8.03±0.30

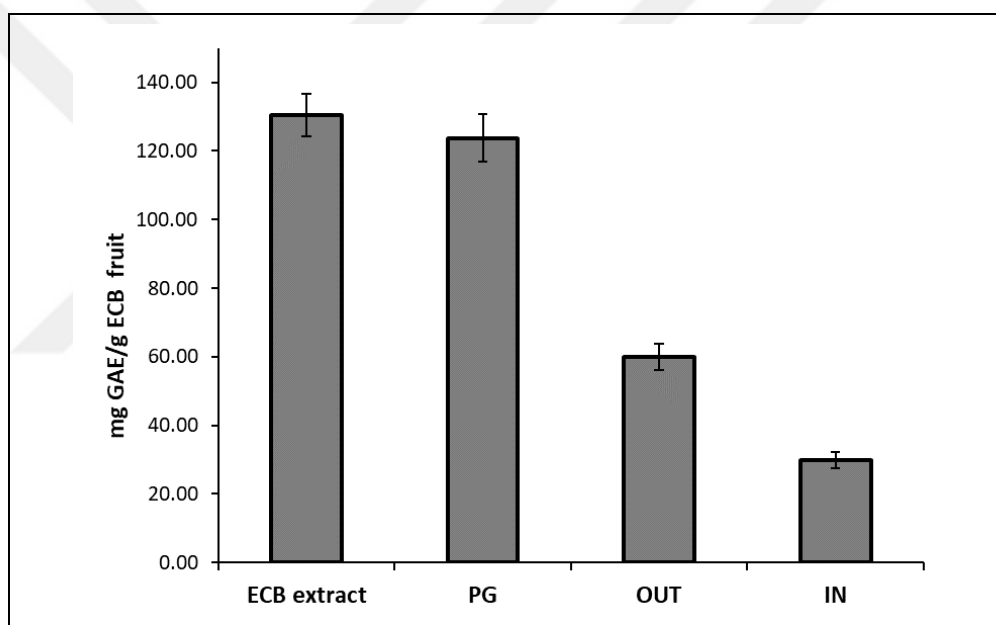


Figure 3.5. Total phenolic content of ECB fruit juice before and after simulated gastrointestinal digestion, expressed as mg gallic acid (GA)/g ECB fruit

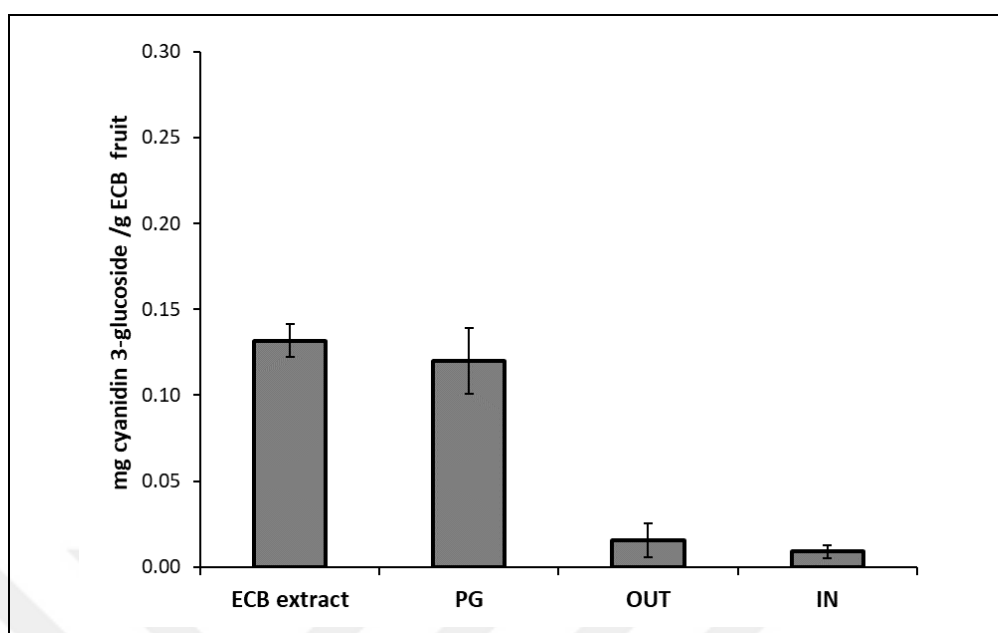


Figure 3.6. Total anthocyanin content of ECB fruit juice before and after simulated gastrointestinal digestion, expressed as mg cyanidin 3-glucoside/g ECB fruit

The pepsin digestion did not affect phenolic composition of ECB extract. Similarly, previously published studies also concluded that the pepsin digestion did not affect PC content dramatically in orange juice, strawberry, strawberry jam and pomegranate juice [189–191]. Anthocyanin content of ECB extract is highly stable when exposed to stomach conditions. Similarly, McDougall et al. (2005) concluded that red wine anthocyanins was stable under gastric conditions which is in agreement with our result [17]. Pérez-Vicente et al. (2002) documented a slight increase in anthocyanin content after gastric digestion of pomegranate juice due to pH value 2 after pepsin digestion, lower than the fresh juice (pH 3.8) and caused a rise in the flavylum cation concentrations in the solution [192]. Despite the stability of anthocyanin is affected by several factors such as pH, temperature, light, oxygen, presence of enzymes, proteins and so on, the stable of ECB anthocyanins would be due to low pH in stomach because low pH mainly contributes to the high stability of anthocyanins because chemical structure of a stable flavylum cation [193]. However, after the pancreatin bile salt digestion, anthocyanin content dramatically decreased and the bioaccessibility of anthocyanin was low. The loss of anthocyanin could be related to structural rearrangements due to pH changes in molecular structures [194]. The pH value of the environment increases to higher than 7.5 in small intestine digestion and therefore this pH value was the main factor in the irreversible breakdown of the anthocyanins [17]. In

literature, it has been reported that the bioaccessibility of anthocyanins were found to be lower than other flavonoids [195]. In accordance to our results, in the study of Ozkan et al. (2022) observed the decrease in anthocyanin content after digestion in ECB fruit puree, ECB juice mixed with water, ECB juice blended with bovine and almond milks [196].

In order to imitate the intestinal digestion process, samples obtained from after stomach digestion were then transferred to a mildly alkaline intestinal environment and mixed with a solution of pancreatin and bile salts. The use of sodium bicarbonate solution in dialysis tubing is done so that pancreatin digestion can take place while allowing for a gradual rise in pH. This procedure thereby simulates the passage of food from the stomach into the duodenum. Additionally, as a model for the epithelial barriers, dialysis tubing with a specified pore size enables separation of high and low molecular weight soluble phenolic chemicals [197]. After small intestine digestion, a clear decrease of the TPC and TAA content up to 27.03 mg GA/ml and 0.009 mg cyanidin 3-glucoside/ml were observed in bioavailable IN samples, respectively.

Gastrointestinal tract is similar to extractor because a part of PC present in food matrix can pass and become available for the uptake in the intestine [198]. The bioavailability index were calculated as the ratio of amount of phenolics in the IN (serum-available) sample to the phenolic content of nondigested ECB extract [199]. In our study, bioavailability index of ECB extract were found as 22.88% for phenolic compounds and 6.81% for anthocyanins. Barak et al. (2019) also calculated bioavailability of methanol and water extracted ECB fruit samples resulting that TPC of nondigested methanol and water extracts were 40.17 mg GA/g and 25.64 mg GA/g, respectively and after digestion, the IN samples were 14.77 mg GA/g and 15.23 mg GA/g. Bioavailability index of TPC was 36.77% and 59.40% in methanol and water ECB extracts, respectively which were higher than our result. The reason might be the initial TPC content of our extract was approximately 3-5 fold higher than the study of Barak et al. (2019). Similar bioavailability percentage results were found in literature with different PC-rich juice such as 29% of pomegranate juice [192], 21.75% of blueberry wine and 30.70% of black mulberry wine [160]. Bioavailability of anthocyanin content of ECB extract (6.81%) was lower than phenolic content after digestion. Similarly, in the study of Pérez-Vicente et al. (2002), only 2.4% anthocyanin in pomegranate juice was bioavailable. The 0.34% of mulberry anthocyanin [200], 3.7% of red wine anthocyanin [17], and blueberry [201] were recovered in serum available sample.

3.4. CONCLUSION

Our findings showed valuable biological activities of ECB fruit juice rich in PC that were considered the main responsible for obtained results. Considering the functional ingredient role of ECB juice, this study analyzed its role of them in preventing microbial growth and inhibition biofilm formation. Current results of the antimicrobial activity of ECB fruit juice have shown the promising potential to use probiotic foods applications. Moreover, our mutagenicity and antiproliferative studies indicated ECB juice is a safe and effective cancer prevention agent, showing ECB juice cytotoxic properties against AGS cells. Besides, bioavailability of PC present in ECB juice results would be helpful for the next study to understand the effect PC present in ECB fruit on gut microbiota.

In conclusion, it was possible to use ECB fruit juice in different industries with its rich source of PC for using as functional foods or dietary supplements.

4. EVALUATION OF SURVIVAL OF PROBIOTIC LAB AND *L. MONOCYTOGENES* IN ECB FRUIT JUICE ADDED WHEY AND MILK MEDIA

4.1.INTRODUCTION

In recent years, growing consumer interest toward the improving their state of health and well-being is the driving force for growth of functional food and beverage in worldwide market [202,203]. Among these products, the number of functional dairy products enriched with various probiotic strains, mainly *Lactobacillus* and *Bifidobacterium* species, has remarkably increased with the preference of health interested consumers [204–207]. As defined by Food and Agriculture Organization (FAO)/World Health Organization (WHO), “probiotics are live microorganisms when administered in adequate amounts, confer a health benefit on host” [208]. And they should be maintained the viability during the gastrointestinal passage, food processing, packaging and storage of the product until the consumptions time, thus showing beneficial health effect [209,210]. The number of viable probiotic microorganisms required at the time of consumption is also important in order to provide the target health benefit. When the food products supplemented with probiotics, it must contain at least 10^6 colony forming unit (CFU)/g as a minimum therapeutic level of viable probiotic microorganism throughout the shelf life for the labelling as a probiotic functional food [211]. Even there is no consensus on daily amount adequacy of probiotics, suggesting that a minimum of 10^8 - 10^9 CFU probiotic bacteria per gram food was recommended by some countries to cause positive physiological functionals for human health [209,212–214].

Apart from probiotics as well as prebiotics are increasingly searched in the literature. The scope of prebiotics was expanded by International Scientific Association for Probiotics and Prebiotics (ISAPP). The association defines as “a substrate that is selectively utilized by host microorganisms conferring a health benefit”. With this updated definition, phenolic compound (PC) has been also recognized as prebiotics in the consensus statement of ISAPP apart from currently dominate fructooligosaccharides/inulin and galactooligosaccharides [213]. Prebiotics shows beneficial effect on host modulating gut microbiota by selectively stimulating the growth or increasing the number of specific microorganisms thus they can

improve the health of host [215]. In many recent studies have shown that there is a mutual relation between PC and probiotic microorganisms. The PC are ability to modulate gut microbiota by exerting stimulation of beneficial microorganisms and inhibition of pathogenic microorganisms as a prebiotic [65,216–218].

Beverages are the most acceptable growing sector among functional food categories because of the ease for dissolution of functional components and convenience for consumers. Extensive research has focused on the potential use of probiotic, prebiotic as well as symbiotic activities in the production of functional beverages. Among all types of beverages, especially one-shot or daily dose probiotic beverages are gaining popularity for available nutrients, specific amino acids, PC, or other components in ready-to-consume drinks. Especially, dairy-based beverages are the most effective food matrix in which to deliver probiotics that are ideal media due to their essential nutrient content as well as the easiness of incorporating PC as a functional ingredient [219,220].

Dairy-based probiotic beverages generally consist of milk and whey-based probiotic beverages [221]. The addition of fruit juices is also an ideal substrate for growth/ survival of probiotic cultures and has a healthy source of PC, micronutrients, and fibers and good sensory properties. Because of the presence of biological and functional valuable proteins in whey, a by-product of the cheese industry, utilization of whey in probiotic dairy beverages without negatively affecting acceptability and functionality making better use of this by product is gaining interest. The suitability of different probiotic cultures in fruit juice added whey environment for the development of probiotic beverage have been evaluated in different studies [222,223]. There has been an increased interest in the development of whey or milk-based beverages blend using optimum concentration of various fruit juices. Previous studies showed good survival of various probiotic lactic acid bacteria (LAB) cultures (*L. acidophilus*, *L. bulgaricus*, *Streptococcus. thermophilus*, *L. rhamnosus* and *B. lactis*) incorporated into dairy beverages [203,220,224,225].

Besides the health benefits of PC, they are closely associated with the organoleptic properties of foods. The impact of PC on color, flavor, astringency, and bitter sensation may affect positively or negatively sensorial characteristics of food products [226]. Therefore, the amount/concentration of incorporated PC-rich fruit juice/extract into a functional beverage is an significant issue to obtain the best condition due to directly responsible for sensorial attributes. Moreover, PC-rich extracts exhibit differential effects on pathogenic bacteria and

probiotic LAB [167–169,227], therefore, fruit juice that is high in PC may offer an alternative strategy for assuring beverage safety. In present study, the percentage of ECB fruit juice added to beverages was determined by both the fact that ECB fruit has a bitter taste and the antimicrobial activity of ECB fruit juice selectively inhibited the growth of foodborne pathogens and spoilage yeasts, however not probiotics and LAB. The high dose of ECB fruit juice inhibited all microorganisms at a different level, however, the inhibition zone and bactericidal effect (MBC values) of ECB fruit juice on beneficial microorganisms were lower than the others (explained in detail section 3). At the same time, our previous study proved that the growth of LAB and probiotic cultures were stimulated when their growth media contained 5-10% of freshly squeezed ECB fruit juice by analyzing the growth kinetic performance [228].

In this study, impact of ECB fruit juice on the viability and growth performance of probiotic cultures *L. rhamnosus* Lr-32 and *B. lactis* BI-04 co-fermented with yogurt cultures, *Streptococcus thermophilus* and *L. bulgaricus* were evaluated in presence of different combinations of inulin and ECB fruit juice added whey-based environments. Then, since ECB fruit juice was found to be strongly inhibitory to *Listeria monocytogenes* (Section 3), antimicrobial effect of ECB fruit juice was investigated in milk with probiotics cultures by challenging the product with pathogenic *L. monocytogenes* ATCC 15313.

4.2.MATERIALS AND METHODS

4.2.1. Microbial Cultures

Freeze-dried probiotic (*Lactobacillus rhamnosus* Lr-32, *Bifidobacterium lactis* BI-04, *L. plantarum* Lp-115 and *Streptococcus thermophilus* St-21) and yogurt cultures Yo-Mix 528 (*Streptococcus thermophilus* and *L. bulgaricus*) were kindly supplied from Danisco (USA). All cultures were stored in a freezer at -20°C until they were used in the formulations.

Listeria monocytogenes ATCC 15313 was used to contaminate ECB fruit juice added milk-based environment.

4.2.2. Materials

High protein whey powder manufactured from sweet whey by using spray-drying method was kindly provided from Maybi (Türkiye). The whey powder, a by-product of cow's, sheep's, and goat's cheese production, composed of 12.33% protein, 77.94% lactose, 8% ash, 0.33% fat, and 2.63% salt. Whey powder was used as reconstituted in water during experiment. The inulin and glucose were supplied from Beneo Orafiti (Belgium) and Fluka (Germany), respectively. The supplied ECB fruits and fruit juice preparation were previously explained in Section 3.2.1. The UHT cow's milk (3.3% milk fat, 3% protein, 4.5% sugar) was used in the milk-based environment study was purchased from supermarket (Pınar, Türkiye).

4.2.3. Preparation of Whey-Based Beverages

Reconstituted whey powder based beverages with different combinations were prepared as shown in Table 4.1 with their codes to evaluate the growth and viability of all cultures. In this study, the whey content (6% w/v) was determined according to Chr. Hansen "My Whey" one-shot whey-based probiotic drink [229]. Preliminary sensory evaluations were done to formulate the beverage. The 10% (w/w) of freshly squeezed ECB fruit juice was used. Glucose (2% w/v) was added to obtain a sweet taste to mask the astringent taste of ECB fruit and also helped the growth of cultures as an energy and carbon source. Inulin (1% w/v) was used to evaluate the prebiotic effect on probiotic cultures by comparing it with ECB fruit juice.

In the preparation of beverages, firstly, whey powder was reconstituted in distilled water and then, glucose, and inulin were added depending on the beverages and pasteurized at 90 °C for 5 min in a water bath. Then, mixtures were cooled at 42°C. Both probiotics were separately added into environments. Probiotic *L. rhamnosus* Lr-32 (1%) (10^{10} CFU/ml) and yogurt culture (0.02%) were added into all environments. *B. lactis* (1%) (10^9 CFU/ml) cultures and yogurt culture (0.02%) were added into environments. ECB fruit juice (10% v/v) was added into determined formulations. All whey-based formulations were fermented at 37°C and stopped when pH values were reached to 4.5. After fermentation, the beverages

were cooled 4°C and stored for 7 days for further analysis. the appearance of beverages was presented in Figure 4.1.

Table 4.1. The ingredients of different combinations of whey-based beverages

Samples		Ingredients present in beverages
Lr-32 + YoMix	WG	Lr-32 and Yo-Mix cultures + Whey Powder (6% w/v) + Glucose (2% w/v)+ water
	WGI	Lr-32 and Yo-Mix cultures + Whey Powder (6% w/v) + Glucose (2% w/v)+ Inulin (1% w/v) + water
	WGECB	Lr-32 and Yo-Mix cultures + Whey Powder (6% w/v) + Glucose (2% w/v)+ ECB fruit juice (10% v/v) + water
	WGIECB	Lr-32 and Yo-Mix cultures + Whey Powder (6% w/v) + Glucose (2% w/v)+ Inulin (1% w/v) + ECB fruit juice (10% v/v) +water
BI-04 + YoMix	WG	BI-04 and Yo-Mix cultures + Whey Powder (6% w/v) + Glucose (2% w/v)+ water
	WGI	BI-04 and Yo-Mix cultures + Whey Powder (6% w/v) + Glucose (2% w/v)+ Inulin (1% w/v) + water
	WGECB	BI-04 and Yo-Mix cultures + Whey Powder (6% w/v) + Glucose (2% w/v)+ ECB fruit juice (10% v/v) + water
	WGIECB	BI-04 and Yo-Mix cultures + Whey Powder (6% w/v) + Glucose (2% w/v)+ Inulin (1% w/v) + ECB fruit juice (10% v/v) +water

Lr-32 *Lactobacillus rhamnosus*, BI-04 *Bifidobacterium lactis*, Yo-Mix Yogurt cultures W:whey, G:glucose, I:inulin, ECB:European cranberrybush fruit juice.

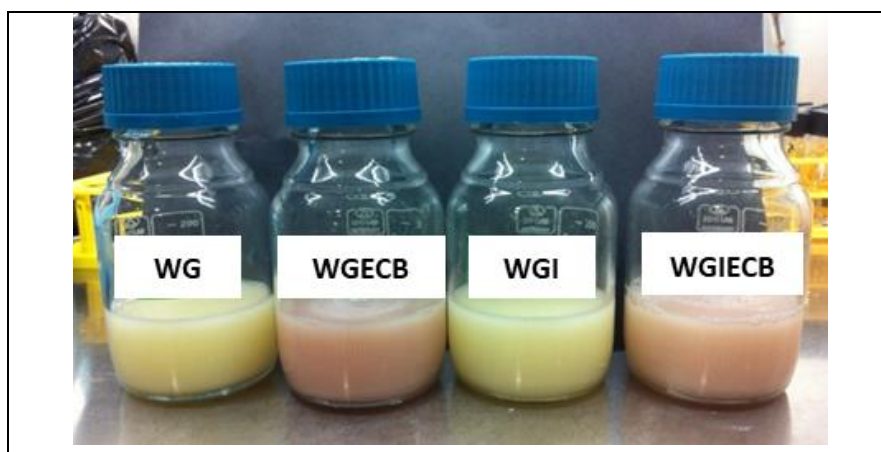


Figure 4.1. The appearance of formulated whey-based beverages

4.2.3.1. Counts of Probiotic and Yogurt Cultures in Whey-Based Beverages

The variation in the microbial population (log CFU/ml) were analyzed during fermentation (0, 1, and 2 h) and storage time (1 and 7 day) period in whey-based beverages. The growth media of probiotics, MRS, were adjusted as selective by changing pH to differentiate the microorganisms because both *L. rhamnosus* and *L. bulgaricus* are ability to grow in MRS media. For the enumeration of *B. lactis*, the MRS medium was supplemented with L-cysteine (Merck, Germany) which is a reducing agent. The pH adjusted MRS (pH 5.4) and cysteine supplemented MRS (contain 0.05% cysteine) agar media were used to count the number probiotic cultures of *L. rhamnosus* and *B. lactis* after incubation at 37°C for 48 h anaerobic environment. The MRS (pH 6.5) and M17 agar media were used to count the number of yogurt cultures of *L. bulgaricus* and *S. thermophilus* after incubation aerobically at 37°C for 48 h.

4.2.3.2. Measurement of pH and Sugar Consumption in Whey-Based Beverages

The change in the pH and the sugar consumption during fermentation (0, 1, and 2 h) and storage time (1 and 7 day) period were analyzed in whey-based beverages. The pH values of all samples were measured at both during fermentation and storage time with a pH meter (Hanna Instruments, Italy).

The sugar consumption of probiotic and yogurt cultures in different combinations of whey-based beverages during fermentation and storage time period were analyzed by using HPLC-Refractive Index (RI) detector [230]. A HyperREZ XP Carbohydrate Ca⁺⁺ column (300 x 7.7mm, 8µm; Thermo Scientific, England) was operated at 50°C while the detector

temperature was kept at 35°C. 0.005 M H₂SO₄ (Sigma- Aldrich, Germany) was used as the mobile phase, following at a rate of 0.6 ml/min. Before injection, all samples were filtered using a 0.45 µm RC filter (Minisart Sartorius, Germany) before injection and 10 µl volume sample injected for each run. Glucose, fructose and lactose (Supelco, USA) were used as external standards to detect sugar compounds present in samples. Six different concentrations were prepared from the standard to draw a calibration curve to calculate the amount of each sugar. Results were expressed as mg sugar per ml.

4.2.4. Preparation of milk-based environment for the evaluation of growth inhibition of *L. monocytogenes*

Firstly, growth and viability of probiotic *L. plantarum* Lp-115, *S. thermophilus* St-21 and pathogenic *L. monocytogenes* ATCC 15313 were monitored by assessing optical density at 600 nm at 37°C for 48 h with 96-well microplate reader in MRS for *L. plantarum*, M17 for *S. thermophilus*, and Brain Heart Infusion (BHI) broth for *L. monocytogenes* supplemented with different concentrations of freshly squeezed ECB fruit juice (100, 50, 25, 12.5, 6.25, 3.13, 0.78, and 0.39% v/v) to ensure optimum ECB fruit juice concentration for functional beverage composition.

UHT whole milk, ECB fruit juice (10% v/v), and glucose (2% w/v) were mixed and then probiotic *L. plantarum* Lp-115 and *S. thermophilus* St-21 cultures (0.1%, 10⁹ CFU/ml) were added into mixture (Table 4.2). While probiotics could grow in determined ECB fruit juice in growth media, before fermentation, pH values of milk samples were adjusted to 6.0 by using 0.1N sterile NaOH and HCl solutions due to the low pH (2.90) of ECB fruit juice. Then, *L. monocytogenes* (10⁵ CFU/ml) were contaminated both in presence and absence of ECB fruit juice added beverages. Samples were held at 37°C for the fermentation until the pH value of the mixtures reached to 4.5, then they were stored at 4°C for 14 days to evaluate the changes during storage period. The appearance of milk sample was presented in Figure 4.2.

Table 4.2. The ingredient of milk-based environments

Samples	Ingredients in environments
No ECB added	Probiotic <i>L. plantarum</i> Lp-115 and <i>S. thermophilus</i> St-21 cultures + <i>L. monocytogenes</i> + milk + Glucose (2% w/v)
ECB added	Probiotic <i>L. plantarum</i> Lp-115 and <i>S. thermophilus</i> St-21 cultures + <i>L. monocytogenes</i> + milk + Glucose (2% w/v) + ECB fruit juice (10% v/v)

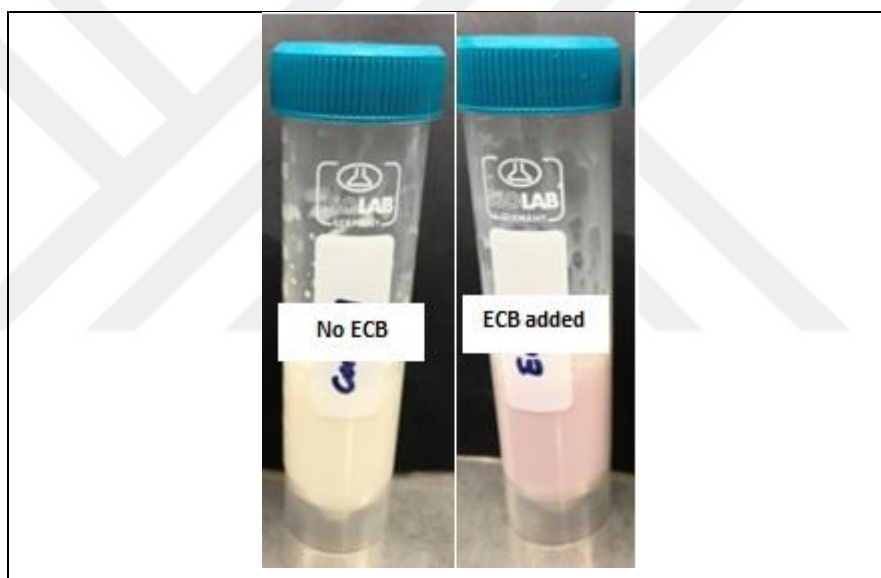


Figure 4.2. The appearance of control and ECB fruit juice added milk environments

4.2.4.1. Survival of Probiotic Cultures and Growth Inhibition of *L. monocytogenes*

The change in the numbers (CFU/ml) of *L. plantarum* Lp-115, *S. thermophilus* St-21 and *L. monocytogenes* ATCC 15313, and pH values were monitored during fermentation (0, 1, 2, and 3 h) and storage period (1, 7, and 14 days). The number of microorganisms were determined using selective growth media MRS, M17 and Oxford agar and incubation at 37°C for 24 h for *L. plantarum* Lp-115, *S. thermophilus* St-21 and *L. monocytogenes* ATCC 15313, respectively.

4.3. RESULTS AND DISCUSSION

4.3.1. Evaluation of the Growth and Viability of Probiotic and Yogurt Cultures in ECB Fruit Juice added Whey-Based Beverages

The initial pH value of ECB-added beverages was lower than others due to low pH of fruit juice (2.90). Survival of probiotic cultures is challenging in presence of fruit juice matrix because of the adverse effect of pH and organic acid content on their viability [231,232]. While PC present in berries can exhibit antimicrobial activity on probiotic microorganisms dose-dependent, these compounds may contribute to the survival of probiotics in food products and exert a prebiotic-like effect on gut microbiota [233]. As we analyzed in Section 3, high concentrations of ECB fruit juice showed inhibitory effect against not only pathogenic and spoilage microorganisms, but also inhibiting the growth of probiotic cultures slightly. However, at low concentrations of ECB fruit juice still inhibited pathogens and did not exert inhibition on probiotic cultures. In literature, both probiotics *L. rhamnosus* and *B. lactis* were co-culture with *S. thermophilus* showing synergistic effect between microorganisms due to mutual interactions [205,206]. In addition, when they were used as co-culture, fermentation time decreased compared to mono-cultures. In our study, same probiotic cultures were used and co-fermented with yogurt cultures. Fermentation was stopped after 2 hours which is very short time. The reason of short fermentation might be the use of yogurt cultures, Yo-mix 528, having fast fermentation properties.

After 2 h fermentation, the pH values of *L. rhamnosus* added WG, WGI, WGECEB and WGIECEB were 4.20, 4.29, 4.19 and 4.28, respectively and the pH values of *B. lactis* added beverages were 4.43, 4.40, 4.18 and 4.19, respectively (Table 4.3.). The addition of 4% inulin was also accelerated the fermentation time apart from co-culture microorganism in the study of de Souza Oliveira et al (2012a and 2012b) [205,206]. However, we did not observe the effect of inulin on fermentation time by comparing pH values of with and without inulin added beverages. The reason may be the use of inulin was low in our study. While the pH values decreased to 3.5 during storage in the refrigerator after 7 days in *L. rhamnosus* added to all types of beverages, there was no significant change in *B. lactis* added beverages and the pH values changed between 4.29-4.52 after 7 days of storage in refrigerator (Table 4.3.). The pH value measurements of *B. lactis* added beverages decreased

to about 3.70 in all types of beverages at the end of 14-day storage (data not shown). Decrease in pH value of *L. rhamnosus* added samples was suggesting that the occurrence of post-acidification during storage which is normally found in fermented dairy products because of the active metabolism of added cultures. Although the pH reduction in the beverages did not cause the reduction of viability of microorganisms, storage was stopped after 7 days due to the acidity from a sensory point of view. Generally, shelf life of whey-based beverages are maximum of 21-28 days, so the pH values of beverages could be adjusted prior to fermentation.

Regardless of the added ingredients (inulin and ECB fruit juice) in whey-based beverages, the number of *L. rhamnosus* was between 9.13 to 9.63 log CFU/ml and the number of *B. lactis* was between 10.0 to 10.45 log CFU/ml (Figure 4.3.). Addition of inulin and ECB fruit juice did not interfere or stimulate number of probiotic cultures in these environments. Both probiotic cultures showed good stability that remained the same number during both fermentation and storage period with respect to the number of counts. The number of probiotic cultures in all beverages were presented between 10^9 - 10^{10} CFU/ml, showing a probiotic level that is both high enough to benefit consumers and high enough to make up for any probable loss during the digestive process [234]. Apart from probiotic cultures, the number of yogurt cultures (*L. bulgaricus* and *S. thermophilus*) were also stayed same number (10^9 CFU/ml) during fermentation and storage in presence of *L. rhamnosus* and *B. lactis* added beverages (data not shown).

Inulin was used as a prebiotic agent to promote the growth and viability of probiotic cultures in whey-based beverages, but the addition of 1% of inulin did not promote growth of probiotic *L. rhamnosus* and *B. lactis* and also yogurt cultures. Similar to our result, 1% of inulin addition into reconstituted whey (6%) did not affect on the number of probiotic cultures (*L. acidophilus* La-5, *B. bifidum* Bb-12 and *L. casei* Lc-01) during fermentation and storage time [235]. The growth of *S. thermophilus* was not significantly impacted by the addition of inulin (4%) in the study of de Souza Oliveira et al. (2012) when it was fermented by *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus* and *B. lactis*, either in pure cultures or in binary co-cultures with *S. thermophilus* or in a cocktail. Among *Lactobacillus* strains, apart from *L. bulgaricus*, inulin stimulated growth of other LAB and number of *B. lactis* significantly grew from 7.5 to 9.1 log CFU/ml after 1 day and 7 days storage at refrigerator temperature [236]. The reason of different results compared to our study might be the amount

of inulin was low in our study and also the initial count of all culture was 10^6 CFU/ml which was lowering than our study. However, comparable to our study, in the study of Allgeyer et. al (2010), the growth pattern of *B. animalis* Bb-12 and *L. acidophilus* La-5 which had 10^8 CFU/ml as the initial number of cultures, was not affected by addition of inulin (1.24 and 2.48% used) in yoghurt base drink [237]. In another study, the growth promoting effects of three different prebiotics mixture (inulin, polydextrose and oligofructose) were examined in fermented whey beverages on *B. animalis* Bb-12, *L. acidophilus* La-5 and *S. thermophilus*. It was found that the addition of 1% of each prebiotic had no effect the growth of *B. animalis* Bb-12, but had a positive effect on *L. acidophilus* La-5 and *S. thermophilus* growth [238]. Variable results have been observed when incorporating inulin with probiotic beverages. The prebiotic substance's nature and the characteristics of food matrix may play a role in the stimulatory impact of prebiotics.

Table 4.3. Changes in pH values of *L. rhamnosus* Lr-32 and *B. lactis* BI-04 probiotics co-fermented with yogurt cultures in different combinations of whey-based beverages during fermentation and storage time

Probiotics co-fermented with yogurt cultures		Time (h)				
		Fermentation			Storage	
		0	1	2	24	168
Lr-32	WG	5.90±0.75	4.79±0.28	4.20±0.14	3.97±0.21	3.50±0.30
	WGI	6.18±0.42	4.92±0.32	4.29±0.08	3.99±0.17	3.46±0.37
	WGECB	4.90±0.35	4.51±0.27	4.19±0.18	3.99±0.22	3.47±0.40
	WGIECB	5.13±0.05	4.63±0.15	4.28±0.12	4.02±0.18	3.52±0.33
BI-04	WG	5.83±0.25	4.72±0.17	4.43±0.12	4.47±0.25	4.52±0.03
	WGI	5.84±0.08	4.71±0.08	4.40±0.06	4.48±0.22	4.51±0.04
	WGECB	5.04±0.27	4.41±0.16	4.18±0.15	4.29±0.29	4.31±0.06
	WGIECB	5.04±0.23	4.42±0.04	4.19±0.01	4.29±0.23	4.29±0.07

As expected with our previous study results [12], the addition of 10% ECB juice into whey-based beverages did not show inhibitory effect on neither probiotic nor yogurt cultures suggested that the potential of ECB fruit juice in whey beverage as appropriate food matrix for probiotic microorganisms. In literature, there are many different studies on the

incorporation of whey into the development of PC-rich fruits. For example, pineapple, reservoir of gallic acid, chlorogenic acid and ferulic acid, blended with whey for developing probiotic whey-pineapple beverage [239]. Probiotic *L. acidophilus* could preserve the number during shelf life at refrigeration temperature. Number of *L. acidophilus* and *B. longum* increased in anthocyanin source of acai supplemented whey-based beverage [240]. A similar effect was demonstrated in dos Santos et al (2017) study, *L. rhamnosus* grew more quickly when PC source grape pomace extract was added to the goat milk used to make probiotic fermented dairy products.

Whey is one of the potential substrated for the production of various whey-based probiotic beverages due to the presence of critical nutrients such as lactose and proteins, for the growth of bacteria. According to HPLC-RI sugar analysis results, it was stated that microorganisms primarily used glucose and fructose. The sugar content of ECB fruit juice was determined in our previous study found that glucose (28.59 mg/ml) and fructose (38.74 mg/ml) are sugar sources [12]. Sugars are the primary carbon and energy sources for microorganisms that are grown in formulated beverages. LAB generally prefer to consume glucose but can also metabolize other hexoses strain dependent manner [241]. Lactose was the other sugar source coming from whey, but microorganisms did not metabolize in this process (Table 4.4.). Due to lack of β -galactosidase enzyme, *L. rhamnosus* does not have ability to ferment lactose [242]. For *B. lactis*, it can degrade lactose together with increase of added prebiotic concentration in milk [243], however, we did not observe difference in lactose consumption with or without inulin added beverages in our study. Even microorganisms prefer to consume firstly glucose, the amount of fructose was also decrease which were an additional carbon and energy source for microorganisms. Presence of glucose and fructose might be priority for sugar consumption rather than lactose in the same environment. The source of fructose came from both inulin and ECB fruit juice, therefore fructose did not detected in WG samples. The amount of fructose was higher in inulin added beverages (WGI and WGIECB) compared to WGE CB (Table 4.4.). The amount fructose decreased during fermentation and storage resulting microorganism were able to consume inulin. For example, the amount of fructose in WGI decreased from 6.26 to 5.43 mg/ml by *L. rhamnosus* co-fermented with yogurt cultures and decreased from 7.48 to 5.24 mg/ml by *B. lactis* co-fermented with yogurt cultures.

Table 4.4. Sugar (lactose, glucose, fructose) consumption (mg/ml) of *L. rhamnosus* Lr-32 and *B. lactis* BI-04 probiotics co-fermented with yogurt cultures in different combinations of whey-based beverages

Samples	Time	Sugar consumption of probiotics co-fermented with yogurt cultures (mg/ml)					
		Lr-32			BI-04		
		Lactose	Glucose	Fructose	Lactose	Glucose	Fructose
WG	0	45.54±2.21	23.32±1.41		42.54±1.54	22.39±0.96	
	1	46.66±1.33	22.02±1.12		42.09±0.87	20.74±0.62	
	2	46.48±2.02	19.75±1.35		41.49±0.30	19.72±0.32	
	24	48.43±2.46	19.60±1.48		42.44±1.12	19.91±0.66	
	168	49.12±0.20	16.98±0.58		45.20±3.93	18.44±2.16	
WGI	0	49.78±1.18	28.77±2.76	6.26±1.28	43.18±0.13	25.96±1.86	7.48±4.32
	1	48.32±1.48	29.27±1.87	6.63±0.81	43.54±0.44	25.29±2.19	5.59±2.20
	2	47.45±1.67	27.36±2.09	5.67±0.75	42.78±0.97	23.87±1.20	5.25±1.76
	24	48.65±0.81	26.60±0.98	5.35±0.14	41.05±3.28	22.82±0.11	5.03±1.55
	168	47.82±1.81	23.21±0.85	4.53±0.26	46.19±0.52	22.11±6.64	5.24±3.55
WGECB	0	47.46±1.51	24.49±0.30	2.22±0.76	40.32±1.01	21.76±0.64	2.25±0.16
	1	45.41±1.21	21.77±0.78	2.19±0.70	41.93±0.27	21.09±0.41	1.99±0.27
	2	46.75±0.28	20.41±0.11	2.14±0.70	42.30±0.26	20.50±0.08	1.88±0.05
	24	46.00±0.62	14.55±8.52	2.05±0.67	39.42±4.57	19.03±2.39	1.90±0.37
	168	46.68±0.33	16.27±0.53	1.76±0.48	42.06±1.44	16.76±3.05	2.01±0.23
WGIECB	0	45.59±0.50	27.60±1.18	6.86±0.98	44.12±0.42	27.02±1.40	7.29±1.82
	1	46.00±1.19	26.60±0.25	6.14±0.69	40.43±4.32	23.87±1.42	6.33±1.03
	2	45.97±0.23	25.13±0.59	5.49±0.60	43.85±0.70	25.10±1.00	6.91±1.54
	24	46.30±0.90	24.23±1.43	5.46±0.81	39.93±1.27	22.37±0.96	5.82±2.12
	168	46.69±1.16	21.96±1.92	4.88±0.87	42.79±0.87	21.57±0.27	6.62±1.71

Interestingly, the amount fructose was not higher in presence of both inulin and ECB juice (WGIECB) compared to WGI in the beginning of fermentation. At the end of storage period, remained fructose in *L. rhamnosus* fermented samples were higher than *B. lactis* fermented samples. Therefore, the reason might be the pH values of *B. lactis* samples were higher than *L. rhamnosus* at the end of 7 days storage. Our findings were consistent with the literature; fructose released from inulin's partial hydrolysis and subsequent metabolization as an extra carbon and energy source enhanced the metabolism of LABs [244]. Oliveira et al. (2011)

found the stimulation of inulin on milk fermented by pure cultures of *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus* and *B. lactis*, binary co-cultures with *S. thermophilus* or in a combination of all of them. Inulin stimulated both metabolism and biomass growth of *B. lactis* co-culture with *S. thermophilus* confirmed bifidogenic impact of inulin [205]. In our study, although we did not observe the stimulation of inulin on the number of *B. lactis*, *B. lactis* released fructose by partial hydrolysis of inulin and used it in its metabolism as an additional carbon and energy source.

As a result of these findings showed that probiotic bacteria, *L. rhamnosus* and *B. lactis* with yogurt cultures could maintain their viability in the presence of ECB fruit juice and inulin added whey-based beverages. Although these findings indicated that they have the potential to be an useful beverage and that producing one by using probiotic cultures to ferment whey could be innovative alternative for using whey, however, there are some problems that occur in whey-based beverages. Fermented whey beverages have low viscosity and mild flavor compared to fermented milk products. The reason might be using a low percentage of whey. Whey has an astringent taste, therefore using high content whey did not recommend. In addition, after pasteurization step, heat-triggered sedimentation occurs and this is the major challenges of whey-based beverages. For these reason, we continued to our study with milk.

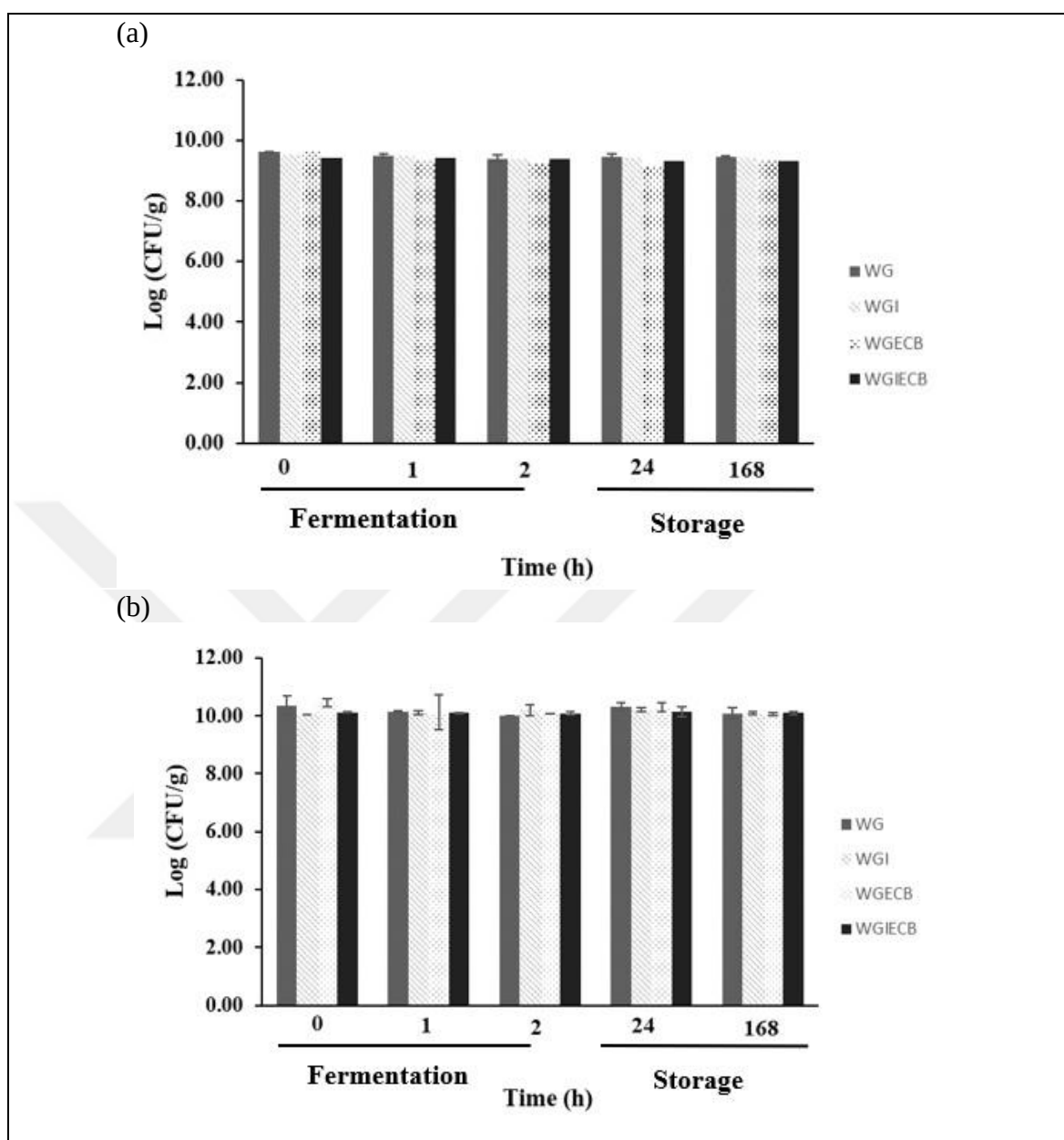


Figure 4.3. The number (log CFU/ml) of *L. rhamnosus* Lr-32 (a) and *B. lactis* BI-04 (b) in different combinations of whey-based beverages during fermentation and storage time period. Error bars are standard deviation with respect the mean values of repeated analysis of fermentations

4.3.2. Evaluation of Growth Inhibition of *L. monocytogenes* in ECB Fruit Juice Added Milk

As mentioned previously, ECB fruit contain high amount of PC, known as their beneficial health effects such as antioxidant and antimicrobial activities. PC may not only act as

antimicrobial agent for pathogens, but also lead to tolerate or promote of growth of probiotic cultures depend on structure and concentration of PC and microbial strain that mention in previous Section 3. In literature, mutual and synergistic interactions were observed between probiotic *L. rhamnosus* and *B. lactis* cultures and *S. thermophilus* in fermented milk [205]. In a previous whey-based beverage study, we used these strains, but a different probiotic strain was used in this study.

The results of optical density values of probiotic *L. plantarum* Lp-115 and *S. thermophilus* St-21 cultures obtained from microplate reader in growth medium added various concentration of ECB fruit juice was shown in Figure 4.4. The concentration of 100% and 50% of freshly squeezed ECB fruit juice were inhibited growth of both probiotic cultures. When the concentration decreased to 25% ECB fruit juice, both probiotic cultures could growth in the presence of 25% ECB fruit juice added growth media. Optical density values of probiotic *L. plantarum* Lp-115 showed that growth of *L. plantarum* Lp-115 in presence of 12.5% and 6.25% of ECB fruit juice was higher than control which is no ECB fruit juice added growth medium. However, the growth pattern of probiotic *S. thermophilus* St-21 was different than the *L. plantarum* Lp-115. The highest growth was observed in control which is no ECB fruit juice added media, but the maximum growth of *S. thermophilus* St-21 was observed at 25% and 12.5% of ECB fruit juice among the ECB juice concentrations.

On the other hand, optical density values of *L. monocytogenes* were decreased when the ECB fruit juice concentration increases in the growth medium. Moreover, there was no growth observed at 100-50-25, and 12.5% of ECB fruit juice added growth media (Figure 4.5.). When the concentration of ECB fruit juice d to 6.25%, *L. monocytogenes* started to grow but the the period of lag phase, adaptation time for microorganism in order to initiate exponential growth in the environment, was significantly longer than the control. In presence of 6.25% ECB fruit juice, absorbance values of *L. monocytogenes* increased after 14 h as seen Figure 4.5.

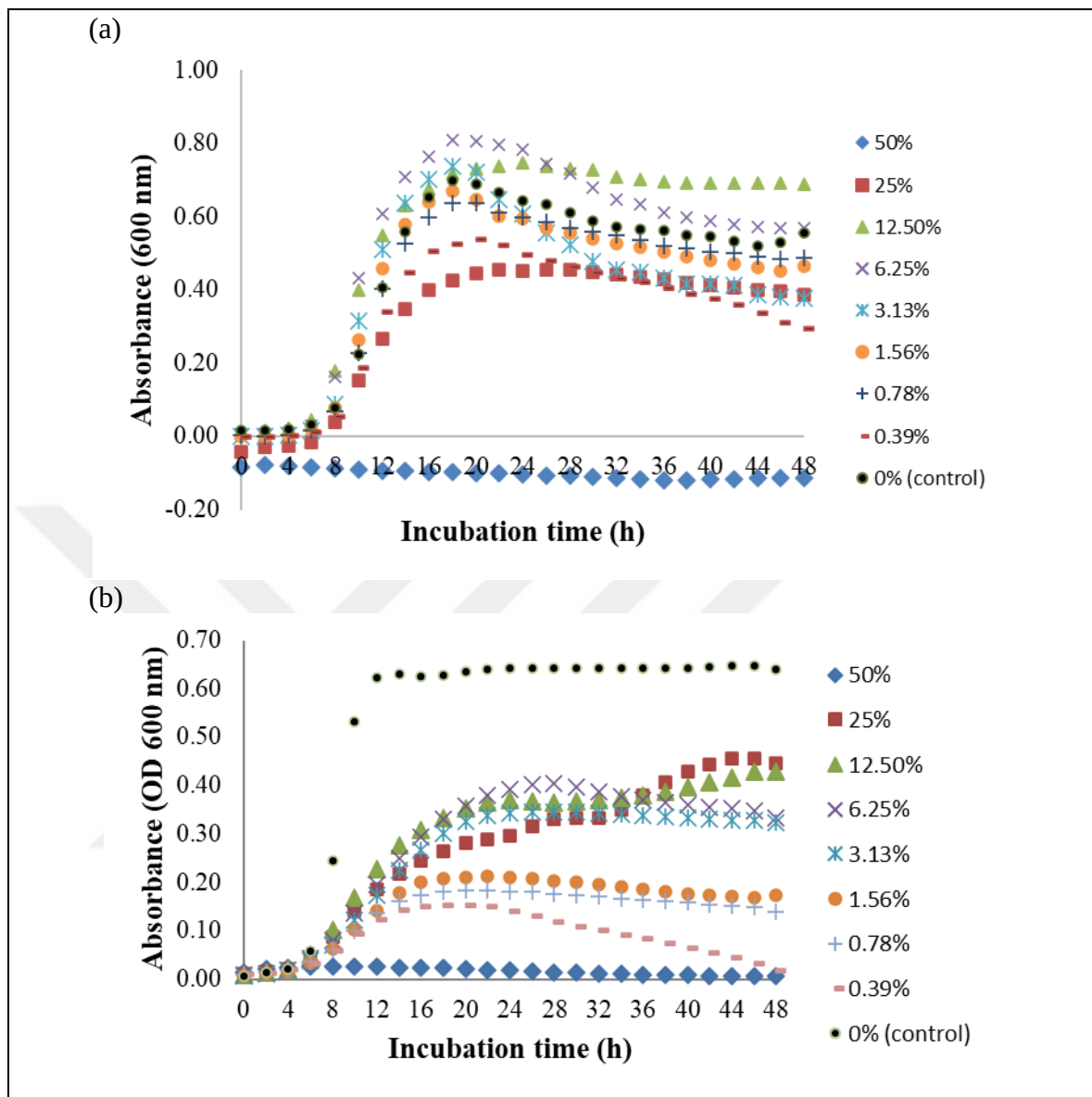


Figure 4.4. Growth curve of (a) *L. plantarum* Lp-115, (b) *S. thermophilus* St-21 in growth medium containing different concentrations (100-50-25-12.5-6.25-3.13-1.56-0.78-0.39% v/v) of ECB fruit juice at 37°C during 48 h of incubation

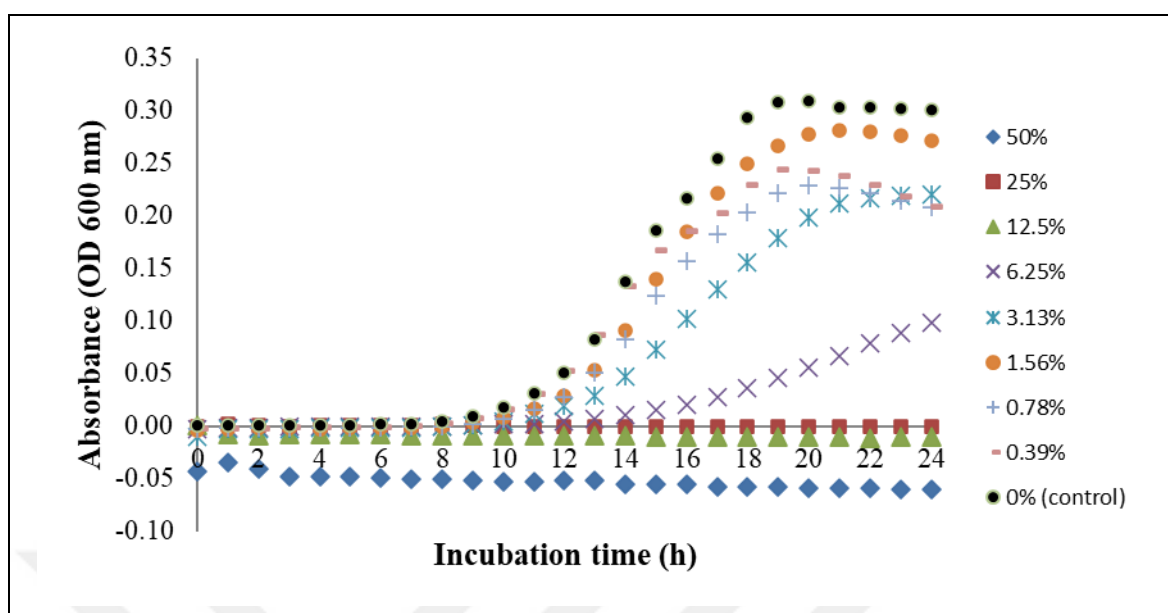


Figure 4.5. Growth of *L. monocytogenes* in the presence of different (100-50-25-12.5-6.25-3.13-1.56-0.78-0.39% v/v) ECB fruit juice concentrations added media and in the absence of ECB fruit juice at 37°C during 24 h of incubation

Hence, the addition of 10% ECB fruit juice was found as optimum concentration according to absorbance values of each probiotic and pathogenic bacteria for functional beverage composition. The reason for the selection of this ECB juice concentration is that at this concentration both probiotic cultures were able to grow and also inhibit the growth of *L. monocytogenes*, and also the added concentration was more desirable for consumer based on sensorial attributes of ECB fruit juice.

The changes in pH and number of probiotic cultures were analyzed during fermentation and storage time period. The numbers of probiotic cultures (10^9 CFU/ml) in presence and absence of ECB fruit juice added samples were similar and did not change significantly during fermentation and even after 14 days of storage. On the other hand, pH values of the samples decreased around 4.27 to 4.17 at the end of storage in presence and absence of ECB juice (Figure 4.6.). Storage time period could be extended after 14 days.

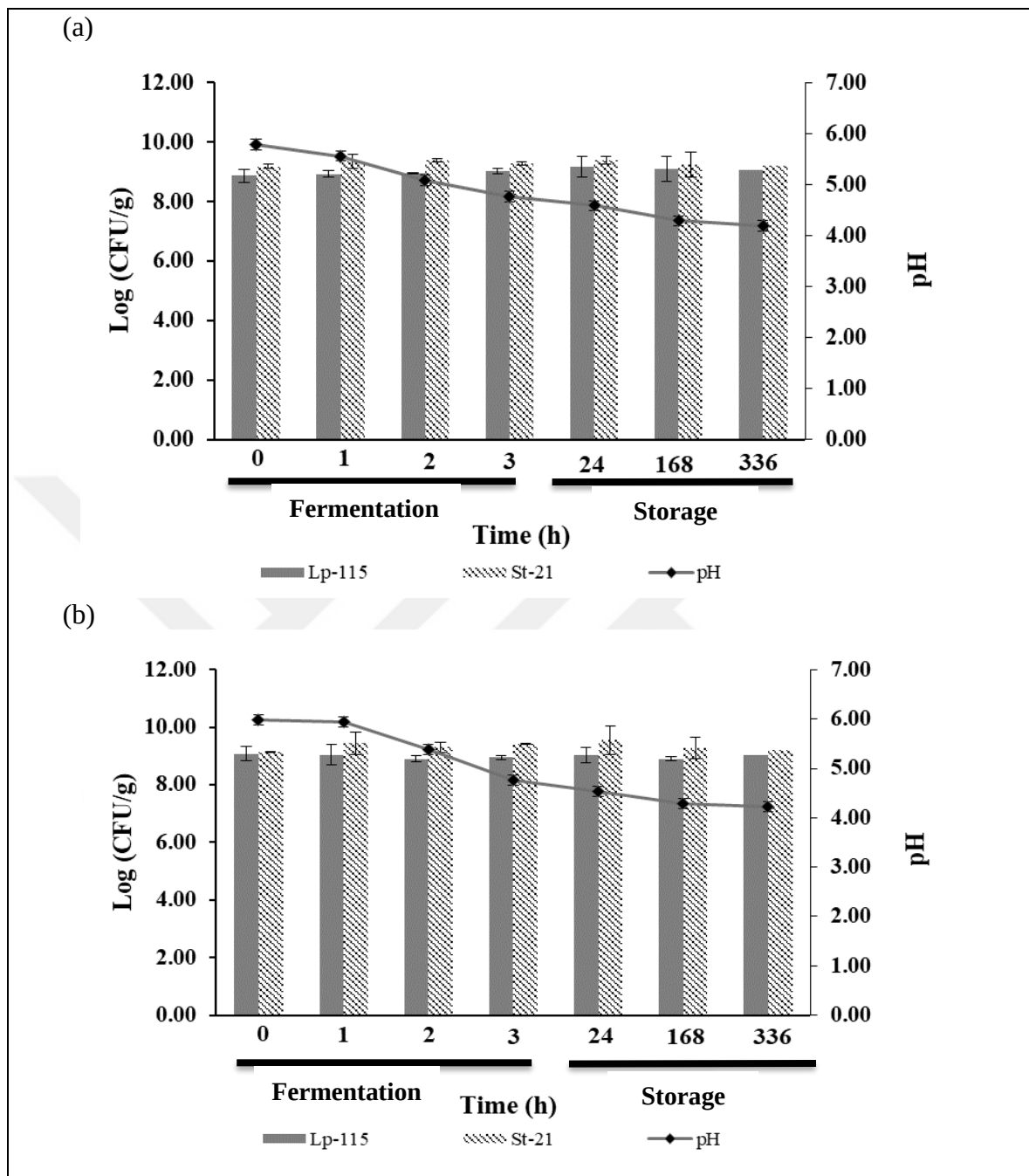


Figure 4.6. The change in pH values and the number of *L. plantarum* Lp-115, *S. thermophilus* St-21 (log CFU/ml) containing (a) and without (b) 10% of ECB fruit juice during fermentation and storage time period. Error bars are standard deviation with respect to the mean values of repeated analysis of fermentations

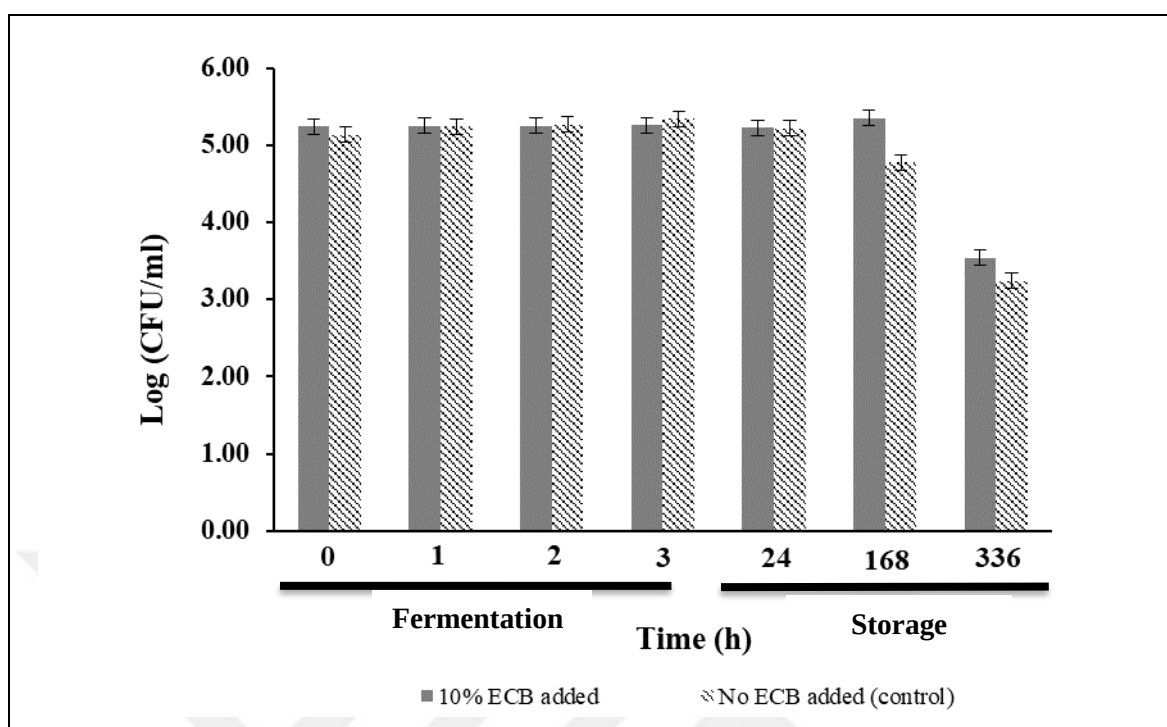


Figure 4.7. The change in the number of *L. monocytogenes* (log CFU/ml) in the presence and absence of ECB juice during fermentation and storage time period. Error bars are standard deviation with respect the mean values of repeated analysis

Even though presence of 10% ECB fruit juice in growth medium inhibited *L. monocytogenes* growth, neither significant increase nor inhibition was observed in the presence of ECB juice added milk during fermentation time that the amount of bacteria persisted at 5.13-5.34 log CFU/ml on samples. To detect antimicrobial activity in milk, it may be useful to utilize fruit juice with an ECB of greater than 10%, however, a high level of ECB juice will be unacceptable to use as an antimicrobial agent in milk due to its strong astringency and bitterness. During storage time period, after 1 day storage the number of *L. monocytogenes* decreased from 5 log CFU/ml to 3.54 -3.24 log CFU/ml in both ECB fruit juice and without added samples. *L. monocytogenes* is a psychrotrophic organism which means that it is able to grow under refrigeration temperature, therefore refrigeration temperature is not effective way to control the growth. However, the number of *L. monocytogenes* did not increase in our study, the number of pathogenic bacter decreased after 7 days storage conditions (Figure 4.7.).

This findings indicated that number of viable counts of *L. monocytogenes* was not significantly different between ECB fruit juice added media and the control during both

fermentation and storage time suggesting that food matrices might have different complexities with respect to growth medium, thus behavior of microorganisms can be affected differently. Complex food matrices containing fats, proteins and carbohydrates, may lead to interfere with antimicrobial activity [245]. Therefore, although PC-rich extracts exhibit antimicrobial activity against foodborne pathogens and spoilage microorganisms, the efficacy of these substances against bacteria varies between food systems and laboratory settings [120]. Protein and/or fat molecules in milk could bind bioactive compounds and/or form a protective coating around bacterial cells by reducing their availability for antimicrobial activity [50]. In this study, the fat content of used milk sample had 3.3%, thus the fat in milk and also containing protein, minerals probably decreased the antimicrobial potential of PC-rich ECB fruit juice. In one of the study examination the effect of antimicrobial activity of essential oils from clove and cinnamon against *L. monocytogenes* in presence of different fat levels of milk showing that antimicrobial activity reduced when the fat levels in milk increased [246]. PC-rich jasmine and green tea extracts (10%) showed antibacterial activity against foodborne pathogens in laboratory medium, however, the addition of these extracts on ground beef, has high fat content, failed to inhibit *L. monocytogenes* [247]. The results of this study were comparable with our results, viable cell counts of bacteria were not significantly different between tea extracts and control, remained same growth pattern during storage at 7 °C for 7 days. Similarly, antibacterial activity of tea and its application to a milk as a model food system showed no significant difference in inhibitory effect between 10% of extract and control against *L. monocytogenes* in milk stored at 7 °C [248]. However, growth inhibition observed in milk at 25 °C that affected the growth kinetics of bacteria, prolonged the lag phases and reduced growth rate. In the study of Yang et al (2014), the number of *L. monocytogenes* stayed almost same number at 24, 48 and 72 h fermentation in 10% blackberry juice added whole milk, but the number of *L. monocytogenes* in whole milk without added blackberry juice increased about 2 log CFU/ml. Therefore, the authors concluded that the addition of 10% of blackberry juice in milk demonstrated inhibitory effects against *L. monocytogenes* and other foodborne pathogens and stimulatory effects on beneficial bacterial strains which were *L. casei*, *L. plantarum* and *L. rhamnosus* [167].

In our study, milk was an ideal environment for the growth of both probiotic *L. plantarum* Lp-115 and *S. thermophilus* St-21 cultures that could grow and keep their viability during

fermentation and storage period. However, in the production of probiotic dairy functional beverages, the addition of PC are able to affect the viability of probiotic cultures due to their antimicrobial activity and also interfere with the fermentation process. Therefore, knowledge about the optimal percentage of functional ingredients that takes place in food matrix to be added is necessary [249–251]. An enough amount to generate a positive result without adversely influencing other functional components is considered to be using an optimal percentage of functional ingredients. Therefore, in our study, we firstly investigated to find the optimal dosage while formulating a functional beverage before the incorporation of ECB fruit juice with milk to ensure functional ingredients of PC in presence of ECB juice's antimicrobial, antioxidant activities and viability of probiotic culture will remain active. PC are able to play a crucial function in increasing viability and promoting the metabolic activity of microorganisms as well as improving viability of probiotic cultures [252].

4.4.CONCLUSION

Nowadays, increased demand for probiotic beverages and application of different food matrices on the viability of probiotic cultures and also in large quantities at the moment of intake to have the desired effects represents a great challenge [203,220,232,252]. Our studies showed that formulated beverages have promising functional properties with respect to antioxidant potential of ECB juice and enough number of viable probiotic cultures that beneficially modify gut microbiota.

5. ASSESSMENT OF *IN VIVO* SAFETY OF ECB FRUIT JUICE: ACUTE AND SUBACUTE ORAL TOXICITY STUDIES

5.1.INTRODUCTION

European cranberrybush (*Viburnum opulus* L.) (ECB) fruits are rich in phenolic compounds (PC), containing high amount of phenolic acids, flavonoids and anthocyanins that have associated with promoting health benefits [3,12,103,106]. In fold medicine, ECB fruits were traditionally used for the treatment of some disease such as ulcer, kidney, stomach and menstrual pain, coughs and colds [3,103,105], but in recent decades ECB fruit have gained significant attention in different research studies for their beneficial health properties. The results of *in vitro* studies of ECB fruit or extracts confirmed antioxidant, antimicrobial, and anticancer activities [105,106,115,116,122,125]. Moreover, being a cytoprotective agent [122,127], anti-obesity [8], anti-diabetic [126], osteogenic potential [5] and protective activity against hepatic lipotoxicity [128] of ECB fruit juice and its extracts were investigated in cell-based studies to understand the use of ECB fruit juice/extract as a diet component. *In vivo* animal studies, ECB juice/extracts showed ability to decrease gastrointestinal mucosal damage [129], prevent initiation stage of colon cancer [4], have anti-urolithiatic effect [7], use in the treatment of endometriosis [6], prevent or reduce structural and functional damages in reproductive organs [11].

PC-rich ECB fruit extracts have been of great interest to obtain extracts with high concentrations from them, hence it is of high interest for applications in functional foods and nutraceuticals. In Türkiye, there are commercially available various products such as “Gilaburine” which is a film-coated tablets of *V. opulus* extract (Natural Health Products, NHP), “Viburnum plus” liquid and powder extracts (Talex Pharma) and “Gilaburu Extract” (Immu-nat) on the internet under dietary supplement category for the treatment of kidney stone. Gok et al. (2021) and Kızılay et al. (2019) studies effect of gilaburine on patients at this suggested dose and time period (1000 mg tablet/2 tablet/3 times a day for maximum 4 weeks) were applied and they concluded that gilaburine can be used as an alternative herbal extract that facilitates the passage of distal ureteral stones smaller than 10 mm [131,132]. The effect of gilaburu liquid extract (Immu-nat) was studied on on hyperglycemia and oxidative damage in kidney of diabetic rats [253]. At the end of 20 days, they found that the

consumption of extract (300 mg/kg) by oral gavage once a day could reduce possible nephropathy which is a kidney failure for diabetic people.

Nevertheless, the use of concentrated high-dose bioactive compounds could render PC consumption potentially problematic depending on consumption dose and PC types [71,254–258]. Lambert et al. (2007) reviewed prooxidative versus antioxidant potentials of tea catechins, high dose of tea catechins caused hepatic and intestinal toxicities [259]. Similarly, high dose of PC present in tea, epigallocatechin-3- gallate (EGCG) showed hepatotoxicity to mice [256], high dose of quercetin (2-4%) showed chronic nephropathy, kidney disease, in rats [89] and the addition of 2% of caffeic acid in the diet of rats and mice was induced forestomach and kidney tumors [91]. Hence, the consumption of PC-rich supplements or extracts is a major health concern for the safety and the toxicological evaluations required to ensure safe levels of intake. There were no systematically study on safety of ECB fruit juice and more in-depth studies should be conducted to assess the safety for human consumption because even the consumption of ECB fruit promotes some health effects, the safety of concentrated PC intake may be a major health concern and adverse effects. Therefore, acute and subacute oral toxicity of PC-rich ECB extract were evaluated with *in vivo* animal studies to establish a toxicity profile of ECB extract by detecting lethal dose (LD₅₀) and no observed adverse effect level (NOAEL).

5.2.MATERIALS AND METHODS

5.2.1. Preparation and Phenolic Composition of ECB Fruit Extract

ECB fruit juice preparation was done as described previously at section 3.2.1. The lyophilized ECB fruit juice was stored at -80°C until analysis. Lyophilized form of juice was dissolved with water daily to obtain desired concentration of extract prior to gavage administration to animals.

The two main PCs in ECB fruit juice were catechin and chlorogenic acid [12]. Total phenolic and anthocyanin contents of ECB extracts were determined by using the Folin-Ciocalteu and pH differential methos, respectively [161,162]. Total phenolic content was expressed as mg gallic acid equivalents (GAE) per g ECB extract (dry weight) and total anthocyanin content was expressed as cyanidin 3-glucoside equivalents per g ECB extract in dried basis. Total

PC of lyophilized ECB juice extract was 27.9 mg GAE/g ECB extract which was approximately 5-fold higher than freshly squeezed ECB fruit juice. Total anthocyanin content was found as 0.13 mg cyanidin 3-glucoside/g ECB extract which was 1.5-fold higher than freshly squeezed ECB fruit juice.

5.2.2. Experimental Animals and Housing Conditions

Sprague-Dawley rats (200-250 g body weight) and BALB/c mice (25±3 g body weight) were provided by Experimental Animal Production and Research Laboratory of Yeditepe University (YUDETAM), Istanbul, Türkiye. Before conducting experiments, animals were kept in their cage for 5 days prior to dosing to allow acclimatization to laboratory conditions. All animals were separated by sex and housed steel cages under temperature-controlled environment (21±2°C), relative humidity of 60% with 12-hour light/dark cycle. All animals were provided with free access to standard diet and water *ad libitum*.

All tests were carried out in accordance with international standards for the ethical use of animals. The experimental protocols were approved by Experimental Animals Ethics Committee of Yeditepe University (Decision Numbers: 2019/751 and 2020/02-9) (Appendix A).

5.2.3. Acute Toxicological Assessment

Acute oral toxicity-fixed dose procedure study was performed according to Organization for Economic Co-operation and Development (OECD) guideline 420 for testing chemicals [260]. In this study, five male Sprague-Dawley rats were provided by YUDETAM. Prior to administration, overnight fasted rats were weighted. According to OECD 420 guideline, the 2000 mg/kg was the highest fixed dose and therefore, ECB extract was given orally administered to animals at a dose of 2000 mg/kg body weight (bw). All rats were monitored closely for 1 hour after receiving the ECB extract and then periodically for 4 hours. Clinical observations such as the changes in skin, fur, eyes, oral mucosa, respiration, circulation and behavior were done for 24 h and once daily for 14 days to check any signs of mortality and toxicity. At the end of 14 day, the body weights of rats were measured and then, they were euthanized.

5.2.4. Subacute Toxicological Assessment

The 28-days repeated oral dose subacute toxicity analysis was carried out in compliance with OECD 407 guideline [261]. BALB/c male and female mice were randomly separated into three different groups and all mice within each cage were marked to permit individual identification. The experimental groups (n = 10 animals/group, 5 animals per sex) received two different doses of ECB extract (500 mg/kg (low dose) body weight and 2000 mg/kg (high dose) body weight) and control group (n= 5 male mice) received 0.9% saline by oral gavage daily repeatedly given for 28 days.

All animals have been observed for mortality and in clinical signs on daily basis, body weight, food and water intake were recorded on weekly basis. After 28 days, the control and experimental groups were fasted overnight and sacrificed. Each mouse's blood was drawn through a heart puncture and divided into two groups of test tubes, half of which contained the anticoagulant EDTA and the other half of which did not. After the blood collection, internal organs and adipose tissue of all were immediately removed for the histopathological examination.

5.2.4.1. *Measurement of Body Weight and Relative Organ Weight*

Body weights of the mice were noted weekly, before administration ECB extracts during the experimental investigation. Brain, liver, spleen, kidney, heart and testis were excised immediately and then organ weights were recorded. Relative organ weight was computed by dividing individual weight of each organ with final body weight of each mouse as follows:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)} \times 100}{\text{Body weight of mice on sacrifice day}} \quad (6.1)$$

5.2.4.2. *Hematological Analysis*

Blood samples were placed in test tubes containing EDTA to analyse the hematological parameters. Firstly, samples were allowed to coagulate at ambient temperature for 20 min. Serum was separated by centrifugation at 4 000 rpm, 15 min at 4°C. Using a fully automated hematology analyzer (Prokan PE-6800 Vet, China), hematological parameters for the control and treatment groups were examined. Total blood was subjected to the evaluation of white blood cells (WBC), lymphocyte (LYM), granulocyte (GRAN), red blood cells (RBC),

hemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean platelets volume (MPV), red blood cell distribution width (RDW), platelet counts (PLT), platelet volume distribution width (PDV), plateletcrit (PCT), platelets larger cell ratio (P-LCR).

5.2.4.3. *Biochemical Analysis*

In test tubes with no anticoagulant, blood samples were collected and centrifuged at 4000 rpm, 15 min at 4°C to obtain serum for analyses. Then, serum was taken into new tubes and stored at -20°C until analyzes. Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), urea, cholesterol, creatinine, amylase, lactate dehydrogenase (LDH), lipase and triglycerides were measured by using VetTest kits (IDEXX Laboratories, USA).

5.2.4.4. *Histopathological Analysis*

Samples of the brain, liver, heart, spleen, kidney, pancreas, stomach, small intestine, colon, testis, ovary, adipose tissue, uterus and urinary bladder were collected for histopathological examinations. The 10% neutral formaldehyde were used for fixation of all tissue samples and stored at 4°C. Samples were put into automatic tissue processor (Leica TP1020) for the fixation as indicated in Table 5.1.

Table 5.1. The flow of fixation of tissues in automatic tissue processor

Reagents	Time period (h)	Process
Neutral formaldehyde	2	Fixation
Neutral formaldehyde	2	
Ethanol (70%)	1	Dehydration
Ethanol (80%)	1	
Ethanol (90%)	1	
Ethanol (96%)	1	
Ethanol (96%)	1.5	
Ethanol (100%)	1.5	
Xylene	1	Clearing
Xylene	1	
Paraffin	1	Paraffin Infiltration
Paraffin	3	

After fixation of tissue processing, all tissue samples were embedded in paraffin wax blocks (Leica embedding station, EG1160; Leica, Germany). The paraffin-embedded tissues were

section at 5 μm thickness by using a rotary microtome (Leica RM 2245 model; Leica Instruments, Germany). All sections of each tissue were mounted on poly-L-lysine coated slides and stained (Leica Autostainer XL) with Hematoxyline&Eosine (H&E) staining method as indicated in Table 5.2.

Table 5.2. Hematoxyline and Eosine (H&E) staining procedure

Steps	Staining reagent	Time (min)
1	Oven at 70°C	15
2	Xylene	3
3	Xylene	3
4	Xylene	3
5	Ethanol (100%)	2
6	Ethanol (96%)	2
7	Ethanol (96%)	2
8	Wash in running tap water	2
9	Distilled water	1
10	Hematoxyline	0.45
11	Wash in running tap water	1
12	Wash in running tap water	1
13	Acid alcohol	1
14	Wash in running tap water	1
15	Ammonia	0.1
16	Wash in running tap water	1
17	Ethanol (96%)	0.5
18	Eosine	1.10
19	Ethanol (96%)	0.5
20	Ethanol (96%)	0.5
21	Ethanol (96%)	1
22	Ethanol (100%)	1
23	Xylene	1
24	Xylene	1
25	Xylene	1

Apart from H&E staining, special staining procedures were also applied on selected tissues. These were cresyl violet staining for brain, Masson's trichrome (TCM) staining for liver, Periodic Acid-Schiff (PAS) and Alcian Blue staining for heart, kidney, stomach, intestine tissue samples and, oil red staining was done for adipose tissue staining by using Bio-Optica (Milano, Italy). All special staining procedures were given in Appendix B.

All tissue sections were assessed and photographed in different scale under a Leica DM6000B microscope by using Leica Application Suite image analysis program. All histopathological changes were examined by the help of pathologist. The microscopic features of all tissue samples administrated ECB fruit juice were compared with control group.

A semi-quantitative scoring system was applied for the evaluation of tissue damage based on the severity of lesion changes observed in each mouse by an experienced pathologist. Briefly, histopathological signs of each tissue were graded on a three-degree scale ranging from 0 to 3 (0 indicates no abnormality, 1 indicates mild injury, 2 indicated moderate and 3 indicates severe injury) for each tissue according to different evaluation parameters presented in Table 5.3. [262].

Table 5.3. Histopathological scoring parameters used to semi-quantify for each tissue

Tissues	Indications*
Brain	Edema in nervous tissue Bleeding in nervous tissue Capillary congestion in nervous tissue Degeneration in nervous tissue Mononuclear cell infiltration
Heart	Myocardial Edema Bleeding Cardiac muscle fibers with irregularity Capillary congestion Mononuclear cell infiltration
Stomach	Gastric glands degeneration Capillary congestion Epithelial degeneration Mucosal edema Mononuclear cell Infiltration
Jejunum and colon	Shortening of the villus structure Epithelial degeneration Tunica mucosa edema Mucosal capillary congestion Intestinal glands degeneration
Spleen	Lenfoid necrosis Hemorrhage Red and white pulp degeneration Capillary congestion Mononuclear cell infiltration
Liver	Interstitial edema Sinusoid capillary congestion Hepatocellular necrosis Hepatocyte vacuolation Leukocyte infiltration
Kidney	Interstitial edema Epithelial degeneration Tubular degeneration Capillary congestion Mononuclear cell infiltration
Urinary bladder	Epithelial degeneration Congestion Edema Mononuclear cell infiltration
Adipose tissue	Cellular Shape Interstitial edema Capillary Congestion Leukocyte Infiltration Fibrosis
Ovary	Germinal epithelium degeneration

	Follicles degeneration Edema in connective tissue Capillary congestion Mononuclear cell infiltration
Uterus	Uterus glands degeneration Capillary congestion Epithelial degeneration Mucosal edema Mononuclear cell infiltration
Testis	Interstitial edema Capillary congestion Seminiferous tubule basement membrane degeneration Spermatogenesis series degeneration Seminiferous tubules lumen irregularity Loss of seminiferous tubule Giant cell formation

*Each tissue was scored as 0(none); 1(mild); 2(moderate) and 3(severe) according to indications.

5.2.4.5. Statistical Analysis

All values were expressed as the mean $\pm$ standard deviation. Comparison between groups were done with a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test in all analysis except, independent t-test analysis was applied for the histopathological scoring results of ovary and uterus tissues using the SPSS statistical software version 20. Statistically significant differences defined as $p < 0.05$.

5.3. RESULTS

5.3.1. Acute Toxicity Study

All rats that administered a single oral dose of ECB extract at 2000 mg/kg bw were not observed any signs of either toxicity or death during 14 days. They were normal and did not show any alterations in behavior, breathing, impairment in food and water consumption, skin, postural abnormalities and hair loss. There were increase in body weight (Table 5.4.) that was initial mean body weight of rats was 217.6 ± 12.9 g and at the end of experiment their final mean body weight was 280.4 ± 22.8 g. According to the OECD 420 guideline, the results of acute toxicity showed that lethal dose (LD₅₀) of the ECB fruit juice was greater than 2000 mg/kg body weight.

Table 5.4. Body weight of animals (g) before and after feeding a single dose of 2000 mg/kg ECB extract for the acute oral toxicity study

The number of animals	Weight of before feeding ECB juice (g)	Weight of at the end of 14 days (g)
1	218	285
2	211	278
3	226	294
4	200	243
5	233	302
avg $\pm$ std	217.6 $\pm$ 12.9	280.4 $\pm$ 22.8

5.3.2. Subacute Toxicity Study

Administration of ECB extracts at doses 500 mg/kg (low dose) and 2000 mg/kg (high dose) did not cause any mortality during and at the end of the subacute toxicity studies in any mice. There were no obvious abnormal clinical signs such as changes in skin color, eyes, mucous membranes, loss of fur or behavior patterns in any dose of ECB extract groups. All mouse groups displayed a modest increase in body weight during the subacute experiment period. Changes in body weights recorded on a weekly basis were presented as shown in Figure 5.1. Throughout the study, even no significant changes in body weight in low dose administered ECB extract in comparison with control, body weight gain in administrated high dose of extract was significantly lower than control ($p=0.018$). Initial mean body weight of control group was 24.54 ± 3.8 g, and final mean body weight was 28.52 ± 2.1 g. While the initial mean body weight of mice administered low and high doses were 25.07 ± 5.1 and 22.66 ± 2.7 g, the final mean of body weights was 27.40 ± 4.5 and 24.70 ± 4.0 g, respectively.

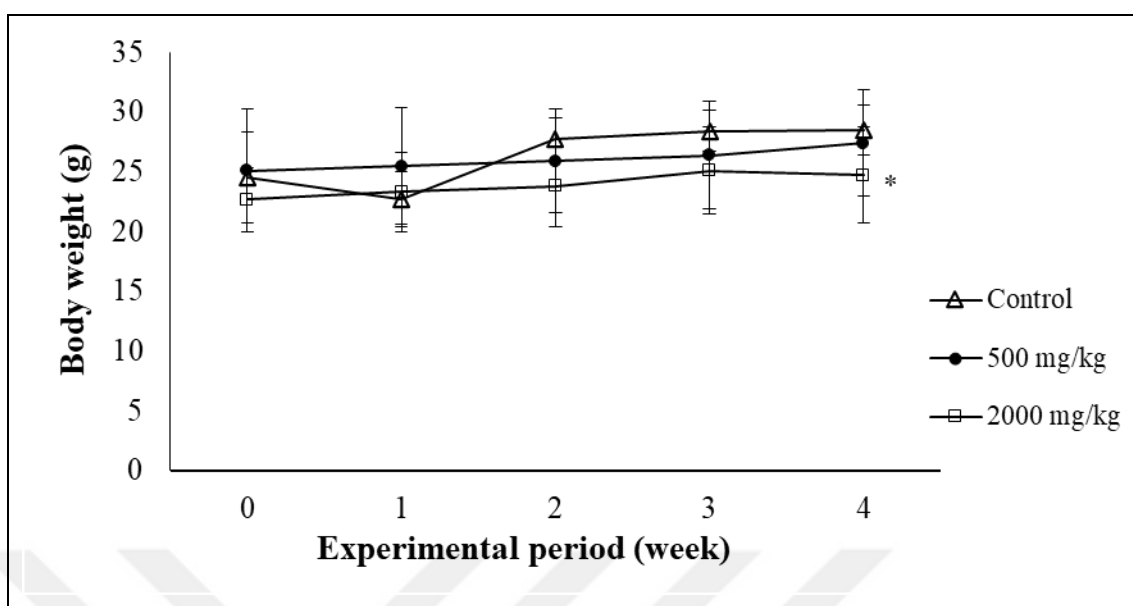


Figure 5.1. Body weight changes of mice during administration of low (500 mg/kg) and high (2000 mg/kg) doses of ECB extract and control group for 28 days. All values represent the mean $\pm$ S.D. *Significantly different from the control ($p < 0.05$)

Food and water consumption of experimental groups were comparable with control group. In the macroscopic analysis of organs, there were no significant differences in the mean absolute and the relative weights of the brain, liver, spleen, kidney, heart and testis of mice compared to control that was presented in Table 5.5. Hematological parameters of subacute oral toxicity were indicated in Table 5.6. Except PLT level, all hematological parameters showed no significant changes in the mice which were administered low and high doses of ECB extracts compared to control group. PLT level of both ECB doses ($476\text{--}760.50 \times 10^3/\mu\text{L}$) was higher than the reference range ($200\text{--}450 \times 10^3/\mu\text{L}$) for mice and the high dose of PLT level was significantly higher than control group ($p < 0.05$). The results of MID, MHC, MHCH, and MPV levels of both ECB doses and control group were higher than reference intervals, but there were no significant differences between groups. Moreover, both ECB doses of MCV levels ($53.68\text{--}54.23 \text{ fL}$) were within the reference interval ($45.0\text{--}55.0 \text{ fL}$), but the level of control group (59.76 fL) was not within the range and had a much more high level. In contrast, the LYM percent of all experiment groups ($47.65\text{--}55.80\%$) was lower than the reference interval ($57.0\text{--}93.0\%$).

Table 5.5. Absolute and relative organs weights in mice administered ECB extracts

Final body weight (g)	Control	Dose Groups (mg/kg body weight/day)	
		Low dose (500 mg/kg)	High dose (2000 mg/kg)
	28.52±2.1	27.40±4.5	24.70±4.0
Absolute organ weights (g)			
Brain	0.380±0.04	0.415±0.06	0.399±0.03
Liver	1.289±0.12	1.102±0.24	1.226±0.17
Spleen	0.147±0.03	0.105±0.02	0.151±0.04
Kidney	0.490±0.07	0.426±0.13	0.398±0.09
Heart	0.193±0.02	0.192±0.03	0.194±0.04
Testis*	0.174±0.05	0.214±0.02	0.196±0.02
Relative organ weight (g/100 g body weight)			
Brain	1.34±0.16	1.55±0.29	1.66±0.34
Liver	4.55±0.64	4.03±0.64	5.02±0.68
Spleen	0.50±0.09	0.40±0.13	0.63±0.19
Kidney	1.68±0.15	1.55±0.29	1.62±0.32
Heart	0.68±0.09	0.71±0.08	0.81±0.20
Testis*	0.62±0.21	0.72±0.28	0.74±0.13

All values represent the mean ±S.D. No significant difference from control ($p > 0.05$).

*only male animals mean ±S.D value

Serum biochemical analysis was reported in Table 5.7. Results indicated that mice administered with low and high doses of ECB extracts did not significantly alter in levels of ALT, AST, urea, amylase, lipase, triglycerides, and cholesterol, except for creatinine and LDH levels. The obtained data from creatinine and LDH levels of low dose ECB extract administered group was significantly lower compared to the control ($p < 0.05$). However, measured level of creatinine in all experiment groups were low compared to the range of reference interval. LDH levels of high dose and control groups were within the reference intervals while the LDH level of mice administered low dose ECB extract was significantly low.

Table 5.6. Hematological analysis results of mice administered ECB extracts for 28 days

Parameters	Reference intervals	Control	Dose Groups (mg/kg body weight/day)	
			500	2000
WBC (10³/uL)	6.0-15.0	5.20±2.74	8.18±2.75	8.34±4.29
LYM (%)	57.0-93.0	55.80±7.53	54.28±6.31	47.65±6.70
MID (%)	1.8-6.0	13.22±4.22	14.29±2.23	17.16±2.03
GRAN (%)	8.0-48.0	30.98±4.78	31.43±5.52	35.19±6.89
LYM # (10³/uL)	3.4-7.4	2.78±1.32	4.49±1.76	4.51±1.78
MID # (10³/uL)	0.1-0.3	0.74±0.47	1.17±0.44	1.63±0.65
GRAN # (10³/uL)	0.5-3.8	1.68±1.05	2.52±0.82	3.20±1.23
RBC (10⁶/uL)	7.00-12.00	7.12±1.71	7.95±0.44	7.53±2.68
HGB (g/dL)	12.2-16.2	13.70±4.15	15.89±1.03	15.39±5.64
HCT (%)	35.0-45.0	38.30±9.61	43.00±1.94	40.51±14.50
MCV (fL)	45.0-55.0	59.76±13.57	54.23±1.96	53.68±1.42
MCH (pg)	11.1-12.7	18.94±1.39	19.94±0.53	19.79±1.84
MCHC (g/dL)	22.3-32.0	35.32±2.31	36.89±1.07	37.03±2.98
RDW-SD (fL)	0.1-99.9	26.74±2.52	26.38±0.95	27.46±2.41
RDW-CV (%)	13-17	15.10±1.29	14.77±0.53	15.50±1.33
PLT (10³/uL)	200-450	419.60±122.11	476.00±162.54	760.50±199.53*
MPV (fL)	3.8-6.0	7.90±1.09	8.07±1.14	9.17±1.05
PDV (%)	0.1-30.0	8.26±0.80	8.44±0.90	9.47±1.32
PCT (%)	0.01-9.99	0.53±0.55	0.38±0.16	0.73±0.36
P-LCR (%)	0.1-99.9	12.50±2.72	15.23±5.96	15.67±6.92

All values represent the mean ±S.D. *Significant difference between high dose ECB extract administered group compared to control groups ($p < 0.05$).

Table 5.7. Biochemical parameters result of mice administered ECB extracts for 28 days

Parameters	Reference intervals	Control	Dose Groups (mg/kg body weight/day)	
			500	2000
Aspartate aminotransferase (AST) (U/L)	59-247	146.00±51.2	159.20±143.96	171.44±78.09
Alanine aminotransferase (ALT) (U/L)	28-132	89.00±31.4	62.50±24.87	66.38±16.39
Urea (mmol/L)	6.4-10.4	10.08±1.8	7.58±2.26	7.89±1.47
Creatinine (μmol/L)	18-71	4.80±2.4	1.40±1.71*	2.44±1.88
Amylase (U/L)	1691-3615	1508.20±314.2	1609.00±205.82	1373.11±329.56
Lipase (U/L)	1105-3993	1244.60±346.2	1116.00±500.52	1020.43±399.73
Triglycerides (mmol/L)	0.62-1.63	1.59±0.3	1.51±0.39	1.39±0.28
Cholesterol (mmol/L)	0.93-2.48	2.11±0.4	1.94±0.70	1.56±0.36
Lactate Dehydrogenase (LDH) (U/L)	1105-3993	2800±0.0	2099.30±758.85*	2800.00±0.0

All values represent the mean ±S.D. *Significant difference between low dose ECB extract administered group compared to control groups ($p < 0.05$).

Although assessment of histopathological results showed that no obvious macroscopic findings in any tissue after administered with low and high dose of ECB fruit juice extract, all tissues were examined control versus ECB extracts administered groups and the total histopathological scores were calculated for each tissue that were shown in Table 5.8.

Table 5.8. Total histopathological score of all tissues in mice administered ECB extracts

Tissues	Control	Low dose (500 mg/kg)	High dose (2000 mg/kg)	<i>p</i>-value
Brain	1.00±0.71	1.40 ±0.70	1.50±0.85	0.492
Heart	1.40±1.67	2.10±1.37	2.60±0.70	0.216
Stomach	1.0±1.22	1.4±0.84	1.50±0.85	0.613
Jejunum	1.00±1.22	1.60±0.84	2.10±0.88	0.118
Colon	1.60±1.14	1.50±0.85	1.80±0.79	0.899
Spleen	0.80±0.84	0.90±0.74	1.10±0.57	0.051
Liver	1.40±0.89	2.00±1.25	7.30±2.83*	0.000
Kidney	1.20±1.10	2.10±1.10	6.70±1.49*	0.000
Urinary bladder	1.40±0.55	1.70±0.67	1.90±0.88	0.481
Adipose tissue	4.00±2.55	6.40±3.44	11.10±2.08*	0.000
Uterus	1.60±1.14	2.00±1.41	2.20±0.84	0.481

The results are indicated as the mean ±SD. *Significantly different from each group ($p<0.001$; One-way ANOVA, Tukey test).

The tissue sections of brain, heart, stomach, jejunum, colon, spleen, testis, ovary, uterus and urinary bladder from mice at both doses showed normal histological features in comparison with control and therefore no significant changes between groups. The histopathological images of all tissue sections were given in Appendix C. Histopathological examinations of various tissues administered ECB extracts compared to normal appearance of tissues apart from liver, kidney and adipose tissue were summarized in Table 5.9 by experienced pathologist.

Table 5.9. Evaluation of histopathological changes in tissues after administered ECB extracts

Tissues	Histopathological observations
Brain	Gray matter and white matter in the brains of all groups were observed as usual and no pathological alterations in the neurons.
Heart	Cardiac muscle fibers of myometrium in the heart were observed as usual.
Stomach	The layers of tunica mucosa, submucosa and muscularis externa in the stomach of all groups were observed as usual. Glands structural and connective tissue components of mucosa of stomach were seen as normal histological structure.
Jejunum and colon	The layers of tunica mucosa, submucosa and muscularis externa in the jejunum and colon of all groups were observed as usual. Intestinal glands structural and connective tissue components of mucosa were observed normal histological structure.
Spleen	Connective tissue and parenhyme tissue in the spleen of all groups were observed usual and no pathological alterations in the white and red pulpa were observed.
Ovary	Germinal epithelium, cortex and medulla in the ovary of all groups were observed as usual and follicle structures and connective tissue of ovary were observed as usual.
Uterus	Endometrium, myometrium and perimetrium of uterus were observed usual. No pathological changes in the uterine glands and epithelium of uterus were observed in all groups.
Testis	Tunica albuginea and parenchyme of testis were observed usual and seminiferous tubules and intersitital tissue of testis of all groups were normal.
Urinary bladder	Layer of mucosa, muscularis and adventitita of urinary bladder were observed usual and no pathological changes in the transitional epithelium in all groups.

However, the total histopathological score of liver, kidney and adipose tissue showed significant changes in their structures at high dose ECB extract administered groups showed in Table 5.8. and Figure 5.2. ($p < 0.001$). Vacuolization in hepatocytes, the presence of edema and bleeding in central veins in the liver tissue of animals was remarkable compared to controls. Similarly, the formation of new blood vessels and bleeding edema, infiltration,

bleeding and tubular degeneration was observed on kidney sections and adipose tissue of mice was observed. Although significantly change was observed in high dose ECB, there were slight alterations observed in low dose groups of kidney and adipose tissue. Finally, in histopathological examination of the adipose tissue of mice was observed new blood vessels and bleeding. Even histopathological score of adipose tissue showed no statistically significant difference with control and low dose ($p=0.279$), there was a significant difference between control and high dose groups ($p=0.003$).

Total histopathological damage scores in the liver, kidney and adipose tissue of mice administered with low and high doses of ECB extracts were plotted on the graph (Figure 5.2.). The high dose of ECB administered to all tissues was significantly different compared to the control and low dose groups ($*** p<0.001$).

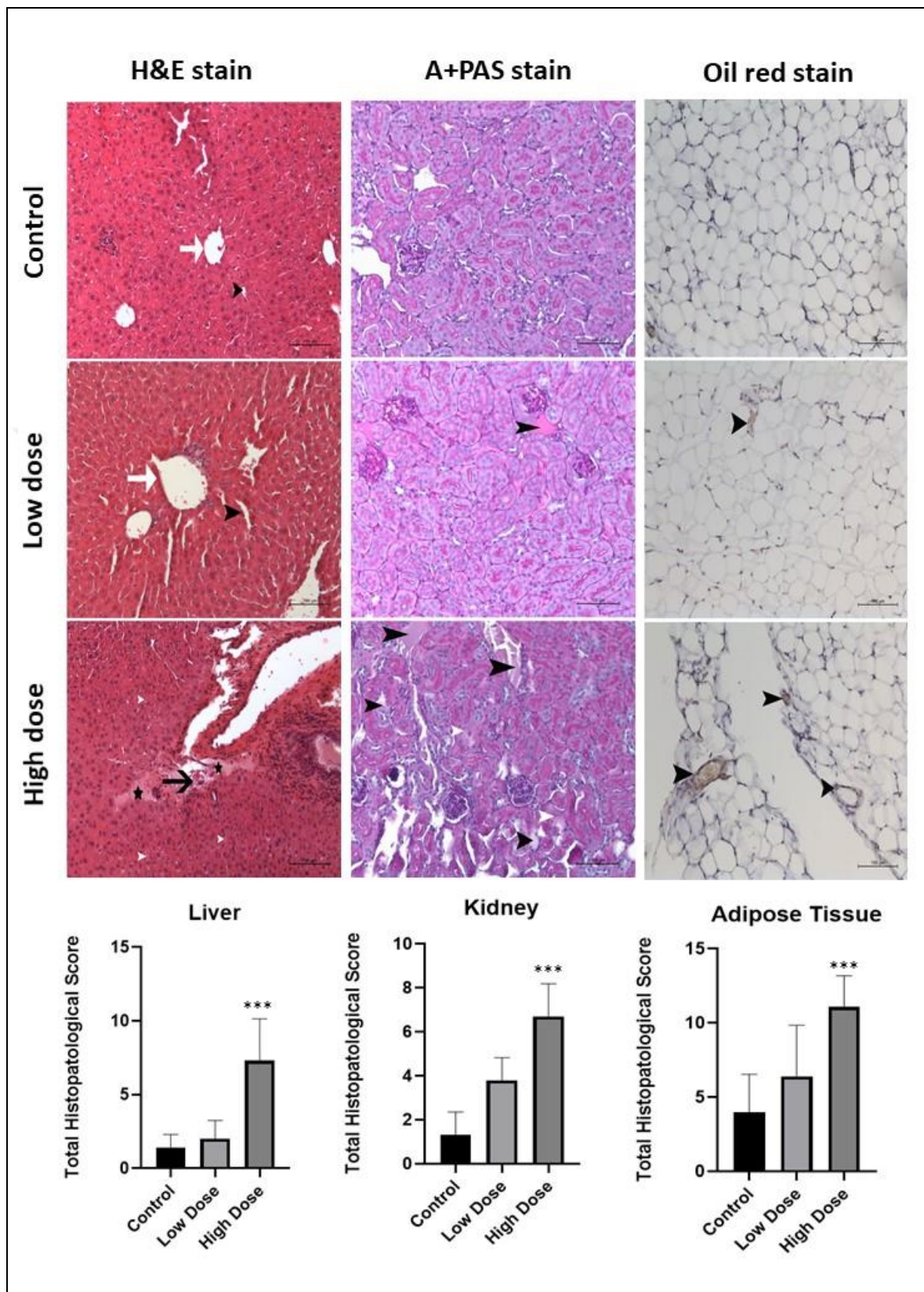


Figure 5.2. Representative histological sections of H&E-stained liver, A+PAS-stained kidney and oil red stained-adipose tissue of animals ECB extract (low (500 mg/kg) and high (2000 mg/kg) dose) groups and control group in subacute toxicity study. Sinusoids

(arrowhead), vacuoles (white arrowhead), central veins (white arrow), bleeding (black arrow) and edema (star) were indicated in the liver parenchyma; Proximal tubule (arrowheads), and distal tubule (white arrowheads) were indicated in the kidney parenchyma; New blood vessels and bleeding was indicated as arrowheads in the adipose tissue. Scale Bars: 100 μ m

5.4. DISCUSSION

Despite the beneficial health effect of PC and PC-rich fruits, their safety in functional foods or dietary supplement use may be a main concern especially to make decision on dose and to avoid the untoward effects. Hence, the safety of ECB fruit juice extract was evaluated in this study, because this kind of information is lacking in the scientific literature.

During the 14 days of acute oral toxicity testing, rats exposed to a single dosage of ECB extract (2000 mg/kg) did not experience any mortality and changes in body weight. The ECB extract can therefore be considered safe at the largest dose recommended in the study by OECD Guideline 420. The oral LD₅₀ value of ECB extract was determined to be more than 2000 mg/kg body weight. Although it's crucial, the absence of toxicity after a single encounter does not imply that the ECB extract is safe.

As a continuation of the acute toxicity study, subacute toxicity was done with different doses of ECB extracts (2000 mg/kg and 500 mg/kg) because these doses may cause an accumulation in the body that will gradually affect the tissues and organs. Because these effects are typically not visible in acute toxicity, this study is helpful in determining the effects of extracts on target organs as well as hematological or biochemical impacts.

Determination of NOAEL is an important issue for the establishing human safety. Hence, in this study, dose-related subacute toxicity profile of ECB extract was evaluated in mice using measurement of body weight, hematological, biochemical and histopathological parameters. Administration of neither high (2000 mg/kg) nor low dose (500 mg/kg) of ECB extracts did not result in mortality and did not lead to changes in animal behavior, food and water consumption of animals in comparison with control group. Although no weight loss was observed in our study, the weight gain of high dose animals throughout the study was lower than other groups ($p < 0.05$) (Figure 5.1.). Percent gain of body weight was calculated

as the ratio between final weight against initial weight of mice at both administered ECB dose group. The results showed that the percent weight gain was 9% in high dose and 9.29% in low dose ECB administered groups which was lower than control (16.22%) group suggesting that PC in ECB fruit juice might get to have a potential to help control body weight gaining regardless of how much dose administered.

In toxicity studies, the relative organ weight is considered as primary indicators of organs toxicity because of their sensitivity to harmful compounds. In our study, no significant difference in animal organ weights of brain, liver, kidney, heart, spleen, and testis were observed in contrast to control group (Table 5.5), suggesting that administration of ECB extracts at subacute oral doses produces no effect on normal growth. Toxicity-related changes in major organ weight are generally accompanied by corresponding histopathological findings. However, in the present study the results did not correlate with histopathological analysis of liver and kidney organs of the highest ECB dose administered group.

Hematological parameters results, good index of physiological and pathological status, of ECB administered mice were not different than control, except PLT (Table 5.6). Otherwise, the results of some hematological parameters, MID, MHC, MHCH, and MPV levels measured from ECB extracts administered to mice and control groups, were outside the reference range that could be considered abnormal, but the reference intervals were given for mouse and is not specific for animal species such as BALB-c. Nevertheless, the results were indicated that the ECB extracts did not cause abnormalities in hematological parameters for low dose ECB. However, PLT level in high dose ECB extract administered mice was significantly higher than control group and also higher than reference range for mouse (Table 5.6). The PLT level, play a crucial role in inflammatory disease, of high dose of ECB administered animals was showed that the ECB extract may induce thrombocythemia that does not known accompanying any disease, but thrombocythemia may develop due to the anemia, cancer, or infectious diseases and may cause serious complications. Bleeding or clotting abnormalities lead to platelet dysfunction [263,264]. The measured PLT level in this study may imply that the ECB extract has toxic effect taken at high dose and could attribute the reason of bleeding observed in liver, kidney and adipose tissue in high dose of ECB administered animal tissues.

Certain blood biochemical parameters give an idea to understand the mechanism of toxicity especially the hepatic and renal function examinations are the paramount importance to assess the toxic properties of extracts. Our study result showed that the level of AST and ALT, are main biochemistry marks related with liver damage, were within the normal ranges and were not statistically difference in both high and low dose ECB extract administered groups compared to control showing that ECB extracts did not exert adverse effect on liver. However, histopathological analysis of high dose of ECB animals' liver result did not correlate with biochemical analysis results. Creatinine and urea levels, representative of renal injury, analysis supply an important knowledge about the function of kidney. Results showed that the creatinine level of low dose of ECB administered group was significantly lower compared to control group ($p < 0.05$), but all group values were found as low and not within the normal range (Table 5.7.). The kidney dysfunction is generally seen due to the increased level of creatinine [265]. The level of creatinine usually use for as an indicator of renal function, but it does not always reflect kidney function and varies with age, sex and race [266]. In accordance with this information, the result of low creatinine level in low dose group was compatible with histopathological result and there was no toxicological significance observed. Even the creatinine level of high dose ECB administered group did not significantly different compared to control, histopathological results of kidney samples were showed edema, infiltration, and tubular degeneration on kidney sections.

On the other hand, neither amylase nor lipase levels of ECB administered animals did not showed any changes and were within the normal range that are marker of pancreatic abnormality [267]. Generally, amylase and lipase parameters check to understand the hepatic and pancreatic damage and function. Although histopathological analysis of the pancreas was not examined, significantly alteration was observed at liver tissue of high dose ECB extract administered animals in histopathological analysis.

The lipid profile of ECB administered animals demonstrated that the LDH level of low dose group was significantly lower than high and control groups, but the measured levels of all groups were in normal ranges. The LDH, widely distributed in the body, is used to investigate damage to liver, cardiac or skeletal muscle, but this test is not a specific or sensitive indicator of damage to any organ or tissue [268]. An elevated LDH value demonstrates a variety of disorders such as hepatic, cardiac, skeletal muscle and renal diseases. Even the highest dose of ECB extract led to alteration in the liver with an obvious

microscopic change, the results of measured LDH level of high dose and control group were the same. Therefore, we could not associate this value with toxicity. The cholesterol levels of ECB extract administered to animals did not show any changes and the levels were within the normal range. Furthermore, to detect abnormalities in lipid metabolism the level of triglycerides in ECB administered animals at the high dose were lower than other groups, but all of them were in the normal range.

Even biochemical parameters are decisive indicators of organ dysfunction or damage, there is an inadequacy of correlation between results of liver enzyme parameters and histopathological damage results due to sensitivity and specificity limitations of markers [269]. Therefore, the relationship between toxin concentrations in the body and their impact on the target organs can be better understood with the use of histopathological tissue analysis. In our histopathological examination showed that obvious microscopic changes such as edema, infiltration and bleeding were observed in kidney, liver and adipose tissue of high dose ECB extract administered animals (Figure 5.2.). Therefore, findings indicated that PC-rich ECB extract might show a toxic effect at high dose of ECB fruit juice (2000 mg/kg body weight) and 500 mg/kg bw ECB extract was found as NOAEL.

The obtained results demonstrated that hepatotoxicity and nephrotoxicity observed on animals at high dose of ECB extract the reason might be pro-oxidative activity of high dose of ECB polyphenols causing oxidative stress. Parallel to our study, Yang and Zhang (2018) resulted that EGCG and other catechins are ability to quench reactive oxygen species (ROS) and prevent their formation at low doses due their antioxidant activity. At moderate levels of catechins may produce ROS, however, high levels of ROS would produce at the high dose of catechin intake and this situation leads to oxidative stress causing cellular and tissue damage [257]. Correlated to this study, ECB extract has also high content of catechin that might be causing damage to liver and kidney. Furthermore, in this study histopathological evaluation of adipose tissue showed an inflammation on high dose ECB extract administered mice. The reason of inflammation might occur due to oxidative stress. Due to occurring imbalance in protective mechanism, oxidative stress gives rise to inflammation and damage of cellular molecules such as DNA, proteins and lipids [270]. Regarding the contribution of PC to the cellular antioxidant defense, they may induce toxicity at high concentrations meaning that they may have prooxidative activities and can cause oxidative stress [71,255,259,271,272]. Adipose tissue is generally considered as an energy reservoir of body,

but it has also functioned as an endocrine organ to control vascular system, immune response and metabolism through the secretion of growth factors, hormones, cytokines, and enzymes [273]. Inflammation is a natural defense mechanism against toxic chemicals and our study showed that feeding animals with high dose of ECB extract might cause inflammation, so administration dose is very crucial [274].

5.5. CONCLUSION

As a conclusion, based on the toxicological parameters measured and conditions in this study, ECB extract was not toxic after a single high dose exposure. However, consuming high dose of ECB extract in a prolonged period affected histopathological parameters indicating that precautions should be taken when it consumes over a longer period of time. High-dose ECB extract (2000 mg/kg) was shown to be deleterious as hepatotoxic and nephrotoxic by changes in liver, kidney, and adipose tissue architectures. It was concluded that the extract does not cause any obvious toxicity when used at lower doses. Based on the findings, the NOAEL for ECB extract is considered as 500 mg/kg/day. Hence, safety evaluation and risk assessment of PC are needed to determine consumption amount by humans.

When the NOAEL of ECB extract translated to human dose, human equivalent dose (HED) was calculated based on body surface area and weight [275]. The calculated HED value was 38.33 mg/kg and dose was found as 2300 mg for a 60kg human. Then, HED value was further divided by a safety factor of 100-fold found as 23 mg lyophilized ECB extract. Safety factor represents the two separate 10-fold factors: a factor of 10 to cover interspecies differences between mice and human, the second 10 factor to cover inter-individual differences between humans.

Consequently, the use of supplements containing rich PC should be pondered and therefore users need to be caution to avoid overdosing.

6. CONCLUDING REMARKS

According to all these findings showed that functional properties make ECB fruit important in the food industry as an additive for developing products in medical, cosmetic and pharmaceutical fields. Among biological activities, the properties of ECB fruit juice inhibited selectively the growth of foodborne pathogens but not probiotic LAB suggesting that the promising potential to use ECB fruit extracts in probiotic food applications. Therefore, in the second part of this study, investigation of the viability and growth performance of different probiotic cultures in presence of ECB juice added whey-based and milk-based environments were done. Both environments were ideal for the growth of probiotic cultures that could grow and keep their viability during fermentation and storage time. For both studies, the addition of (10%) ECB fruit juice did not affect the survival of probiotics and also interfere with the fermentation process which is a critical issue to produce a beneficial effect without negatively affecting other components. Our studies showed that formulated environments have promising functional properties of ECB juice with respect to enough numbers of viable probiotic cultures that beneficially modify the gut microbiota. In this section, microbial safety in a food model which was milk environment was also studied. ECB fruit juice was found to be strongly inhibitory to *Listeria monocytogenes*, microbial safety of ECB fruit juice was investigated in a milk-based environment fermented with probiotics by challenging with pathogenic *L. monocytogenes* ATCC 15313. The growth of *L. monocytogenes* did not inhibit in the presence of ECB juice added to milk that might be food matrices having different complexities with respect to growth medium may lead to interfering with antimicrobial activity. This study revealed that ECB extracts have a selective antibacterial activity, which may be of significant relevance to the food industry, particularly for the application to probiotic products.

Some polyphenols are reported to be pro-oxidant, mutagenic and hazardous at large doses despite the many positive effects, therefore the intake of phenolic-rich food or extract is a major health concern for the safety and the toxicological evaluations required to ensure safe levels of intake. Related to this topic, bioavailability plays a crucial role in determining whether a substance will be more hazardous or provide additional benefits. In the study of *in vitro* bioavailability of PC present in ECB fruit showed that while the content of PC was stable in gastric conditions, the content decreased in intestinal conditions. However, they are

still available during digestion and might contribute to biological activities after consumption. It is necessary to look at the safety implications of long-term ingestion of large dosages of PC as food additives or dietary supplements. There was no systematic study on safety of ECB fruit juice and this study showed that although the consumption of ECB fruit promotes some health effects, safety of prolonged consumption of ECB fruit was dose dependent.

In light of the expanding trend for natural, safe and health-promoting ingredients, it can be said that ECB fruit as a natural component of food products or dietary supplements fits perfectly into new trend and has a good chance to increase its role as a beneficial component of a healthy diet to consider safe dose.

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APPENDIX A: ETHICAL APPROVAL FORMS



**T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU
(YÜDHEK)
ETİK KURUL KARARI**

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
30.04.2019	751	02.04.2019	Prof. F. Yeşim EKİNCİ
'European cranberrybush (Viburnum opulus L.) meyve suyunun akut oral ve subakut toksisite değerlendirmesi ve bağırsak mikrobiyotası üzerine etkisinin fareler üzerinde incelenmesi' adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.			
Etik Onay Geçerlilik Süresi: 3 Yıl		Hayvan Türü ve cinsiyeti: Fare ♂♀	Hayvan Sayısı: 20

GÖREVİ	ADI SOYADI	
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	
Raportör	Vet. Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Doç. Dr. Soner DOĞAN	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Prof. Dr. Gamze TORUN KÖSE	
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	



T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2020-827	18.02.2020	2020/02	2020/02-9	Prof. F. Yeşim Ekinci
"European cranberrybush (<i>Viburnum opulus</i> L.) meyve suyunun akut oral ve subakut toksisite değerlendirmesi ve bağırsak mikrobiyotası üzerine etkisinin fareler üzerinde incelenmesi" isimli proje oy birliğiyle etik açıdan uygun görülmüştür.				
Hayvan Türü / Irkı		Toplam Hayvan Sayısı		Hayvanın Cinsiyeti
Fare / BALB/C		10		Erkek ve Dişi

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Veteriner Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Prof. Dr. Gamze TORUN KÖSE	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Doç. Dr. Burea GEMİCİ BAŞOL	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	

APPENDIX B: SPECIAL STAINING PROCEDURES USED IN HISTOPATHOLOGICAL ANALYSIS

Special staining procedures were also applied on selected tissues. These stains were cresyl violet staining for brain, Masson's trichrome (TCM) staining for liver, Periodic Acid-Schiff (PAS) and Alcian Blue staining for heart, kidney, stomach, intestine tissue samples and, oil red staining was done for adipose tissue staining by using Bio-Optica (Milano, Italy). All staining procedures were given in Table B.1-B.6.

Table B.1. Cresyl violet staining procedure for brain tissue

Steps	Staining reagent	Time (min)
1	Oven at 70 °C	15
2	Xylene	1
3	Xylene	1
4	Xylene	1
5	Distilled water	3
6	Cresyl violet solution (1%, Abcam)	18
7	Distilled water	1
8	Ethanol (70%)	1
9	Ethanol (80%)	1
10	Ethanol (90%)	1
11	Ethanol (96%)	1
12	Ethanol (100%)	1
13	Xylene	2
14	Xylene	2

Table B.2. Masson's trichrome (TCM) staining procedure for liver tissue

Steps	Staining reagent	Time (min)
1	Oven at 70 °C	15
2	Xylene	3
3	Xylene	3
4	Ethanol (100%)	2
5	Ethanol (96%)	2
6	Ethanol (96%)	2
7	Ethanol (70%)	2
8	Distilled water	1
9	Hematoxyline	4
10	Acid alcohol (1%)	1
11	Wash in running tap water	1

12	Acid fuchsin	5
13	Distilled water	1
14	Phosphomolybdic acid	5
15	Light green	6
16	Distilled water	1
17	Acid alcohol (1%)	2
18	Ethanol (70%)	1
19	Ethanol (80%)	1
20	Ethanol (96%)	1
21	Ethanol (100%)	1
22	Xylene	5
23	Xylene	5

Table B.3. Periodic Acid-Schiff (PAS) and Alcian Blue staining procedure for hearth, kidney, stomach, intestine

Steps	Staining reagent	Time (min)
1	Oven at 70 °C	15
2	Xylene	5
3	Xylene	5
4	Xylene	5
5	Ethanol (100%)	2
6	Ethanol (96%)	2
7	Ethanol (96%)	2
8	Distilled water	1
9	Alcian Blue solution (1% in 3% acetic acid)	5
10	Wash in running tap water	2
11	Distilled water	1
12	Periodic acid (0.5%)	5
13	Distilled water	1
14	Schiff solution	15
15	Wash in running tap water	10
16	Hematoxyline	0.30
17	Wash in running tap water	2
18	Ethanol (100%)	2
19	Xylene	2
20	Xylene	2

Table B.4. Oil red staining procedure for adipose tissue samples

Steps	Staining reagent	Time (min)
1	Oven at 70 °C	15
2	Xylene	5
3	Xylene	5
4	Xylene	5
5	Ethanol (96%)	3
6	Ethanol (90%)	3
7	Ethanol (80%)	3
8	Ethanol (70%)	3
9	Distilled water	1
10	Reagent A	20
11	Wash quickly in tap water	1
12	Reagent B	0.30
13	Wash quickly in tap water	3
14	Ethanol (70%)	1
15	Ethanol (80%)	1
16	Ethanol (90%)	1
17	Ethanol (96%)	1
18	Xylene	1

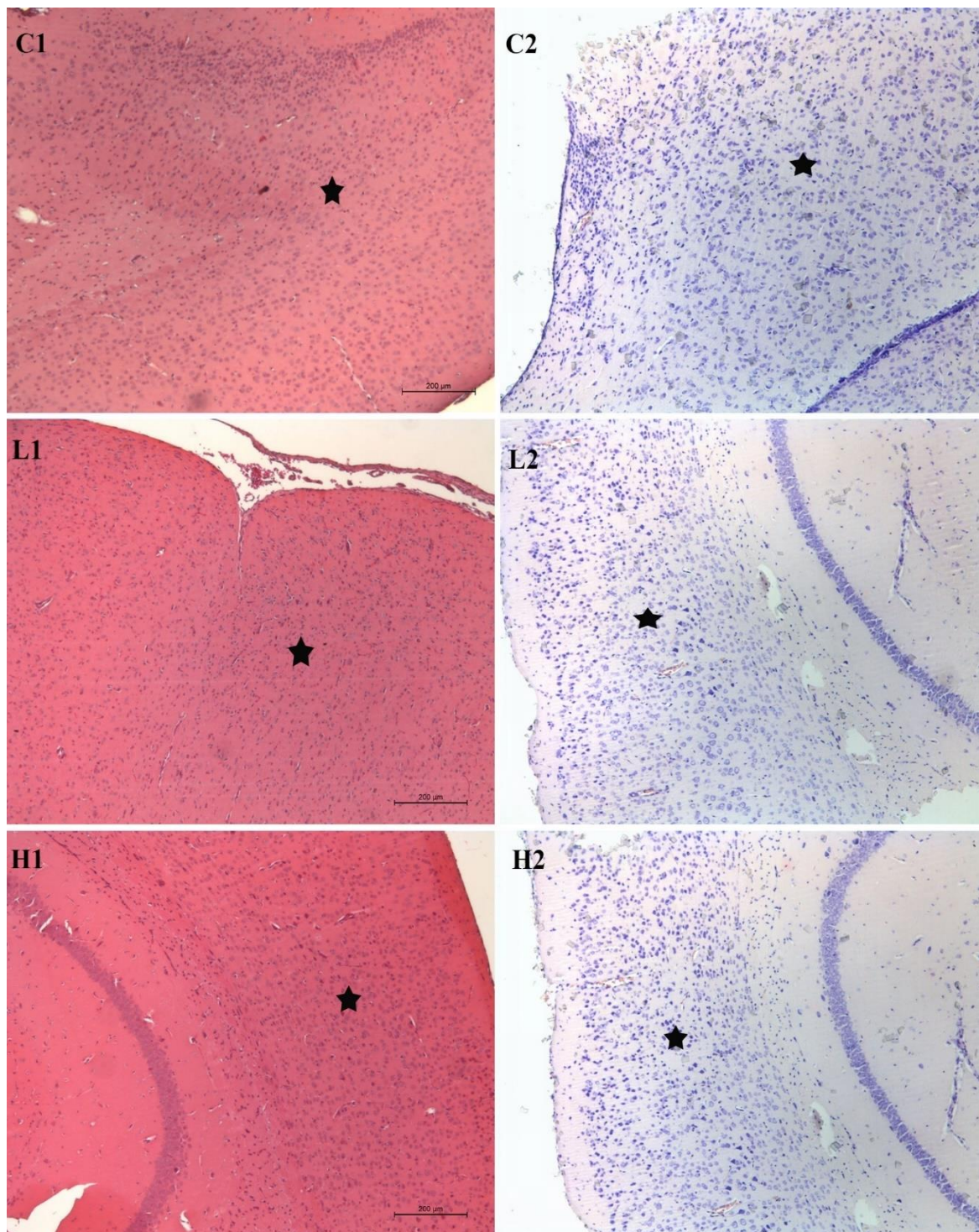
APPENDIX C: HISTOPATHOLOGICAL IMAGES OF ALL TISSUES

Figure C.1. Histopathological images of brain tissue in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (10 $\times$, H&E stained) and C2-L2-H2 (10 $\times$, cresyl violet stained). Stars shows the normal cortex in brain tissue. Scale Bars:200 μ m.

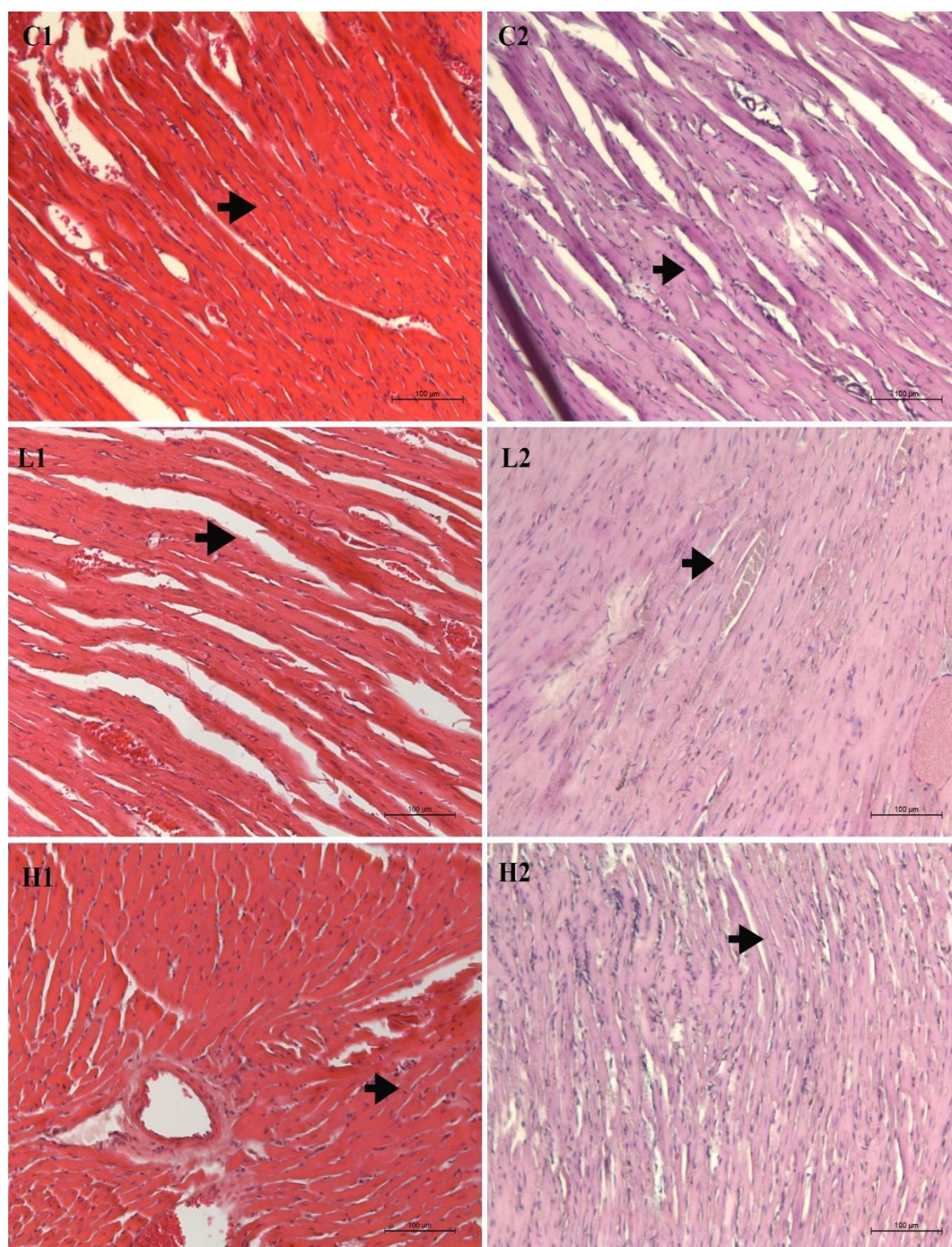


Figure C.2. Histopathological images of heart tissue in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (20×, H&E stained) and C2-L2-H2 (20×, PAS stained). Arrows show normal muscle fibers. Scale Bars: 100 μm.

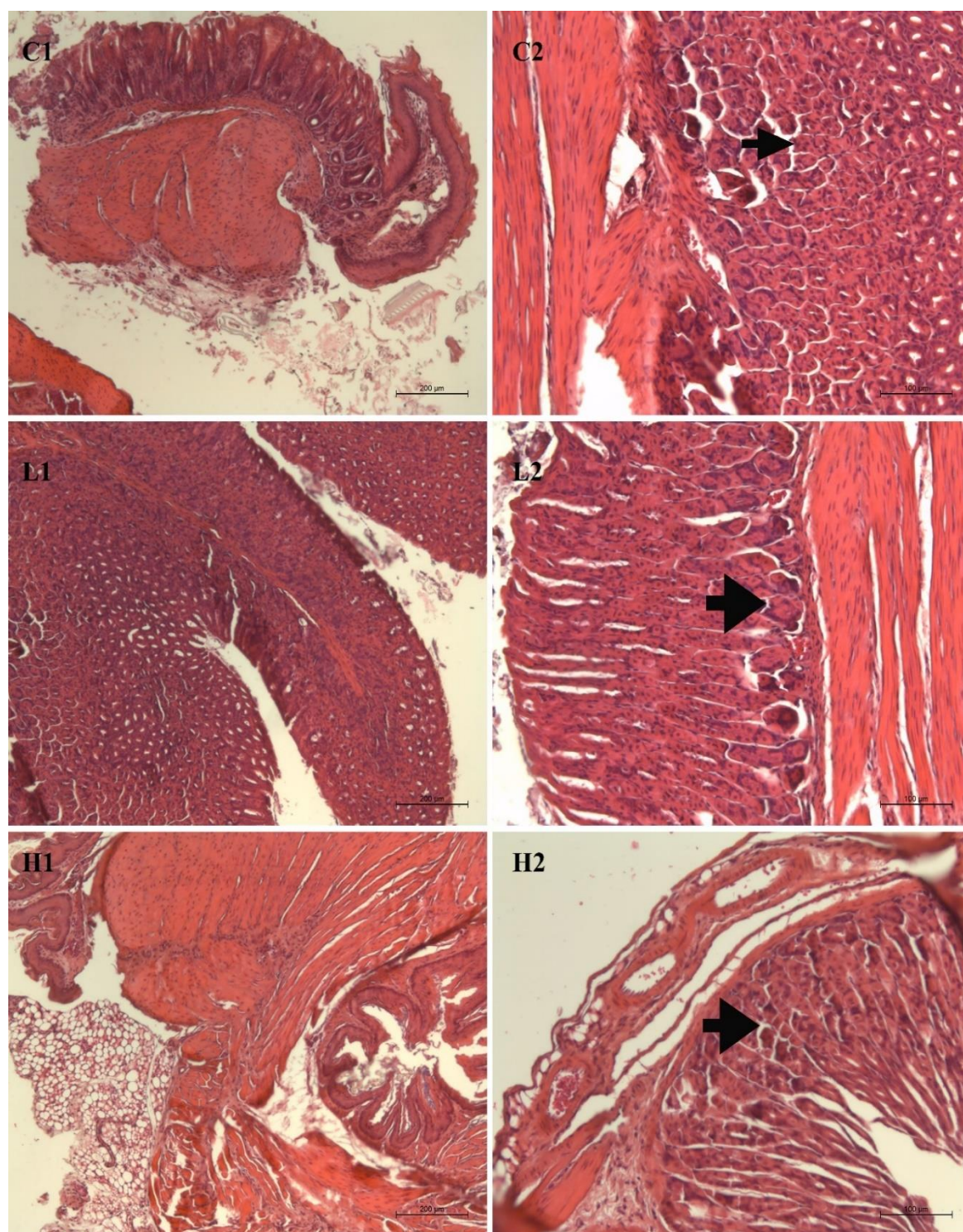


Figure C.3. Histopathological images of stomach tissue in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (10 $\times$, H&E stained) and C2-L2-H2 (20 $\times$, H&E stained). Arrows show gastric glands in the mucosa. Scale Bars: 200 μ m and 100 μ m.

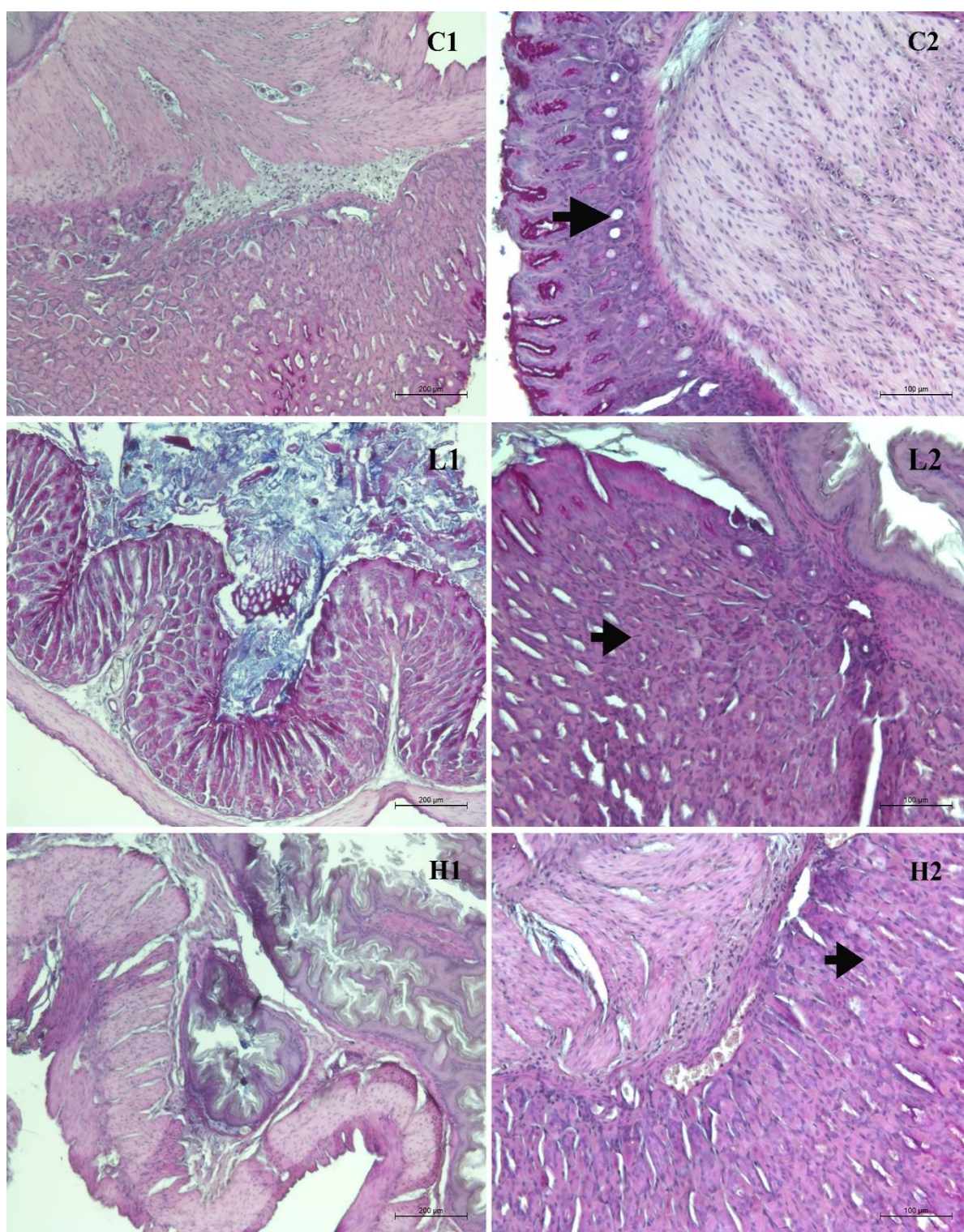


Figure C.4. Histopathological images of stomach tissue in control experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (10 $\times$, PAS stained) and C2-L2-H2 (20 $\times$, PAS stained). Arrows show gastric glands in the mucosa. Scale Bars: 200 μ m and 100 μ m.

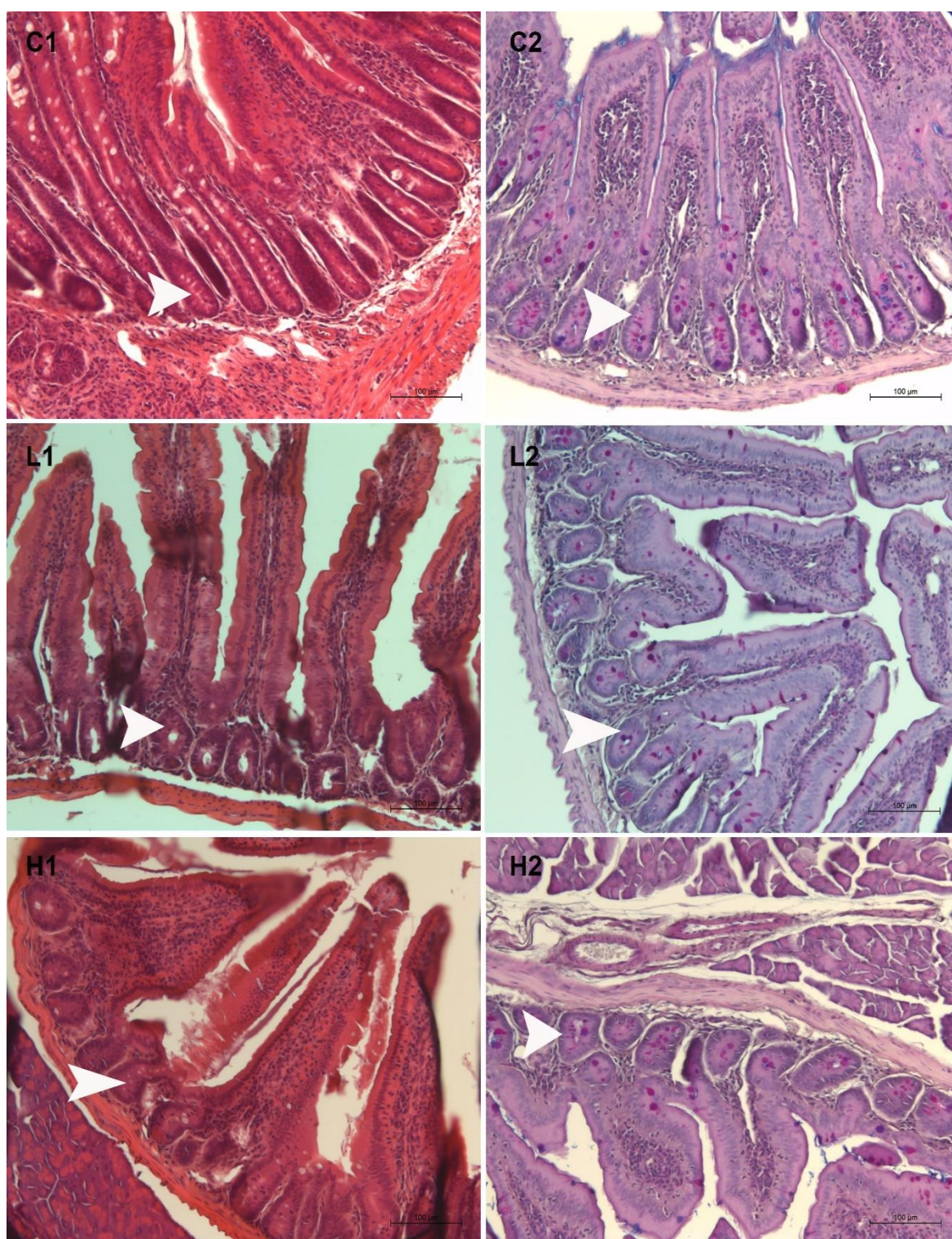


Figure C.5. Histopathological images of jejunum in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (20 $\times$, H&E stained) and C2-L2-H2 (20 $\times$, PAS stained). White arrowhead shows intestinal glands in the mucosa. Scale Bars: 200 μ m and 100 μ m.

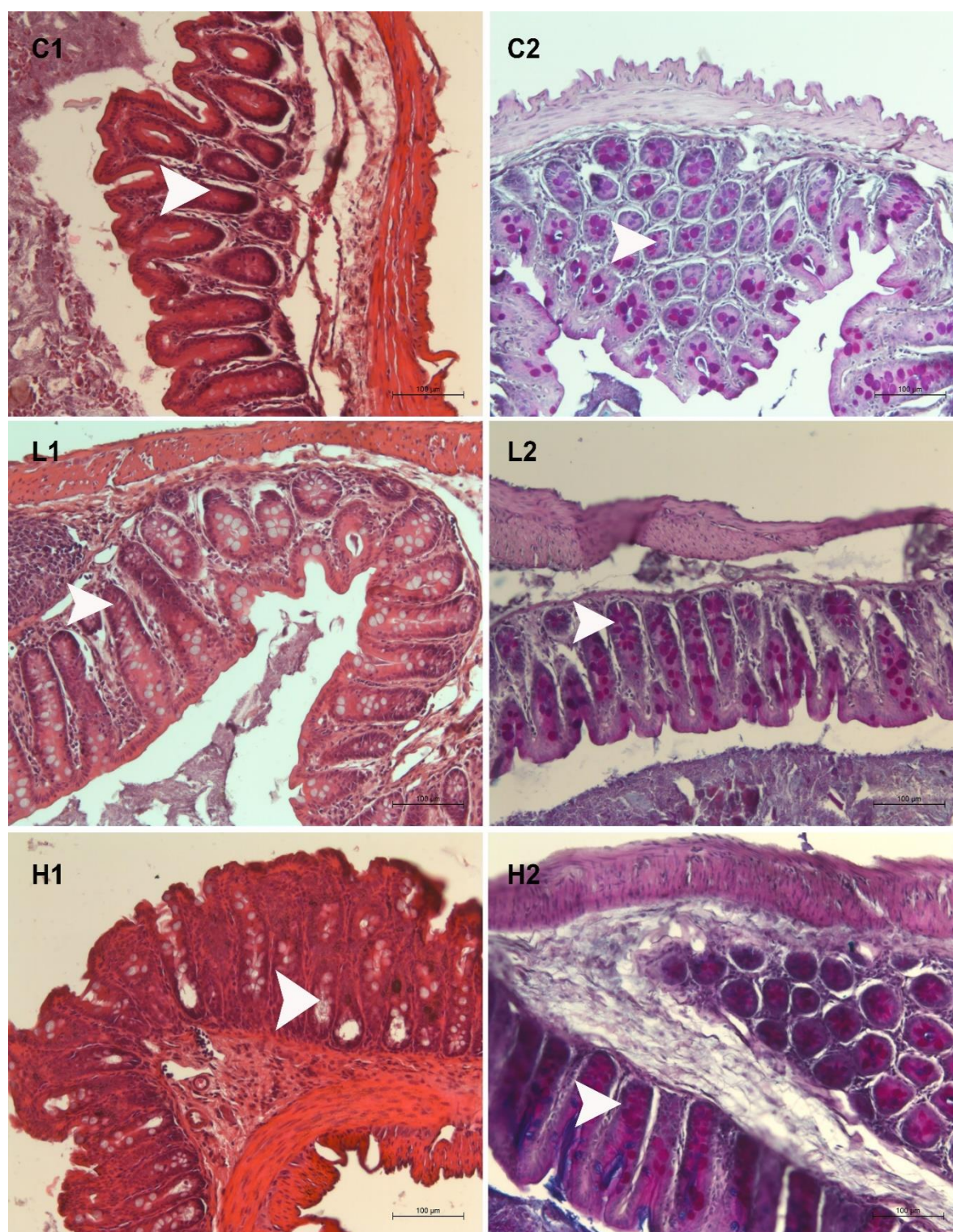


Figure C.6. Histopathological images of colon tissue in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (20 $\times$, H&E stained) and C2-L2-H2 (20 $\times$, PAS stained). White arrowhead shows intestinal glands in the mucosa. Scale Bars:100 μ m.

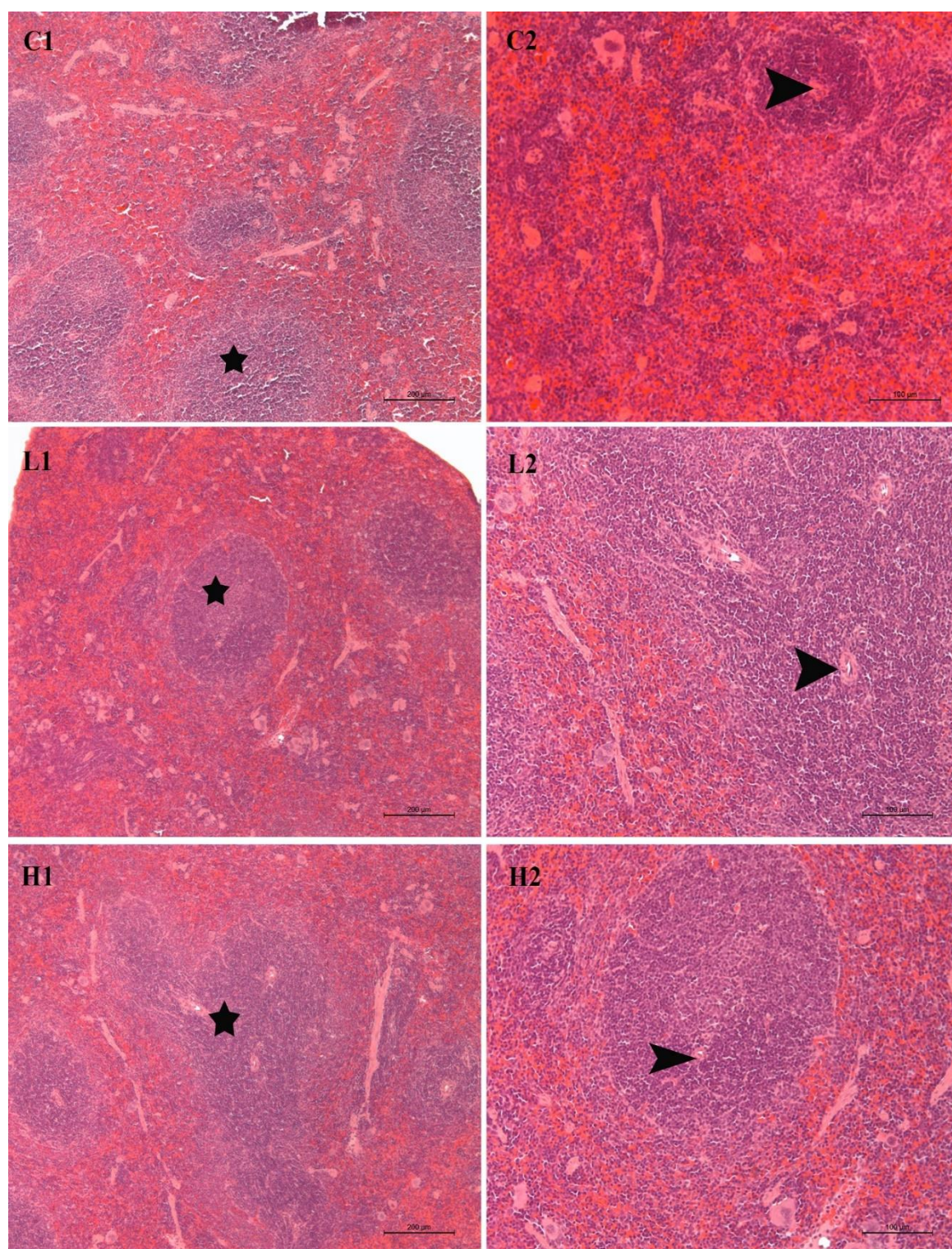


Figure C.7. Histopathological images of spleen tissue in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (10 $\times$, H&E stained) and C2-L2-H2 (20 $\times$, H&E stained). Star and arrowhead show the white pulp and central arteries in the parenchyme of spleen. Scale Bars: 200 μ m and 100 μ m.

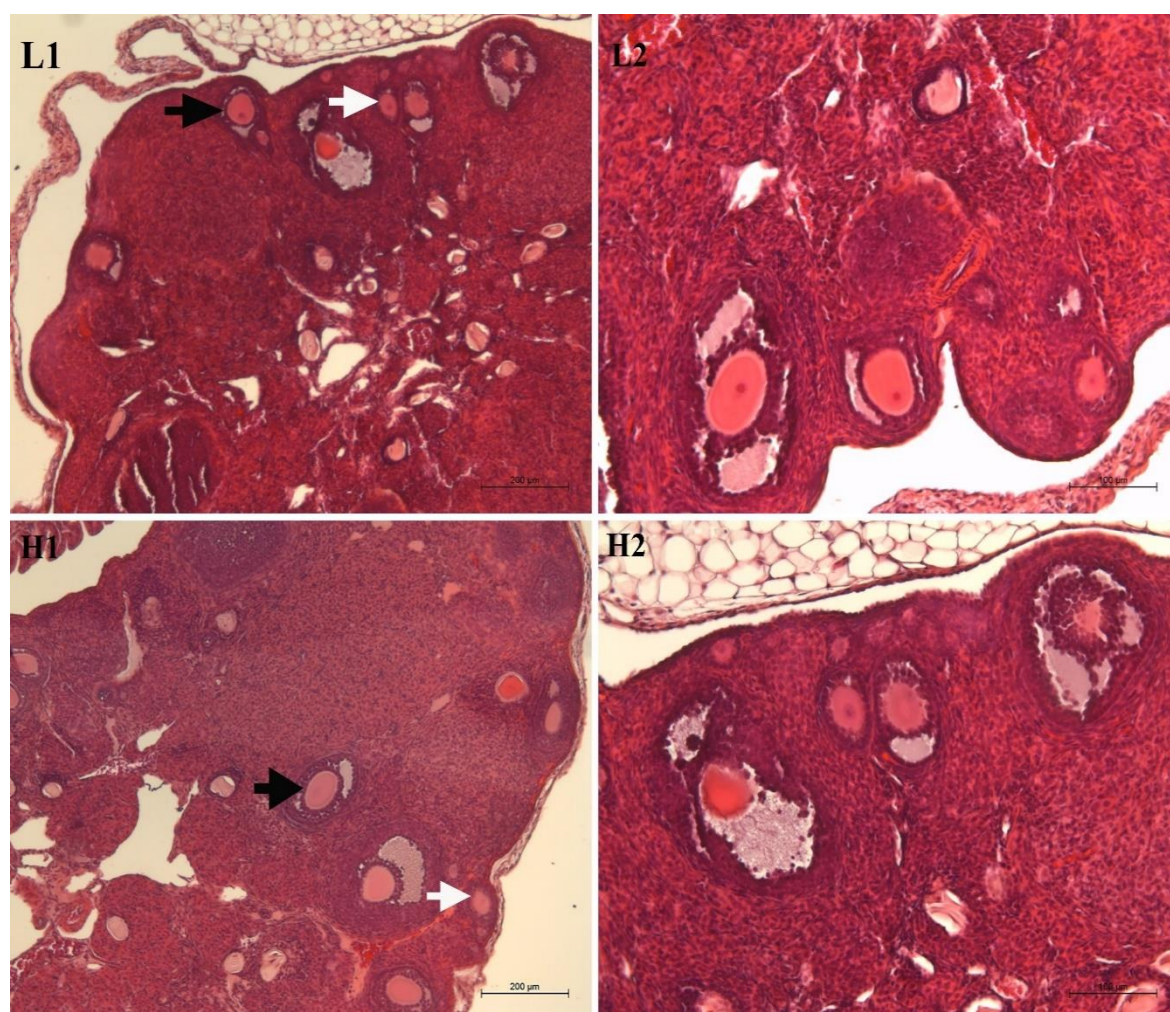


Figure C.8. Histopathological images of ovary tissue in experimental groups. L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). L1-H1 (10 $\times$, H&E stained) and L2-H2 (20 $\times$, H&E stained). Black arrow shows secondary follicle and the white arrow shows the primary follicle in the cortex of ovary. Scale Bars: 200 μ m and 100 μ m.

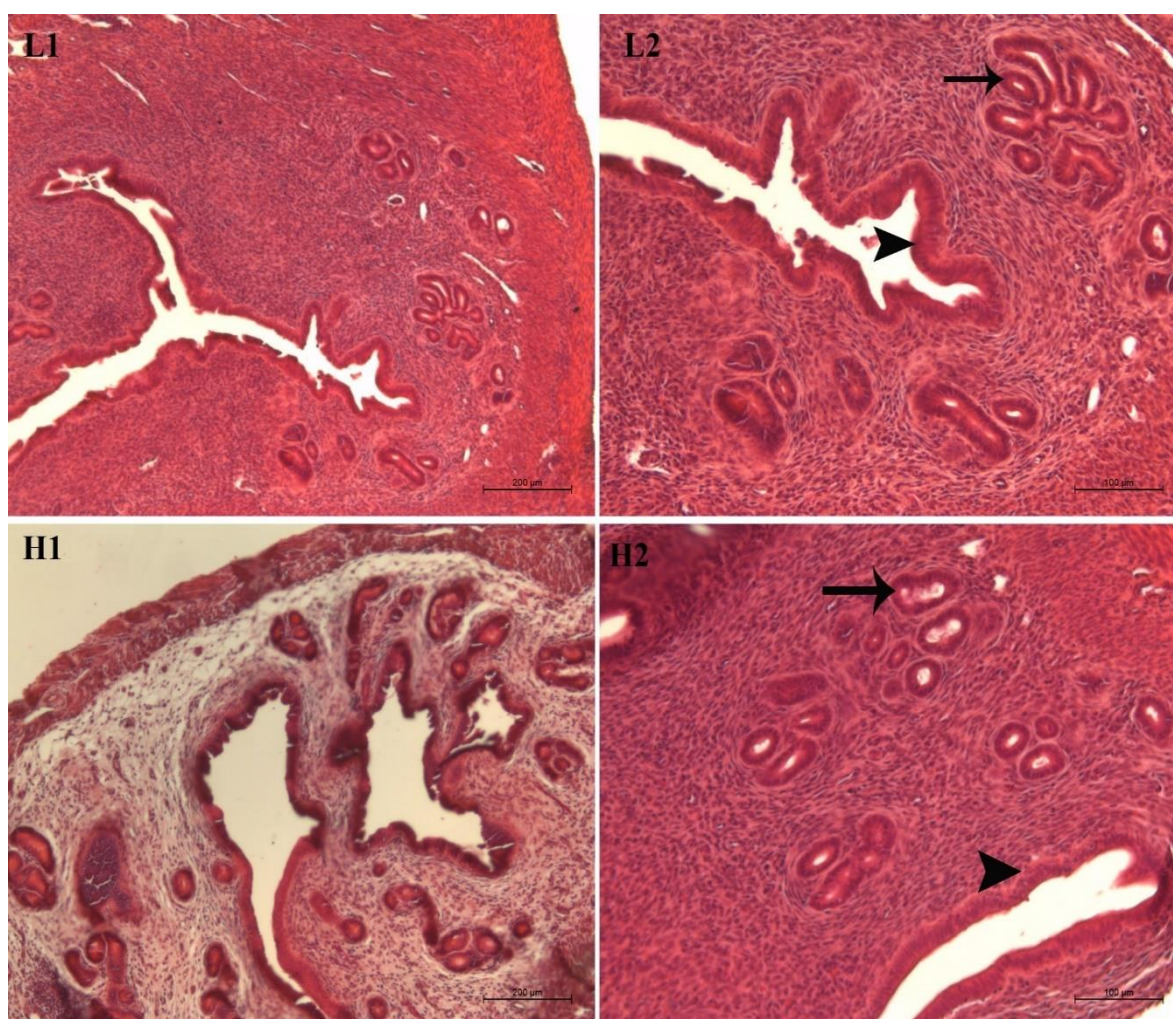


Figure C.9. Histopathological images of uterus tissue in experimental groups. L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). L1-H1 (10 $\times$, H&E stained) and L2-H2 (20 $\times$, H&E stained). Arrow shows normal glands and the arrowhead shows the epithelium of tunica mucosa. Scale Bars: 200 μ m and 100 μ m.

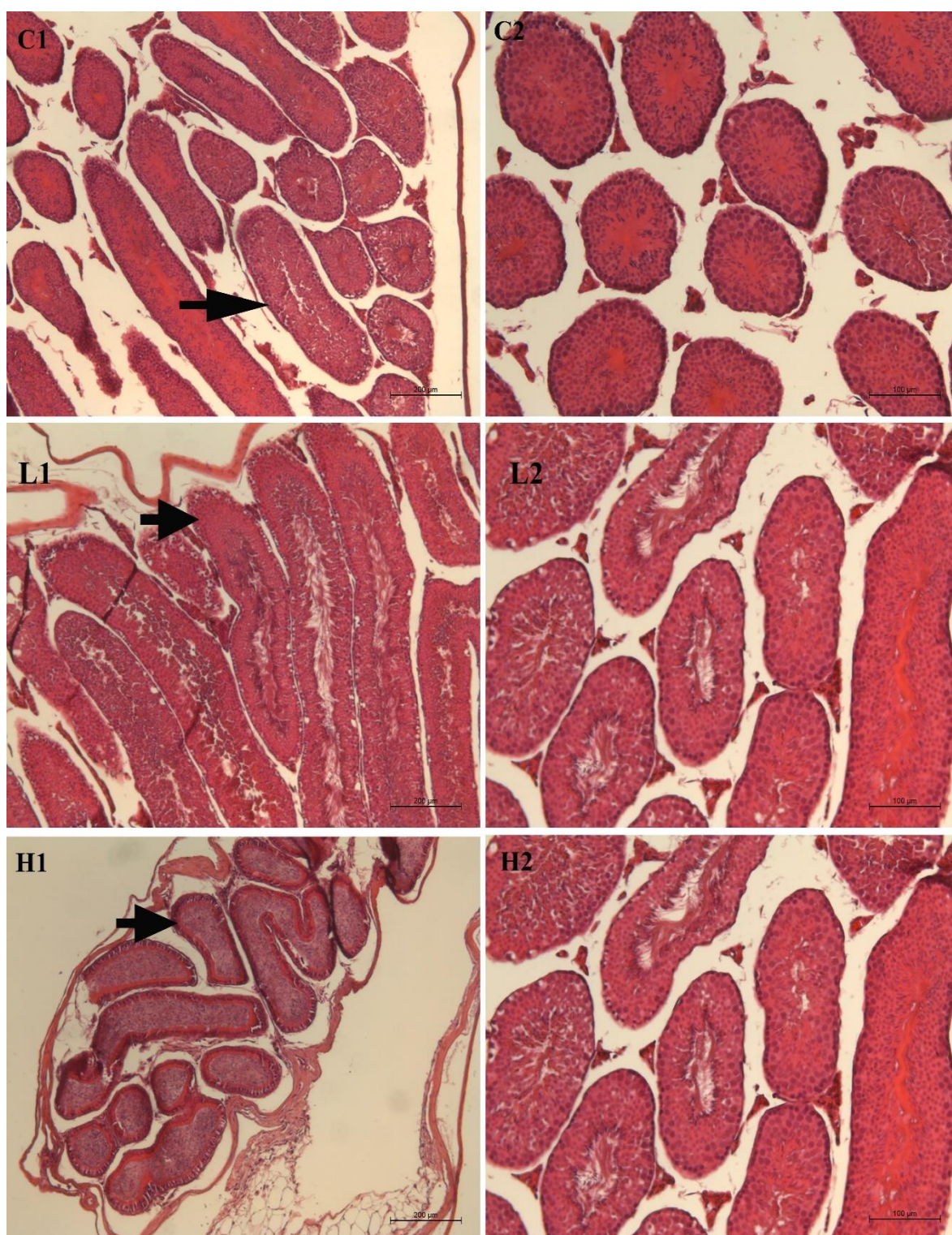


Figure C.10. Histopathological images of testis in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (10 $\times$, H&E stained) and C2-L2-H2 (20 $\times$, H&E stained). Arrow shows normal seminiferous tubules of the parenchyme of testis. Scale Bars: 200 μ m and 100 μ m.

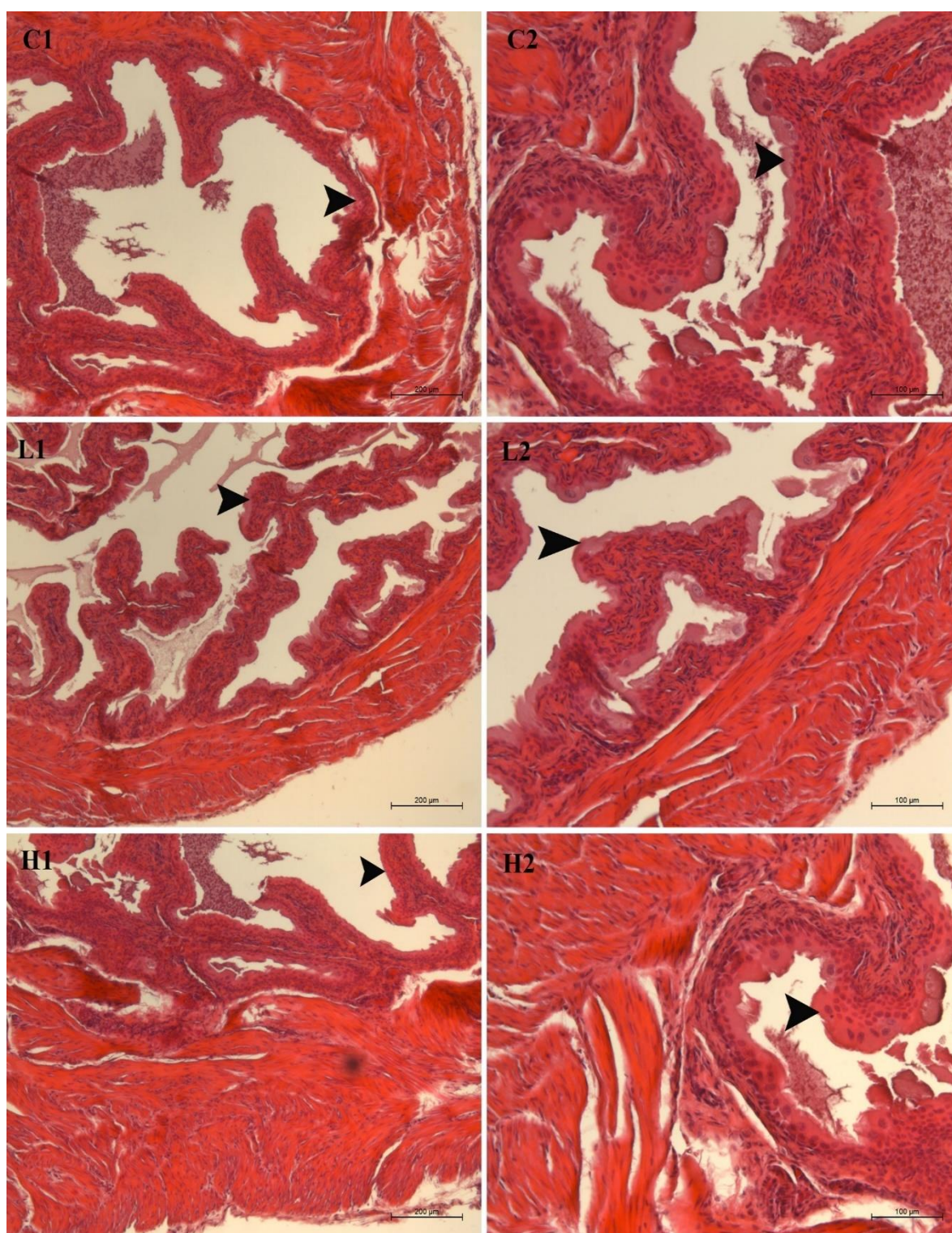


Figure C.11. Histopathological images of urinary bladder tissue in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (10 $\times$, H&E stained) and C2-L2-H2 (20 $\times$, H&E stained). Arrowhead shows transitional epithelium of the mucosa. Scale Bars: 200 μ m and 100 μ m.

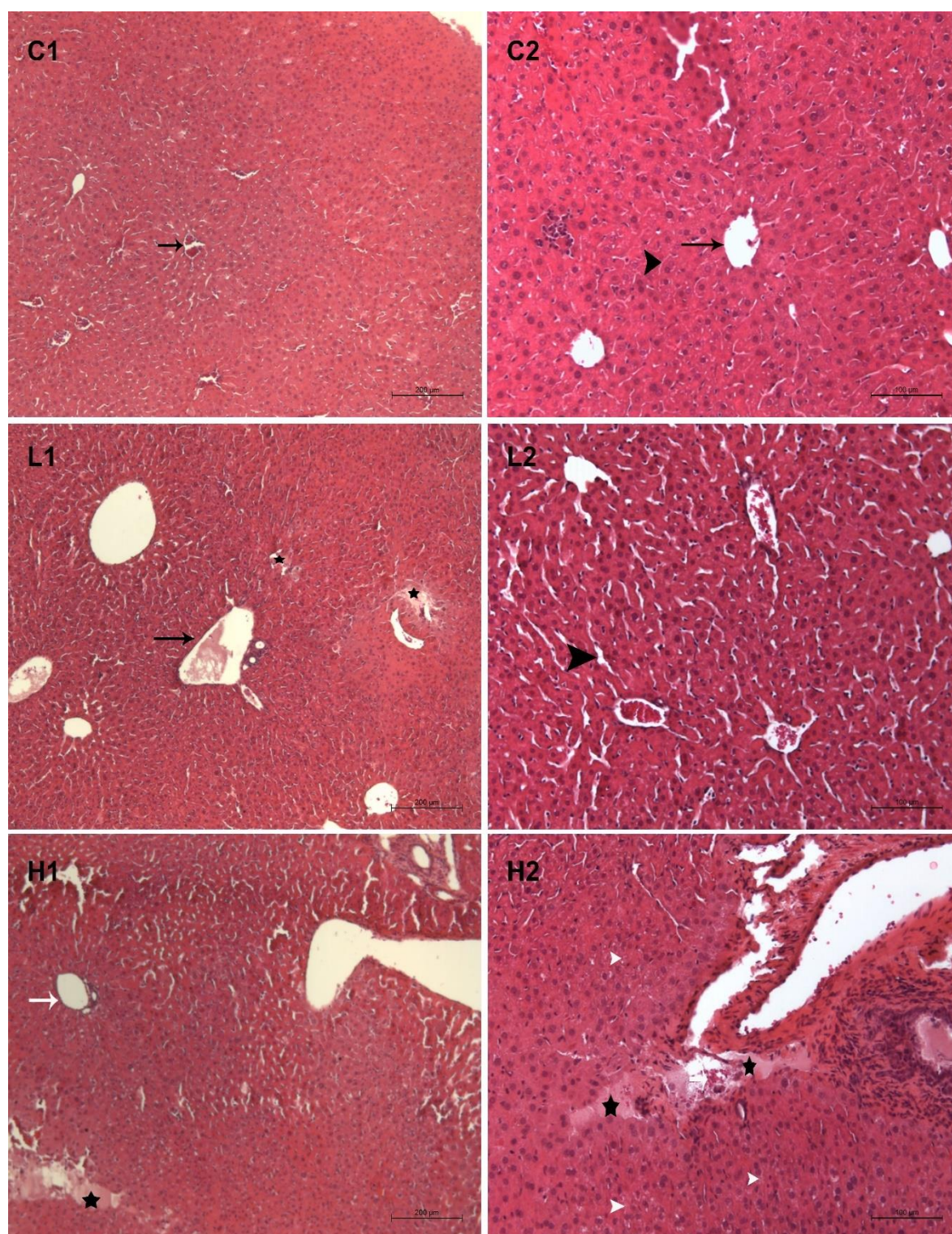


Figure C.12. Histopathological images of liver tissue in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (10 $\times$, H&E stained) and C2-L2-H2 (20 $\times$, H&E stained). Sinusoids (black arrowheads), vacuoles (white arrowheads), central veins (arrows) and edema (stars) show in the parenchyme of the liver. Scale Bars: 200 μ m and 100 μ m.

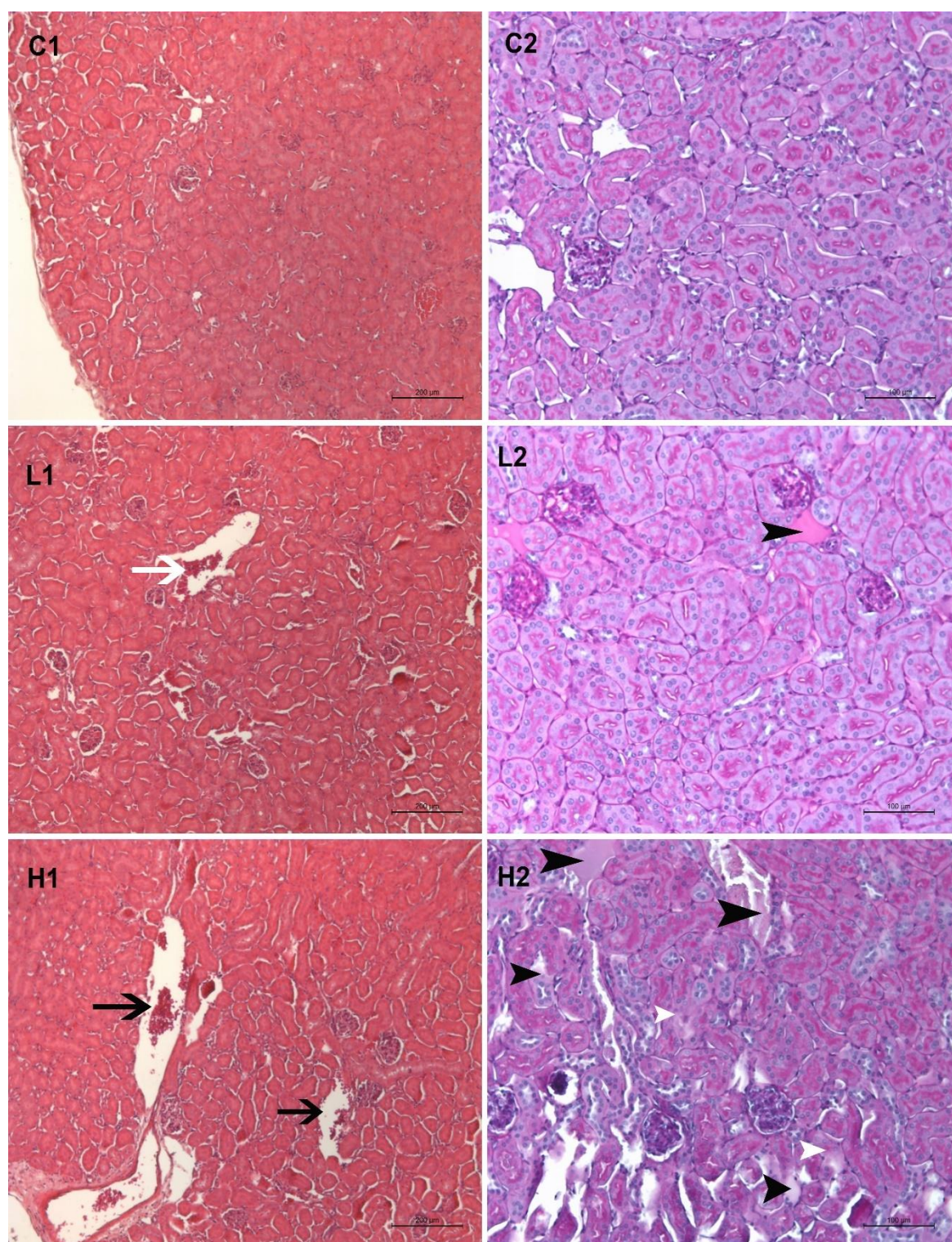


Figure C.13. Histopathological images of kidney tissue in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (10 $\times$, H&E stained) and C2-L2-H2 (10 $\times$, PAS stained). Proximal tubule (arrowheads), distal tubule (white arrowheads) and renal corpuscle (arrows) show in the parenchyme of the kidney. Scale Bars: 200 μ m and 100 μ m.

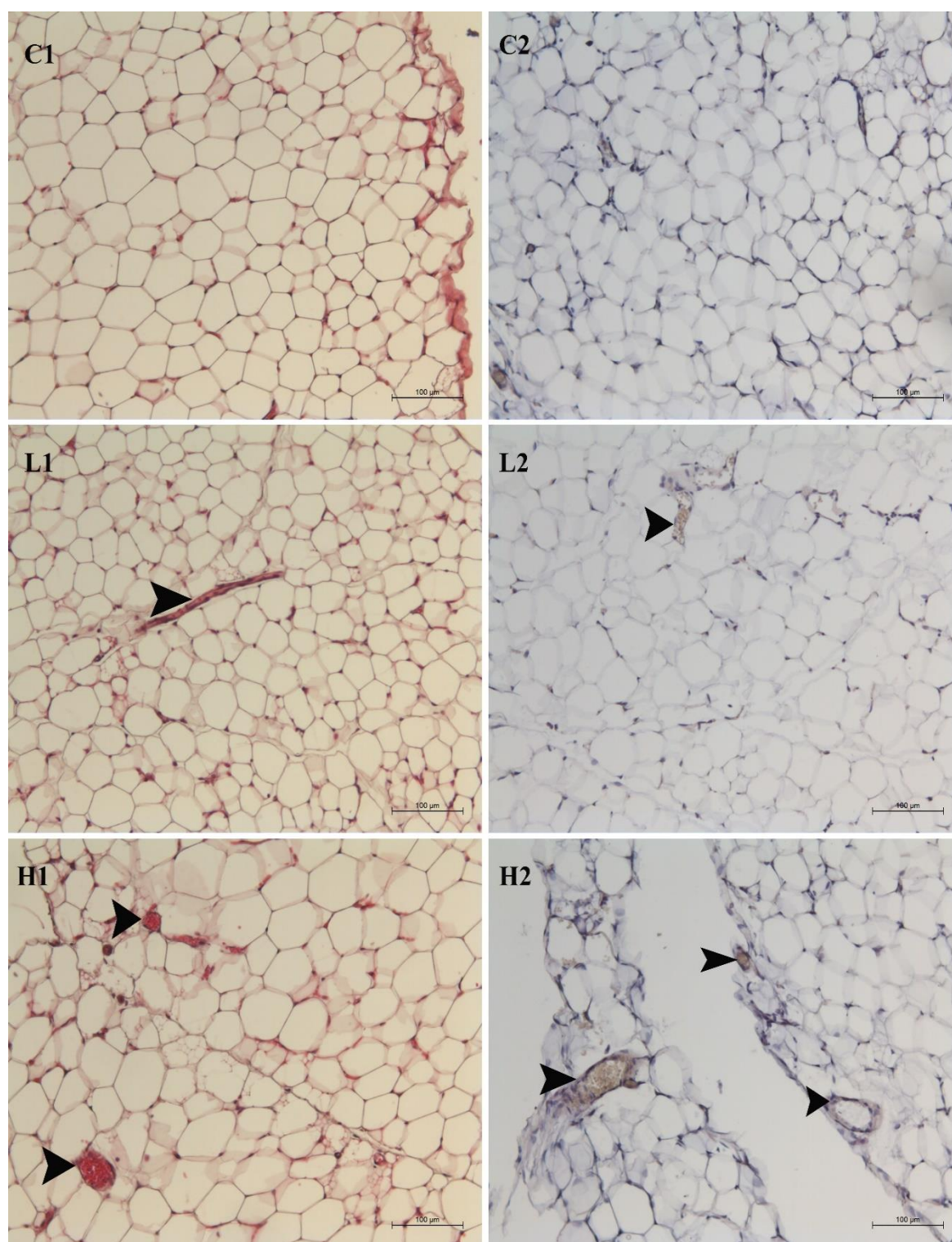


Figure C.14. Histopathological images of adipose tissue in control and experimental groups. C. control, L. low dose ECB (500 mg/kg) and H. high dose ECB (2000 mg/ml). C1-L1-H1 (20 $\times$, H&E stained) and C2-L2-H2 (20 $\times$, oil red stained). Arrowheads show new blood vessels and bleeding of the adipose tissue. Scale Bars: 100 μ m