PROBIOTIC METABOLITES OF OLIGOMERIC PROANTHOCYANIDIN REDUCE NICOTINE-DERIVED NITROSAMINE KETONE ACETATE-INDUCED DNA DAMAGE IN HUMAN LUNG EPITHELIAL AND FETAL HEPATIC CELLS *IN VITRO*

by

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I dedicate this thesis to my family

and those who thrive against cancer

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ABSTRACT

Potential of proanthocyanidin (PAC) in cancer risk reduction is dependant on the biotransformation process by colonic microbiotia. Seven selected microbial metabolites of proanthocyanidins (MMP) were evaluated for their ability to reduce 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone (NNKOAc)-induced DNA damage in human lung epithelial (BEAS-2B) and fetal hepatic (WRL-68) cells. Pyrogallol (PG) in BEAS-2B cells and pyrocatechol (PC) in WRL-68 cells reduced the DNA damage as measured by the γ-H2A.X assay. Western blot analysis suggested that PG- and PC-mediated DNA damage reduction is driven by alterations in the DNA damage response (DDR) pathways. Oligomeric apple peel (AP)-PAC and commercial grape seed (GS)-PAC were biotransformed by *Lactobacillus rhamnosus* and *Lactobacillus casei*. Phenolic metabolites identified after biotransformation of GS-PAC by *L. rhamnosus* were PG, PC, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, methyl hippuric acids, 4-hydroxyphenylvaleric acid, and sinapic acid. To confirm the PAC-mediated chemoprevention, an experimental animal model of NNKOAc-induced A/J mice can be recommended.

LIST OF ABBREVIATIONS AND SYMBOLS

AFC	Aberrant crypt foci
AKT	Protein kinas B
AMPK	AMP-activated protein kinase
ASK1	Apoptosis signal-regulating kinase 1
ATCC	American type culture collection
ATM	Ataxia-telangiectasia mutated protein
ATR	Ataxia telangiectasia and Rad 3 related protein
BAX	BCL-2 associated X protein
BEAS-2B	Human bronchial epithelial cells
BEBM	Bronchial epithelial basal medium
BEGM	Bronchial epithelial growth medium
BHI	Brain heart infusion
BNIP3	BCL-2 Interacting protein 3
BSA	Bovine serum albumin
CE	Catechin equivalence
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CIN	Chromosome instability
COX-2	Cyclooxygenase-2
CYP	Cytochrome P
DAPK2	Death-associated protein kinase 2
DDR	DNA damage response
DMAC	4-dimethylaminocinnamaldehyde
DMH	1, 2-Dimethylhydrazine
DMSO	Dimethyl sulfoxide
DSB	DNA double-strand breaks
DSS	Dextran sulphate sodium
ECG	Epicatechin gallate
ECL	Enhanced chemiluminescence
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulating kinase
ESI	Electrospray ionization
ESI^-	Negative ion mass
FACS	Fluorescence-activated cell
GS	grape seed
GSTT2	Glutathione-S-transferase theta 2

HRMS	High resolution mass spectrometry
IFN-γ	Interferon-γ
IL-12	Interleukin 12
IL-4	Interleukin 4
JAK2	Janus kinase 2
MEK	Mitogen-activated protein kinase kinase
MEME	Minimum essential medium eagle
MIN	Microsatellite instability
MMP	Microbial metabolites of proanthocyanidin
MRS	De Man, Rogosa, and Sharpe
MS/MS	Tandem mass spectrometry
mTOR	mechanistic target of rapamycin
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNKOAc	4-[(acetoxymethyl)nitrosamino]-1-(3-pyridyl)-1-butanone
ºC	Celsius
p53	Tumour protein
PAC	Proanthocyanidin
PBS	Phosphate buffered saline
PC	Pyrocatechol
PG	Pyrogallol
PI3K	Phosphatidylinositol-3-kinase
PMS	1-Methoxy phenazine
POB-DNA	Pyridyloxobutylation of DNA
PVDF	Polyvinylidene difluoride
QToF	Quadrupole time of flight mass spectrometry
RhOA	Ras homolog gene family, member A
RIPA	Radio-immunoprecipitation assay
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulphate
ssDNA	Single stand DNA
STAT3	Signal transducer and activator of transcription 3
TBST	Tris-buffered saline with tween 20
TNF-α	Tumour necrosis factor-α
UHPLC	Ultra high-performance liquid chromatography
UPLC	Ultra performance liquid chromatography
WRL-68	Human fetal hepatic cells

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CHAPTER 1: INTRODUCTION

Cancer, the fastest growing and second deadliest disease on the planet, is truly a collection of diseases sharing similar characteristics. Humans are susceptible to over 277 different types of cancers, narrowing the chance to discover effective treatments (Hassanpour and Dheghani, 2017). The global cancer burden was reported high in the year 2012 with 14.1 million new cancer cases and 8.2 million deaths. The number of new cancer cases is expected to increase in the future due to the aging population (Torre *et al*., 2015), growing the need for effective cancer preventive strategies.

Many of the cancer preventive strategies suggest simple life style changes such as being physically active, maintaining of healthy body weight, and confinement of tobacco use (Brawley, 2017). However, the cancer preventive capacity of such strategies varies among individuals, highlighting the strong relationship of cancer with genetic inheritance (Hojman *et al*., 2018). Cancer chemoprevention involves more complex strategies tied to molecular genetics. Cancer chemoprevention is the utilization of chemical agents (natural or synthetic) to reverse, suppress or prevent cancer progression into metastatic stage (Tsao *et al*., 2004). The chemopreventive strategies are categorized as primary, secondary, and tertiary approaches. Primary strategies prevent cancer initiation in the first place while secondary and tertiary approaches reduce cancer progression and relapse, respectively (Correll and Barqawi, 2011).

An alteration in genomic materials (DNA) is a key reason for carcinogenesis. Such damages are a result of exogenous stimuli, endogenous metabolic process or combination of both (De Bont and Larebeke, 2004). DDR system of eukaryotic cells is a sophisticated signal transduction pathway which enables the cellular level detection and responses for

DNA damages. ATM-Chk2 and ATR-Chk1 are the main cellular DDR pathways. Repairing of DNA damages are performed by carefully regulated enzymatic processes to restrict undesired changes for healthy DNA molecules (Ciccia and Elledge, 2010). The enzymes involved in DNA rectifying mechanisms include nucleases, helicases, polymerases, topoisomerases, recombinases, ligases, glycosylases, demethylases, kinases, and phosphatases (Ciccia and Elledge, 2010). Proper measures in prevention of DNA damage, especially the damage of endogenous origin are substantially important in cancer chemoprevention (De Bont and Larebeke, 2004).

The diet is an easily modifiable, yet a significant cancer risk determinant. A diet rich in sugar, salt, and processed red meat increases cancer risk. Consumption of fruits, vegetables, whole grains, and pulses can considerably reduce cancer risk by providing the body with phytochemicals potent in chemoprevention (Norat *et al*., 2015). Phenolic compounds make the largest group of phytochemicals with over 50,000 heterogeneous compounds (Ziaullah and Rupasinghe, 2015). Dietary polyphenols exhibit chemopreventive and therapeutic activities (George *et al*., 2016) through reduction of cellular DNA damage by acting as antioxidants (Pandey and Rizvi, 2009) and interfering carcinogenesis by manipulation of signal transduction pathways important for proliferation, metastasis, and angiogenesis (Ramos, 2008).

However, the most abundant dietary polyphenols including proanthocyanidins (PAC) show limited bioavailability in the human body, hindering the potential for chemoprevention (Hu *et al*., 2017). PAC or condensed tannins are the oligomers and polymers of polyhydroxy flavan-3-ol monomers, especially catechins and epicatechins. PAC larger than four monomeric units are not absorbed in the small intestine (Ou and Gu,

2014). The majority of ingested PAC are collected in the colon to undergo microbial catabolism yielding a variety of phenylvalero lactones and simple phenolic acids (Lamuel-Raventos and Onge, 2017; Ou and Gu, 2014) that are easily absorbable and contribute in cancer prevention (Nemec *et al*., 2017; Kong *et al*., 2013). For example, biotransformation of PAC by *Lactobacillus casei-01* produces *p*-coumaric acid (Li *et al*., 2013) which is rapidly absorbed (Konishi *et al.,* 2004) and proficient in inhibiting angiogenesis (Kong *et al*., 2013). Simple metabolites produced by the gut microbiota are capable of the reprogramming of the epigenome through influencing of enzymes such as methyltransferases, acyltransferases, and phosphotransferases (Bhat and Kapila, 2017). These enzymes are involved in the methylation, acetylation, and phosphorylation of DNA and histones, thus controlling the gene expressions. Also, the ability of dietary polyphenols to promote the growth of beneficial gut microbes is appreciated in many studies (Bhat and Kapila, 2017). Therefore, dietary polyphenols and gut microbiota resemble a relationship of prebiotics and probiotics which could result in important health promotion in humans.

The constituent of gut microbiome greatly varies among individuals depending on diet, life style (Teixeira *et al*., 2017) and disease conditions (Margalef *et al*., 2017). Therefore, dietary ingestion of polyphenols may not be equally beneficial for every individual. The interdependency of dietary polyphenols and gut microbiome to contribute to cancer prevention can be overcome by the development of "synbiotics." A synbiotic is a single package containing both prebiotics and probiotics (Gourbeyre *et al*., 2011), mitigating the limitations caused by unavailability of corresponding prebiotics or probiotics. This study is focussed on biotransformation of PAC using common probiotic

bacteria and evaluating the cancer prevention potential of the resulted simple phenolic metabolites.

1.1 Hypothesis

Biotransformation of PAC by probiotic bacteria generates simple phenolic metabolites potent in significant reduction of carcinogen-induced DNA damage in vitro.

1.2 Research objectives

Overall objective

To evaluate the potential of probiotic metabolites of PAC in the reduction of carcinogen-induced DNA damage using experimental cell models of NNKOAc-induced DNA damage in BEAS-2B and WRL-68 cells.

Specific objectives

1) To extract oligomeric and polymeric PAC from apple peels.
2) To biotransform extracted PAC into simple phenolic metabolites by using probiotic bacteria, *Lactobacillus rhamnosus* and *Lactobacillus casei*.
3) To characterize the phenolic metabolites from biotransformation using ultra high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS).
4) To evaluate the reduction of carcinogen-induced DNA damage by probiotic metabolites of PAC using NNKOAc-induced cytotoxicity and genotoxicity in BEAS-2B and WRL-68 cells.

CHAPTER 2: LITERATURE REVIEW

2.1 Carcinogenesis

Carcinogenesis is the multi-stage process of cancer generation (Fernando and Rupasinghe, 2013). This process is possible to elaborate on four conceptual phases, initiation, promotion, malignant conversion, and progression (Weston and Harris, 2003). Initiation phase is involved with the rapid introduction of irreversible and/or epigenetic changes to the cell's genome, commonly as a result of being exposed to a carcinogen (Rupasinghe *et al*., 2014). Epigenetic effects are characterized by methylation of the promoter region of genes with tumor suppression potential, making it impossible for transcription, thus eliminating the gene's expression (Arita and Costa, 2009). Activation of proto-oncogenes (conversion of proto-oncogene into oncogene) often by producing DNA-chemical carcinogen adducts, has been identified as one of the major factors for cancer initiation (Weston and Harris, 2003). Changes in proto-oncogenes lead for production of abnormal proteins which may interfere with proper regulation of cell activities such as growth and apoptosis (Ward *et al*., 1994). Thus, cancer initiation is a result of human genome instability. An instable genome is more likely to undergo genomic alterations, attributed by gene mutations, rearrangement or deletion of chromosome segments, repetition of nucleotide sequences and changes in the number of chromosomes (Shen, 2011). Genomic instability is contributed by both endogenous metabolic processes, for example, oxidation and alkylation, and exposure to carcinogens (Jia, 2015). Genomic alterations are categorized as microsatellite instability (MIN) and chromosome instability (CIN) (Charames and Bharati, 2003). MIN results in the loss of cellular ability for correction of mismatched nucleotides during DNA replication and therefore is associated

with alterations in DNA strand (Boland and Goel, 2010). However, CIN is associated to much larger alterations, including a differed number of chromosomes and modifications to the chromosomal structure (Heng *et al.,* 2013).

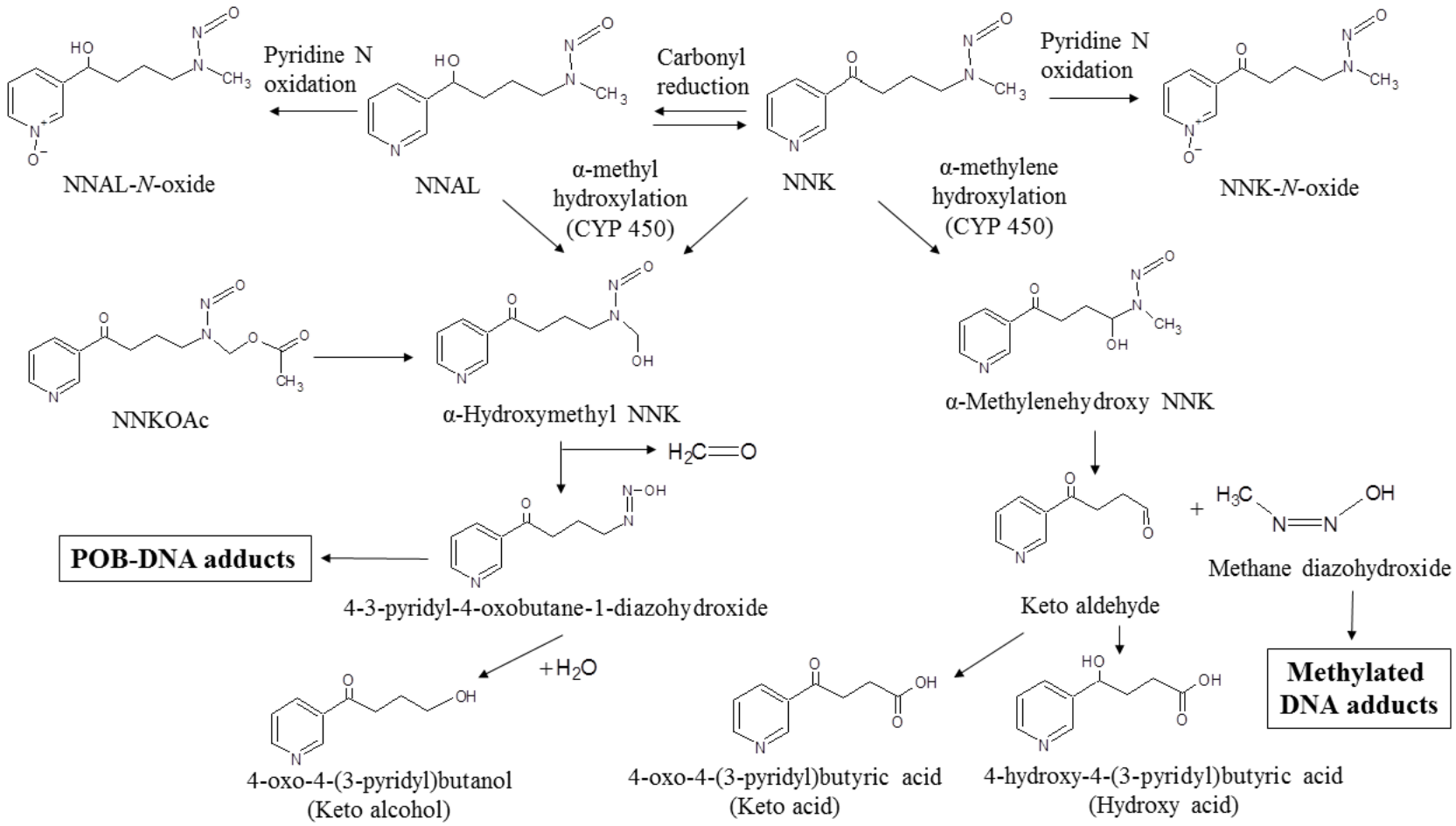
During cancer promotion, cells with genetic defects replicate and form a colony of cells (preneoplastic cells) which are susceptible to further genetic changes and ready to experience malignant conversion. Functions of tumor promoters must be taken into consideration during this phase as they are capable to reduce the cancer latency period. Also, they are potent in increasing number of tumors in diseased tissue. Tumor promoters are not carcinogens but boost the functionality of real carcinogens allowing them to produce initiator cells under lower dosages than what is actually required (Weston and Harris, 2003). The cancer promotion phase takes a longer time to be completed and the cellular changes during this period are suspected for reversibility, especially during early stages. Therefore, most of the cancer prevention strategies aim to be active during promotion stage (Murakami, 1996).

In the malignant conversion phase, the preneoplastic cells are further subjected to genomic changes and converted into malignant phenotypes which are highly uncontrollable and invasive. The degree of malignant cell conversion is a function of replication rate and the ability of preneoplastic cells to replicate. Availability of tumor promoters during this entire phase is more important in promoting malignant conversion rather than exposure to larger doses for a shorter period. If the promoters are not available at the beginning of this phase, there is a significant opening in degeneration of preneoplastic lesion (Weston and Harris, 2003). Furthermore, erroneous DNA replication and exposure of preneoplastic cells to carcinogens contribute greatly to this phase (Yuspa

et al., 1988; Loeb and Cheng, 1990). The final stage of carcinogenesis, cancer progression, is associated with the malignant phenotype expression and gearing of malignant cells with higher virulent characteristics (Weston and Harris, 2003). Malignant phenotypes are characterized by genomic instability and unregulated cell growth (Lengauer *et al.,* 1998). Some malignant cells acquire the ability in the secretion of proteases which in turn provide them with the ability to invade other healthy tissues of the body, making cancer a metastatic disease (Weston and Harris, 2003).

2.1.1 NNKOAc-mediated carcinogenesis

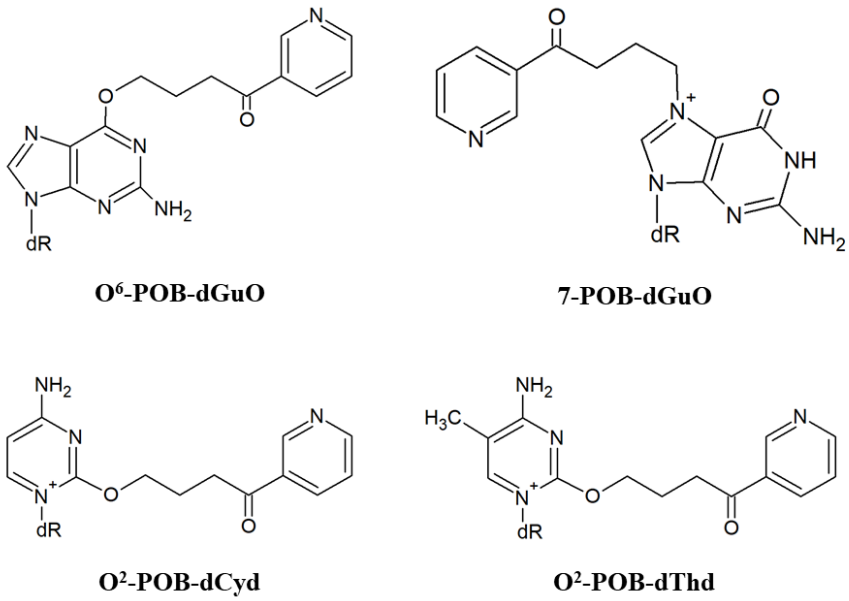
NNKOAc is a commonly used chemical carcinogen to resemble the carcinogenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is a confirmed chemical procarcinogen in cigarette smoke and tobacco products. Thus, NNK and NNKOAc are frequently used in studies related to lung cancers (Hecht *et al*., 2016; Cloutier *et al*., 2001). Activation of the procarcinogen NNK is complex and involves a series of enzyme regulated reactions (Fig.1) that make the detection and quantification of cellular DNA damage more difficult (Cloutier *et al*., 2001). Cellular metabolism of NNK occurs through three major chemical reactions, carbonyl reduction, pyridine nitrogen oxidation and methyl or methylene carbon hydroxylation (Hecht *et al*., 2016). Carbonyl reduction of NNK yields 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which then undergo α-hydroxylation aided with cytochrome P450 (CYP 450) to generate electrophilic mediators (diazohydroxides).



(Sources: Hecht *et al*., 2016; Yalcin and Monte, 2016; Maertens *et al*., 2010; Hecht *et al*., 1993)

Figure 1: Cellular metabolism of 4-[(acetoxymethyl)nitrosamino]-1-(3-pyridyl)-1-butanone (NNKOAc) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

These electrophilic mediators cause pyridyloxobutylation of DNA (POB-DNA) forming bulky DNA adducts (Fig. 2), altering genomic expression (Hecht, *et al*., 1993). Oxidation of pyridine nitrogen of NNK and NNAL yields non-toxic -*N*- oxides, excreted from the body with urine (Hecht *et al*., 1993). α-Hydroxylation (contributed by CYP 450) of NNK and NNAL produce more electrophilic metabolites (methane diazohydroxides) that promote DNA damage through methylation, pyridyloxobutylation and pyridylhydroxybutylation-mediated adduct formation (Yalcin and Monte, 2016).



(Source: Brown *et al*., 2010)

Figure 2: Chemical structures of POB-DNA adducts formed by NNK and NNKOAc. O^6-POB-dGuo, O^6-[4-(3-pyridyl)-4- oxobut-1-yl]-2′-deoxyguanosine; 7-POB-dGuo, 7-[4-(3-pyridyl)-4-oxobut-1-yl]-2′-deoxyguanosine; O^2-POB-dCyd, O^2-[4- (3-pyridyl)-4-oxobut-1-yl]-2′-deoxycytidine; O^2-POB-dThd, O^2-[4-(3- pyridyl)-4-oxobut-1-yl]thymidine

Cytochrome P450 activity is necessary for the activation of NNK to yield metabolites capable of DNA damage (Hecht *et al*., 1993). However, CYP 450 activity of

in vitro cell lines greatly varies rising concerns on the direct use of NNK to induce DNA damage. CYP activity of BEAS-2B and human alveolar basal (A549) epithelial cells is significantly weak (Garcia-Canton *et al*., 2013). NNKOAc is commonly used with such cell lines to induce DNA damage as the activation does not require CYP activity. Conversion of NNKOAc into α-hydroxymethyl NNK, the common intermediate metabolite shared by both NNK and NNKOAc, requires the activity of cellular esterase enzymes (Ma *et al*., 2015). Therefore, the carcinogenicity of NNKOAc is mainly explained by its ability to disturb normal genomic expression by the formation of bulky POB-DNA adducts. DNA adducts resulted by NNK can activate oncogenes such as *KRAS* (Keohavong *et al*., 2011) and inactivate tumor suppressor genes such as TP53 (Ding *et al*., 2008), eventually arming the cells with cancerous characteristics, unregulated proliferation, apoptosis resistance, unregulated cell cycle, and deterred DNA damage repair mechanisms.

2.2 Cancer chemoprevention

Studies carried-out for the past three decades have convinced researchers that prevention of cancer is comparatively easier than treatments to cure or extend the five-year relative survival rate (Yu and Kong, 2007). By definition cancer chemoprevention is the "use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer" (Tsao *et al.,* 2004). According to this definition chemoprevention of cancer is stretched till the point of malignant phenotype generation, the state in which cancer become more virulent and metastatic. Even though chemoprevention is valid before the detection of cancer and chemotherapy comes into action after clear diagnosis, both aspects share common targets such as apoptosis activation, growth factor signaling suppression, and reduction of cellular anti-apoptotic

protein level (Aggarwal *et al.,* 2004). The need for better chemo-preventive approaches is mainly based on the increasing cancer-related deaths and harmful side effects of currently practiced treatments (George *et al*., 2017; Fernando and Rupasinghe, 2013; Sporn and Suh, 2002). Chemo-preventive agents are categorized as blocking and suppressing agents. The blocking agents are active in the inhibition of cancer initiation and the suppressing agents in the prevention of cancer progression mainly by inhibition of signal transduction pathways. However, most of the chemopreventive agents especially the plant food-derived ones are capable of acting as both blocking and suppressing agents (Steward and Brown, 2013). Resveratrol, diallyl sulphide, lycopene, eugenol, 6-gingerol, silymarin, and anethol are few of the well-studied phytochemicals for chemoprevention properties (Dorai and Aggarwal, 2004). Chemo-preventive approaches are divided as primary, secondary, and tertiary approaches. The primary approaches are focused to reduce the risk of cancer initiation while secondary approaches are tuned to reduce the risk of cancer progression. Tertiary approaches are to minimize the reoccurrence of new cancers in previously diagnosed but currently cured patients (Correll and Barqawi, 2011).

2.3 Polyphenols

Polyphenols, the largest group of phytochemicals, are consisted of 30,000 heterogeneous natural compounds (Ziaullah and Rupasinghe, 2015). Essentially every single plant is comprised of a mixture of these compounds to ensure plant's capability to adapt to ever-changing and challenging biotic and abiotic stress of their environment. Chemically a phenol composed of a combination of one or multiple hydroxyl groups with a C_6 aromatic ring. Simple phenolics; the phenolic acids and glycosylated variants are water soluble. Combination, polymerization and/or condensation of simple phenolic

monomers provide polyphenolic structures which are of higher molecular weight and mostly insoluble in water (Ziaullah and Rupasinghe, 2015).

Phenolic compounds are classified based on their source of origin, biological functions, and chemical structure (Tsao, 2010). More often the classification is based on the chemical structure of the phenolic compound. All phenolic acids are derivatives of either hydroxybenzoates or hydroxycinnamates (Heleno *et al.,* 2015; Tsao, 2010). The benzoic variants are based on simple carbon backbone structure of $C_6 – C_1$ while same for cinnamic variants is $C_6 – C_3$, which resembles the carbon backbone of phenylpropanoid unit; the end product of major polyphenol cellular biosynthesis process termed as shikimic acid pathway (Ziaullah and Rupasinghe, 2015). Derivatives of these polyphenols differ from each other only by slight structural changes on positional variations in hydroxylation and methoxylation of the basic C_6 aromatic ring (Mattila *et al.,* 2006). Phenolic acids are available from a wide range of commonly consumed fruits and vegetables as well as from seeds or grains, especially associated with outer layers of seed kernels (Kim *et al.,* 2006; Tsao, 2010). *p*-Coumaric, caffeic, ferulic, and sinapic acids are the most common hydroxycinnamates found in fruits. *p*-Hydroxybenzoic, protocatechuic, vanillic, and syringic acids are the most common hydroxybenzoate derivatives of fruit origin. Phenolic acids are often available in conjugated forms, where the hydroxycinnamates esterify with quinic acid or glucose, and hydroxybenzoates produce glycosides (Mattila *et al.,* 2006).

The flavonoids are derivatives of benzo-gamma-pyrone and exist ubiquitously in plants as major pigments (Havsteen, 2002). All flavonoids share similar carbon backbone structure of $C_6–C_3–C_6$ which is evidently consisted of two C_6 aromatic rings termed as ring A and B (Fig. 3).

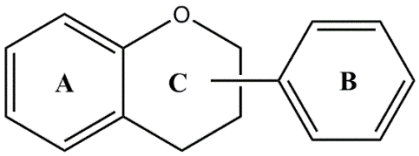


Figure 3: Flavonoid backbone structure of C_6–C_3–C_6.

The short C_3-chain which links the two aromatic rings is arranged to a heterocyclic ring (ring C), except in chalcones. The chalcones or (E)-1,3-diphenyl-2-propene-1-one acts as precursors of other flavonoids and isoflavones (Mahapatra *et al.,* 2015). Chalcones are available in fruits such as apples and hops; therefore, in the products derived by them such as wine and hop flavored beers (Tsao *et al.,* 2003; Zhao *et al.,* 2005). Isoflavones are phytoestrogens which can be found in around 300 suitable plant sources (Markovic *et al.,* 2015). In isoflavones the aromatic ring B is combined with the ring A through the C_3 position of the heterocyclic ring C. 7-*O*-Glucosides and 6"-*O*-malonyl-7-*O*-glucosides are the predominantly found forms of isoflavones in plant sources, notably in legumes such as soy beans and in red cloves (Tsao, 2002; Wang and Murphy, 1994; Mazur *et al.,* 1998).

Flavonoids are further sub-divided as isoflavones, flavones, flavonols, flavanones, flavanonols, and anthocyanidins. Structural differences in these sub groups are resulted by hydroxylation, methylation/acylation, isoprenylation, dimerization and/or glycosylation. Flavonols are the 3-hydroxy derivatives of flavones. Flavanols are also termed as catechins and distinct from other 3-hydroxy flavonoids by not being unsaturated at C_2 of aromatic ring B. The trans-configured monomeric isomers of this arrangement are referred to as catechins and the cis configuration as epicatechins. Generally, flavonoids are available in fruits such as apples, cherries, grapes, plums, and citrus fruits (Ziaullah and Rupasinghe, 2015; Tsao, 2002; Prior *et al.,* 2001).

It is the glycosidic forms of anthocyanidins common in plant materials which are literally referred to as anthocyanins. Anthocyanidins are important components in plant pigments, especially the pigments of red, blue, and purple colors. The differences in hydroxylation and methoxylation of aromatic ring B yield a variety of anthocyanins. The stability of anthocyanins is dependent on the pH of the medium, and as a result, the color expressed by anthocyanins is a function of media pH (Tsao, 2002). High temperature, light, enzymatic activity, and availability of oxygen also contribute to the instability of anthocyanin (Loypimai *et al*., 2016). Anthocyanins are available in a variety of plant sources; fruits, vegetables, grains, flowers, etc. (Tsao, 2002).

PAC, also referred to as condensed tannins are the oligomeric or polymeric forms of flavan-3-ols, especially catechins and epicatechins. Polymerization of monomeric units occurs through interflavan linkages. PAC falls into two main categories based on the nature of structural linkages. Both A- and B-type PAC consist of C4-C8 and/or C4-C6 interflavan linkages. Only the A-type PAC have C2-O7 ether bonds in polymeric structure (Fig. 4). PAC consisted of a single type of monomeric units (e.g., catechin, epicatechin, gallocatechin, epigallocatechin, afzelechin, and epiafzelechin) are especially termed as "procyanidins" (Ou and Gu, 2014).

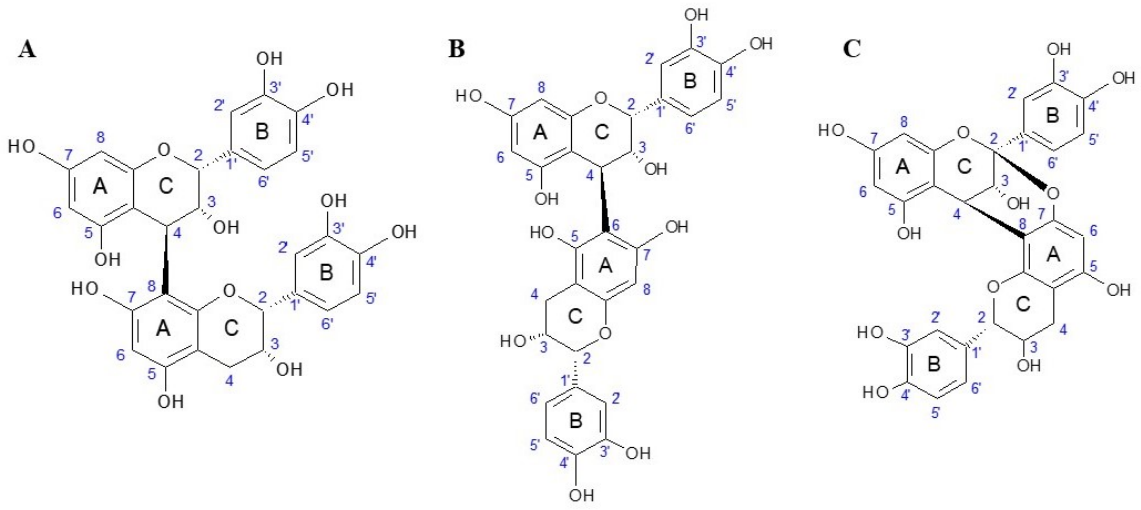


Figure 4: Different types of procyanidins based on the chemical bonds present between monomers (A and B: B-type procyanidins and C: A-type procyanidin). A: procyanidin B2 with the 4β-8 interflavan linkage between two epicatechin monomers. B; procyanidin B5 with the 4β-6 interflavan linkage between two epicatechin monomers. C; procyanidin A2 with 4β-8 interflavan linkage and 2β-O-7 ether bond between two epicatechin monomers.

Bioavailability of PAC depends on the structural degree of polymerization. However, even with a low degree of polymerization PAC depicts weak bioavailability. Only 5 – 10% of ingested dimers (procyanidins) pass through the blood-membrane barrier, compared to the absorption rate of epicatechin (Ou and Gu, 2014). About 40% of the daily consumed PAC is absorbable, and their degree of polymerization is limited only up to four monomeric units (Gu *et al*., 2003). However, only the PAC in the aqueous phase is biologically accessible. Due to the large size of PAC molecules, the intestinal absorption does not directly occur through cellular lipid bilayers, but rather through a transcellular pathway that utilizes intercellular spaces coupled with passive diffusion (Deprez *et al*.,

2001). Absorbed PAC undergo intestinal and hepatic phase 2 metabolism to yield glucuronidated, sulfated and methylated conjugates (Romanov-Michailidis *et al*., 2012; Parmar and Rupasinghe, 2014). The majority of the ingested PAC reaches colon intact and undergoes depolymerization by colonic microbiota. Microbial degradation of PAC yield easily absorbable simple phenolic acids (Deprez *et al*., 2000) and phenylvalerolactones (Sanchez-Patan *et al*., 2012). Thus, the bioavailability of PAC is greatly dependent on colonic microbial degradation process.

2.4 Polyphenols in cancer prevention

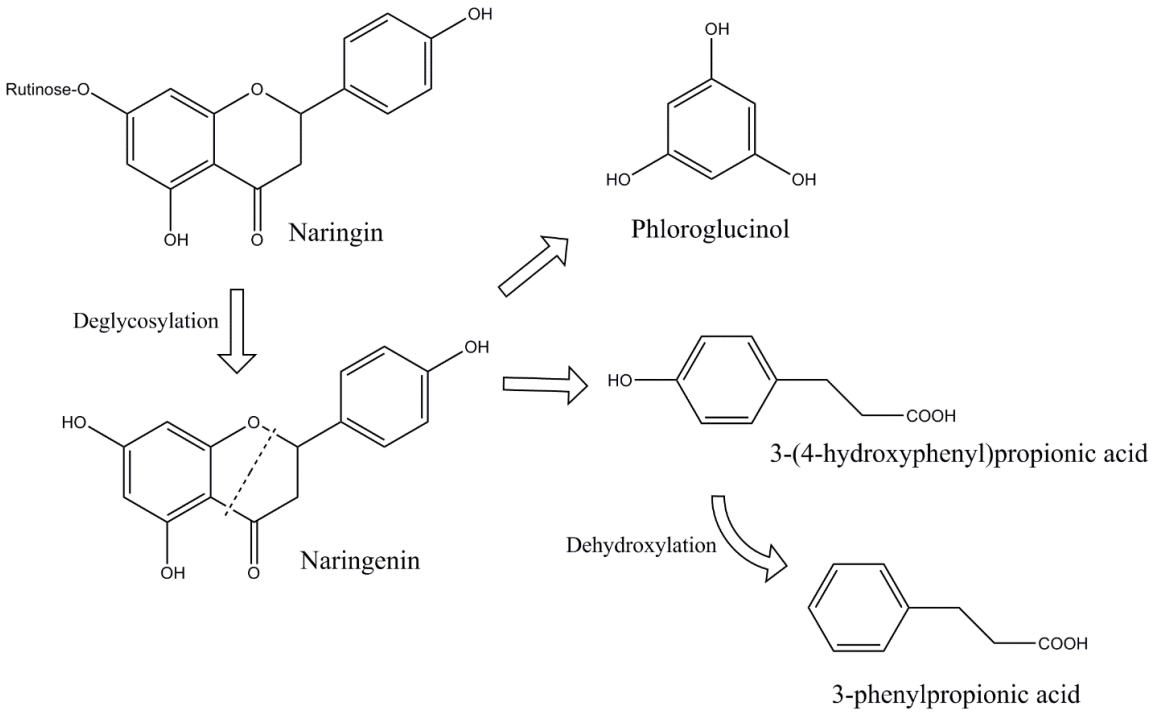
Recently published investigations, particularly *in vitro* studies provide promising evidence on cancer chemopreventive ability of dietary polyphenols. Neutralization of carcinogens and mutagens is a basic chemopreventive property of polyphenols. Polyphenols are especially useful in preventing carcinogenic substances such as heterocyclic amines, nitrosamine, and radical carbonyl derivatives resulted by oxidation of lipids and protein during food processing (Xiong, 2017). Polyphenols are also capable of protecting cells and tissues from inevitable oxidative damages (Rupasinghe *et al*., 2014). For example, a polyphenol rich extract from grape by-products and its main hydroxybenzoic acid derivatives, gallic, and benzoic acids protect Caco-2 cells against pro-oxidant tert-butylhydroperoxide-induced toxicity (Wang, *et al*., 2016).

Among the multiple mechanisms of polyphenols in chemoprevention, enhancement of xenobiotic metabolism, regulation of cell apoptosis and proliferation (Rupasinghe, *et al*., 2012), improved body immunity, restriction of angiogenesis and metastasis (Walczak, *et al*., 2017) must be appreciated as effects at the cellular level. Resveratrol, a stilbene

commonly found in grapes and grapes derived products, have exhibited the ability to suppress tumor growth *in vivo* (Singh *et al.,* 2015). Even though cancer prevention mechanisms involving resveratrol are not yet confidently established, studies suggest that anti-inflammatory functions of resveratrol occupy a mechanism involving estrogen receptor-α. Apoptosis functionality of resveratrol includes restriction of fatty acid synthase activity and promoting expression of proapoptosis genes (DAPK2 and BNIP3) (Singh, *et al.,* 2015). Flavonoids are also potent in cancer chemoprevention and depict structure dependent unique effectiveness. George and Rupasinghe (2017) observed improved repair of DNA damage in BEAS-2B cells pretreated with apple flavonoids and exposed to oxidative stress using known carcinogens, NNK, NNKOAc, methotrexate, and cisplatin. Green tea catechins, especially (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin (EGC) have shown to be potent in cancer prevention. Apart from being anti-mutagenic and anti-oxidative, these catechins are engaged in inhibition of tumor necrosis factor-α (TNF-α), a vital need in tumor suppression (Kuzuhara *et al.,* 2008). Curcumin, a well-studied polyphenol of turmeric has demonstrated potential in the prevention of breast cancer. The potential of curcumin in down-regulation of metastasis and angiogenesis is mainly described by effects on cellular signaling pathways, especially inhibition of NFκB, down-regulation of RhOA and inactivation of JAK2/STAT3 pathways (Banik *et al*., 2017).

The expected bioactivities of polyphenols in the human body are highly dependent on the dose and their bioavailability (Thilakarathna and Rupasinghe, 2018; Holst and Williamson, 2008). Chlorogenic acid, the most abundant hydroxycinnamate is only 33% absorbed in the small intestine. Major fraction of ingested chlorogenic acid reached colonic microbiota

and metabolized into hippuric and *m*-coumaric acids (Rupasinghe, *et al.,* 2012). The first step in the microbial degradation of complex polyphenols is de-glycosylation or cleavage of glycosidic bonds to produce aglycones (Fig. 5). The resulting aglycones are further catabolized into easily absorbable simple metabolites (Fig. 6) (Rechner *et al*., 2004).



(Source: Rechner *et al*., 2004)

Figure 5: Colonic microbial metabolism of naringin. Naringin is de-glycosylated to aglycon, naringenin which is catabolized to phloroglucinol and 3-(4-hydroxyphenyl)propionic acid. The latter is further dehydroxylated into 3-phenylpropionic acid.

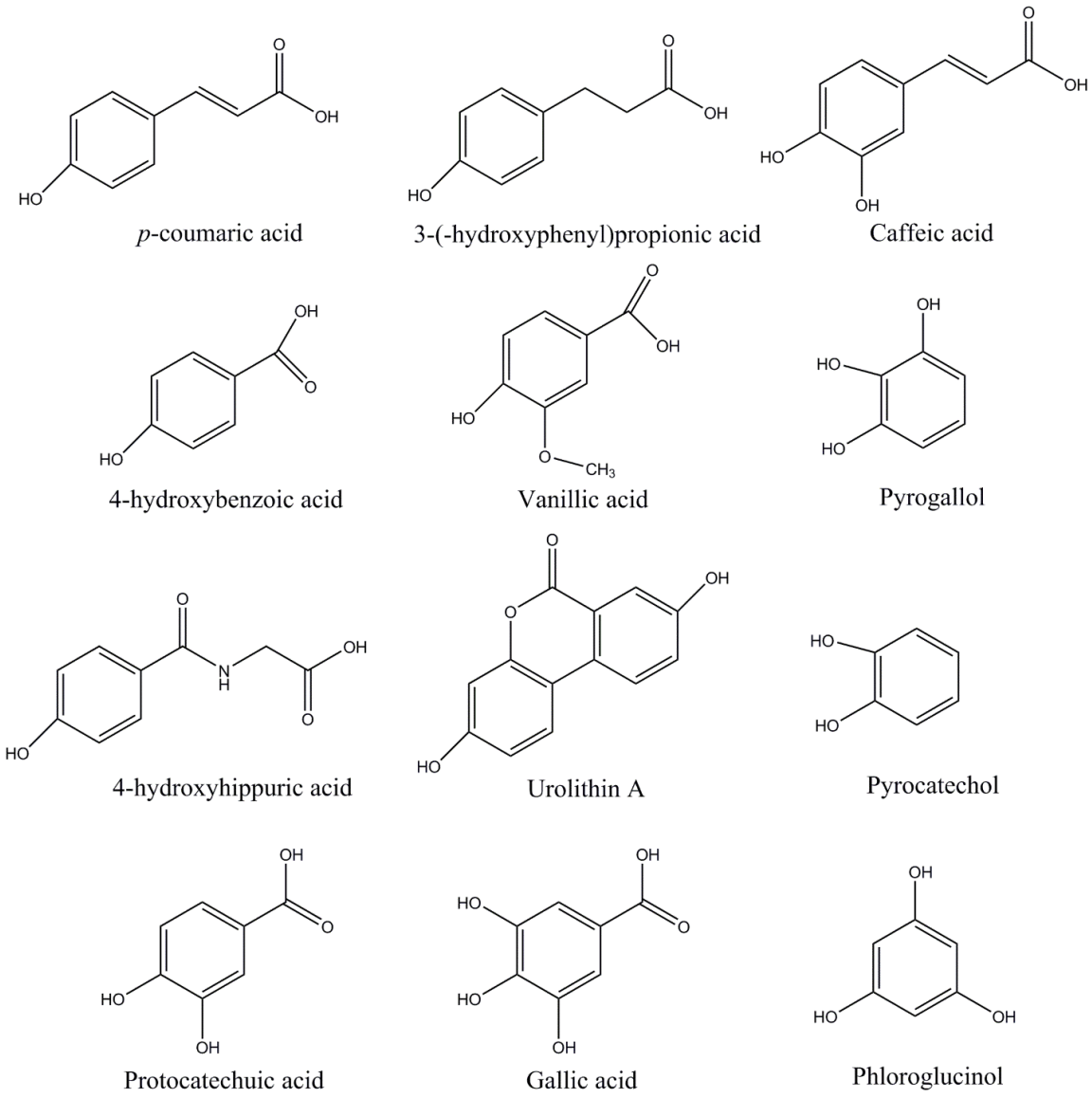


Figure 6: Chemical structures of common colonic microbial metabolites of polyphenols.

Other than basic antioxidant properties, these phenolic metabolites exhibit a wide range of mechanisms in cancer chemoprevention (Table 1) (Rastmanesh, 2011; Rechner *et al.,* 2004).

Table 1: Chemopreventive actions of common metabolites by microbial metabolism of polyphenols.

Parent polyphenols	**Metabolite**	**Health benefits**	**Reference**
Tea polyphenols	3,4-dihydroxyphenyl acetic acid	Anti-proliferative effects against HCT-116 colon cancer cells.	Henning *et al.*, 2013
Tea polyphenols	3-O-methylgallic acid	Anti-proliferative effects against HCT-116 colon cancer cells.	Henning *et al.*, 2013
	p-Coumaric acid	Inhibition of angiogenesis in mouse models by down regulation of AKT and ERK signaling pathways.	Kong *et al.*, 2013
Anthocyanin	Protocatechuic acid	Proapoptotic and anti-proliferative effects against cancer in mice.	Semaming *et al.*, 2015
	Ferulic acid	Anti-cancer effects by limiting endogenous nitrosamine formation and preventing mutagenesis.	Srinivasan *et al.*, 2007
	Sinapic acid	Prevention of 7, 12 dimethyl benz(a)anthracene induced buccal pouch	Kalaimathi and Suresh, 2015

Parent polyphenols	Metabolite	Health benefits	Reference
		carcinogenesis in hamsters by detoxification and antioxidant effects.	
		Inhibition of the proliferation and induction of apoptosis in colonic cancer cell lines HT-29 and SW480.	Balaji *et al.*, 2014
Daidzin	Equol in plasma from Daidzein	Induce apoptosis in human cervical HeLa cancer cells by promoting tumor necrosis factors. Activate caspase 3, 8, and 9.	Kim and Kim, 2013
Naringin	Naringenin	ASk-1 induced apoptosis in SNU-213 pancreatic cancer cells and unbalanced cellular redox homeostasis by lowering expression of peroxiredoxin-1.	Park *et al*., 2017
Red grape seeds extract (Rutin)	Leucocyanidin	Protect HaCaT keratinocyte against UVB induced DNA damage and lipid peroxidation.	Prede-Schrepler *et al*., 2013

PG, the major metabolite from microbial metabolism of mango (*Mangifera indica* L. cv. Keitt) restricted the proliferation of breast cancer cells (MCF10DCIS.com) by reactive oxygen species (ROS)-dependent upregulation of AMPK pathway and down-regulation of AKT/mTOR pathway (Nemec *et al*., 2017). Microbial metabolites of quercetin and caffeic acid are 3,4-hydroxyphenyl acetic acid and 3-(3,4-dihydroxyphenyl)propionic acid, respectively. Both microbial metabolites limited the DNA damage in human adenoma cells LT97, by upregulation of cellular glutathione S-transferase T2 (GSTT2) and down-regulation of cyclooxygenase (COX-2) levels (Miene *et al*., 2011).

2.5 Polyphenol-based synbiotics for cancer prevention

Beneficial health effects of probiotics, prebiotics, and synbiotics have been intensively studied and proven for desired health effects (Thilakarathna *et al.* 2018). Probiotics are the live microorganisms capable of delivering health benefits when adequately administered (Pandey *et al*., 2015). Prebiotics are the non-digestible dietary components, selectively stimulating growth or biological activity of probiotics, to promote host's health (Gibson *et al.*, 2017; Tomasik and Tomasik, 2003). Non-digestible food carbohydrates such as fermentable dietary fibers, resistant starches, oligosaccharides (especially from milk), polyols (lacitol, mannitol, sorbitol, and xylitol), inulin with low degree of polymerization, and pectin are often considered as common prebiotics (Krumbeck *et al.*, 2016; Pandey *et al.*, 2015). However, the prebiotic properties of other biologically unavailable dietary components, including phytochemicals must be appreciated (Gibson *et al*., 2017).

2.5.1 The role of probiotics in cancer prevention

The positive correlation between the probiotic administration and reduced risk of cancer is evident by many studies describing the possible mode of actions. Catabolism of complex indigestible dietary components to release simple and easily absorbable metabolites with anticarcinogenic properties is appreciated as a major role of probiotics in cancer risk reduction (Kumar *et al*., 2010). Polymerized polyphenols such as ellagitannins and PAC depict considerably limited absorption in the human small intestine and catabolized by probiotic bacterial strains including *Bifidobacterium* and *Lactobacillus*, naturally found in human microbiome (Lamuel-Ravntos and Onge, 2017). Probiotics also contribute to cancer risk reduction by minimizing the colon exposure to carcinogens and toxins. Probiotics reduce the levels of *β*-glucuronidase, nitro-reductase and azo-reductase enzymes by colonic cells to prevent the conversion of pro-carcinogens into carcinogens (Hijova *et al*., 2016; Verma and Shukla, 2013). Furthermore, probiotics are involved in the uptake of nitrites and secondary bile salts which act as the substrates for these enzymes. Probiotics are also capable of detoxification of toxins, including the mycotoxins such as aflatoxin B_1, proven to aid in colon and liver cancers (Rajoka *et al*., 2017). Oxidative stress created by ROS is a primary cause of cancer. Sufficient availability of antioxidants and antioxidant enzymes is important to neutralize ROS before damaging cellular components, more importantly, DNA. Probiotics produce antioxidants such as glutathione, and vitamin E and C. Enzymes such as superoxide dismutase, glutathione peroxidase and catalases activated by probiotics further contribute in ROS neutralization (Bhat and Kapila, 2017; Raman *et al*., 2013).

Probiotics also exhibit cancer prevention mechanisms of a higher level of interaction with the host at the cellular level. Several studies suggest the ability of probiotics in the induction of apoptosis, necrosis, and restriction of proliferation and migration of colonic cancer cell lines (Dallal *et al*., 2015; Shyu *et al*., 2014; Chen *et al*., 2009). As suggested by Altonsy *et al*. (2010) *L. rhamnosus* GG and *B. lactis* Bb12 bacterial strains induce apoptosis in Caco-2 cells by increasing BAX translocation, releasing of cytochrome C, and activation of caspase-3 and -9. *Saccharomyces boulardii* limits the proliferation and induces apoptosis in human colonic cancer cells, HT29, SW480, and HTC-116 by inactivating EGFR-Mek-Erk pathway signaling (Chen *et al*., 2009). Furthermore, *L. acidophilus* and *L. casei* is capable of limiting the migration and invasiveness of Caco-2 cells by reducing the cellular potential for degradation of tissue collagen matrix (Dallal *et al*., 2015). Potential of probiotics in host immunomodulation could be a possible intervention in effective colonic cancer prevention. Wistar rats induced for colonic cancer using 1, 2-dimethylhydrazine (DMH), demonstrated a lesser number of aberrant crypt foci (AFC) and cancer incidence when supplemented with *Enterococcus faecium* bacteria. Improved immune response expressed by elevated production of IL-4, TNF-α, and IFN-γ production (confirmed by histological experiment) protected the rats from DMH-induced colonic cancer (Sivieri *et al*., 2008).

Probiotics-mediated restriction of pathogenic invasions significantly contributes to limitation of cancer risk (Young *et al*., 2017). Pathogenic microbes cause tumorigenesis in 15 to 20% of cancer cases. Dysbiosis or alteration to the natural microbiome is a common characteristic among such cases (Bhatt *et al*., 2017). However, further investigations are required to understand the capacity of probiotics in limiting of pathogenic invasions.

Supplementation of female *Swiss* mice with *Saccharomyces boulardii* caused no reduction in *Toxocara canis* larvae infection in the intestinal mucosa. Moreover, this supplementation was incapable of improving IL-12 expression in mice intestinal mucosal cells, suppressed by *T. canis* (de Moura, *et al*., 2017), showing the limited potential in securing host immune functions.

2.5.2 Polyphenols as prebiotics in cancer prevention

The capacity of polyphenols in cancer prevention is considerably lessened by indigestibility. However, polyphenols are capable of acting as prebiotics by favoring the growth of beneficial gut microbes such as *Bifidobacterium* and *Lactobacillus* spp. (Lamuel-Raventos and Onge, 2017). More than 95% of dietary polyphenols remain intact during the digestion process and accumulates in the colon and fermented by gut microbiota (Clifford, 2004). Colonic biotransformation of polyphenols is extensive. Rats fed with grape seed extract (0.25% w/w of diet ad libitum) rich in oligomeric and polymeric PAC showed only 11% of PAC recovery from feces analysis (Choy *et al*., 2013). Potential of dietary polyphenols in selective growth stimulation of gut microbes is demonstrated by *in vitro* and animal studies. Kemperman *et al*. (2013) experimented the effect of red wine grape extract and black tea polyphenols on gut microbes using a simulated intestinal microbial ecosystem. Treatment with red wine extract stimulated the growth of *Klebsiella*, *Alistipes*, *Cloacibacillus*, *Victivallis*, and *Akkermansia* microbes, while restricting *Bifidobacteria*, *B. coccoides*, *Anaerolobus*, *Subdoligranulum*, and *Bacteroides*. Similarly, treatment with black tea also promoted and limited the growth of certain groups of microbes. However, microbial growth promotion and restriction was not exactly same for the two treatments. Alterations to the microbial ecosystem with polyphenols lowered the

Firmicutes: *Bacteroidetes* ratio (Kemperman *et al*., 2013). Dietary supplementation of caffeic acid (1 mM) suppressed the inflammation and prevented the increase of *Firmicutes*: *Bacteroidetes* ratio in C57BL/6 mice induced for colitis by dextran sulfate sodium (DSS) (Zhang *et al*., 2016). Dietary supplementation of crossbred female pigs with PAC rich grape seed extract (1% w/w) can significantly promote the growth of *Lachnospiraceae*, *Clostridales*, *Lactobacillus*, and *Ruminococcacceae* spp. microbes. Major phenolic metabolites identified in pig feces were 4-hydroxyphenylvaleric and 3-hydroxybenzoic acids (Choy *et al*., 2014). *Lachnospiraceae* and *Ruminococcacceae* spp. are known butyrate and propionate producers in human gut microbiome (Reichardt *et al*., 2014). Butyrate is a short chain fatty acid capable of cancer suppression by inducing apoptosis in cancer cells and limiting histone deacetylase activity. Sodium butyrate is capable of inducing apoptosis and restricting the proliferation of Huh-7 hepatocarcinoma cells, by increasing cellular ROS production through overexpression of intracellular micro RNA miR-22 (Pant *et al*., 2017).

2.5.3 Polyphenols for cancer preventive synbiotics

The composition of the healthy gut microbiota is not well-established and varies among different populations and depends on individual's lifestyle (Teixeira *et al*., 2017). Variation in heathy gut microbiota questions the usefulness of prebiotics as only the individuals with beneficial gut microbes can utilize administered prebiotics. Furthermore, the fermentation of prebiotics by probiotic microbes is strain specific, and only the strains with the ability to yield useful metabolites and overcome the challenges by other species of microbiota are significant in the useful administration of prebiotics and probiotics (Krumbeck *et al.*, 2016). Out of all the studies carried out on the ability of *Lactobacillus*

bacteria to improve body immunity, only 10% has come up with strong results, suggesting the limited availability of useful probiotic strains (Bengmark and Martindale, 2005). Their effectiveness is depending on the supply of prebiotics convertible into metabolites with desired effects. Better results are possible to achieve by designing synbiotics; an association of prebiotics and probiotics in a single system. According to the definition postulated by Gourbeyre *et al.*, (2011), "a combination of probiotic(s) and prebiotic(s) constitutes a synbiotic, which can stimulate and increase the survival of probiotic and autochthonous-specific strains in the intestinal tract". Intestinal degraded products of phenolic microbial metabolites and short chain fatty acids are capable in the reduction of colonic cancer risk, signifying the potential for successful application of phenolic compounds in designing of synbiotics. For example, degraded phenolic metabolites of substrate tert-butoxycarbonyl-lysine (acetylated)-4-amino-7-methyl coumarin; *p*-coumaric acid, 3-(4-hydroxyphenyl)-propionate and caffeic acid, suppressed the histone-deacetylase activity in the HT-29 human colon cancer cell nuclear extract. Inactivation of histone-deacetylase is important in the prevention of malignant conversion and apoptosis induction in the pre-neoplastic cell (Waldecker *et al*., 2008).

Kim *et al*. (1998) provide a list of specific gut microbes involved in the bioconversion of flavonoid glycosides which may become handy in designing of synbiotics from flavonoids (Table 2). The study has further extended to determine the cytotoxicity of flavonoid glycosides, their aglycones and aglycone microbial metabolites in several cancer cell lines (e.g., human colon cancer cells (SNU-C4), human stomach cancer cells (SNU-1), etc.) Cytotoxicity of metabolites has determined by measuring the IC_{50} values; sample concentrations resulted in 50% inhibition of cell growth. Baicalin has registered with

considerably low IC_{50} values compared to other flavonoid glycosides. The monomeric flavonoids, except catechins, are effective cytotoxins for cancer cells. However, their cytotoxicity is low for HepG2, A549, and MA-104 cell lines. Microbial metabolites of polyphenol aglycones demonstrate similar cytotoxicity for cancer cells. 3,4-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde, 3,4-hydroxyphenylacetate, phenyl acetate and PG are the aglycone metabolites that have exhibited higher cytotoxicity in cancer cell lines. Considerably low cytotoxicity was observed for 2,4-dihydroxyacetophenone, 3-hydroxyphenylacetate, 4-hydroxyphenylacetate and 2-hydroxy-4-methoxyacetophenone, suggesting their low effectiveness in inhibition of cancer cell growth.

Table 2: Phenolic metabolites of flavonoid glycosides by specific human intestinal bacteria

Flavonoid glycoside	**De-glycosylation bacteria**	**Aglycone**	**Aglycone transforming bacteria**	**Phenolic metabolite**
Baicalin	*E. coli* HGU-3	Baicalein	*Streptococcus* S-2	4-Hydroxybenzoate
	Bacteroides J-37		*Lactobacillus* L-2	3,4-Dihydroxybenzoate
			Eubacterium A-44	Pyrogallol
			Bifidobacterium B-9	Phenylacetic acid
Puerarin	*Peptostreptococcus* YK-10			4-Hydroxybenzoate
				2,4-Dihydroxybenzoate
Daidzin	*Bacteroides* J-37	Daidzein	*Bacteroide* JY-6	Resorcinol
	Eubacterium A-44			2,4-Dihydroxyacetophenone
	Fusobacterium K-60			4-Hydroxyphenylacetate

Flavonoid glycoside	**De-glycosylation bacteria**	**Aglycone**	**Aglycone transforming bacteria**	**Phenolic metabolite**
Rutin	*Bacteroides* JY-6	Quercetin	*Streptococcus* S-2	4-Hydroxybenzoate
	Fusobacterium K-60		*Lactobacillus* L-2	3,4-Dihydroxybenzoate
	Eubacterium YK-4		*Bifidobacterium* B-9	3,4-Dihydroxyphenylacetate
Naringin	*Bacteroides* JY-6	Naringenin	*Streptococcus* S-2	4-Hydroxybenzoate
	Eubacterium YK-4		*Lactobacillus* L-2	Phloroglucinol
	Feptostreptococcus YK-10		*Bacteroides* JY-6	2,4,6-Trihydroxybenzoate
	Fusobacterium K-60			4-Hydroxyphenylacetate
Hesperidin	*Fusobacterium* K-60	Hesperetin	*Streptococcus* S-2	Resorcinol
	Eubacterium YK-4		*Lactobacillus* L-2	Phloroglucinol
	Bacteroides JY-6		*Bifidobacterium* B-9	2,4-Dihydroxy phenylacetate

Flavonoid glycoside	De-glycosylation bacteria	Aglycone	Aglycone transforming bacteria	Phenolic metabolite
			Bacteroides JY-6	
Poncirin	*Fusobacterium* K-60	Ponciretin	*Streptococcus* S-2	4-Hydroxybenzoate
	Eubacterium YK-4		*Lactobacillus* L-2	2,4-Dihydroxyacetophenone
	Bacteroides JY-6		*Bifidobacterium* B-9	Phloroglucinol
			Bacteroides JY-6	Pyrogallol
Catechin		Catechin	*Lactobacillus* L-2	4-Hydroxybenzoate
			Bifidobacterium B-9	2,4,6,-Trihydroxybenzoate
			Bacteroides JY-6	Phloroglucinol
				4-Methoxysalicylate

(Source: Kim *et al*, 1998)

Biotransformation of purified polymeric PAC (extracted from willow tree leaves) by using human fecal bacteria has resulted in an array of simple metabolites, namely; *m*-hydroxyphenylpropionic, *m*-hydroxyphenylacetic, *p*-hydroxyphenylpropionic, *p*-hydroxyphenylacetic, *m*-hydroxyphenylvaleric, phenylpropionic, phenylacetic, and benzoic acids (Deprez *et al*., 2000). Also, bioconversion of purified PAC dimers from grape seeds by using human feces bacteria has yielded 2-(3,4-dihydroxyphenyl) acetic acid, 5-(3,4-dihydroxyphenyl)-gamma-valerolactone, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenyl propionic acid, phenylvaleric acid, monohydroxylated phenylvalerolactone and 1-(3',4'-dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl) propan-2-ol (Appeldoorn *et al.,* 2009). Cytotoxic effect of 3 and 4-phenyl acetic acids is possible to assume by considering the results of the study by Kim *et al.* (1998).

Potential of polyphenols to be utilized in synbiotics for cancer prevention is possible to elaborate using more examples. Tea is a rich source of polyphenols, especially of epicatechin, catechin, 3-O-methyl gallic acid, gallic acid and caffeic acid (Lee *et al.,* 2006). Microbes of human feces are capable in metabolizing these tea polyphenols into simpler forms including 3-phenylpropionic acid, 4-hydroxyphenylacetic acid and 3-(4-hydroxy phenyl) propionic acid. Tea polyphenols as well as their metabolites by fecal bacteria are potent in suppressing the growth of bacterial pathogens such as *C. perfringens* and *Bacteroids*. The effect on desirable *Bifidobacterium* and *Lactobacillus* strains by these polyphenols and their metabolites is minimal. Management of gut microbial content by enhancing beneficial bacterial strains and suppressing of pathogenic strains are supportive in minimizing colorectal cancers (Lee *et al.,* 2006).

Prenylated chalcone xanthohumol is a polyphenol available in hops used in beer production. Kim and Lee (2006) was able to convert xanthohumol into isoxanthohumol, xanthohumol 4-O-β-glucopyranoside, xanthohumol 4,4-O-β-glucopyranoside, 5-methoxy-8-prenylnaringenin and 7-O-β-glucopyranoside by using series of bacteria. Xanthohumol and isoxanthohumol are potent in the suppression of angiogenesis (Negrao *et al.,* 2010) which is an important aspect of cancer prevention. Anti-angiogenesis ability of 8-prenylnaringenin is controversial as different studies report opposing results (Brunelli *et al.,* 2009; Negrao *et al.,* 2010). However, the chemopreventive ability of 8-prenylnaringenin is suggested by its effect in restricting of epidermal growth factor-induced proliferation of MCF-7 breast cancer cells by inhibition of PI(3)K/AKT pathway (Brunelli *et al.,* 2009).

Even though a number of studies are available to describe the potential of utilizing microbial metabolites of polyphenols in cancer prevention, comprehensive studies where the polyphenol is administered together with specific probiotics is unavailable. Studies with properly designed polyphenol based synbiotics are a necessity in the development of functional foods for cancer prevention.

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals and reagents

Microbial metabolites of PAC (MMP); 3-phenylpropionic acid, 4-hydroxyphenyl acetic acid, 3-(4-hydroxyphenyl) propionic acid, *p*-coumaric acid, benzoic acid, PG and PC were purchased in pure form from Sigma-Aldrich (Oakville, ON, Canada). Oligomeric GS-PAC was also purchased (Catalog No. 1298219, USP, Rockville, MD, USA) to use in the same experiments as of extracted AP-PAC.

Bronchial Epithelial Basal Medium (BEBM) and Bronchial Epithelial Growth Medium (BEGM) kit (Catalog No. CC-4175) were purchased from Lonza (Walkersville MD, USA). Minimum Essential Medium Eagle (MEME), fetal bovine serum (FBS), penicillin-streptomycin, bovine collagen type-1, fibronectin (from human plasma), L-glutamine, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), trypan blue stain, phenazine methosulphate (PMS) were purchased from Sigma-Aldrich (Oakville, ON, Canada). All chemicals and reagents were of research grade and suitable in cell culture experiments where applicable.

3.2 Cell lines and culture conditions

Two human non-cancerous lines were used in this study. BEAS-2B (ATCC® CRL-9609™) and WRL-68 (ATCC® CL-48™) were purchased from American Type Culture Collection (Manassas, USA). BEAS-2B cells were cultured in BEGM prepared by the addition of BEGM kit into BEBM. Gentamycin-amphotericin B mix provided with the BEGM kit was replaced with 100 U/mL penicillin, and 100 µg/mL streptomycin (as per

the instructions by ATCC®). Culture flasks and well plates were pre-coated overnight with 0.01 mg/mL fibronectin, 0.03 mg mL bovine collagen type-1 and 0.01 mg/mL BSA prior to the plating of cells. WRL-68 cells were cultured in MEME supplemented with 10% FBS, 4 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cell cultures were maintained at 37 ºC and 5% CO_2 in a humidified incubator and sub-cultured before reaching confluence.

3.3 MMP-mediated protection against NNKOAc-induces DNA damage in BEAS-2B and WRL-68 cells

3.3.1 MTS cell viability assay for evaluation of concentration-dependant MMP cytotoxicity

Concentration-dependant cytotoxicity of the MMP were evaluated using CellTiter 96® AQ_{ueous} MTS reagent powder (Promega, Madison, WI, USA) to identify sensible MMP concentrations for future experiments. Cells were seeded in 96-well plates at a density of 6000 cells/well and incubated overnight at 37 ºC in a humidified incubator with 5% CO_2. Following incubation, cells were treated with 10 concentrations (0.1, 1, 10, 25, 50, 75, 100, 250, 500, and 1000 µM) of each MMP separately, and incubated at 37 ºC in a humidified incubator with 5% CO_2. for 24 h. Treated cells were exposed to MTS/PMS solution (MTS, 333 µg/mL; PMS, 25 µM of final concentration) and incubated for 3 h at 37 °C. After incubation absorbance was measured at 490 nm using the Infinite® M200 PRO multimode microplate reader (Tecan Trading AG, Mannedorf, Switzerland).

3.3.2 Amplex® Red assay to investigate H_2O_2 production by MMP in cell culture medium

The possibility of H_2O_2 production in culture medium (cell-free system) by PG and PC was investigated using Amplex® Red assay kit (Catalog No. A22188, Life Technologies^{TM}, Eugene, OR, USA). Standards of H_2O_2 (up to 1000 µM) and the MMP treatments used in MTS assay were prepared (in 2× concentrations) in phenol red-free complete growth medium and aliquoted (100 µL) into 96-well plate. The Amplex ® Red master mix was made by adding Amplex® Red solution (final concentration; 25 µM) and horseradish peroxidase (final concentration; 0.005 U/mL) into phenol red free growth medium. Standards and the MMP treatment aliquots in 96-well plates were mixed with 100 µL of master mix and incubated in the dark at 37 ºC for 2 h. After incubation absorbance for wells were measured at 570 nm using the Infinite® M200 PRO multimode microplate reader (Tecan Trading AG, Mannedorf, Switzerland). H_2O_2 production by each MMP treatment was calculated by creating a standard curve.

3.3.3 Oregon Green® assay for measuring of MMP promoted cell proliferation

Initially, the cells were synced-up (bringing all cells into the same phase of cell cycle) by incubating in serum-free medium for 24 h to bring all cells to the same phase of cell cycle. Following synchronization, cells were seeded in 6-well plates at 1×10^5 cells/well density using complete growth medium and incubated under normal culture conditions overnight. Oregon Green® 488 (Carboxy DFFDA-SE, Catalog No. C34555) was purchased from Life Technologies Corp. (Eugene, OR, USA). Oregon Green® stock solution was prepared by addition of 16.8 µL of DMSO to a vial (contained 50 µg). The stock solution was diluted in serum-free culture medium (0.25 µL/ mL) to produce a

working solution. Wells were washed with warm 1× PBS and added with 1 mL of Oregon Green® working solution prior to incubation at 37 ºC in the dark for 45 min. After incubation, wells were washed with warm complete growth media and cells allowed to recover in the dark for 2 h at 37 ºC in complete growth media (2 mL/well). After cell recovery period, cells in control wells (non-proliferative controls) were harvested and fixed in 0.5 mL of 1% paraformaldehyde in 1× PBS. Non-controls of BEAS-2B cells were treated with 1, 10, and 25 μM concentrations of PG and PC while WRL-68 cells treated with 10, 25, and 50 μM concentrations of same MMP. After 72 h of MMP treatment cells were harvested and fixed in 1% paraformaldehyde in 1× PBS. Non-proliferative controls and treatment cells were stored in dark soon after fixation. Finally, the prepared samples were analyzed by fluorescence-activated cell sorting (FACS) and data were processed using FCS Express 6 plus Research Edition software.

3.3.4 Assessment of MMP-mediated cytoprotection against NNKOAc by MTS assay

MTS protocol identical to section 3.3 was followed with an additional treatment step. Based on MTS results for evaluation of concentration-dependant cytotoxicity of MMP, cells were treated with 4 concentrations (10, 25, 50, and 250 μM) of each MMP separately for 24 h and challenged for cytotoxicity by NNKOAc (300 μM, 4 h). After incubation with NNKOAc % cell viability was measured using the MTS assay.

3.3.5 MMP-based protection in NNKOAc induced DNA damage

Degree of protection provided by MMP in NNKOAc induced DNA damage was measured using H2A.X immunofluorescence assay and comet assay (COMET SCGE assay kit, ADI-900-166, Enzo Life Sciences Inc., NY, USA). As per MTS assay test results by

the assessment of MMP-mediated cytoprotection against NNKOAc, 10 and 25 μM concentrations of PG were selected as the treatments for BEAS-2B cells. For WRL-68 cells 25 and 50 μM concentrations of PC were the selected treatments.

3.3.5.1 H2A.X immunofluorescence assay

Cells were seeded in 6-well plates (2×10^5 cells/well) included with 95% ethanol sterilized glass cover slips. Cells were allowed to adhere overnight on the coverslip surfaces and treated with MMP for 24 h. Following MMP treatment cellular DNA damage was induced using NNKOAc (300 μM) for 4 h. Cells were allowed to recover for 4 h to facilitate maximum phosphorylation at DNA damage sites and washed with 2 mL of PBS to reduce background staining. Cells were fixed onto cover slips by adding 2 mL of 3.7% formaldehyde and incubating in dark for 20 min at room temperature (RT). Fixed cells were permeabilized by exposing to 0.5% Triton X-100 in PBS for 15 min at RT. Humidifying chambers were prepared by applying PBS wetted filter papers in bottom of 15 cm culture Petri dishes. A sheet of parafilm was used to separate cover slips from wet filter paper. Triton X-100 was thoroughly removed from wells by washing with PBS. Cells were blocked by transferring cover slips onto drops (55 μL) of 4% BSA in PBS on parafilm in humidifying chambers and incubating at RT for 20 min. Blocking with 4% BSA ensures binding of primary antibody only with the phosphorylated H2A.X at DNA damage sites. Incubation with primary antibody (anti-phospho-histone H2A.X) was done similarly by transferring cover slips onto drops (55 μL) of primary antibody diluted 1:250 in 4% blocking solution for 1 h in dark at RT. Cover slips were transferred back into respective wells and excess of primary antibody was washed-off using PBS. Cells were incubated with secondary antibody (Alexa fluorophore® 594 donkey anti-mouse IgG) diluted 1:500

in 4% BSA blocking solution by transferring onto drops of secondary antibody in humidifying chambers. Excess of secondary antibody was washed off similar to washing after incubation with primary antibody. Cover slips were wet-mounted on glass slides using mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA, USA). All slides were imaged by fluorescence microscopy and number of phosphorylated H2A.X foci were counted using Image J software (Version 1.51j8, National Institute of Mental Health, Bethesda, MD, USA).

3.3.5.2 Comet assay

Cells were seeded (3×10^5 cells/well) overnight in 6-well plates and treated with MMP for 24 h. MMP treated cells were induced for DNA damage using NNKOAc (300 µM for 4 h). Following NNKOAc treatment cells were harvested and suspended in ice-cold PBS (1×10^5 cells/ mL). Cell suspension was mixed with molten low melting agarose provided with the assay kit (50 µM of cell suspension with 500 µL of molten agarose) and 75 µL was immediately spread evenly over comet slide. Slide was placed at 4 ºC in dark for 30 min to set agarose. Slides were immersed in pre-chilled lysis solution and kept in dark at 4 ºC for 45 min. Slides were then immersed in freshly prepared alkaline solution (pH > 13; 1.2 g/ 100 mL NaOH and 1mM EDTA in deionized water) and incubated in dark at RT for 1 h. Slides were washed with 1 × TBE buffer (pH = 8) and subjected to electrophoresis (1 volt/cm) in 1 × TBE buffer for 10 min. Excess of TBE buffer was tapped off and slides were immersed in 70% ethanol for 5 min. Slides were air dried and stained with cygreen dye (1 µL in 999 µL deionized water) by placing 100 µL of dye on agarose gels. Slides were placed in dark at RT for 30 min. Excess of stain was taped off from the slides and gently rinsed in deionized water. Finally, the slides were dried at 37 ºC and

imaged by fluorescence microscopy using FITC filter (489/515 nm) at × 40. Images were analyzed by OpenComet software and results presented as olive moment.

3.3.6 Effect of PG and PC in NNKOAc-induced DNA damage response mechanisms

The influence of PG and PC on cellular DNA damage response (DDR) mechanisms was studied by using western blotting technique. Cellular expression of DDR proteins; *p*-ATM, *p*-ATR, *p*-p53, and *γ*-H2A.X was measured. Activation of cell cycle checkpoint kinases Chk1 and Chk2 was monitored by measuring the levels of *p*-Chk1 and *p*-Chk2. Levels of β-actin were measured for each treatment as the housekeeping protein to eventually normalize the expressions of the measured proteins (George and Rupasinghe, 2017). Primary and secondary antibodies required for detection of these proteins were purchased from Cell Signaling Technology (DNA damage antibody sampler kit, catalog number: 9947, cell signaling technology, Inc., Danvers, MA, USA).

BEAS-2B and WRL-68 cells were seeded at a density of one million cells in 75 cm^2 cell culture flasks. Cells were allowed to attach overnight before treating with PG and PC. BEAS-2B cells were treated with 10 and 25 μM concentrations of PG for 24 h. WRL-68 cells were treated with 25 and 50 μM concentrations of PC for 24 h. After PG and PC treated cells were exposed to 300 μM NNKOAc for 4 h to induce DNA damage. Each experimental model consisted of a DMSO control, NNKOAc control, and controls from respective concentrations of PG and PC. After the treatments cells were harvested by using TrypLE Express and centrifuged at 1000 rpm for 5 min to obtain a cell pellet. The cell pellet was washed with 1× cold PBS and centrifuged at 1000 rpm for 5 min in a micro-centrifuge (Legend MICRO 21R, catalog number: 75002446, ThermoFisher Scientific,

Osterode, Germany) at 4 ºC. Radio-immunoprecipitation assay (RIPA) buffer was prepared by mixing 50 mM Tris HCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, and 10 mM NaF. Protease inhibitor cocktail (Catalog number: P8340, Sigma-Aldrich, Oakville, ON, Canada) was added to the RIPA buffer (10 µL/1 mL of RIPA buffer) just before using. Cell protein was extracted by mixing the cell pellets with 100 µL of RIPA buffer and incubation on ice for 30 min. After incubation, the sample was centrifuged in micro-centrifuge (4 ºC) at 12,000 rpm for 20 min to separate protein containing RIPA buffer from cell debris. The pellets of cell debris were discarded, and the protein dissolved RIPA buffer samples were collected.

The protein contents of extracted samples were estimated by Pierce™ Coomassie (Bradford) protein assay kit (Catalog number: 23200, ThermoScientific, Rockford, IL, USA). Standard albumin solutions of 0, 25, 125, 250, 500, 750, 1000, and 1500 µg/mL concentrations were prepared by using albumin stock solution (2 mg/mL) provided with the assay kit. A volume of 5 µL from each extracted protein sample and albumin standard solution were pipetted on a 96-well plate in triplicates. Each well was added with 200 µL of Coomassie solution and incubated at 37 ºC for 30 min. Absorbance values for wells were measured at 595 nm, and protein content of extracted samples was calculated by creating a standard curve for albumin solutions.

Denaturation of extracted protein was performed by using Blue Loading Buffer Pack by New England BioLabs™ Inc (Catalog number: B7703S, New England BioLabs™ Inc., Ipswich, MA, USA). Fresh 3× reducing Blue Loading Buffer was prepared by mixing 30× reducing agent with 3× Blue Loading Buffer in 1:10 ratio. Extracted protein samples were mixed with 3× reducing Blue Loading Buffer in 2:1 ratio and heated on a heating

block up to 97 ºC for 5 min. After denaturation protein samples were diluted with RIPA buffer so that 10 µL from each sample contains 20 µg of protein. Then, the protein samples were subjected to SDS-gel electrophoresis using BIO-RAD Mini PROTEAN® Tetra Cell gel electrophoresis unit equipped with Bio-Rad PowerPac™ Basic (made in Singapore) power unit. Precast gel cassettes (Mini-PROTEAN® TGX Stain-Free™ Gels, Catalog number: 456-8094, Bio-Rad Laboratories Inc., Hercules, CA, USA) were fixed to the running module in tetra cell unit and filled with 1× SDS running buffer (Catalog number: 4050S, cell signaling technology, Danvers, MA, USA). The first well of precast gel cassettes was loaded with 10 µL of protein dual color marker (Precision Plus Protein™ Dual Color Standards, catalog number: 161-0374, Bio-Rad Laboratories Inc., Hercules, CA, USA) and other wells with 10 µL of protein samples (20 µg of protein). SDS-gel electrophoresis was executed for 90 min at 80 V and 400 mA. Protein separated on SDS-gels by electrophoresis were transferred onto polyvinylidene difluoride (PVDF) membranes (Catalog number: 88518, ThermoScientific, Rockford, IL, USA) by using BIO-RAD Trans-Blot® Turbo™ system (made in the USA). The PVDF membrane was activated with 100% methanol for 10 sec and equilibrated in transfer buffer (0.557% w/v TRIS, 0.293% w/v glycine, 20% v/v methanol, in distilled H_2O) for 15 min. A transfer sandwich was built on Trans-Blot system platform by placing SDS-gel on activated PVDF membrane and sandwiching with thick blot papers (catalog number: 1703966, Bio-Rad Laboratories Inc., Hercules, CA, USA) soaked in transfer buffer. Protein transferring was carried-out for 30 min at 20 V and 1 A.

PVDF membranes were blocked with blocking solution; non-fat milk powder (5% w/v) in 1× Tris-buffered saline with tween 20 (TBST) (Catalog number 9997S, Cell

Signaling Technology, Danvers, MA, USA), for 1 h at room temperature. Primary antibody solutions were prepared by dissolving antibodies in 5% w/v bovine serum albumin (BSA) in TBST at a concentration of 1:1000 (v/v). Blocked PVDF membranes were incubated with primary antibody solutions (10 mL) at 4ºC overnight. The PVDF membranes were washed 3 × 20 min using 1× TBST solution (10 mL). Secondary antibody solutions were prepared by dissolving antibodies in 5% non-fat milk powder (w/v) in 1× TBST at a concentration of 1:5000 (v/v). PVDF membranes were incubated with secondary antibody solutions (10 mL) for 1 h at room temperature. After probing with secondary antibodies, the PVDF membranes were washed 3 × 20 min using 1× TBST solution (10 mL).

The membranes were developed by using enhanced chemiluminescence (ECL) based Clarity™ and Clarity Max™ Western ECL Substrates Kit (Catalog number: 1705060, Bio-Rad Laboratories Inc., Hercules, CA, USA) to be imaged by BIO-RAD Chemidoc MP™ imaging system (Model number: Universal hood III, Bio-Rad, Hercules, CA, USA. The ECL working solution was made by mixing two substrate components provided with the kit in 1:1 ratio. Membranes were developed for imaging by incubating in 7 mL of ECL working solution. After incubating for 5 min, membranes were imaged in signal accumulation mode. Membrane images were analyzed for band intensities using ImageJ software (version 1.51j8). The results were expressed in relative protein levels compared to the DMSO control. Protein levels were compared with the NNKOAc control where DMSO control failed to generate a measurable band.

3.4 PAC biotransformation by probiotic bacteria

3.4.1 Extraction and purification of PAC

PAC required for the experiments in this study was extracted from Northern Spy apple peels. Powdered apple peel (500 g) was washed with 3 L of hexane to remove lipids and ground with 3 L of extraction solvent (70% acetone, 29.9% deionized water, and 0.1% acetic acid v/v) into a slurry using a glass blender (Model No. BL3000SC, Black and Decker, Applica consumer products Inc., Miramar, FL, USA). This slurry was subjected to ultra-sonication (Model No. 750D, VWR International, West Chester, PA, USA) for 30 min × 3 with 10 min intervals in between to maintain the slurry temperature at 30 ºC. After sonication, the slurry was filtered through 8 layers of cheese cloth followed by vacuum aided filtration through Fisherbrand™ P8 grade filter papers. The filtrate was collected, and rotary evaporated (Rotavapor HR-200, Buchi, Flawil, Switzerland) at 37 ºC until acetone was completely removed. The acetone-free thick slurry was freeze-dried to obtain crude PAC extract ready to undergo purification.

Purification of crude extract was performed by flash chromatography with Sorbent beads (Sorbent SP207-05 Sepabeads resin brominated styrenic adsorbent; 0.25 mm diameter, 630 m^2/g surface area; Sorbent Technologies, Atlanta, GA, USA). A chromatography column (6.5 × 45 cm, Sati International Scientific Inc., Dorval, QC, Canada) was loaded with 400 g of sorbent beads and conditioned with deionized water and equilibrated overnight using 50% ethanol-water mixture. Crude PAC extract was dissolved in 300 mL of 50% ethanol-water mixture and loaded into the chromatography column. Sugars in the loaded extract were eluted with deionized water, measuring the Brix value to

reach below 0.1% using a digital hand-held refractometer (Digital refractometer 300016, SPER SCIENTIFIC, Scottsdale, AZ, USA). Phenolic acids, flavonol, flavan-3-ol, and anthocyanin were eluted using 3 L of 95% ethanol-water mixture. PAC was eluted using 3 L of 100% acetone. The acetone eluate was collected, and rotary evaporated at 37 ºC for complete removal of acetone followed by freeze-drying to obtain isolated PAC.

PAC extraction efficiency of the described method was compared with two other methods, supercritical CO_2 extraction (Yilmz *et al*., 2011) with and without 20% ethanol (%Wt) modification (Extraction conditions: 50 ºC, 300 bar, 5 g/min CO_2 flow rate, 1 h extraction cycle).

3.4.2 Quantification of PAC content in extracts

PAC content in the extracts were quantified using modified (96-well plate format) 4-dimethylaminocinnamaldehyde (DMAC) method described by Prior *et al*. (2010) and modified by Bhullar and Rupasinghe (2014). Ethanol (80% in water) was acidified by adding 2.5 mL of 6 N H_2SO_4 into 100 mL. DMAC working reagent was prepared by adding 50 mg of DMAC into 100 mL of acidified ethanol. DMAC working reagent was mixed with PAC extracts dissolved in acetone at 3:1 ratio (210 µL of DMAC reagent + 70 µL of PAC extract). A standard curve was created using 1 - 500 µg/mL concentrations of catechin. After incubation for 30 min, absorbance values for sample wells were measured at 640 nm wavelength. Results were expressed in mg catechin equivalence (CE/ g dry weight of the sample.

3.4.3 Bacterial strains and culture conditions for PAC biotransformation

Probiotic *Lactobacillus* bacterial strains; *L. rhamnosus* GG (ATCC® 53103™, Manassas, VA, USA) and *L. casei* (ATCC® 393™, Manassas, VA, USA) were used for PAC biotransformation. The bacterial cultures were maintained in De Man, Rogosa and Sharpe broth (MRS) (Catalog No. 69966, Sigma-Aldrich, St. Louis, MO, USA) at 37 ºC. culture conditions for PAC biotransformation were established by incubation of probiotic bacteria with different concentrations of PAC (10 – 500 µg/mL) under aerobic and anaerobic conditions. Anaerobic conditions were created by using an anaerobic chamber (Catalog No. 27310, Stem cell technologies, Vancouver, BC, Canada) inserted with three AnaeroGen™ sachets (Catalog No. 68061, Sigma-Aldrich, St. Louis, MO, USA). PAC stock solution (50 mg/mL) was prepared by dissolving extracted PAC in DMSO. Different PAC concentrations were created by combining PAC stock solution with MRS broth, which then pipetted (200 µL) into a 96-well plate in triplicates. Wells with different concentrations of PAC were inoculated (1%) with a fresh bacterial suspension of 1×10^7 colony forming units (CFU)/mL, incubated for 16 h. Bacterial growth under different PAC concentrations was determined by measuring optical density at 600 nm wavelength (OD_{600}) after incubating at 37 ºC for 24 h.

3.4.4 Biotransformation of PAC

AP and GS-PAC were dissolved in DMSO to prepare 50 mg/ mL stock solutions. These PAC stock solutions were filter sterilized by passing through 0.22 nm DMSO resistant syringe filters. Sterilized PAC stock solutions were added into heat sterilized MRS broth to form 500 mL aliquots of 250 µg/mL AP-PAC in MRS and 50 µg/ mL GS-PAC in

MRS broths. Each PAC-MRS aliquot was inoculated (1%) with a fresh bacterial suspension of 1×10^7 colony forming units/mL, incubated for 16 h. Experimental controls were prepared by following the same steps with the exception of PAC addition. PAC biotransformation was completed under both aerobic and anaerobic conditions. Anaerobic conditions were established as described above by using an anaerobic chamber. PAC biotransformation was carried-out with continuous shaking (100 rpm) at 37 ºC for 12, 24, 48 and 72 h. Furthermore, the same experiment was repeated with MRS broth modified for protein and sugar content (Table 3), and limiting incubation time only for 48 h.

Table 3: Percent protein and sugar combinations used for the bioconversion of PAC

Protein % (relative to original MRS)	**Sugar % (relative to original MRS)**
0	0, 5, 10, and 20
50	0, 5, 10, and 20
100	0, 5, 10, and 20

Biotransformation was performed only under aerobic conditions as a considerable difference was not observed between aerobic and anaerobic biotransformation processes. PAC biotransformation was also performed with brain heart infusion broth (BHI) (Catalog No. CM1135, Oxoid Ltd., Hampshire, England) using GS-PAC (50 µg/mL), repeating the same experiment protocol only with *L. rhamnosus* under aerobic conditions for 72 h.

After biotransformation process, the PAC-broth aliquots were ultra-sonicated for 30 min at 32 ºC to lyse the bacterial cells and release phenolic compounds from bacterial cells. Then, the broth aliquots were centrifuged at 3000 × g for 15 minutes to separate lysed

bacterial cells from the culture broth. The bacterial cell pellet was discarded, and the broth was collected to separate simple phenolic metabolites resulting from the biotransformation process. Liquid-liquid extraction technique using ethyl acetate was utilized to separate phenolic metabolites from the culture broth. The broth with phenolic metabolites (500 mL) was combined with an equal volume of ethyl acetate (500 mL) and mixed well in a 2 L separatory funne1. The mixture was allowed to settle for 24 h in the separatory funnel to ensure complete separation of the two immiscible phases. Phase separation was allowed in the dark to prevent the loss of light-sensitive phenolic compounds. Following phase separation, ethyl acetate fraction was collected, and rotary evaporated at 32 ºC. The resulted concentrate was nitrogen purged at 32 ºC to completely remove moisture and obtain a mixture of simple phenolic metabolites from the biotransformation process.

3.4.5 Characterization of phenolic metabolites from PAC biotransformation

Phenolic metabolites generated by biotransformation were characterized using UPLC-ESI-MS/MS. Samples for analysis were prepared by dissolving the phenolic metabolites cocktail in methanol (20,000 µg/mL) and filtered through 0.22 µm nylon filters. Each sample was analyzed for 39 ion masses (ESI^-) using single ion monitoring mode of UPLC-ESI-MS/MS (Table 4) of commonly reported phenolic metabolites by previous studies. The retention times (Rt) of phenolic metabolites suspected for detection were matched with Rt of their pure standards to ensure false-free characterization.

Table 4: Expected phenolic metabolites after biotransformation and their ESI⁻

Expected phenolic metabolites	Ion mass ESI⁻
1. Fumaric acid	115.07
2. Succinic acid	117.088
3. Pyrocatechol	109.1
4. p-Hydroxybenzaldehyde	121.123
5. Pyrogallol	125.11
6. 4-Hydroxybenzoic acid	137.024
7. 3-Hydroxybenzoic acid	137.024
8. *trans*-Cinnamic acid	147.161
9. Hydrocinnamic acid (3-phenyl propionic acid)	149.177
10. 4-Hydroxyphenylacetic acid	151.04
11. 3-Hydroxyphenylacetic acid	151.04
12. Vanillin	151.149
13. Protocatechuic acid	153.012
14. *m*-Coumaric acid	163.04
15. *p*-Coumaric acid	163.04
16. 3-Hydroxyphenylpropionic acid	165.056
17. 3-(4-Hydroxyphenyl)propionic acid	165.176
18. 3,4-Dihydroxyphenylacetic acid	167.035
19. Vanillic acid	167.035
20. 2,5-Dihydroxyphenylacetic acid (homogentisic acid)	167.148
21. Gallic acid	169.12
22. 5-Phenylvaleric acid	177.092
23. Hippuric acid	178.051
24. Caffeic acid	179.035

Expected phenolic metabolites	Ion mass ESI^-
25. Homovanillic acid	181.051
26. Methyl 3,4,5-trihydroxybenzoate	183.14
27. Monohydroxylated phenylvalerolactone	191.21
28. Methyl hippuric	192.067
29. 4-Hydroxyphenylvaleric acid	193.087
30. Isoferulic acid	193.186
31. Ferulic acid	193.186
32. 4-Hydroxyhippuric acid	194.046
33. Syringic acid	197.174
34. 5-(3,4-Dihydroxyphenyl)-gamma valerolactone	207.21
35. 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid)	223.212
36. (+)-Catechin	289.072
37. (−)-Epicatechin	289.072
38. Ellagic acid	301.19
39. Chlorogenic acid	353.311

The UHPLC system (Model H-class system, Waters, Milford, MA, USA) was equipped with a 1.7 µm Acquity UPLC BEH C_{18} column (2.1 × 100 mm) (Waters, Milford, MA, USA). UPLC separation was performed with a linear gradient solvent profile using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), where proportion of solvent A was changed with time (min); (time, solvent A%): (0, 94%), (2, 83.5%), (2.61, 83%), (3.17, 82.5%), (3.63, 82.5%), (4.08, 81.5%), (4.76, 80%), (6.75, 20%), (8.75, 94%), and (12, 94%). The injection volume was regulated at 0.2 µL, and the flow rate was maintained at 0.2 mL/min. Mass spectrometry analysis was done by using

Micromass Quattro micro API MS/MS system (Micromass, Cary, NC, USA) controlled by MassLynx V4.1 data analysis software. The analysis was conducted in single ion monitoring (SIM) mode using electrospray ionization negative ion mode (ESI^-), with applied 3000 V capillary voltage and nebulizing gas (N_2) at 375 ºC with a flowrate of 0.35 mL/min.

UPLC-ESI-MS/MS Waters Quattro Micro API system was unable to detect expected metabolites in biotransformed PAC samples due to the limitation of sensitivity. Therefore, a sample of GS-PAC biotransformed by *L. rhamnosus* (72 h under aerobic conditions in BHI broth) was analyzed using UPLC ESI Xevo G2-XS QTof high-resolution mass spectrometry system at Waters Corporation (Beverley, MA, USA). The biotransformed PAC sample and its control were analyzed by non-targeted mode of UPLC-ESI-QTof system.

3.5 Protection by biotransformed and pure GS-PAC against NNKOAc-induced cytotoxicity

The potential of biotransformed and pure GS-PAC in protecting BEAS-2B and WRL-68 cells against NNKOAc-induced cytotoxicity was compared.

3.5.1 Cytotoxicity of biotransformed and pure GS-PAC

Toxicity of biotransformed and pure GS-PAC in BEAS-2B and WRL-68 cells were assessed by the MTS assay. Selection of non-toxic levels of PAC was required for the experiments investigating the potential of PAC (biotransformed and pure) in protection against NNKOAc-induced cytotoxicity. BEAS-2B and WRL-68 cells were seeded in 96-well plates at a density of 6000 cells/well and allowed to attach overnight. Cells were

treated with 10, 25, 50, 100, 250, and 500 µg/mL concentrations of the biotransformed and pure PAC for 24 h. MTS assay was conducted as described in section 3.3.1 to assess the cell viability.

3.5.2 Protection by biotransformed and pure GS-PAC against NNKOAc-induced cytotoxicity

The potential of biotransformed and pure GS-PAC for cytoprotection in NNKOAc-treated BEAS-2B, and WRL-68 cells were assessed by the MTS assay. Cells were seeded in 96-well plates at a density of 6000 cells/ well and allowed to attach overnight. Cells were treated with biotransformed GS-PAC (50 and 100 µg/mL) and pure GS-PAC (5 and 10 µg/mL) concentrations for 24 h. Then the cells were treated with 300 µM NNKOAc for 4 h. MTS assay was conducted as described in section 3.3.1 to assess the cell viability.

3.6 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Minitab statistical software (version 17) at 0.1% significance level ($p < 0.01$). Results were expressed as the mean ± standard deviation of three individual experiments performed in triplicates. Means were compared by Tukey's multiple mean comparisons at $p < 0.05$ level.

CHAPTER 4: RESULTS

4.1 Specific microbial metabolites protect BEAS-2B and WRL-68 cells from NNKOAc-induced cytotoxicity and genotoxicity

4.1.1 Concentration-dependent cytotoxicity of MMP

The concentration-dependent cytotoxicity of MMP was investigated, using MTS assay of cell viability, for the selection of non-cytotoxic concentrations in BEAS-2B and WRL-68 cells. Tested MMP; 3-phenylpropionic, 4-hydroxyphenyl acetic, 3-(4-hydroxyphenyl) propionic and *ρ*-coumaric acid were not cytotoxic in the concentration range of 0.1 to 1000 µM (≥ 80 % cell viability) for both cell lines. Benzoic acid depicted cytotoxicity in BEAS-2B cells at extremely high 1000 µM concentration (56.1 % cell viability) with no cytotoxicity in WRL-68 cells (≥ 80 % cell viability). Cytotoxicity by PG and PC was concentration-dependent in both cell lines (Fig. 7). Interestingly, both PG and PC promoted the % cell viability in BEAS-2B cells at 1, 10, and 25 µM concentrations as measured by MTS assay. PC promoted the % cell viability of WRL-68 cells in the same concentration range. However, no promotion in % cell viability was observed for PG in WRL-68 cells. DMSO control in all experiments maintained the % cell viability over 96%. Based on these results, 10, 25, 50, and 250 µM concentrations of the MMP were selected for the further experiments on cytoprotection induced by NNKOAc.

4.1.2 PG- and PC-mediated H_2O_2 production in cell culture media

Experimental results on cytotoxicity of phenolics *in vitro* are often misguided by the production of H_2O_2 and other ROS in the culture media (Lapidot, *et al*., 2002). Potential

production of H_2O_2 by PG and PC in cell-free BEGM and complete MEME media was evaluated by Amplex® Red assay, to ensure the selected concentrations cause no misguided results in this study. PG and PC produced H_2O_2 in a concentration-dependent manner in both BEGM and complete MEME media (Fig. 8), especially at higher concentrations (≥ 250 μM). However, 250 μM concentration was retained for the experimentation of MMP-mediated cytoprotection, as tested MMP other than PG and PC showed no cytotoxicity at this concentration.

4.1.3 Effect of PG and PC on cell proliferation

Promoted % cell viability is related to increased cell proliferation. Oregon Green® cell proliferation assay was conducted to measure the effect of PG and PC on the proliferation of BEAS-2B and WRL-68 cells. In contrast to the MTS results, PG limited the proliferation of BEAS-2B and WRL-68 cells in a concentration-dependent manner (Fig. 9 A and C). PC showed similar activity in BEAS-2B cells, being highly effective at 25 μM concentration (Fig. 9 B). Potential of PC in limiting of WRL-68 cells proliferation was statistically evident, especially at 50 μM concentration (Fig. 9 D). However, its effectiveness in limiting of WRL-68 cells proliferation is low compared to PG.

4.1.4 Cytoprotection by MMP against NNKOAc-induced cytotoxicity

Potential of MMP in mitigation of NNKOAc-induced cytotoxicity was investigated by MTS assay of cell viability. MMP concentrations capable of protecting cells against NNKOAc-induced cytotoxicity were identified to be used in the experiments evaluating MMP-mediated DNA damage reduction. Tested MMP other than PG and PC were incapable of protecting of BEAS-2B and WRL-68 cells against NNKOAc-induced

cytotoxicity at tested concentrations (Fig. 10). PG and PC protected BEAS-2B and WRL-68 cells respectively, exhibiting the cell line specificity of two MMP in cytoprotection. PG depicted the highest cytoprotection at 10 and 25 μM concentrations in BEAS-2B cells, while PC at 25 and 50 μM concentrations in WRL-68 cells. Cytoprotection in BEAS-2B cells by PC was significantly higher ($P< 0.01$) than other MMP excluding PG. However, the PC-mediated cytoprotection in BEAS-2B cells was less than 50% at the tested concentrations and therefore, not considered for further experimentation. DMSO control maintained the % cell viability over 80% in all experiments. Cytotoxicity of NNKOAc in BEAS-2B cells was more intense than in WRL-68 cells. Based on the potential of MMP in mitigation of NNKOAc-induced cytotoxicity, 10 and 25 μM concentrations of PG were selected to investigate their ability in the prevention of NNKOAc-induced DNA damage in BEAS-2B cells. Similarly, 25 and 50 μM concentrations of PC were selected for WRL-68 cells.

4.1.5 Potential of PG and PC in the prevention of NNKOAc-induced cellular DNA damage

Potential of PG and PC in the prevention of NNKOAc-induced DNA damage was assessed using γ-H2A.X immunofluorescence assay and comet assay. The γ-H2A.X immunofluorescence assay specifically detects double-strand breaks in cellular DNA by detecting DNA damage repair protein, phosphorylated histone H2A.X, at the damaged site (Ivashkevich, *et al*., 2012). These damaged sites appear in fluorescence microscopic images as red dots in DAPI stained blue colour cellular nuclei (Fig. 11). DNA damage by NNKOAc in BEAS-2B cells pre-exposed to PG was significantly reduced ($p < 0.01$) at 10 and 25 μM concentrations (Fig. 11 A). The tested PG concentrations induced DNA damage in cells independent of NNKOAc. Similarly, PC reduced the NNKOAc-induced DNA

damage in WRL-68 cells, yet showing increased protection with the concentration (Fig. 11 B). PC was capable of inducing DNA damage in WRL-68 cells independent of NNKOAc. DMSO controls showed no signs of DNA damage in both cell lines while NNKOAc exhibited high damage.

The comet assay evaluates the single and double strand breaks in cellular DNA by taking advantage of loss of super coil structure in damaged DNA (Collins, 2004). Statistical analysis of software generated olive moment data suggested no potential of PG and PC in reducing of NNKOAc-induced DNA damage in BEAS-2B and WRL-68 cells, respectively. Statistical insignificance is common in comet assay experiments due to the high olive moment variation among comets from the same treatment. However, the fluorescence microscopic images of comet assay suggested the potential of PG and PC in reducing NNKOAc-induced DNA damage (smaller comets compared to the NNKOAc control) in BEAS-2B and WRL-68 cells, respectively (Fig. 12). Analogous to the results by γ-H2A.X immunofluorescence assay PG and PC caused DNA damage in cells independent of NNKOAc. DMSO controls depicted low DNA damage visually in both cell lines, while NNKOAc caused higher DNA damage.

4.1.6 PG and PC alters the expression of DNA damage response proteins

PG in BEAS-2B cells and PC in WRL-68 cells significantly altered the expression of DDR proteins (Fig. 13 and 14). NNKOAc upregulated the expression of all tested phospho (*p*)-proteins in BEAS-2B and WRL-68 cells. PG downregulated the expression of *p*-ATM at 10 μM level in BEAS-2B cells. However, at 25 μM concentration of PG depicted no significant difference with NNKOAc control for the expression of *p*-ATM (Fig. 13 B).

PG upregulated the expression of *p*-ATR in BEAS-2B cells at both 10 and 25 μM concentrations (Fig. 13 C). Expression of *p*-Chk1, *p*-Chk2, and *p*-p53 was downregulated in BEAS-2B cells by both 10 and 25 μM concentration of PG (Fig. 13 D, E, and F). Downregulation of *p*-Chk1 was dependant on the PG concentration. A significant reduction in the expression of *p*-p53 was observed (compared to NNKOAc control) in BEAS-2B cells when treated with 10 and 25 μM concentrations of PG. The effect by PG on *γ*-H2A.X expression in BEAS-2B cells was concentration-dependent, and only 25 μM level of PG could suppress *γ*-H2A.X expression (Fig. 1 G).

In contrast to PG in BEAS-2B cells, PC upregulated the expression of *p*-ATM, *p*-p53, and *γ*-H2A.X in WRL-68 cells (Fig. 14 B, F, and G). PC enhanced the expression of *p*-ATM in WRL-68 cells at both 25 and 50 μM concentration. The levels of *p*-p53 and *γ*-H2A.X in WRL-68 cells were only improved at 50 and 25 μM concentrations of PC, respectively. Furthermore, in contrast to the influence of PG in BEAS-2B cells, PC restricted the expression of *p*-ATR in WRL-68 cells at 25 μM level (Fig.14 C). The activity of PC on the regulation of *p*-Chk1 and *p*-Chk2 in WRL-68 cell was similar to the activity of PC in BEAS-2B cells. PC downregulated *p*-Chk1 and *p*-Chk2 expression in WRL-68 cells at both 25 and 50 μM concentrations. Downregulation of *p*-Chk2 by PC was concentration-dependant.

4.2 Biotransformation of AP and GS-PAC by probiotic bacteria *L. rhamnosus* and *L. casei*

4.2.1 Efficiency of PAC extraction by different extraction methods

The efficiency of different extraction methods was studied with the intention of selecting the most suitable method to extract PAC in sufficient quantities. The conventional PAC extraction method which involves solvent extraction together with purification by flash chromatography was used as the primary extraction method. The quantity of PAC in final extracts greatly varied depending on the extraction method (Table 5). The supercritical CO_2 extraction methods generated excessive quantities of final extract (g PAC/100g dry sample) with higher PAC content (mg CE/ g dried extract) compared to the solvent extraction method, indicating the possible inclusion of non-PAC materials. Thus, PAC extraction for experiments was continued with the primary extraction method.

4.2.2 PAC extracts show toxicity in probiotic bacteria with increasing concentrations

Quantification of the lowest threshold toxic concentrations of PAC extracts for *L. rhamnosus,* and *L. casei* was essential in designing PAC biotransformation protocol. AP and GS-PAC caused concentration-dependant toxicity in both bacterial strains (Figure: 15) under aerobic and anaerobic conditions. GS-PAC limited the growth of *L. rhamnosus* and *L. casei* at and above 100 µg/mL concentrations. Toxicity of apple PAC on two bacterial strains was low compared to the toxicity of GS-PAC. AP-PAC considerably limited the bacterial growth at 500 µg/mL concentration. The toxicity concentrations of PAC remained similar for bacteria culturing under aerobic and anaerobic conditions. Interestingly, GS-

PAC stimulated the growth of both bacterial strains at 50 µg/mL concentration increasing the % bacterial growth over DMSO control (Fig. 15 B).

4.2.3 PAC biotransformation generates new metabolites

The ability of *L. rhamnosus* and *L. casei* in the biotransformation of AP and GS-PAC was studied under varying conditions. The two bacterial strains were unable to biotransform PAC in MRS broth under both aerobic and anaerobic conditions. Thus, the chromatograms for PAC biotransformed samples, and respective controls were identical to each other (Figure 16) signifying insignificant generation of new phenolic metabolites. Furthermore, no difference was observed between the samples incubated for different time periods and the samples incubated under aerobic and anaerobic conditions. As the biotransformation under aerobic and anaerobic conditions yielded similar results, the study was continued by performing biotransformation process only under aerobic conditions. The next set of biotransformation experiments were designed by modifying original MRS broth recipe to limit sugar (dextrose) and protein, forcing the bacteria to depend on PAC as a carbon source to sustain continuous growth.

Modified MRS media with 0% and 100% of protein (relative to the original MRS recipe) was unsuccessful in contributing PAC biotransformation. Only the GS-PAC biotransformation performed with MRS containing 50% protein yielded a variety of metabolites depending on the sugar % (Table 6). The Rt of these metabolites (suspected for PC, vanillic acid, hippuric acid, homovanillic acid, and ellagic acid) were matched with the Rt of pure phenolic PAC metabolites with same ion mass (ESI^-). However, the Rt of

known PAC metabolites did not match with the metabolites generated by biotransformation, requiring further experimentation for structure characterization.

Another PAC biotransformation experiment was performed with *L. rhamnosus* in BHI broth using GS-PAC under aerobic conditions. Even though BHI was not the recommended growth medium for *Lactobacillus* bacteria, PAC biotransformation with BHI yielded metabolites (suspected for PC, fumaric acid, succinic acid, 3-hydroxybenzoic acid, 3-hydroxyphenylpropionic acid, 5-phenylvaleric acid, homovanillic acid, methyl-3,4,5-trihydroxy benzoate, and syringic acid) not previously observed when biotransformation was performed with MRS broth (Table 7). The Rt of biotransformation derived metabolites were matched with pure phenolic metabolites (reported by previous studies) with similar ion masses. Similar, to the previous experiment, the Rt of these metabolites did not match (Figure 17), requiring further experiments for structure characterization. Therefore, high-resolution mass spectrometry (HRMS) using UPLC-ESI-QTof was performed at Waters Corporation laboratory (Beverly, MA, USA) to identify phenolic metabolites in biotransformed PAC (in BHI media for 72 h). HRMS was able to identify over 150 compounds, and most of these compounds were common to biotransformed and pure GS-PAC samples. However, higher quantities of PG, PC, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, methyl hippuric acids, 4-hydroxyphenylvaleric acid, and sinapic acid were present in the biotransformed PAC sample compared to the control (Figure 18).

4.3 Potential of pure and biotransformed GS-PAC in the reduction of NNKOAc-induced cytotoxicity

4.3.1 Pure and biotransformed GS-PAC induce cytotoxicity

Pure and biotransformed GS-PAC induced concentration-dependent cytotoxicity in BEAS-2B and WRL-68 cells. Pure GS-PAC developed toxicity (< 80% cell viability) in both cell lines at and above 25 µg/mL levels (Fig. 19 A and C). Biotransformed GS-PAC induced significant toxicity in both cell lines at 250 µg/mL level (Fig. 19 B and D). Thus, the cytotoxicity of biotransformed GS-PAC in BEAS-2B and WRL-68 cells is low compared to pure GS-PAC.

4*.3.2 Pure and biotransformed GS-PAC do not exhibit cytoprotection*

Pure and biotransformed GS-PAC was incapable of protecting BEAS-2B and WRL-68 cells from NNKOAc-induced cytotoxicity (Fig. 20). In BEAS-2B cells pure GS-PAC reduced the % cell viability even lower than NNKOAc control.

Table 5: Comparison of different methods for PAC extraction

Extraction method	Yield (g PAC/ 100 g dried sample)	PAC content (mg CE/g dried extract)
Solvent extraction: Acetone: water: acetic acid (70: 29.9: 0.1) extraction + column chromatography	0.504 ± 0.137	161.7 ± 12.3
Supercritical CO_2	3.795	276.3 ± 9.3
Supercritical CO_2 modified with 20% ethanol (Wt%)	6.30	322.4 ± 13.9

Table 6: Ion masses (ESI^-)/charge of the metabolites observed after GS-PAC biotransformation with 50% protein-sugar media broth combinations

Ion mass/charge (ESI^-)	Expected PAC metabolite	Sugar %	
		L. rhamnosus	***L. casei***
109.1	Pyrocatechol	0, 5	–
167.03	Vanillic acid	5	0, 5, 10
178.05	Hippuric acid	5	–
181.05	Homovanillic acid	5	0, 5, 10
301.19	Ellagic acid	5, 10, 20	0, 5, 10

Table 7: Ion masses (ESI^-)/charge of the metabolites resulted by biotransformation of GS-PAC with L. rhamnosus under aerobic condition in BHI broth

Expected PAC metabolite	**Ion mass (ESI^-)/charge**
Pyrocatechol	109.01
Fumaric acid	115.07
Succinic acid	117.08
3-hydroxybenzoic acid	137.03
3-hydroxyphenylpropionic acid	165.05
5-phenylvaleric acid	177.09
Homovanillic acid	181.05
Methyl-3,4,5-trihydroxy benzoate	183.14
Syringic acid	197.04

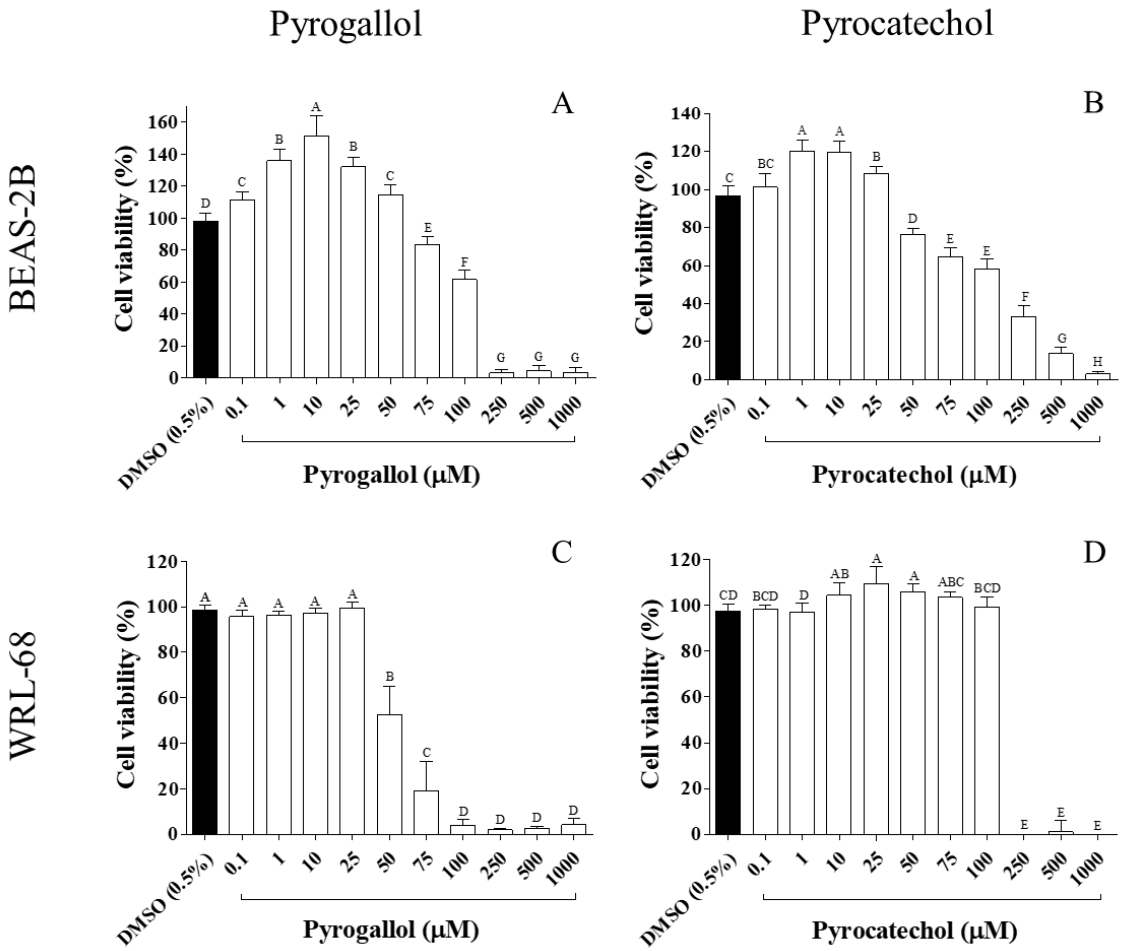


Figure 7: Concentration-dependent cytotoxicity of pyrogallol and pyrocatechol in BEAS-2B (A, B) and WRL-68 cells (C, D). Cells were treated with microbial metabolites of proanthocyanidins for 24 h and measured for % cell viability using the MTS assay. Results were presented as mean ± SD of three independent experiments. Means that do not share a similar letter are significantly different ($p \leq 0.01$).

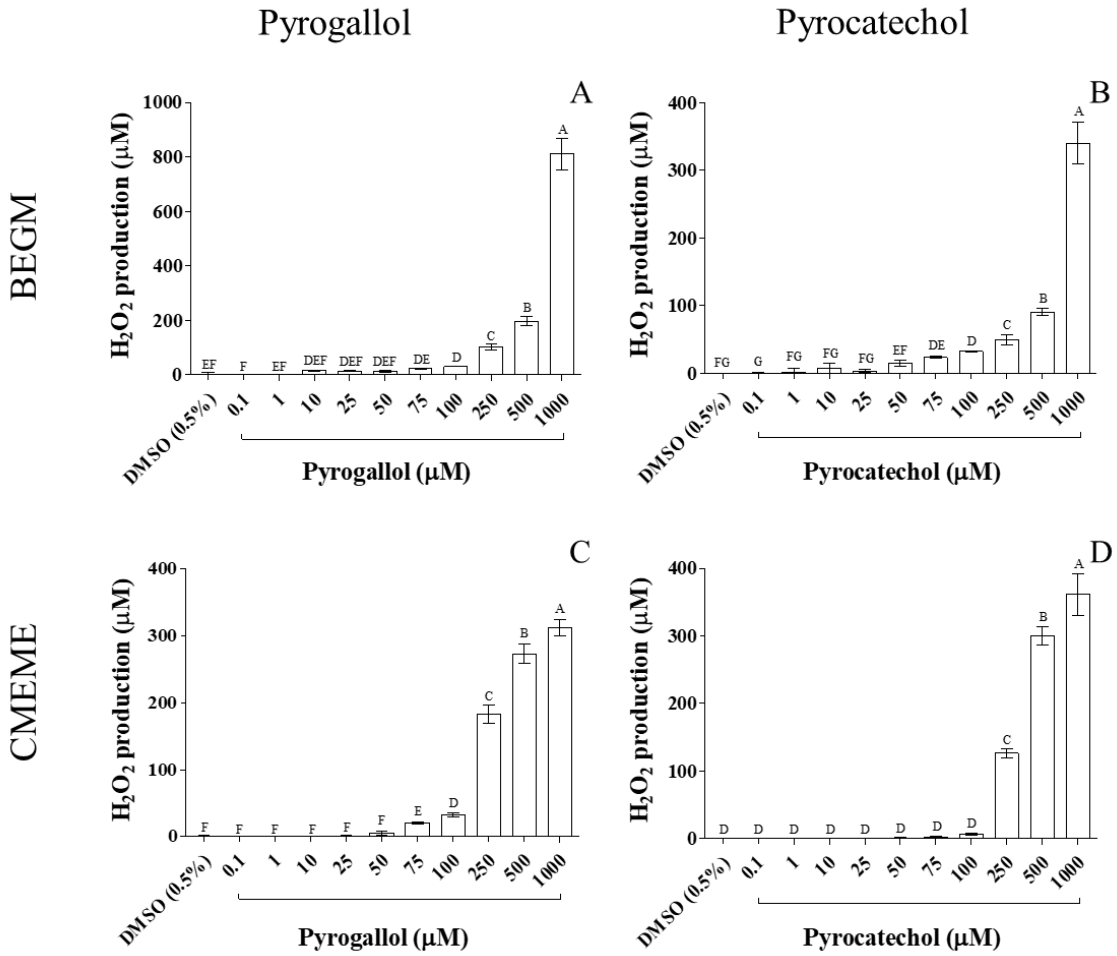


Figure 8: Pyrogallol and pyrocatechol-mediated H_2O_2 production in BEGM (A, B) and CMEME (C, D) media. Treatment concentrations (0.1 – 1000 µM) of pyrogallol and pyrocatechol were prepared in cell culture media, and H_2O_2 production was measured by Amplex® Red assay (2 h of incubation at 37 ºC). Results were presented as mean ± SD of three independent experiments. Means that do not share a similar letter are significantly different ($p \leq 0.01$).

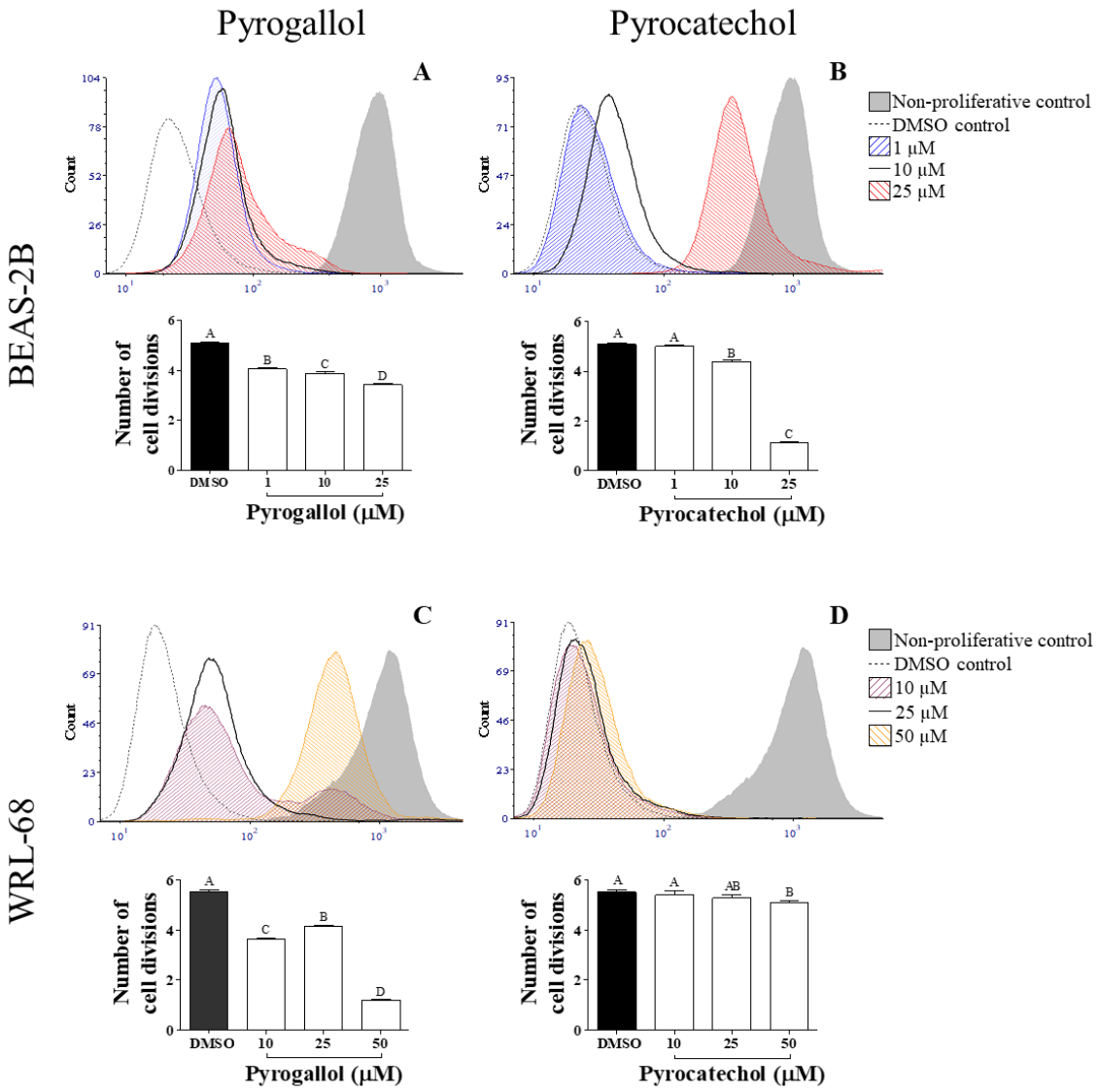


Figure 9: Effect of pyrogallol and pyrocatechol on the proliferation of BEAS-2B and WRL-68 cells. Cells were treated with low concentrations (10, 25, and 50 µM) of pyrogallol and pyrocatechol for 72 h and assessed for effect on cell proliferation by Oregon green-flow cytometry assay. Each experiment was repeated for three times to ensure reproducibility of data. Data were processed and plotted using FCS Express 6 plus Research Edition software. Means that do not share a similar letter are significantly different ($p \leq 0.01$).

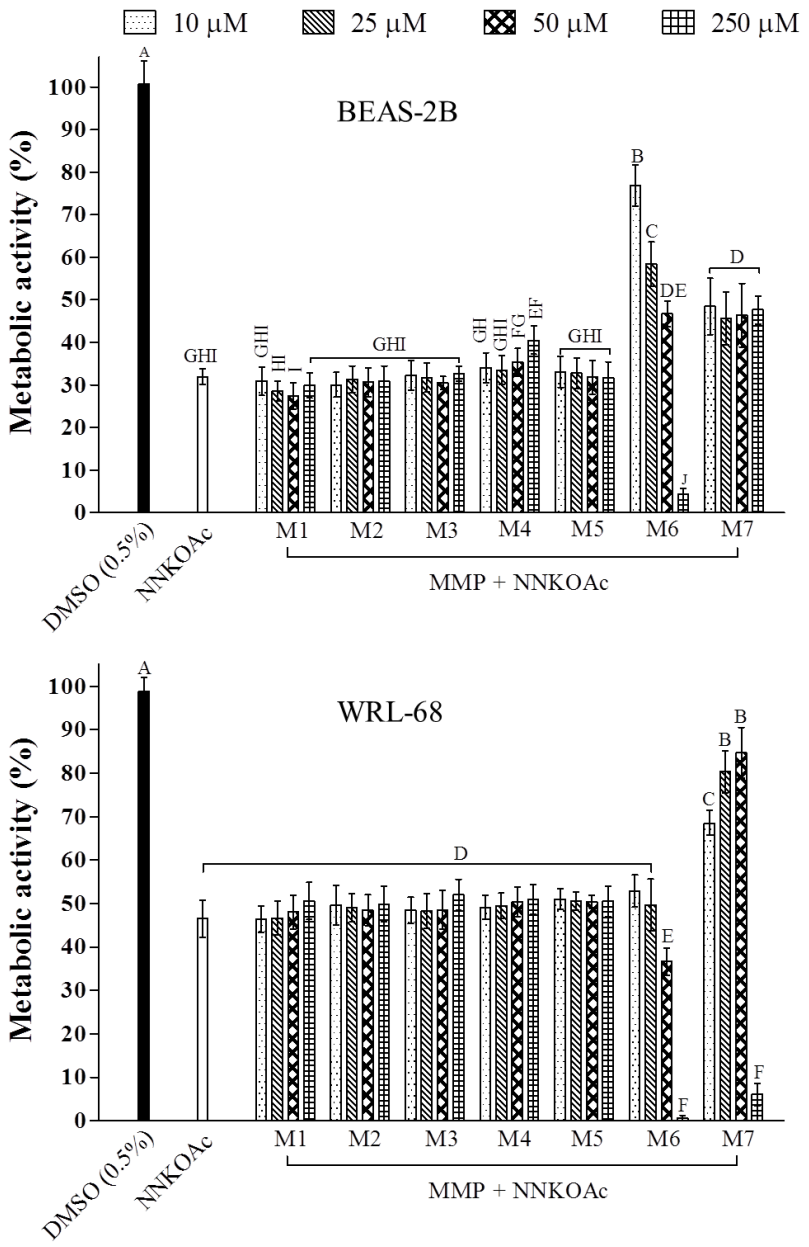


Figure 10: Potential of microbial metabolites of proanthocyanidins (MMP) in the prevention of NNKOAc-induced cytotoxicity in BEAS-2B and WRL-68 cells. Cells were pre-exposed to MMP (10, 25, 50, and 250 µM) for 24 h and treated with NNKOAc (300 µM) for 4 h after washing with PBS. The capacity of MMP in cytoprotection was measured as % cell viability by MTS assay. Results were presented as mean ± SD of three independent experiments. Means that do not share a similar letter are significantly different ($p \leq 0.01$). M1, 3-phenylpropionic acid; M2, 4-hydroxyphenyl acetic acid; M3, 3-(4-hydroxyphenyl) propionic acid; M4, *p*-coumaric acid; M5, benzoic acid; M6, pyrogallol; M7, pyrocatechol.

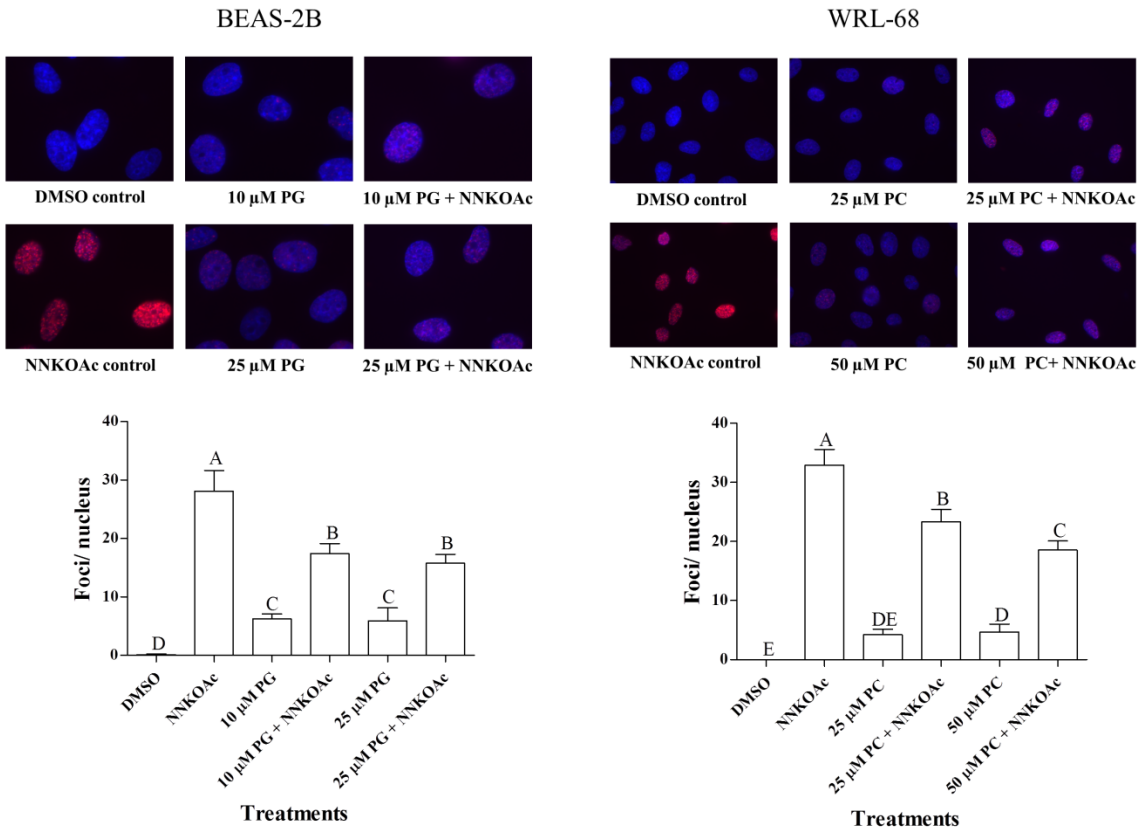


Figure 11: Reduction of NNKOAc-induced DNA damage by PG in BEAS-2B cells and PC in WRL-68 cells as measured by the γ-H2A.X assay. BEAS-2B and WRL-68 cells were pre-exposed to selected MMP for 24 h and induced for DNA damage using NNKOAc (300 µM) for 4 h. Phosphorylated histone γ-H2A.X foci were labeled with specific antibodies and nuclei stained with DAPI. At least 100 nuclei of each treatment were imaged by fluorescence microscopy (×40). A number of γ-H2A.X foci was counted by ImageJ software and results averaged as foci/nucleus. Results were presented as mean ± SD of three independent experiments. Means that do not share a similar letter are significantly different ($p \leq 0.01$). PG, pyrogallol; PC, pyrocatechol.

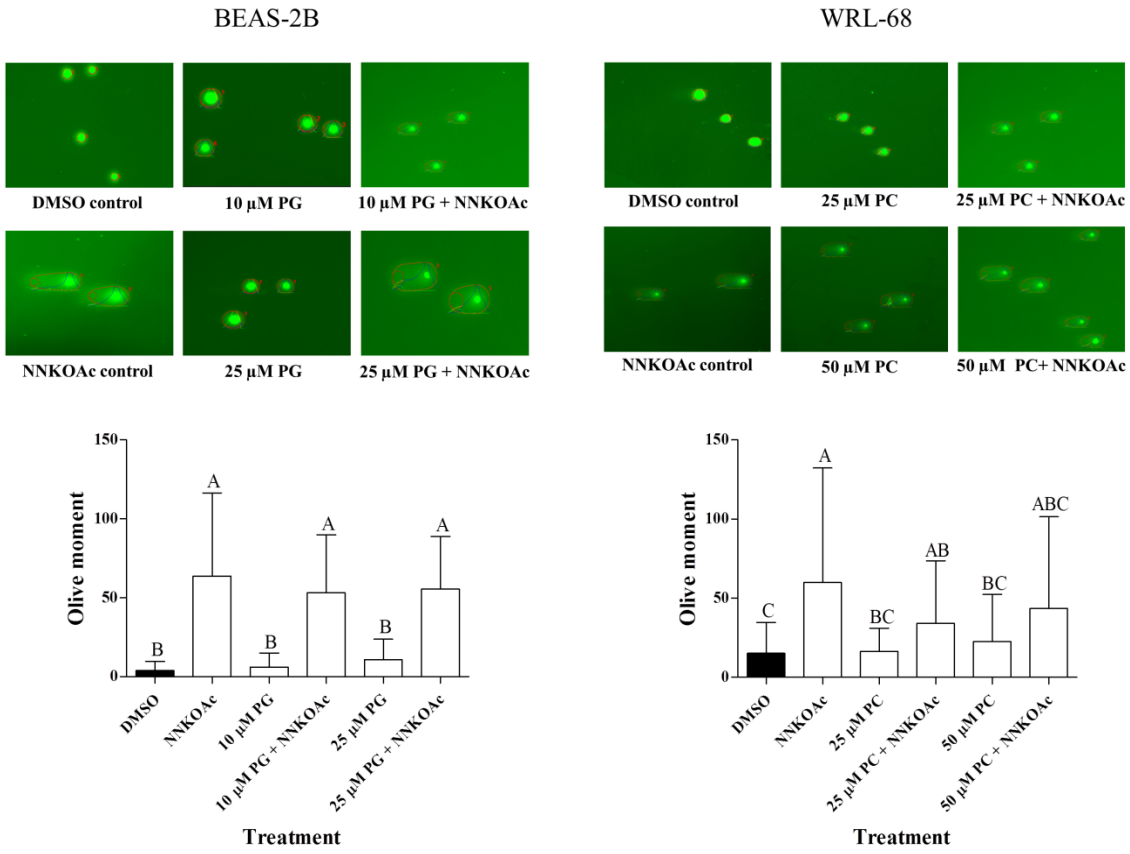


Figure 12: The effect of PG in BEAS-2B cells and PC in WRL-68 cells on NNKOAc-induced DNA damage as measured by comet assay. BEAS-2B and WRL-68 cells were pre-exposed to the selected MMP for 24 h and induced for DNA damage using NNKOAc (300 µM) for 4 h and subjected to alkaline comet assay. At least 100 comets of each treatment were imaged by fluorescence microscopy (×40). Images were analyzed by OpenComet software and results were presented as mean olive moment ± SD. Means that do not share a similar letter are significantly different ($p \leq 0.01$). PG, pyrogallol; PC, pyrocatechol.

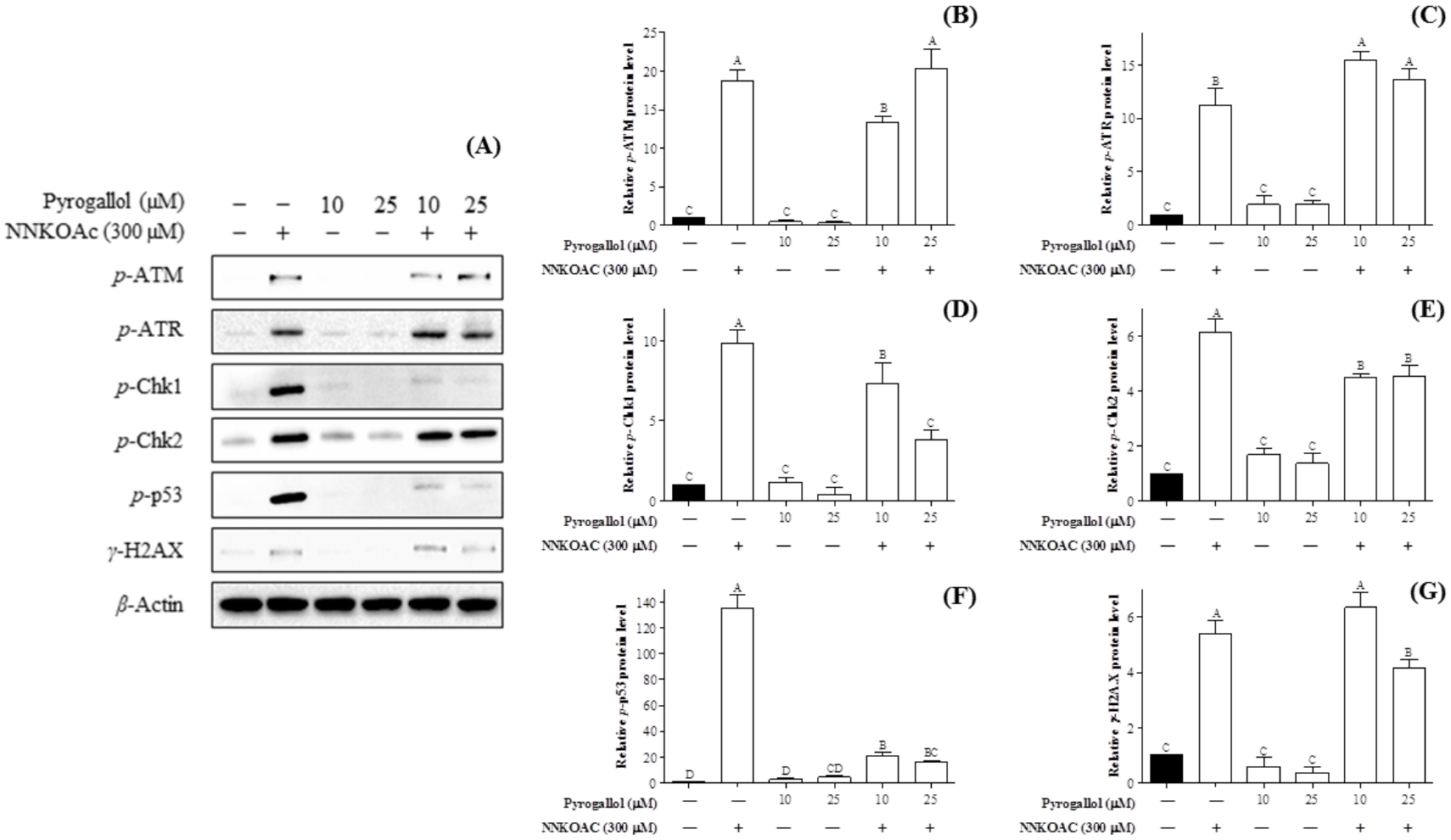


Figure 13: Expression of phosphorylated (*p/γ*) ATM (B), ATR (C), Chk1 (D), Chk2 (E), p53 (F), and H2A.X (G) proteins in BEAS-2B cells. Cells were treated with 10 and 25 μM concentrations of pyrogallol and exposed to 300 μM NNKOAc for 4 h. The

level of expression of DNA damage repair proteins were quantified by western blotting. Results were expressed as relative protein levels compared to the DMSO control. At least three western blotting experiments were performed, and the results were expressed with means ± standard deviations. Results were statistically analyzed by one-way ANOVA and Tukey's mean separation ($\alpha = 0.01$) using Minitab statistical software.

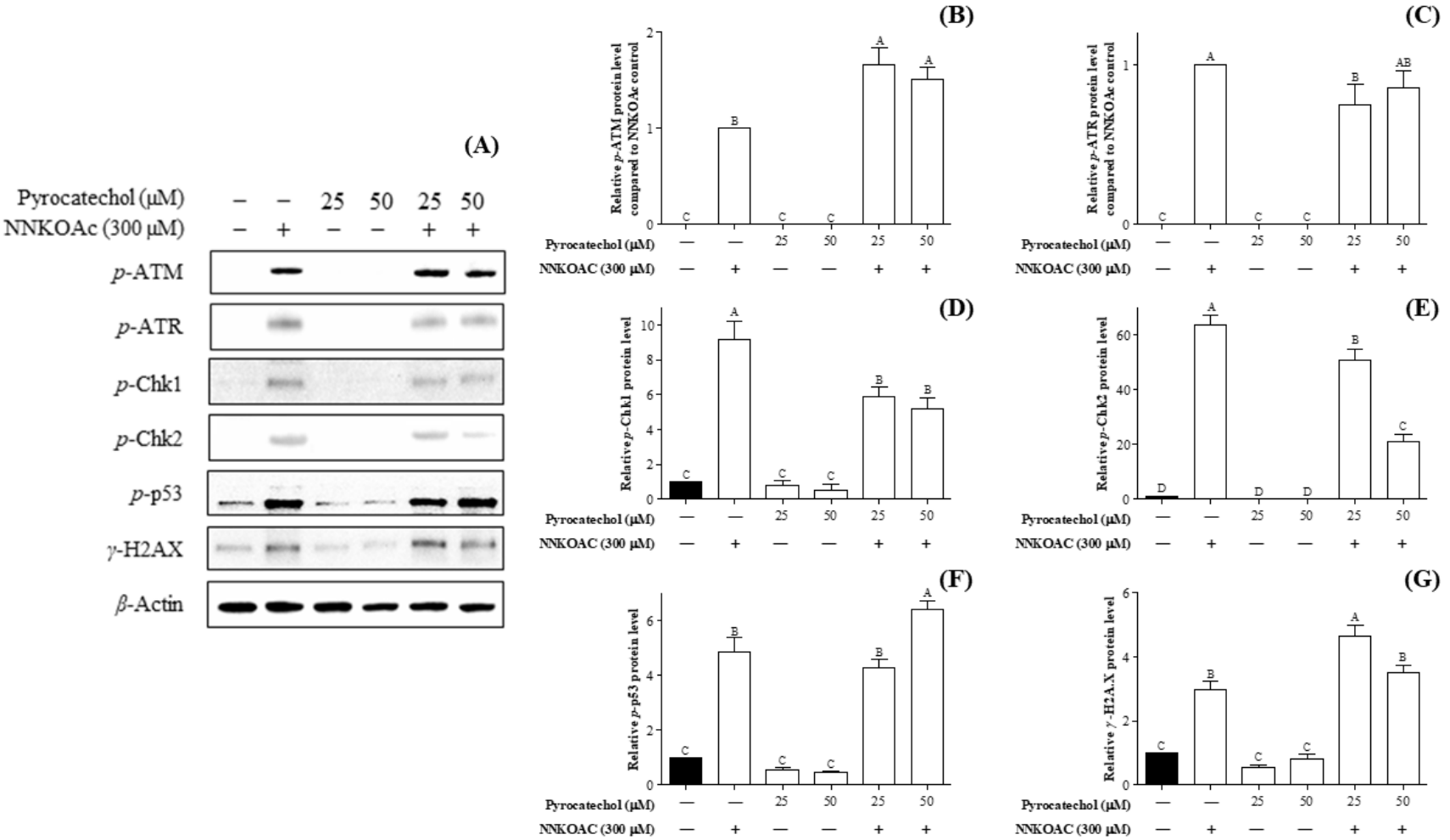
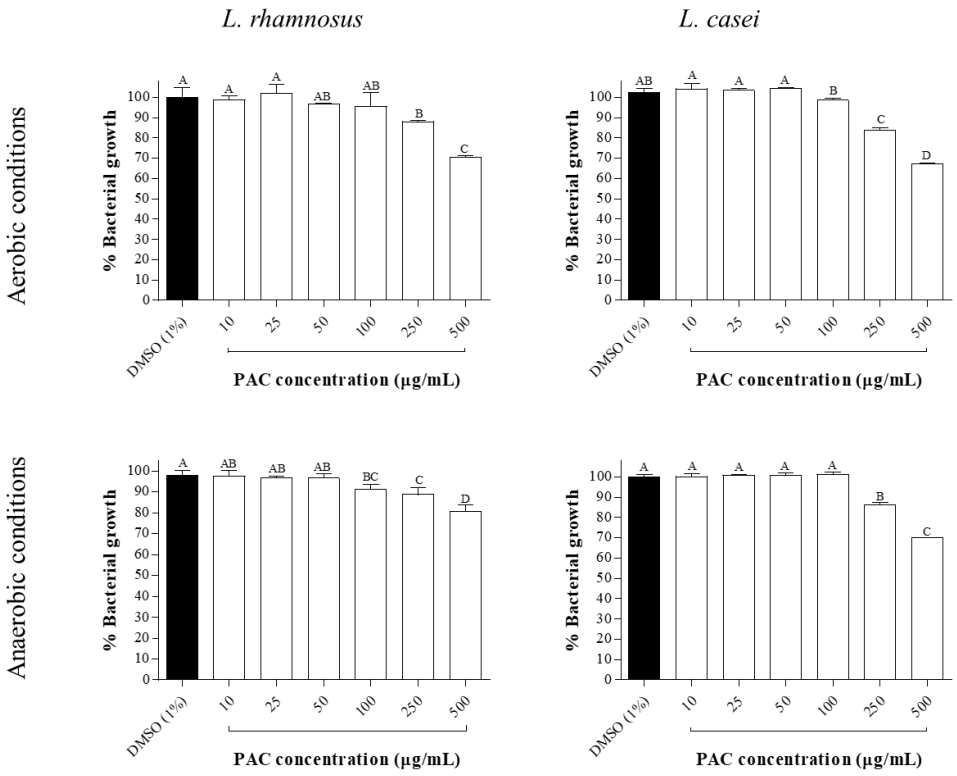


Figure 14: Expression of phosphorylated (*p/γ*) ATM (B), ATR (C), Chk1 (D), Chk2 (E), p53 (F), and H2A.X (G) proteins in WRL-68 cells. Cells were treated with 10 and 25 μM concentrations of pyrocatechol and exposed to 300 μM NNKOAc for 4 h. The

level of expression of DNA damage repair proteins were quantified by western blotting. Results were expressed as relative protein levels compared to the DMSO control. Protein levels were compared with the NNKOAc control when DMSO control failed to generate a measurable band. At least three western blotting experiments were performed, and the results were expressed with means ± standard deviations. Results were statistically analyzed by one-way ANOVA and Tukey's mean separation ($\alpha = 0.01$) using Minitab statistical software.

A. Apple peel PAC



B. Grape seed PAC

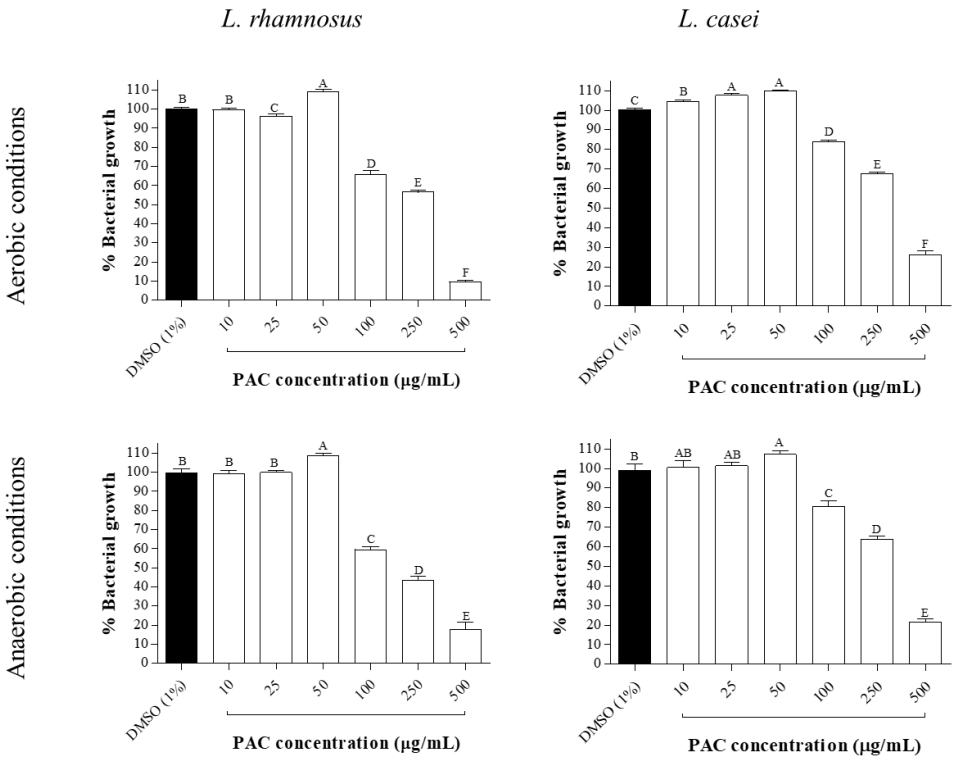


Figure 15: Concentration-dependent toxicity of apple peel PAC (A) and GS-PAC (B) in *L. rhamnosus* and *L. casei* probiotic bacteria. Probiotic bacteria were cultured with 10 – 500 µg/mL concentrations of PAC in De Man Rogosa and Sharpe broth. Cultures were incubated for 24 h at 37 ºC, and optical density was measured at 600 nm (OD_{600}).

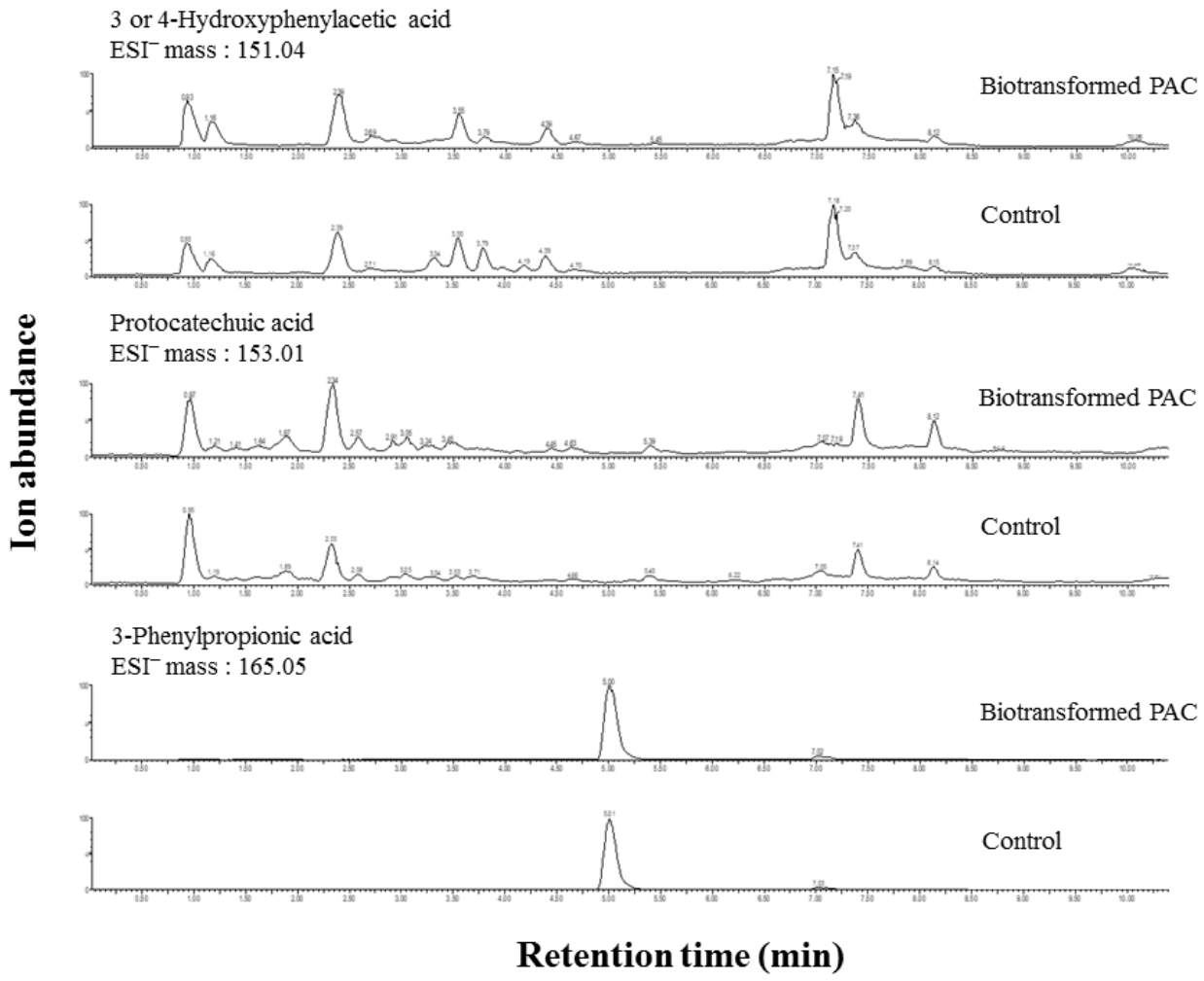


Figure 16: Comparison of chromatograms by UHPLC-ESI-MS/MS for selected target phenolic metabolites. PAC, Proanthocyanidin.

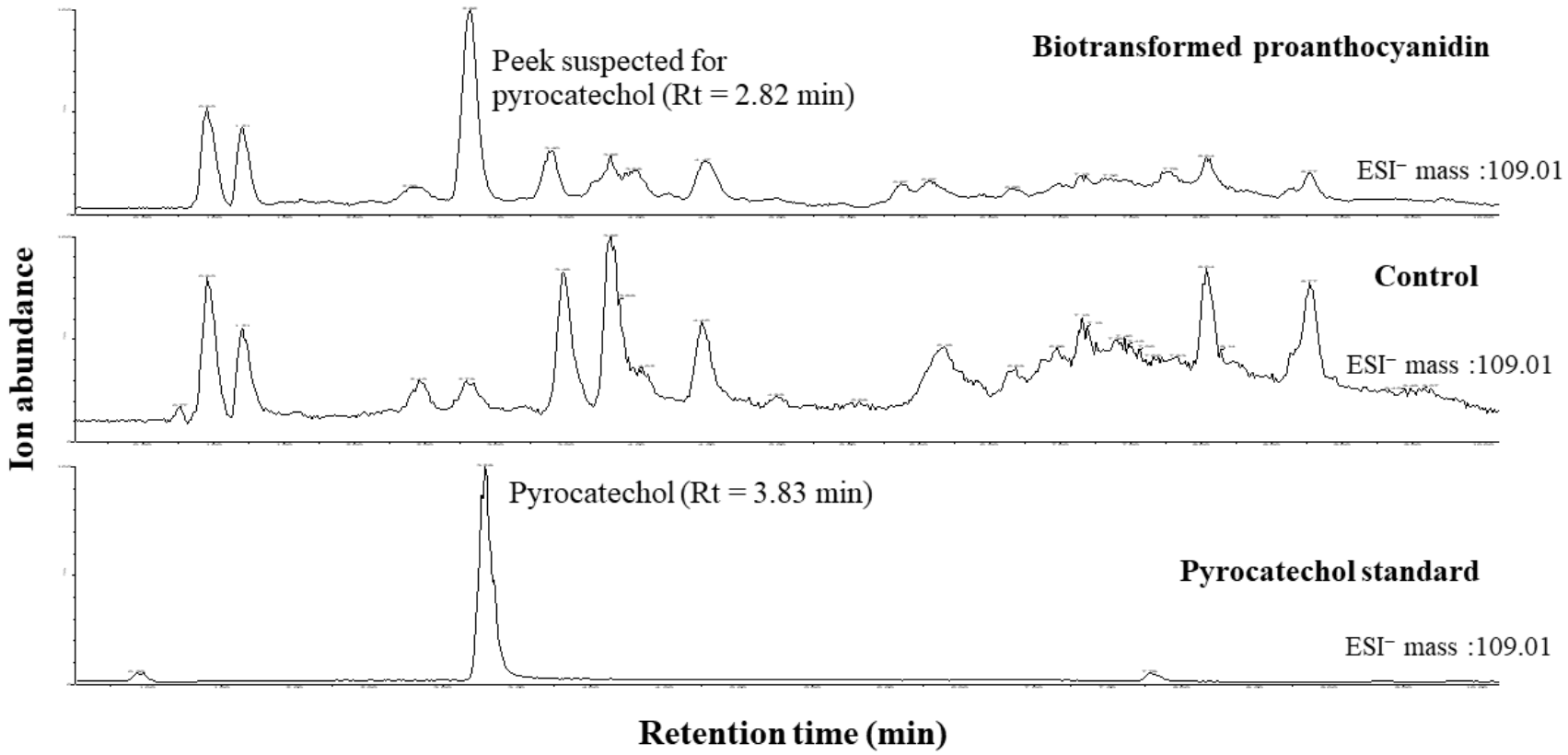


Figure 17: Comparison of chromatograms by UHPLC-ESI-MS/MS for biotransformed GS-PAC, control sample (bacterial culture with no GS-PAC), and pyrocatechol standard.

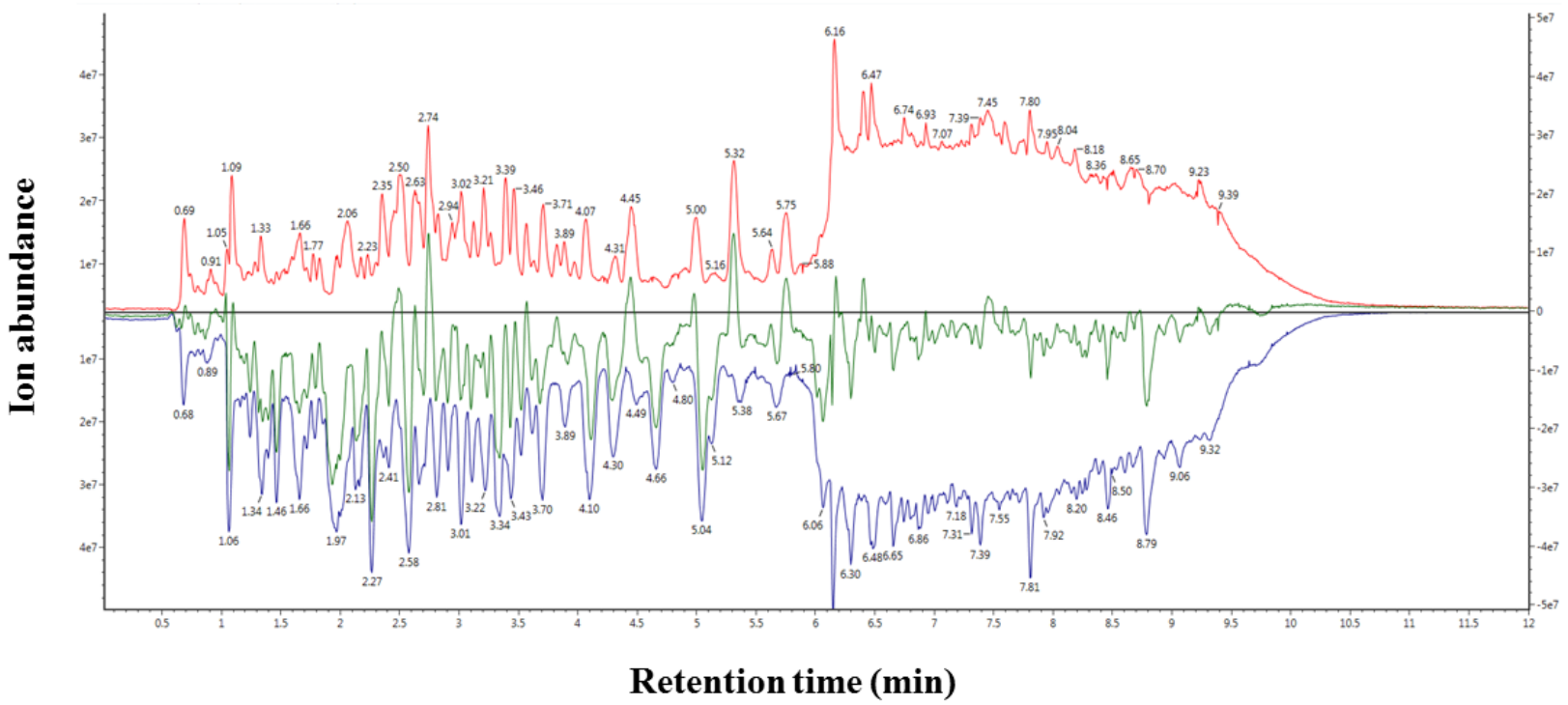


Figure 18: Total ion chromatogram by non-targeted mode of analysis of microbial metabolites of PAC measured by UPLC-ESI-QTof mass spectrometry. Red line, PAC-fed *L. Rhamnosus*; Blue line, control without PAC; green line, difference of metabolites between the PAC-fed Vs. Control.

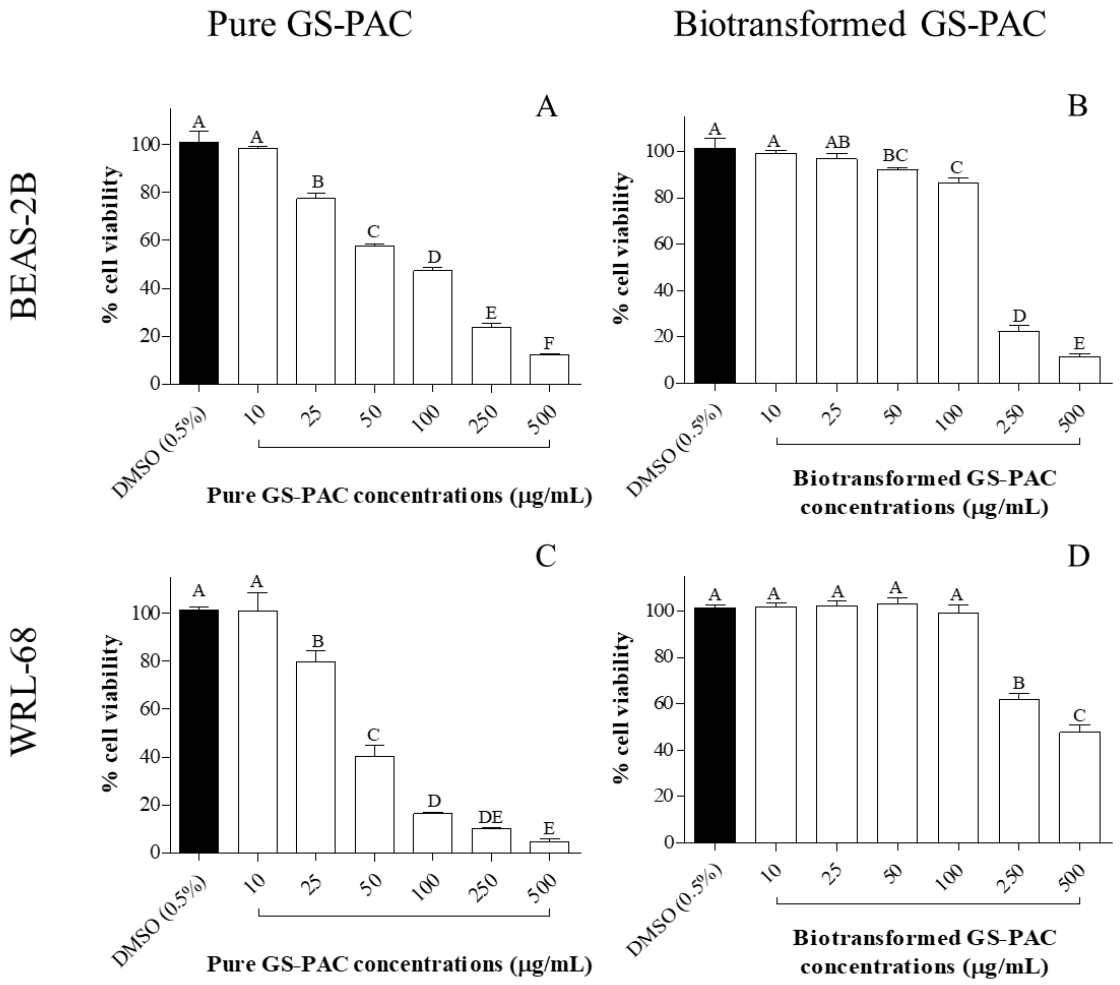


Figure 19: Cytotoxicity of pure and biotransformed GS-PAC in BEAS-2B and WRL-68 cells. Cells were treated with 10 – 500 µg/mL concentrations of pure and biotransformed grape seed proanthocyanidin (GS-PAC) for 24 h. Cell viability was measured using the MTS assay. Results were presented as mean ± SD of three independent experiments. Means that do not share a similar letter are significantly different ($p \leq 0.01$).

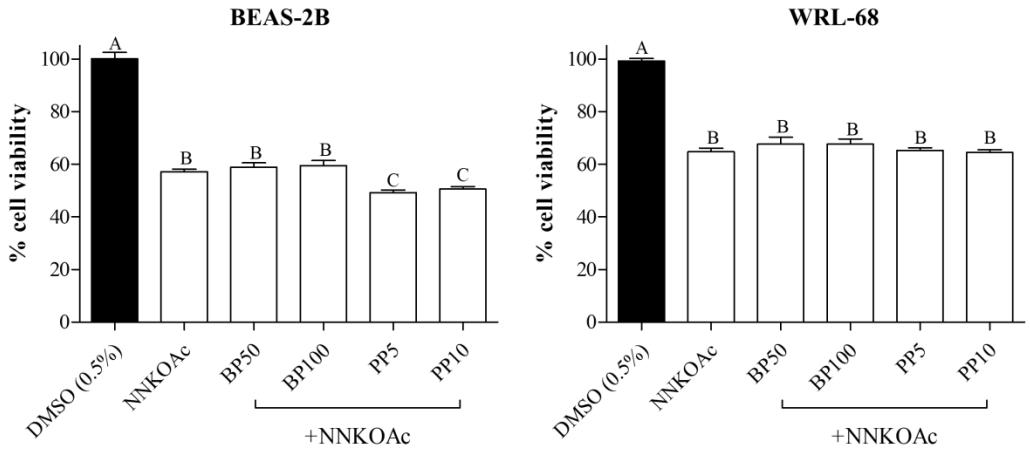


Figure 20: Potential of pure and biotransformed grape seed proanthocyanidin (GS-PAC) in prevention of NNKOAc-induced cytotoxicity in BEAS-2B and WRL-68 cells. Cells were pre-exposed to biotransformed GS-PAC (50 and 100 µg/mL) and pure GS-PAC (5 and 10 µg/mL) for 24 h and treated with NNKOAc (300 µM) for 4 h after washing with PBS. Capacity of cytoprotection was measured as % cell viability by MTS assay. Results were presented as mean ± SD of three independent experiments. Means that do not share a similar letter are significantly different ($p \leq 0.01$). BP50, biotransformed grape seed proanthocyanidin 50 µg/mL; BP100, biotransformed grape seed proanthocyanidin 100 µg/mL; PP5, pure grape seed proanthocyanidin 5 µg/mL; PP10, pure grape seed proanthocyanidin 10 µg/mL

CHAPTER 5: DISCUSSION

The cell experimental model of this study was developed by considering the well-studied positive relationship between tobacco smoking and lung carcinogenesis, to investigate the potential of MMP in prevention or reduction of carcinogen-induced DNA damage *in vitro*. Human bronchial epithelial cells (BEAS-2B) pre-exposed to MMP were challenged for cyto-and genotoxicity caused by NNKOAc. Chemical carcinogen NNKOAc mimics the effects of NNK, a major nicotine-derived carcinogen in cigarette smoke. Cellular cytochrome P450 activity is essential in the metabolism of NNK to generate secondary metabolites capable of carcinogenesis (Garcia-Canton *et al*., 2013). Cyto-and genotoxicity of NNK can be limited in BEAS-2B cells due to low cytochrome activity (Garcia-Canton *et al*., 2013). Carcinogen NNKOAc requires no cytochrome P450 activity for cellular metabolism (Peterson *et al*., 2001) and has been successfully used in a number of studies to induce DNA damage in BEAS-2B cells. Human fetal hepatic cells (WRL-68) were used as the second cell line for this study after considering the positive relationship between tobacco smoking and liver carcinoma. NNK is the predominant systemic carcinogen in tobacco smoke (Pfeifer *et al*., 2002) that may reach hepatocytes to induce carcinogenesis.

Tested MMP except for PG and PC showed no cytotoxicity in BEAS-2B and WRL-68 cell lines. Benzoic acid demonstrated cytotoxicity at an extremely high concentration (1000 μM). Low cytotoxicity of simple phenolics in normal human cells is demonstrated in a number of experiments by the extremely high concentrations used to induce cytotoxicity (Galati *et al*., 2006; Bachi and Visioli, 2003). A study by Yang *et al*. (2009)

demonstrates the PG-mediated cytotoxicity in human lung cancer cells using MTT assay. Cytotoxicity of PG is caused by cell cycle arrest at the G2 phase and induction of apoptosis (Yang *et al*., 2009). Furthermore, PG-treated cells are characterized by elevated level of intracellular superoxide (Park *et al*., 2007; Han *et al*., 2008). Cytotoxicity by PC is expressed as induction of apoptosis, evident by morphological disruption and DNA damage (De Oliveira *et al*., 2010).

It is important to consider the ability of PG and PC in production of ROS in cell-free culture media. Polyphenols, especially with catechol moiety, tend to experience rapid oxidation in commonly used culture media and produce H_2O_2 (Long *et al*., 2010). Generation of H_2O_2 in culture media leads to concentration-dependent cytotoxicity by rapid depletion of cellular ATP (Varani *et al*., 1990). A study by Kelts *et al*. (2015) demonstrated the potential of PG in the production of H_2O_2 in cell-free minimal essential media (MEM). Phenolics are also reported to alter cell proliferation by H_2O_2 production in cell culture media (Lapidot *et al*., 2002). Therefore, the potential of PG and PC for production of H_2O_2 in cell-free BEGM and complete MEME media was evaluated by Amplex® Red H_2O_2 assay. Both PG and PC showed concentration-dependent production of H_2O_2 in BEGM and complete MEME media. Excessive H_2O_2 production was observed at higher concentrations ($\geq$ 250 μM) of PG and PC. However, the concentrations (1–50 μM) of PG and PC used for the evaluation of cyto-and geno-protective effects in this study did not produce significantly high ($p \geq 0.01$) concentrations of H_2O_2 compared to the DMSO control. Moreover, preliminary MTS test results on cytotoxicity of PG in BEAS-2B and PC in WRL-68 cells, showed no signs of cytotoxicity, at these concentrations, signifying the negligible effect of H_2O_2.

Preliminary MTS test results for the investigation of MMP-mediated cytotoxicity, revealed the ability of PG and PC in the promotion of % cell viability in BEAS-2B cells at lower concentrations. Augmentation of % cell viability may relate with promoted cell proliferation, an undesirable effect for mitigation of cancer occurrence. Therefore, the effect of PG and PC in cell proliferation was further studied by Oregon Green® flow cytometry assay. The proliferation of BEAS-2B cells was limited by both PG and PC in a concentration-dependent manner. Similar results were obtained for PG and PC in WRL-68 cells. However, antiproliferative activity of PG in WRL-68 cells was more prominent compared to PC. Antiproliferative properties of PG and PC are investigated in a number of cancer cell lines. A study by Yang *et al*. (2009) experimented the potential of PG in limiting the proliferation of human lung cancer cells (H441 and H520). PG limits the proliferation of lung cancer cells by arresting cell cycle at G2/M phase (Yang *et al*., 2009). Both PG and PC-type phenolics limit the cell proliferation in estrogen responsive (ER+) MCF-7 human breast cancer cells (Fernandes *et al*., 2010). Apart from arresting of the cell cycle at different phases, phenolics express antiproliferative properties by blocking and regulation of kinases (e.g., c-Jun *N*-terminal kinase), inhibition of transcription factors and suppression of pathways important for cell growth (Dai and Mumper, 2010). Intense antiproliferative effect of PG over PC may be possible to describe by the number of hydroxyl groups bound with the phenyl ring. Structure of PG consists of two hydroxy groups and the PC with one hydroxyl groups bound to a single phenyl ring. Trihydroxylated phenolic acids demonstrate higher antiproliferative effect over dihydroxylated variants. The difference between antiproliferative effects may be due to the higher radical

scavenging activity of trihydroxy phenolic acids compared to that of dihydroxylated phenolic acids (Gomes *et al*., 2003).

The pseudo % cell viability promotion by PG and PC at lower concentrations resembles a hormetic dose-response relationship. "Hormesis is a dose-response relationship that is generally characterized as a biphasic dose-response with a low dose stimulation and a high dose inhibition" (Calabrese *et al*., 2013). The basics of hormesis concept recognize lower concentrations of a detrimental substance as a biological stimulation for cellular survival mechanism, that results in a net beneficial effect for cells (Calabrese *et al*., 2013; Chirumbolo, 2011). The % cell viability measured by MTS assay is truly a product defined by its proportionality with cell metabolic activity (Chan *et al*., 2013). Therefore, a high metabolic activity always accounts for higher % cell viability value regardless of the number of live cells. PG and PC induce toxicity at higher concentrations in BEAS-2B and WRL-68 cells by generating H_2O_2 in the cell culture media. Peters *et al*. (2017) observed the increased availability of free cellular nicotinamide adenine dinucleotide hydride (NADH) in cultured Müller astrocytes after exposing the cells to H_2O_2 for two hours (Peters *et al*., 2017). Cellular NADH converts the MTS into formazan detectable at 490 nm wavelength (Chan *et al*., 2013). Therefore, the metabolic activity (indirectly % cell viability) measured by MTS assay is depending on the availability of NADH. Thus, the pseudo % cell viability promotion and reduction by PG and PC may be a result of balance between increased availability of NADH and the H_2O_2-mediated cytotoxicity, resembling a hormetic dose-response relationship for PG- and PC-mediated cell viability as measured by the MTS assay.

Evaluation of the quantitative structure activity relationship (QSAR) for antioxidant and biological activities of the seven selected MMP, can be useful in justification of MMP-mediated cyto- and geno-protection against NNKOAc. Investigation of QSAR of antioxidant activity involves the consideration of molecular properties such as presence of hydroxyl and carbonyl groups, methylation and acylation, molecular size, and configuration of 2D and 3D structure (Rastija and Medic-Saric, 2009). The antioxidant activity of gallic acid derivatives increase with the number of hydroxyl groups attached to the aromatic ring. Acylation of hydroxyl groups and increase of the length of esterified carbon chain significantly reduce the antioxidant capacity among gallic acid derivatives (Lu *et al*., 2006). The ability of PG and PC to reduce NNKOAc-induced cytotoxicity over other tested MMP may be due to the higher antioxidant activity attributed by higher number of hydroxyl groups directly attached with the ortho-positions of the phenol rings. Studying on QSAR of cellular enzymes, especially CYP and esterase for NNK and NNKOAc metabolism, may also become handy in the justification of difference in MMP-mediated DNA damage reduction in BEAS-2B and WRL-68 cells. A review by Basheer and Kerem (2015) discusses the QSAR of polyphenols on CYP3A4 activity. CYP3A4 is a major CYP enzyme involved in the drug and polyphenol metabolism. Many of the polyphenols including gallolcatechins, inhibit the activity of CYP3A4 while isoflavone, genistein depicts modest activation in clinical trials (Basheer and Kerem, 2015). As discussed earlier, BEAS-2B cells depict only limited CYP enzyme activity. The cell line specific DNA damage reduction by PG and PC in BEAS-2B and WRL-68 cells may be attributed by the different activity levels of cellular enzymes. Therefore, investigation of QSAR of MMP on

cellular enzyme activity, especially NNKOAc activating esterases, is also important in understanding of MMP-mediated and cell line specific DNA damage reduction.

Cytoprotection by MMP for NNKOAc-induced toxicity was investigated by MTS assay to select MMP-concentration to be used in the assays of DNA damage prevention. Tested MMP except for PG and PC showed no cytoprotection in both cell lines. A study by Kling *et al*. (2013) investigated the cytoprotection by 19 different flavonoids, flavonoid metabolites, phenolic acid and methyl esters of phenolic acids on the neuronal cells exposed to *tert*-butyl hydroperoxide. Only the phenolics with catechol units and 4-keto group provided significant cytoprotection in the study (Kling *et al*., 2013). Antioxidant capacity of polyphenols is dependent on their structure. Availability of hydroxyl groups in structure (in certain positions and the combination of other structural significances) increases the antioxidant capacity of polyphenols (Rice-Evans *et al*., 1997). Antioxidant capacity of polyphenols is an important parameter determining the degree of cytoprotection. Cyto-and geno-toxicity by NNK is positively related to the extremely increased oxidative stress *in vivo* (Bhagwat *et al*., 1998). Cytoprotection by phenolics in cellular systems under oxidative stress is probably due to the prevented cellular depletion of glutathione (Lima *et al*., 2006). Glutathione is an intracellular antioxidant and co-factor of the cytoprotective enzyme such as glutathione peroxidase and glutathione reductase (Hudson *et al*., 2005). Therefore, it is important to investigate the effects of PG and PC on cellular glutathione levels in future cytotoxicity studies.

The degree of cellular DNA damage induced by NNKOAc, under different concentration treatments of PG and PC was measured by γ-H2A.X immunofluorescence and comet assays. Cellular metabolism of NNK produces electrophilic metabolites capable

in DNA damage (Proulx *et al*., 2007), characterized by DNA strand breakage and adducts formation (Hecht, 1998; Weitber and Corvese, 1993). The phosphorylated histone protein H2A.X (γ-H2A.X) is useful in detection of DNA damage at histone protein level, particularly the double strand breaks (DSB) (Zhou *et al*., 2006). Comet assay is useful in the detection of both single and double strand breakages in DNA structure that causes to lose its supercoiled nature and extend towards the anode, resembling a comet when subjected to gel electrophoresis (Collins, 2004).

Cellular DNA damage protection by PG in BEAS-2B cells and PC in WRL-68 cells was evaluated considering their performance in cytoprotection against NNKOAc. The γ-H2A.X test results showed that both PG and PC protected the cells from NNKOAc-induced DNA damage and the protection by PC was concentration-dependent. However, the comet assay results showed no statistically significant ($p \geq 0.01$) DNA damage protection by PG and PC. The statistical insignificance is common in many of the experiments evaluating DNA damage by comet assay, where the trend is more appreciated than clean-cut statistical distinction. This is evident in a study by Gyori, *et al*. (2014) where the reliability of OpenComet comet image analysis software (the same software used to analyze comet images in this study) is evaluated. Mean olive moments reported for the PG and PC treatments in this study was low compared to that of DMSO controls, suggesting their potential in mitigation of cellular DNA damage. There are many studies focused on the mechanisms of polyphenols-mediated DNA damage reduction. The ability of polyphenols in DNA damage reduction by acting as antioxidant agents is appreciated by many researchers using different polyphenols with chemical and metallic carcinogens (George and Rupasinghe, 2017; Gill *et al*., 2010). The metal chelating ability of phenolics is an

important mechanism for reducing metal ions-mediated oxidative stress (Garcia *et al*., 2012). Phenolics are also capable in the prevention of DNA adducts and interstrands cross-links formation by trapping of causative agents (Wang and Hao, 2015). Cyto- and geno-protective effects of PG and PC can be predominantly due to their antioxidant activity. Antioxidant activities of PG and PC are well documented (Sarikaya, 2014; Kosobutskii, 2014). Anticancer properties of phenolics are also demonstrated by more complex interventions with metabolic pathways and gene modulation (Gill *et al*., 2010). Therefore, it is important to further investigate the mechanisms of PG and PC in the prevention of NNKOAc-induced cyto- and geno-toxicity in future studies as the two MMP demonstrated cell line specific protection for the same chemical carcinogen, suggesting possible interventions at the molecular level.

The influence of phenolic compounds in DDR mechanisms is emphasized by a number of studies (George *et al*., 2017; Sharma *et al*., 2017). Activation/phosphorylation of ATM, ATR, Chk1, Chk2, p53, and histone protein H2A.X is common in cells experiencing DNA damage and replication stress (Yan *et al*., 2014). These proteins are capable in the modulation of cellular DDR mechanisms for cell proliferation, cell cycle arrest and apoptosis (George *et al*., 2017). DDR is initiated with the decondensation or relaxation of chromatin to ensure increased accessibility to the DNA damage site (Murga *et al*., 2007). The MRN complex with MRE_{11}, RAD_{50}, and NBN proteins is an essential component in two major DNA DSB repair mechanisms; nonhomologous end joining (NHEJ) and homologous recombination (HR). MRN is responsible for the activation and recruitment of ATM, ATR, and DNA-PKcs (DNA-dependant protein kinase) kinases at damage site (Czornak *et al*., 2008). ATM and ATR are the key regulatory proteins of DDR

mechanisms. DNA-PKcs is mainly involved with NHEJ and activates an only small number of DDR proteins. Activation of ATM and ATR is dependent on the nature of DNA damage, and the functionality of the two kinases is distinct from each other. ATM activation is predominantly resulted by DNA DSB while ATR activation occurs through a number of DNA damages especially bulky lesions by methylation (Marechal and Zou, 2013; Goodarzi *et al*., 2003). Repair of DNA DSB through ATR activation is achieved through conversion of DSB into single strand DNA (ssDNA) by resection with the help of ATM (Marechal and Zou, 2013). ATM-Chk2 checkpoint activation and ATR-Chk1 checkpoint activation are two important pathways in DDR (Yan *et al*., 2015). Progression of ATM and ATR powered DDR mechanisms involve further activation of proteins, especially cell cycle check point protein Chk1 and Chk2, p53, and H2A.X (Zhao *et al*., 2009). ATR-Chk1 checkpoint pathway, once activated is capable in the modulation of nucleic acid metabolism, protein metabolism, and cell cycle arrest by activation of DDR proteins such as Cdc25, BLM, and FANCD2/FANCE (Yan *et al*., 2015).

BEAS-2B and WRL-68 cells exposed to NNKOAc showed substantial upregulation of targeted DDR proteins, ensuring the activation of DDR pathways. Treatment with PG resulted in significant upregulation of *p*-ATR and concentration-dependant downregulation of *p*-Chk1 in BEAS-2B cells exposed to NNKOAc. Thus, the predominant DDR pathway influenced by PG in NNKOAc exposed BEAS-2B cells can be ATR-Chk1 pathway. This can be further explained by DNA damaging mechanism of NNKOAc and characteristic DNA damage required for ATR activation. DNA damage by NNKOAc causes the formation of bulky DNA lesions and adduct formation. Bulky DNA lesions is a major drive for ATR activation. However, further experimentation for the

detection of other proteins activated or inactivated by ATR (e.g., Cdk1) is recommended to establish a clear connection between PG treatment and activation of the ATR-Chk1 pathway. PG also influenced the ATM-Chk2 checkpoint pathway in NNKOAc exposed BEAS-2B cells by downregulation of *p*-ATM, *p*-Chk2, *p*-p53 and *γ*-H2A.X expression. George and Rupasinghe (2017) reported similar results (with exception of upregulation of *p*-ATR) for BEAS-2B cells treated with flavonoids and challenged for genotoxicity by NNKOAc. Furthermore, the study suggests ATR-dependant DNA damage prevention potential of the flavonoids. However, the ATM-Chk2 pathway is the predominant DDR mechanism recognized in the normal human bronchial epithelial cells challenged for genotoxicity by cigarette smoke carcinogens (Zhao *et al*., 2009). In contrast to PG in NNKOAc-treated BEAS-2B cells, PC upregulated the expression of *p*-ATM and restricted *p*-ATR expression in WRL-68 cells exposed to NNKOAc. Also, PC-mediated downregulation of *p*-Chk2 in NNKOAc exposed WRL-68 cells was concentration-dependant. Thus, the PC-mediated DNA damage reduction in WRL-68 cells may be achieved through the activation of the ATM-Chk2 pathway. Intensified expression of *p*-p53 and *γ*-H2A.X reassure the activity of ATM-Chk2 pathway in NNKOAc exposed WRL-68 cells. Furthermore, the influence of PC on the ATR-Chk1 pathway is demonstrated by the downregulation of *p*-ATR and *p*-Chk1.

Apple peels were used for the extraction of PAC, considering the high phenolic compound content in peels compared to the flesh (Henriquez *et al*., 2010; Wolfe *et al*., 2003). PAC extraction efficiency of three different extraction methods was compared to identify a suitable method to extract PAC in sufficient quantities. Yield by the two supercritical CO_2 extraction methods was excessively high as these methods are not fine-

tuned only to extract PAC. Also, the PAC samples from supercritical CO_2 extraction showed higher PAC content as measured by DMAC method. The DMAC colorimetric method is widely used for the quantification of PAC. However, the method is not specific for the quantification of PAC only (Dooren *et al*., 2018) and the results can be inflated by reaction with non-PAC phenolic compounds in test samples. The total flavonoid contents of commonly used North American apples; Rome beauty, Idared, Cortland and Golden Delicious varies from 167.4 ± 20.2 to 306.1 ± 6.7 mg CAE/ 100 g of fresh peel (Wolfe *et al*., 2003). A study by Vieira *et al*. (2011) evaluated the total flavonoid content among 11 apple cultivars grown in Brazil using the DMAC method. The total flavonoid content of the peels from these apple cultivars varied from 58.24 ± 1.44 up to 147.75 ± 3.66 mg CAE/ 100 g of fresh weight. As the water content of an apple is around 85% (Gu *et al*., 2003) the total flavonoid values reported in the literature can be speculated to fall well behind the PAC content of samples extracted by the supercritical CO_2 methods.

L. rhamnosus and *L. casei* were selected for the biotransformation of PAC. Both bacterial species are heterofermentative, and therefore capable in utilizing different carbon sources to sustain continuous growth (Turgay and Erbilir, 2006). Thus, both bacterial species may be able in utilization of PAC and production of phenolic metabolites. Also, these bacterial species are commonly used in food industry, especially in dairy sector, to produce yoghurt, cheese, and fermented milk (Turgay and Erbilir, 2006). Versatile use in food industry as common probiotic bacteria qualifies *L. rhamnosus* and *L. casei* to be used in the designing of functional foods (synbiotics). Furthermore, the side effects because of lactobacillus supplementation are rare and reported only to be associated with immune-

suppressed individuals and infants (Armuzzi *et al*., 2001). Thus, the functional foods with *L. rhamnosus* and *L. casei* can be recommended for the consumption by general population.

The growth of probiotic bacteria, *L. rhamnosus,* and *L. casei* was inhibited by AP and GS-PAC at higher concentrations. Similar results are reported by a number of previous studies mostly evaluating the antimicrobial properties of PAC against human pathogenic bacteria (e.g., *E. coli* and *S. aureus*). These studies further suggest that the PAC exhibit moderate antimicrobial properties. However, antimicrobial efficacy of PAC varies for different bacterial strains, even within the same bacterial species (Navarro-Hoyos *et al*., 2017; Mayer *et al*., 2008). Furthermore, flavonoids are more effective in inhibiting the growth of Gram-positive bacteria over Gram-negative bacteria (Cushnie and Lamb, 2011). Proposed antibacterial mechanisms of action for flavonoids (including PAC) target the key biological functions required for bacterial survival. Inhibition of bacterial cell membrane and cell wall synthesis appears to be major contributors to bacterial growth inhibition. Cytoplasmic membrane damage by H_2O_2 production is another profound mechanism for the inhibition of bacteria (Cushnie and Lamb, 2011). We demonstrated the potential of PG and PC for H_2O_2 production by autoxidation (only at higher concentrations) in cell culture media. Resveratrol, a flavonoid abundantly found in grapes is capable of inhibiting *Salmonella typhimurium* growth by pro-oxidant activity. Resveratrol causes bacterial cell membrane disruption by depolarization and increased membrane permeability (Lee and Lee, 2017). PAC extracted from American cranberry (*Vaccinium macrocarpon*) inhibits the growth of *E. coli* through disrupting cell membrane by significantly enhancing the permeability (Alshaibani *et al*., 2017). Flavonoids also influence bacterial energy metabolism and nucleic acid synthesis. Flavonoids disrupt bacterial energy metabolism by

the inhibition of key enzymes such as ATP synthase (Chinnam *et al*., 2010). Topoisomerase and dihydrofolate reductase enzymes are inhibited by flavonoids to mitigate bacterial growth by blocking nucleic acid synthesis. However, it is important to note that all flavonoids are not capable of exhibiting antibacterial properties. The flavonoids with antibacterial properties may adopt one or more of the mechanisms of action described above (Cushnie and Lamb, 2011).

GS-PAC stimulated the growth of *L. rhamnosus* and *L. casei* at the concentration of 50 µg/mL. Potential of phenolic compounds in bacterial growth stimulation is reported in previous studies. Hervert-Hernandez *et al*. (2009) studied the effect of grape pomace polyphenols and selected standards of phenolic compounds on the growth of *L. acidophilus* CECT 903. Growth stimulation of *L. acidophilus* by grape pomace polyphenols is weak. However, GS extractable polyphenols and tannic acid strongly stimulated the growth of *L. acidophilus* (Hervert-Hernandez *et al*., 2009). Bacterial growth stimulation by polyphenols is dependent on the ability of bacteria to utilize polyphenols in the growth phase to fulfill energy requirements. Also, polyphenols can support *Lactobacillus* growth by inducing energy metabolism to enhance sugar and malic acid utilization (Garcia-Ruiz *et al*., 2007).

Biotransformation of PAC by bacteria is dependent on the ability of bacteria to utilize PAC in cellular metabolism. Also, limitation of energy in growth media may encourage bacteria to utilize PAC, as some bacterial strains (e.g., *Lactobacillus*) are capable in utilizing polyphenols for energy production (Garcia-Ruiz *et al*., 2007). MRS is the recommended enrichment media for growing *Lactobacillus* cultures. Therefore, the MRS media is comprised of all nutritional requirements required for comfortable growth of *L. rhamnosus* and *L. casei*, relinquishing any need to utilize PAC in the growth media.

Thus, the metabolite profile of biotransformed PAC sample and the control sample was identical. This is further described by the yielding of new metabolites when energy supply to bacteria is limited by reducing sugar content in growth media. Limitation of energy may force the bacteria to utilize PAC, resulting in the formation of new metabolites not present in control. This factor remains unchanged when BHI media is used for the PAC bioconversion. BHI media is not designed to enrich *Lactobacillus* cultures and may create a nutritional gap for *L. rhamnosus* and *L. casei* growth, encouraging the bacteria to utilize PAC to form new phenolic metabolites.

PAC exhibits cytotoxic effects when administered in high dosages. Cytotoxic effects of PAC are predominantly proven for a number of cancer cell lines *in vitro* (Navarro *et al*., 2017; Lai *et al*., 2017). More importantly, PAC is potent in exerting cytotoxic effects selectively on cancer cell lines. PAC extracted from a medicinal fern (*Blechnum orientale* Linn) triggers strong cytotoxic effects in HT29, HepG2, and HCT116 cancer cells while showing limited cytotoxicity in normal liver cells (Lai *et al*., 2017). In human gastric cancer cell line MGC-803, GS-PAC mediated cytotoxicity is driven by promoted apoptotic cell death or autophagy. Interestingly, inhibition of PAC-induced autophagy leads to the significantly improved apoptotic death of MGC-803 cells (Nie *et al*., 2016). The GS-PAC-induced apoptosis in ventricular myocytes by chick embryos is resulted by increased production of nitric oxide through activation of cellular NO synthase enzyme. NO is a key regulator of cellular apoptosis (Shao *et al*, 2006).

The biotransformed GS-PAC was incapable of protecting BEAS-2B and WRL-68 cells against NNKOAc-induced cytotoxicity. The potential of PG and PC (both metabolites present in biotransformed GS-PAC) is previously proven in this study. Therefore, the

mitigated potential of biotransformed GS-PAC in cytoprotection against NNKOAc can be partly due to the severity of treatment (300 μM NNKOAc) or insufficient dose of phenolic metabolites in biotransformed GS-PAC mixture.

As a summary of this study, only selected PAC metabolites are capable of reducing cancer risk. Tested MMP except for PG and PC at high concentrations are not cytotoxic in BEAS-2B and WRL-68 cells. Cytotoxicity triggered by PG and PC is due to the concentration-dependent H_2O_2 production in culture media by autoxidation. The promoted % cell viability in BEAS-2B cells by lower concentrations of PG and PC is not characterized by induced cell proliferation. The proliferation of BEAS-2B cells is limited by PG and PC in a concentration-dependent manner. PG and PC limit the proliferation of WRL-68 cells. However, PC is less effective in restriction of WRL-68 cell proliferation compared to PG. Cytotoxicity induced by NNKOAc is mitigated only by PG in BEAS-2B cells and PC in WRL-68 cells. Cytoprotection by PG and PC is cell line specific. Cellular DNA damage induced by NNKOAc is reduced by PG in BEAS-2B cells and PC in WRL-68 cells. According to western blot analysis PG contribute to DNA damage repair predominantly through ATR-Chk1 DDR pathway in BEAS-2B cells. PC-mediated DNA damage repair in WRL-68 cells is driven by ATM-Chk2 DDR pathway. *L. rhamnosus* can utilize GS-PAC and generate simple phenolic metabolites. However, the biotransformation efficiency is dependent on the restriction of energy supply by limiting culture media nutrients. Biotransformed GS-PAC shows low cytotoxicity in BEAS-2B and WRL-68 cells compared to pure GS-PAC. Biotransformed GS-PAC was incapable of protecting cells against NNKOAc-induced cytotoxicity. This can be due to the low dose of phenolic metabolites in biotransformed GS-PAC mixture or high severity of NNKOAc treatment.

Identification of effective probiotic bacterial strains capable of PAC biotransformation is a necessity to establish effective chemoprevention strategies through the use of probiotic metabolites of PAC. In future experiments, synergism between probiotic bacterial strains for PAC biotransformation can be studied to establish more efficient biotransformation protocols. Furthermore, it is important to commence biotransformation process with larger capacity using bioreactors as the metabolites in low concentrations are difficult to detect. This was evident when HRMS used to detect PAC metabolites instead of UPLC-ESI-MS/MS. An experimental animal model can be used to confirm the occurrence of biotransformation by probiotic bacteria and the chemoprevention potential of resulting phenolic metabolites *in vivo*. A/J mice of high spontaneous adenocarcinoma can be supplemented with probiotic bacteria to establish a microbiome fluent in PAC biotransformation. Tumorigenesis can be chemically-induced in mice to study the potential interventions of biotransformation resulted in PAC metabolites in chemoprevention. Finally, it is possible to create functional foods that contribute to cancer prevention, through designing of synbiotics. Synbiotics, with active probiotic bacteria and sensible PAC substrates to undergo biotransformation, can be included in food to introduce chemoprevention properties. Biotransformation of polyphenols using probiotic bacteria for cancer prevention is still a new research area that combines the aspects of medical sciences, microbiology, and food science. Results of this study unveil the potential to utilize probiotic metabolites of PAC in cancer prevention. Thus, this study is the first preliminary step of a scientific investigation for the use of PAC biotransformed metabolites in disease prevention.

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