CITRUS BIOACTIVE COMPOUNDS: ISOLATION, CHARACTERIZATION AND MODULATION OF BACTERIAL INTERCELLULAR COMMUNICATION AND VIRULENCE

A Dissertation

by

AMIT VIKRAM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Horticulture

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May 2011

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ABSTRACT

Citrus Bioactive Compounds: Isolation, Characterization and Modulation of Bacterial Intercellular Communication and Virulence. (May 2011)

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The secondary metabolites of citrus such as limonoids and flavonoids constitute an important part of human diet. The present work was undertaken to elucidate the effect of citrus limonoids and flavonoids on the bacterial cell-cell signaling in *Vibrio harveyi*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium LT2. The first experiment was focused on purification of limonoids from grapefruit and sour orange seeds. The limonoids were extracted using organic solvents and purified by chromatographic techniques. A total of ten limonoids (7 aglycones and 3 glucosides) were purified.

Currently, simultaneous measurement of aglycones and glucosides of limonoids is not available. To address this limitation, an analytical method using high performance liquid chromatography was developed with the capability of measuring both aglycones and glucosides in a single run. Furthermore, its applicability in the fruit and juice samples was demonstrated.

The third study investigated the *V. harveyi* cell-cell signaling inhibitory potential of purified limonoids. Isolimonic acid, ichangin, obacunone and nomilin showed potent

inhibitory activity. Furthermore, isolimonic acid and ichangin inhibit the signal transduction pathway by up-regulating the response regulator *luxO*. Isolimonic acid was also found to be a potent inhibitor of *Escherichia coli* O157:H7 cell-cell signaling in the fourth study. The results demonstrated that isolimonic acid inhibits the autoinducer/epinephrine mediated cell-cell signaling, biofilm and virulence in QseBC and QseA dependent fashion. Further investigations using limonin analogues, in the fifth study, demonstrated that the analogue limonin-7-methoxime inhibited the *E. coli* biofilm in type 1 pili and antigen 43 dependent-fashion, by preventing the binding of the adhesins to plastic surfaces. Another limonoid, obacunone was demonstrated to attenuate the *Salmonella* virulence by repressing Salmonella Pathogenicity Island 1 (SPI-1) in EnvZ/OmpR dependent mecahnism.

The seventh study showed that naringenin, among the flavonoids, was the most potent inhibitor of *V. harveyi* and *E. coli* O157:H7 cell-cell signaling. Furthermore, naringenin was found to repress the (SPI-1) in PstS-HilD dependent fashion in the eighth study. The results of the study suggest that several limonoids and flavonoids possess the ability to interfere with cell-cell signaling and virulence in three bacterial species.

DEDICATION

This dissertation is dedicated to the spirit, who visited us on this planet earth.

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This work would not have seen the daylight without the support and guidance of many people, who shared their experiences, knowledge and passion for science. The foremost among them is my mentor, Dr. Bhimanagouda S. Patil, whose thoughtful guidance and measured 'challenges' allowed me to grow as a scientist. I would like to thank him for his keen support and assistance through the completion of my doctoral degree program. I would also like to thank members of my graduate committee, Dr. Pillai, Dr. Lombardini, Dr. Jayaraman, Dr. Yoo and Dr. Jayaprakasha, for their productive criticism and support through the thick and thin of my graduate life, especially for letting me test my crazy ideas and using the lab facilities without hindrance. I also would like to thank Dr. Jayaprakasha and Dr. Jesudhasan for teaching me the techniques I have used in my research. Additionally, I would like to recognize my collegues and fellow labmates, Ram, Priyanka, Jinhee, HaeJeen, Murthy and Kranthi, for their support. I would also like to extend my special thanks to Ms. Connie Sebesta and her team for taking excellent care of all the administrative formalities and providing immense help in coping with the not-so-routine non-research stuff. I would like to thank the entire Vegetable and Fruit Improvement Center family where multidisciplinary researchers and students exchange ideas and conduct experiments, which provided me the foundation to face future challenges as a scientist. I appreciate the financial support from the project, "Designing Foods for Health" through the Vegetable and Fruit Improvement Center.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Background

The project explored the effect of citrus secondary metabolites on bacterial cell-cell signaling. The limonoids were isolated from *Citrus* species and investigated for their potential to interfere with cell-cell signaling and associated properties with an emphasis on the food borne pathogens *Escherichia coli* O157:H7 and *Salmonella* Typhimurium LT2. In addition, cell-cell signaling modulatory properties of flavonoids was also studied. The following introduction is intended to serve as a background for the experimentation presented in the subsequent chapters. Since the project involves multi-disciplinary science, a number of different fields and sub-fields relevant to the project are presented.

1.2 Citrus

Citrus fruits originated in South East Asia. The oldest known reference to citrus appears in Indian literature, "Vajasaneyi Samhita" in "Yajurveda", a text dated prior to 800 B.C (322). Citrus belongs to family Rutaceae, subfamily Aurantoideae and tribe Citrae. In common parlance, the word 'Citrus' refers to all species of sexually compatible genera, *Citrus*, *Fortunella* and *Poncirus* (351). The genus *Citrus* is suggested to contain between 16 and 162 species based on different taxonomic classification systems (350, 354). However, the recent phenotypical and molecular data indicate presence of three basic taxa, *C. maxima* (pummelos), *C. medica* (citron), and *C.*

This dissertation follows the style of Applied and Environmental Microbiology.

reticulata (mandarins) (247, 351). Rest of the species originated either by hybridization of basic taxa in complex pattern or through mutations (247, 351).

Citrus is one of the most widely grown and consumed fruits on the planet with a total global production of 124.4 million tons (104). USA is the second leading producer and consumer of citrus fruits after Brazil, with annual production of 10.74 million tons. The major citrus crops grown in USA are Oranges (8.28 million tons) followed by grapefruit (1.18 million tons). According to the U.S. Dept. of Agriculture database, fresh and processed citrus were the highest consumed fruit in 2008.

1.2.1 Nutritional Content and Health Benefits of Citrus

Mature citrus pulp is comprised of 85-90 % water. Additionally, more than 300 naturally occurring compounds are present in edible portion (83). However, the composition of citrus fruits varies depending on the species, cultivar, agro-climatic conditions, cultivation practices and rootstocks (83).

Role of citrus in human health is known since the amelioration of scurvy disease by lime juice (253). In the early 20^{th} century, citrus flavonoids were found to help in capillary fragility (324). More recent data indicate a positive correlation between consumption of citrus fruits and reduced incidence of ischemic stroke (179). In addition to the preventive effect against cardiovascular diseases, the emerging evidence, primarily from in vitro and animal studies, suggests that certain citrus secondary metabolites may be helpful as anti-cancer agents (242, 277, 355, 368). In addition, the citrus secondary metabolites also demonstrate antimicrobial, antioxidant, anti-inflammatory, antiviral and other beneficial activities (26, 51, 67, 366, 383).

1.2.2 Citrus Secondary Metabolites

Citrus accumulate flavonoids, terpenoids, carotenoids and coumarins as major secondary metabolites in edible as well as inedible fruit tissues. Monoterpenes are the principal components of essential oil and contribute to aroma and flavor. Many of these monoterpenes are believed to exhibit health beneficial properties owing to their antioxidant and anti-cancer activities (336, 383). However, the recent enthusiasm about health beneficial properties of citrus is centered on the flavonoids and the limonoids.

1.2.2.1 Flavonoids

Flavonoids are polyphenolic compounds comprised of 15 carbons. Flavonoids contain two aromatic rings connected by a three-carbon bridge. A series of condensation reactions between hydroxycinnamic acid (B-ring and carbon atoms 2, 3 and 4 of the C-ring) and malonyl residues (A-ring), give rise to a C_6—C_3—C_6 flavonoid base structure (Fig. 1.1). The three-carbon bridge between the phenyl rings is commonly cyclized to form a third ring (C-ring). According to the cyclization pattern, degree of unsaturation and oxidation of the three-carbon segment, the flavonoids are classified into (a) flavonols, (b) flavones, (c) flavan-3-ols, (d) anthocyanidins, (e) flavanones and (f) isoflavones, (g) dihydroflavonols, (h) flavan-3,4-diols,(i) chalcones (j) dihydrochalcones and (k) aurones. Citrus species particularly accumulate high concentrations of flavanones. The most common flavanone glycosides are hesperidin (hesperetin-7-O-rutinoside), narirutin (naringenin-7-O-rutinoside) and naringin (73).

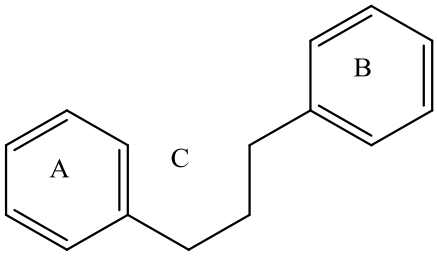


FIG. 1.1 Flavonoid skeleton

Multiple roles have been ascribed to flavonoids in plants including plant-defense and regulation of plant development. In addition, flavonoids demonstrate anti-cancer (31, 199), cardiovascular health maintenance (159-160), anti-dementia (65) and anti-atherosclerosis (14) properties. The emerging evidences indicate an inverse relation between flavonoid intake and incidence of the coronary heart disease (CHD) (159-160, 189). The interaction of flavonoids with various enzymatic systems such as cyclooxygenase and lipoxygenase (186) to decrease platelet activation and aggregation may partly explain the inverse relationship with CHD. However, because of the reducing capacity and modulation of intracellular redox status, the anti-oxidant activity of flavonoids was proposed as the primary mode of the biological activities (388). At least four basic mechanisms for antioxidant activity of flavonoids were speculated: (a) free radical scavenging, (2) singlet oxygen quenching, (3) transition metals chelation and (4) enzyme inhibitory activity (66). However, the recent data does not support the anti-oxidant activity as the sole explanation for the observed cellular effects (121).

The flavonoids are extensively metabolized in human gut, resulting in significant alteration in redox potential. The hydroxyl groups are conjugated with glucuronic acid, a sulfate or a methyl group (121, 163). Furthermore, microorganisms can degrade unabsorbed flavonoids in the colon and conjugated flavonoids excreted in bile (318). Generally, the heterocyclic C-ring is opened by the colonic microflora (42, 390), and the resultant byproduct is further metabolized leading to the formation of simple phenolic compounds such as phenylpropionic acid, phenylacetic acid and benzoic acid derivatives (275). In addition, recent studies indicate that flavonoids and other secondary metabolites may influence the colonic microflora (266).

1.2.2.2 Citrus Limonoids

Limonoids are tetracyclic, highly oxygenated and degraded triterpenoids (Fig 1.2), classified as tetranortriterpenoids. The occurrence of limonoids is confined to the plant order Rutales, with families Rutaceae and Miliaceae demonstrating widespread distribution. A limited number of genera in Cneoraceae and Simaroubaceae, were also reported to synthesize limonoids (80, 260). In family Simaroubaceae, limonoids are reported only from one species, *Harrisonia abbysinica* (260). The family Meliaceae accumulates a very diverse array of limonoids, with over 200 different structures reported in the literature (81). A limited number of structures are reported from Rutaceae. *Citrus* and related genera contain 36 different variations of limonoid skeleton (144).

Limonin (Fig 1.2) was the first limonoid identified as the bitter constituent of citrus seeds in 1841 by Bernay (162). Limonin was isolated from Valencia and navel

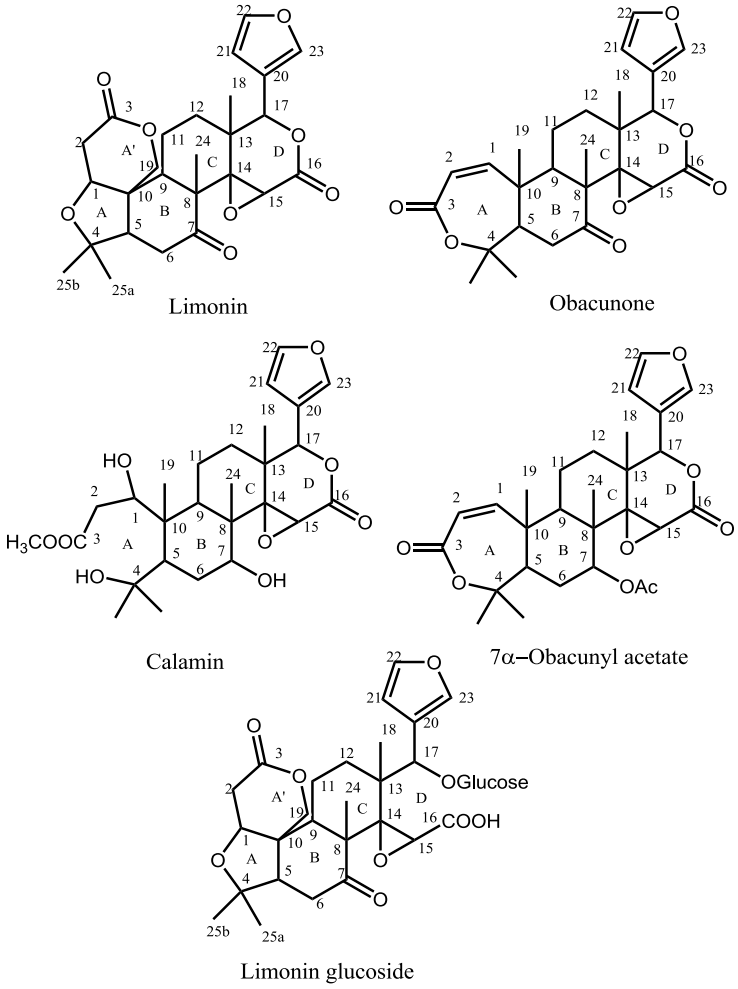


FIG. 1.2 Structures of representative limonoids

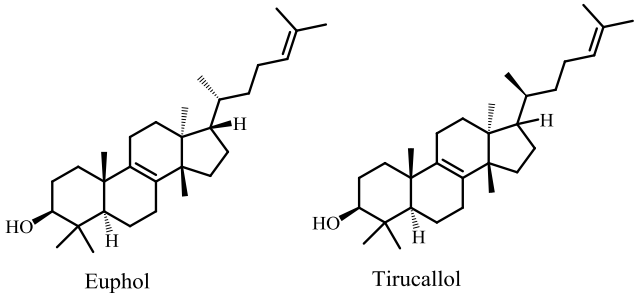


FIG. 1.3 Structures of limonoid precursors

oranges in 1938 (162), while several other limonoids such as obacunone, nomilin, obacunoic acid, isolimonic acid and dictomnolide were identified around the same time (98-99, 114). The dilactone nature of limonin, presence of a β-substituted furan ring, a ketonic oxygen atom and two ethereal oxygen rings were elucidated through degradation experiments, X-ray crystallography and NMR studies (10, 12, 127, 306). Rutaceae accumulate three forms of limonoids; monolactone, dilactone and glucosides (140).

Limonoids are biosynthesized via the cytosolic mevalonate pathway (80, 82). The triterpene alcohols, Δ^7-tirucallol or Δ^7-euphol (Fig. 1.3) are considered to be the immediate precursors of limonoids in the Rutales (80). Limonoid skeleton is believed to be generated by epoxidation of Δ^7 bond to 7-epoxide, which undergoes a series of transformations, resulting in formation of 7-OH and C-14/C-15 double bond (330). Subsequently, the side chain at C-17 is cyclized to form the β-furan ring. After the formation of limonoid skeleton, numerous skeletal rearrangements and oxidations generate a variety of structures (80-81, 330). The oxidation of A, B, C and D-rings generate several lactones (A-, B-, C-, D-,A,B-, A,D-, B,D-*seco* limonoids) (58). In Rutaceae, rearrangements of A-ring (Fig 1.2) and/or the structural modifications at C6-C7 results in diverse structures of A,D-*seco* limonoids (80-81).

A total of 36 aglycones and 17 glucosides have been reported from various *Citrus* species till date (140). *Citrus* species accumulate limonin, nomilin, obacunone, and deacetylnomilin as major limonoids (140). A list of major limonoids in economically important Citrus fruits is presented in Table 1.1. The first precursor of limonoid biosynthesis, nomilin and/or deacetylnomilinic acid is synthesized in the

phloem region of stem from mevalonate or farnesyl pyrophosphate and translocated to other tissues, where it is converted to other products (140, 263). Active metabolism of limonoids takes place in young and immature leaves which are near the immature fruits, while older leaves, which are far from the fruit, do not synthesize the limonoids (148). The primary reason for such differential biosynthesis may be the synthesis and subsequent translocation of precursor deacetylnomilin/nomilin in the young stem (146, 263). In addition, immature fruits convert nomilin predominantly into monolactones, whereas immature seeds synthesize and accumulate mono- as well as dilactones (140). During the fruit maturation process, the monolactones accumulated in fruit tissues and seeds are glucosidated by UDP-D-Glucose: limonoid glucosyltransferase (109, 149).

TABLE 1.1. Current status of limonoids in economically important *Citrus* fruits

Species/cultivars	Limonoids	Reference
Grapefruit (*Citrus paradisi*)	Limoin, nomilin, obacunone, deacetylnomilin, isoobacunoic acid, isolimonic acid, limonin 17-β-D glucopyranoside (LG), deacetyl nomilinic acid 17-β-D glucopyranoside (DNAG), nomilin 17-β-D glucopyranoside (NG), deacetylnomilin 17-β-D glucopyranoside (DNG), and obacunone 17-β-D glucopyranoside (OG).	(33, 141)
Oranges (*Citrus sinensis*)	Limoin, nomilin, obacunone, deacetylnomilin, LG, NG, OG, nomilinic acid 17-β-D glucopyranoside (NAG) and DNAG	(109, 145, 158)
Mandarin/Tangerine (*Citrus reticulata*)	Limoin, nomilin, obacunone, nomilinic acid, LG, NG, OG, NAG	(90, 175)
Lemon (*Citrus limon*)	Limoin, nomilin, obacunone, deacetylnomilin, nomilinic acid, deacetylnomilinic acid and their glucosides	(142, 310)

In recent years, several reports have suggested probable anti-cancer activity of limonoids using in vitro assays and animal studies. Specifically, limonin and nomilin were shown to induce GST enzyme in the small intestine mucosa (195). Limonoids inhibited the proliferation of colon cancer, ovarian cancer, human neuroblastoma, estrogen receptor-negative and –positive human breast cancer cells in in vitro study (277, 362). In vivo studies in animals further support the results obtained in vitro for colon cancer in rat (355, 368), forestomach neoplasia in mice (194) and oral tumors in hamster cheek pouch model (242). Limonin and nomilin also showed antiviral activity against HIV-1 (26). Furthermore, the limonoids were reported to reduce LDL level in rabbit model and apo-B production in HepG-2 cells (192-193).

The limonoids also contribute to the plant defense by demonstrating anti-feedant activity against a number of economically important insects. However, structurally complex limonoids present in citrus are less active than simpler limonoids of Meliaceae family (58, 236, 313). Recently, the citrus limonoids, limonin and nomilin were demonstrated to possess larvicidal activity against the Asian tiger mosquito, *Aedes albopictus* (136). However, the antibacterial activities of citrus limonoids were not reported and were speculated to not possess the antibacterial activity (58).

In recent years, several novel mode of antimicrobial activity have been identified. One of the mechanisms known as anti-pathogenic/anti-infective targets the virulence and infection processes such as type three secretion system, biofilm and quorum sensing. It is suggested that targeting pathogenesis may be a viable strategy to counter the bacterial pathogens. The bacterial pathogenesis is a complex process and regulated by several

environmental and genetic factors. Quorum sensing or bacterial cell-cell signaling is one such mechanism, which is involved in regulation of virulence in several pathogenic bacteria.

1.3 Quorum Sensing

Tomasz (363) and Nealson et al. (254) suggested that individual bacteria can communicate and coordinate multicellular behavior using the small molecules. Tomasz et al (363) demonstrated that the *Streptococcus pneumoniae* regulates the genetic competence by self- produced chemical cues, now known as the "competence factor". Nealson et al. made a important observation about the disconcerted production of luminescence with population growth in *Vibrio fischeri* (254), and referred to the phenomenon as "autoinduction. Autoinduction was defined as an environmental sensing mechanism which allows bacteria to monitor their own population density (117). The details of the cell-to-cell signaling mechanism in *V. fisheri* and *S. pnueumoniae* are now well understood. This cell-to-cell signaling, which was termed as quorum sensing (117), is, in essence, a two-component signaling, evolved to interpret the extra-cellular signals in density dependent gradient. The signaling molecules are produced by the bacteria at basal rate and secreted to the local environment. When a sufficient concentration of these molecules, called autoinducers (AI), is attained in the niche, the bacterial population at large responds by altering the expression of target gene in a coordinated fashion. The result is simultaneous alteration in gene expression in bacterial cells mimicking a population wide behavior (88, 117). Quorum sensing has become a much studied phenomena in recent years as it regulates various physiological processes in

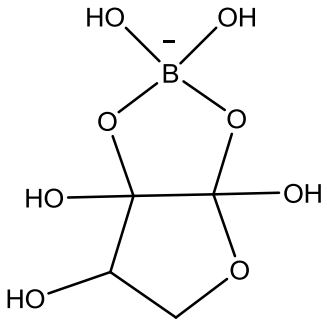
different bacteria such as virulence, biofilm formation, bioluminescence, conjugation, production of secondary metabolites etc. (88).

1.3.1 Quorum Sensing in *Vibrio harveyi*

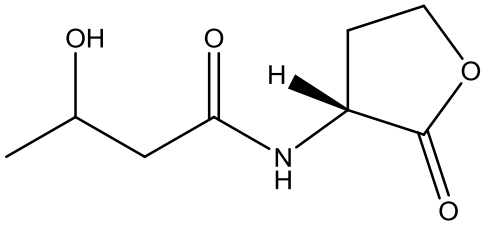
Quorum sensing in *Vibrio harveyi,* a marine bacterium, is well understood and the signal transduction cascade has been elucidated (24, 154, 204, 208, 382). Several studies have used *V. harveyi* as model system to investigate the quorum sensing inhibitory properties of natural and synthetic compounds (86, 225). Quorum sensing in *V. harveyi* regulates bioluminescence, biofilm formation, type III secretion system and protease production (138, 246). The production of bioluminescence in *V. harveyi* does not occur in concert with growth and is pronounced in the later stages of growth (254). The luminescence is regulated by the AIs in concentration dependent fashion. In *V. harveyi* the luminescene production is determined by interaction of three AIs with their cognate receptors (LuxN, LuxQ and CqsA) and subsequent integration of the information. The three AIs are N-(3-hydroxybutanoyl) homoserine lactone (AHL), 3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2) dioxaborole-2,2,6,6,A-tetraol (AI-2) and CAI-1 (154). These AIs are produced at low basal level during the early stages of cell-growth (corresponding to low cell density) and secreted to the local environment. The concentration of these autoinducers increases with the increase in the cell density and at critical threshold level the autoinducers initiate the signaling cascade resulting in the production of bioluminescence, observed as population wide phenomena.

AHL is produced by LuxM (25) and belongs to a broad class of N-acyl homoserine lactones produced by bacteria believed to be species specific autoinducers

(25, 52, 245). AI-2 is a furanosyl borate diester (Fig. 1.4), and was identified as bound ligand to periplasmic protein LuxP (59). The AI-2 synthase LuxS is widely conserved in bacteria (347) and speculated to be a cross-species communication molecule. LuxS converts S-ribosylhomocysteine to 4,5-dihydroxy-2,3-pentanedione (DPD). DPD undergoes further rearrangements to yield AI-2 (59). AI-2 binds to periplasmic protein LuxP and the resulting complex then binds to sensor kinase LuxQ (24). The third autoinducer CAI-1 was identified first in *V. cholerae*, and subsequent search revealed CAI-1 like activity in *V. harveyi*. The structure of CAI-1 is still unknown. CAI-1 is produced by synthase CqsA and detected by CqsS.



Furanosyl borate diester



3-hydroxy-C4-homo serine lactone

FIG. 1.4 *Vibrio harveyi* signaling molecules

The signal from three detectors converge on a phosphotransferase protein LuxU and rest of the signal transduction pathway is shared (382). LuxN, LuxQ and CqsS are

histidine kinases and at low cell densities the three detectors have intrinsic kinase activity and autophosphorylate at conserved His residue. The phosphoryl group is subsequently transferred to the conserved Asp in the C-terminal phosphotransferase domain. This Asp group transfers its phosphoryl group to a conserved His on phosphotransferase protein LuxU, which passes the phosphoryl group to Asp47 on response regulator LuxO (154). LuxU and LuxO are encoded in the same operon with luxO termination codon overlapping with initiation codon of luxU (112). At low cell densities phosphorylation of LuxO by LuxU causes activation of LuxO. Phospho-LuxO activates the expression of five small regulatory RNAs (*qrr1*, *qrr2*, *qrr3*, *qrr4*, and *qrr5*) in σ^{54} dependent fashion (204, 208). These small regulatory RNAs are stabilized by the chaperon Hfq, and subsequently destabilize the LuxR mRNA (204).

LuxR is a master transcriptional regulator controlling the expression of all quorum sensing regulated gene in *V. harveyi* known so far (349). LuxR binds to the promoter region of lux operon and activates the transcription of *luxCDABE* as well as its own gene (100). *luxCDABE* encodes for luciferase enzyme which oxidizes a reduced flavin and log-chain aldehyde to oxidized flavin and fatty acid. The conversion emits the energy in terms of visible light (100). Destabilization of *luxR* mRNA curbs the emission of light resulting in dark phenotype during early growth stages. At high cell densities, the three autoinducers bind to their cognate receptors, possibly altering their conformation in the process. This conformational change switches the predominant activity of the receptors from kinase to phosphatase. The result is dephosphorylated LuxO, which cannot drive the expression of small RNAs. In absence of small RNA, *luxR*

mRNA is stabilized and translated into protein. LuxR protein then drives the expression of *luxCDABE* operon encoding luciferase.

The accumulated evidence suggests that the three receptors integrate the information from three autoinducers to regulate the gene expression at different levels of signal intensity. (382). The AHL and AI-2 act synergistically and required to drive the expression of target genes (246). CAI-1 has minimal effect on the LuxR controlled genes (154). Furthermore, at low cell densities each of five small RNAs are expressed to different levels and act additively (365). Together, integration of signals from three quorum sensing systems results in three levels of gene expression by regulating LuxR affinity at low, medium and high DNA binding ability (382).

1.3.2 Quorum Sensing in *Escherichia coli*

Escherichia coli is a harmless commensal bacterium, present in the gut of humans and warm blooded animals. However, several pathogenic strains of *E. coli* are now known to cause diseases in humans and animals. *E. coli* O157:H7 (EHEC) was identified in 1983 following an outbreak in United States (182). EHEC produces a lethal shiga toxin and cause diarrhea and hemolytic uremic syndrome (396). Quorum sensing in *E. coli*, particularly in pathogenic strains such as EHEC and enteropathogenic *E. coli* has been of great interest primarily as a regulatory factor of virulence and shiga toxin production (333, 340). In *E. coli* several different intercellular signaling systems have been identified: a system mediated by SdiA (a LuxR homolog), the luxS/AI-2 system, an AI-3 system, and indole mediated signaling system. In *E. coli* SdiA was shown to regulate AcrAB multidrug efflux pump (397). Indole is an stationary phase signal in *E.*

coli (202), which has been implicated with regulation of biofilm, virulence and multidrug transporters in *E. coli* (202). The focus of current project was on the luxS/AI-2 and AI-3 systems, therefore, these systems are discussed in detail.

The DPD, synthesized by LuxS, cyclizes spontaneously in solution, to form two epimeric furanones, (2R,4S)-2,4-dihydroxy-2methyldihydrofuran-3-one(R-and S-DHMF, respectively). (320, 391). Hydration of R- and S-DHMF gives rise to R- and S-THMF, respectively. The LsrB, receptor in *E. coli*/*Salmonella* Typhimurium, detects R-THMF (395).

The synthesized AI-2 is transported outside the cell by the transporter YdgG (TqsA) (161). The extracellular AI-2 is then transported back into the cells by another transporter, Lsr, composed of LsrBCDA encoded by *lsr* operon (352-353, 394). LsrR is the repressor of the *lsr* operon (394). Upon internalization, intracellular AI-2 is phosphorylated by the cytoplasmic kinase, LsrK. Phosphorylated AI-2 binds to LsrR and derepresses *lsr* expression. Activation of *lsr* operon results in increased production and assembly of the Lsr transporter followed by rapid AI-2 internalization. The *lsrF* and *lsrG* genes, encoded in the *lsr* operon are involved in the destruction of the internalized AI-2 (395). LsrG cleaves P-DPD, producing 2-phosphoglycolic acid (PG) terminating the AI-2 signaling cycle (395).

In addition to regulation by AI-2, the expression of *lsr* operon is also subject to catabolite repression by glucose through the cAMP-CRP complex. The cAMP-CRP complex directly stimulates the expression of *lsr* operon and indirectly represses *luxS* expression (221, 379). The presence of glucose in the growth medium suppresses the

expression of *cya* and *crp*, resulting in decreased levels of cAMP and CRP (169, 221). This low level of cAMP-CRP amounts to almost no transcription of *lsr* operon but a high expression of *luxS* (395). The net effect is a higher extracellular accumulation of AI-2, but little internalization. Furthermore, accumulation of the downstream metabolites of glucose metabolism, glycerol-3-phosphate and dihydroxyacetone phosphate, in cytoplasm is suggested to repress the *lsr* operon in catabolite dependent and independent fashion (395).

In case of *E. coli* O157:H7 pathogenesis, the role of AI-2 is debated. It was reported that AI-2 regulate only genes involved in its own transport and metabolism but not the Locus of Enterocyte Effacement (LEE), which is central to EHEC virulence (184). However, an elegant study by Bansal et al. (17), demonstrated that AI-2 is involved in regulation of LEE, and consequently in the EHEC pathogenesis in temporal fashion. In addition to LEE, AI-2 mediated signaling regulates two more important pathogenic determinant of *E. coli*, biofilm and motility (91, 133). The AI-2 was demonstrated to directly stimulate *E. coli* biofilm formation by positively regulating the flagellar operon (133).

The autoinducer-3 (AI-3) was identified while attempting to study the role of purified AI-2 in the activation of EHEC LEE (342). In addition, epinephrine was shown to enhance the expression of LEE. Later on quorum sensing regulator QseA was identified and implicated in regulation of LEE (314, 325). QseA was demonstrated to activate LEE by binding to the promoter site in response to AI-3/epinephrine (325). Another two component system, QesBC was identified in EHEC and demonstrated to

regulate flagella and motility (343). It was suggested that QseC, the sensor kinase, detects AI-3/epinephrine/Norepinephrine. Detection of Epi/NE by QseC causes QseC to phosphorylate response regulator QseB. Phosphorylated QseB is active and activates transcription of key virulence genes (64, 342-343) governing flagella, motility and biofilm formation in EHEC. Another AI-3/Epi/NE regulated two component system identified in EHEC was QseEF and QseG. QseEF was shown to activate transcription of EspFU (298), whereas QseG contributes to the pedestal formation during EHEC pathogenesis (297).

The regulation of various pathogenic traits by cell-cell signaling or quorum sensing in EHEC, *S.* Typhimurium and *V. harveyi*, makes the bacterial communication system an attractive target for drug development in these bacterial species. Targeting of EHEC QS becomes more important in the light of the CDC's recommendation against use of antibiotics and antidiarrheal agents like immodium (392). Identification of new molecules, which can serve as a lead compound, will significantly contribute in development of an effective preventive and treatment strategy.

1.3.3 Quorum Sensing in *Salmonella* Typhimurium

Every year an estimated 40,000 cases of salmonellosis are reported in the United States, though the actual incidence is estimated to be more than 1 million cases (319). Infection by *Salmonella* spp., the causal organisms of salmonellosis produce symptoms of diarrhea, fever and abdominal cramps. Usually the infection clears by itself, however in children and immunocompromised persons, the infection may become very severe, resulting in hospitalization.

Salmonella spp. colonizes the small intestine and invades normally non-phagocytic epithelial cells. *S.* Typhimurium remodels the host actin cytoskeleton and manipulates the signal transduction pathways to induce membrane ruffling and triggers uptake by macropinocytosis. Once internalized, the bacteria multiply in specialized intracellular endosomal compartments called *Salmonella*-containing vacuoles (SCVs) (68). Invasion is mediated by Type III Secretion System (TTSS) encoded in *Salmonella* Pathogenicity Island 1 (SPI1) (288). TTSS is a supramolecular structure spanning both outer and inner bacterial membranes (190) and mediates the delivery of virulence proteins and effectors into the host cell cytoplasm (120). SPI1 is required for the invasion and colonization of intestine (55), as well as maturation of SCVs (119). Therefore, SPI plays an important role during pre- and post-invasion efficiency of pathogen in the epithelial cells (183).

Regulation of SPI1 is a complex process, and among other factors, luxS/AI-2 and QseBC mediated cell-cell communication is reported to play a role in expression of SPI1 (60). *Salmonella* Typhimurium has three known quorum sensing systems. *Salmonella* recognizes the AHL molecules produced by other bacterial species in SdiA, a *luxR* homolog (240), dependent manner. The *sdiA* regulates *rck* and *srg* operons in *Salmonella* (6) and the expression of AcrAB multidrug efflux pump (286). Similar to *E. coli*, in *S.* Typhimurium AI-2 controls the expression of the transporter, Lsr, responsible for internalizing, phosphorylating, and processing of the AI-2 signal molecule (352-353, 394). Furthermore, the LuxS/AI-2 system was demonstrated to be a global regulator in *S.* Typhimurium LT2 strain and regulated important pathogenic processes such as motility,

flagella and biofilm formation (176, 337). In addition to LuxS/AI-2, the two component system, QseBC, was demonstrated to regulate expression of flagellar operon in *Salmonella* (29). However, in a contrasting study, overexpression of PreA (QseB) in *S.* Typimurium did not affect the expression of virulence and motility, suggesting PreA/PreB (QseBC) regulon did not regulate these processes in *Salmonella* (239). Further, *qseC* mutant in *Salmonella* was demonstrated to depict a motility defect in response to epinephrine and QseBC was suggested to regulate flagellar operon (29). In addition, *qseC* mutant exhibited a defective phenotype for intestinal colonization in swine model (29). Furthermore, a recent study demonstrated the involvement of *qseBC* in *Salmonella* pathogenesis (248). The study further elucidates the role of AI-3/epinephrine/norepinephrine in the expression of SPI1/SPI2. It is possible that the apparent contradictions observed in various study could be due to the labile nature of the epinephrine/norepinephrine (248). Furthermore, the evidence supports the notion that *Salmonella* biofilm formation is regulated by QseBC hence AI-3/epinephrine/norepinephrine mediated cell-cell signaling.

1.4 The Under-utilized Potential of Plants

Historically, plants have served as great source of active ingredients used as drugs (257). Concoctions and plant extracts are still used in many of the traditional medicine systems. However, discovery and success of antibiotics from microbial source in the 1950s, limited the use of plants and plant-derived compounds as antimicrobials (70). However, the advent of antibiotic resistant microbes has renewed interest in the plant source to search for new antimicrobials. Plants produce a wide array of different

secondary metabolites which are involved in plant defense and regulate several important processes (37). The majority of the secondary metabolites are derived from isoprenoid, phenylpropanoid, alkaloid and fatty acid/polyketide pathways (89). The plant may accumulate the plant defense compounds (phytoanticipins) or the pathway may be inducible (phytoalexins) (89). Phytochemicals may act as phytoanticipin or phytoalexin depending on the mode of synthesis in a particular species. For instance, flavanones sakuranetin is accumulated constitutively in blackcurrent, while in rice leaves, it is a major inducible antimicrobial (89).

Antimicrobial activity of phytochemicals may range from non-specific to specific. Several phenolics, flavonoids, quinones were reported to exhibit non-specific activity by disrupting the cell membrane (70). Terpenoids such as carvacrol and thymol were reported to disrupt bacterial membrane resulting in membrane expansion, increased fluidity/permeability, damaged membrane proteins including ion channels and inhibition of respiration (47, 53, 71, 134, 237). On the other hand, examples of specific interactions include: interaction of catechol, catechin, epicatechin, cholrogenic acid and caffeic acid with beta-glucan synthase (231); inhibition of DNA gyrase by Clerocidin, quercetin and apigenin (232); topoisomerase IV-dependent DNA cleavge by rutin (74). Although several metabolites with antimicrobial activity have been identified, various aspects of phytochemical-microbe interaction are still not completely understood (30).

1.5 Interference with Quorum Sensing

One of the alternative methods for plants to successfully deal with diverse pathogens may be disruption of cell-cell signaling involved in regulation of pathogen

virulence factors. Such a strategy may equip the plant to protect itself from a large number of bacterial and fungal species, and only selective pathogens, which may overcome the plant defenses, will successfully colonize the plant. Indeed, inhibition of quorum sensing has also been suggested as potential mechanism to control bacterial and fungal pathogenicity (292-293). The first evidence of successful interference of bacterial quorum sensing was identified in marine alga *Delisea pulchera* (225). Halogenated furanones from *D. pulchera* disrupt *V. harveyi* quorum sensing system by interfering with the DNA binding activity of the master regulator LuxR (86, 225). In addition, the halogenated furanones were reported to inhibit quorum sensing systems in *Pseudomonas aeruginosa*, *Chormobacterium violaceum*, and *Serratia liquefaciens* (156, 230, 294). Since the discovery of anti-quorum sensing activities of furanones several phytochemicals produced by terrestrial plants such as epigallocatechin gallate, ellagic acid, tannic acid, ellagitannis and sulfur containing compounds (2-vinyl-4H-1,3-dithiin and 3-vinyl-4H-1,2-dithiin) from garlic (3, 165, 271) were reported to interfere with quorum sensing systems in different model organisms. Furthermore, toluene extract of garlic was demonstrated to inhibit biofilm formation in *P. aeruginosa* as well as helped in clearing of the bacteria in mice model (39, 291). However, the active principles effecting the biofilm inhibition and clearing of lung were not purified.

In addition to few purified compounds, several extracts from medicinal plants *Conocarpus erectus*, *Quercus virginiana*, *Tetrazygia bicolor*, *Bucida buceras*, *Callistemon viminalis*, and *Chamaecyce hypericifolia* (4), *Vanilla planifolia* (vanilla) (61) seedlings and root exudates of *Pisum sativum* (359) and *Medicago truncatula* (122)

were reported to interfere with quorum sensing system in *C. violaceum* and *Agrobacterium tumefaciens*. Furthermore, the anti-quorum sensing activities of bean sprout, carrot, chamomile, water lily and various peppers extracts were demonstrated using QSI selector *P. aeruginosa* strain possessing *luxI*-gfp reporter (291). Similarly, another study showed the inhibition of quorum sensing system by polyphenol rich fruit extracts of raspberry, blueberry, blackberry, cranberry, and grape, and herbs such as thyme, ginger, basil, kale, oregano and turmeric (370). Although, the inhibitory potential of various plant extracts were reported, the active principles in these plants were not identified.

The interference with the quorum sensing system can be achieved in a number of ways including competitive inhibition of binding of signal molecule to the receptor, degradation of signal, interference with the downstream signaling cascade, production and transport of the signal molecule (293). For instance the *D. pulchra* furanone was demonstrated to inhibit the downstream signaling cascade by inhibiting LuxR binding activity (86, 225). In addition, several synthetic antagonists were designed based on AHL structure and were subsequently demonstrated to inhibit LuxR- and LasR/RhlR-based quorum sensing systems (271). Furthermore, a synthetic molecule, LED209, was developed by modifying an organic molecule identified in a screen to inhibit the QseC (290). In addition, several bacterial species produce lactonases – enzymes which can cleave the lactone ring and deactivate the AHLs (92).

The search for quorum sensing inhibitors has gained momentum because of two coinciding factors- emergence of antibiotic resistant strains of pathogens coupled with

decreased number of new antibiotics being discovered. Targeting quorum sensing and pathogenicity is suggested to put bacteria under less selective pressure and decrease the emergence of resistant strains (292-293).

1.6 Main Hypothesis and Aims of the Project

Hypothesis: Since plants have coexisted with the bacteria throughout evolution, it is reasonable to assume that plants may produce anti-quorum sensing compounds. Secondary metabolites being most often involved in plant defense are the most likely candidates to exhibit anti- quorum sensing activity. Therefore, the secondary metabolites such as limonoids and flavonoids are speculated to possess the anti-quorum sensing and antivirulence properties and contribute to the defense of citrus plants against bacterial pathogens. Furthermore, these secondary metabolites by virtue to their anti-quorum sensing and antivirulence properties are likely to affect virulence and biofilm formation in human pathogenic organisms such as *E. coli* O157:H7 and *S.* Typhimurium LT2.

Specifically this research explored the following aims:

I. Isolation and Purification of limonoids from grapefruit and sour orange

II. Development of an analytical method to measure limonoids

III. Elucidation of cell-cell signaling inhibitory activity of limonoids in the model organism *V. harveyi*.

IV. Elucidation of cell-cell signaling inhibitory activity of limonoids in human pathogen *E. coli* O157:H7

V. Investigation of cell-cell signaling and biofilm inhibitory activity of limonin analogues

VI. Study of antivirulence properties of limonoids against *Salmonella* Typhimurium LT2

VII. Elucidation of cell-cell signaling inhibitory activity of flavonoids in the model organism *V. harveyi*.

VIII. Effect of flavonoids on the virulence and pathogenicity of *Salmonella* Typhimurium LT2

The research project to accomplish the above objectives was carried to provide evidences for anti-quorum sensing and antivirulence activities of citrus secondary metabolites against human pathogens such as *E. coli* O157:H7 and *S.* Typhimurium LT2. The results of the undertaken projects not only identify the limonoids and flavonoids with anti-quorum sensing and antivirulence properties and in-turn novel health benefits of Citrus, but could also be used to design new therapeutic strategies for *E. coli* O157:H7 and *S.* Typhimurium LT2.

CHAPTER II

ISOLATION AND PURIFICATION OF LIMONOIDS*

2.1 Synopsis

In recent years, health beneficial properties of limonoids have been focus of investigation. In vitro and animal studies indicate that citrus limonoids demonstrate anti-cancer, anti-viral and cholesterol lowering properties. However, investigation of biological activities requires purified compounds. Therefore, unavailability of commercial sources for citrus limonoids warrants their isolation in pure form and adequate quantities. The current study focused on to isolate limonoid aglycones and glucosides from grapefruit and sour orange. Seeds were used as raw material for isolation procedure, as they were suggested to contain relatively high amount of limonoids compared to other fruit tissues. The limonoids were extracted using different solvents and fractionated on silica gel or adsorbant resin to obtain purified limonoid aglycone and glucosides, respectively. Furthermore, 10 limonoids in sufficient quantity were obtained for further biological investigations.

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2.2 Introduction

Grapefruit is a major citrus crop grown and consumed in United States. In recent years, grapefruit and other citrus crops have gained attention due to their potential role in sustenance of health. Grapefruit contains a number of bioactive compounds. The wide variety of limonoids (Table 1.1) is speculated to be among the primary contributors towards health maintaining property of grapefruit. Limonin, nomilin, obacunone and deacetylnomilin are the major limonoids accumulated in grapefruit (35) with limonin being the dominant limonoid (142). To investigate the contributions of various limonoids to the health maintaining properties, purified limonoids are required. Since limonoids are not commercially available, therefore, in order to investigate their biological activity, it is necessary to purify the limonoids. However, compared to flavonoids, limonoids are present in smaller quantities. Citrus seeds are usually better source of limonoids for purification purposes, as they contain relatively higher concentration of limonoids (142). In addition, flavonoids frequently demonstrate similar polarity as limonoids, especially limonoid glucosides, and therefore, interfere with the limonoid purification process.

Limonoid aglycones were separated on a variety of stationary phases such as silica gel, alumina (34, 94, 142). In addition, numerous mobile phases comprising of ethanol, chloroform, benzene, acetone etc, were combined with different stationary phases. For instance, ichangin was chromatographically separated on alumina (94), while isolimonic acid was purified by methylating the mother liquor and subsequent chromatography on silica gel (34). The elution of ichangin and isolimonic acid was

carried out with by 10% acetone in chloroform and ether in dichloromethane, respectively. Limonin was isolated from Navel orange juice by extracting with benzene and precipitation by petroleum ether (98). Similarly nomilin was separated from limonin by its higher solubility in hot isopropyl alcohol, while obacunone was separated from nomilin by virtue of higher solubility in hot toluene (98). The limonoid glucosides are relatively polar and can be extracted using polar solvents such as water or methanol. However, isolation of limonoid glucoside is a cumbersome process and usually involves two or more chromatographic steps. Limonoid glucosides from sour orange were reportedly purified by extracting with water and separation on XAD-2 column followed by purification by preparative HPLC on C_{18} column (36). Further, Bennett et al (35) isolated limonoid glucosides by fractionating the methanol extracts of grapefruit seeds on XAD-2 coarse resin followed by purification on fine XAD-2 resin. The fractions containing single glucoside were further purified on DEAE Sephacel and sunsequently freed of hydrochloric acid (HCl), used for elution of glucosides from XAD-2 resin, on C_{18} adsorbant column.

Recently, a process for purification of limonoid glucosides was developed in our laboratory. This process involves fewer steps and does not require the salt and acid solution generally used for elution of limonoid glucosides (174). The process involves use of a cation exchange resin in combination with an adsorbant resin coupled with elution of limonoids by water and acetonitrile (ACN).

Citrus seeds are better source of limonoids for purification purposes, as they contain relatively higher concentration of limonoids- approximately 1 % on dry weight

basis (142). However, compared to flavonoids, the limonoids are present in smaller quantities. In addition, flavonoids frequently demonstrate similar polarity as limonoids, especially limonoid glucosides, and therefore, interfere with the limonoid purification process. The current study aimed at isolating major limonoid aglycones and glucosides from grapefruit and sour orange in sufficient quantity for subsequent biological activity.

2.3 Materials and Methods

2.3.1 Materials

Reagent and HPLC grade methanol (MeOH), ethyl acetate (EtOAc), acetonitrile (ACN), dichloromethane (DCM), and thin Layer chromatographic silica gel 60F-254 plates were purchased from Fisher Scientific (Hampton, NH).

2.3.2 Plant Material

Sour orange (*Citrus aurantium*) and grapefruit (*Citrus paradisi*) fruits were collected from the Texas A&M University Kingsville Citrus Center in Weslaco, Texas. The seeds were separated from the fruits and air-dried under shade at 25ºC for 7- 8 days to obtain ≈ 2% moisture level and then ground to powder (40-60 mesh size).

2.3.3 Extraction of Plant Material

Defatted seed powder (2.0 kg) was extracted successively for 8 h each with ethyl acetate (EtOAc), acetone, and methanol (MeOH) in a Soxhlet apparatus as depicted in Fig. 2.1. The extracts were filtered, concentrated under vacuum (Buchi, Switzerland) to get a viscous concentrate and freeze dried.

2.3.4 Purification of Limonoids from Grapefruit Seed

2.3.4.1 Purification of Acetone Extract

The limonoids were purified according to previously published methods from our laboratory (223). In brief, the acetone fraction (42 g) was partitioned between dichloromethane (DCM) and water (2:1). Compound **1** crystallized in the DCM. *In vacuo* dried supernatant (27 g) was chromatographed on silica-gel (300 g) column (3.5 x 90 cm) and eluted with linear gradient of 1 % ethyl acetate (EtOAc) in DCM. Compounds **2**, **1, 3** and **4** were eluted with DCM/EtOAc (99:1), (95:5), (97.5: 2.5), (80:20) respectively.

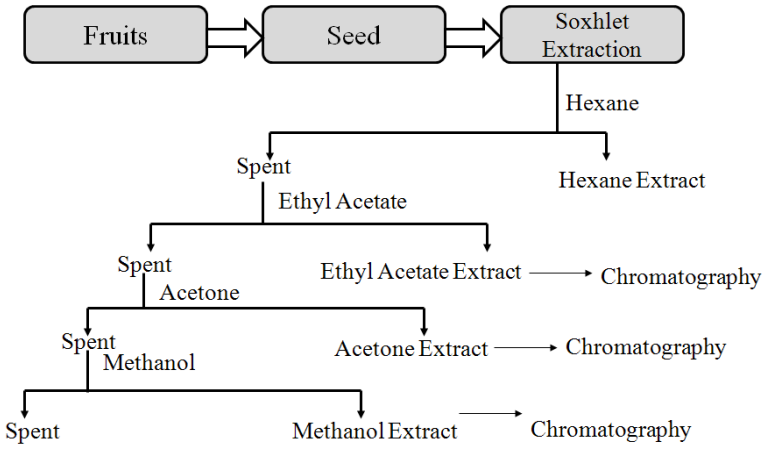


Fig 2.1 General scheme for extraction of limonoids from seeds

2.3.4.2 Purification of MeOH Extract

The methanol extract was processed according to our previous published method (174). First column was packed with Dowex [H^+] resin, while second column was

packed with Sepabeads adsorbant resin. The two columns were connected in tandem as presented in Fig 2.2. Vacuum dried methanol extract was loaded on the Dowex resin column. The column was washed thoroughly with deionized water. The wash from Dowex colum was passed over Sepabeads resin column to adsorb the limonoids. Sepabeads column was later separated from Dowex column and eluted with stepwise linear gradient of ACN in water. The fractions depicting similar profile were pooled and concentrated under vacuum. The concentrated fractions were stored under 4ºC for crystallization. Compound **5** was obtained as crystallized product from acetonitrile:water (1:9) fractions.

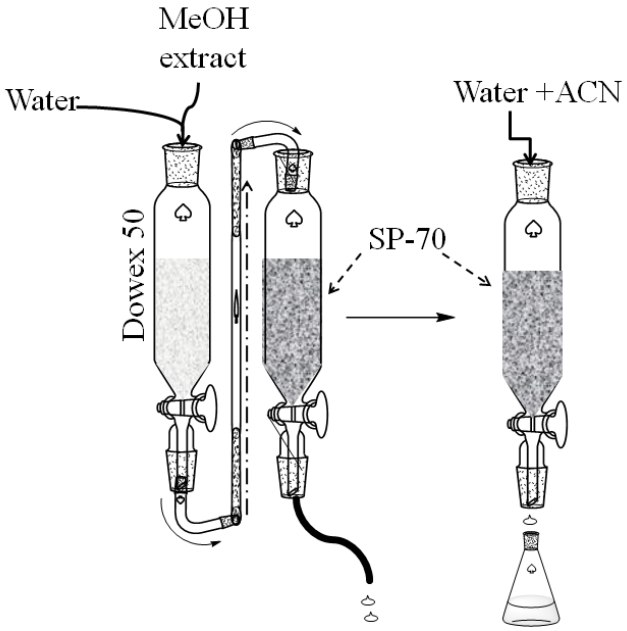


Fig. 2.2 Purification scheme of MeOH extract

2.3.5 Purification of Limonoids from Sour Orange Seed

2.3.5.1 Purification of Ethyl Acetate Extract

The EtOAc extract (30 g) was dissolved in 500 ml DCM and extracted with 250 ml water. Compound 1 (1.42 g) crystallized in DCM. The vacuum dried DCM fraction (28.1 g) was chromatographed on a silica gel column (300 g) with stepwise linear gradient of EtOAc in DCM. Compounds **1**, **6**, **7**, and **8** were eluted with DCM/EtOAc (99:1), (92.5: 7.5), (85:15), (80:20) respectively.

2.3.5.2 Purification of MeOH Extract

The MeOH extract was purified as described in the section **2.3.4.2.** Compounds **9** and **10** were eluted with 12.5:87.5 and 15:85 (ACN/Water). Fractions containing compound **9** in higher concentration were pooled and concentrated to yield 1.47 g of colorless crystalline product. The fraction with higher concentrations of compound **10** were pooled, dried under vacuum and re-dissolved in water-acetonitrile (1:1) to yield 350 mg of colorless compound.

2.3.6 Analysis

2.3.6.1 TLC Analysis

Purified compounds were spotted on silica gel 60F-254 plates. The plates were developed using DCM/EtOAC (3:2) for aglycones and EtOAC/methyl ethyl ketone/formic acid/water (5:3:1:1) for glucosides, respectively. The plates were sprayed with Ehrlich's reagent (2 % N,N –dimethyl amino benzaldehyde in ethanol) and developed in a HCl gas chamber. Typical pink/reddish colored spots were obtained for

limonoids (220). In addition plates were sprayed with 10% sulfuric acid in MeOH followed by heating at 100ºC for 10 min.

2.3.6.2 High Performance Liquid Chromatography

HPLC system consisted of Perkin Elmer Series pump 2000 coupled with Perkin Elmer Series 2000 Autosampler and Perkin Elmer Diode Array detector 235C. Limonoids were separated on Waters Prevail C_{18} analytical column (Alltech, Deerfiels, IL, USA), 5 µm particle size, (250 × 4.6 mm) and detected at 210 nm. The compounds were quantified using TurboChrome Navigator Software ver. 6.1.2.0.1 (Perkin Elmer, Boston, MA). All the column fractions and compounds **1-4** were filtered through 0.45 µm filter and then subjected to HPLC analysis. The gradient mobile phase used for (A) aglycones was isocratic 40% acetonitrile in water for 30 min, while (B) for glucosides a linear gradient consisted of 10% acetonitrile in 0.03M phosphoric acid to 24% acetonitrile in 30 min.

2.3.7 Identification

The compounds were identified by matching the retention time and absorption spectra with standards. Identities of the purified compounds were confirmed by APCI/ESI-mass spectrometry. The spectra of the purified compounds were compared with the published data (223, 227).

2.4 Results

2.4.1 Purification and Identification of Limonoids from Grapefruit

Freeze-dried acetone extract was purified by chromatographic techniques to obtain four limonoids. Compound **1** (4.7 g) was obtained as crystals when acetone

extract was partitioned in DCM and water. The remaining part was dried and further purified to obtain compound **2** (54 mg), **3** (632 mg), **1** (685 mg) and **4** (357 mg). The total yield of compound **1** was 5.385 g. Isolation and identification of compound **2** was reported previously from our lab (223). Compound **5** was obtained from methanol:water extract with a yield of 6.25g. Compound **1**, **2**, **3, 4** and **5** were identified as limonin (m/z=471, M+1), obacunone (m/z=454.9, M+1), nomilin (m/z=515, M+1), deacetyl nomilin (DAN) (m/z=473, M+1) and limonin 17-β D glucopyranoside (LG) (m/z=650.2) (Fig. 2.3). Limonin was found to be predominant aglycone present in the seeds while LG was predominant glucoside.

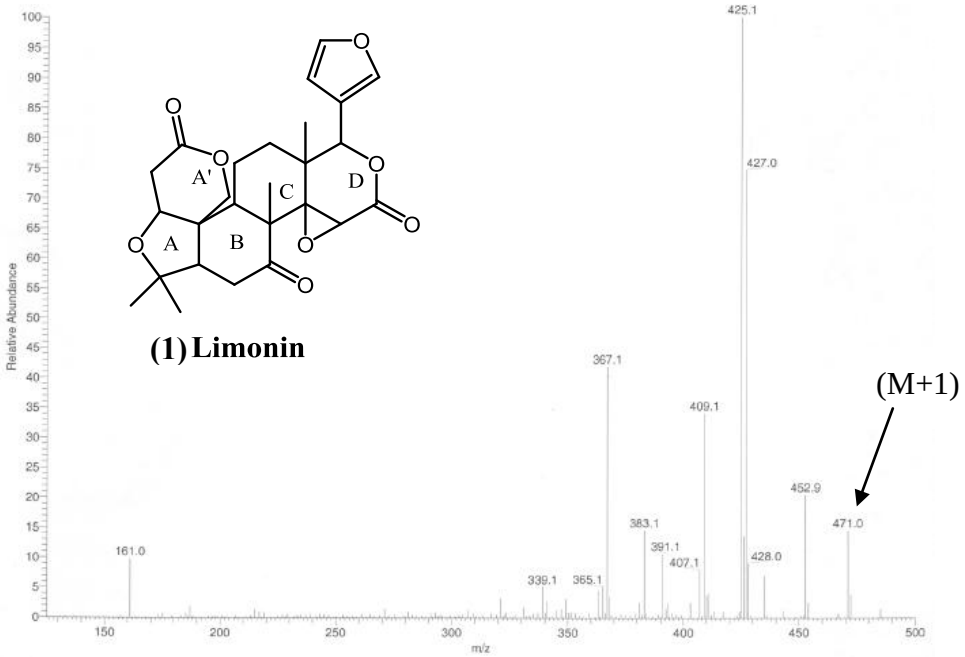
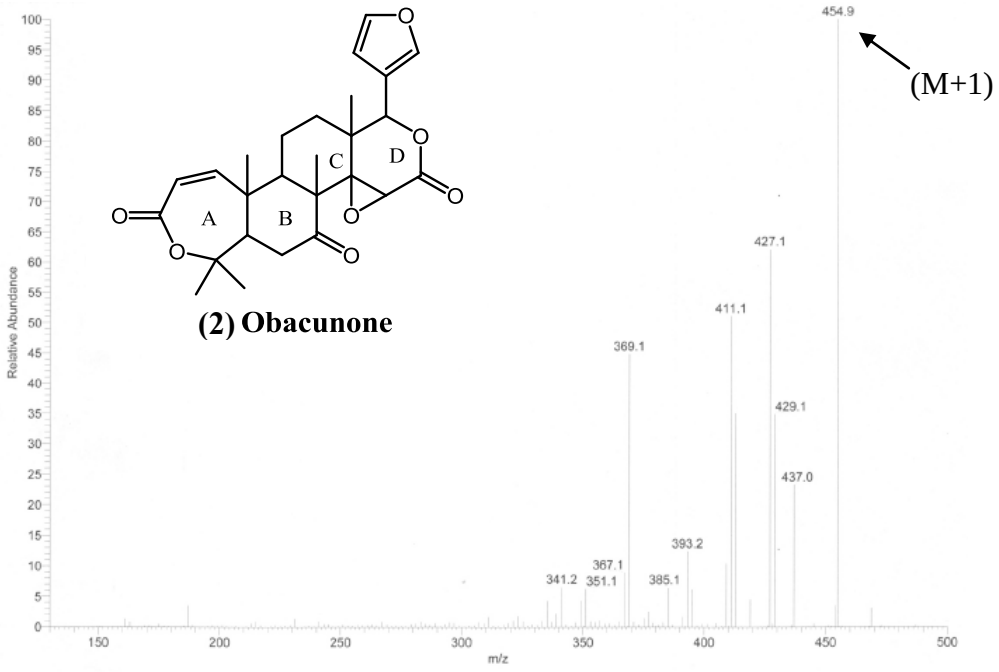


FIG. 2.3 Mass spectra of limonoids purified from grapefruit seeds. (1) limonin, (2) obacunone, (3) nomilin, (4) deacetyl nomilin (DAN) and (5) limonin 17β-D-glucopyranoside (LG)



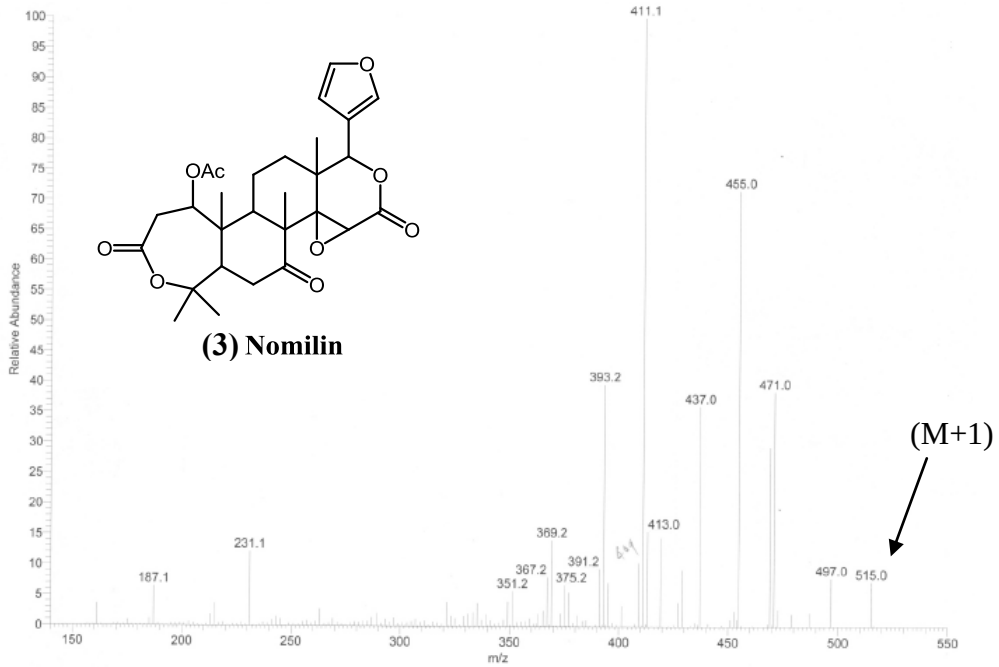
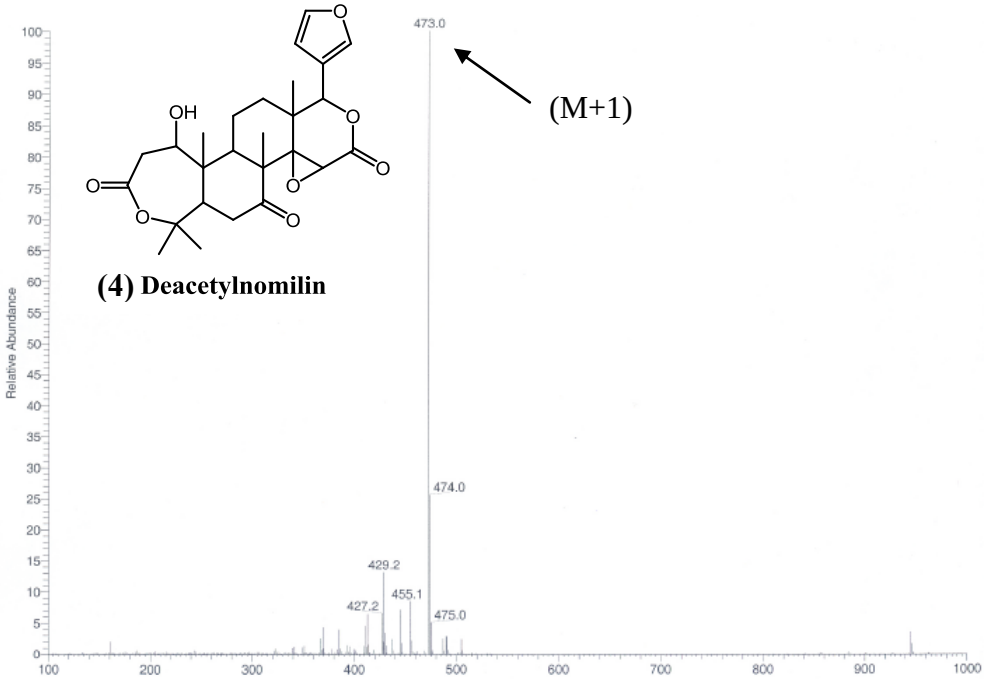


FIG. 2.3 Continued.



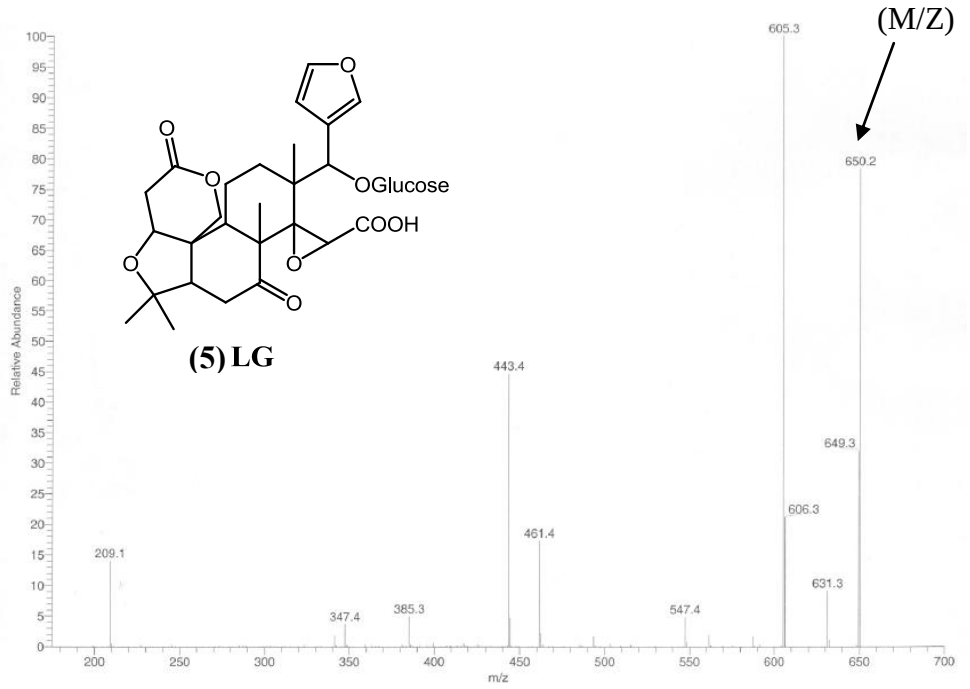


FIG. 2.3 Continued.

2.4.2 Purification and Identification of Limonoids from Sour Orange

Ethyl acetate extract of sour orange seed yielded three aglycones. The purities of the compounds were analyzed by HPLC according to our previous research (372) and found to be >96 % pure. Further, identities of purified compounds were confirmed by positive ion APCI-mass spectra for aglycones and negative ion APCI for glucosides. Compound **6**, **7**, and **8** were identified as ichangin (m/z 489, M+1), isoobacunoic acid (m/z 473, M+1) and isolimonic acid (m/z 489, M+1) (Fig. 2.4). The mass spectra of these compounds are presented in Fig. 2.4. The yields of the compounds **1**, **6**, **7**, and **8** were 3102, 413, 805 and 24 mg, respectively.

The methanol extract yielded two glucosides **9** and **10** which were identified as deacetyl nomilinic acid 17β-D-glucopyranoside (DNAG) (m/z 669.3), and isoobacunoic acid 17β-D-glucopyranoside (IOAG) (m/z 651.2) respectively (Fig 2.4). The negative ion mass spectra of DNAG and IOAG are presented in Fig. 2.4. All the compounds were isolated as crystallized products. The total yield of compound **9** and **10** was 1.47 g and 350 mg respectively.

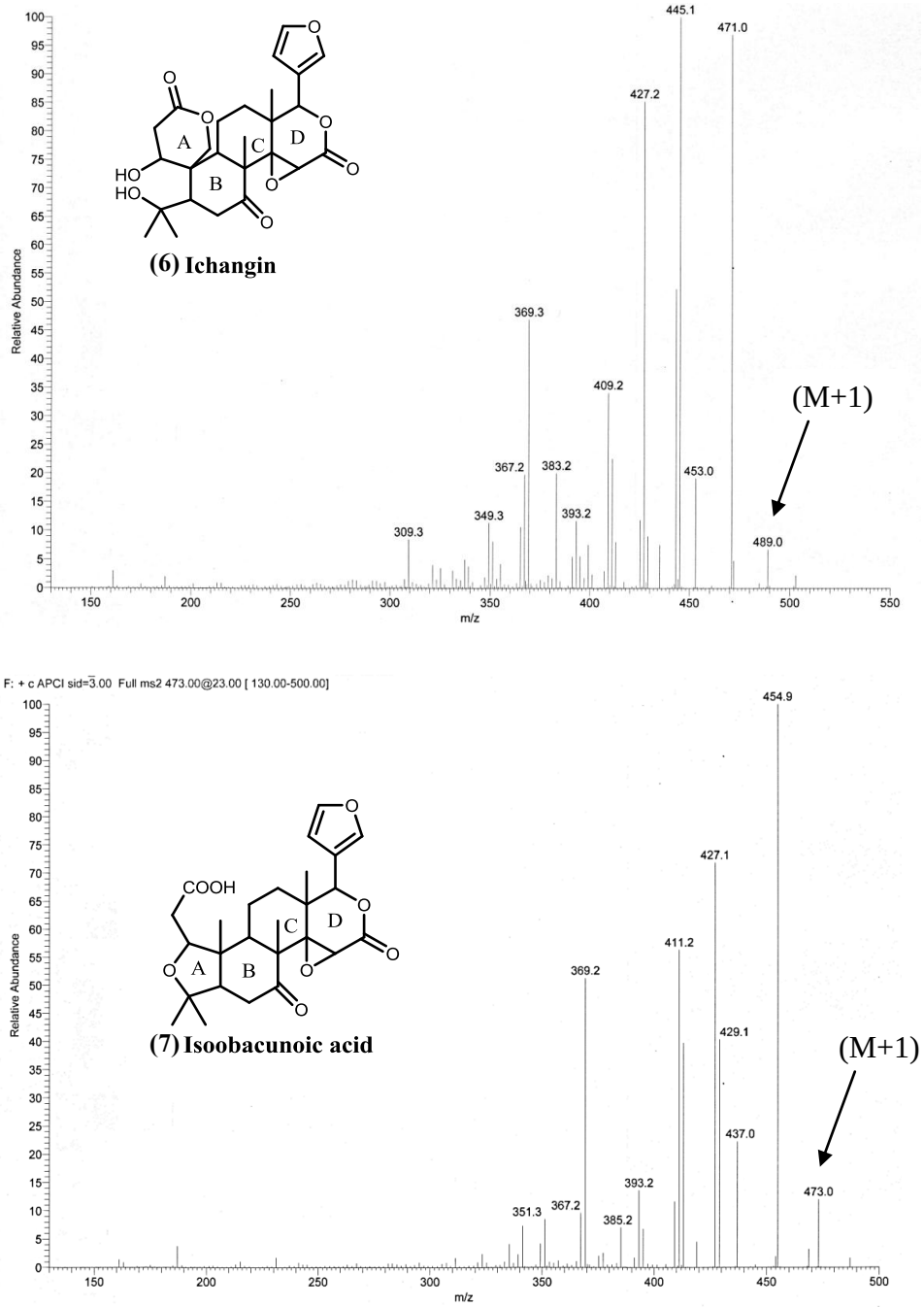
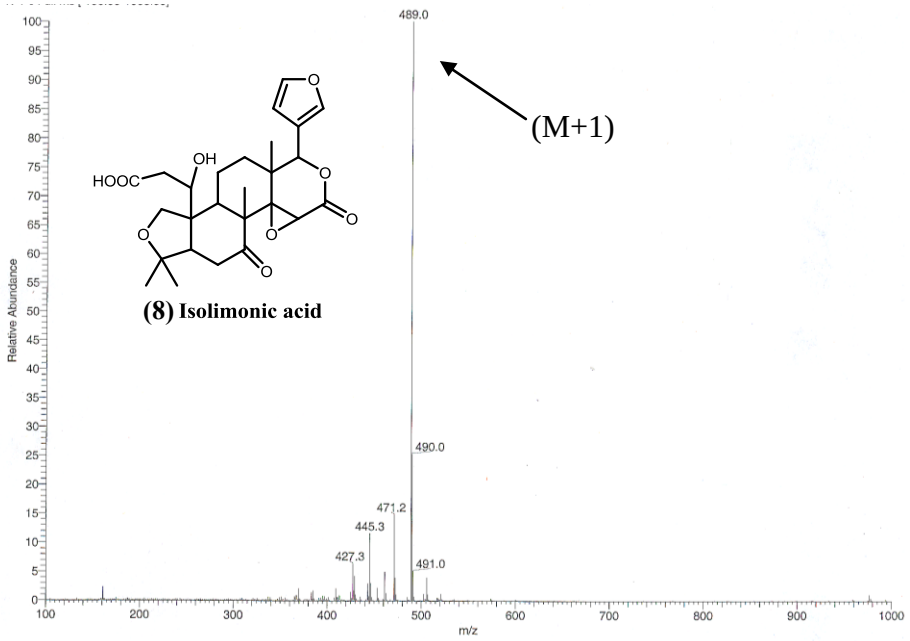


FIG. 2.4: Mass spectra of limonoids purified from sour orange seeds. (6) ichangin, (7) isoobacunoic acid, (8) isolimonic acid, (9) deacetyl nomilinic acid 17β-D-glucopyranoside (DNAG) and (10) isoobacunoic acid 17β-D-glucopyranoside (IOAG)



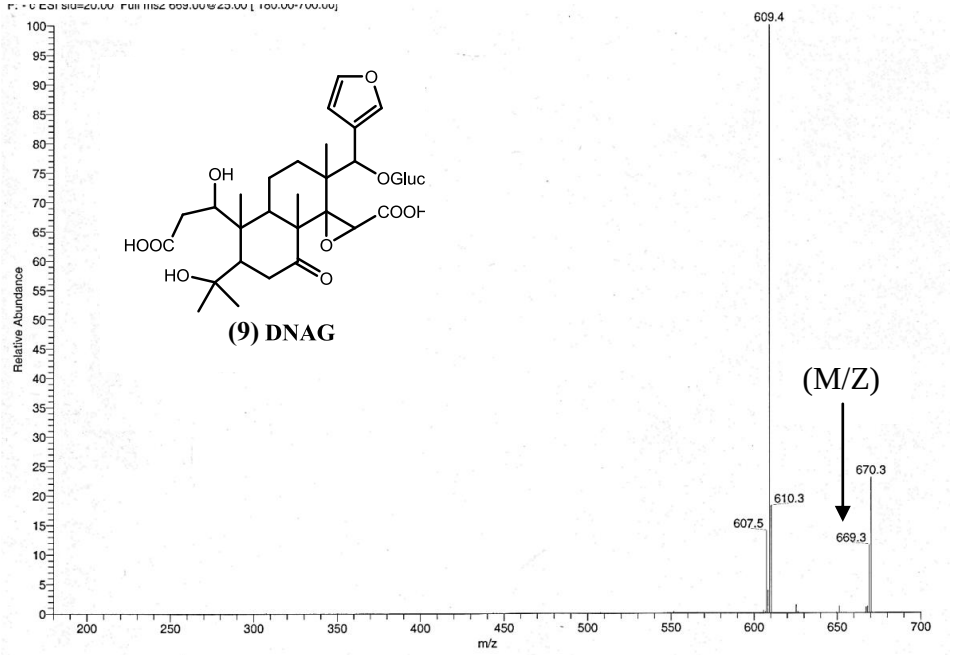


FIG. 2.4: Continued.

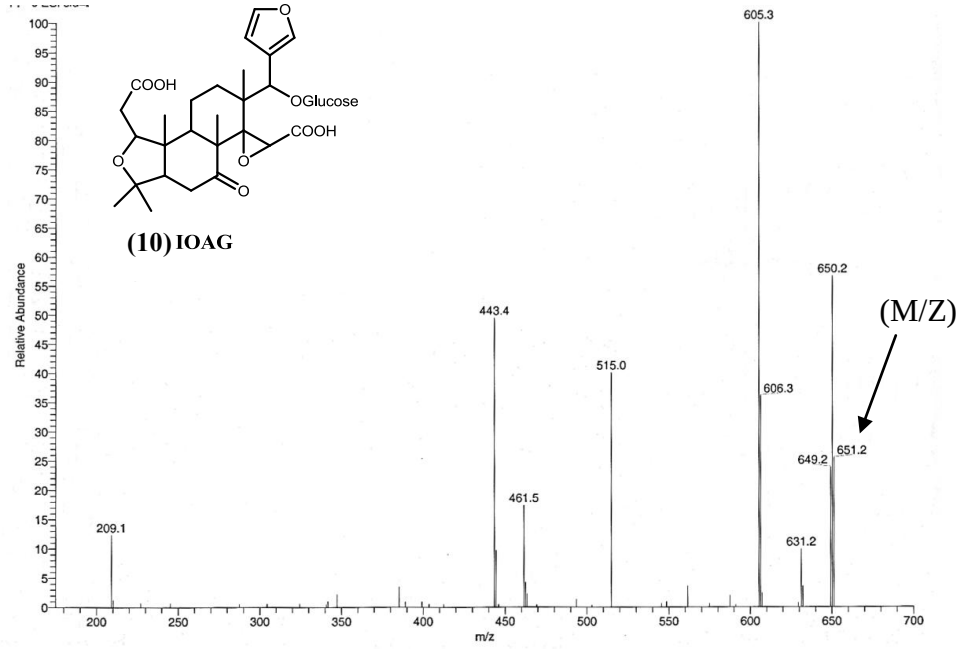


FIG. 2.4: Continued.

2.5 Discussion

In the present study, a total of ten limonoids were purified. Grapefruit seeds yielded 4 aglycones and one glucoside while three limonoid aglycones and two limonoid glucosides were purified from sour orange seeds. Despite their higher concentration in citrus juice, limonoid glucosides are difficult to purify owing to their very similar polarities. In addition, flavonoid glycosides, which are present relatively at higher concentration, interfere with the purification of limonoids. Seeds contain relatively higher concentration of limonoids, speculated to be as much as 1 % of dry weight. Therefore, seeds, instead of the fruits, were chosen for purification of limonoids. In the current study, we have employed a cation [H^+] exchange column. Flavonoid glycosides

have weak ionization (150) and they are absorbed on the cation exchange column (Dowex), whereas limonoids were adsorbed on the Sepabeads column. The limonoid glucosides were eluted using water and ACN to obtain DNAG and IOAG as a crystallized product.

The predominant limonoids in grapefruit seeds are limonin and LG, reported to be as much as 60 % of total limonoid content (142). During the purification procedure the limonin and LG were obtained in major quantity, which was consistent with previous report (142). Furthermore, neutral limonoids such as limonin are usually the end products of the biosynthetic pathway thus accumulate in higher concentration compared to other limonoids (142). The biosynthesis of limonin starts with deacetylnomilinic acid and proceeds via nomilin, obacunone, ichangin (140). During the purification procedure, relatively small amounts of nomilin and obacunone were also isolated, indicating that the biosynthetic pathway from nomilin to limonin was the most active in grapefruit. In addition, purification of deacetylnomilin, which is synthesized from the deacetylnomilinic acid (140), indicates that this pathway is also active in grapefruit.

Sour orange is related to grapefruit via pummello, which is the common ancestor for both species. Sour orange is widely used as a common rootstock for citrus varieties. In addition, sour orange juice is a common adulterant in grapefruit juice (251). Furthermore, anti-oxidant and health promoting properties of sour orange were the focus of several studies (173). Limonoids are prominent constituents of sour orange. In particular, sour orange was reported to contain high concentrations of isolimonic acid and deacetylnomilinic acid (34, 140). During isolation procedure significant quantities of

DNAG and isolimonic acid were purified from sour orange seeds. DNAG is the glucoside of deacetylnomilinic acid, and may have accumulated during the fruit maturation process. During isolation procedure, DNAG comprised ≈23.8 %, while limonin content was 50.32 % of total limonoid yield. Although, the purification process inevitably results in some losses, DNAG and limonin seems to be the major limonoid accumulated in sour orange seeds. Since deacetylnomilinic acid is the precursor of limonoid biosynthesis, the observed accumulation suggests that deacetylnomilinic acid was possibly converted into glucoside form during early fruit development stage which was unavailable for synthesis of other limonoids. However, further experiments are required to establish the conversion of aglycone to glucoside at an early fruit development stage. In addition, limonin and ichangin were isolated in significant quantities indicating the limonin biosynthesis pathway was active in sour orange. Accumulation of significant quantities of isolimonic acid also suggests that another pathway is also active. Interestingly, we also obtained significant quantities of isoobacunoic acid and its corresponding glucoside during the purification procedure. Isoobacunoic acid was previously reported from grapefruit (35), but this is the first report of its presence in sour orange seeds. Presence of isoobacunoic acid further strengthens the reported taxonomic relationship between sour orange and grapefruit.

Finally, the primary objective of this project was to purify limonoids for subsequent studies. Since biological activity elucidation frequently requires large quantities, 10 limonoids were purified from grapefruit and sour orange in sufficiently large quantities to use them in biological assays.

CHAPTER III

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS MEASUREMENT OF CITRUS LIMONOID AGLYCONES AND GLUCOSIDES*

3.1 Synopsis

High performance liquid chromatography (HPLC) method has been developed for simultaneous quantification of limonoid aglycones and glucosides on a reversed phase C_{18} column using a binary solvent system, coupled with diode array detector. Seven limonoids such as limonin, nomilin, isolimonic acid, ichangin, isoobacunoic acid, limonin 17-β-D glucopyranoside and deacetyl nomilinic acid 17-β-D glucopyranoside were separated and detected at 210 nm. Furthermore, limonoids were separated, identified and quantified in four varieties of citrus fruits and seeds using developed method. Limonin and limonin glucoside were found to be the predominant limonoid aglycone and glucoside respectively in all tested samples. The sensitivity of the method was found to be 0.25µg - 0.50µg for tested limonoids.

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*Reprinted with permission from "Simultaneous determination of citrus limonoid aglycones and glucosides by high performance liquid chromatography" by A. Vikram, G. K. Jayaprakasha, and B. S. Patil, 2007, Anal. Chim. Acta 590:180-186, Copyright 2007 Elsevier.

3.2 Introduction

Limonoids are highly oxygenated modified triterpenes derived from a precursor with 4, 4, 8-trimethyl-17-furanylsteroid skeleton biosynthesized from acetate-mevalonate pathway in Citrus (147). Limonoids occur in significant amounts as aglycones and glycosides in seeds and fruit of *Citrus* species (226). Limonin was discovered as a bitter principle in navel orange juice in 1949 (144). While certain limonoids cause bitterness, some of them are being investigated intensively due to their wide range of biological activities (26, 192, 195, 242, 276-277, 362, 368). Considering the potential applications, the citrus limonoids needs to be studied more thoroughly, and it will be pragmatic to determine the limonoid profile of different citrus varieties and their juices. While, several methods are available for the analysis of limonoids in the literature, currently, there are no methods for screening of limonoid aglycones and glucosides simultaneously in citrus fruits and seeds.

Majority of reported analytical methods targeted at separation and quantification of the limonin owing to its contribution to bitterness (1, 106-107, 326-328, 385-386). A variety of techniques were explored to determine the limonin content in citrus varieties. Previous methods explored various techniques such as fluorometery [21], gas chromatography (196, 386), thin layer chromatography (143), radioimmunoassay (234), enzyme linked immunoassay (1, 180, 386), high performance liquid chromatography (HPLC) (1, 105-107, 226, 228, 309, 326-328, 367), HPLC-Electron Ionization/Mass Spectroscopy and HPLC-Atmospheric Pressure Chemical Ionization /Mass Spectroscopy (226, 361).

High performance liquid chromatography is a prominent analytical method for the analysis of limonoids in citrus (227) due to ease of operation and versatility. Table 3.1 summarizes the major studies conducted in the development of HPLC method for limonoid analysis. From Table 3.1 it is clear that historically, limonoids were analyzed and quantified separately as aglycones and glucosides. Further, both reversed phase and normal phase chromatographic procedures and HPLC columns have been widely used in the limonoid analysis (1, 106-107, 158, 228, 259, 309, 326-328, 385). Normal phase HPLC method was reported by Rouseff and Fisher for analysis of limonin in citrus juices (309). More recently, another normal phase HPLC method was described for separation of neutral limonoid aglycones (228). For normal phase separations cyanide or C_{18} columns were generally used in conjuction with a mobile phase comprising of a combination of organic solvents such as hexane, chloroform, acetonitrile, methanol, tetrahydrofuran, and cyclohexane (Table 3.1). Whereas, the C_{18} column with a combination of acetonitrile and 3mM phosphoric acid was used for the analysis of glucosides (158, 259). In recent years, mass spectroscopy coupled with liquid chromatography was also employed in determination of citrus limonoids. A screening method to detect limonoid glucosides was developed by Tian and Ding (360) employing high performance liquid chromatography on C_{18} reversed phase column with diode array detector and interfaced with electron spray mass spectroscopy.

The reported HPLC methods have targeted for the quantification of limonoid aglycones or limonoid glucosides separately. Since the citrus fruit juice or seeds contain both the aglycones as well as glucosides, separate measurement of these bioactive

limonoids is time consuming and requires separate analytical methods to determine both aglycones and glucosides content in the particular tissue. The present study describes an HPLC method for successful simultaneous separation and identification of five limonoid aglycones and two glucosides (Fig. 3.1) simultaneously in citrus fruits and seeds.

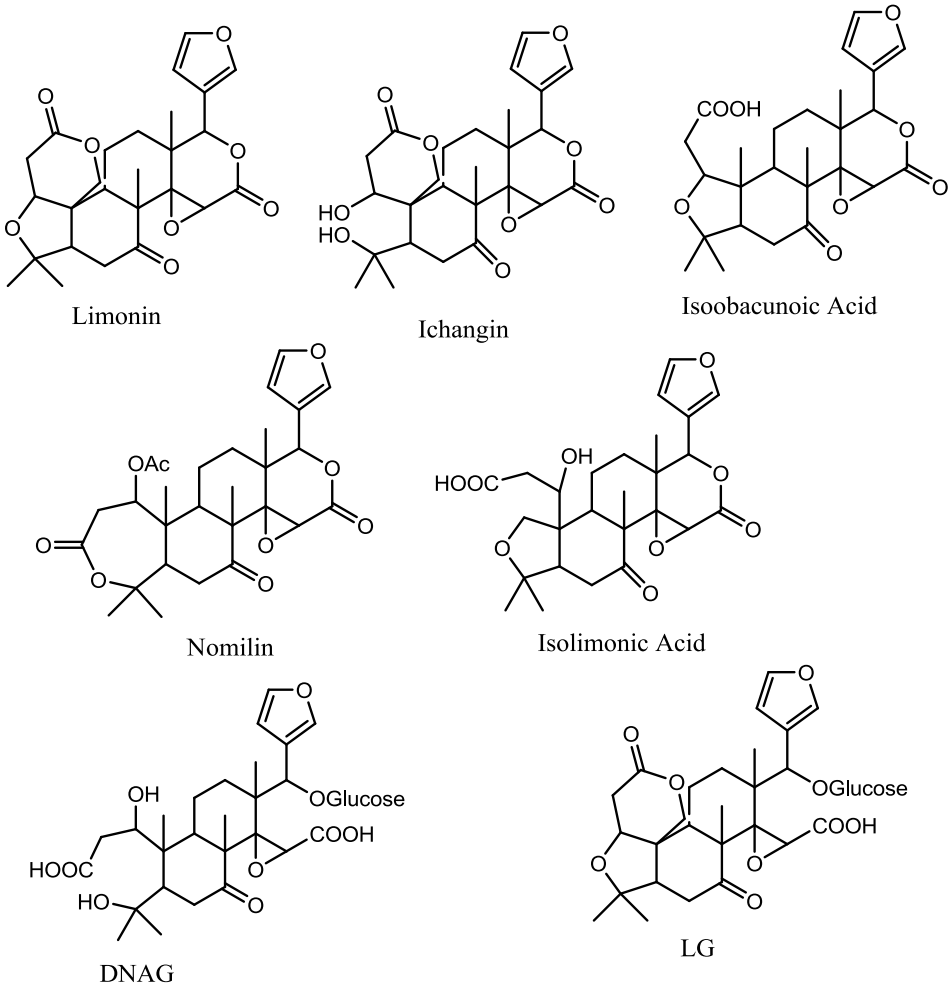

FIG. 3.1 Strucures of the limonoids used for simultaneous HPLC separation (LG= Limonin glucoside, DNAG=Deacetyl nomilinic acid glucoside)

TABLE 3.1: Published HPLC methods for citrus limonoid analysis

Compounds analyzed	Column & Mobile phase	Disadvantages	Ref.
limonin	10µ porous silica; isocratic, chloroform : acetonitrile (95:5)	Cumbersome maintenance of temperatures the column was maintained at 35^{o}C in an insulated water jacket; Problems with baseline due to presence of 1 % ethyl acetate in chloroform and temperature fluctuation and problems associated with refractive index detection; but the procedure was found better over the fluorometric analysis.	(107)
limonin	10µ CN; isocratic; methyl alchohol: water (35:65 and 40:60)	Faster loss of resolution on CN column; clean up required after every third injection; though use of UV detector reduced the effect of temperature, pressure and flow rate to some extent.	(106)
Limonin, deoxylimonin, nomilin, obacunone	5µ CN; Reverse phase-water: methanol (45:55) normal phase – hexane (or heptane) : isopropanol: methanol (6:3:1)	In reverse phase the resolution was poorer than normal phase and baseline was irregular in normal phase forcing the substitution of hexane with heptane and back.	(309)
Limonin, nomilin	5µ C_{18} and C_8; acetonitrile-tertrahydrofuran-water (17.5-17.5-65) for C-18 and 17.5-15-67.5 for C-8)	Tetrahydrofuran is very unstable and needs constant degassing and cause unstable baseline	(327)
Limonin, nomilin	5µ C-18; used 7 different mobile phases	Found out that mobile phase comprising of methanol, acetonitrile and water is most suitable in terms of baseline stability and freedom from negative peaks while lengthening the analysis time.	(326)
Limonin	5µ C-18; acetonitrile(30-32%)-water	Claims the ability to control separation of limonin on C_{18} column in a predictable manner by varying the composition of mobile phase	(328)
Limonin, nomilin	5µ C_{18}, acetonitrile-methanol-water (28.5:13:58.5 and 31.8:22.7:45.5), methanol –water(65:35)	In the first mobile phase the flow rate was very high at 2 ml/min but nomilin cannot be determined while the second combination detected both limonin and nomilin but sacrificed the resolution.	(367)

TABLE 3.1 Continued.

Compounds analyzed	Column & Mobile phase	Disadvantages	Ref.
Limonin, nomilin	5µ CN preceded by 0.45 µm in-line filter which was maintained at 30 ^{o}C; reverse phase acetonitrile: water (38:62); normal phase- 2-propanol:heptane:methanol (48:44:8)	Baseline was irregular making the quantitation difficult, even though CN column may have better selectivity for limonin but were susceptible to contamination and thus lose the efficiency faster	(385)
Limonin, nomilin, deacetylnomilin, obacunone	5µ C_{18}; acetonitrile:methanol:water (10:41:49)	Separate mobile phases were used for limonoid aglycone and glucoside analysis and analysis time was long as compared to the previous methods for four aglycones.	(259)
17 Limonoids	5µ Sperisorb silica; cyclohexane-tetrahydrofuran	Tetrahydrofuran is difficult to handle as it requires constant degassing and causes irregular baseline therefore reproducibility of the method is difficult. The analysis had a long equilibration time.	(228)
Limonin	5µ C-18; acetonitrile-tertrahydrofuran-water(17.5-17.5-65), acetonitrile-water (32-68)	Tetrahydrofuran is very unstable causing irregular baseline; the method concentrated only on limonin and did not investigated the separation in presence of other limonoids or flavonoids	(1)
Limonin glucoside, nomilinic acid glucoside, nomilin glucoside, deacetylnomilinic acid glucoside	5 µ C18; acetonitrile and 0.003M phosphoric acid	Poor peak resolution and drifting baseline resulting in poor quality chromatograms.	(158)
Limonin glucoside, nomilinic acid glucoside, nomilin glucoside, deacetylnomilin glucoside, and obacunone glucoside	5 µ C_{18}; acetonitrile and 0.003M phosphoric acid	Nomilin glucoside and nomilinic acid glucoside peaks did not have good resolution.	(259)

3.3 Materials and Methods

3.3.1 Materials

3.3.1.1 Plant Materials

The seeds of four varieties of citrus - nova tangerine (*Citrus reticulata* Blanco), cleopatra mandarin (*Citrus reticulata* Blanco), sour orange (*Citrus aurantium*) and rio red grapefruit (*Citrus paradisi* Macf.) were collected from the Texas A&M University, Kingsville, Citrus Center (Weslaco, TX). The seeds were cleaned and dried completely under shade. The dried seeds were ground and used for the extraction of the limonoids. The fruits of rio red grapefruit, tangerine, valencia orange and navel oranges were also collected from Texas A&M University, Kingsville, Citrus Center, (Weslaco, TX).

3.3.1.2 Analytical Reagents

All the solvents used were of analytical or HPLC grade and were obtained from Sigma (St. Louis, USA).

3.3.2 Instrument

HPLC system consisted of Perkin Elmer Series pump 2000 coupled with Perkin Elmer Series 2000 Autosampler and Perkin Elmer Diode Array detector 235C. Limonoids were separated on C_{18} Phenomenex Gemini series column (Torrence, CA, USA), 5 µm particle size, (250 × 4.6 mm) and detected at 210 nm.

3.3.3 Preparation of Standards

All citrus limonoids are not available commercially in market. Hence, we have isolated, identified five limonoid aglycones *viz.* limonin, nomilin, isoobacunoic acid, ichangin and isolimonic acid and two limonoid glucosides - limonin glucoside and

deacetyl nomilinic acid glucoside as described in chapter II (174, 277, 374-375). Stock solutions of standards were prepared by dissolving 5 mg of each compound in 5 mL of acetonitrile or acetonitrile:water (1:1 v/v). Furthermore, the stock solutions were diluted to six different concentrations *viz.* 0.025, 0.05, 0.1, 0.2, 0.3 and 0.4 mg mL^{-1} with acetonitrile for aglycones and acetonitrile:water (1:1) for limonoid glucosides.

3.3.4 Chromatographic Conditions

Binary solvent system used was 3mM phosphoric acid (solvent A) and acetonitrile (solvent B). The elution of binary solvent was conducted in gradient fashion, starting at 85 % of solvent A, reduced to 77 % in 5 min, 74 % after 25 min, which was further reduced to 60 % at 30 min and completing the gradient at 54 % at the end of 45 min. The column was equilibrated for 5 min with 85% solvent A and 15% solvent B before next run. The flow rate was kept at 1.0 mL min^{-1}. All standards and samples were filtered through 0.45µm Millipore filter and 50 µL loop was used in this study. The compounds were quantified using TurboChrome Navigator Software ver. 6.1.2.0.1 (Perkin Elmer, Boston, USA).

3.3.5 Calibration Curve

The linearity of the method was evaluated by analyzing a series of limonoids such as limonin, nomilin, isolimonic acid, ichangin, isoobacunoic acid, limonin glucoside and deacetyl nomilinic acid glucoside. About 20 µl of each of the six working standard limonoids solutions containing 0.5 – 8.0 µg were injected on to the HPLC; elution was carried out as discussed above and peak area responses were obtained. The

calibration curve for each limonoid was prepared by plotting concentration of limonoid versus peak area (average of three runs).

3.3.6. Limit of Detection

Limit of detection was defined as the lowest limonoids concentration that can be determined with an accuracy and precision <10%. Limit of detection of the system for each limonoid was tested by injecting different amount of the limonoids replicating each injection at least three times. For variation in the amount of compound injected in the column the different injection volumes were used. Injection volume varied from 5 µl to 20 µl.

3.3.7 Sample Preparation

3.3.7.1. Seeds

Four varieties of citrus seeds (20 g each) were ground and subjected to Soxhlet extraction with hexane (80 mL) at 60-70 ºC for 4 h for the removal of fatty material. The spent material was further extracted with 80 mL of ethyl acetate for 4h. The extract was cooled to room temperature and filtered through Whatman paper No 1. This extract was diluted appropriately and filtered through 0.45 micron filters and used for the quantification of limonoid aglycones.

3.3.7.2. Juice

Edible part of rio red Grapefruit, navel orange, valencia orange and tangerines were homogenized thoroughly in blender and freeze dried separately. The dried powder (500 mg each) was extracted three times with 10 mL of methanol using separating funnel for 15 min separately. All the respective extracts were pooled and made up to 35

mL and filtered. These samples (1.0 mL each) were further filtered through 0.45 micron filter and injected to HPLC analysis for the quantification of limonoid glucosides.

3.3.8. Quantification of Limonoids in Samples

A known volume (20 µl) of the different samples prepared above was injected into the HPLC and concentration of each limonoid was obtained directly from the peak area and by application of the dilution factor. The limonoids in the samples were expressed as mg/100g of seeds or fruit.

3.3.9. Statistical Analysis

All the experiments and HPLC analysis were carried out in triplicates and the results were expressed as average and SD values. Regression analysis and confidence interval (95 %) for intercepts of each limonoid was constructed using SPSS 14.0.

3.4 Results and Discussion

Citrus fruits and juice contains both limonoid aglycones and glucosides. Most of the developed methods describe either aglycone or glucoside quantification (106-107, 228, 309, 326-328, 367). The present study was focused for the simultaneous separation of aglycones and glucosides in a single run. The three neutral aglycones (limonin, nomilin, and ichangin), two acidic aglycones (isoobacunoic acid and isolimonic acid) and two glucosides (limonin glucoside and deacetyl nomilinic acid glucoside) were chosen for the study. All the standards used in this study were purified and identified as described in chapter II and previously published papers (277, 374-375). The interest in the selected limonoids stems from their biological activity (374-375). In addition, these compounds have been reported from the sour orange, grapefruit, orange and tangerines

(142, 158). Hence, fruits and seeds from these citrus varieties were used in the present study.

In order to separate the limonoids, a C_{18} reversed phase column (Phenomenex Gemini) of 5µ particle size (25 × 0.5mm) with a flow rate of 1.0 mL min^{-1} was used. A binary gradient mobile phase comprising of 3 mM phosphoric acid and acetonitrile was used. The glucosides were eluted before aglycones with limonin glucoside being the first at a retention time of 12.73 min followed by deacetyl nomilinic acid glucoside at 13.67 min (Fig. 3.2). Elution order of aglycones was isolimonic acid> ichangin> isoobacunoic acid> limonin> nomilin with retention time of 24.19, 32.69, 35.20, 37.22 and 41.15 min respectively (Fig. 3.2).

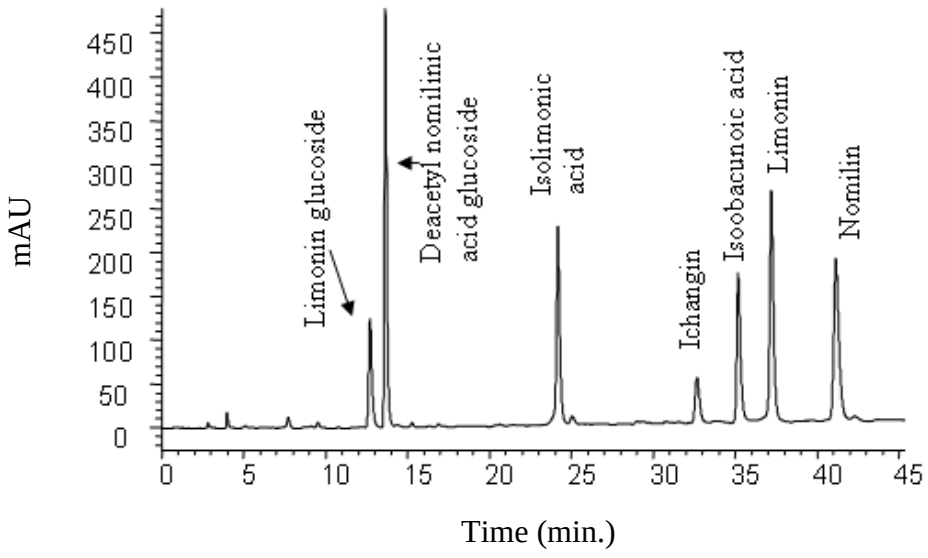

Fig. 3.2. Chromatogram of standard limonoids separated simultaneously obtained by high performance liquid chromatography, using C-18 column at 210 nm

In the present investigation all the tested compounds were resolved efficiently. The combination of Phenomenex Gemini C_{18} reversed phase column with 3mM phosphoric acid/acetonitrile and the unique gradient imparted the ability to analyze the limonoid aglycones and glucosides simultaneously. Rouseff and Fisher (309) reported low efficiency of resolution of limonoid aglycones on the reversed phase column. However, a very good separation of limonin and nomilin with retention times of 15.1 and 26.3 min respectively, was observed on a reversed phase column by Ohta et al (259). In present investigation, all of the tested aglycones were resolved very efficiently with limonin and nomilin eluting at an interval of about 4 min *viz.* at 37.22 and 41.15 min respectively, while other compounds were also resolved efficiently.

Citrus seeds are rich in limonoid aglycones as well as glucosides and considered to be very good source for the limonoids as compared to whole fruit. The limonoids profile in seeds of four different citrus varieties was analyzed using the developed HPLC method. Fig. 3.3 depicts the HPLC chromatograms of four varieties of citrus seeds. The individual peaks were identified by their retention time and spiking with the corresponding standards. The sour orange showed peaks corresponding to two limonoid aglycones (limonin and isoobacunoic acid) and two glucosides (limonin glucoside and deacetyl nomilinic acid glucoside). Limonin was found in significant amount in all four varieties of seeds. The isolimonic acid was present in significant amount in Nova tangerine and Cleopatra mandarin seeds whereas corresponding peak was absent in Rio Red grapefruit (Fig. 3.3; Table 3.2). Similar observation has been reported by Hasegawa et al (142) and Ozaki et al (265). Limonin was found to be the highest concentration

followed by nomilin in three varieties except sour orange seeds (Fig. 3.3). Ohta et al. (259) reported a similar observation in seeds of Shikuwasha (*Citrus depressa* hayata) variety with limonin being the predominant limonoid aglycone. Limonin concentration in the Rio Red grapefruit at 616.6 mg/100g seed was highest among the samples studied (Table 3.2).

TABLE 3.2 Concentration of limonoid agylcones and glucosides quantified simultaneously in citrus seeds (mg 100 g^{-1}). Results are expressed as mean ± SD of three experiments consisting of three individual analyses

varieties	Limonin	Nomilin	Isolimonic acid	Isoobacunoic acid	Limonin glucoside	Deacetyl nomilinic acid glucoside
Sour orange	116.73 ± 7.96	-	-	32.71 ± 2.9	85.95 ±3.06	76.27 ± 3.98
Cleopatra mandarin	211.87 ± 1.71	59.38 ± 0.39	6.25 ± 0.25	-	-	-
Nova tangerine	282.17 ± 2.00	35.33 ± 1.22	32.96 ± 0.54	-	-	-
Rio Red grapefruit	616.60 ± 0.73	41.81 ± 0.27	-	-	-	-

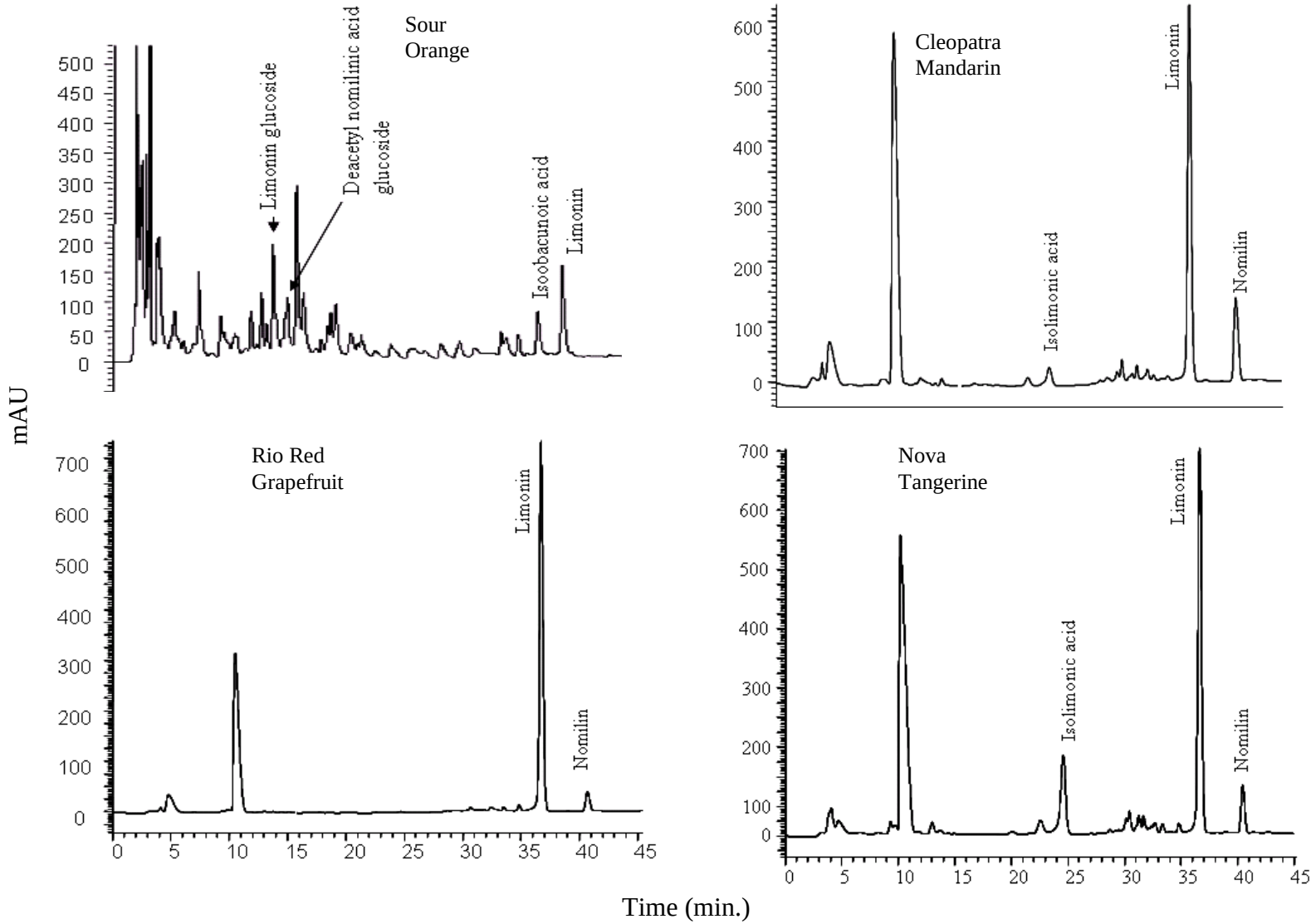

Fig. 3.3 Chromatograms of four varieties of citrus seed extracts obtained by high performance liquid chromatography using C_{18} column at 210 nm

Limonoids content in fruits of the four citrus species such as rio red grapefruit, navel oranges and tangerines were analyzed. HPLC chromatograms of four varieties of citrus fruits are presented in Fig. 3.4. Limonin 17-β-D glucopyranoside was found in all four varieties of citrus fruits, whereas deacetyl nomilinic acid 17-β-D glucopyranoside was observed in three varieties except valencia oranges (Table 3.3; Fig. 3.4). Limonin glucoside was present in higher concentration than deacetyl nomilinic acid glucoside in three varieties rio red grapefruit, navel orange and tangerines. Similar type of observation was reported earlier (265). In valencia oranges - limonin, ichangin and limonin glucoside were detected and quantified with limonin as the major component (Table 3.3; Fig. 3.4). Fong et al (108) has reported similar values for valencia orange fruit samples. Rio red and tangerines showed a peak at 24.6 min, which was found to be different than isolimonic acid upon spiking with the standard. Taken together, simultaneous separation and quantification of limonoid aglycones and glucosides was demonstrated in case of sour orange seeds and valencia orange fruit samples. The aglycones were not detected in three of the samples, which may be due to seasonal variation as aglycones content in fruits reduces with the advancement of maturity (149).

Most of the earlier methods utilized C_{18} bonded silica column with different solvent mixtures of acetonitrile, methanol, terahydrofuran and water as eluents for aglycone analysis (227). In our method, the binary solvent system used, imparted the ability to manipulate gradient mixtures to maximize the separation of limonoids. Separation of limonoid aglycones and glucosides was evaluated using a range of gradient systems. Since limonoid aglycones are relatively less polar compared to glucosides, a wide range of combinations of solvents were investigated. Most of the combinations

tested were efficient to resolve neutral and acidic limonoid aglycones but provided moderate resolution with limonoid glucosides owing to the very similar polarity. Limonin glucoside and deacetyl nomilinic acid glucoside had a tendency to elute together. Previous reports also had the problem of poor peak resolution of glucosides (158, 259).

TABLE 3.3 : Concentration of limonoid agylcones and glucosides quantified simultaneously in citrus fruits (mg 100 g^{-1}). Results are expressed as mean ± SD of three experiments consisting of three individual analyses

Varieties	Limonin	Ichangin	Limonin glucoside	Deacetyl nomilinic acid glucoside
Valencia orange	7.22 ± 0.34	0.32 ± 0.01	0.31 ± 0.02	-
Rio Red grapefruit	-	-	653..02 ± 7.73	160.88 ± 7.11
Navel Orange	-	-	785.44 ± 10.61	213.12 ± 12.78
Tangerine	-	-	573.11 ± 12.47	278.96 ± 8.19

In this experiment, we were able to resolve the limonoid glucosides with the solvent combination comprising of 3 mM phosphoric acid and acetonitrile coupled with a unique gradient system. It was observed that replacement of water with 3 mM phosphoric acid improves the resolution of aglycones besides imparting the ability to provide simultaneous separation. Earlier reported methods had problems of irregular baseline and poor resolution (1, 106-107, 228, 259, 309, 326-328, 367, 385). In the present study, it was found that the peaks were shifting depending upon the slope of the

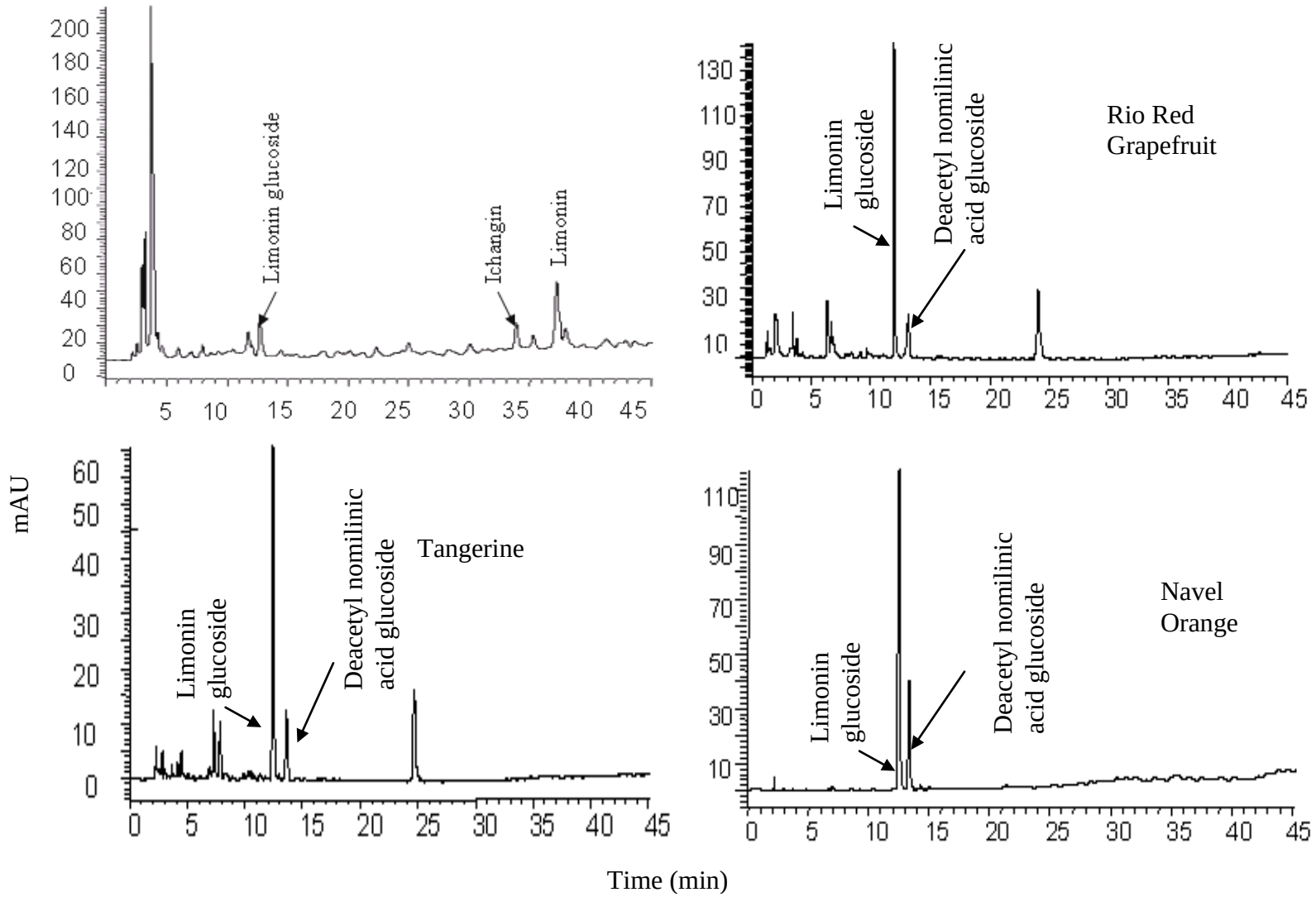

FIG 3.4. Chromatograms of four varieties of citrus fruit extracts obtained by high performance liquid chromatography using C_{18} column at 210 nm

gradient. After analyzing many gradient systems by their resolving capacity, we chose one gradient system with better baseline separation in the limonoid glucosides and aglycones. Thus after many investigations, an analytical procedure with optimal and reproducible separation was obtained.

To check the linearity of relationship between limonoid concentration and the peak area with the developed gradient system, six different concentrations *viz.* 0.5, 1, 2, 4, 6, and 8 µg of each compound was injected in triplicate in the HPLC column. The correlation coefficients for seven limonoids were found to be >0.99 (Table 3.4). Regression equations for limonin glucoside, deacetyl nomilinic acid glucoside, isolimonic acid, ichangin, isoobacunoic acid, limonin, and nomilin are presented in Table 3.4. In order to validate specificity of the method 95% confidence intervals for intercept for all compounds were calculated. It was found that the intercepts are not statistically different from zero (Table 3.4). The gradient system and Gemini column used in the present study was found to be highly precise and reproducible for seven limonoids tested. The method was linear in the range of 0.5 - 8 µg for all the limonoids.

The limit of detection for each limonoid was detected by injecting each compound in various concentrations in triplicate. The lower limit for detection was found to be 0.25 µg for isoobacunoic acid and isolimonic acid, while 0.50 µg was observed for limonin glucoside, deacetyl nomilinic acid glucoside, ichangin, limonin and nomilin.

TABLE 3.4: Regression equations and confidence intervals for intercepts of limonoids

Compound	Regression equations β (± SD) X + C (±SD)	95 % Confidence Interval for Intercept		R^2
		Lower limit	Upper Limit	
Limonin	733.76 (± 5.24) X - 60.07 (± 23.56)	-123.680	23.794	0.9999
Nomilin	697.76 (± 3.31) X - 38.39 (± 14.89)	-65.770	2.642	0.9998
Ichangin	808.68 (± 2.77) X -18.83 (± 12.47)	-69.110	16.238	0.9998
Isoobacunoic acid	1064.5 (± 3.89) X + 22.088 (± 17.48)	-14.297	88.397	0.9998
Isolimonic acid	1775.8 (± 5.39) X - 66.13 (± 24.21)	-95.140	7.516	0.9999
Limonin glucoside	394.38(± 3.96) X - 18.74 (± 17.80)	-63.912	11.557	0.9977
Deacetyl nomilinic acid glucoside	559.21(± 4.23) X+ 31.52 (± 19.00)	-26.070	54.498	0.9994

X= Concentration of the respective limonoids in micrograms

Previous authors have reported separation of either limonoid aglycones or the limonoid glucosides (106-107, 259, 326-328, 367, 386). The developed method was found to be powerful even for the separation of acidic and neutral limonoid aglycones simultaneously as compared to a more recent normal phase HPLC method using a linear gradient mixture of cyclohexane and tetrahydrofuran (228). The present analytical procedure was very useful in separating the acidic limonoids (isoobacunoic acid and isolimonic acid), neutral limonoids (limonin, nomilin and ichangin) and limonoid glucosides (limonin glucoside and deacetyl nomilinic acid glucoside) simultaneously.

The present reversed phase HPLC method is simple and accurate for the determination of limonoid aglycones and limonoid glucosides simultaneously in citrus fruits and seeds. It is possible that faster screening of samples may reduce cost of analysis by reducing the analysis time and solvent consumption. In addition, this method showed higher reproducibility and reliability in the separation of neutral, acidic aglycones and limonoid glucosides. With the increasing importance of citrus limonoids as health promoting agents, this method can be used for the demonstration of routine analysis of large number of citrus samples simultaneously for aglycones and glucosides.

CHAPTER IV

INTERFERENCE OF *VIBRIO HARVEYI* CELL-CELL SIGNALING AND BIOFILM FORMATION BY LIMONOIDS*

4.1 Synopsis

Citrus limonoids are unique secondary metabolites, characterized by triterpenoids skeleton with furan ring. Several studies have demonstrated health beneficial properties of limonoids. In addition, certain citrus limonoids play a role in plant defense against insect-pests. In the present study, five limonoids were purified from sour orange and evaluated for their ability to inhibit cell-cell signaling. The purified limonoids were tested for their ability to interfere with cell-cell signaling and biofilm formation in *Vibrio harveyi*. Isolimonic acid, deacetylnomilinic acid glucoside and ichangin demonstrated significant inhibition of autoinducer mediated cell-cell signaling and biofilm formation. Furthermore, isolimonic acid and ichangin treatment resulted in induced expression of response regulator *luxO*. In addition, *luxR* promoter activity was not affected by isolimonic acid and ichangin. Therefore, the ability of isolimonic acid and ichangin to interfere with cell-cell signaling and biofilm seems to stem from modulating *luxO*

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expression. The results of the study suggest that isolimonic acid and ichangin are potent modulators of bacterial cell-cell signaling.

4.2 Introduction

Terpenoids are the largest group of plant secondary metabolites, and are suggested to act in diverse array of plant physiological processes including plant defense (129, 197). Several triterpenoids such as betulinic acid possess potent antimicrobial activity (70). A unique class of triterpenoids, termed limonoids, is present in *Citrus* species. Early research focused on the antifeedant activity of citrus limonoids (38). However, in recent years these secondary metabolites are intensively investigated for their potential health benefits. Several reports from our lab (277, 362, 368) and by others (135, 242, 355) suggest a potential of reducing risks of various cancers using citrus limonoids. Limonoids are postulated to exert their effect by induction of GST enzyme activity (195, 268), apoptosis (277), antioxidant activity (400) and suppression of Cox2/iNOS (368).

Limonoids are distributed throughout the plant including leaves, branches and fruit (235), suggesting a possible defense function of these secondary metabolites in plants (234). Concurrent with this hypothesis, antifeedant activity of citrus limonoids was reported by several studies (58, 313). It was suggested that increasing oxidation and skeletal rearrangement is associated with increased activity against insects. However, structurally simple limonoids present in family Meliaceae were more potent insect-repellents compared to complex citrus limonoids (58). Furthermore, limonoids isolated from neem tree (*Azadirachta indica*, family Meliaceae) also demonstrated antibacterial

activity (13). On the contrary, reports elucidating impact of citrus limonoids on microoraganisms are relatively limited. One study demonstrated the impact of limonin and nomilin on HIV-1 replication (26). Furthermore, it is suggested that citrus limonoids did not inhibit bacterial growth (58).

Elucidation of novel targets for drug development provides an opportunity to screen compounds, which otherwise do not possess growth inhibitory property. A recently identified target is bacterial cell-cell signaling (also termed as quorum sensing). Quorum sensing has been defined as the coordinated gene expression in response to cell-density (23). Bioluminescence production in *V. harveyi* is a quorum sensing controlled process and the various events in the signaling pathway has been well elucidated. The bioluminescence production in *V. harveyi* is controlled by three coincidence detectors which are part of typical two-component pathway. The three coincidence detectors LuxN, LuxPQ and CqsS converge on the phosphorelay protein LuxU which in turn activates response regulator LuxO. LuxO activates the transcription of small antisense RNAs which regulate the production of luminescence by regulating master regulator LuxR. Cell-cell signaling regulates various pathogenic processes in several bacteria (101, 333, 377). It is postulated that interference with bacterial cell-cell signaling may attenuate the bacterial pathogenicity (293). Consistent with this hypothesis, several synthetic and natural products were demonstrated to interfere with cell-cell signaling and consequently impact bacterial pathogenicity (157, 225, 271, 295). Furthermore, plant secondary metabolites such as halogenated furanones (132), polyphenols (165) and

organosulfur compounds from garlic (271) were reported to interfere with cell-cell signaling.

Several phytochemicals are reported to interfere with cell-cell signaling (165, 225, 271) and biofilm formation (165, 302-303). Citrus limonoids are characterized by high degree of oxygenation and presence of a furan ring. In comparison, autoinducer molecules HAI-1, AI-2 are furanones. These observations led us to investigate antagonistic activities of grapefruit limonoids against quorum sensing and regulated processes such as bioluminescence and biofilm formation. We were interested if certain unique limonoids present in sour orange posses the ability to interfere with bacterial cell-cell signaling. The present study provides the evidence that certain citrus limonoids are potent inhibitors of cell-cell signaling in the model organism *V. harveyi*.

4.3 Materials and Methods

4.3.1 Materials

All restriction enzymes and Deep Vent DNA polymerase was purchased from New England Biolabs, Inc. (Ipswich, MA).

4.3.2 Preparation of Limonoid Stock Solution

The limonoids purified from grapefruit and sour orange as described in chapter II were dissolved in DMSO at 20 mg ml^{-1} concentration and stored at -20^{o}C until further use.

4.3.3 Bacterial Strains and Media

V. harveyi strains BB170 (*luxN*::Tn5), BB886 (*luxPQ*::Tn5), BB120 (Wild type), JAF483 (*luxO* D47A), JAF553 (*luxU* H58A) and BNL258 (*hfq*::Tn5*lacZ*), JAF548

(*luxO* D47E, black) were kindly provided by B. L. Bassler (Princeton University, Princeton, N.J.) (111-112, 204, 346). *E. coli* #5, an environmental isolate (284) was used as a positive control for Autoinducer-2 (AI-2) activity. The autoinducer bioassay (AB) or Luria Marine (LM) medium were used to culture the *V. harveyi* strains (212, 346).

4.3.4 Plasmids

The molecular biology techniques were used as described (317). Cml^R gene was amplified from vector *pBAD33* and cloned into vector *pFZY1* by partial digestion with PstI, the resultant vector was termed as *pAV09*. Next, *luxR* promoter (+400 to -56) was cloned into *pAV09* resulting in plasmid *pAV10*. DNA primers for *luxR* promoter were designed with one base modification to generate *EcoR1* and *HindIII* restriction sites. The fragment was amplified by polymerase chain reaction using Deep Vent DNA polymerase and cloned in plasmid *pAV09* ahead of *lacZ* to generate plasmid *pAV10*. The *V. harveyi* BB120 was transformed with *pAV10* electroporation at 2100 V (317). The transformed colonies were selected and maintained on agar plates supplemented with 10 µg/ml Cml.

Furthermore, *luxU*, *luxO* and *hfq* were amplified with primers carrying restriction sites for KpnI, SalI (*luxU*), SmaI, PstI (*luxO*) and SacI, SalI (*hfq*). The amplified fragments were digested with respective pairs of restriction enzymes and cloned into *pBAD33*. The new plasmids were termed *pAV05* (*luxU*), *pAV06* (*hfq*) and *pAV07* (*luxO*). The plasmids were maintained in *E. coli* DH10B and electroporated in *V. harveyi* JAF553 (*luxU*), JAF483 (*luxO*) and BNL258 (*hfq*). The resulting strains were then called

as AVS40 (JAF483 + *pAV07*), AVS41 (BNL258 + *pAV06*) and AVS42 (JAF553 + *pAV05*).

4.3.5 Growth and Metabolic Activity

The effect of limonoids purified from sour orange was measured in the following manner. Overnight culture of *V. harveyi* BB120 was diluted 100 fold in AB media and exposed to100 µg/ml limonoids or equivalent volume of DMSO. The cultures were grown for 16 hours and OD at 600 nm was measured every 15 min using the Synergy™ HT Multi-Mode Microplate Reader (BioTek, Instruments, Winooski, VT). The instrument was set to maintain a temperature of 30^{o}C and plates were constantly shaken at medium speed between readings. The data was presented as mean of three biological replicates. To independently verify the results from the optical density experiment, metabolic activity of *V. harveyi* in presence of limonoids was measured by AlamarBlue (Invitrogen Inc., Carlsbad, CA). Metabolic activity of *V. harveyi* was measured in similar fashion as growth curve with addition of 25 µl of AlamarBlue and absorption at 570 and 600 nm were monitored. Mean percent reduction of AlamarBlue (380) from three biological replicates was calculated and plotted against time.

The effect of limonoids purified from grapefruit on *V. harveyi* BB120 growth rate was measured by diluting the overnight culture (100 fold) in LM media and grown in presence of 100 µg/ml limonoids at 30^{o}C in an incubator. The OD_{600} was measured every 2 h upto 16 h using NanoDrop Spectrophotometer 1000 and presented as mean of three replicates.

4.3.6 Bioluminescence Assay

The bioluminescence assay was carried out as described previously (371). In brief, cell free culture supernatants (CFS) were prepared from *E. coli* #5 and *V. harveyi* BB120 by culturing in LB and LM media, respectively, to achieve high concentrations of autoinducer activity followed by centrifugation at 10,000 rpm for 10 min in a microcentrifuge. The supernatants were collected and filtered through 0.2 µm membrane filter and stored at -20^{o}C. Inhibition of autoinducer (harveyi autoinducer [HAI] and autoinducer-2 [AI-2]) mediated bioluminescence was measured in 96 well-plate assay (212). The final concentrations of citrus limonoids tested were 6.25, 12.5, 25, 50 and 100 µg ml^{-1}. Diluted (5000 fold) overnight cultures (90 µl) of reporter strains BB886 (for HAI) and BB170 (for AI-2) were incubated with 5 µl CFS, 0.5 µl of limonoids or DMSO, 4.5 µl sterile AB media at 30^{o}C; 100 rpm. Light production was measured by a Victor^{2} 1420 multilabel counter (Beckman Coulter, Fullerton, CA) in luminescence mode. The values were recorded as relative light units (RLU) and used in calculation (212).

Overnight cultures of strains JAF553, JAF483, BNL258, AVS40, AVS41 and AVS42 were diluted 5000 fold and treated with 100 µg/ml isolimonic acid, ichangin or DMSO (Control). The 100 µl of each was placed in 96 well plate and grown for 4 h at 30^{o}C, 100 rpm. Luminescence was measured as described above. The relative light units from 3 biological replicates were presented as mean ± SD. In addition, OD_{600} values in presence of DMSO, isolimonic acid and ichangin for strains JAF553 (0.38 ±0.1, 0.36 ±0.1 and 0.38 ±0.07), JAF483 (0.33 ±0.07, 0.32 ±0.03 and 0.35 ±0.02), BNL258 (0.27

±0.11, 0.26 ±0.04 and 0.28 ±0.03), AVS40 (0.24 ±0.05, 0.25 ±0.02 and 0.24 ±0.18), AVS41 (0.2 ±0.08, 0.21 ±0.04 and 0.2 ±0.02) and AVS42 (0.28 ±0.07, 0.27 ±0.13 and 0.28 ±0.05) at the time of measurement of luminescence were recorded.

4.3.7 Biofilm Assay

Biofilm assay was conducted as described previously (371). Overnight culture of *V. harveyi BB120* was diluted1:50 in LM media and 190 µl of this fresh culture was incubated with 9 µl sterile media and 1µl DMSO or 6.25, 12.5, 50, or 100 µg/ml of limonoids dissolved in DMSO. *V. harveyi* JAF548 was used as negative control for the assay. The culture was placed in polystyrene 96-well plates (Fischer Scientific, Pittsburg, PA, USA) and incubated at 26°C for 24 hours without shaking. Total biofilm mass was quantified by washing with phosphate buffer (0.1 M, pH 7.4), followed by staining with 0.3% crystal violet (Fisher, Hanover Park, IL) for 20 min. The dye associated with biofilm was dissolved with 200 µl of 33% acetic acid, and measured at 570 nm. Mean ±SD of three biological replicates are presented.

4.3.8 RNA Extraction and Relative Quantification of Transcripts

Overnight culture of *V. harveyi* BB120 was diluted 100 fold with fresh AB medium, treated with 100 µg/ml of isolimonic acid, 100 µg/ml of ichangin or equivalent volume of DMSO and grown further at 30°C and 200 rpm for 4 h. Bacterial cells were collected by centrifugation at 5000g for 10 min. RNA was purified using the RNeasy minikit (Qiagen Inc.) and used as template for cDNA synthesis as described previously (371). In case of quantification of *qrr1*, the RNA was extracted using TRIZOL (Invitrogen Inc.) according to manufacturer's instructions. For quantification, 25 ng

cDNA was amplified with 10 pmol of primers and 10 µl of SYBR green (Applied Biosystems, Foster City, CA) for 40 amplification cycles (371). All the measurements were done on three biological replicates. Amplification of target sequences was done on ABI-Prism 7000 HT (Applied Biosystems, Foster City, CA). Relative quantification of target genes was calculated by $2^{(-\Delta\Delta Ct)}$ (210) and expressed as fold change ±SD. Primers (Table 4.1) were designed by Primer3 software (312).

TABLE 4.1: List of *V. harveyi* primers

Gene Name		Sequence 5'-3'
Real Time PCR		
hfq	Forward	ATGGCTAAGGGGCAATCTCT
	Reverse	ACTGTGGTGGCTCACTGGAC
luxO	Forward	GGCACTTTACCGCTCTTACC
	Reverse	GTCTGGGTGGCTTTTCTTCA
luxP	Forward	GATTTGACCACGCAGAAGG
	Reverse	CTTTCGCAGCATCATAGCC
luxQ	Forward	CGTTCACTCGGTTGTCTTCC
	Reverse	GGGCTTGGTCTTGCGATT
luxR	Forward	GTCAATGCCCTCAACCAAGT
	Reverse	TCTTCACGCCCAGTTTACG
luxS	Forward	AAAAGGAGACACCATCACGG
	Reverse	TGCTGCTCTGAAGGCGTT
qrr1	Forward	CCCCTCGGGTCACCTATCCAACT
	Reverse	GGCAGTCGGATCTATTGGCTCGTTC
rpoA	Forward	CACAGGGCCCGAGCCTGATTT
	Reverse	ACCACAGGGCCCGAGCCTG
Cloning		
luxR	Forward	CGAAGCCAAACTGCAAGAATTCGTTCTTCTGCA
	Reverse	TGTACCGATTGGAACAAGCTTGCGGAACAG
luxU	Forward	GCAAAATTCATGGTACCTGACATTATGACGG
	Reverse	TTACTGTCTAACTAGTCGACAAGCATTGAG
luxO	Forward	ACATCTAATGCCCGGGTGATTATGTCTACGA
	Reverse	ATCGCTACCAATTTCTGCAGACAGTTCTTCAA
hfq	Forward	AGACAGATGTGGGAGCTCTTAGATGGGGA
	Reverse	TGTGGGGATTGTCGACTGCCAGTCACAA
Cml	Forward	ATGCCATAGCATTTTTATCC
	Reverse	GATTTAATCTGTATCAGG

4.3.9 β-Galactosidase Assay

Overnight culture of *V. harveyi* BB120 containing plasmid pAV10 was diluted 100 fold in AB media. The diluted culture was treated with 100 µg/ml of isolimonic acid, ichangin or equivalent volume of DMSO and grown for 4 h at 30°C and 200 rpm. β-Galactosidase assay was performed as described previously (243).

4.3.10 Statistical Analysis

AI activity was measured as a relative AI activity. The inhibition of AI activity was calculated from the formula 100- [(relative AI activity/relative activity of positive control) x 100] (212, 371) and expressed as percentage and SD values. The percent inhibition of biofilm formation was calculated as 100- [(OD_{620} of sample well/ OD_{620} of positive control) x 100] and expressed as percentage and SD values.

The effects of different limonoids for each activity were analyzed with an analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test on SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The effect was considered significant at $p < 0.05$. The percent inhibition and Log_{10} (Concentration in µM) data from AHL and AI-2 and biofilm assays were fitted to a 4-parameter sigmoid model y= y0+a/(1+exp(-(x-x0)/b)) using SIGMAPLOT 11.0 (Systat Software, Inc.). The data for *V. harveyi* biofilm was fitted to a 3-parameter sigmoid models y= a/(1+exp(-(x-x0)/b)). The IC_{90} and IC_{50} values were calculated from the model.

4.4 Results

4.4.1 Effect of Citrus Limonoids on Cell Growth and Viability

Cell growth was measured by monitoring optical density at 600 nm for 16 h. Limonoids did not affect growth of *V. harveyi* at 100 µg/ml (Fig. 4.1A and C). In order to further validate the findings, cell viability of *V. harveyi* in presence of 100 µg/ml limonoids, was monitored for 16 h using alamar blue. The results indicate no impact on the cell metabolic activity by limonoids (Fig. 4.1B).

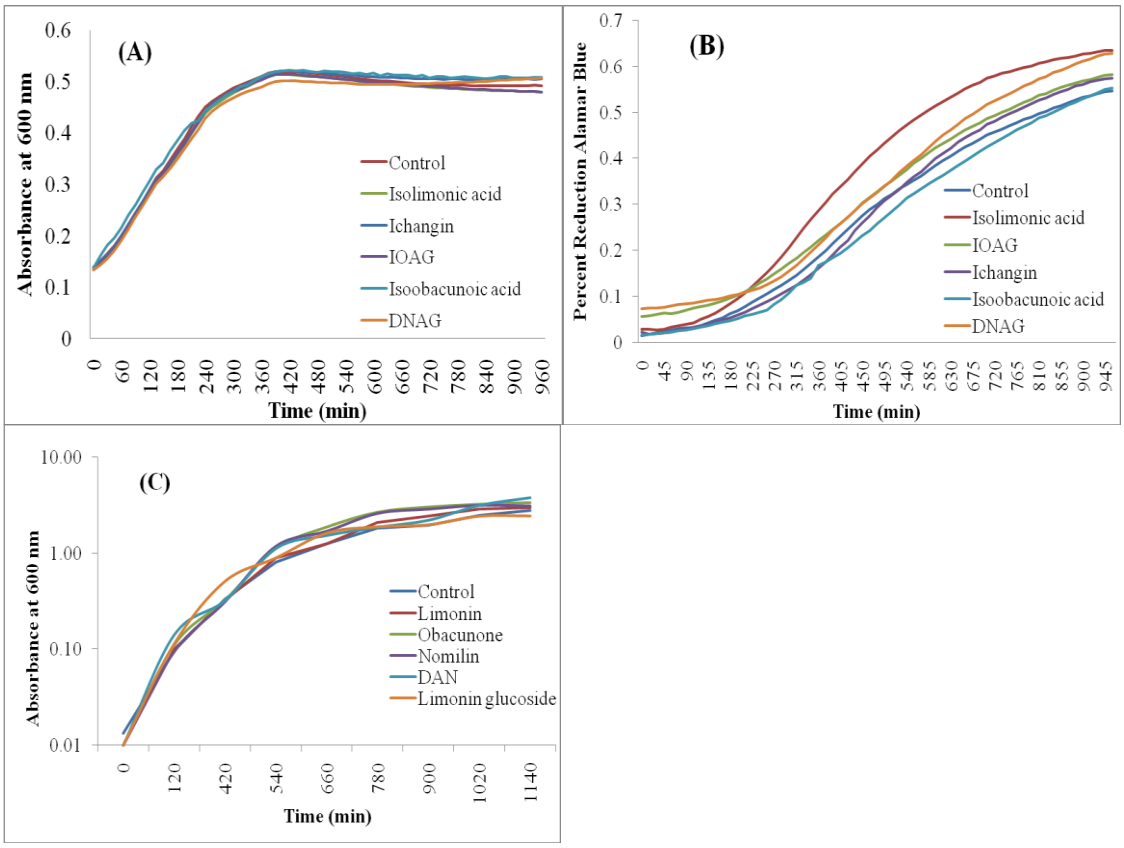


FIG. 4.1. Effect of 100 µg/ml limonoids on *Vibrio harveyi* growth curve and metabolic activity. (A) and (C) Growth curves of *Vibrio harveyi* (B) metabolic activity of *V. harveyi* as measured by AlamarBlue reduction

4.4.2 Inhibition of HAI and AI-2 Mediated Bioluminescence by Citrus Limonoids

Bioluminescence produced by *V. harveyi* mutant strains was utilized as readout for the HAI and AI-2 mediated cell-cell signaling (22). All the tested compounds inhibited HAI and AI-2 mediated bioluminescence in concentration dependent manner (Fig. 4.2). Four of the ten tested limonoids - isolimonic acid, ichangin, nomilin and DNAG demonstrated greater than 90 % inhibition of AI-2 activity at 100 µg/ml, while obacunone depicted inhibition in the range of 73-87 % therefore; IC_{90} or IC_{75} values for AI-2 inhibitory activity were compared. In contrast, IC75 or IC50, values were compared for HAI activity. Isolimonic acid was the most potent inhibitor of HAI and AI-2 activity (Fig. 4.2B and 4.3) among the tested limonoids and inhibited 99.23 % AI-2 mediated bioluminescence at 100 µg/ml (Fig. 4.2B). IC_{90} values for isolimonic acid, DNAG and ichangin for AI-2 activity were 38.90 µM, 123.03 µM and 194.98 µM, respectively (Fig. 4.3B). IC_{50} value of isolimonic acid, DNAG and ichangin against HAI activity were 148.59 µM, 197.70 µM and 79.43 µM, respectively (Fig. 4.3A). Isoobacunoic acid (Fig 4.2C) depicted the lowest IC_{50} value of 65.01 µM against HAI activity; however, the 4-parameter sigmaoid plot demonstrated that the activity of isoobacunoic acid was saturated at ≥100 µM (Fig. 4.3A). Ichangin (Fig. 4.2A) depicted more linear response against HAI inhibition, while inhibited the AI-2 activity by 9.63 to 91.58 % (Fig. 4.2A).

Obacunone and nomilin were compared using IC_{75} because obacunone demonstrated > 70% inhibition in autoinducer bioassays in the tested concentration range. Obacunone was the more effective inhibitor of AHL and AI-2 induced

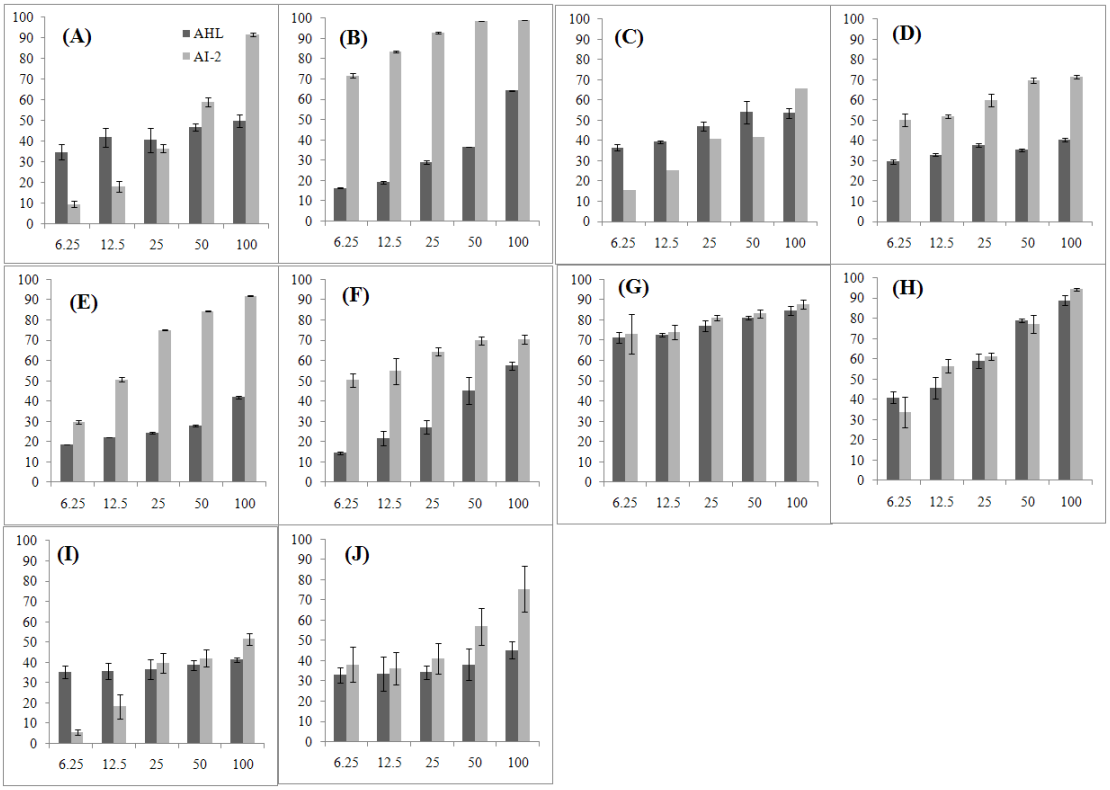
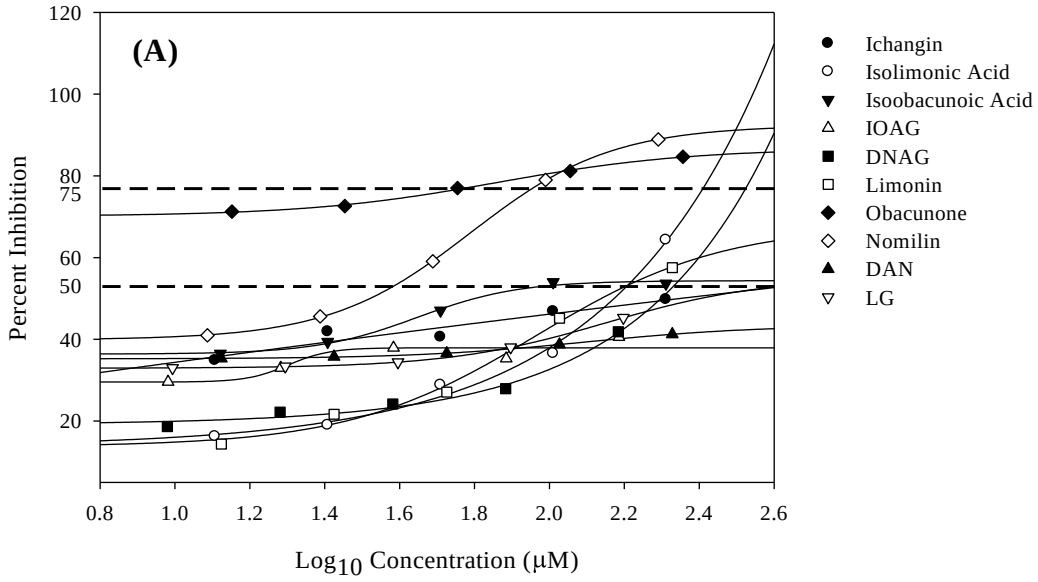


FIG. 4.2. HAI-1 and AI-2 induced bioluminescence in *V. harveyi* BB886 and BB170. (A) ichangin, (B) isolimonic acid, (C) isoobacunoic acid, (D) isoobacunoic acid 17β-D-glucopyranoside (IOAG) and (E) deacetyl nomilinic acid 17β-D-glucopyranoside (DNAG), (F) limonin, (G) obacunone, (H) nomilin, (I) deacetylnomilin and (J) limonin 17β-D-glucopyranoside. The relative light units for each strain were normalized by dividing with the negative control (see text). The data is presented as percent inhibition of bioluminescence ± SD



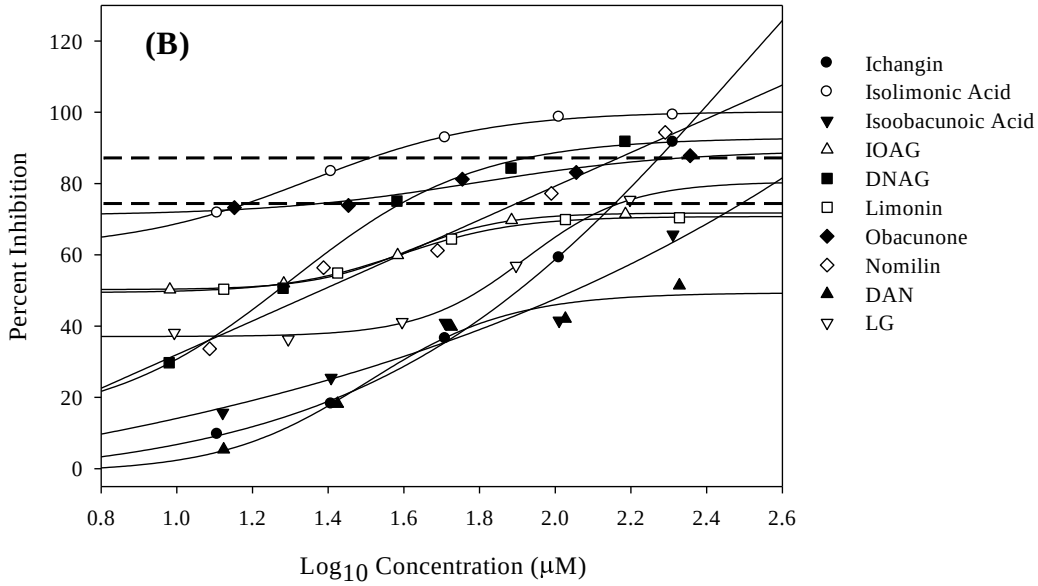


FIG. 4.3. Four-parameter sigmoidal models of bioluminescence in presence of limonoids. (A) HAI-1 and (B) AI-2

bioluminescence compared to nomilin (FIG. 4.2G) with IC_{75} values of 42.66 and 28.18 μM (FIG. 4.3 A,B), demonstrating 71.25-84.67 % and 73.24-87.81 % inhibition of AHL and AI-2 mediated bioluminescence, respectively (FIG. 4.2G). In comparison, nomilin inhibited 40.97-88.91% and 33.63-94.35% (Fig. 4.2H) with IC_{75} values calculated as 83.18 and 81.28 μM for AHL and AI-2 mediated bioluminescence, respectively (FIG. 4.3 A,B).

For limonin and limonin glucoside, IC_{50} values were compared as IC_{75} values could not be obtained in the tested concentration range. The IC_{50} values for limonin and limonin glucoside against AHL activity were 138.04 and 223.87 μM respectively. Since, DAN demonstrated <50 % inhibition in the autoinducer bioassays, IC_{50} value for DAN was not calculated. In order to draw conclusion, inhibition percentage and nonlinear regression curves were compared. Limonin, deacetyl nomilin and limonin glucoside (FIG. 4.2 I,F,J) were relatively less effective in inhibiting the autoinducer mediated bioluminescence. Limonin and DAN showed poor antagonistic activity against AI-2, demarcated by distinct plateau at low concentrations. The IC_{50} values for limonin and limonin glucoside were 11.48 and 61.66 μM respectively. Collectively, these results suggest significant ($p< 0.05$) inhibitory activity by isolimonic acid, ichangin, obacunone, nomilin and DNAG against cell-cell communication in *V. harveyi* model.

4.4.3 Inhibition of *V. harveyi* Biofilm

Quorum sensing is suggested to positively regulate the biofilm formation in *V. harveyi* (40). Since citrus limonoids demonstrated inhibition the quorum sensing regulatory system, influence on *V. harveyi* biofilm was further investigated. All the

tested limonoids demonstrated concentration dependent inhibition of *V. harveyi* biofilm formation (FIG. 4.4). The inhibitory activities of various limonoids significantly differed from each other ($p<0.05$) for *V. harveyi* biofilm. IC_{50} and IC_{25} values were calculated from nonlinear regression models. Isolimonic acid (IC_{50} = 94.18 μM) and obacunone (IC_{50} = 91.2 μM) were the most effective inhibitor of *V. harveyi* biofilm (Fig. 4.4 and 4.5) with. The IC_{50} values for ichangin, isoobacunoic acid, DNAG, IOAG, limonin, LG and DAN could not be calculated, as these limonoids demonstrated ≤ 50 % biofilm inhibition under experimental conditions. Moreover, ichangin, isoobacunoic acid and DNAG demonstrated similar level of inhibitory effect on *V. harveyi* BB120 biofilm, whereas IOAG was the least effective for inhibiting the *V. harveyi* biofilm (Fig. 4.4 and 4.5). The, limonin, limonin glucoside and DAN were compared using IC_{25} value, as these limonoids showed <50 % inhibition. In comparison, QS-negative strain JAF548 formed very low amount of biofilm.

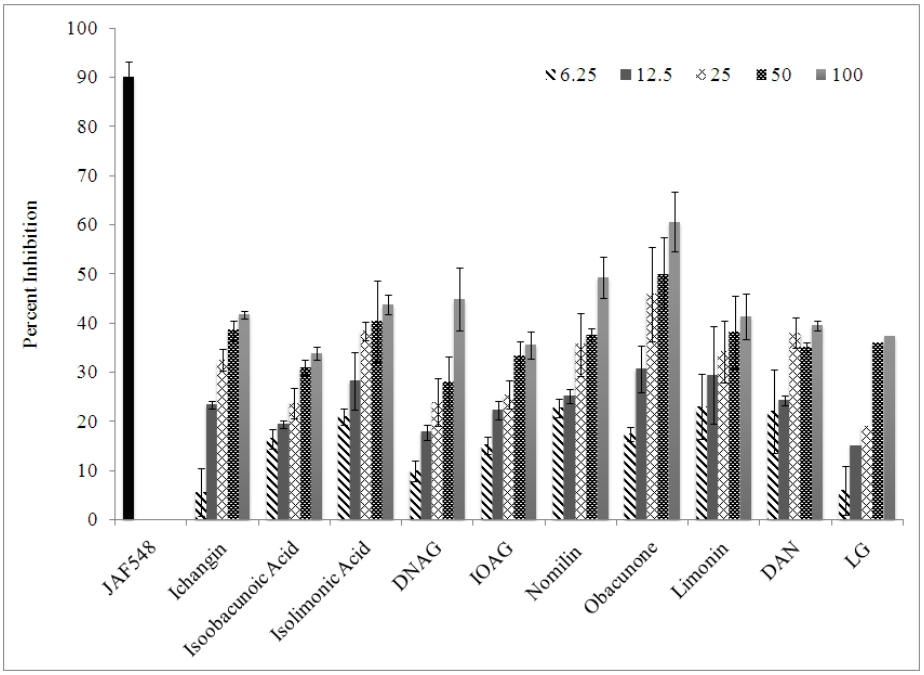


Fig. 4.4. Inhibitory activity of limonoids against *V. harveyi* BB120 biofilm. The *V. harveyi* biofilm was measured in presence of 6.25, 12.5, 25, 50 and 100 µg/ml of limonoids.The data is presented as mean ± SD

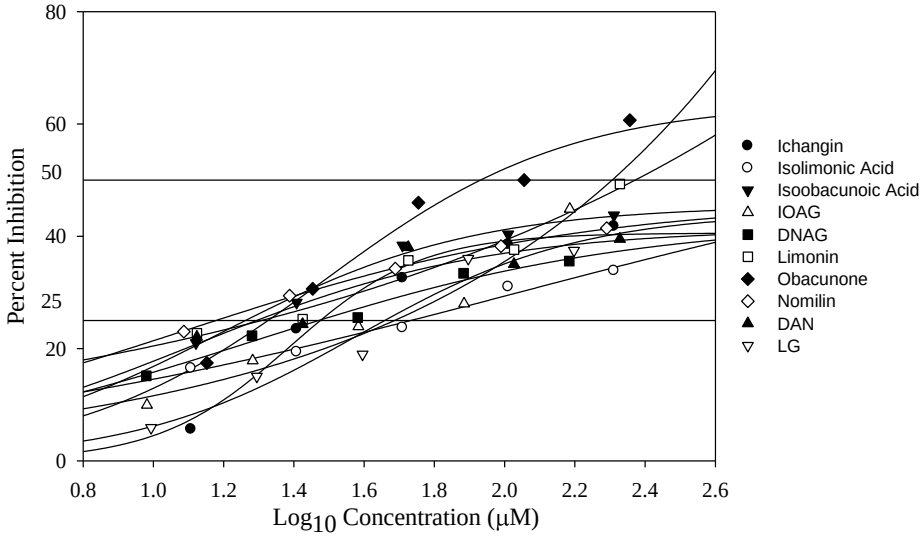


Figure 4.5: Four parameter sigmoidal model for *V. harveyi* BB120 biofilm inhibition by limonoids

4.4.4 Effect of Limonoids on Constitutively Luminescent *V. harveyi* Mutants

Because all the tested limonoids demonstrated inhibitory activity in HAI and AI-2 under experimental conditions, it is possible that these limonoids are non-specific inhibitors of cell-cell signaling. To test the hypothesis, effects of the limonoids was investigated in constitutively bioluminescent mutants JAF553, JAF483 and BNL258 (111-112). Based on the potency in bioluminescence assay and structural features, isolimonic acid and ichangin were selected for further studies. The strains JAF553 and JAF483 carry point mutations in *luxU* and *luxO*, rendering them defective in phosphorelay (111-112), while BNL258 contains a Tn5 insertion in *hfq* gene rendering a non-functional Hfq protein (204). We reasoned that a diminished luminescence in these strains will indicate interference with signal transduction downstream of non-functional gene. Interestingly, isolimonic acid treatment enhanced the luminescence production in LuxU (6.46 %) and LuxO (64.63 %) mutants significantly, but did not affect luminescence production in *hfq* mutant (Fig. 4.6). Similarly, ichangin application induced the bioluminescence in JAF483 (17.64 %), but reduced the luminescence in LuxU and *hfq* mutants (Fig. 4.6). Furthermore, to determine if the increase in the luminescence production can be negated by expression of functional copy of mutant or disrupted gene, the *luxO*, *luxU* and *hfq* were expressed in-trans under the control of arabinose promoter. Expression from the plasmid resulted in decreased levels of luminescence production in the transformed strains. The bioluminescence levels for isolimonic acid or ichangin treated strains were not significantly different from control.

The results suggest that isolimonic acid and ichangin are likely to affect the signal transduction pathway at or downstream of LuxO but upstream of Hfq (Fig. 4.6).

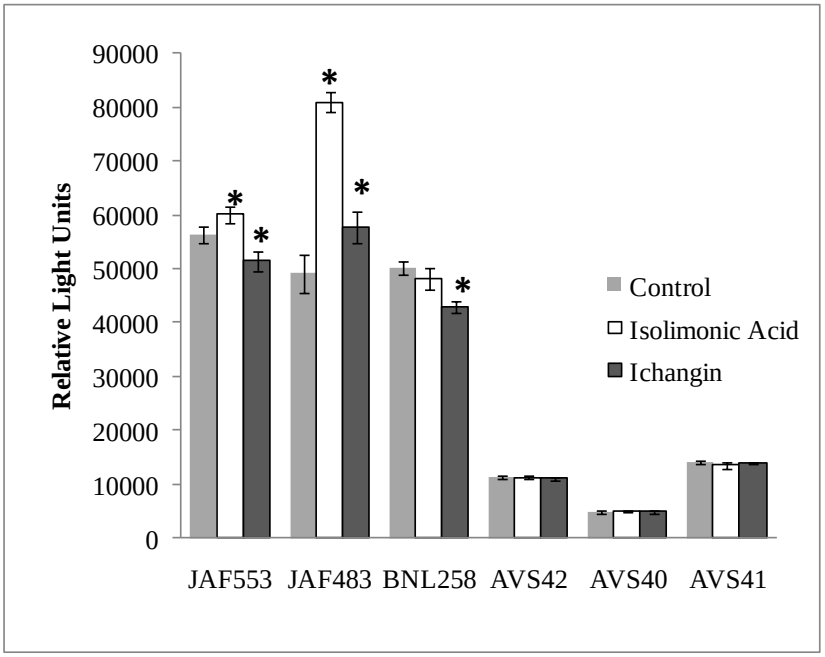


FIG. 4.6. Impact of isolimonic acid and ichangin (100 µg/ml) on bioluminescence of *Vibrio harveyi* mutants JAF553 (*luxU* H58A), JAF483 (*luxO* D47A) BNL258 (*hfq*::Tn5lacZ), AVS40 (JAF483 complemented with *luxO*), AVS41(BNL258 complemented with *hfq*) and AVS42 (JAF553 complemented with *luxU*)

4.4.5 Expression Analysis of Signal Transduction Pathway

To further understand the enhanced bioluminescence observed in *luxO* and *luxU* mutants by isolimonic acid and ichangin, expression of *luxP, luxQ, luxO, hfq*, *luxR* and *luxS* was investigated in *V. harveyi* strain BB120 using qRT-PCR.

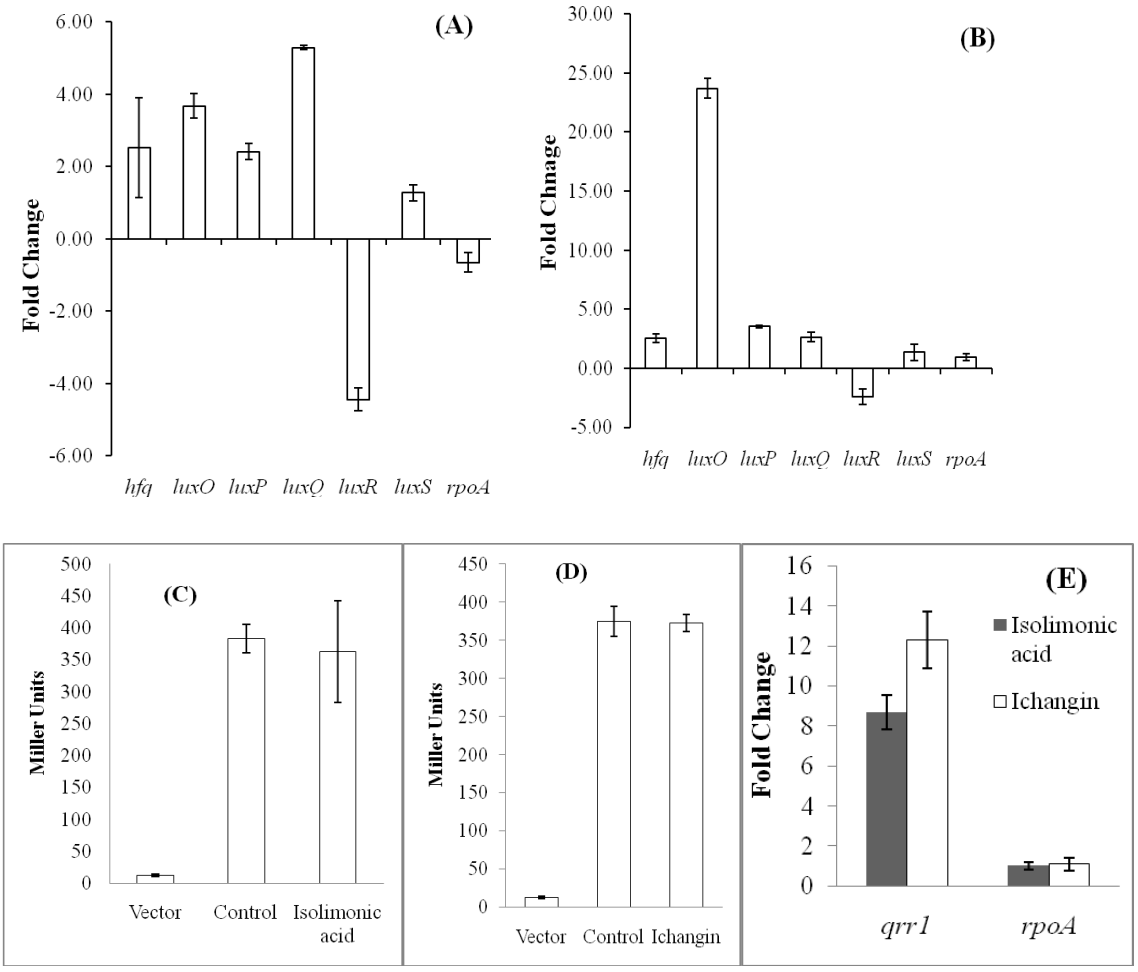


Fig. 4.7. Expression of signal transduction pathway and AI-2 synthase in *V. harveyi* strain BB120 as measured by qRT-PCR. (A) isolimonic acid, (B) ichangin LuxR promoter activity measured in *V. harveyi* BB120 in presence of (C) isolimonic acid and (D) ichangin. (E) Relative quantification of *qrr1* in *V. harveyi* BB120, upon exposure of 100 µg/ml isolimonic acid and ichangin

Isolimonic acid treatment induced the expression of *luxO* by 3.68 fold after 4 h (Fig. 4.7A). In addition, *hfq*, *luxP*, and *luxQ* (2.53, 2.42 and 5.29 fold respectively) were also induced by isolimonic acid treatment. In contrast, *luxR* (-4.44 fold) was suppressed, whereas *luxS* (1.27 fold) was not affected by isolimonic acid. In presence of ichangin, expression of *luxO*, *luxP*, *luxQ* and *hfq* was induced 23.7, 3.54, 2.64 and 2.53 fold (Fig.

4.7B) respectively. In addition, ichangin treatment resulted in 2.4 fold suppression of *luxR*.

In wild type *V. harveyi* strains LuxO regulates the levels of *luxR* mRNA by activating the transcription of sRNA (*qrr1-5)* (208, 365). Isolimonic acid and ichangin seems to induce *luxO*, however, at the same time suppressed *luxR*. To further confirm that the isolimonic acid and ichangin act by upregulating *luxO*, *luxR* promoter activity was measured. Isolimonic acid (Fig. 4.7C) and ichangin (Fig 4.7D) treatment did not affect the β-galactosidase activity. The empty vector was used as control and did not demonstrate appreciable β-galactosidase activity. In addition, the *qrr1* mRNA levels were quantified using relative qRT-PCR. The *qrr1* was induced by 8.7 and 12.3 fold by isolimonic acid and ichangin (Fig. 4.7E). These results further suggest that isolimonic acid and ichangin influence the cell-cell signaling pathway by modulating *luxO* expression.

4.5 Discussion

The bioluminescence production in *V. harveyi* is regulated by two component signal transduction pathway via three coincidence detectors (154). The three coincidence detectors LuxN, LuxPQ and CqsS converge on the phosphorelay protein LuxU and share rest of the pathway (154). In order to measure HAI and AI-2 inhibitory activity, limonoids were tested in two *V. harveyi* strains carrying mutations in receptors. Strain BB886 is defective in cell signaling mediated by AI-2, whereas, strain BB170 (LuxN::*Tn5*) does not detect N-hydroxybutanoyl-L homoserine lactone (22). *E. coli* #5 does not produce acylhomoserine lactone (corresponding to HAI), but produces AI-2.

Addition of CFS from *E. coli* #5 in AI-2 activity bioassay ensured that measured activity is due to presence of AI-2. Furthermore, the reporter strains BB886 and BB170 produce and sense their own autoinducers. Therefore, the time to measure the AI activity was chosen carefully. The assay was carried out only till the negative control showed a luminescence reading of 100, which was taken as a final value and analyzed.

Limonoids demonstrated a dose dependent inhibition of HAI and AI-2 activity. In order to rule out that the observed effect is due to inhibition of bacterial growth, cell density at OD_{600} in presence of limonoids was measured. Citrus limonoids did not show growth inhibitory effect on *V. harveyi* (FIG. 4.1) which is consistent with the previous study (58). Isolimonic acid, ichangin, nomilin, obacunone and DNAG demonstrated significant inhibition of AHL and AI-2 induced bioluminescence. Collectively, the data seems to suggest inhibition of autoinducer mediated cell-cell signaling. Since, citrus limonoids are structurally very similar, it is likely that their mode of action may be similar. The inhibitory potential of the citrus limonoids was comparable to the reported activity of furanones from *D. pulchera* (302). Since furanones are highly reactive molecules, it is suggested that they can only serve as model compounds in research (165). Furthermore, citrus limonoids inhibited both AHL and AI-2 mediated bioluminescence in this study, suggesting a probable non-specific antagonistic activity.

The suppression of luminescence production suggests that limonoids probably act by a mechanism, which mimics the phosphorylated LuxO or low-density phenotype. LuxO mutant, locked in phosphorylated condition, is further shown to be defective in biofilm formation indicating positive regulation of biofilm formation in *V. harveyi* by

quorum sensing (9). Since certain citrus limonoids demonstrated inhibition of autoinducer mediated cell-cell signaling; we further hypothesized that the inhibitory limonoids may also influence the biofilm formation in *V. harveyi*. Therefore, the biofilm formed by wild type strain *V. harveyi* BB120 in presence of various concentrations of limonoids was measured. Corroborating with the hypothesis, a dose dependent inhibition of biofilm formation was observed. In addition, isolimonic acid, which was the most potent inhibitor in autoinducer assay, demonstrated significant inhibition of biofilm formation under experimental conditions. This observation indicates that the tested limonoids inhibit the biofilm formation possibly by interfering with cell-cell signaling.

Obacunone, which was the most effective agent against autoinducer mediated cell-cell signaling, also demonstrated most potent activity against *V. harveyi* and EHEC biofilms. Furthermore, obacunone was found to inhibit the biofilm formation by as much as 69% and 47.5 % at 100 and 50 μg/ml. In comparison, *Delisea pulchera* derived furanone is reported to inhibit 55% biofilm at 60 μg/ml (302). The non linear regression analysis predicted a value of > 56 % reduction in biofilm formation by 60 μg/ml obacunone (Fig. 4.5). Together, these results seem to suggest that the citrus limonoids; specifically obacunone and isolimonic acid suppress biofilm formation in non-growth inhibitory fashion and plausibly in quorum sensing dependent manner. However, biofilm development and establishment is a complex multi-factorial process, which is governed by a combination of environment and genotype (187). Therefore, the possibility of limonoids acting via some other factors cannot be negated.

Isolimonic acid and ichangin were chosen for further investigation. Isolimonic acid and ichangin demonstrated significant inhibition of HAI/AI-2 activity and biofilm. In addition, ichangin is structurally different from other tested limonoids (Fig 2.5). We were interested to see if isolimonic acid and ichangin differed in their mode of action. In order to determine the probable site of action, effect of limonoids on constitutively luminescent *V. harveyi* mutants was investigated. Both, isolimonic acid and ichangin enhanced the luminescence production in *luxO* mutant JAF483. The *luxO* in JAF483 contains a point mutation and is incapable of phosphorelay, and hence did not activate the transcription of sRNA (111). Furthermore, isolimonic acid did not influence the luminescence production in *hfq* mutant BNL258. In contrast, ichangin treatment caused a small but significant reduction of luminescence in BNL258. The Hfq protein is nonfunctional in strain BNL258, consequently *luxR* mRNA is stable and produces luminescence (204). Taken together, data indicated possible site of action of isolimonic acid is between LuxO and Hfq, whereas, ichangin seems to affect at LuxO. In order to gain further insight, *luxO* mRNA transcript levels were measured by qRT-PCR (Fig. 4.8A). An induction of *luxO* suggested that isolimonic acid and ichangin modulate the signal transduction pathway by affecting *luxO* expression. Since, *luxO* regulates the stability of the *luxR* mRNA, and higher expression of *luxO* will result in lower transcript levels of *luxR*. In addition, induction of *hfq* by isolimonic acid treatment was also observed. In wild type *V. harveyi*, *luxO* in conjunction with alternative sigma factor σ^{54} regulates the expression of sRNAs *qrr1*, *qrr2*, *qrr3*, *qrr4* and *qrr5* (208, 365). In turn, these sRNAs with the help of Hfq, destabilize the *luxR* mRNA (204). Consistent with

these observations, an induction of *qrr1* and suppression of *luxR* mRNA transcript was observed in *V. harveyi* BB120 in presence of isolimonic acid and ichangin. Together, higher expression levels of *luxO* and *hfq* plausibly explain the enhanced luminescence observed in *luxO* (JAF483) and *luxU* (JAF553) mutants. Furthermore, induced expression of *luxPQ* may possibly a response of induced luxO. In addition, the results indicate isolimonic acid did not impact AI-2 production, as *luxS* expression was unaltered under these experimental conditions. Interestingly, ichangin treatment also produced similar expression profile as that of isolimonic acid, indicating a closely related mode of action. Like isolimonic acid, ichangin also seems to exert its action primarily by inducing *luxO* and *hfq* (Fig. 4.8B).

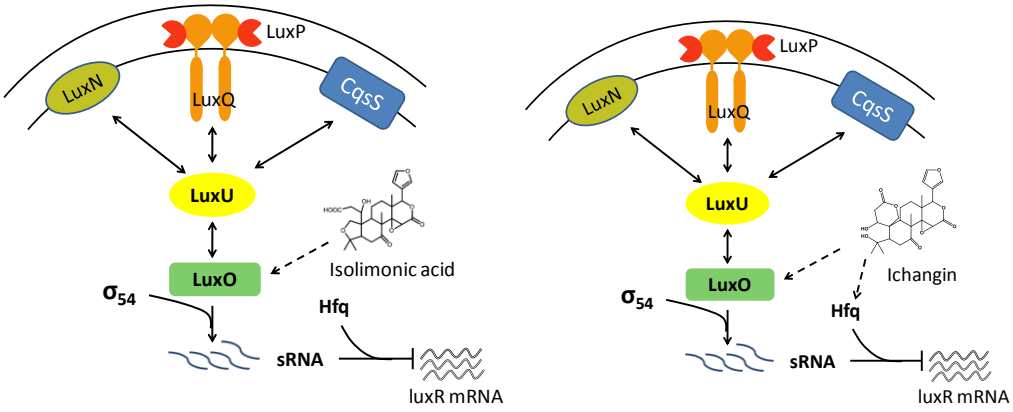


Fig. 4.8. Speculated model of impact of isolimonic acid and ichangin on signal transduction pathway in *V. harveyi*. The broken line represents unknown mode of action on expression of *luxO*. The figure was adapted from (154) and modified to depict impact of compounds

The quantitative PCR results suggest that isolimonic acid and ichangin treatment resulted in decreased *luxR* transcript levels. It is possible that the observed reduction in *luxR* transcripts is due to decreased transcriptional activity of *luxR* and not due to induced expression of *luxO*. To further understand, we constructed a plasmid carrying *luxR* promoter fused with *lacZ*, and measured the β-galactosidase activity in presence of isolimonic acid and ichangin. The results demonstrate that isolimonic acid and ichangin treatment did not affect the *luxR* promoter activity. This observation suggests that the decreased level of *luxR* mRNA transcript level is due to induced expression of *luxO*. All together the results suggest that isolimonic acid and ichangin interfere with *V. harveyi* cell-cell signaling by modulating *luxO* expression (Fig. 4.8). However, at this point it is not known if this effect is direct or indirect. Furthermore, a negative feedback loop mechanism is suggested to regulate the levels of transcriptional regulator HapR in V. cholerae and aid in switching from high to low cell density state (348). However, isolimonic acid and ichangin were only tested for their inhibitory effect under low density conditions. Further the proposed mechanism (Fig. 4.8) seems to operate at low density conditions. It is possible that the isolimonic acid and ichangin may have different effect at the transition phase between high to low cell density. During early growth phase LuxT was suggested to regulate the levels of luxO (209), it is possible that the isolimonic acid and ichangin may affect the cell-cell signaling by affecting LuxT.

In conclusion, citrus limonoids specifically, isolimonic acid and ichangin exhibited a strong inhibitory activity against bacterial cell-cell signaling as measured using the *V. harveyi* reporter strain model system. Furthermore, inhibition of quorum

sensing regulated processes, such as biofilm was observed. Finally, isolimonic acid and ichangin seems to exert their effect by modulating the expression of response regulator *luxO* and RNA chaperone *hfq*. However, whether isolimonic acid and ichangin exert direct or indirect effect, remains to be elucidated. The current study provides the evidence that certain citrus limonoids possess the ability to interfere with the bacterial cell-cell signaling in the model organism *V. harveyi*. However, anti-quorum sensing activity of citrus limonoids remains to be elucidated for human pathogens.

CHAPTER V

INHIBITION OF *ESCHERICHIA COLI* O157:H7 CELL-CELL COMMUNICATION AND VIRULENCE BY LIMONOIDS*

5.1 Synopsis

The cell-cell signaling is used by bacteria to regulate various important processes including biofilm formation, bioluminescence and virulence. Therefore, cell-cell signaling has been hypothesized to be a novel target for controlling the virulence with lesser risk of developing the resistance. Limonoids are prominent secondary metabolites of *Citrus* spp. and demonstrate anti-cancer activity. The objective of the current investigation was to investigate the effect of citrus limonoids against cell-cell signaling, biofilm formation and bacterial virulence in *E. coli* O157:H7. Citrus limonoids assayed for their potential to interfere AI-3 mediated cell-cell signaling and biofilm formation. Two of the tested limonoids, isolimonic acid and ichangin demonstrated high antagonistic activity towards bacterial cell-cell signaling and biofilm formation in E. coli O157:H7. Moreover, these limonoids also repressed the expression of LEE encoded genes in *E. coli* O157:H7. Furthermore, the evidence suggests that isolimonic acid activity is dependent upon QseBC and QseA.

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5.2 Introduction

Secondary metabolites such as terpenoids contribute to the systemic and induced plant defense system and respond to various stimuli including insect, bacterial and fungal infestation (197). Although, produced by plants, the secondary metabolites frequently demonstrate activities beyond the natural enemies of plants. Limonoids are characteristic triterpenoids in plant order Rutales. They are particularly abundant in family Meliaceae where >200 different limonoid structures were reported (58). A limited number of limonoids were reported from *Citrus* and related genera (148). Limonoids are biologically active compounds (313, 345) and several studies reported antifeedant activities of citrus limonoids for a number of economically important insects such as colorado beetle, corn earworm, fall armyworm, spruce budworm and tobacco budworm (148). However, citrus limonoids were weak anti-feedant agents compared to limonoids present in Meliaceae (11, 58, 313). In recent years, citrus limonoids have attracted much attention due to their potential to improve human health. Several reports have suggested potential anticancer activity (98, 242, 268, 272, 368), antiviral activity (26), and cholesterol lowering properties (192). However, antibacterial activities of citrus limonoids were not reported (58, 375).

During the co-evolution with bacteria, plants have developed several novel mechanisms to ward off the bacterial infection (77). One such recently identified mechanism was inhibition of QS or cell-cell signaling in bacteria. Quorum sensing (QS) is a collective term for the intercellular signaling systems used by bacteria to regulate its gene expression in density dependent fashion (50). This intercellular signaling is

facilitated by small molecules and regulates various cellular processes depending upon the needs of the particular bacterial species (50, 117). These small molecules, termed as autoinducers (AI), belong to several different classes (154, 387) such as acyl-homoserine lactones (AHLs), oligopeptides, furanosyl diester, indole, quinolones and a yet unidentified molecule named as autoinducer-3 (AI-3) (50, 342). Several important pathogenic traits such as virulence, conjugation of plasmids, spore formation, biofilm formation and transformation competence (50, 381, 397) are regulated by QS. In Enterohemorrhagic *E. coli*, AI-2 and AI-3 mediated cell signaling regulates the transcription of Locus of Enterocyte Effacement (LEE) and flagellar genes (17, 341). In addition, AI-2 plays a role in stimulation of biofilm formation in *E. coli* (133, 161).

Biofilms are highly organized and dynamic habitats (69) characterized by high population density and slow growth. Cells in the biofilms are inherently resistant to antimicrobial agents and host defenses and therefore, are the root cause of many persistent infections (118). Biofilms formed by pathogenic *E. coli* strains can pose serious health problems such as prostatitis, biliary tract infections, and urinary catheter cystitis (69). Owing to control of expression of virulence and pathogenic traits in many pathogenic bacterial species, quorum sensing is suggested as a possible new therapeutic target with low risk of developing drug-resistance (270, 292). Several plant secondary metabolites and extracts rich in phytochemicals were identified as inhibitors of cell-cell signaling (4, 61, 225, 271, 371, 375).

During our study with *V. harveyi*, we observed that certain limonoids such as isolimonic acid, ichangin, obacunone and nomilin interfere with autoinducer mediated

cell-cell signaling (374). The present study was conducted to understand the potential of citrus limonoids to inhibit the QS and associated phenotypes in *E. coli* O157:H7 (EHEC).

5.3 Materials and Methods

5.3.1 Materials

The limonoids isolimonic acid, ichangin, isoobacunoic acid, IOAG and DNAG were purified earlier in our lab (374). The purity of the individual limonoids was measured by high performance liquid chromatography (HPLC) technique. The limonoids were separated using HPLC as described (372), and purity was assessed by calculating percent peak area. A stock solution was prepared by dissolving 20 mg of each purified limonoid in 1 ml of dimethyl sulfoxide (DMSO).

5.3.2 Bacterial Strains and Plasmids

Bacterial strains and plasmids used in the study are listed in Table 5.1. Unless otherwise specified, bacterial cultures were grown at 37^{o}C in Luria-Bertani (LB) medium supplemented with 0.5 % glucose (LB Glu). When appropriate, the medium was supplemented with 10 µg of chloramphenicol, or 100 µg of ampicillin per ml media. The colony forming antigen (CFA) medium was used to culture the *E .coli* O157:H7 strain for biofilm studies (102, 172). Plasmids *pVS150* and *pVS178* were purified from strains VS151 and VS179 respectively, using Qiagen Plasmid Purification Kit (Qiagen) and electroporated into EHEC. The transformed strain were designated as AVS43 (EHEC containing *pVS178*) and AVS45 (EHEC containing *pVS151*). In addition, *pVS150* was electroporated into strains TEVS232 and TEVS21. These strains were designated as

AVS46 and AVS47 respectively. Further, *qseB* and *qseC* were amplified from EHEC genomic DNA using primer pairs AV25/AV26 and AV29/30. The primers were designed by altering one base to create restriction sites for the respective enzymes. The amplified fragment of *qseC* was digested with SacI and SalI and cloned into pBAD33 generating the plasmid *pAV11*. The *qseB* fragment was digested with SacI and HindIII and cloned into pBAD33 generating plasmid *pAV12*. Plasmids *pAV11* and *pAV12* were subsequently electroporated into EHEC and the strains were designated as AVS48 and AVS49, respectively.

TABLE 5.1: EHEC strains and plasmids

Strain/Plasmid	Genotype	Reference/Source
Strains		
E. coli O157:H7 EDL933	Wild type	ATCC (#43895)
TEVS232	*LEE1:lacZ*	(340)
TEVS21	*LEE2:lacZ*	(340)
VS145	EHEC 86-24 *qseA* mutant	(339)
VS151	VS145 with plasmid pVS150 harboring *qseA*	(339)
VS138	EHEC 86-24 *qseC* mutant	(343)
VS179	VS138 with plasmid pVS178 harboring *qseBC*	(343)
AVS43	EHEC harboring plasmid *pVS178*	This study
AVS45	EHEC harboring *pVS150*	This study
AVS46	TEVS232 harboring *pVS150*	This study
AVS48	EHEC harboring *pAV11*	This study
AVS49	EHEC harboring *pAV12*	This study
Plasmids		
pVS150	*qseA* into *pACYC177*	(339)
pVS178	*E. coli* K12 *qseBC* in *pBAD33*	(343)
pAV11	EHEC *qseC* in *pBAD33*	This Study
pAV12	EHEC *qseB* in *pBAD33*	This study
pBAD33	*pBAD33*	ATCC

5.3.3 Growth and Metabolic Activity

The growth and metabolic activity of EHEC was measured as previously described (374). Briefly, overnight culture of EHEC was diluted 100 fold in LB-glu media and exposed to100, 50, 25, 12.5 and 6.25 µg/ml of isolimonic acid, ichangin, isoobacunoic acid, DNAG and IOAG or equivalent volume of DMSO. The cultures were grown for 16 at 37^{o}C and plates were constantly shaken at medium speed. OD at 600 nm was measured every 15 min using the Synergy™ HT Multi-Mode Microplate Reader (BioTek, Instruments, Winooski, VT). Metabolic activity of EHEC was measured in similar fashion as growth curve with addition of 25 µl of AlamarBlue and absorption at 570 and 600 nm were monitored.

For limonin, obacunone, nomilin, DAN and LG overnight culture of *E. coli* O157:H7 was diluted 100 fold in LB media and treated with 100 µg/ml limonoids or equivalent volume of DMSO. The cultures were incubated at 37^{o}C with shaking for 16 h. OD_{600} was recorded at every 2 h and presented as mean of three replicates.

5.3.4 Biofilm Assay

The biofilm assay was conducted as previously described (375). Briefly overnight culture of EHEC strain ATCC 43895, AVS43, AVS48 and AVS49 were diluted 100 fold in LB medium (102) and 200 µl/well culture was incubated in presence of 6.25, 12.5, 50, or 100 µg/ml of limonoids in polystyrene 96-well plates at 26°C for 24 h (48 h in case of VS138 and VS179) without shaking. The biofilms were stained with 0.3 % crystal violet (Fisher, Hanover Park, IL) for 20 min. The extra stain was washed with phosphate buffer (0.1 M, pH 7.4) and dye associated with the attached biofilm was

dissolved with DMSO. An absorbance at 570 nm was used to quantify the total biofilm mass.

5.3.5 In vitro Adhesion Assay

Human epithelial Caco-2 cells were grown in Dulbecco's Minimal Essential Medium (DMEM) with nonessential amino acids and 10 % fetal bovine serum without antibiotics. Caco-2 cells were seeded at 1 x 10^5 cells/well in 6-well plates and infected with approximately 5 x 10^6 cells/well of freshly grown EHEC in presence or absence of 100 µg/ml isolimonic acid, ichangin, isoobacunoic acid, IOAG and DNAG. The plates were incubated for 3 h at 37^{o}C in 5 % CO_2 environment. After completion of incubation, the plates were washed with sterile PBS to remove any loosely attached cells. The Caco-2 cells were lysed with 0.1 % Triton-X in PBS to release the bacteria and serial dilutions were plated on LB-agar and incubated at 37^{o}C for 24 h. Colonies were counted after incubation period and presented as $\log_{10}$CFU/ml.

5.3.6 Caco-2 Cell Survival Assay

The Caco-2 cells (1 X 10^4/well) were seeded in 96-well plate and exposed to 100 µg/ml of isolimonic acid, ichangin, isoobacunoic acid, IOAG and DNAG for 6 h in humidified incubator at 5 % CO_2, 37^{o}C. The cell survival was determined by measuring released lactate dehydrogenase using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega Corp., Madison, WI)

5.3.7 Quantitative PCR

Relative transcript amount of selected genes (Table 5.2) was measured by qRT-PCR as described (375). Briefly, overnight cultures of *E. coli* O157:H7 was diluted 100

fold with fresh LB-glu medium or DMEM+10% FBS (referred as DMEM henceforth), treated with limonoids (100μg/ml) or DMSO and grown further at 37°C and 200 rpm. Bacterial cells were collected at OD_{600} at ≈0.5, 1.0 and 2.0. RNA was extracted using the RNeasy minikit (Qiagen Inc., Valencia CA) and converted to cDNA using MuLV reverse transcriptase enzyme and random hexamer in a Reverse-Transcriptase polymerase chain reaction (RT-PCR) (317) at 42˚ C for 1 h. The cDNA product was purified with QIAquick PCR-purification kit (Qiagen Inc.). Twenty five nanogram cDNA was amplified with 10 pmol target primers using SYBR Green PCR master mix (Life Technologies Corporation, Carlsbad, CA) for 40 amplification cycles. After completion of 40 PCR cycles, melt curve data was generated. All the measurements were done on three biological replicates consisting of three technical replicates each. Amplification of target sequences was done on ABI-Prism 7000 HT (Applied Biosystems, Foster City, CA). The C_t values for primers were normalized against that of 16S rRNA. Fold change in the gene expression was calculated by $2^{(-\Delta\Delta Ct)}$ (210) and expressed as fold change ±SD of three replicates.

5.3.8 AI-3 Reporter Assay

Preconditioned media (PM) was prepared as described (340). Overnight cultures of TEVS232, TEVS21 and AVS45 were diluted 100 fold in LB medium and grown till OD600 ≈0.2. The cells were collected by centrifugation at 2500 x g for 10 min and resuspended in either fresh LB media supplemented with 50 μM epinephrine or PM and treated with 100 μg/ml isolimonic acid and equivalent amount of DMSO. The β-galactosidase activity was measured after 30 min incubation at 37^{o}C using *o*-nitrophenyl

β-D-galactopyranoside as previously described (243) and reported as mean ± SD of three replicates .

TABLE 5.2: Sequences of the EHEC Primers used. The underlined segment in AV25/26 and AV29/30 indicate the restriction enzyme sites

Primer		**Sequence (5'-3')**	**Reference**
cesD	F	GTTTATCAAATCATGAAGATGCACAA	(375)
	R	GCCCTGGGATCTTGCATAAC	
escJ	F	CCAATGATGTCAATGTTTCCAAA	(375)
	R	GCGCGAACAAAATCCTCTTT	
escR	F	GCCAGCCTCCAACAAGAATG	(375)
	R	ATTGGCCTTGGGTATGATGATG	
escU	F	TCCACTTTGTATCTCGGAATGAAG	(375)
	R	CAAGGATACTGATGGTAACCCTGAA	
flhC	F	CGCTTTCCAGCATCTGCAA	(375)
	R	CGGGATATTCAGCTGGCAAT	
flhD	F	TCATTCAGCAAGCGTGTTGAG	(375)
	R	TCCCGCGTTGACGATCTC	
ler	F	CGACCAGGTCTGCCCTTCT	(375)
	R	TCGCTCGCCGGAACTC	
sepZ	F	CGGAGACGAGCAGCACAGA	(375)
	R	CCGCCAACCGCAGTAAGA	
stx2	F	ACCCCACCGGGCAGTT	(375)
	R	GTCAAAACGCGCCTGATAGAC	
rpoA	F	GTTGCCGCACGACGAATCGC	This study
	R	CCCAATCGGCCGTCTGCTGG	
AV25	F	GTGCTGTACA<u>GAGCTC</u>GTTACAAC	This study
AV26	R	CCAGGCGACA<u>AAGCTT</u>GAAAGCA	
AV29	F	TGCGTCTGG<u>GAGCTC</u>ACGATTATC	This study
AV30	R	GGTGAGACGTTT<u>GTCGAC</u>TATAGTACG	

5.3.9 Statistical Analysis

The percent inhibition of biofilm formation was calculated from three replicates consisting of three replicate wells using the formula 100- [(OD_{570} of sample well/ OD_{570} of positive control) x 100] and expressed as percentage and SD values. The effects of different limonoids for each activity were analyzed with an analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test on SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The effect was considered significant at $p < 0.05$. The data for EHEC biofilm was fitted to a 3-parameter sigmoid models y= a/(1+exp(-(x-x0)/b)) using SIGMAPLOT 11.0 (Systat Software, Inc.). In order to conduct the analysis, concentration of each limonoids was converted to Log_{10} µM and plotted against percent inhibition values.

5.4 Results

5.4.1 Effect of Citrus Limonoids on EHEC Growth and Biofilm Formation

The growth of EHEC in LB-glu was not inhibited at 100 µg/ml of tested limonoids (Table 5.3 and Fig. 5.1B). In addition, limonoids at 100 µg/ml did not reduce the viability of the EHEC (Fig. 5.1A) as measured by AlamarBlue. Higher reduction of AlamarBlue was observed in presence of DNAG, ichangin and isoobacunoic acid, indicating an increased metabolic activity of EHEC.

All the limonoids depicted inhibition of biofilm formation in concentration dependent manner (Fig. 5.2A). To compare the relative efficacy of different limonoids, the data was fitted to 3-parameter sigmoid equation. Nonlinear regression models depicted a typical sigmoidal response (Fig. 5.2B). Since, the limonoids except

obacunone demonstrated <50 % inhibition of biofilm formation, IC_{25} values were calculated and compared.

TABLE 5.3: Generation time of *E. coli* O157:H7 upon exposure of different concentrations of limonoids. The mean ± SD of three replicates are presented

Concentration (µg/ml)	Control	IL	IBA	Ichangin	DNAG	IOAG
100	23.56 ±0.71	23.11 ±0.76	22.97 ±0.96	23.65 ±0.95	23.58 ±1.06	22.96 ±1.06
50	24.90 ±1.82	22.97 ±0.97	23.12 ±0.92	23.16 ±0.93	23.27 ±1.09	23.64 ±1.08
25	23.62 ±2.47	23.58 ±1.19	23.26 ±1.23	22.58 ±1.26	23.68 ±0.91	23.51 ±1.26
12.5	23.68 ±1.84	23.54 ±1.01	22.69 ±1.09	23.12 ±1.08	23.97 ±1.31	23.69 ±1.32
6.25	23.91 ±0.63	23.70 ±1.09	23.90 ±1.02	23.55 ±1.05	23.61 ±1.05	23.76 ±1.01

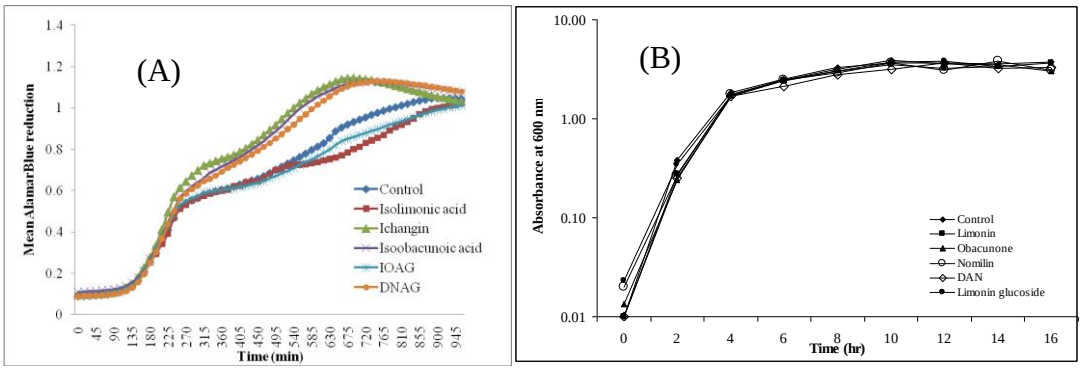

FIG. 5.1 Effect of 100 µg/ml limonoids on growth and metabolic activity of *E. coli* O157:H7. (A) Metabolic activity and, (B) Growth rate

Obacunone and isolimonic acid were the most effective antagonists of (Fig. 5.2A) EHEC biofilm. Only obacunone demonstrated >50 % inhibition of EHEC O157:H7 biofilm with 9- 68 % inhibition (IC_{50} ≈116.68 µM). Isolimonic acid depicted

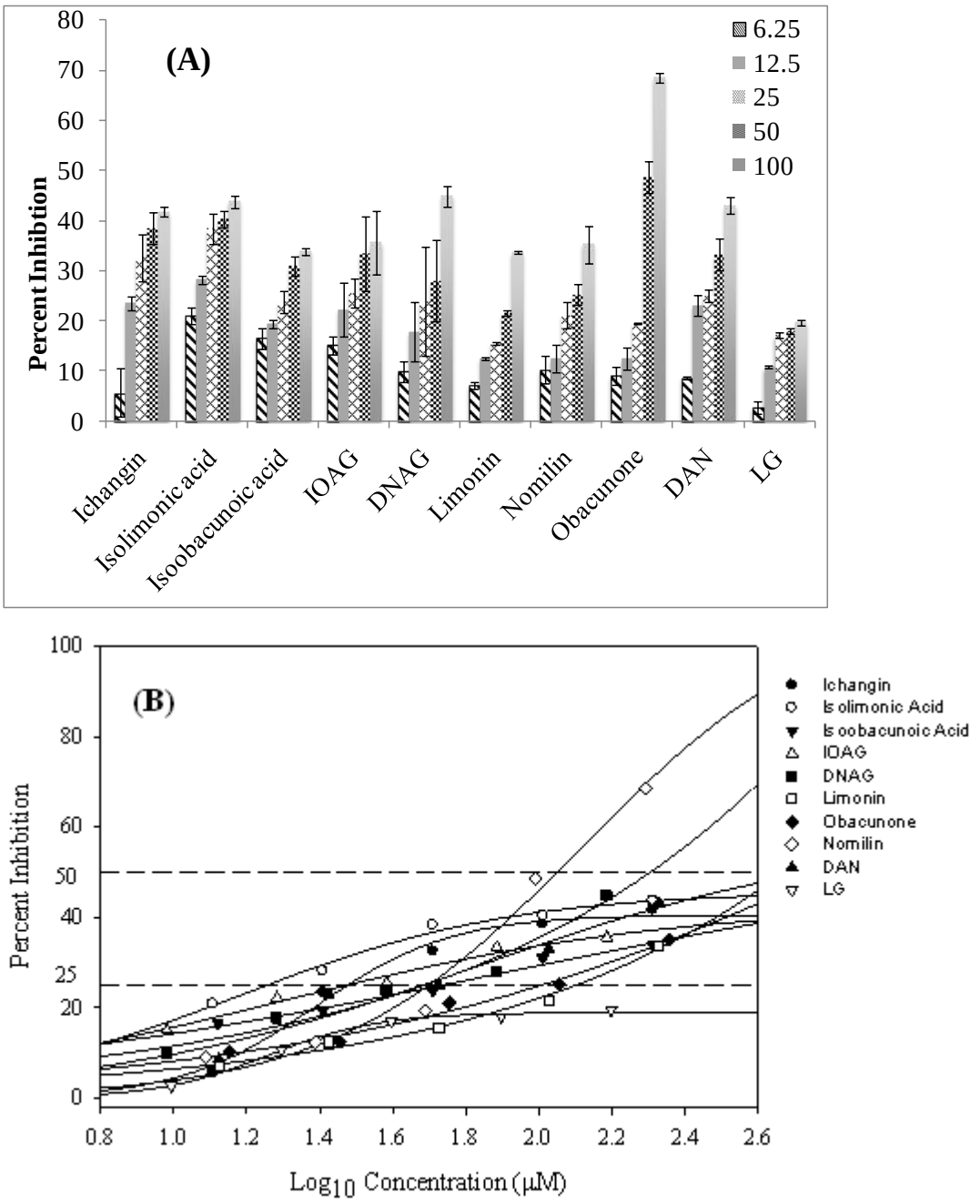

FIG. 5.2. (A) Inhibition of *E. coli* O157:H7 biofilm formation by limonoids. Percent inhibition was calculated over DMSO treated samples as described in Materials and Methods section. Mean and standard deviation of 3 independent experiments with 3 replicates each are presented. (B) Response curves of biofilm formation inhibition to the concentration of citrus limonoids. Curves at 50 % and 25 % represent the IC_{50} and IC_{25} values for compounds

an IC_{25} of 19.7 μM (Fig. 5.2B). Ichangin demonstrated the inhibitory activity in range of 5-41 % respectively with an IC_{25} of 28.3 μM (Fig. 5.2A and B). In comparison, isoobacunoic acid was less effective antagonist of biofilm formation with inhibition values ranging from 16.45 % to 33.9 % (Fig. 5.2A). Interestingly, IOAG was more potent (IC_{25}= 29.54 μM) than its aglycone isoobacunoic acid (IC_{25}= 57.2 μM). Similar level of EHEC biofilm inhibition was observed for DAN, limonin and nomilin (Fig. 5.2B). DAN, limonin, nomilin and limonin glucoside inhibited the EHEC biofilm by 8.67- 43.11 %, 7.15- 33.64 %, 10.25-35.19 % and 0-19.55 % respectively, in the tested concentration range. However, LG showed a very poor response, depicting plateau at 39.8 μM (Fig 5.2B). The R^2 values for fitted model of isolimonic acid, ichangin, isoobacunoic acid, IOAG, DNAG, limonin, obacunone, nomilin, DAN and LG were 0.99, 0.96, 0.92, 0.88, 0.99, 0.99, 0.99, 0.98, 0.96 and 0.99 respectively.

5.4.2 Citrus Limonoids Repress the LEE Region and *stx2* Gene Expression

Adherence of EHEC to epithelial cells is facilitated by LEE encoded type three secretion apparatus, effector proteins and intimin (335). To determine if the limonoids affect the transcription of LEE, gene expression pattern of 6 LEE encoded genes *ler*, *escU*, *escR* (LEE1 encoded), *escJ*, *sepZ* and *cesD* (LEE2 encoded) in presence of 100 μg/ml limonoids was studied. In LB media, isolimonic acid, obacunone and ichangin exerted the strongest effect on the LEE. Expression of *ler* was downregulated by ≈5 fold by isolimonic acid and ≈3 fold by ichangin. Exposure of obacunone decreased the transcription of genes encoded in the LEE region by 2.23 – 5.16 fold and *stx2* by 3.37 fold (Table 5.4). The genes encoded within *LEE1* (*escRU*) (Table 5.4) and *LEE2* (*escJ*,

TABLE 5.4: Differential expression of selected genes by limonoids

Gene	Fold Change in Gene Expression by Citrus limonoids									
	Ichangin	Isolimonic acid	Isoobacunoic acid	IOAG	DNAG	Obacunone	Nomilin	DAN	Limonin	LG
stx2	-2.48 ± 0.8	-4.87 ± 0.9	-1.62 ± 0.4	-2.2 ± 0.8	-1.17 ± 0.	-3.37 ± 0.3	-1.35 ± 0.1	-2.58 ± 0.1	1.08 ± 0.2	-1.53 ± 0.1
ler	-3.2 ± 2.1	-5.04 ± 0.8	-1.44 ± 0.3	-1.8 ± 0.4	-0.7 ± 1.5	-2.23 ± 0.1	-1.34 ± 0.3	-1.54 ± 0.5	-1.23 ± 0.3	1.00 ± 0.1
escU	-5.34 ± 0.8	-6.63 ± 1.0	-1.63 ± 0.1	-3.17 ± 0.3	-1.96 ±0.6	-3.17 ± 0.4	-2.01 ± 0.4	-1.65 ± 0.1	-1.24 ± 0.2	-1.06 ± 0.4
escR	-2.55 ± 0.7	-6.25 ± 1.3	-1.67 ±0.3	-2.54 ± 1.2	-2.32 ± 0.5	-2.77 ± 0.1	-1.59 ± 0.8	-1.31 ± 0.4	1.13 ± 0.6	-1.01 ± 0.1
escJ	-6.16 ± 0.9	-12.44 ± 2.1	-2.38 ± 1.3	-3.65 ± 1.9	-1.23 ± 2.3	-5.16 ± 0.1	-1.06 ± 0.2	-1.01 ± 0.1	-1.53 ± 0.1	-1.04 ± 0.2
sepZ	-2.67 ± 0.1	-6.87 ± 1.1	-0.67 ± 1.5	-1.67 ± 0.6	-1.62 ± 0.8	-3.31 ± 0.5	-1.28 ± 0.5	-1.10 ± 0.1	1.50 ± 0.6	1.02 ± 0.2
cesD	-3.54 ± 0.7	-9.95 ± 1.5	-2.96 ± 1.2	-2.61 ± 1.7	-1.64 ± 0.8	-3.04 ± 0.3	-1.24 ± 0.1	-1.19 ± 0.2	-1.19 ± 0.2	-1.34 ± 0.3
flhC	-2.81 ± 0.9	-4.5 ± 1.3	-1.52 ± 0.3	-2.11 ± 0.4	-1.29 ± 0.3	-2.21 ± 0.1	-2.51 ± 0.2	-1.18 ± 0.1	-1.04 ± 0.4	-1.54 ± 0.3
flhD	-2.8 ± 0.5	-6.91 ± 0.4	-1.77 ± 0.5	-2.28 ± 0.4	-1.71 ± 0.5	-3.28 ± 0.8	-2.21 ± 0.1	-1.24 ± 0.2	-1.06 ± 0.1	-1.38 ± 0.1
rpoA	-0.35 ± 1.8	-0.45 ± 1.6	1.82 ± 0.8	1.32 ± 0.4	1.71 ± 0.5					
ptsN						-1.05 ± 0.5	-1.02 ± 0.1	-1.66 ± 0.8	1.51 ± 0.1	-1.08 ± 0.4

cesD, sepZ) (Table 5.4) were downregulated to various extents by the treatment of different limonoids. IOAG repressed the *escU, escR, escJ* and *cesD* by 3.2, 2.5, 3.7 and 2.6 fold respectively. Isoobacunoic acid and DNAG did not affect the expression of *LEE1* and *LEE2* genes under investigation (Table 5.4).

Flagella is implicated in biofilm formation and adherence of pathogenic *E. coli* to mammalian cells (131). Because certain limonoids inhibited the biofilm formation, expression of flagellar transcriptional regulators *flhDC* upon exposure of 100 µg/ml limonoids was measured. Isolimonic acid repressed the *flhC* and *flhD* by ≈4.5 and 6.9 fold respectively (Table 5.4). In comparison, ichangin exposure resulted in ≈ 2.8 fold repression of both *flhC* and *flhD*, while obacunone downregulated the flagellar genes by 2-3 fold. IOAG demonstrated ≈ 2.1 and 2.3 fold downregulation of *flhC* and *flhD*, respectively. Isoobacunoic acid and DNAG did not affect the expression of *flhCD* (Table 5.4). Furthermore, gene expression pattern of *rpoA*, was unaffected by treatment of isolimonic acid and ichangin whereas isoobacunoic acid (1.75 fold), IOAG (1.30 fold) and DNAG (1.65 fold) slightly induced the expression of *rpoA* (Table 5.4).

Nomilin had profound influence on *flhDC* while moderately affected the expression of LEE encoded genes and *stx2*. In addition, obacunone and nomilin do not affect the expression of *ptsN*. Interestingly, DAN exerted strong effect on *stx2* but had moderate to no effect on LEE encoded genes and *flhDC*, while moderately affected the *ptsN*. Limonin and LG had moderate to no effect on the transcription of the genes studied, however limonin induced the expression of *ptsN* moderately (Table 5.4).

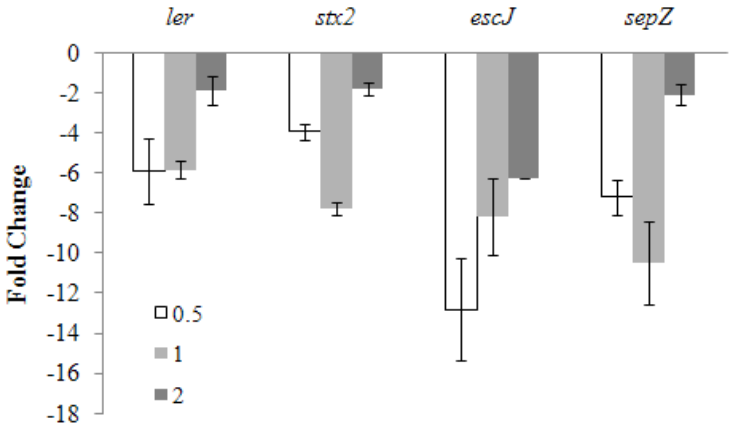

FIG. 5.3 Expression of LEE encoded genes in DMEM in response to isolimonic acid. The data represents mean of three biological replicates and SD. The samples were collected at OD600 of 0.5, 1.0 and 2.0 processed as described in Materials and Methods

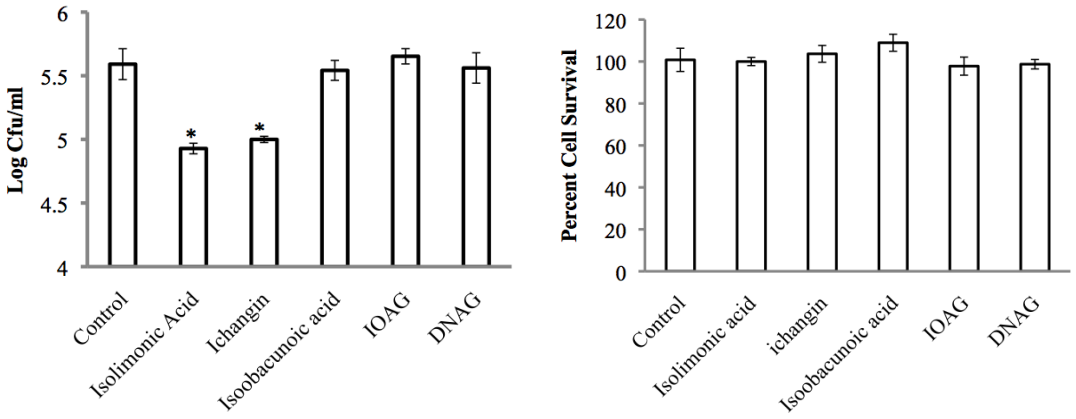

FIG. 5.4. (A) Adhesion of EHEC to Caco-2 cells. (B) Survival of Caco-2 cells in presence of 100 µg/ml limonoids. The data represents mean of three biological replicates and SD

Shiga toxin produced by EHEC is the responsible for HUS (356). We were further interested if limonoids modulate expression of *stx2*. Exposure of 100 μg/ml isolimonic acid, ichangin and IOAG repressed the *stx2* by 4.9, 2.5 and 2.2 fold, respectively (Table 5.4). Further, the effect of isolimonic acid on expression of *ler, stx2*, *escJ* and *sepZ* in DMEM grown EHEC was investigated. Culture of *E. coli* O157:H7 in DMEM is suggested to activate LEE expression (340) in response to epinephrine. Consistent with the results in LB medium, isolimonic acid treatment downregulated the expression of *ler, stx2, escJ* and *sepZ* in DMEM media (Fig. 5.3).

5.4.3 Effect of Limonoids on Adhesion of EHEC to Caco-2 Cells

In order to further understand the effect of limonoids, adherence of EHEC to colon epithelial Caco-2 cells was studied. Exposure of 100 μg/ml isolimonic acid and ichangin reduced the number of EHEC cells attached to Caco-2 cells by 0.66 and 0.59 Log_{10} cfu/ml ($p<0.05$) respectively (Fig. 5.4A). However, isoobacunoic acid, IOAG and DNAG exposure did not affect the adhesion of EHEC to Caco-2 cells. To determine if the observed reduction in adhesion of EHEC is due to reduced cell viability of Caco-2 cells by limonoids, survival of Caco-2 upon exposure of 100 μg/ml limonoids at 6 h was measured by assaying extracellular LDH. The results demonstrate that the limonoids (100 μg/ml) did not affect the cell survival of Caco-2 cells (Fig. 5.4B).

5.4.4 Effect of Isolimonic Acid on AI-3/Epinephrine Induced LEE Expression

AI-3 mediated cell-cell signaling regulates biofilm, motility and expression of LEE in EHEC (325, 342-343). To ascertain the involvement of AI-3 signaling in modulation of EHEC biofilm and virulence by isolimonic acid, AI-3 induced expression

of *LEE1* and *LEE2* was measured. Exposure of 100 µg/ml isolimonic acid resulted in 46.05 and 34.23 % reduction of β-galactosidase activity in TEVS232 and TEVS21 (Fig. 5.5A and B). Further, effect of isolimonic acid on epinephrine-induced expression of *LEE1* was measured by supplementing the LB media with 50 µM epinephrine. The isolimonic acid repressed the epinephrine-induced expression of *LEE1* by ≈3 fold (Fig. 5.5C).

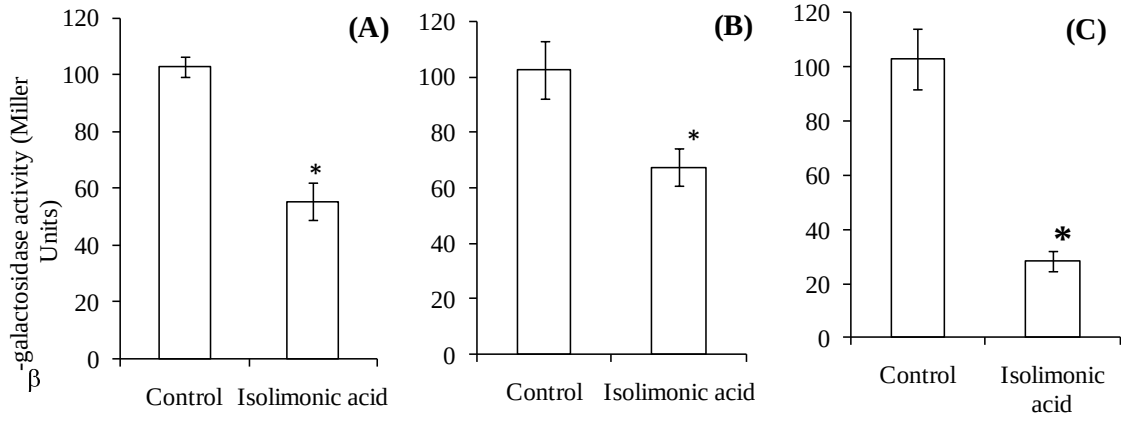

FIG. 5.5: Inhibition of AI-3 induced β-galactosidase activity by 100 µg/ml isolimonic acid. (A) TEVS232 and, (B) TEVS21. (C) Epinephrine induced β-galactosidase activity in TEVS232 in presence of 100 µg/ml isolimonic acid

5.4.5 QseBC Dependent Inhibition of Biofilm by Isolimonic Acid

Since AI-3 regulates the biofilm and motility in EHEC via QseBC (343), role of QseBC in biofilm inhibition by isolimonic acid was investigated. To determine the involvement of QseC in inhibition of biofilm formation by isolimonic acid, biofilms

formed by strains VS138 and VS179 at 48 h was measured. Isolimonic acid did not affect the biofilm formed by strain VS138 (Fig. 5.6). In contrast, 61.33 % reduction in total biofilm of VS179 was observed in presence of 100 µg/ml isolimonic acid. In order to further determine the effect of isolimonic acid on *qseBC* mediated biofilm formation, *pVS178* was purified from VS179 and introduced into EHEC. In addition, *qseB* and *qseC* were amplified from EHEC genomic DNA, cloned into pBAD33 vector and introduced into EHEC. The expression of *qseBC*/*qseB*/*qseC* was induced by addition of 0.2 % arabinose in the media. No significant difference in total biofilm was observed between control and isolimonic acid treatment after 24 h in AVS43, AVS48 and AVS49. Furthermore, supplementation of *qseBC* and *qseC* in EHEC resulted in ≈2 fold increase ($p< 0.05$) in total biofilm as measured by crystal violet, while *qseB* supplementation demonstrated a mild (1.3) fold but significant ($p< 0.05$) increase in biofilm. These data indicate involvement of QseBC in isolimonic mediated inhibition of biofilm.

5.4.6 QseA Dependent Inhibition of *ler* by Isolimonic Acid

Repression of LEE by isolimonic acid prompted us to investigate the role of QseA. Expression of *ler* was measured using qRT-PCR in VS145 and VS151 in presence of 100 µg/ml isolimonic acid. The mean fold change of -1.9 in *ler* expression was observed upon exposure of VS145 to isolimonic acid. In comparison, isolimonic acid repressed the *ler* expression by 7.4 fold in VS151 (Fig. 5.7A). To further confirm the repression of *ler*, plasmid *pVS150* was introduced into strain TEVS232 and expression of *LEE1* (β-galactosidase activity) was measured. Expression of *qseA* from a multicopy plasmid negated the inhibitory activity of isolimonic acid (Fig. 5.7B).

Altogether, the results indicate that isolimonic acid repressed EHEC virulence in *qseA* dependent manner.

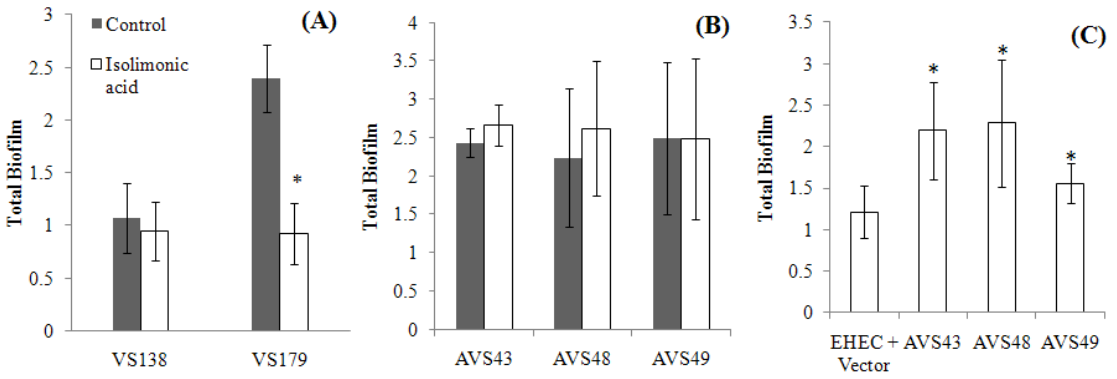

FIG. 5.6. Inhibition of biofilm in (A) QseC mutant and QseC supplemented with *pVS178*. (B) EHEC supplemented with *pVS178* (AVS43), *pAV11* (AVS48) and *pAV12* (AVS49) by 100 µg/ml isolimonic acid. The experiment was conducted in triplicate and mean ± SD are presented

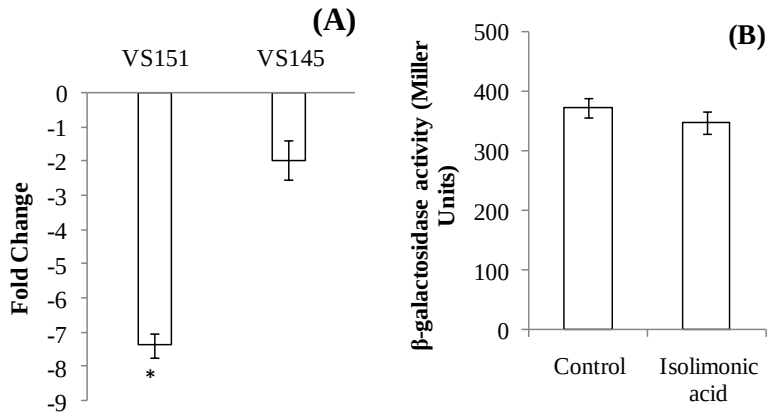

FIG. 5.7. Expression of *ler* in QseA mutant and QseA supplemented with pQseA. The expression was monitored 30 min after addition of preconditioned media and 100 µg/ml isolimonic acid. The data represents mean ±SD of triplicate experiment. (B) AI-3 induced β-galactosidase activity in TEVS232 supplemented with *qseA* (AVS46)

5.5 Discussion

During our studies on bacterial cell-cell signaling, several citrus limonoids such as obacunone, nomilin, isolimonic acid and ichangin as well as flavonoids demonstrated the ability to interfere with the autoinducer mediated signaling and biofilm formation (371, 374-375) in *V. harveyi*. Furthermore, isolimonic acid and ichangin seems to modulate response regulator *luxO* in *V. harveyi* (374). Therefore, the current study investigated the possibility of EHEC QS interference by the citrus limonoids purified in our earlier work and reported to interfere with *V. harveyi* QS system (374).

EHEC is known to form biofilm on various surfaces and in different conditions (201, 398). Biofilm formation in *V. harveyi* and EHEC are suggested to be positively regulated by quorum sensing, specifically by AI-2 mediated cell-cell signaling (40, 133, 161, 206). Since, certain citrus limonoids demonstrated inhibition of autoinducer mediated cell-cell signaling; we further hypothesized that the inhibitory limonoids may also influence the biofilm formation in *E. coli*. To determine the effect on biofilm formation, EHEC biofilms were grown for 24 h in 96-well plates in presence of limonoids. The results demonstrate that obacunone and isolimonic acid which were the most effective agent against autoinducer mediated cell-cell signaling, also demonstrated most potent activity against EHEC biofilms. Furthermore, the isolimonic acid and other tested limonoids inhibit EHEC biofilm without affecting the growth rate of EHEC (Table 5.1 and Fig. 5.1B) or metabolic activity (Fig. 5.1A). Since metabolic activity is a robust measure of cell viability (273), measurement of respiratory activity by AlamarBlue was used to independently confirm the effect of limonoids on the growth of

EHEC. An increased rate of metabolic activity was observed in presence of ichangin, isoobacunoic acid and DNAG. Implications of such increased metabolic activity by these limonoids, is not clear as of yet and require further study.

Furthermore, obacunone was found to inhibit the biofilm formation by as much as 69% and 47.5 % at 100 and 50 μg/ml. In comparison, *Delisea pulchera* derived furanone is reported to inhibit 55% biofilm at 60 μg/ml (302). The non linear regression analysis predicted a value of > 56 % reduction in biofilm formation by 60 μg/ml obacunone. Together, these results seem to suggest that the citrus limonoids; specifically isoobacunoic acid, ichangin, obacunone and nomilin suppress biofilm formation in non-growth inhibitory fashion and plausibly in quorum sensing dependent manner. However, biofilm development and establishment is a complex multi-factorial process, which is governed by a combination of environment and genotype (187). Therefore, the possibility of limonoids acting via some other factors cannot be negated.

In EHEC, the initial attachment to epithelial cells is regulated by several factors including TTSS, flagella and fimbriae (217, 357, 364). LEE encoded type three secretion apparatus, effector proteins and intimin (335) plays an important role in adhesion of EHEC to gastrointestinal tract surface. EHEC utilizes AI-2 and AI-3 mediated quorum sensing to regulate expression of its virulence, flagellar and motility genes (17). Since citrus limonoids were found to interfere with AHL, AI-2 mediated cell-to-cell signaling and biofilm formation, their influence on the expression of type III secretion and flagellar genes was studied. Since limonoids interfered with autoinducer mediated cell-cell signaling and biofilm, it was possible the limonoids influence the expression LEE,

flagella and shiga toxin gene. To investigate this hypothesis, expression of *LEE1* and *LEE2* encoded genes upon exposure of limonoids was measured. The isolimonic acid and ichangin treatment repressed the *LEE1* and *LEE2* (Table 5.4) suggesting that isolimonic acid and ichangin may reduce the adhesion of EHEC to Caco-2 cells by repressing the LEE.

In addition to LEE, flagella was reported to play an important role in adhesion of EHEC to epithelium (217). We wondered if the limonoids also affect the expression of genes encoding flagella and measured the expression of flagellar transcriptional regulators *flhDC* using qRT-PCR. The isolimonic acid, ichangin and IOAG repressed the expression of flagellar master regulators (Table 5.4) indicating repression of flagellar operon may also contributes to the reduced adhesion of EHEC to Caco-2 cells in presence of isolimonic acid and ichangin. It is pertinent to note that AI-2/AI-3 mediated cell-cell signaling regulates the expression of flagellar operon and LEE, which subsequently contribute to adhesion and biofilm formation. Therefore, repression of *LEE1*, *LEE2* and *flhDC* indicated a possibility of interference of EHEC QS. Furthermore, Obacunone, which demonstrated potent antagonistic activity in autoinducer and biofilm formation assay, was also a strong inhibitor of type III secretion and flagellar transcription (Table 5.4). This observation is consistent with the idea that limonoids inhibiting the quorum sensing will affect the expression of type III secretion and flagellar genes.

Shiga toxin, produced by the late genes of λ-like bacteriophage (274), is primarily responsible for the EHEC infection associated mortality. Applications of

conventional antibiotics trigger an SOS response causing bacteriophage to enter its lytic cycle, resulting in release of shiga toxin. In addition to *LEE* and flagella, shiga toxin is an important virulence factor in EHEC (356). Furthermore, Sperandio et al (341) demonstrated that expression of *stx2* is positively regulated by quorum sensing. This prompted us to explore the effect of limonoids on the expression of *stx2* gene. We speculated that if limonoids under investigation interfere with EHEC QS system, they may also affect the expression of *stx2*. To test the hypothesis expression of *stx2* upon exposure of 100 µg/ml limonoids was measured by qRT-PCR. Consistent with the hypothesis isolimonic acid, ichangin and obacunone repressed the *stx2*. In addition, IOAG also seems to modulate the expression of *stx2* (Table 5.4).

In order to verify QS dependent effect of limonoids, relative change in expression of *rpoA*, which is not regulated by QS (341), was measured using qRT-PCR. No significant change in the expression of *rpoA* (Table 5.4) by isolimonic acid and ichangin was observed. In contrast, DNAG, which had moderate to no effect on the expression of LEE, *flhDC* and *stx2*, induced *rpoA*, indicating a different mechanism of action. The glucoside IOAG was more effective than its aglycone isoobacunoic acid in suppressing LEE encoded genes, *stx2* and *flhDC*. However, both IOAG and isoobacunoic exposure resulted in induction of *rpoA* suggesting possibly a different mechanism than interaction with QS system. Furthermore, the obacunone and nomilin did not affect the expression of the *ptsN*, which is not regulated by quorum sensing. These results suggest that the observed effect of obacunone and nomilin is probably through inhibition of cell-to-cell signaling. In contrast, the effect of limonin and DAN

may be attributed to the influence on other cellular processes, since *ptsN* was also affected. DAN repressed the *ptsN*, while limonin induced the *ptsN*.

Adhesion of EHEC to intestinal epithelium is an essential early event in colonization of gastrointestinal tract and subsequent initiation of infection (357, 364). The adhesion process in EHEC seems to proceed via two distinct stages, diffuse adherence and microcolony development (357). A potential strategy to counter the effect of EHEC may be reduction of adhesion to intestinal epithelium. Therefore, effect of various limonoids (100 µg/ml) on adhesion of EHEC to colon epithelial cells using in vitro Caco-2 cell model was investigated. The results demonstrated that isolimonic acid and ichangin exposure significantly ($p < 0.05$) reduced the number of EHEC cells attached to Caco-2 cells.

In EHEC, multiple environmental and genetic factors including autoinducer mediated cell-cell signaling (QS), regulates biofilm formation, expression of LEE and flagellar operons (17, 133, 342). In addition, shiga toxin gene *stx2* was reported to be regulated in QS dependent fashion (341). Collectively, with the results of biofilm, adhesion and gene expression assays, repression of LEE, *flhDC* and *stx2* by limonoids seems to suggest that limonoids, in particular isolimonic acid exert their effect through inhibition of EHEC QS system. Since isolimonic acid demonstrated the potent inhibition of biofilm formation, adhesion and LEE, further studies were focused on it.

DMEM + 10% FBS contains epinephrine and was reported to induce the transcription of LEE (314, 342). Since isolimonic acid demonstrated repression of LEE in LB media as well as reduced adhesion of EHEC to Caco-2 (carried out in DMEM +

10 % FBS), it was possible that isolimonic acid represses epinephrine-induced expression of LEE. Repression of relative transcript levels of *ler*, *escJ*, *sepZ* and *stx2* in DMEM grown EHEC by isolimonic acid (Fig. 5.3) indicated an interference with epinephrine-mediated induction of LEE. Collectively, the data suggested that isolimonic acid and ichangin reduce the attachment of EHEC to Caco-2 cells by repressing LEE and flagellar operons.

EHEC utilizes AI-2 and AI-3 mediated cell-cell signaling to regulate biofilm formation, LEE expression, and flagellar operon. Biofilm formation is a complex process and regulated by several factors. In *E. coli*, AI-2/AI-3 mediated signaling is suggested to influence biofilm formation through MsqR and QseBC (133, 161). It was possible that isolimonic acid inhibit the various processes via AI-2/AI-3 mediated cell-cell signaling. To directly test if the isolimonic acid acts as an inhibitor of AI-3 mediated QS in EHEC, activation of LEE1 and *LEE2* in reporter strains was measured (340). Since the purified AI-3 and its structure is not yet available, a preconditioned medium described earlier (340) was used as a source of AI-3 molecules. The isolimonic acid suppressed the activation of both *LEE1* and *LEE2* in response to preconditioned medium indicating inhibition of AI-3 mediated cell-cell signaling. Furthermore, isolimonic acid also repressed the epinephrine mediated induction of *LEE1* by ≈3 fold in the reporter strain TEVS232 (Fig 5.5C). AI-3 binds to its cognate receptor QseC to regulate motility and biofilm formation, whereas interaction with AI-3 results in *qseA* dependent expression of LEE encoded genes (64, 325). Taken together, the biofilm, adhesion assay

and expression studies indicate QseBC and QseA dependent regulation of biofilm and virulence by isolimonic acid.

In order to verify QseBC dependent inhibition, biofilm formation by EHEC strains VS138 and VS179 in presence of 100 µg/ml isolimonic acid was measured. Strain VS138 is a *qseC* mutant, while VS179 is VS138 supplemented with *qseC* (343). We hypothesized that isolimonic acid exposure should not impact the biofilm in *qseC* mutant. As expected, no difference in biofilms formed by VS138 was observed. In contrast, isolimonic acid exposure resulted in a significant decrease in VS179 (*qseC* supplemented strain) biofilm as measured by crystal violet (Fig. 5.6A) indicating QseC dependent inhibition of biofilm. Furthermore, it was speculated that higher expression levels of *qseB* and/or *qseC* will negate the inhibitory effect of isolimonic acid in EHEC. To determine the effect of *qseBC*, the *qseBC, qseB* and *qseC* were expressed under the control of arabinose operon. Induction of *qseBC, qseC* and *qseB* from plasmid relieved the inhibitory effect of isolimonic acid on EHEC biofilm indicating QseBC dependent mechanism. To understand the effect of *qseBC* supplementation on EHEC biofilm, the biofilm formed by different strains was compared. As expected, supplementation of *qseBC/qseB/qseC* resulted in increased biofilm (Fig. 5.6C). However, supplementation of *qseBC/qseC* demonstrated ≈2 fold increase compared to *qseB.* The result indicates that supplementation of *qseB* or *qseC* is sufficient to increase the biofilm formation in EHEC. Based on the current data it was not possible to differentiate if the effect of isolimonic acid is dependent solely upon *qseB* or *qseC*, as supplementation of EHEC by both *qseB* and *qseC* relieved the inhibitory effect. Further studies are required to

precisely determine if the target of isolimonic acid is *qseB* or *qseC*. Altogether the data suggest inhibitory activity of isolimonic acid is dependent on *qseBC*.

Furthermore, we suspected that suppression of *LEE1* and *LEE2* by isolimonic acid was QseA dependent. To verify this hypothesis, a similar approach as used for biofilm was employed. QseA mutant strain VS145 and *qseA* supplemented strain VS151 were employed. The expression of *ler* was measured by qRT-PCR in the two strains, exposed to the 100 µg/ml isolimonic acid. The results demonstrated that expression of *ler* was not significantly altered in *qseA* mutant (VS138), whereas a 7.4 fold repression (Fig. 5.7A) of *ler* was observed in *qseA* supplemented strain (VS179). Furthermore, expression of *qseA* from multicopy plasmid *pVS150* in AVS46 countered the repressive effect (Fig. 5.7B) of isolimonic acid on *LEE1* observed in Fig. 5.5A. These data suggest that repression of LEE by isolimonic acid is mediated by *qseA*. Altogether the results of the study provide evidence that isolimonic acid is a potent modulator of AI-3/epinephrine mediated cell-cell signaling in EHEC and its effect is *qseBC/qseA* dependent. A speculative model of possible interaction of isolimonic acid is presented in Fig. 5.8. It is possible that it actually interferes with the signal transduction process, however such a possibility require further investigations.

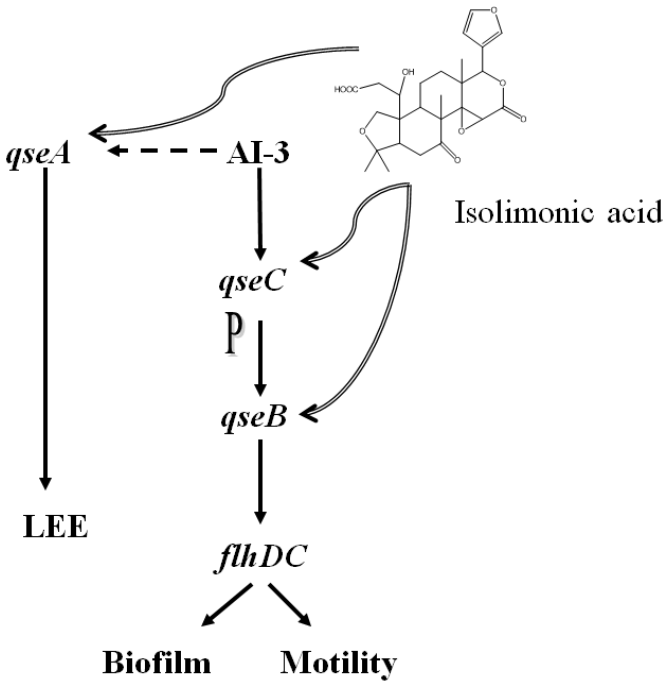

FIG. 5.8. Speculative model of isolimonic action on EHEC. Broken arrow indicate unknown mode of interaction of AI-3 with *qseA.* Wavy arrows indicate interaction of isolimonic acid and *qseC* and *qseA* is unknown

Citrus limonoids have a common parent structure with B, C, and D rings except the variation in A and A' rings. This may be responsible for differences in antagonistic activity of the limonoids. In limonin (bitter compound) and limonin glucoside (non-bitter compound), the skeleton is a collection of five rings A, A, B, C and D. In contrast, A (five member) and A′ (six member) rings are modified into a seven member A ring in case of nomilin, DAN and obacunone. Furthermore, in case of isolimonic acid and ichangin, the A ring is modified. Together, the data suggests that, change in A ring may be important determinants of the effectiveness of the activity. Obacunone contains a double bond between C1 and C2, while in case of nomilin and DAN, hydroxyl and

acetoxy groups are attached at C2 position, respectively. Presence of double bond at C2 position enhances the antagonistic activity against TTSS. In other words, the loss of activity may be due to change in the conformation of large part of the molecule due to the saturation of ring A by introduction of acetoxy (nomilin) and hydroxyl group (DAN) at C-1. On the other hand, presence of A′ ring seems to diminish the activity for limonin and limonin glucoside. Furthermore, modification in D ring does not seem to affect the antagonistic activity against TTSS. Presence of A and A′ rings are important for inhibition of oral carcinogenesis, where limonin was better antagonist compared to obacunone and nomilin (242). In contrast, this study exhibit presence of A′ ring is detrimental to the activity of limonoids against TTSS.

A significant amount of limonoids are ingested by consumption of citrus fruits and juices including grapefruit. The present study elucidates a possible interaction of putative dietary limonoids with EHEC. The results of the current study suggest that the citrus limonoids possess the anti-quorum sensing activity and are antagonistic to biofilm of *E. coli* O157:H7. In the present study, we have shown that the citrus limonoids, particularly isolimonic acid and ichangin inhibit biofilm formation and attachment of EHEC to Caco-2 cells. Furthermore, structure of A ring seems to be critical for the antagonistic activity of limonoids. Further studies involving modification at B and C ring are needed to elucidate the role of these functional groups in relation to antagonistic activity and to elucidate the structure-activity relationship. Furthermore isolimonic acid and ichangin repressed the *LEE1* and *LEE2* operons in EHEC. The results of the study seem to suggest that at least one limonoid *i.e.* isolimonic acid exerts its effect by

inhibiting AI-3/epinephrine mediated cell-cell signaling. Isolimonic acid seems to inhibit the AI-3 mediated cell-cell signaling in QseBC and QseA dependent manner. However, the mechanism by which isolimonic acid affects the QseBC and QseA remains to be elucidated. Apart from LEE and flagella, adhesion of EHEC to epithelium is regulated by several factors such as long polar fimbrae and other unidentified determinants (357, 364). In the present report we have not investigated these factors. It is possible that limonoids may act on EHEC through these factors. Further research is required to elucidate the effect of limonoids on such factors. Moreover, limonoids such as isolimonic acid may serve as lead compound for development of preventive and therapeutic strategies.

CHAPTER VI

INHIBITION OF *E. COLI* BIOFILM BY LIMONIN ANALOGUES

6.1 Synopsis

Citrus limonoids were reported to inhibit autoinducer-mediated bacterial cell-cell signaling, *Escherichia coli* O157:H7 biofilms and the Type III secretion system. The goal of the present study was to understand the effect of specific modifications in limonin molecule upon cell-cell signaling inhibitory activity. Two natural citrus limonoids (limonin, limonin glucoside) and three modified limonoids (de-furan limonin, limonin 7-oxime, limonin 7-methoxime) were examined for their cell-cell signaling and biofilm inhibitory properties. The results of the study suggest that the furan ring and C-7 substitutions are important structural features in the limonin molecule to inhibit cell-cell signaling and biofilm formation in *V. harveyi*. In addition, the C-7 modifications of limonin showed increased inhibitory activity against the *E. coli* biofilm formation. In addition, limonin 7-methoxime seems to reduce the adhesion of *E. coli* to colon epithelial cells. Furthermore the data indicates that limonin-7-methoxime reduce the attachment of *E. coli* to plastic surface possibly by interacting with type 1 pili and Ag43, resulting in reduced biofilm levels.

6.2 Introduction

Hospital acquired infections cause about 1.7 million illnesses and 99,000 associated deaths each year in US (323), with a conservative estimate for cost of treatment being $ 5.7-6.8 billion (323). Majority of these infections are urinary tract infections caused by pathogenic *Escherichia coli* strains (323). Furthermore, 65% of these nosocomial infections are related to biofilms (216). Biofilms demonstrate tolerance/resistance to antimicrobials thereby limiting the treatment options. Several mechanisms were suggested for the observed behavior of biofilms. In the biofilm, the bacterial cells are attached to each other and to a living or non-living surface in a highly organized structure (69). Exopolysaccharide is the most common component of the biofilm and seems to restrict the access of antimicrobials to the microbial cells buried in the matrix (69). In addition, biofilm components may react with antimicrobials or limit the sorption thereby reducing the transport of the compound through the biofilm (216). It was suggested that bacterial cells growing in biofilms experience nutrient limitations and consequently depict a slower growth rate (48). Since majority of the antimicrobials target actively growing cells, they fail to profoundly impact the slow growing cells in biofilm (48). Furthermore, heterogeneous bacterial population and stress factors may also contribute to antimicrobial resistance (216). Given that the biofilm cells demonstrate higher degree of resistance compared to planktonic cells, it is imperative to search for antimicrobials with non-conventional targets.

Bacterial biofilm formation is regulated by several genetic and environmental factors, including surface-adhesion-proteins, pili, flagella, pili and extracellular

polymeric substance matrix (137). In addition, autoinducer (AI)-2 and AI-3 mediated cell- cell signaling was reported to regulate the biofilm formation in *Escherichia coli* (161, 206). AIs are small molecules produced and secreted by the bacteria. At or above threshold concentration of AIs, binding with cognate receptor initiates a signal transduction cascade resulting in change in gene expression profile on population wide scale. This phenomenon is now known as quorum sensing. *V. harveyi* AI-2 molecule, (2S, 4S)-2 methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF-borate) is a furanone (59), whereas LsrB, the AI-2 receptor present in *Salmonella* Typhimurium and *E. coli*, binds to another furanoid, (2R, 4S)-2 methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) (244). Furthermore, cell-cell signaling was suggested as a novel target to control bacterial pathogenesis (293). Altogether, antagonizing AI-2/AI-3 mediated cell-cell signaling may provide a new avenue to modulate *E. coli* biofilms. In support of this idea, several plant extracts and pure compounds from our lab such as limonoids, flavonoids and furocoumarins from *Citrus* spp. (130, 371, 374-375), and elsewhere (garlic extracts, halogenated furanone from *Delissa pulcherra*) (156, 291), were reported to modulate bacterial biofilms by inhibiting cell-cell signaling.

Citrus limonoids are degraded tritepenoids (C_{26}), characterized by high degree of oxidation and presence of furan ring at C-17 (140). Structurally, citrus limonoids can be classified into two groups. First group represented by limonin (Fig. 6.1) contains five rings designated as A, A', B, C and D; whereas second group, exemplified by nomilin, contain 4 rings A, B, C and D. In nomilin A and A' rings are fused and modified to seven-membered heterocyclic A ring containing an oxygen atom. The various structural

features in limonoids are suggested as important determinant for differential biological activities of limonoids. Our recent study demonstrated that furan ring and C7 position ketone group are involved in enhanced expression of phase II enzymes glutathione-S-transferase (GST) and quinone reductase (195, 268). Furthermore, a strong induction in GST activity by a limonin analogue, limonin 7-methoxime (LM) (Fig. 6.1) was observed, suggesting C7 position plays an important role in the bioactivity (268). In addition, based on structure-activity studies, the epoxide and furan functional groups were reported as essential features for anti-feedant activity (38).

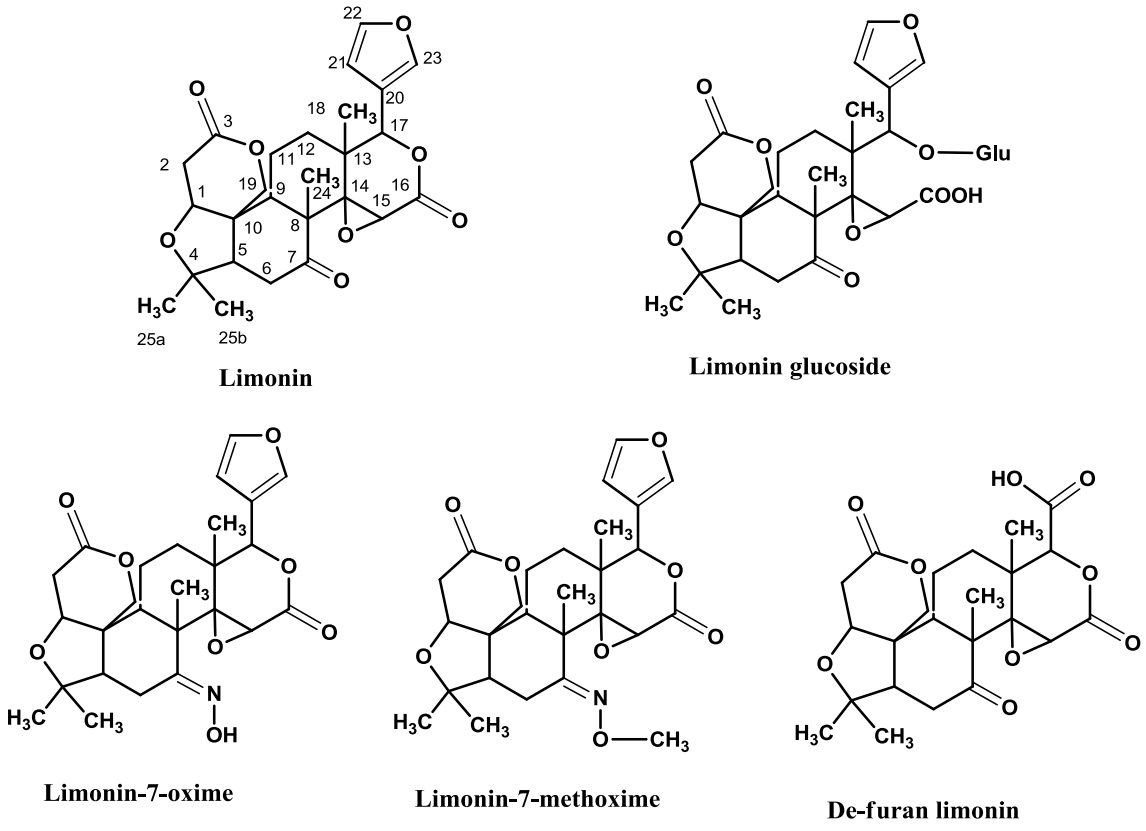

FIG. 6.1. Structure of purified limonoids and limonin analogues

Recently our investigation of cell-cell signaling inhibitory activities of citrus limonoids, a differential inhibition of cell-cell signaling by various limonoids was observed (374-375). We were further interested in studying the effect of these limonin analogues containing furan ring and C7 modifications on cell-cell signaling and biofilm. The current study investigated the effect of modifications at key positions in limonin molecule on the cell-cell signaling and biofilm formation.

6.3 Materials and Methods

6.3.1 Materials

All chemicals/HPLC grade solvents and thin Layer chromatographic silica gel 60F-254 plates were purchased from Fisher Scientific (Pittsburgh, PA). Molecular biology grade dimethyl sulfoxide (DMSO), pyridine and hydroxylamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO).

6.3.2 Isolation and Modification of Limonoids

Limonin and limonin glucoside were purified as reported earlier (223, 375). LM and DFL were prepared as per our recently published method (268).

6.3.3 Preparation of Limonin 7-Oxime (LOX)

Limonin (1.12 g) was dissolved in pyridine (20 mL), and hydroxylamine hydrochloride (1.26 g) was dissolved in absolute ethanol (20 mL) and transferred to the round-bottom flask. The reaction mixture was refluxed for 6 h at 75 ^{o}C - 80 ^{o}C and cooled to room temperature. The completion of the reaction was determined by the disappearance of the limonin spot on TLC using chloroform:acetone (9:1) as the mobile phase. Saturated solution of sodium chloride was added to the cold reaction mixture until

the complete precipitation of pyridinium chloride, which was removed by filtration. The filtrate was extracted using ethyl acetate (4 x 25 mL). The pooled ethyl acetate layer was washed with 2 N HCl (2 x 50 mL), followed by washing with sodium bicarbonate (2 x 50 mL), and finally water. The organic phase was concentrated under vacuum to obtain the precipitate. Further purification was performed by silica gel column chromatography. The column was eluted with chloroform and acetone as the mobile phase. Limonin-7-methoxime was eluted at 10% acetone in chloroform.

6.3.4 HPLC Analysis

The analysis of purified and modified compounds was performed using Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA). The HPLC system consisted of a quaternary pump, a photodiode array detector, degasser and an autosampler. A reverse phase Gemini C_{18} column (4.6 × 250 mm, 3.5 micron) was used for the chromatographic separation with a C_{18} security guard column from Phenomenex (Torrance, CA) was used to protect the column. ChemStation LC software was used for data acquisition and analysis.

6.3.5 Identification of Limonin 7-Oxime

The structures of the purified limonoids and modified compounds were elucidated by 1H, ^{13}C NMR, and DEPT studies using a JEOL ECS-400 spectrometer at 298 K using a 5 mm broadband probe equipped with a shielded z-gradient and Delta software version 4.3.6 using TMS as an internal reference. One-dimensional ^{1}H and ^{13}C spectra were obtained using one pulse sequence.

6.3.6 Bacterial Strains and Media

E. coli O157:H7 ATCC 43895 (EHEC), *Vibrio harveyi* MM30 and *V. harveyi* MM32 were purchased from ATCC (Manassas, VA, USA). The *E. coli* K12 mutant strains BW29659 (404), JW3350, JW3933, JW4275 and JW4283 (15) were obtained from Coli Genetic Stock Center, Yale University (New Haven, CT). *E. coli* #5 (environmental isolate) a known producer of AI-2, was used as a positive control for AI-2 bioassay. *V. harveyi* BB120 was a kind gift from Dr. B. B. Bassler, Princeton University, USA. The autoinducer bioassay (AB) medium was used to culture the *V. harveyi* strains (212, 346). *E. coli* biofilms were grown in Luria Broth (LB), M63 and colonization factor antigen (CFA) (233) media (102, 172).

6.3.7 Assay for Inhibition of Autoinducer Molecules

The bioluminescence assay was carried out as described previously (371, 375). In brief, cell free culture supernatants (CFS) were prepared from environmental isolate of *E. coli* (#5) and *V. harveyi* BB120 as described (371). The bioluminescence assay was conducted in 96 well plates with five different concentrations of limonoids viz. 6.25, 12.5, 25, 50 and 100 µg/mL. Overnight cultures of reporter strains MM30 (for HAI) and MM32 (for AI-2) were diluted 5000 fold. The fresh culture (90 µl) was incubated with 5 µl CFS, 0.5 µl of limonoids or DMSO and 4.5 µl sterile AB media. The reactions were placed in Nunc 96 –well plates (Fischer Scientific, Pittsburg, PA, USA) and incubated at 30^{o}C; 100 rpm. Light production was measured by using a Victor^{2} 1420 multilabel counter (Beckman Coulter, Fullerton, CA) in luminescence mode. The values were recorded as relative light units (RLU) and used in calculation (212).

6.3.8 Growth of *V. harveyi* BB120 and *E. coli* K12

Growth of *V. harveyi* BB120 and *E. coli* K12 was monitored for 16 h in presence of 100 µg/ml LM, LOX and DFL or equivalent volume of DMSO as previously described (374). Briefly, overnight cultures were diluted 100 fold in appropriate media and placed in 96-well plates along with LM, LOX and DFL. The OD_{600} was measured every 15 min using the Synergy™ HT Multi-Mode Microplate Reader (BioTek, Instruments, Winooski, VT) on three biological replicates consisting three technical replicates. The mean OD_{600} values were plotted against time using EXCEL 2007.

6.3.9 Biofilm Assay

Biofilm assays were conducted in 96-well plates as previously described (375). Briefly, overnight cultures *V. harveyi* BB120, *E. coli* O157:H7 strain ATCC 43895 and *E. coli* K12 were diluted 50 fold and incubated with 6.25, 12.5, 50, or 100 µg/ml of test compounds. The appropriate amount of DMSO was added to negative and positive controls to eliminate solvent effects. Biofilm mass was quantified by staining with 0.3% crystal violet (Fisher, Hanover Park, IL) for 20 min. The wells were washed with phosphate buffer (0.1 M, pH 7.4) to remove extra dye. The biofilm associated dye was dissolved with 200 µl of DMSO, and absorbance was recorded at 570 nm. Data from three plates, each containing three replicate wells, for each experiment were averaged and expressed as mean and one standard deviation.

6.3.10 Adhesion of *E. coli* K12 to Epithelial Cells

Colon adenocarcinoma epithelial Caco-2 (ATCC # HTB-37) cells were routinely maintained in DMEM supplemented with 10 % fetal bovine serum. For adhesion assay,

Caco-2 cells were plated in 6 well plates at a density of 1 x 10^5 cells/well. The Caco-2 cells were infected with *E. coli* K12 cells at a multiplicity of infection (moi) 10, in presence of 100 µg/ml LM or equivalent amount of DMSO. The plates were incubated for 4 hr at 37^{o}C, 5 % CO_2. The excess *E. coli* K12 cells were washed three times with PBS. The Caco-2 cells were lysed by 0.1 % TritonX-100 solution. The resulting culture was then serially diluted using 0.9 % saline solution and plated on the LB Agar plates. Colonies were counted after 24 hrs and recorded as Colony Forming Units (CFU).

6.3.11 Attachment *E. coli* K12 to Plastic Surface

The assay was essentially conducted as described Genevaux et al (128). The overnight culture was diluted 100 fold in LB-glu. In first set of experiments, LM (100 µg/ml) or DMSO was added to the diluted cultures and incubated at 37^{o}C, 200 rpm until $OD_{600} \approx 0.6$. At this point 200 µl aliquot was dispensed in 96-well plates. While in the second set, the diluted overnight culture was grown to $OD_{600} \approx 0.6$, and the LM (100 µg/ml) or DMSO was added just before placing the culture in the 96-well plate. The cultures were then allowed to adhere for 30 at 37^{o}C without shaking. The supernatant was decanted and attached cells were fixed at 80^{o}C for 30 min followed by staining with 0.3 % crystal violet. The plates were then processed as described in the biofilm assay.

6.3.12 Uptake of LM and Limonin by *E. coli* K12

Freshly diluted (100 fold) overnight culture was treated with LM (100 µg/ml) or limonin (50 µg/ml) and incubated at 37^{o}C, 200 rpm. Sample (1 ml) was collected at 0, 2, 4, 6 and 8 h and centrifuged at 10,000 rpm for 5 min in a microcentrifuge (Eppendorf). The cell pellet was washed with 500 µl PBS, which was collected by centrifugation and

mixed with 1 ml supernatant in earlier step. As a control, tubes with heat-killed (5 min at 80^{o}C) *E. coli* K12 cells were treated in similar manner as live cells and samples were collected and processed. All the samples were filtered through 0.2 µ membrane filter (Nalgene) and analyzed as described in 'HPLC Analysis' section.

6.3.13 Statistical Analysis

The inhibition of AI activity was calculated from the formula 100- [(relative AI activity/relative activity of positive control) x 100] (212) and expressed as percentage and SD values. The per cent inhibition of biofilm formation was calculated as 100- [(OD_{620} of sample well/ OD_{620} of positive control) x 100] and expressed as percentage and SD values. Nonlinear regression analysis and t-test were conducted with SIGMAPLOT 11.0 (Systat Software, Inc., San Jose, CA). The data for biofilm and luminescence assay were fitted to a 3-parameter sigmoid equation $y= a/(1+\exp^{(-(x-x0)/b)})$ using SIGMAPLOT 11.0 (Systat Software, Inc.). In order to conduct the analysis, concentration of each limonoids was converted to Log_{10} µM and plotted against percent inhibition values.

6.4 Results

6.4.1 Purification and Modification of Limonoids

Ethyl acetate extract of citrus seeds yielded two limonoids i.e. limonin and limonin 17-β-D-glucoside (LG). Limonin was further modified to obtain de-furan limonin (DFL), limonin 7-methoxime (LM) (268) and limonin 7-oxime (LOX). Purity of the isolated limonoids was analyzed by HPLC as per our published method (372). The chromatograms of purified and modified limonoids are presented in Fig. 6.2. Further,

structures of the purified limonoids and modified compounds (Fig. 6.1) were determined by spectrometric studies. Chemical shifts of limonin, limonin 17-β glucopyranoside (LG), DFL and LM were recorded as described in 'Methods' and compared with previously reported data (223, 268, 278). The ^{1}H and ^{13}C spectra of LOX with assignments of various signals are presented in TABLE 6.1.

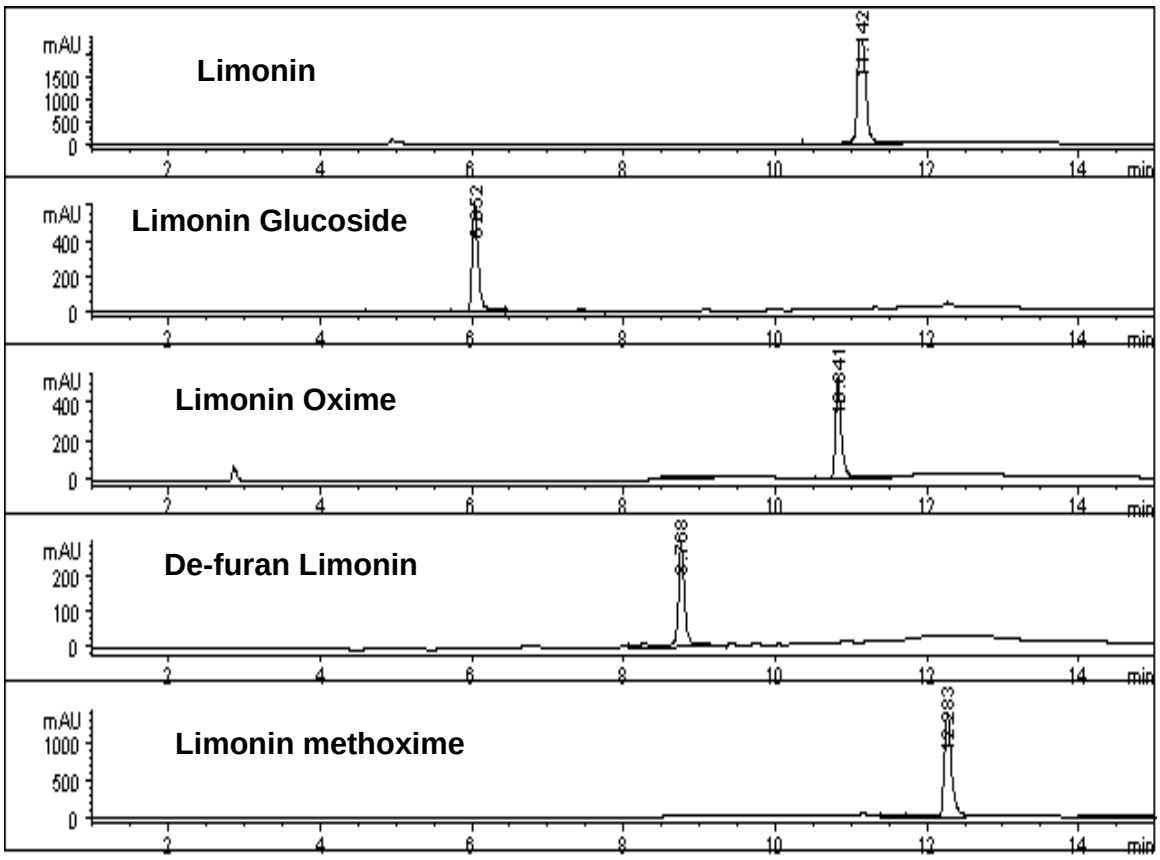

FIG. 6.2. HPLC chromatograms of purified limonoids and limonin derivatives

TABLE 6.1. Chemical shifts of limonin 7-oxime in DMSO-d6. (s=singlet, d=doublet, Br.S= Broad singlet)

#	**Limonin oxime**		
	Carbon type	^{13}C	^{1}H
1	CH	79.1	4.09 (d, 2H)
2	CH2	32.7	1.49 (d, 1H), 1.80 (d, 1H)
3	C	170.8	
4	C	80.3	
5	CH	59.8	1.80 (d,2H)
6	CH2	36.8	2.85 (d, 1H), 2.78 (d, 1H)
7	C	159.4	
8	C	45.7	
9	CH	49.7	2.42 (d, 2H)
10	C	45.9	
11	CH2	19.3	2.22 (d, 2H)
12	CH2	19.1	2.10 (d, 2H)
13	C	39.2	
14	C	66.0	
15	CH	54.7	3.76 (s, 1H)
16	C	167.7	
17	CH	78.3	5.46 (s, 1H)
18	CH3	18.1	1.13 (s, 3H)
19	CH2	65.7	4.87 (d, 1H), 4.55 (d, 1H)
20	C	120.8	
21	CH	143.6	7.47 (d, 1H)
22	CH	111.6	6.63 (Br.s, 1H)
23	CH	142.3	7.49 (d, 1H)
24-	CH3	21.4	1.01 (s)
25a	CH3	22.5	0.93 (s)
$25b_3$	CH3	30.7	1.21 (s)

6.4.2 Inhibition of Autoinducer Mediated Bioluminescence

V. harveyi reporter strains MM30 and MM32 were employed to understand the effect of various modifications of limonin on N-(3-hydroxybutanoyl) homoserine lactone (AHL) and AI-2 mediated cell-cell signaling. Limonin analogues were prepared as described in ‘Methods’ and assayed for interference of AHL and AI-2 induced

bioluminescence. Inhibitory activities of modified limonoids were compared to limonin. A loss of activity for all the three modified limonoids compared to limonin was observed (Fig. 6.3). Limonin demonstrated an inhibition of ≈14-57% AHL induced bioluminescence. In contrast, LG, LM (4-26 %), LOX and DFL (7-27 %) demonstrated <50 % inhibition of AHL-induced bioluminescence, hence IC_{50} values could not be calculated (Fig. 6.3A, B and D). LOX did not inhibit the AHL induced bioluminescence. Furthermore, limonin inhibited the AI-2 induced bioluminescence by ≈50-70 %. The LM and LOX inhibited AI-2 induced bioluminescence by 1-45 % and 7-39 % respectively. DFL was ineffective against AI-2 mediated bioluminescence (Fig. 6.3B). However, for LOX and LM, greater loss in AHL inhibitory activity compared to limonin was observed. In contrast, D-furan limonin demonstrated greater loss in AI-2 inhibitory activity. LG demonstrated an overall loss in AHL and AI-2 mediated activity compared to limonin (Fig. 6.3 B and D).

6.4.3 Growth rate of *E. coli* K12 and *V. harveyi* BB120

To ascertain the effect of various limonin analogues on the growth of *E. coli* K12 and *V. harveyi* growth, the OD_{600} was monitored upto 16 h. The results demonstrate that LOX, LM and DFL did not affect the growth of *E. coli* K12 (Fig. 6.4A) and *V. harveyi* (Fig. 6.4B) at 100 µg/ml.

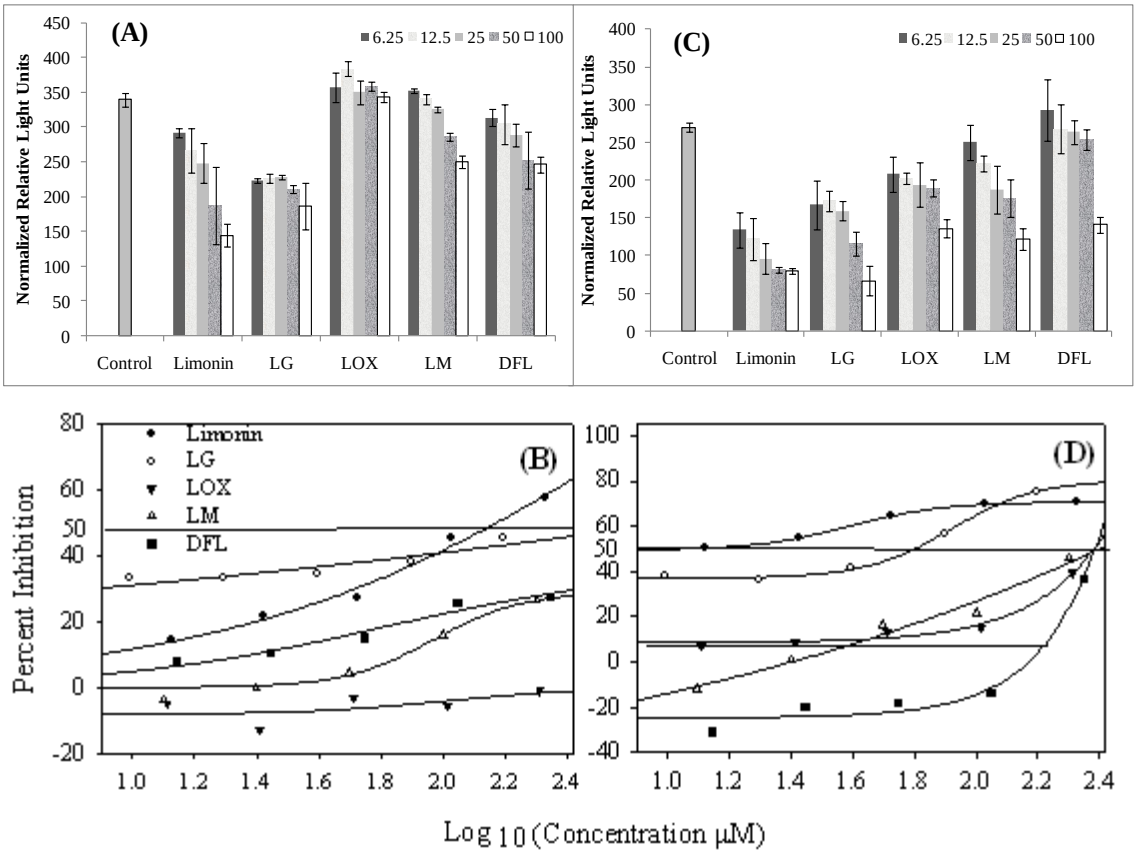

FIG. 6.3. (A) Inhibition of AHL and (C) AI-2 mediated bioluminescence by limonin and its derivatives as measured by *V. harveyi* reporter assay. Three-parameter sigmoidal model of (B) AHL and (D) AI-2 mediated bioluminescence by limonin and its derivatives. The data is presented as mean and standard deviation of three independent experiments consisting of three replicates each. The legend of (A) and (C) presents the different tested concentrations in µg/ml

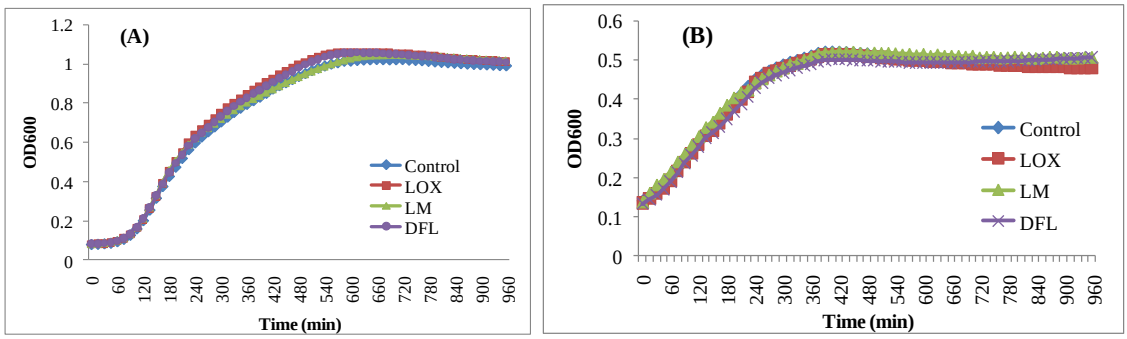

FIG. 6.4. Growth rate of (A) *E. coli* K12 and (B) *V. harveyi* BB120 in presence of LOX, LM and DFL

6.4.4 Inhibition of *V. harveyi* BB120 and *Escherichia coli* O157:H7 Biofilm

The present study compared the potential of limonin and limonin-derivatives to inhibit EHEC and *V. harveyi* BB120 biofilms grown in 96-well plates. *V. harveyi* BB120 biofilm were grown in Luria-marine media at 26^{o}C. Compared to limonin, the limonin derivatives were less effective in inhibiting *V. harveyi* BB120 biofilm formation (Fig. 6.5A and B). DFL, LM and LOX demonstrated 23-46 %, 4-41 % and 6-38 % inhibition of *V. harveyi* BB120 biofilm, respectively, compared to limonin (37-49 %) and LG (22-52 %) (Fig. 6.5A).

In case *E. coli* O157:H7, LM, LOX and DFL depicted a significantly ($p < 0.05$) higher inhibition of biofilm (grown in LB-glu at 26^{o}C) formation (Fig. 6.5C and D) compared to limonin. LM and LOX inhibited *E. coli* O157:H7 biofilm by ≈ 49 and 51 % at 100 µg/ml, whereas limonin, LG and DFL demonstrated < 50 % inhibition. In order to compare the activities of different compounds, data was fitted to 3-parameter sigmoid plots and IC_{25} values were calculated. The IC_{25} values of limonin, LM, LOX and DFL were 125.53, 19.04, 41.61 and 64.91 µM, respectively (Fig. 6.5D). The IC_{25} value of LG was not calculated since LG demonstrated <25 % inhibition in the tested concentration range.

6.4.5 Effect of Limonin and Derivatives on *E. coli* K12 Biofilm Grown Under Different Conditions

To understand the effect of limonin and its derivatives on different adhesins, *E. coli* K12 biofilms were studied under varying media and temperature conditions. All the tested compounds demonstrated significant ($p < 0.05$) inhibition of *E. coli* K12 biofilm

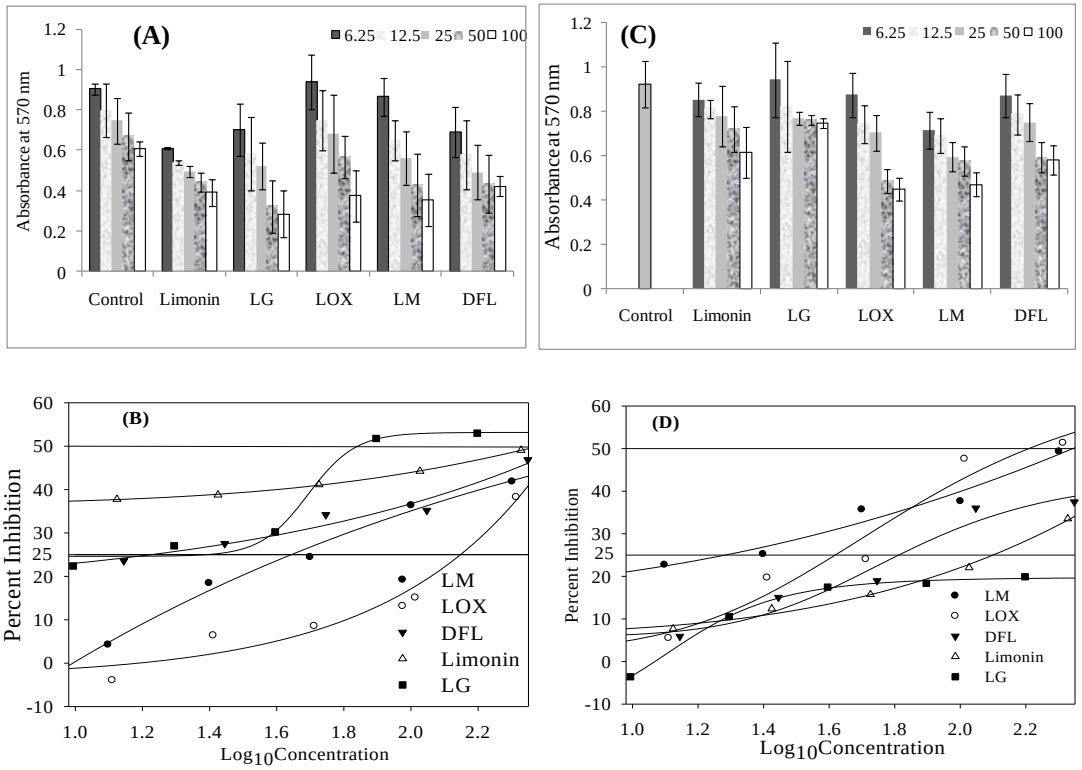

FIG. 6.5. (A) Inhibition of *V. harveyi* BB120 biofilm by limonin and its derivatives. (B) 3-parameter sigmoid plots of *V. harveyi* BB120 biofilm formation at 24 hrs in presence of limonin and its derivatives quantified by crystal violet. (C) Inhibition of *E. coli* O157:H7 biofilms in presence of modified limonoids. The data represents mean of three independent experiments and standard deviation. (D) 3-parameter sigmoid plots of *E. coli* O157:H7 biofilm inhibition by limonin and its derivatives. Concentration values were converted in micromolar quantities and plotted as $\log_{10}$. The legend of (A) and (C) presents the different tested concentrations in µg/ml

grown in LB-glu medium (Fig. 6.6A). However, no difference among the various compounds was discernible ($p > 0.05$) for biofilms grown in LB-glu at tested concentrations. In minimal glucose medium M63 (Fig. 6.6B), limonin did not inhibit *E. coli* K12 biofilm M63 ($p > 0.05$). In contrast LG, DFL, LM and LOX demonstrated significant ($p < 0.05$) inhibition of the M63 grown *E. coli* K12 biofilm. LM was the most

potent inhibitor of *E. coli* biofilm in minimal medium and demonstrated 17-34 % inhibition (Fig. 6.6B) followed by LOX (10-22%). However, none of the compounds inhibited *E. coli* K12 biofilms grown in CFA at 26^{o}C (Fig. 6.6C). In contrast, significant ($p < 0.05$) inhibition of *E. coli* K12 biofilms grown in CFA medium at 37^{o}C, by LM (16-42%) was observed (Fig. 6.6D). Furthermore, inhibition by LM was significant only at concentrations of 50 μg/mL and 100 μg/mL.

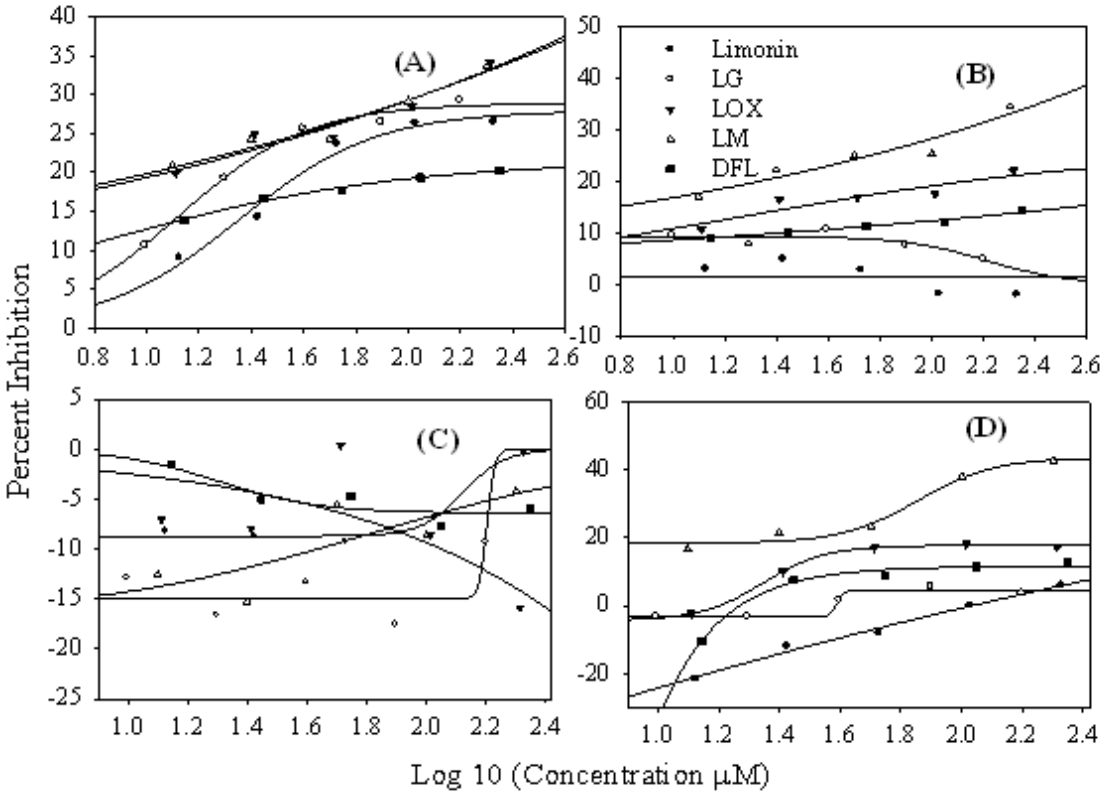

FIG. 6.6. (A) Inhibition of *Escherichia coli* K12 biofilms, grown in LB-glucose, by limonin and its derivatives. (B) Inhibition of *Escherichia coli* K12 biofilms, grown in minimal medium M-63, by limonin and its derivatives. (C) Inhibition of *Escherichia coli* K12 biofilms, grown in CFA medium at 26^{o}C, by limonin and its derivatives. (D) Inhibition of *Escherichia coli* K12 biofilms, grown in CFA medium at 37^{o}C, by limonin and its derivatives. The data represents mean of three independent experiments

6.4.6 Differential Effect of Limonin Methoxime in Pili and Antigen 43 Mutant Strains

To determine the effect of mutations in pili and Ag43 on biofilm inhibitory activity of limonin analogues, deletion mutant in *fimB*, *fimH*, *dam* and *oxyR* from Keio collection (15) and *fimZ* (404) were used. The biofilms of strains BW29659 (Δ*fimZ*), JW4275 (Δ*fimB*) and JW4283 (Δ*fimH*) were grown in LB-glu media as described in "Methods" in presence of limonin analogues. LOX, LM and DFL did not affect the biofilm formation ($p > 0.05$) in BW29659, JW4275 and JW4283 (Fig. 6.7A). Further, JW3350 (Δ*dam*) and JW3933 (Δ*oxyR*) biofilm were grown in M63 glucose minimal medium. LOX, LM and DFL were not inhibitory to JW3350 biofilm (Fig. 6.7B). However, LM significantly ($p < 0.01$) inhibited the JW3933 biofilm grown in 96-well plates, whereas LOX and DFL did not demonstrate differential effect on JW3933 biofilm (Fig. 6.7B).

6.4.7 Adhesion of *E. coli* K12 to Caco-2 Cells

E. coli cells use pili to attach to epithelial cells. Inhibition of *E. coli* to plastic surface in pili dependent mechanism by LM indicated that LM may reduce the adhesion to epithelial cells. To investigate this possibility, adhesion of *E. coli* K12 to colon epithelial cells (Caco-2) in presence of 100 µg/ml LM was measured. A small (0.59 Log_{10} CFU) but significant ($p<0.05$) reduction in number of adhered *E. coli* K12 to Caco-2 cells was observed (Fig. 6.8).

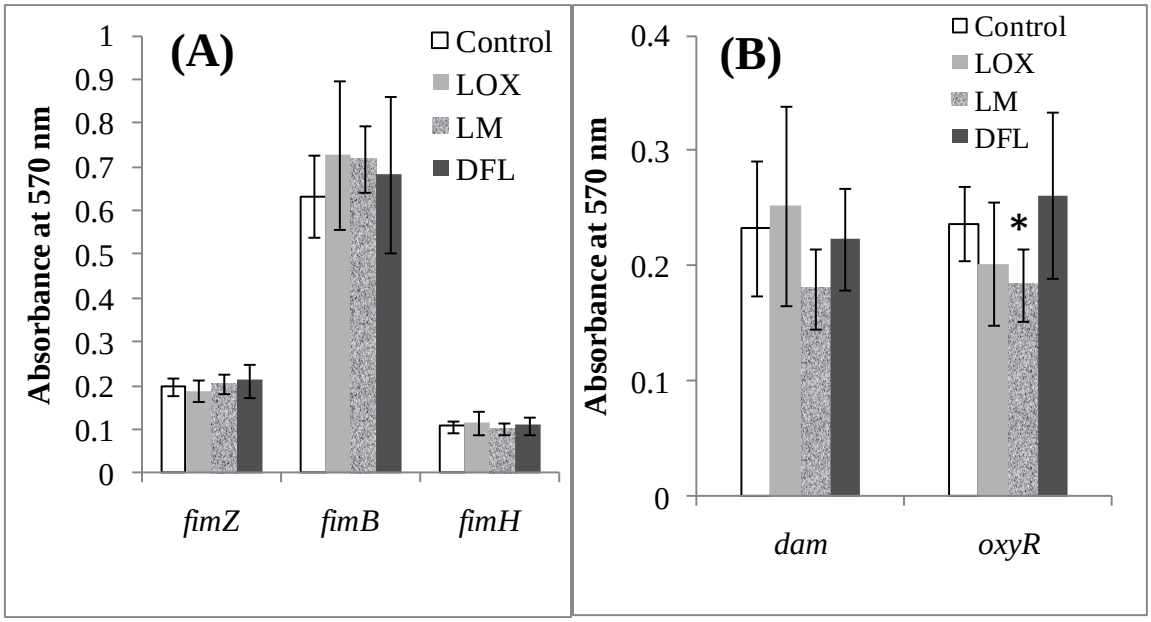

FIG. 6.7. Biofilm formation by mutant strains affecting (A) type 1 pili and (B) Ag43 in presence of 100 µg/ml of limonin analogues

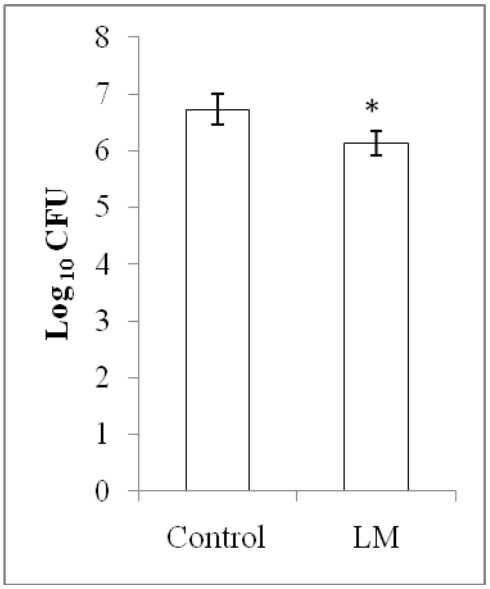

FIG. 6.8. Adhesion of *E. coli* K12 to Caco-2 cells in presence of 100 µg/ml of LM

6.4.8 Effect of LM on Attachment of *E. coli* K12 to Plastic Surface

In order to further understand the possible mechanism through which LM inhibits the biofilm formation, attachment of *E. coli* K12 cells to plastic surface in 96-well plate was studied. In first case, the *E. coli* K12 was grown to OD600 ≈ 0.6 in presence of LM or DMSO and then placed in 96-well plates (Condition A), while second set of experiment LM was added just prior to placement in 96-well plates (Condition B). A significantly ($p < 0.05$) lower number of cells were attached to the plastic surface when LM was added to the culture prior to attachment of *E. coli* to 96-well plate (Fig. 6.9, Condition B). However, no difference in total attached cells was observed (as measured by crystal violet) when *E. coli* K12 was grown in presence of LM (Fig. 6.9, Condition A).

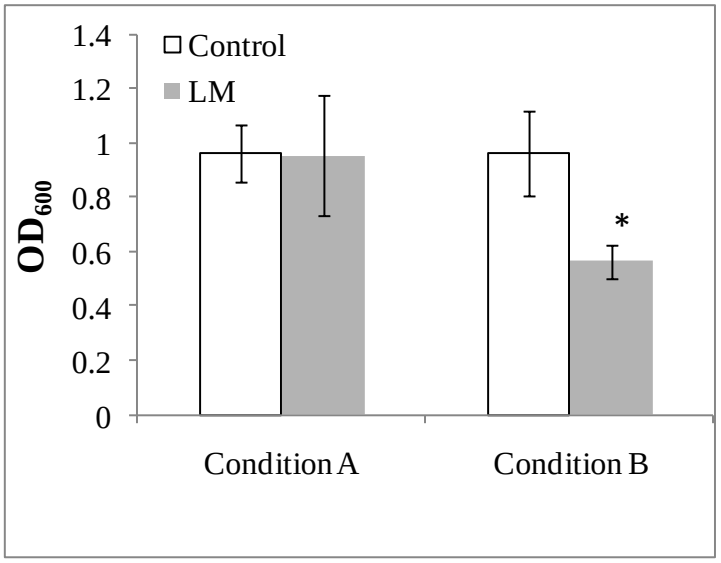

FIG. 6.9. Attachment of *E. coli* K12 to plastic surface in 96-well plate (see text for details). In Condition A *E. coli* cells were grown in presence of LM and DMSO to OD600 ≈ 0.6, while in Condition B the *E. coli* cells were exposed to LM or DMSO after they reached OD600 ≈ 0.6 and just prior to placement in 96-well plate

6.4.9 Uptake of LM and Limonin by *E. coli* K12

The supernatant of *E. coli* K12 cells grown in presence of LM or limonin was collected at different time intervals and levels of LM and limonin were assessed by HPLC. To eliminate the effects of non-specific binding, heat-killed cells were treated with LM or limonin and samples were taken at each time point. The LM detected in supernatant was consistently less until 2 h compared to control. However, at later time point adsorption pattern of live cells and heat killed cells was similar (Fig. 6.10A), indicating no uptake of LM by *E. coli* K12 cells. In contrast, limonin seems to be actively adsorbed by *E. coli* cells (Fig. 6.10B).

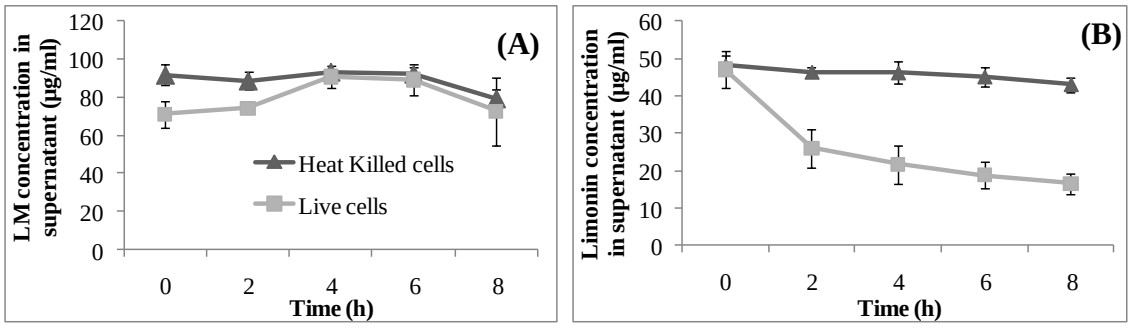

Fig. 6.10. Uptake of (A) LM and (B) limonin by *E. coli* K12 cells

6.5 Discussion

Recently, we reported that limonin and LG inhibit cell-cell signaling in *V. harveyi* reporter assay. In addition, limonin and LG inhibited biofilm formation in *V. harveyi* BB120 and *E. coli* O157:H7 (375). The results of the present study for limonin

and LG were comparable to previous reported result (375). In this study, we were interested in understanding the role of structural components of limonin involved in the cell-cell signaling inhibition. For this purpose, four derivatives of limonin along with the parent limonin were evaluated in bioluminescence and biofilm assay. A general loss in activity in bioluminescence assay was observed with each modification. In addition, inhibition of bioluminescence due to effect of growth was ruled out as limonin analogues did not alter the growth of *V. harveyi* and *E. coli* K12.

Furan ring and C7 position on the limonoid molecule were reported to enhance phase II enzyme, GST and QR, activities (268). In addition, methoxylation at C7 position was reported to enhance that activity against breast cancer cells (135). We were interested in the effect of these various modifications on bacterial cell-cell signaling. To investigate the effect of furan ring, limonin was modified to generate DFL and this molecule was tested for inhibitory activity in autoinducer mediated bioluminescence model. Replacement of furan ring with carboxylic acid group at C17 position resulted in loss of inhibitory activity for AHL and AI-2 induced bioluminescence. However, the loss in AI-2 mediated signaling inhibition for DFL was more pronounced than AHL induced bioluminescence. Furthermore, in order to determine the role of ketone group, two limonin derivatives LOX and LM were prepared. LOX and LM demonstrated a loss of activity in AHL- and AI-2 induced bioluminescence assay (Fig. 6.3B and D) suggesting that C7 position also plays a key role in cell-cell signaling inhibitory activity of limonin.

Biofilm formation in *Vibrio harveyi* is positively regulated by autoinducer mediate cell-cell signaling (9). Therefore, inhibition of autoinducer mediated cell-cell signaling will result in inhibition of *V. harveyi* biofilm formation. Alternatively, loss in inhibition of cell-cell signaling will result in loss of activity against the biofilm formation. The hypothesis was tested by comparing the inhibition of *V. harveyi* BB120 biofilm formation in presence of LM, LOX and DFL with the limonin. Consistent with the hypothesis, the LM, LOX and DFL demonstrated significantly ($p<0.05$) less inhibition of *V. harveyi* BB120 biofilm compared to limonin. Collectively, results suggest that furan ring and C7 position play an important role in the inhibitory activity of limonin against bacterial cell-cell signaling.

AI-2 positively regulates *E. coli* biofilm formation (91, 161, 206). We were further interested if the modified limonoids and limonin affect the *E. coli* O157:H7 biofilm in analogous manner as *V. harveyi* biofilm. Interestingly, a higher inhibition of *E. coli* O157:H7 biofilm by modified compounds compared to limonin was observed (Fig. 6.5C and D). Furthermore, methoxylation (compare LOX with LM) seems to enhance the potency against E. coli O157:H7 biofilm. The cell-cell signal transduction pathway for AI-2 response in *E. coli* does not share homology with the *V. harveyi* pathway. Therefore, it is likely that compounds will have different targets in these two vastly different systems. As a result, there may or may not be a direct correspondence between inhibitions of AI-2 signaling as measured by *V. harveyi* reporter assay and inhibition of *E. coli* O157:H7 biofilm formation. In addition, the situation becomes more complicated with biofilm being regulated simultaneously by another autoinducer

molecule, AI-3 and several environmental factors. It is possible that limonin analogues may have higher inhibitory activity against AI-3 mediated cell-cell signaling, which may explain the observed biofilm inhibition. However, this hypothesis needs further experimentation.

Biofilm formation and maturation is a complex process and various factors such as cell-cell signaling, nutrient availability, temperature and stress regulate biofilm formation (279). Furthermore, cell surface appendages and adhesins such as flagella, type 1 pili, Ag43 protein and curli, help in development of *E. coli* biofilm under different conditions (76, 279-282). The evidence presented in literature supports a role of AI-2 mediated cell-cell signaling in *E. coli* biofilm formation. Pili and Ag43 are in turn regulated by AI-2 mediated cell-cell signaling (161). In addition, pili mediated cell-cell adherence is suggested as necessary and sufficient for the *E. coli* K-12 biofilm maturation (301). Therefore, the effect of LM, LOX and DFL was investigated under the conditions reported to induce or suppress various adhesins in *E. coli* K12. LB medium supplemented with glucose is a rich medium and supports induction of type 1 pili. In contrast, glucose minimal medium with low osmolarity assists in induction of Antigen 43 (76, 262). The curli fibers are produced at low temperature and low osmolarity while temperature >32^{o}C inhibit their production (261). A significant ($p<0.05$) inhibition of *E. coli* biofilm by LM and LOX under various conditions (Fig. 6.6) suggested that LM and LOX act in type 1 pili and Ag43 dependent manner. Since none of the limonoids inhibited biofilm under curli inducing conditions, it was concluded that limonin and limonin analogues action is not dependent on curli.

In order to understand the role of type 1 pili and Ag43 in LM, LOX and DFL mediated inhibition of *E. coli* K12 biofilm, knockout mutant in *fimZ*, *fimB*, *fimH*, *dam* and *oxyR* were employed. The *fimZ* positively regulates fim operon by activating *fimA*, which regulates the expression of fim structural genes (316). FimB is a recombinase, which regulates the phage variation and expression of fim operon positively. Furthermore, AI-2 was suggested to regulate the both *fimZ* and *fimB* (161). The third pili gene *fimH* is a mannose-binding adhesin component of type 1 pili (2). It was speculated that if limonin and limonin analogues inhibit *E. coli* biofilm via adhesins, mutant strain for the respective adhesin will not demonstrate differential biofilm formation. Results for the *fimZ*, *fimB* and *fimH* knockout mutant strain biofilm assay suggest type 1 pili dependent inhibition of *E. coli* biofilm by LM, LOX and DFL (Fig. 6.7A). As our data indicated involvement of Ag43 in biofilm inhibition by limonin analogues, the role of Ag43 was further probed using two mutants. The mutations in *dam* and *oxyR* affect the *E. coli* biofilm by regulating the *agn43*. Specifically, *dam* mutation was reported to decrease the number of cells producing Ag43, while *oxyR* mutation increased the number of cells producing Ag43 (87, 152). Using a similar hypothesis as for type 1 pili, biofilm formation by *dam* mutant was measured in presence of limonin analogues. An exposure of 100 μg/ml LOX, LM and DFL did not affect biofilm formation in *dam* mutant strain in glucose minimal medium suggesting Ag43 dependent activity. To further confirm the Ag43 dependent mode of action, biofilm formation by *oxyR* mutant strains was measured. However, only LM demonstrated a significant inhibition ($p<0.01$) of *oxyR* mutant biofilm (Fig. 6.7B). Altogether the results suggest that LM inhibits *E.*

coli biofilm in type 1 pili and Ag43 dependent fashion. Since type 1 pili and Ag43 are regulated by AI-2 mediated cell-cell signaling, the data is suggestive of AI-2 mediated cell-cell signaling inhibition by LM. Although an inhibition of *oxyR* mutant biofilm by LOX and DFL was not observed, it is pertinent to note that LOX and DFL were weak inhibitors of *E. coli* biofilm compared to LM. It is possible that the higher expression of Ag43 in *oxyR* mutant overcame the inhibitory activity of LOX and DFL.

Type 1 pili bind to α-D-mannosylated protein and contribute to the colonization of bladder (393). Specifically, FimH mediates adhesion to epithelial cells through an N-terminal adhesin domain, which interacts with mannose-containing glycoprotein receptors expressed by different cells (252). In addition Ag43 was reported to assist in attachment of E. coli to epithelial cells (329). Dependence of LM's effect on type 1 pili and Ag43 would then indicate interference in adhesion of *E. coli* to epithelial cells. The possibility was investigated by measuring the attachment of *E. coli* K12 to Caco-2 cells. The results demonstrated that decreased number of *E. coli* cells attached to Caco-2 cells in presence of LM, suggesting LM modulates *E. coli*-Caco-2 interaction through type 1 pili and Ag43.

A possible mode of action of biofilm inhibition by LM may be that LM actually prevents binding of *E. coli* cells to plastic surface, plausibly by interfering with binding of cell-surface appendages with plastic surface. This possibility was investigated by measuring the total cells being attached to plastic surface under two conditions. In Condition A, the *E. coli* cells were grown in presence of LM, thereby representing an effect on synthesis of cell-surface appendages. While in Condition B the LM was added

after *E. coli* cells were grown to predetermined density, allowing them a chance to express the cell-surface appendages normally and LM was added to prevent binding to plastic surface. The data suggest that LM plausibly works by affecting the binding of preformed of cell-surface appendages like type 1 pili to plastic (Fig. 6.7), but apparently did not affect the synthesis of cell-surface appendages. To further understand the interaction of LM with *E. coli* K12, uptake dynamics was studied. The data suggest that LM is possibly not uptaken by the *E. coli* cells (Fig. 6.10A). While, in stark contrast limonin was actively absorbed by the *E. coli* cells (Fig. 6.10B). Collectively, the data from the study suggests that modification to methoxime at C7 position of limonin resulted in loss of absorption. This result also explains the observed losses in *V. harveyi* luminescence and biofilm assay. However, methoxylation at C7 position seems to impart a unique ability to the limonin viz. specifically interact with type 1 pili and Ag43 and preventing their attachment to surface. This interaction possibly resulted in the greater reduction of *E. coli* biofilm as measured by the crystal violet assay. Furthermore, it is possible that LM actually binds to and prevents the attachment of type 1 pili and Ag43. However, this possibility requires further investigation.

In conclusion, C7 position and furan ring significantly contribute towards the cell-cell signaling inhibitory activity of the limonin. The modification at C7 position seems to increase the potency against the *E. coli* biofilm. In addition, methoxylation of C7 further enhanced the potency against *E. coli* biofilms. LM seems to specifically interact with type 1 pili and Ag43 to prevent attachment of *E. coli* cells to surface. In this capacity, the LM may be used as topical agent to prevent the *E. coli* biofilm formation

on various surfaces. Further research is needed to deduce the structure-activity relationship of the limonin and/or limonoids. Structure-activity relationship among natural bioactive compounds provide a preliminary insight into the mechanism of action (151). It is possible that certain other citrus limonoids or bioactive compounds may demonstrate similar mode of action with equal or better potency. Identification of such compounds will greatly facilitate the development of preventive strategies for bacterial biofilms. As the bacterial biofilms are prevalent in nature and affect human health, evidence that limonin analogues are more active against *E. coli* biofilms may have significant implications in preventive and therapeutic use of these compounds.

CHAPTER VII

ANTIPATHOGENIC ACTIVTY OF OBACUNONE AGAINST *SALMONELLA* TYPHIMURIUM LT2

7.1 Synopsis

Obacunone is a limonoids present in Citrus species. We previously reported that obacunone was inhibitory to the cell-cell signaling in *Vibrio harveyi* and *Escherichia coli* O157:H7. In the present work we evaluated the effect of obacunone on the food borne pathogen *Salmonella* Typhimurium LT2 using cDNA microarray. Obacunone exposure resulted in repression of *Salmonella* Pathogenicity Island-1 and 2 (SPI1, SPI2), maltose transporter and hydrogenase operon. Consistent with the repression of SPI1 and SPI2, obacunone application reduced the attachment and subsequent internalization of *S.* Typhimurium LT2 into Caco-2 cells. In addition, obacunone seems to impair the ability of *S.* Typhimurium to uptake maltose. Furthermore, obacunone seems to repress the SPI1 and SPI2 EnvZ dependent fashion. The results of the study indicate that obacunone may have preventive utility and may serve as a lead compound for development of antipathogenic strategies for *S.* Typhimurium.

7.2 Introduction

Obacunone is one of the major limonoids present in *Citrus* species (Fig. 7.1). It is naturally present as glucoside and aglycone in the citrus juices and seeds (158, 164, 264). The citrus juices such as grapefruit and orange juice reportedly contain about 1-11 parts per million (ppm) of obacunone (158, 164, 264). The lemon seeds were reported to contain comparatively high concentration (as much as 29 ppm) of obacunone (142) and may serve as a good source of obacunone for purification purposes. Chemically, obacunone (and other limonoids) is a triterpenoids, characterized by the presence of furan ring and high degree of oxygenation.

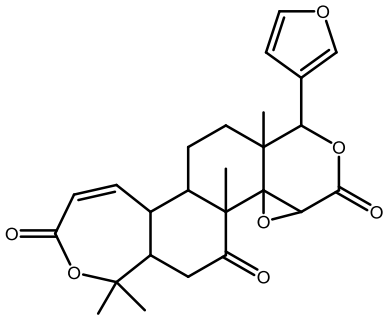

FIG. 7.1 Structure of obacunone

Obacunone and other limonoids serve as part of plant systemic defense system. Anti-feedant activities of obacunone and other citrus limonoids were reported by several studies (313). However, citrus limonoids demonstrate weaker anti-feedant activity compared to simpler limonoids present in family Meliaceae (Neem tree and related species) (58, 313). In recent years, the research on anti-cancer activities suggests that

obacunone and certain other citrus limonoids may have a possible health beneficial effect. Obacunone (aglycone and glucoside) was reported to induce apoptotic and cytostatic responses in SH-SY5Y and Caco-2 cells (276-277). Furthermore, surface and oral application of obacunone resulted in suppression of chemically induced carcinogenesis in animal models of oral and colon cancer (242, 355). In addition, feeding of obacunone reportedly enhanced the glutathione-S-transferase activity in various organs of mice (195).

Autoinducer mediated bacterial cell-cell signaling, commonly known as quorum sensing, is a density dependent phenomenon (50). However, the actual regulation of various processes is dependent on the concentration of autoinducer molecules rather than the bacterial population itself. Several physiological processes are now known to be regulated by quorum sensing in different bacteria. The examples include biofilm formation and expression of TTSS in *E. coli* O157:H7 (17, 133, 161, 341), bioluminescence production, biofilm formation and TTSS in *V. harveyi* (9, 153) and related species. Recently, motility in *Salmonella* Typhimurium LT2 were reported to be regulated by autoinducer-3 mediated cell-cell signaling. Several natural bioactive compounds including flavonoids, furocoumarins and limonoids demonstrate inhibition of quorum sensing (130, 371, 375).

Salmonella species continue to be an important health problem. According to the FoodNet surveillance 2009 preliminary report on infectious diseases, *Salmonella* was the leading cause of food borne diseases in Unites States. A total of 7,039 cases of *Salmonella* infection were reported from only 10 states (57). *S.* Enteritidis and *S.*

Typhimurium were among the 10 major serotypes identified as causative agents. Furthermore, an earlier report estimated that 1.4 million cases of non-typhoidal *Salmonella* infections occur in United States, resulting in 15,000 hospitalizations and 400 deaths annually (376). This grim situation may aggravate further, especially as the spread of antibiotic resistant strains of *Salmonella* to various sources are continually being reported (403). In order to counter the *Salmonella* infections, it is imperative to search for new antimicrobial/antipathogenic compounds.

One of the possible strategy is to identify and develop novel compounds which are antipathogenic but do not affect the bacterial growth. The strength of such a strategy may lie in the low risk for resistance development against the antipathogenic agent. *S.* Typhimurium contains several virulence determinants, such as fimbrial/nonfimbrial adhesions, flagella, virulence plasmid/s, spv gene cluster and *Salmonella* Pathogenicity Islands (SPIs) (304). In particular, SPI-1 was implicated in initial attachment and subsequent internalization of the pathogen to the intestinal cells. SPI-2, on the other hand, plays an important role in intracellular survival and systemic infection. In addition, several SPI-1 encoded effectors contribute to pathogen's successful intracellular existence. Identification of an antipathogenic agent, which influences all or any of these virulence factors, may have preventive and therapeutic potential.

Previously, we reported that obacunone was a potent inhibitor of autoinducer mediated cell-cell signaling and biofilm formation in *Vibrio harveyi* (375). In addition, *E. coli* O157:H7 biofilm and type three secretion system (TTSS) were repressed by obacunone. Since, obacunone demonstrated antipathogenic activity against *E. coli*

O157:H7 possibly via quorum sensing. We wondered if the obacunone has an antipathogenic effect on *Salmonella*. The present investigation was focused on determination of *S.* Typhimurium response to obacunone. The present report demonstrates that obacunone represses the SPI-1 and 2.

7.3 Materials and Methods

7.3.1 Chemicals

Obacunone was purified previously in our lab (375). A stock solution of obacunone was prepared by dissolving 40 mg obacunone in 1 ml dimethyl sulfoxide (DMSO).

7.3.2 Bacterial Growth Conditions

Unless otherwise specified, bacterial cultures were grown at 37^{o}C in Luria-Bertani (LB) medium supplemented with 0.5 % glucose (LB-glu). When appropriate, the medium was supplemented with 10 µg of chloramphenicol or 10 µg of tetracycline per ml media. For *Salmonella* Pathogenicity island 2 studies, the bacteria was cultured in magnesium minimal medium (123).

7.3.3 Growth and Metabolic Activity

Diluted (100 fold in LB-glu) overnight culture of *S.* Typhimurium LT2 (200 µl) was incubated in 96-well plates with 100, 50, 25, 12.5, and 6.25 µg/ml obacunone or equivalent amount of DMSO. OD_{600} was monitored for 16 h, at 15 min interval in Synergy^{TM} HT MultiMode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The instrument was set to maintain 37^{o}C with shaking at medium speed. The data was fitted to a 4-parameter sigmoid model $Abs_t = Abs_{min} + Abs_{max} - Abs_{min}/1 + \exp[-$

μmax ($t-t_i$)] (75) and maximum specific growth rate (μmax) was calculated. Mean generation time was calculated from three biological replicates using the formula g= Ln(2)/μmax, and presented as mean ± SD.

7.3.4 RNA Extraction and Hybridization Conditions

Total RNA was isolated from freshly diluted overnight *S.* Typhimurium LT2 culture (100 fold in LB-glu) supplemented with 100 μg/ml obacunone or DMSO and grown to OD600 ≈1.0 at 37^{o}C. Cultures were stabilized with RNAProtect bacteria reagent (Qiagen Inc., Valencia, CA, USA). RNA was isolated with RNeasy minikit (Qiagen Inc.) according to manufacturer's instructions. RNA quality was analyzed spectrophotometrically (Nanodrop, Fisher Scientific) and with bioanalyser 2100 (Agilent Technologies Inc., Santa Clara, CA, USA).

Total RNA (10 μg) from treatment and control was used to synthesize cDNA using random primer (Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously (176). *S.* Typhimurium LT2 genome microarrays version 4, containing 5405 open reading frames from strain LT2, were obtained from Pathogen Functional Genome Resource Center of the National Institute of Health. Each microarray was hybridized with a pair of Cy-3 and Cy-5 monoreactive dyes (GE Healthcare, Piscataway, NJ, USA) labeled cDNA from obacunone and DMSO treated samples. A total of 6 dye-swapped hybridizations, corresponding to three biological replicates, were conducted. Hybridizations were carried out at 42^{o}C for 18 h. Microarrays were scanned on a GenePix Scanner 4100A (Molecular Devices Corporation, Sunnyvale, CA, USA) at 532 and 635 nm.

7.3.5 Microarray Data Collection and Analysis

Signal intensities from the images were extracted using GenePix 6.0 software (Molecular Devices Corporation). Spots were omitted from further analysis if their median signal value was less than sum total of local background median value plus three standard deviations. LOWESS normalization (285) was applied to median signal intensities for all probes on each array using Acuity 4.0 software and statistical significance was evaluated (Molecular Devices Corporation). Differentially expressed genes between obacunone and DMSO treated samples were identified by Student's t-test. P-values were adjusted for multiple comparisons using Benjamini-Hochberg method (32). Genes with $p<0.05$ were considered significantly differentially expressed and reported. The raw data and complete dataset are available at NCBI Gene Expression Omnibus database (accession no.).

7.3.6 Real Time RT-PCR

For a group of highly induced or repressed genes, measurements of relative transcript amounts were performed by real time RT-PCR. Primers (Table 7.1) were designed by Primer Express program (Applied Biosystems, Foster City, CA). Cells from obacunone or DMSO treated samples were collected at $OD_{600}\approx 1.0$. RNA was extracted using the RNeasy minikit (Qiagen Inc.) according to the manufacturer's instructions. First strand synthesis was carried out using MuLV reverse transcriptase enzyme and random hexamer in a Reverse-Transcriptase polymerase chain reaction (RT-PCR). The RT- PCR was performed on a programmable thermocycler (Applied Biosystems, Gene Amp PCR system 2700), under following conditions: 60 min at 42˚ C for annealing and

elongation followed by 5 min at 99˚C to inactivate the reverse transcriptase enzyme. The cDNA (25 ng) was amplified on ABI-Prism 7000 HT (Applied Biosystems, Foster City, CA) using SYBR® GREEN PCR mix (Applied Biosystems, Warrington, UK) and 10 pmol primers for target sequences. After completion of 35 PCR cycles, melt curve data was generated to ascertain template-independent amplification. The initial concentration of each transcript was normalized to the 16s rRNA. The difference in transcripts was calculated with respect to control using comparative Ct method. The change in gene expression was calculated using the formula: Treatment 2/treatment 1 = $2^{\Delta\Delta CT}$, Where $\Delta\Delta C_T = \Delta C_T$ for treatment 1- ΔC_T for treatment 2 and ΔC_T is the difference between the C_T value of the target gene and the normalization gene (16S rRNA) (210)**.** The mean ±SD of three biological replicates is presented.

TABLE 7.1: Sequence of the *Salmonella* primers used in the study

Primer	Forward Sequence	Reverse Sequence	Ref.
hilA	GTCCGGTCGTAGTGGTGTCT	CGCATACTGCGATAATCCCT	(373)
prgK	GGGTGGAAATAGCGCAGATG	TCAGCTCGCGGAGACGATA	(373)
invB	CTGGGCGCAATTGGGTGCTG	GCTCCCCATTCTGCTCCCCC	(373)
sopE2	ATACCGCCCTACCCTCAGAAG	GCCTGCATCAACAAACAGACA	This study
hyaA	GCCTGCTCCTCCACACGCTG	CCCCACCGTATCGGCGGTTG	This study
malF	ATTGACCAACGGCGGGCCAG	ATTGCCAGCGCGCCTACCAG	This study
malK	AATCGTGGTGCTGGACGCCG	CAATCGCGGTGGCGGTCACT	This study
rpoA	GCGCTCATCTTCTTCCGAAT	CGCGGTCGTGGTTATGTG	This study

7.3.7 Adhesion and Gentamicin Protection (Invasion) Assays

Adhesion assay was performed as described (334). Colon adenocarcinoma epithelial Caco-2 (ATCC # CCL-228) cells were routinely maintained in DMEM supplemented with 10 % fetal bovine serum. For adhesion assay, Caco-2 cells were plated in 6 well plates at a density of 1 x 10^5 cells/well. *S.* Typhimurium LT2 was grown overnight in 25 ml culture. The *Salmonella* cells were centrifuged at 5000 rpm for 30 min and re-suspended in 0.9 % saline solution. The cells were counted by diluting in 0.9 % saline solution and plating on Tryptic Soy Agar plates. The Caco-2 cells were infected with *S.* Typhimurium LT2 cells at a multiplicity of infection (moi) 10, in presence of 100 µg/ml obacunone or equivalent amount of DMSO. The plates were incubated for 3 hr at 37^{o}C , 5 % CO_2. The excess *S.* Typhimurium LT2 cells were washed three times with PBS. The Caco-2 cells were lysed by 0.1 % TritonX-100 solution. The resulting culture was then diluted using 0.9 % saline solution in 6 serial dilutions and plated on the Trytic Soy Agar plates. Colonies were counted after 24 hrs and recorded as Colony Forming Units (CFU).

Invasion assay was performed as described before (288). For gentamicin protection assay, 1 x 10^5 Caco-2 cells were infected with *S.* Typhimurium LT2 at moi 10 in presence of 100 µg/ml obacunone or DMSO. One hour after infection at 37^{o}C and 5 % CO_2, the medium was replaced with DMEM + 10 % FBS and 100 µg/ml gentamicin to kill non-invasive cells and incubation was continued for additional 90 min. After incubation, the Caco-2 cells were washed with three times with PBS and lysed with 0.1

% TritonX-100 solution. The CFUs were determined by plating the appropriate dilution in 0.9 % saline solution on TSA.

7.3.8 β-Galactosidase Assays

Overnight cultures of *S.* Typhimurium strain EE658, EE711 and RL829 were diluted (1:100) in LB-glu and grown upto OD600≈1.0 in presence of 100 µg/ml obacunone or DMSO. β-Galactosidase assay was performed as described previously (214, 243).

7.3.9 Maltose Utilization Assay

To test for the ability to use maltose as carbon source, *S.* Typhimurium was grown on MOPS minimal medium (255). The overnight culture was diluted 100 fold in MOPS minimal media supplemented with 0.2 % maltose or glucose as the sole source of carbon and *S.* Typhimurium growth was measured in presence of 100 µg/ml obacunone as described in "Growth and Metabolic Activity" section. In addition, M63 media was prepared containing 2g $(NH_4)_2SO_4$, 13.6g KH_2PO_4, 0.5 mg $FeSO_4.7H_2O$ and 1mM $MgSO_4.7H_2O$ but without casamino acids and vitamins. The *S.* Typhimurium growth was measured in M63 medium supplemented with 0.2 % maltose or glucose in presence of 100 µg/ml obacunone as described above.

7.4 Results

7.4.1 Effect of Obacunone on *Salmonella* Growth

Growth of S. Typhimurium LT2 in presence of 6.25, 12.5, 25, 50 and 100 µg/ml obacunone or equivalent amount of DMSO (control) was measured upto 16 h.

Obacunone did not seem to impact the *S.* Typhimurium growth at the tested concentrations (Table 7.2).

TABLE 7.2: Mean generation time of *Salmonella* Typhimurium LT2 in presence of different concentrations of obacunone. The values represent mean ± SD of three replicates

	Concentration (µg/ml)				
	6.25	12.5	25	50	100
Control	22.44 ± 0.61	22.40 ± 0.54	21.88 ± 0.92	21.75 ± 0.38	21.87 ± 0.70
Obacunone	21.37 ± 1.05	21.94 ± 1.08	21.91 ± 0.28	22.54 ± 0.97	22.41 ± 1.02
P-value	0.20	0.55	0.96	0.38	0.49

7.4.2 Microarray Expression Profile

To understand the response of *S.* Typhimurium LT2 to obacunone, DNA microarray containing 4518 of 4716 predicted open reading frames (95.8 %) of S. Typhimurium LT2 including its plasmid, was used. Total RNA samples were prepared from three different obacunone treated and control cultures of *S.* Typhimurium. A total of 10 µg RNA from each sample was converted to cDNA and labeled with Cy3/Cy5 monoreactive dyes. Equal quantities of Cy3 and Cy5 labeled cDNAs from sample and treatment were mixed and hybridized to 6 *Salmonella* Typhimurium LT2 version 4 microarrays (PFGRC). The data was lowess normalized using 'R' and evaluated for quality. Student's t-test with Benjamini-Hochberg correction was employed to determine the differentially expressed open reading frames (ORFs) between control and obacunone treatments. A total of 175 genes (3.23 %) were differentially expressed ($p<0.01$) out of

5418 ORFs present upon exposure of 100 μg/ml obacunone. However, only 95 genes demonstrated >2 fold change in the expression in either direction. Several regulons were downregulated by obacunone exposure including *Salmonella* Pathogenicity Island 1 (SPI-1), maltose regulon and hydrogenase-1 operon (Table 7.3).

TABLE 7.3: Differentially regulated gene/ORF of *S.* Typhimurium LT2 upon exposure of 100 μg/ml obacunone

Gene	Putative Identification	Fold Change
Virulence		
prgK	needle complex inner membrane lipoprotein	-9.88
prgJ	needle complex minor subunit	-11.29
hilA	invasion protein regulator	-8.18
iagB	invasion protein precursor	-7.32
sicP	secretion chaparone	-9.01
spaR	needle complex export protein	-7.26
spaP	surface presentation of antigens protein SpaP	-7.31
invB	secretion chaperone	-10.46
invG	outer membrane secretin precursor	-7.95
sopE2	invasion-associated secreted protein.	-10.17
Hydrogenase		
hyaA	hydrogenase-1 small subunit	-9.42
hyaB	hydrogenase 1 large subunit	-8.72
hyaC	hydrogenase 1 b-type cytochrome subunit	-9.10

TABLE 7.3 Continued.

Gene	Putative Identification	Fold Change
hyaD	hydrogenase 1 maturation protease	-8.87
hyaE	hydrogenase-1 operon protein HyaE	-7.90
hyaF	putative hydrogenase-1 protein	-8.93
hycH	hydrogenase 3 large subunit processing protein	-9.71
Maltose		
malF	maltose transporter membrane protein	-5.39
malK	maltose/maltodextrin transporter ATP-binding protein	-6.06
lamB	maltoporin	-6.19
malM	maltose regulon periplasmic protein	-5.26
malE	maltose ABC transporter periplasmic protein	-8.75

7.4.3 Obacunone Represses SPI-1

A downregulation of about 10 genes (≈26 %) of SPI-1 encoded genes by 7.26 – 11.56 fold (Table 7.3) was detected using microarray analysis. Among these was the central transcriptional regulator of SPI-1, *hilA* (downregulated by 8.2 fold) in response to obacunone. Needle complex structural proteins *prgK*, *prgJ* (encoded in prg/org operon) and *invG* were repressed by 9.88, 11.29 and 7.95 fold, respectively. Furthermore, export apparatus protein *spaR* and *spaP* were downregulated. The *iagB*, a muramidase, speculated to help in the passage of needle through peptidoglycan layer, was also repressed (7.32 fold). The chaperons *sicP* and *invB* were downregulated by 9.01 and

10.46 fold, repectively. In addition to the repression of SPI-1 encoded genes, obacunone also downregulated the effector *sopE2*, encoded outside SPI-1. It is pertinent to note that InvF/SicA, encoded in SPI-1 regulate the expression of *sopE2* (79).

7.4.4 Hydrogenase Operon

The nomenclature of *S.* Typhimurium hydrogenases is not well established. Therefore, the annotation assignment presented by Zbell et al (401) was followed in the current study. Specifically, symbols *hyaABCDEF* were used for the genes with locus tags STM1786 – STM1797. The *hyaABCDEF* were suppressed upon obacunone exposure by 7.90 - 9.42 fold (Table 7.3). At present, it is not known if the *hya* operon is transcribed as polycistronic message in *S.* Typhimurium LT2. However, the homologous operon *hya* in *Escherichia coli* was reported to be transcribed as polycistronic message (238). In the present study, the entire operon was down-regulated by obacunone. In addition, suppression of *hycH* (9.71 fold), which encodes large subunit of hydrogenase-3, was observed.

7.4.5 Maltose Transport System

The MalEFG and MalK-LamB-MalM constitute the ABC transport complex, which facilitate the uptake of maltose and maltodextrins (41). Obacunone exposure resulted in down-regulation of *malEFG* (331) and *malK-lamB* (287) operons. Specifically, *malE* and *malF* were down-regulated by 8.78 and 5.39 fold, respectively. The *malK-lamB-malM*, which are transcribed in opposite direction of *malEFG* operon, were downregulated by 6.06, 6.19 and 5.26 fold respectively. The data indicated that obacunone specifically downregulated the translocation complex.

7.4.6 Validation of Microarray Data by Real Time RT-PCR

In order to verify the results obtained from microarray experiment, real time RT-PCR was performed on the selected genes. Based on the interest, one gene from each affected operon was selected (Table 7.1). The housekeeping gene *rpoA* was used as a reference. The Ct values were normalized to levels of *rrsH* and fold change was calculated by $2^{(-\Delta\Delta Ct)}$ method (210). A comparison of microarray data and real time RT-PCR data is presented in Table 7.4. In general, the results from real-time RT-PCR confirm the microarray results. However, the numerical values obtained from real-time RT-PCR were different from the values in microarray analysis. This difference was probably a result of the different sensitivity of the assay and because of the fact that different biological samples were used for real-time RT-PCR analysis.

TABLE 7.4: Validation of microarray-based expression profiles of selected genes by real time RT-PCR

Gene	Relative expression level	
	Microarray	Real Time RT-PCR
hilA	-8.18	-8.02
prgK	-9.88	-8.91
invB	-10.46	-6.83
sopE2	-10.17	-5.90
hyaA	-9.42	-6.09
malF	-5.39	-2.21
malK	-6.06	-2.78
rpoA	-	1.10

7.4.7 Effect of Obacunone on Adhesion and Invasion of Caco-2 Cells by *S.* Typhimurium

Caco-2 cells were grown in DMEM and infected with *S.* Typhimurium LT2 in presence of 100 µg/ml obacunone. The obacunone exposure resulted in significantly reduced number of adhesive *Salmonella* cells ($p < 0.01$). In presence of obacunone, 4.85 logCFU *Salmonella* cells were recovered compared to 7.34 logCFU in control (Fig. 7.2). Furthermore, obacunone reduced the number of internalized *Salmonella* cells by ≈ 1.84 log units ($p < 0.01$) (Fig. 7.2). These results were consistent with the down-regulation of SPI-1.

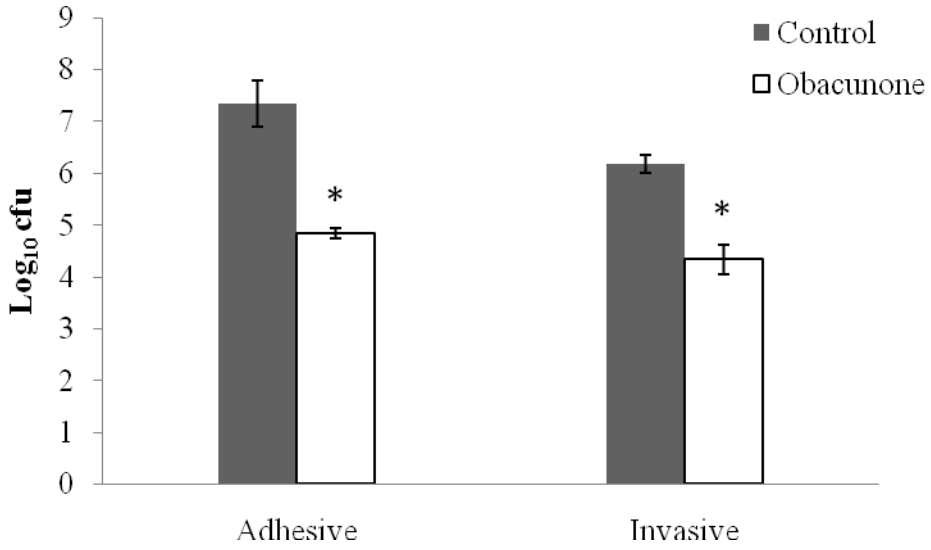

FIG. 7.2: Effect of 100 µg/ml obacunone on adhesion and invasion of *Salmonella* Typhimurium LT2 on Caco-2 cells

7.4.8 Obacunone Impairs the Maltose Uptake

In order determine the effect of obacunone on maltose transport, the MOPS minimal medium and M63 medium were used. Maltose (0.2 %) was used as the sole carbon source. Glucose (0.2%) was used as the positive control for the experiment. The results demonstrate that growth of *S.* Typhimurium was reduced in presence of 100 µg/ml obacunone compared to control when maltose was used as the sole carbon source (Fig. 7.8A and C). In contrast, *S.* Typhimurium growth in presence of obacunone was similar to the control in MOPS minimal and M63 medium when glucose was used as the sole carbon source. Furthermore, M63 medium stripped of casasmino acids and vitamins seems to be more rapid method of determining the maltose utilization (Fig. 7.3).

7.4.9 Obacunone Influences *hilA* Expression in Dose Dependent Fashion

We were further interested in determining the minimum dose of obacunone required to differentially regulate *hilA* by >2 fold. To investigate the dose-effect relationship, *hilA* expression was measured using real-time RT-PCR. The results of the study indicate that obacunone influences the expression of *hilA* in a concentration dependent fashion (Fig. 7.4). Exposure of 12.5 µg/ml obacunone suppressed *hilA* expression by 2.34 fold. The lowest tested concentration 6.25 µg/ml, elicited a response of -1.99 fold. These data indicate that obacunone at a low dose of 12.5 µg/ml may be able to alter the gene expression pattern of *hilA* significantly and possibly *hilA* dependent genes.

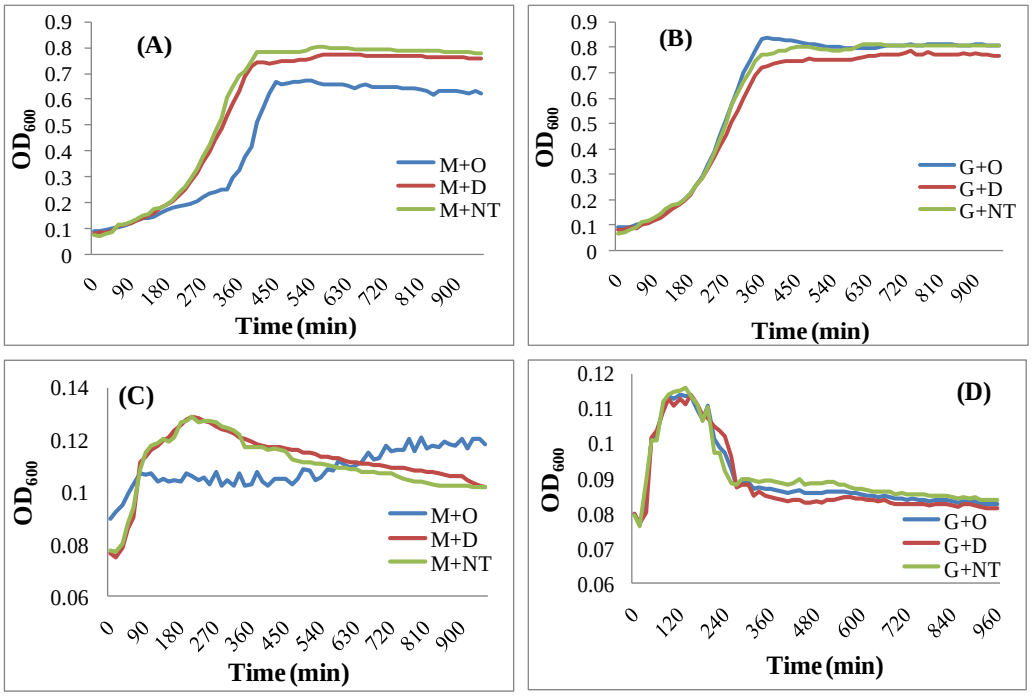

FIG. 7.3: Growth of *Salmonella* Typhimurium LT2 in presence of 100 µg/ml obacunone as determined on MOPS minimal medium. (A) maltose and (B) glucose and M63 minimal medium (C) maltose and (D) glucose as sole carbon source. The data represents the mean of three biological replicates. (Abbr. M= Maltose, G= glucose, O= Obacunone, D= DMSO, NT= no treatment)

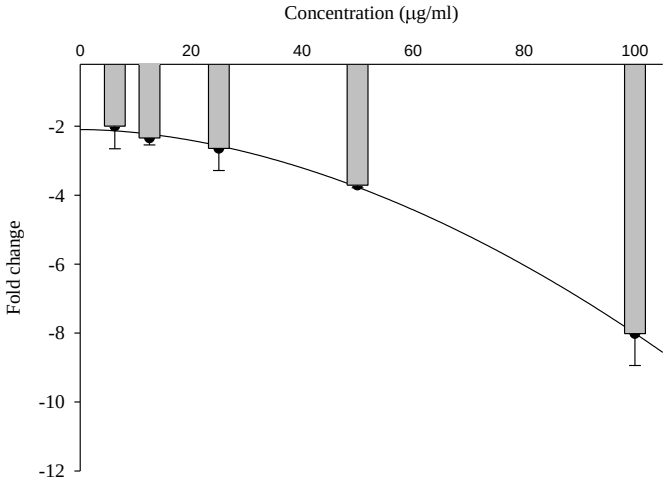

FIG. 7.4. Expression of *hilA* in presence of different concentrations of obacunone. The data is presented as mean of three biological replicates ± SD

7.4.10 Effect of Obacunone on Genes Encoded in SPI-2

In order to understand the impact of obacunone on SPI-2, relative expression of *ssrA*, *ssrB* and *spiC* were measured. MES buffered Mgm medium is reported to induce the expression of SPI-2 (123). *S.* Typhimurium was grown in MES buffered Mgm medium in presence of 100 µg/ml obacunone or DMSO. Exposure of 100 µg/ml obacunone resulted in suppression of SPI-2 encoded *ssrA*, *ssrB* and *spiC* by 9.67, 11.83 and 6.84 fold, respectively (Fig. 7.5).

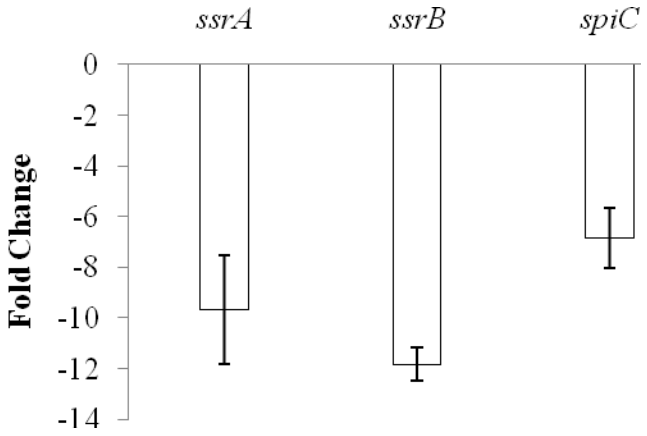

FIG. 7.5. Expression of *ssrA*, *ssrB* and *spiC* upon exposure of 100 µg/ml obacunone under SPI-2 inducing conditions

7.4.11 Impact of Obacunone on Expression of SPI-1 and SPI-2 Encoded Genes in Caco-2 Cells

The Caco-2 cells were infected with *Salmonella* as described for adhesion and invasion assays in presence of 100 µg/ml obacunone and compared to control. Total RNA (Caco-2 +*Salmonella*) was extracted from under adhesion and invasion

experimental conditions. Expression of *hilA* and *ssrA* was measured to determine the expression pattern of SPI-1 and SPI-2 upon exposure of obacunone. Exposure of obacunone resulted in suppression of *hilA* and *ssrA* by 2.55 and 3.16 fold in *Salmonella* cells adhering to and inside Caco-2 cells (Fig. 7.6A). Furthermore, *hilA* and *ssrA* were suppressed by 3.99 and 4.44 fold under invasive conditions (Fig. 7.6B).

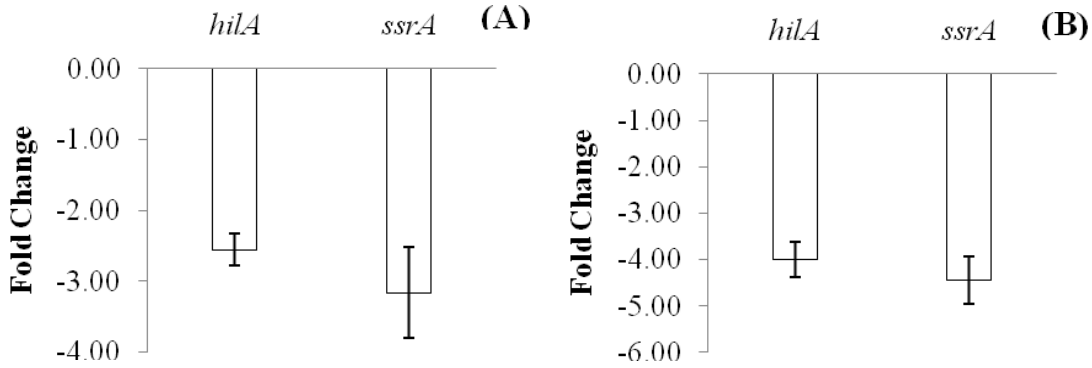

FIG. 7.6. Expression of SPI-1 and SPI-2 encoded *hilA* and *ssrA* under (A) adhesion (B) invasion assay condition (Please see text for details)

7.4.11 *hilA* Expression in Wild Type and *envZ* Mutant

In order to further confirm the differential expression of *hilA* in response to obacunone, *hilA* reporter *S.* Typhimurium strain EE658 (16) was employed. EE658 contains a *lacZY* reporter attached to *hilA* promoter in chromosomal location and therefore reflects the expression of *hilA*. Exposure of 100 µg/ml obacunone resulted in suppression of *hilA* by 2.65 fold (Fig. 7.7). This result was consistent with the microarray analysis results.

Further, role of *envZ* in *hilA* suppression by obacunone was examined. To investigate the role of *envZ*, *S.* Typhimurium strains EE711 and RL829 (214-215) were employed. Strain EE711 has disruption in *envZ*, and defective in *envZ* mediated *hilA* induction. Obacunone did not affect (fold change = 0.92) the expression of *hilA* ($p>0.05$) in *envZ* mutant background (Fig. 7.8A). To further verify the role of *envZ*, expression of *envZ* was induced using arabinose controlled promoter (strain RL829). Induction of controlled expression of *envZ* resulted in suppression of *hilA* ($p <0.01$) by 2.42 fold under experimental conditions (Fig. 7.8B).

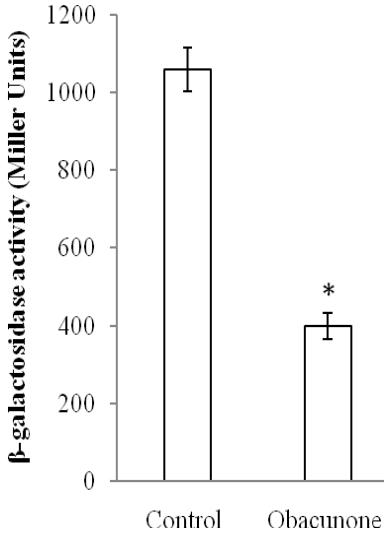

FIG. 7.7: Expression of *hilA* upon exposure of 100 µg/ml obacunone

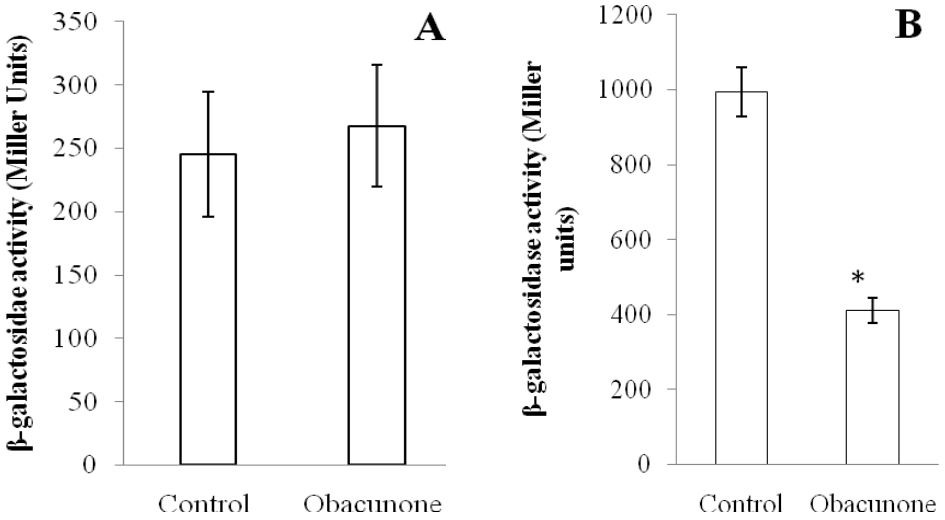

FIG. 7.8: Expression of *hilA* in *envZ* mutants. (A) *S.* Typhimurium strain EE711 and (B) *S.* Typhimurium strain RL829 upon exposure of 100 µg/ml obacunone

7.5 Discussion

The citrus fruits are consumed very widely in USA and world. According to Economic Research Service, USDA, the per capita consumption of total citrus fruits and juices varied between 76-114 lb (fresh weight equivalent) since 2000. A rough calculation using data (1-11 ppm) presented earlier (158, 164, 264) shows that one serving (240 ml) of orange juice may provide 240 µg -2.6 mg of obacunone. Since obacunone demonstrated potential to inhibit *E. coli* O157:H7 virulence, we were interested about its effect on *S.* Typhimurium. As a first measure of effect of obacunone on the pathogen, growth rate of S. Typhimurium was measured in presence of 6.25-100 µg/ml obacunone and OD_{600} was monitored. The data was fitted to a 4-parameter equation as described in 'Methods' and generation times were calculated. The mean generation time for obacunone treated samples were not significantly ($p>0.05$) different

from control suggesting obacunone did not affect *S.* Typhimurium growth. This result was similar to obacunone's effect on *E. coli* O157:H7 growth (375).

To further understand the effect of obacunone, gene expression profile of *S.* Typhimurium in presence of obacunone was studied using cDNA microarrays. Microarrays have become a tool of choice to understand the response of a biological system to a variety of stimuli. Since there were no prior reports investigating the effect of obacunone on *S.* Typhimurium, cDNA microarrays were used to understand the response of *S.* Typhimurium to obacunone exposure. A high concentration of 100 µg/ml obacunone was used to obtain a measurable response in *S.* Typhimurium.

Obacunone exposure resulted in repression of three distinct functional systems, SPI1, hydrogenase operon and maltose transport system. The regulation of SPI1 is complex and several genetic and environmental factors regulate SPI1 encoded genes (215). Majority of the factors regulate the expression of *hilA*, which in turn activates the expression of SPI1 encoded genes as well as several effectors outside SPI1 (16). In particular, HilA regulates the expression of *inv*, *prg*/*org* and *spa* operons in the SPI1 (211). The results of the study demonstrate that obacunone repressed genes encoded in *prg*/*org*, *inv* and *spa* operons. In addition, the effector *sopE2*, which is encoded outside SPI1, but regulated by InvF/SicA (79) (encoded within SPI1), was repressed. SPI1 encoded needle complex is essential for initial attachment and entry of the pathogen into the host cells (304), whereas SPI1 encoded effectors are required for intracellular survival (46). Repression of various operons inside SPI-1 indicated two possibilities (1) impaired assembly of needle complex and export apparatus will result in reduced

adhesion and invasion of animal cells, and (2) repression of SPI-1 by obacunone is mediated through *hilA*. Furthermore, the *hilA* repression by obacunone was dose dependent (Fig. 7.5), indicating a possible competitive nature of inhibition. To determine that obacunone treatment may reduce the attachment and subsequent internalization of *S.* Typhimurium, Caco-2 cell models of adhesion and invasion were employed. Consistent with the observed *hilA* and SPI-1 repression, obacunone significantly reduced the number of *S.* Typhimurium cells adhered to and internalized in Caco-2 cells (Fig. 7.2).

Salmonella contains three hydrogenase enzymes (219), which are important for cellular metabolism. The enzyme Hyc together with formate dehydrogenase oxidizes formate to produce CO_2 and H_2 (308) during fermentative growth of bacteria. The majority H_2 produced by Hyc is recycled by the enzyme Hya (402). Obacunone exposure affected the genes encoded in hydrogenase-1 operon. The *hya* operon was reported to be transcribed as a polycistronic message in *E. coli* and *Salmonella* (219, 238). Obacunone exposure resulted in repression of entire *hya* operon and *hycH* of *hyc* operon. It is noteworthy that the experiment was conducted under aerobic conditions. However, overall hydrogen uptake activity was reported to be higher in the cells grown in rich media such as LB (219), where *hya* is regulated by fumarate nitrate respiration protein (401). Furthermore, hydrogenases were reported to play a role in *S.* Typhimurium virulence (219). It is possible that repression of *hya* operon by obacunone may contribute to reduced virulence of *S.* Typhimurium. However, contribution of *hya* operon in reduced pathogenicity by obacunone requires further study.

The third system affected by obacunone was maltose transporter. The maltose transporter facilitates the uptake and efficient catabolism of maltose and maltodextrins (41). In *E. coli* O157:H7, maltose metabolism was demonstrated to be necessary to initiate infection in mixed culture model (178). Obacunone repressed the maltose transporter suggesting a reduced uptake of maltose and maltodextrin. To determine the reduced uptake of maltose, *S.* Typhimurium was grown in MOPS minimal medium with maltose as the sole source of carbon. In the presence of obacunone the growth rate of *S.* Typhimurium was reduced compared to control. Furthermore, the *S.* Typhimurium growth depicted a typical response in M63 minimal media (prepared without casamino acids and vitamins), where the OD600 reached at maximum at 210 min and decreased thereafter, giving rise to a bell shaped curve. In contrast, in presence of obacunone, the growth rate was slower but steady suggesting that in control the bacteria exhausted the available carbon source in first 210 min and the population faced a nutrient limitation afterwards. Whereas, in presence of obacunone, a reduced rate of maltose uptake prevented the early exhaustion of carbon source and maintained a steady state. However, when glucose was used as carbon source, the growth of *S.* Typhimurium was similar to the control ($p > 0.05$) in both the media. Altogether the data suggest that obacunone exposure resulted in reduced rate of maltose uptake, which may be attributed to downregulation of maltose transporter.

Global regulators EnvZ/OmpR and BarA/SirA in *S.* Typhimurium regulate the SPI-1 and mal regulon (54, 198, 215). We suspected that repression of SPI-1 and *mal* regulon by obacunone is EnvZ and/or CsrA dependent. To verify the role of EnvZ,

expression of *hilA* was measured in EnvZ mutant background. *S.* Typhimurium strains EE711and RL829 were described before (214). The results demonstrate that obacunone did not repress *hilA* expression in EnvZ mutant. Furthermore, controlled expression of functional EnvZ, restores the repression of *hilA* by obacunone, suggesting repression of *hilA* by obacunone is mediated via EnvZ.

EnvZ/OmpR is a two component regulatory system, which responds to osmolarity changes and regulates porin genes *ompC*/*ompF*. In addition, ompR was reported to regulate SsrA-SsrB two component system encoded in SPI-2 (200). Since obacunone seems to exert its effect in EnvZ dependent manner, its exposure was likely to repress *ssrA*/*ssrB* operon and consequently SPI-2. Mgm- minimal medium was reported to be conducive for SPI-2 induction. Therefore, S. Typhimurium was grown in Mgm-medium in presence of 100 µg/ml obacunone and relative change in expression of *ssrA*, *ssrB* and *spiC* were measured using qRT-PCR. Consistent with the hypothesis, obacunone exposure resulted in repression of *ssrA*, *ssrB* and *spiC*, suggesting regulation of SPI-1 and SPI-2. We were further interested in determination of effect of obacunone on the expression of expression of SPI-1 and SPI-2 under adhesion and invasive model conditions. Both SPI-1 and SPI-2 are essential for *S.* Typhiurium pathogenicity. SPI-1 is induced during early invasion stages, whereas SPI-2 gets induced during late stages of *S.* Typhimurium infection. Obacunone exposure resulted in repression of *hilA* and *ssrA* measured under adhesion and invasive conditions. These data suggest that obacunone may help in reducing the severity of *S.* Typhimurium infection. Together data seems to reveal a model where obacunone represses SPI-1 and SPI-2 (and probably mal regulon)

in EnvZ dependent fashion. A summary speculative model of effect of obacunone is presented in Fig. 7.9.

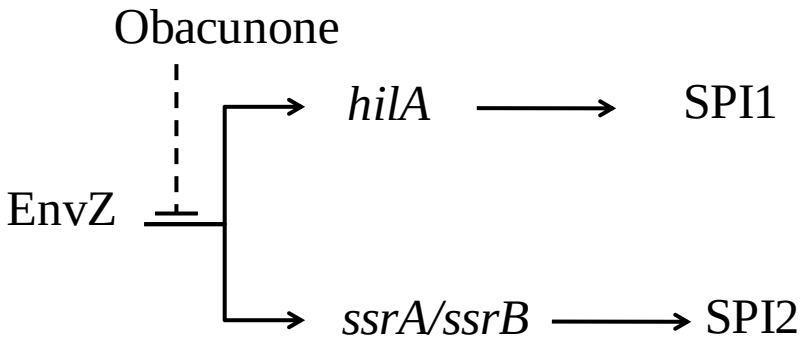

FIG. 7.9: Speculative model of action of obacunone on *S.* Typhimurium LT2

In the nutshell, obacunone exposure repressed the SPI-1 and SPI-2, which resulted in reduced number of *S.* Typhimurium cells attached to and internalized in Caco-2 cells. Furthermore, obacunone seems to exert its action in EnvZ dependent fashion. However, EnvZ also regulates the expression of porins *ompC* and *ompF*. One possible scenario is that obacunone is transported inside the cell through porins OmpC/OmpF and the actual target is different than Env/OmpR. Another possibility is that obacunone regulates the SPI-1 and SPI-2 in SirA/BarA dependent fashion. These possibilities require further investigation, and will be helpful in deducing the mode of action of obacunone. In conclusion, the data suggest that obacunone exerts an antipathogenic effect on *S.* Typhimurium, and may serve as a lead compound in development of antipathogenic strategies to counter *S.* Typhimurium infection.

CHAPTER VIII

INHIBITION OF CELL-CELL SIGNALING BY CITRUS FLAVONOIDS*

8.1 Synopsis

This study investigated the quorum sensing, biofilm and type three secretion system inhibitory properties of citrus flavonoids. Flavonoids were tested for their ability to inhibit quorum sensing using Vibrio harveyi reporter assay. Biofilm assays were carried out in ninetysix well plates. Inhibition of biofilm formation in *Escherichia coli* O157:H7 and *V. harveyi* by citrus flavonoids was measured. Furthermore, effect of naringenin on expression of *V. harveyi* TTSS was investigated by semi-quantitative PCR. Differential responses for different flavonoids were observed for different cell-cell signaling systems. Among the tested flavonoids- naringenin, kaempferol, quercetin and apigenin were effective antagonists of cell-cell signaling. Furthermore, these flavonoids suppressed the biofilm formation in *V. harveyi* and *E. coli* O157:H7. In addition, naringenin altered the expression of genes encoding type three secretion system (TTSS) in *V. harveyi*. The results of the study indicate a potential modulation of bacterial cell-cell communication, *E. coli* O157:H7 biofilm and *V. harveyi* virulence, by flavonoids especially naringenin, quercetin, sinensetin and apigenin. Among the tested flavonoids, naringenin emerged as potent and possibly a non-specific inhibitor of autoinducer mediated cell-cell signaling. Naringenin and other flavonoids are prominent secondary

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metabolites present in citrus species. Therefore, citrus, being a major source of some of these flavonoids and by virtue of widely consumed fruit, may modulate the intestinal microflora. Currently a limited number of naturally occurring compounds have demonstrated their potential in inhibition of cell-cell communications, therefore citrus flavonoids may be useful as lead compounds for development of antipathogenic agents.

8.2 Introduction

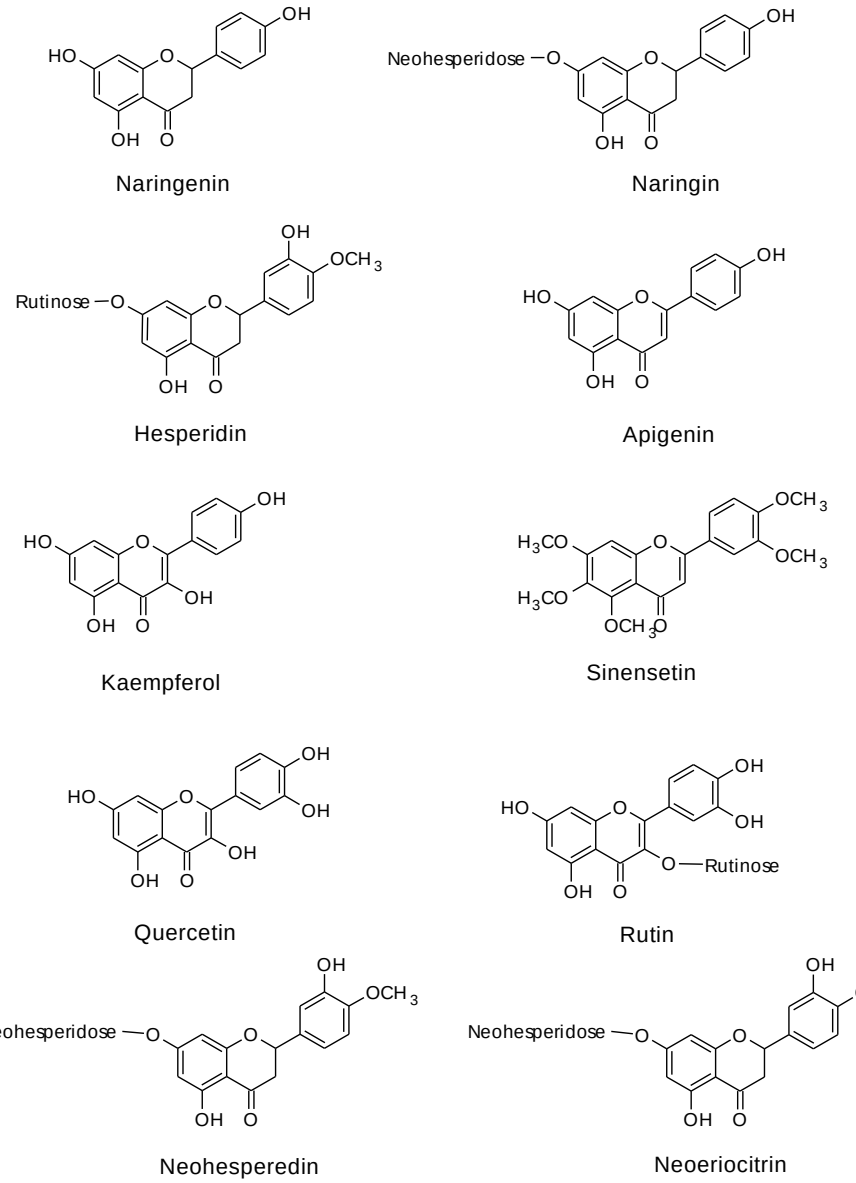
Flavonoids are prominent secondary metabolites ubiquitously present in the plant kingdom. Health promoting properties of flavonoids are focus of intensive research since the beginning of 20^{th} century. Several epidemiological studies have also correlated intake of citrus flavonoids with health benefits (31, 307). Over the years, research in various laboratories has demonstrated several health benefits of flavonoids. Studies in our lab (241, 369, 399) elsewhere (31, 249) have provided numerous evidences, suggesting role of citrus flavonoids in anticancer, antioxidant, anti-inflammatory, cardiovascular diseases and other health benefits.

In recent years, anti-microbial activity of flavonoids against various bacterial species is being explored. However, the published data on the antimicrobial properties of flavonoids do not seem to provide consistent results (74). This is evident from the divergent minimum inhibitory concentration (MIC) values reported for *Escherichia coli* (18-20, 125, 224, 296). This paradoxical situation may be the result of different assay systems employed, and often unreported initial inoculum-size, which significantly affects the outcome of the experiment. Furthermore, it is possible that, some of these flavonoids influence the bacterial cells in non-inhibitory fashion i.e. they modulate

various physiological processes rather than inhibiting the growth. These observations led us to explore the role of flavonoids as modulators of other possible mechanisms. One such intensively investigated anti-virulence mechanism is quorum sensing. It has been postulated that interference with quorum sensing or cell-cell signaling will impact the bacterial pathogenicity (292-293).

Quorum sensing is a coordinated regulation of gene expression as a function of cell-density (23). Bacterial cells produce and secrete small molecules, termed autoinducers, in the local environment. These autoinducers are recognized by specific two-component signaling systems in a concentration dependent fashion. Upon reaching a threshold concentration, binding of autoinducers to specific receptors, elicits adequate response to initiate signaling cascade. Activation of signaling cascade results in simultaneous regulation of several genes across the population (23, 246, 346). This signaling system is used by the bacteria to assess its population as well as density of other bacterial species in a particular niche (50). This phenomenon has gained importance in recent years with the enumeration of tight regulation of pathogenic traits such as virulence and biofilm formation by autoinducer mediated cell-cell signaling (5, 21, 60, 116, 124, 133, 153, 250, 378). Therefore, quorum sensing or cell-cell signaling has emerged as an alternative target to control bacterial virulence (155, 292-293).

(A)



(B)

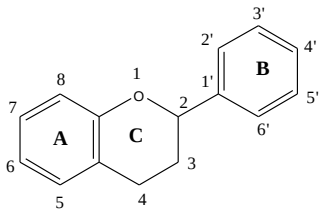


FIG. 8.1 (A) Structure of citrus flavonoids used in the study. (Neohesperidose= 2-O-alpha-L-Rhamnosyl-D-glucose; Rutinose= 6-O-L-rhamnosyl-D-glucose). (B) Basic Structure of Flavonoid nucleus

Biofilms are complex bacterial communities adhered to surfaces, which pose a critical problem in every day life by causing any economic and health problems (69). Biofilms severely afflict immune system, owing to the reduced expression of polysaccharide matrix and phagocytosis resistant biofilm-colonies (218). Even though there has been a great interest in biofilms, effective treatments are still limited. With the increase in the importance of biofilms, it has become imperative to search for alternative antimicrobials with non-conventional targets. Quorum sensing may be one such target, since quorum sensing, in particular autoinducer-2 mediated cell-cell signaling, may be important regulatory factor for biofilm production in *E. coli*, *Vibrio* spp., and *Salmonella* Typhimurium (85, 133, 213, 246, 312). Research in recent years has targeted towards identification of synthetic compounds and analogs with quorum sensing inhibitory properties or quorum quenching. However, only a limited number of natural compounds have been tested for their quorum quenching abilities (4, 43, 61, 170, 207). On the other hand, a few natural products have been demonstrated to possess biofilm inhibitory property (62). Considering the potential of the natural compounds in disease prevention, it is imperative to study their potential as quorum sensing and biofilm inhibitors. Since, certain flavonoids are biologically active in preventing diseases, we were interested in elucidation of their effect on the bacterial system. In the current study, we explored the impact of citrus flavonoids (Fig. 8.1A) on autoinducer mediated bacterial cell-cell signaling. Furthermore, ability of these flavonoids to curtail cell-cell signaling controlled processes, such as biofilm formation and type three secretion system, was investigated.

8.3 Materials and Methods

8.3.1 Flavonoids

Naringin has been purified previously in our laboratory (289). Naringenin, kaempferol, quercetin, rutin, hesperidin, apigenin, neohesperidin and neoeriocitrin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sinensetin was purchased from Chromadex Inc. (Irvine, CA, USA). All compounds were dissolved in DMSO at 10 mg ml^{-1}.

8.3.2 Bacterial Strains and Media

E. coli O157:H7 ATCC 43895 and biosensor strain *Vibrio harveyi* MM32 (luxN::Cm, luxS::Tn5Kan**)** were purchased from ATCC (Manassas, VA, USA). The bioreporter strain *V. harveyi* BB886 (*luxPQ*::Tn5) and *V. harveyi* strain BB120 (Wild type) were kindly provided by B. L. Bassler (Princeton University, Princeton, N.J.) (102). *E. coli* K12 was used as a positive control for AI-2 bioassay. The autoinducer bioassay (AB) medium was used to culture the *V. harveyi* strains (212, 346). The colony forming antigen (CFA) medium was used to culture the *E .coli* O157:H7 strain (102, 172) whereas *V. harveyi* BB120 biofilm was grown in LM medium.

8.3.3 Cell Growth Assay

Overnight culture of *V. harveyi* BB120 and *E. coli* O157:H7 were diluted in 1:100 ratio in LM or LB media and treated with either 100, 50, 25, 12.5 or 6.25 μg/ml flavonoids or equivalent volume of DMSO. The cultures were grown for 16 h and OD at 600 nm was measured every 2 h. In addition, 1:100 dilution of *V. harveyi* BB120 and *E. coli* O157:H7 overnight culture was treated with 100 μg/ml of flavonoids and grown at

30°C or 37°C with shaking. Samples were drawn every two-hour and serial dilution were prepared in saline solution, and plated on LM or LB agar plates. The colonies were counted after 24 h of growth.

8.3.4 Assay for Inhibition of Intercellular Signaling

Cell free supernatant (CFS) was prepared from *E. coli* strain K12 and *V. harveyi* BB120 as described earlier (346). Overnight cultures of *V. harveyi* and *E. coli* were diluted at 1:100 ratio with LM or LB media and grown further at 30°C (for *V. harveyi)* or 37°C (*for E. coli*) to achieve high concentration of autoinducer molecules. CFS was collected by centrifugation of cells at 4000 rpm for 20 minutes followed by filtration through 0.2 µm membrane filter and stored at -20°C.

The citrus flavonoids at five different concentrations (6.25, 12.5, 25, 50 and 100 µg/ml), were tested for their ability to inhibit the luminescence in *V. harveyi* reporter strains BB886 (AHL) or MM32 (AI-2). Bioluminescence assay was used essentially as reported previously (213, 346) with one modification. The reporter strains were cultured overnight at 30°C with aeration in AB media and diluted in 1:5000 ratio with fresh AB medium. Each well received 5 % CFS, 0.5 % flavonoids in DMSO or DMSO, 4.5 % sterile AB media and 90 % freshly diluted culture. In case of negative control CFS was replaced with sterile AB media. The microtiter plates were incubated for 4 h at 30°C at 100 rpm. At the end of incubation period light production was measured by using a Victor^2 1420 multilabel counter (Beckman Coulter, Fullerton, CA) in luminescence mode. The values were recorded as relative light units (RLU) and used in calculation.

8.3.5 Biofilm Formation

Overnight cultures of *E. coli* O157:H7 strain ATCC 43895 and *V. harveyi* BB120 were diluted with fresh CFA media or LM media (102). The diluted culture was placed in 96-well microtiter plates. All the wells received either 0.5 % of DMSO (control) or test compound dissolved in DMSO. The plates were incubated at 26°C for 24 h without shaking. Total biofilm mass was quantified by washing the plates with phosphate buffer (0.1 M, pH 7.4), and staining with 0.3% crystal violet (Fisher, Hanover Park, IL) for 20 min. The extra dye was removed by washing with phosphate buffer (0.1 M, pH 7.4). All of the dye associated with the attached biofilm was dissolved with 200 µl of 33% acetic acid, and an absorbance reading at 570 nm by Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT) was used to quantify the total biofilm mass. Each experiment was carried out at least thrice with three replicated wells per plate. Each data point was averaged from three replicates and expressed as mean ± standard deviation.

8.3.6 Semi-Quantitative Realtime RT-PCR

Three genes encoded in three *V. harveyi* TTSS locus *vopD*, *vscO* and *vscR* were selected for study. Relative transcript amounts of three genes were measured by qRT-PCR. Primers were designed by Primer3 software program (312). Primers designed were targeted to the unique sequences as determined by the alignment with the *V. harveyi* BB120 genomic sequence (Table 8.1).

TABLE 8.1: Sequence of *V. harveyi* primers used in the study

Gene Name		Sequence 5' to 3'
vopD	*forward*	CAGCCAATACGCCTGATACTAC
	reverse	AGCACTGAATGGTGAGCAAG
vcrD	*forward*	CTTTGCTGTTGCGAGATGG
	reverse	TGTTGGTTGGTGGCGTTC
vscO	*forward*	CGACGGTTTTCTGTTTGG
	reverse	TGAGCCCTACACCGACAT
16srRNA	*forward*	CGGACTACGACGCACTTTTT
	reverse	TTGTTTGCCAGCGAGTAATG

One ml samples from naringenin (100 µg/ml), hesperidin (100 µg/ml) or DMSO (10 µl/ml) treated cultures were collected at each time point during 8 hr growth period as mentioned in "Cell Growth Assay" section. Total RNA was extracted from the samples using RNeasy minikit (Qiagen Inc.) according to the manufacturer's instructions. RNA quantity was measured using NanoDrop ND 1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed using MuLV reverse transcriptase enzyme and random hexamer on a programmable thermocycler (Applied Biosystems, Gene Amp PCR system 2700), under following conditions: 60 min at 42˚ C for annealing and elongation followed by 5 min at 99˚C to inactivate the reverse transcriptase enzyme. Amplification of target sequences was done on ABI-Prism 7000 HT (Applied Biosystems, Foster City, CA). A total of 25 ng cDNA was amplified using 10pmol of primers and 10 µl of SYBR® GREEN PCR mix (Applied Biosystems, Warrington, UK). All data was normalized to the 16s gene. After completion of 40 PCR cycles, melt curve data was generated. All the measurements were done on three biological replicates consisting of three technical replicates each.

8.3.7 Statistical Analysis

The inhibition of AI activity was calculated from the formula 100- [(relative AI activity/relative activity of positive control) x 100] (212) and expressed as percentage and SD values. The percent inhibition of biofilm formation was calculated as 100- [(OD_{570} of sample well/ OD_{570} of positive control) x 100] and expressed as percentage and SD values.

For semi-quantitative real-time PCR, the cycle threshold (C_t) was calculated as the cycle number when primary fluorescent curve crossed a threshold of 30 fluorescence units. The C_t values for primers were normalized against that of 16S. Fold change in the gene expression was calculated by $2^{(-\Delta\Delta Ct)}$ (210) and expressed as fold change ±SD.

8.4 Results

8.4.1 Interference with Bioluminescence Production in *Vibrio harveyi*

To evaluate the effect of flavonoids on cell-cell communication system, the bioluminescence production in reporter strains *V. harveyi* BB886 and MM32 in presence of flavonoids were measured. All the flavonoids except hesperidin (Fig. 8.2E) inhibited either HAI-1 or AI-2 mediated bioluminescence, significantly. Naringenin, kaempferol, quercetin and apigenin (Fig. 8.2A, H, F and I) significantly ($p<0.01$) inhibited both HAI-1 and AI-2 induced bioluminescence. Naringin and neohesperidin (Fig. 8.2B and D) depicted stronger inhibitory activity against HAI-1. In contrast, sinensetin (Fig. 8.2J) was more effective antagonist against AI-2 mediated bioluminescence (Fig. 8.2J). Naringin and sinensetin demonstrated maximal response at 100 μg/mlagainst HAI-1 and AI-2 respectively; whereas neohesperidin depicted a saturated response at higher than 25

µg/mldoses against HAI-1. Moreover, naringin and rutin (Fig. 8.2B and G) demonstrated a concentration dependent inhibition of HAI-1 and AI-2. At the same time, hesperidin and neoeriocitrin were relatively ineffective (Fig. 8.2E and C).

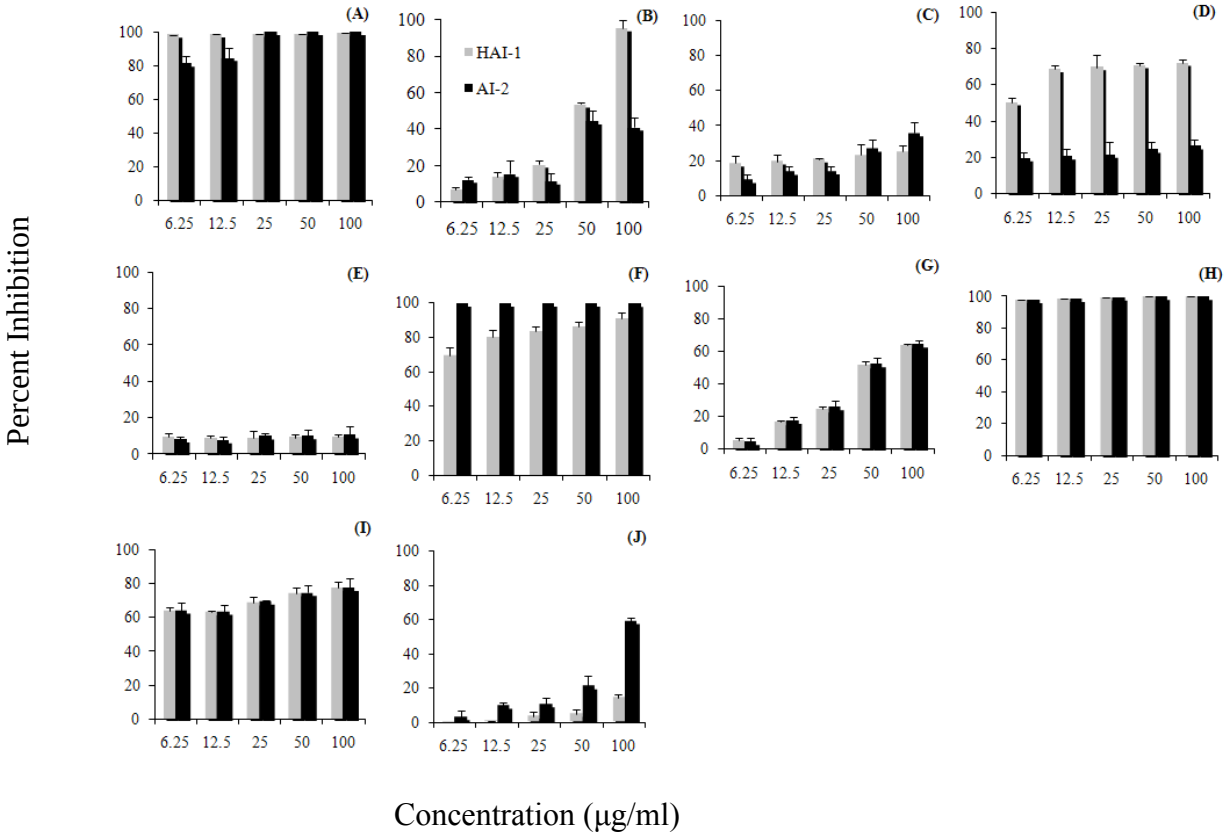


FIG. 8.2 Inhibition of HAI-1 (grey bars) and AI-2 (black bars) mediated bioluminescence in *V. harveyi* mutant strains BB886 and MM32. (A) naringenin, (B) naringin, (C) neoeriocitrin, (D) neohesperedin, (E) hesperidin, (F) quercetin, (G) rutin, (H) kaempferol, (I) apigenin and (J) sinensetin. The bars represent mean of 9 data points and SD

8.4.2 Response of *Vibrio harveyi* and *E. coli* O157:H7 Growth to Flavonoids

Growth rate of *V. harveyi BB120* was measured at OD_{600} over a period of 16 hr (Fig. 8.3A) to determine the inhibitory effect of flavonoids. Apigenin, quercetin and kaempferol inhibited the growth of *V. harveyi* BB120 significantly ($p<0.01$) whereas neoeriocitrin induced the growth rate significantly ($p<0.01$). Naringenin, naringin, neohesperedin, sinensetin, hesperidin and rutin did not show inhibitory effect on *V. harveyi* BB120 growth ($p>0.05$). In order to further confirm the influence of naringenin, quercetin and kaempferol, *V. harveyi* was grown upto 16 h in presence of these flavonoids and viable cell count was determined by plating on LM agar plates every two h (Fig. 8.3B). Quercetin and kaempferol demonstrated significant effect on *V. harveyi* growth, whereas naringenin was ineffective at 100 µg/ml. These results supported the earlier observations using OD_{600}. However, a dose dependent response of quercetin and kaempferol was observed (Fig 8.3 C,D,E,F). In contrast, only quercetin and kaempferol demonstrated growth inhibitory activity against *E. coli* 0157:H7 at 100 µg/ml (Fig 8.4 A, B), but not at lower concentrations (Fig. 8.4 C,D,E,F).

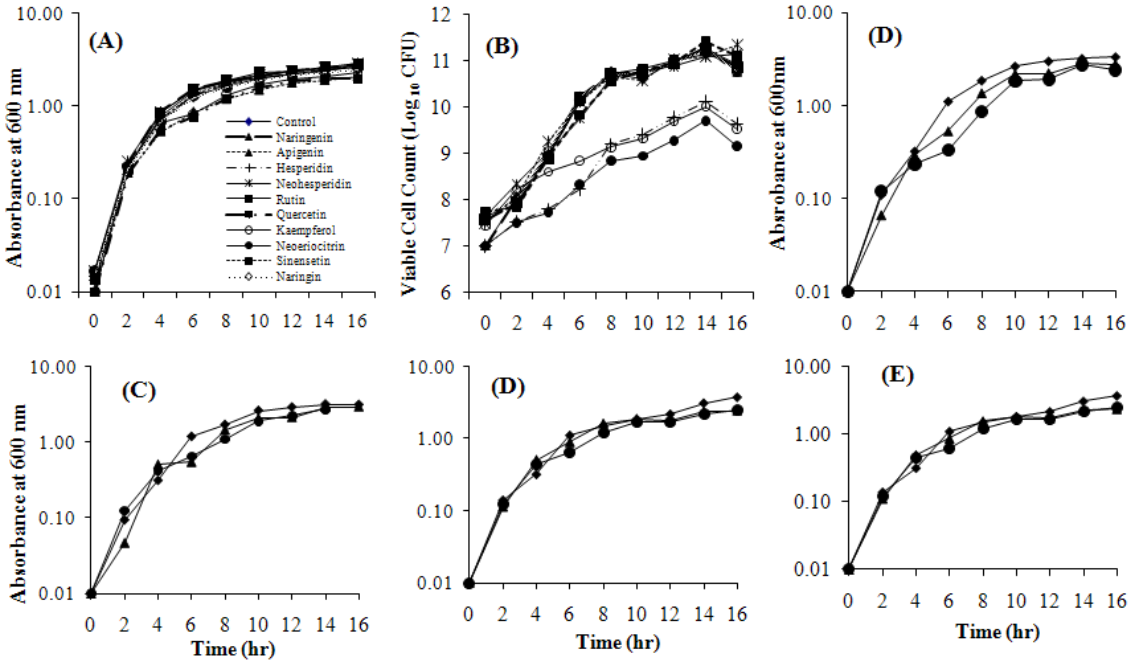


FIG. 8.3 . Growth of (A) *V. harveyi* BB120, (B) Viability of *Vibrio harveyi* BB120 in presence of 100 µg/ml flavonoids measured by colony count method**.** Growth rate of *Vibrio harveyi* in presence of (C) 50 µg/ml, (D) 25 µg/ml, (E) 12.5 µg/ml and (F) 6.25 µg/ml of quercetin and kaempferol was measured upto 16 hrs and compared to control. The data represents mean of three biological replicates

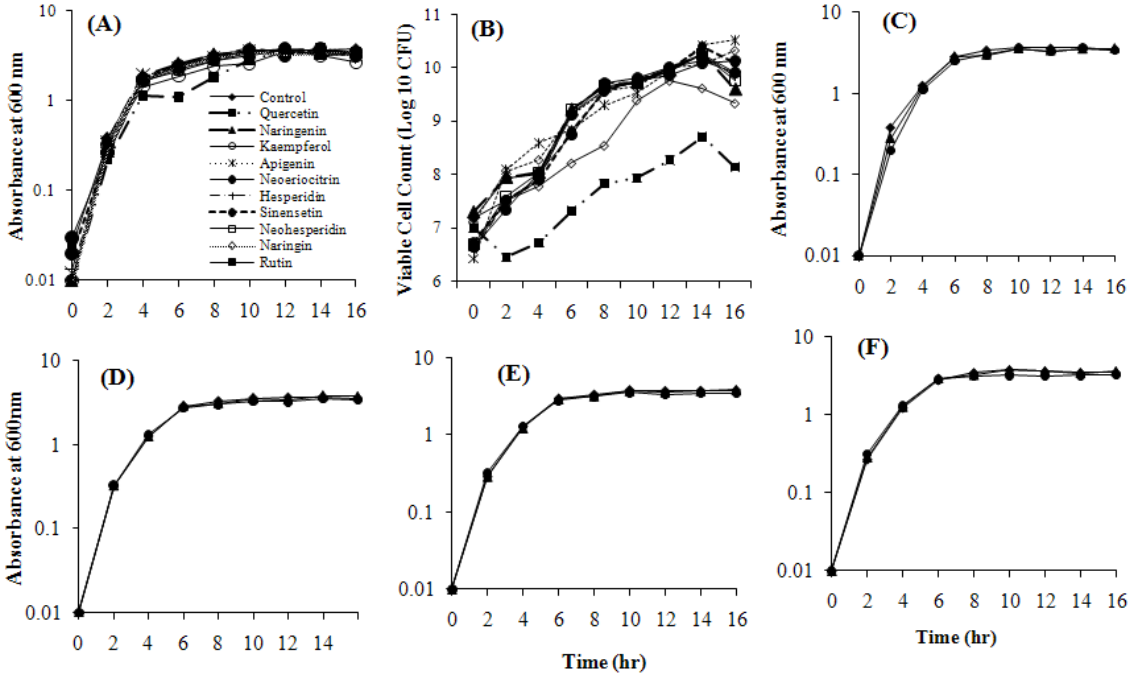


FIG. 8.4 (A) Growth curve of *E. coli* O157:H7 and (B) viable cell count in presence of 100 µg/ml flavonoids. Growth rate of *E. coli O157:H7* in presence of (C) 50µg/ml, (D) 25 µg/ml, (E) 12.5 µg/ml and (F) 6.25 µg/ml of quercetin and kaempferol was measured upto 16 hrs and compared to control. The mean of three biological replicates is presented

8.4.3 Inhibition of Biofilm Formation in *V. harveyi* and *E. coli* O157:H7

Biofilm formation in *Vibrio* spp. and *E. coli* is regulated by quorum sensing particularly by AI-2 mediated cell signaling (72, 133, 138). Therefore, we hypothesized that compounds inhibiting AI-2 mediated bioluminescence may influence the biofilm formation. The biofilm formation by *V. harveyi* and *E. coli* O157:H7 in presence or absence of flavonoids was investigated. All the flavonoids significantly ($p < 0.01$) inhibited *V. harveyi* BB120 biofilm formation, whereas except hesperidin, all the flavonoids demonstrated a significant ($p < 0.01$) inhibition of *E. coli* O157:H7 biofilm

formation in a dose dependent fashion (Fig. 8.5). Quercetin (Fig. 8.5F) and naringenin (Fig. 8.5A) were potent inhibitors of biofilm formation by *V. harveyi* BB120 and *E. coli* O157:H7. Interestingly, sinensetin depicted strong inhibition of *E. coli* O157:H7 and *V. harveyi* biofilm formation (Fig. 8.5J). These results also support the hypothesis that quorum sensing inhibitory flavonoids are likely to inhibit biofilm formation.

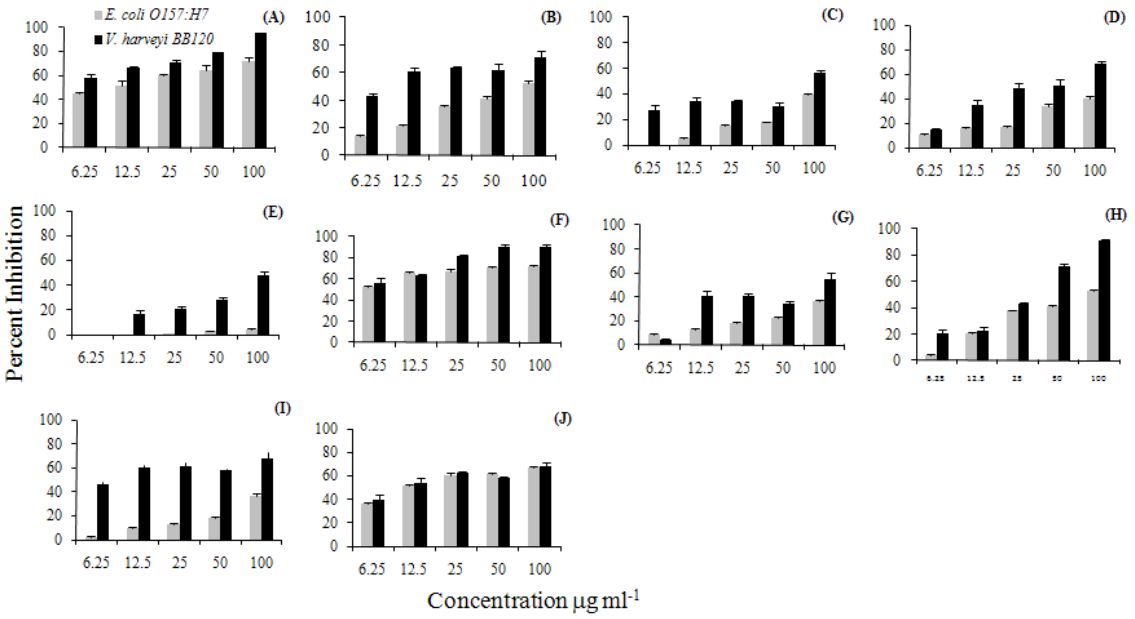


FIG. 8.5 Inhibition of biofilm formation by *E. coli* O157:H7 (grey bars) and *Vibrio harveyi* BB120 (black bars) by flavonoids. (A) Naringenin, (B) naringin, (C) neoeriocitrin, (D) neohesperedin, (E) hesperidin, (F) quercetin, (G) rutin, (H) kaempferol, (I) apigenin and (J) sinensetin. The bars represent average of 9 data points and SD

8.4.4 Response of *V. harveyi* Type Three Secretion System to Naringenin

Since naringenin emerged as potential candidate for cell-cell signaling inhibition, further investigation was focused on expression of *V. harveyi* type three secretion system

(TTSS) in presence of naringenin. In order to understand the behavior of TTSS, the expression of three genes located on three different locus (153) over a period of 8 h was investigated. Since DMSO was used as the solvent to dissolve flavonoids, control experiment consisted of treatment of *V. harveyi* with DMSO. Therefore, expression of the three genes was studied in presence of DMSO (Fig. 8.6A) was studied. Interestingly, *vopD* was upregulated by 151 fold by 2 h over the initiation point (t=0), which was gradually reduced to 23 fold by 8 h. Similar trend was observed for *vcrD* and *vscO*. The expression of *vscO* and *vcrD* increased up to 4 h thereafter, the expression level of both genes was reduced from their high points. This observation was consistent with the results reported by Henke and Bassler (153).

Further, we were interested in the effect of naringenin on the expression pattern of these three genes. The expression pattern of *vopD, vscO* and *vcrD,* in presence of naringenin depicted a similar trend as control i.e. induction during early stages of growth while repression at later phases. Although, expression of three genes depicted similar trend, their induction levels differed greatly. At 8 h *vopD, vscO* and *vcrD* were suppressed as much as 37, 43 and 2.5 fold respectively, over t=0 (Fig. 8.6B).

In addition, the expression of *vscO, vopD* and *vcrD* in response to naringenin over control was determined. The three genes were downregulated by naringenin treatment compared to the control. Interestingly, *vopD* was very strongly suppressed and *vcrD* and *vscO* were moderately downregulated (Fig. 8.6C). Since, the three genes are located on different loci, naringenin treatment may possibly down-regulate the loci involved in production of TTSS. As a negative control, expression of *vopD*, *vcrD* and

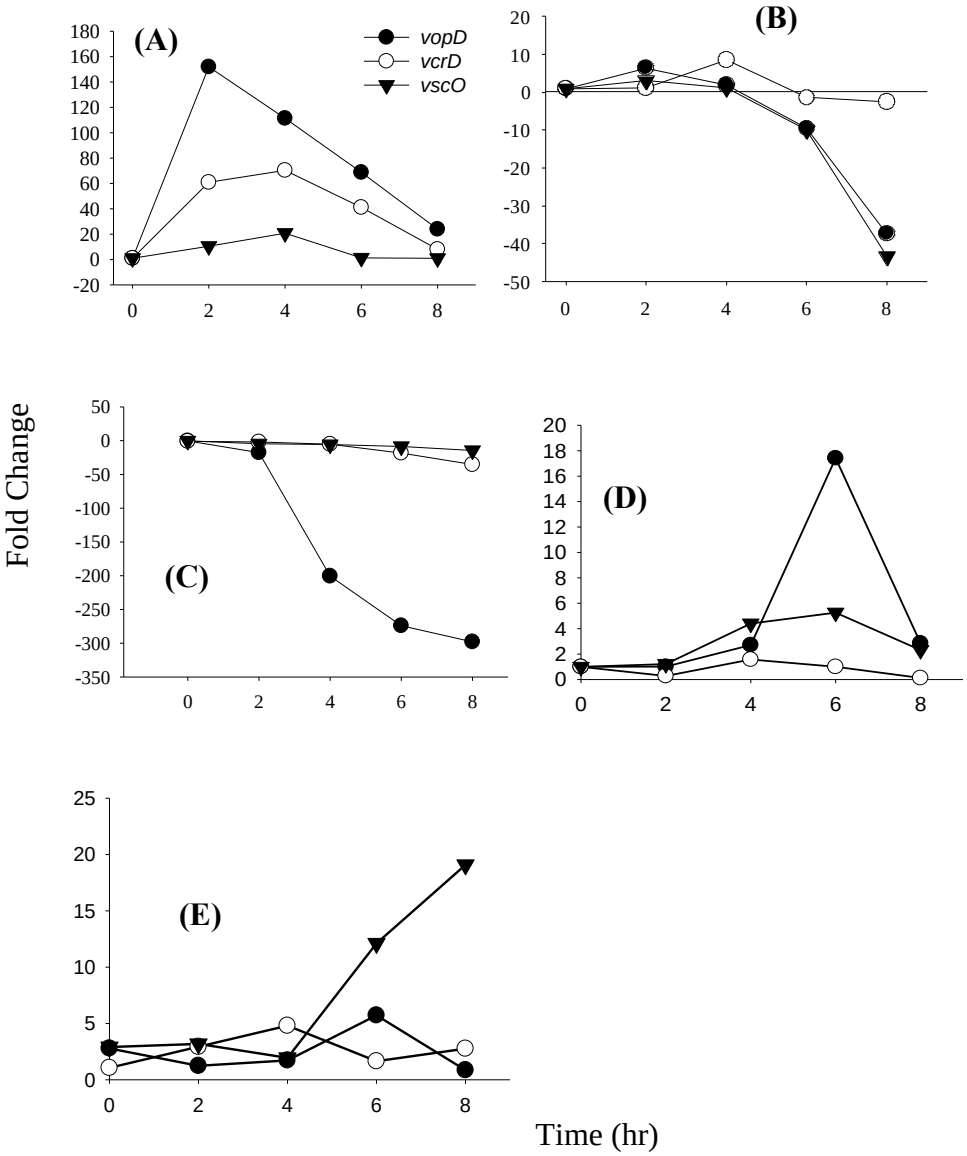


FIG. 8.6. Expression of *vopD*, *vcrD* and *vscO* over a period of 8 h in presence of (A) DMSO, (B) naringenin and (D) hesperidin. The fold change was calculated over the time =0 hr, value for which was taken as 1.0. (C) The relative change in expression of *vopD*, *vcrD* and *vscO* upon exposure of 100 µg/ml naringenin (C) and hesperidin (E) over control is presented

vscO was investigated upon exposure of 100 μg/ml hesperidin. Expression of *vopD*, *vscO* and *vcrD* demonstrated similar pattern as control i.e. all three genes were induced during 2-6 h and expression was decreased from their high level by 8 h (Fig. 8.6D). However, relative change over control demonstrated that *vopD* and *vscO* were upregulated 2.8 and 2.9 fold respectively at t=0. Expression of vopD was decreased at t=2 but increased thereafter upto 6 hr (5.7 fold) then decreased by 8 hr (Fig. 8.6E).*vscO* demonstrated similar trend except that it was induced by 19 fold at t=8. *vcrD* was induced upto 4 h and then decreased (Fig. 8.6E).

8.5 Discussion

In *V. harveyi*, bioluminescence production is regulated by quorum sensing. Three coincidence detectors, LuxN, LuxPQ and CqsS detect three autoinducer molecules HAI-1, AI-2 and CAI-1 respectively. Dephosphorylation cascade from three detectors converges on phosphorelay protein LuxU, resulting in regulation of transcriptional regulator LuxR. LuxR, in turn, regulates all the *V. harveyi* quorum sensing responsive genes known so far (23, 153-154, 246). Our data suggested that quercetin, kaempferol and naringenin are very effective antagonists of HAI-1 and AI-2 mediated cell-cell signaling. However, quercetin and kaempferol suppressed the growth rate of *V. harveyi* (Fig. 8.3A). Taken together, it is likely that the effect of quercetin and kaempferol at higher concentrations is due to their growth inhibiting ability rather than quorum sensing. However, at lower concentrations, activity of quercetin and kaempferol may be due to inhibition of quorum sensing.

These data must be interpreted with a caution, as mode of action of these flavonoids is not known. In contrast to quercetin and apigenin, naringenin did not inhibit the growth rate of *V. harveyi*, but strongly inhibited the HAI-1 and AI-2 induced bioluminescence. These observations suggest a possible quorum sensing inhibitory property for naringenin without affecting the bacterial growth. Since, HAI-1 and AI-2 mediated signaling activate same transcriptional regulator luxR (382) in *V. harveyi*, inhibition of both HAI-1 and AI-2 mediated cell-cell signaling is likely an indication of a nonspecific antagonistic activity. As naringenin inhibited both HAI-1 and AI-2 mediated bioluminescence (Fig. 8.2A), it is possible that naringenin is a nonspecific antagonist of cell-cell signaling, which targets the signal transduction pathway at LuxU or downstream of LuxU.

Furthermore, naringin, neohesperidin were found to be effective against HAI-1 mediated cell signaling whereas sinensetin was more active against AI-2. Differential inhibition of HAI-1 or AI-2 mediated bioluminescence by neohesperidin, naringin and sinensetin seems to suggest specific interactions. In addition, it is unlikely that inhibition of cell-cell signaling by sinensetin, naringin and neohesperidin may be due to growth inhibition, as these flavonoids did not affected the growth of *V. harveyi* (Fig. 8.3). Interestingly, apigenin, which affected the *V. harveyi* growth rate moderately, was weaker antagonist of AI-2 mediated cell-cell signaling compared to naringenin. However, mode of action of apigenin has not been elucidated. A probable cytostatic effect may explain the observed results.

Biofilm formation is highly regulated process and controlled by several factors including autoinducer mediated cell-cell signaling. Since quorum sensing also controls formation of biofilms, study of biofilm formation in presence of flavonoids was hypothesized to provide further insight into the inhibitory property of flavonoids. As previous studies have demonstrated that AI-2 regulates biofilm formation through MsqR (133) in *E. coli*, it was likely that flavonoids inhibiting the AI-2 mediated cell-cell signaling will inhibit also biofilm formation. Since, our data suggested that some of the flavonoids interfere with autoinducer mediated cell-cell signaling, we were further interested if these flavonoids can also influence biofilm formation. The results indicated that naringenin was a potent antagonist of *V. harveyi* BB120 and *E. coli* O157:H7 biofilm formation. Taken together, results of cell-cell signaling inhibition and biofilm inhibition suggest that the ability of naringenin to modulate the biofilm may stem from its antagonistic activity for autoinducer mediated cell-cell signaling. On the other hand, inhibition of biofilm at higher concentrations by quercetin may be attributed to its growth inhibitory property (Fig. 8.3A, B and 4F). Similar to *V. harveyi.*, quercetin and kaempferol may act through inhibition of quorum sensing. Interestingly, sinensetin was found to be very potent antagonist of *V. harveyi* and *E. coli* O157:H7 biofilm. It is pertinent to note that *E. coli* possess *luxS*/AI-2 quorum sensing system but does not produce AI-1 molecule (378). It is possible that the antagonistic activity of sinensetin against biofilm formation is due to inhibition of AI-2 mediated cell-cell signaling. On the contrary, apigenin did not inhibit biofilm formation of *V. harveyi* BB120 or *E. coli* O157:H7 as effectively as quercetin or naringenin. Consistent with published literature,

apigenin reduced the growth rate of *V. harveyi* (74). It is possible that apigenin possess only growth inhibitory property similar to several growth inhibitory agents, which demonstrate poor antagonistic activity against biofilms (205).

Furthermore, the bioluminescence and biofilm inhibitory activities of glycosylated flavonoids did not correspond closely (compare Figs. 8.2B, C, D, E and G with Figs. 8.4B, C, D, E and G, respectively). Natural compounds are reported to inhibit biofilm formation by various mechanisms without affecting the growth rate (115, 303). It is possible that, the various flavonoids may have differential mode of action for inhibition of biofilm formation.

In *V. harveyi* three operons encoding a TTSS were identified (153, 246). TTS system in *V. harveyi* is suggested to be regulated by cell-cell signaling, and both HAI-1 and AI-2 is required for significant differential expression of type III secretion system (103, 124). Based on phenotypic assays, naringenin emerged as most potent cell-cell signaling inhibitor, therefore, its effect on the expression of TTS system was studied. Three genes *vopD*, *vscO* and *vcrD*, encoded by three TTSS operons (124) were selected to investigate the effect of naringenin on three operons. Naringenin suppressed all the three genes, suggesting suppression of corresponding loci. In contrast, hesperidin, which did not demonstrated appreciable activity in bioluminescence assays and was moderately effective against *V. harveyi* biofilm, induced the three genes. Quorum sensing inhibitor furanones isolated from the sea algae *Delisea pulchera*, cinnamaldehyde and their derivatives were shown to inhibit the virulence of *V. harveyi* BB120 (45, 84-85). In the current study, the naringenin disrupted the cell-cell signaling, biofilm formation and

suppressed the virulence genes. Our results were similar to the published reports related to two quorum sensing inhibitors furanone and cinnamaldehyde (45, 86). Based on these observations, naringenin seems to be a natural quorum sensing disruptor molecule.

Flavonoids present in *Citrus* spp. belong to three of six major classes- flavanone, flavone and flavonols (49). Different *Citrus* spp. are characterized by distinct flavonoids profiles; however, flavanones are the dominant class of flavonoids present in many citrus species (126, 153). Further, the bioactivity of flavonoids in vitro depends upon the arrangement of functional groups on the nucleus. In the current study, ten flavonoids, representing flavanone (naringenin, naringin, neoeriocitrin, neohesperedin and hesperidin), flavonol (kaempferol, quercetin and rutin), flavone (apigenin) and polymethoxy flavone (sinensetin) were investigated.

In vitro biological activities of flavonoids were dependent upon the type of sugar moiety and the position. Addition of sugar moiety may be detrimental to the biological activity. Reduction in potency of aglycones by addition of sugar moiety at 7^{th} position (Fig. 8.1A and 1B) (naringin) as well as at 3^{rd} position (rutin) in the three assays was observed. Moreover, neohesperidose was found to be more effective than rutinose.

The spatial arrangement and number of functional groups affects the bioactivity of flavonoids. In particular, presence of a double bond between 2^{nd} and 3^{rd} position enhances the antioxidant and anti-cancer activities of flavonoids (110, 151). In contrast, addition of double bond between 2^{nd} and 3^{rd} position was detrimental in our assays, which is evident from the reduced activity of apigenin compared to naringenin. However, addition of hydroxyl groups was found to increase the activity, with 3^{rd}

position being more responsive. Whereas, modification at 4′ position did not affect the activity dramatically. In case of aglycones, addition of hydroxyl group at 5′ position was found to increase the antimicrobial action.

In the current study we provided the evidence that citrus flavonoids may influence the bacterial cell-cell signaling and biofilm formation. Furthermore several flavonoids modulated the biofilm formation by *E. coli* O157:H7, a pathogen with no known effective treatment at present. Based on our results we propose that naringenin is possibly a non-specific quorum sensing inhibitor. Moreover, naringenin suppressed the biofilm formation in *V. harveyi* and *E. coli* O157:H7 as well as TTSS in *V. harveyi*. These properties may have their implication in human health as naringenin was found to modulate AI-2 mediated signaling and *E. coli* O157:H7 biofilm. It is pertinent to note that intestinal microflora produce AI-2, which may be involved in interspecies signaling among different bacterial species (342). Citrus fruits are very widely consumed and are rich source of dietary flavonoids including naringenin. Therefore, consumption of citrus fruits and juices may influence the cell-cell signaling by gut microflora. In addition, these flavonoids may also serve as the lead compounds for the antipathogenic drug discovery, a critical requirement to counter the effects of pathogenic bacteria.

CHAPTER IX

ANTIPATHOGENIC PROPERTY OF CITRUS FLAVONOID- NARINGENIN AGAINST *SALMONELLA* TYPHIMURIUM LT2*

9.1 Synopsis

Salmonellosis is one of the leading health problems worldwide. With the rise of drug resistance strains, it has become imperative to identify alternative strategies to counter bacterial infection. Natural products were used historically to identify novel compounds with various bioactivities. Citrus species is a rich source of flavonoids. Naringenin, a flavonone, is present predominantly in grapefruit. Previously we have demonstrated that naringenin is potent inhibitor of cell-cell signaling. The current study was undertaken to understand the effect of naringenin on *Salmonella* Typhimurium LT2. The cDNA microarrays were employed to study the response of *S.* Typhimurium to naringenin treatment. Naringenin specifically repressed 24 genes in the *Salmonella* pathogenicity island 1, and down-regulated 17 genes involved in flagellar and motility. Furthermore, phenotypic assays support the result of microarray analysis. In addition, naringenin seems to repress SPI-1 in *pstS/hilD* dependent manner. Altogether the data suggest that naringenin attenuated *S.* Typhimurium virulence and cell motility. This is the first molecular evidence to demonstrate effect of naringenin on bacterial virulence and cell motility.

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*Reprinted with permission from "Citrus flavonoid represses *Salmonella* pathogenicity island 1 and motility in *S.* Typhimurium LT2" by A. Vikram, P. R. Jesudhasan, G. K. Jayaprakasha, S. D. Pillai, and B. S. Patil, 2011, 145:28-36, Int. J. Food Microbiol.

9.2 Introduction

Flavonoids are a very diverse group of secondary metabolites, biosynthesized by plants. Bioactivities demonstrated by flavonoids against chronic diseases such as cancer, cardiovascular disease, neurodegenerative disorders (150, 241, 368) as well as antioxidant properties and modulation of intracellular signaling pathways (305, 388) suggest potential beneficial effects on human health. In addition, antibacterial activities of certain flavonoids are well documented (74). Flavonoids such as quercetin, apigenin, kaempferol, galangin and genistein were reported to possess potent antibacterial activity (18, 113, 258, 296).

In US, tea, citrus juices and wine are the major source of dietary flavonoids (63). *Citrus* species predominantly accumulate flavonones a subclass of flavonoids. The daily consumption of flavonoids through dietary sources was reported to be as high as 1 g/d (191). In addition, mean flavonone intake in the USA and European population was estimated between 14- 46 mg/d (63, 93). Furthermore, bioavailability and absorption profile of flavonoids have been studied extensively. The literature supports a model where a limited amount of dietary flavonoids get absorbed in the small intestine (338), while a large proportion of ingested flavonoids reach colon without degradation (299). The colon contain about 10^{12} micro-organisms per g (332, 389), which interact with dietary components. Dietary flavonoids are extensively metabolized by colonic microflora. Flavonoid glucosides such as naringin are catabolized to yield aglycone naringenin, which may further be metabolized to simpler products (300). On the other

hand, flavonoids were reported to influence the colonic microflora (266, 384) suggesting an important role in maintenance of colon health. However, the interaction of dietary components such as flavonoids with colonic microflora has not been studied extensively.

Salmonella enterica serovar Typhimurium is among the leading cause of food borne diseases. The pathogen colonizes the large intestine and incites gastroenteritis and colitis. *S.* Typhimurium has significant economic burden due to very broad host range and significant morbidity and mortality in human population (44). Several outbreaks of *S.* Typhimurium infection were reported including a recent outbreak in the year 2008-09, causing 714 illnesses spread over 46 states in USA (56). In addition, emergence of antimicrobial resistant *S.* Typhimurium strains pose significant public hazard (256). In order to prevent such outbreaks, identification and development of new antimicrobial agents is warranted.

Salmonella spp. invades normally non-phagocytic epithelial cells, remodels the host actin cytoskeleton and manipulates the signal transduction pathways to induce membrane ruffling resulting in uptake by macropinocytosis. Once internalized, the bacteria multiply in specialized intracellular endosomal compartments called *Salmonella*-containing vacuoles (SCVs) (68). Invasion is mediated by Type III Secretion System (TTSS) encoded in *Salmonella* pathogenicity island 1 (SPI1) (288). SPI1 is required for the invasion and colonization of intestine (55), as well as maturation of SCVs (119). Therefore, SPI plays an important role during pre- and post-invasion efficiency of pathogen in epithelial cells (183). In addition to SPI1, flagella also contributes to the pathogenesis and motility of *S.* Typhimurium (267).

Naringenin (Fig. 9.1), a flavonoid present in *Citrus* species, seems to possess anticancer, anti-inflammatory and antioxidant activities (203, 368). In addition, several in vitro studies reported antimicrobial activities of naringenin, however, the possible mode of action was not elucidated (74). In our previous studies, naringenin was identified as a non-specific quorum sensing inhibitor (371). Specifically, naringenin inhibited autoinducer mediated cell-cell signaling and biofilm formation in *V. harveyi* and *E. coli* O157:H7 as well as production of type three secretion system (TTSS) in *V. harveyi*. We wondered if the naringenin has an anti-pathogenic effect on *S.* Typhimurium. In the present study, effect of naringenin on *S.* Typhimurium transcriptome was explored using cDNA microarrays.

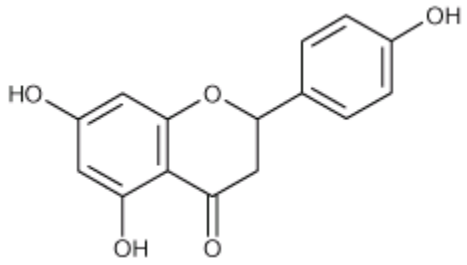

FIG. 9.1 Structure of naringenin

9.3 Materials and Methods

9.3.1 Chemicals

Naringenin was purchased from the Sigma (St. Louis, MO). A stock solution of naringenin was prepared by dissolving 40 mg of naringenin in 1 ml dimethyl sulfoxide (DMSO).

9.3.2 Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in Table 9.1. Unless otherwise specified, bacterial cultures were grown at 37^{o}C in Luria-Bertani (LB) medium supplemented with 0.5 % glucose (LB-glu). When appropriate, the medium was supplemented with 10 µg of chloramphenicol, 50 µg of kanamycin, or 10 µg of tetracycline per ml media.

TABLE 9.1 *Salmonella* Typhimurium strains and sequence of primers used in the study

Strain	**Genotype**	**Reference**
S. Typhimurium LT2	Wild Type	ATCC-15277
EE658	*hilA080*::Tn*5lacZY* (Tet^{r})	(214)
RL21	*fadD1*::Tn*5 hilA080*::Tn*5lacZY* (Kan^{r} Tet^{r})	(214)
RL119	*fliA51*::Tn*5 hilA080*::Tn*5lacZY* (Kan^{r} Tet^{r})	(214)
RL147	*pstS55*::Tn*5 hilA080*::Tn*5lacZY* (Kan^{r} Tet^{r})	(214)
EE711	*envZ182*::*cam hilA080*::Tn*5lacZY* (Cam^{r} Tet^{r})	(214)
LM401	*hilD1::kan hilA080*::Tn*5lacZY* (Kan^{r} Tet^{r})	(214)
Gene Name	***Sequence (5'- 3')***	
acrA-F	ACTTTGCGCGCCATCTTC	This Study
acrA-R	CGTGCGCGAACGAACAT	This Study
fimZ-F	TGCCACTATCTAACCGCGAAGT	This Study
fimZ-R	GGCAATTTCTTTGTTAGACATTCCA	This Study
hilA-F	GTCCGGTCGTAGTGGTGTCT	This Study
hilA-R	CGCATACTGCGATAATCCCT	This Study
hilC-F	GCTGAGGTGGCAGGAAAGC	This Study
hilC-R	CCTCTTCAGCGGCCAGTTT	This Study
hilD-F	CGACTTGGCGCTCTCTATGC	This Study
hilD-R	TCTCTGTGGGTACCGCCATT	This Study
invA-F	GGCGCCAAGAGAAAAAGATG	This Study
invA-R	CAAATATAACGCGCCATTGCT	This Study
marR-F	CGATCTCGGCGCATTGAC	This Study
marR-R	TTTCGATCCAGCCTTTGCA	This Study
orgA-R	CCCTGATGCATTGCCAAAA	This Study
orgB-F	ACCATCCCGAAACGCTTTTA	This Study

9.3.3 Growth and Metabolic Activity

Diluted (100 fold in LB-glu) overnight culture of *S.* Typhimurium LT2 (200 µl) was incubated in 96-well plates with 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/ml naringenin or equivalent amount of DMSO. OD_{600} was monitored for 16 h, at 15 min interval in SynergyTM HT MultiMode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The instrument was set to maintain 37^{o}C temperature with shaking at medium speed. The data was fitted to a 4-parameter sigmoid model $Abs_t = Abs_{min} + Abs_{max} - Abs_{min}/1+ \exp[-\mu max\ (t-t_i)]$ (75) and maximum specific growth rate (µmax) was calculated. Mean generation time was calculated using the formula $g = Ln(2)/\mu max$, and presented as mean of three biological replicates ± SD. Metabolic activity was measured by adding 25 µl AlamarBlue per well (Invitrogen, Carlsbad, CA, USA) and monitoring at 570 and 600 nm as described for growth curve.

9.3.4 Biofilm Assay

Biofilm assay was conducted in a 96-well plate (Fisher Scientific, Pittsburg, PA, USA) as described (371). In brief, freshly diluted overnight culture (100 fold in LB-glu) of *S.* Typhimurium LT2 was incubated with 200 to 1.5625 µg/ml naringenin or equivalent volume of DMSO at 26^{o}C for 24 h without shaking. Biofilms were stained with 0.3 % crystal violet (Fisher Scientific), dissolved in DMSO and quantified at 570 nm.

9.3.5 Microarray Analysis

9.3.5.1 RNA Extraction and Hybridization Conditions

Freshly diluted (100 fold in LB-glu) overnight *S.* Typhimurium LT2 culture was treated with 100 µg/ml naringenin or DMSO (control) and grown to OD600 ≈1.0 at 37^{o}C. Cultures were stabilized with RNAProtect bacteria reagent (Qiagen Inc., Valencia, CA, USA). Total RNA was isolated with RNeasy minikit (Qiagen Inc.) according to manufacturer's instructions. RNA quality was analyzed spectrophotometrically (Nanodrop, Fisher Scientific) and with bioanalyser 2100 (Agilent Technologies Inc., Santa Clara, CA, USA).

Total RNA (10 µg) from treatment and control was used to synthesize cDNA using random primer (Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously (176). The cDNA was purified with Qiaquick PCR purification kit (Qiagen Inc.) and labeled with Cy-3 or Cy-5 monoreactive dyes (GE Healthcare, Piscataway, NJ, USA). *S.* Typhimurium LT2 genome microarrays version 2, containing 5405 open reading frames from strain LT2, were obtained from Pathogen Functional Genome Resource Center of the National Institute of Health. Each microarray slide was hybridized with equal amount of labeled cDNA from naringenin and control samples. A total of 6 dye-swapped hybridizations, corresponding to three biological replicates, were conducted. Hybridizations were carried out at 42^{o}C for 18h. Microarrays were scanned on a GenePix Scanner 4100A (Molecular Devices Corporation, Sunnyvale, CA, USA) at 532 and 635 nm.

9.3.5.2 Data Collection and Analysis

Signal intensities from the images were extracted using GenePix 6.0 software (Molecular Devices Corporation). Spots with median signal value less than sum total of local background median value plus three standard deviations were omitted from further analysis. The median signal intensities for all probes were LOWESS normalized (285) using Acuity 4.0 software (Molecular Devices Corporation) and statistical significance was evaluated. Differentially expressed genes between naringenin and DMSO treated samples were identified by Student's t-test. P-values were adjusted for multiple comparisons using Benjamini-Hochberg method (32). Genes with $p<0.05$ were considered significantly differentially expressed and reported. The raw data and complete dataset are available at NCBI Gene Expression Omnibus database (accession no. GSE21873).

9.3.6 Quantitative PCR

For a group of highly induced or repressed genes, measurements of relative transcript amounts were performed by qRT-PCR. Primers (Table 9.1) were designed by Primer Express program (Applied Biosystems, Foster City, CA). Overnight culture of *S.* Typhimurium was diluted 100 fold in LB-glu and grown in presence of 100 μg^{-1} naringenin or DMSO (control). The samples were collected at $OD_{600} \approx 0.5$, 1.0 and 2.0 and RNA was extracted using the RNeasy minikit (Qiagen Inc.). First strand synthesis was carried out using MuLV reverse transcriptase enzyme and random hexamer in a Reverse-Transcriptase polymerase chain reaction (RT-PCR). The RT- PCR was performed on a programmable thermocycler (Applied Biosystems, Gene Amp PCR

system 2700), under following conditions: 60 min at 42˚ C for annealing and elongation followed by 5 min at 99˚C to inactivate the reverse transcriptase enzyme. The cDNA (25 ng) was amplified on ABI-Prism 7000 HT (Applied Biosystems, Foster City, CA) using SYBR® GREEN PCR mix (Applied Biosystems, Warrington, UK) and 10 pmol primers for target sequences. After completion of 35 PCR cycles, melt curve data was generated to ascertain template-independent amplification. The relative change in transcripts due to naringenin with respect to control was calculated using comparative Ct method. The change in gene expression was calculated using the formula: Naringenin/control= $2^{\Delta\Delta CT}$, Where $\Delta\Delta C_T = \Delta C_T$ for naringenin- ΔC_T for control and ΔC_T is the difference between the C_T value of the target gene and the normalization gene (16S rRNA) (315)**.** The mean ±SD of three biological replicates is presented.

9.3. 7 Motility Assay

Motility assay was performed on LB-agar (0.3 %) plates supplemented with 100 µg/ml naringenin or DMSO (176). Diluted overnight culture of *S.* Typhimurium LT2 was inoculated with sterile tooth-prick in the center of the assay plate and incubated at 37^{o}C. Motility halos were measured after 8 h. Data from five replicates, inoculated on different days was averaged and expressed as mean ±SD.

9.3.8 SW480 Cells Adhesion and Gentamicin Protection (Invasion) Assays

Adhesion assay was performed as described (334). Colon adenocarcinoma epithelial SW480 (ATCC # CCL-228) cells were routinely maintained in DMEM supplemented with 10 % fetal bovine serum (referred as DMEM henceforth). For adhesion assay, SW480 cells were seeded in 6 well plates at a density of 1 x 10^5

cells/well. The SW480 cells were infected with *S.* Typhimurium LT2 cells at a multiplicity of infection (moi) 10 and 100, in presence of 100 μg/ml naringenin or equivalent amount of DMSO. The plates were incubated for 3 h at 37^{o}C, 5 % CO_2. The excess *S.* Typhimurium LT2 cells were washed three times with PBS. The SW480 cells were lysed by 0.1 % TritonX-100 solution. The resulting culture was then serially diluted using 0.9 % saline solution and plated on the Tryptic Soy Agar plates. Colonies were counted after 24 h and recorded as Colony Forming Units (CFU).

Invasion assay was performed as described before (288). For gentamicin protection assay, 1 x 10^5 SW480 cells were infected with *S.* Typhimurium LT2 at moi 10 in presence of 100 μg/ml naringenin or DMSO. One hour after infection at 37^{o}C and 5 % CO_2, 100 μg/ml gentamicin was added to kill non-invasive cells and incubation was continued for additional 90 min. After incubation, the SW480 cells were washed with three times with PBS and lysed with 0.1 % TritonX-100 solution. The CFUs were determined by plating the appropriate dilution in 0.9 % saline solution on TSA.

9.3.9 β-Galactosidase Assays

Overnight culture of *S.* Typhimurium mutant strains (Table 9.1) were diluted (1:100) in LB-glu and grown up to OD600≈1.0 in presence of 100 μg/ml naringenin or DMSO. β-Galactosidase assay was performed as described previously (214, 243).

9.3.10 Statistical Analysis

Analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test on SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used to determine the effect of naringenin for each activity except microarray analysis. The effect was

considered significant at $p < 0.05$. The statistical analysis for microarray is provided in section 2.5.2.

9.4 Results

9.4.1 Effect of Naringenin on *S.* Typhimurium Growth and Biofilm

Growth rate of *S.* Typhimurium in presence of 1.56, 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/ml of naringenin was determined by measuring OD_{600} upto 16 h. Mean generation times were calculated as described in Methods and compared with control. Mean generation time for naringenin treatments were not significantly ($p>0.05$) different from the control (Table 9.2). Further, viability of *S.* Typhimurium in presence of different concentrations of naringenin was assessed using AlamarBlue. The mean generation times were calculated using percent reduced values of AlmarBlue over 16 h period. The effect of naringenin was determined by comparing the mean generation times of naringenin treated samples with control. Similar to turbidimetric assessment, mean generation times of naringenin treated cultures were not significantly ($p>0.05$) different from control (Table 9.2) at the tested concentrations.

To further study the effect of naringenin on *S.* Typhimurium, biofilm formation in 96-well plates was measured. *Salmonella* biofilms were grown in presence of 1.56-200 µg/ml of naringenin. Naringenin did not inhibit the *S.* Typhimurium biofilm grown on plastic surface in 96-well plates at 1.56 -100 µg/ml (Fig. 9.2). However, exposure of 200 µg/ml naringenin resulted in increased biofilm formation by *S.* Typhimurium. For further investigations, 100 µg/ml concentration was chosen.

TABLE 9.2 Generation times of *Salmonella* Typhimurium LT2 in presence of different concentrations of DMSO (Control) and naringenin calculated from OD_{600} and AlamarBlue reduction

Concentration (µg/ ml)	Generation times measured by OD^{600}			Generation times measured by AlamarBlue		
	Control	Naringenin	P value	Control	Naringenin	P value
200	23.04 ± 0.50	24.94 ± 3.28	0.38	21.75 ± 0.69	21.93 ± 0.36	0.77
100	23.56 ± 0.71	23.11 ± 0.76	0.50	24.93 ± 0.49	24.79 ± 2.54	0.37
50	24.90 ± 1.80	22.97 ± 0.97	0.18	23.34 ± 2.19	22.77 ± 0.17	0.08
25	25.32 ± 2.47	23.58 ± 1.19	0.33	24.55 ± 2.01	22.99 ± 0.11	0.26
12.5	23.68 ± 1.84	23.54 ± 1.01	0.91	25.81 ± 0.61	21.68 ± 0.25	0.44
6.25	23.91 ± 0.63	23.70 ± 1.09	0.78	26.22 ± 2.44	25.57 ± 0.49	0.47
3.125	23.71 ± 0.56	23.55 ± 0.96	0.80	26.05 ± 1.30	25.89 ± 1.19	0.27
1.5625	23.61 ± 1.03	23.80 ± 1.19	0.84	23.67 ± 0.51	23.47 ± 0.31	0.85

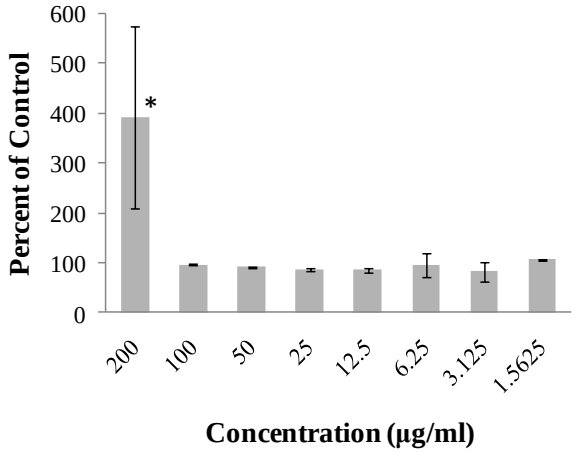

FIG. 9.2 Percent inhibition of *S.* Typhimurium biofilm by naringenin

9.4.2 Microarray Expression Profile

To understand the response of *S.* Typhimurium to naringenin exposure, the transcriptomic expression profile was compared to control. Of the approximately 5405 ORFs present on the array, 151 genes depicted a statistically significant ($p \leq 0.05$) differential expression (119 genes repressed, 32 genes induced) in response to

naringenin treatment. However, only 34 genes demonstrated >2 fold change in expression. Majority of differentially expressed genes were encoded in *Salmonella* Pathogenicity Island I (SPI1). Although a 2-fold change is generally reported as differential response for microarray results, a less than 2-fold change may also be biologically relevant (166, 168). In the present experiment, naringenin exposure affected 14 genes involved in flagellar biosynthesis and 3 chemotaxis genes. Although flagellar genes were down-regulated <2-fold under experimental conditions (Table 9.3), together with the results of motility assay, the regulation seems to be biologically relevant. Further, an induction of AcrAB efflux pump, which plays a role in antibiotic resistance, was observed. In addition, molecular chaperone *grpE* was down-regulated.

9.4.3 SPI1 Encoded Genes Down-Regulated by Naringenin

A total of 24 genes encoded by SPI1 were significantly repressed (Table 9.3) upon exposure to naringenin. Transcriptional regulators encoded within SPI1 - *hilC*, *hilD*, and *invF* (3.94, 3.73 and 7.7 fold respectively) - were down-regulated significantly ($p<0.05$). InvF positively regulates effector proteins within SPI1 (*sipA*, *sipB*, *sipC* and *sptP*) and located outside (*sopB*, *sopD* and *sopE*) (78-79) with the help of chaperone SicA (188). Naringenin exposure down-regulated InvF-dependent genes of the sip operon *sipA*, *sipB*, and *sipC* and *sptP* (Table 9.3). The inner membrane ring proteins PrgK/PrgH and genes *prgI*, *prgJ*, *orgA* and *org B* are essential for the formation of macromolecular complex to facilitate bacterial entry into the host cells (97). Our results indicate that this whole operon was repressed by naringenin significantly by 3 to 12 fold (Table 9.3). In addition, SPI1 encoded chaperones *sicA* and *sicP* were highly repressed.

TABLE 9.3 Differentially regulated genes by naringenin

Gene Name	Putative identification	Fold Change
Virulence genes		
avrA	secreted effector protein	-3.34
sprB	Transcriptional regulator	-3.94
hilC	invasion regulatory protein	-3.94
orgB	needle complex export protein	-5.64
orgA	needle complex assembly protein	-6.99
prgK	needle complex inner membrane lipoprotein	-12.56
prgJ	needle complex minor subunit	-11.68
prgH	needle complex inner membrane protein	-10.11
hilD	invasion protein regulatory protein	-3.73
iagB	invasion protein precursor	-6.48
sptP	tyrosine phosphatase (associated with virulence)	-3.79
sicP	secretion chaperone	-3.49
sipA	secreted effector protein	-11.46
sipB	translocation machinery component	-9.97
sipC	translocation machinery component	-10.54
sipD	translocation machinery component	-7.48
sicA	surface presentation of antigens secretory proteins	-4.31
invB	secretory protein (associated with virulence)	-9.72
invA	needle complex export protein	-8.97
invB	secretion chaperone	-9.70
invC	type III secretion system ATPase	-7.29
invE	invasion protein	-8.22
invF	invasion regulatory protein	-7.71
invH	needle complex outer membrane lipoprotein	-4.04
sopE	Invasion associate secreted protein	-9.74
pipC	pathogenicity island-encoded protein C	-5.46
Flagellar genes		
fliT	flagellar protein FliT	-1.32
fliF	flagellar M-ring protein	-1.41
fliK	flagellar hook-length control protein	-1.37

TABLE 9.3 Continued.

Gene Name	Putative identification	Fold Change
fliL	flagellar biosynthesis protein	-1.53
fliM	flagellar motor switch protein	-1.38
fliP	flagellar biosynthesis protein	-1.48
flgB	flagellar basal body rod protein	-1.32
flgE	flagellar hook protein	-1.44
flgF	cell-proximal portion of basal-body rod	-1.35
flgG	flagellar basal-body rod protein	-1.38
flgH	flagellar L-ring protein precursor	-1.36
flgI	flagellar P-ring protein precursor	-1.43
flhC	flagellar transcriptional activator	-1.37
flhD	transcriptional activator FlhD	-1.40
fimA	major type 1 subunit fimbrin (pilin)	-1.64
fimZ	fimbrial protein Z, putative transcriptional regulator	-3.03
Chemotaxis		
cheM	methyl-accepting chemotaxis protein II	-1.66
STM3138	putative methyl-accepting chemotaxis protein	-4.64
STM3216	putative methyl-accepting chemotaxis protein	-1.66
Efflux pump/Defense		
acrB	RND family, acridine efflux pump	3.21
acrA	RND family, acridine efflux pump	3.40
acrF	RND family, multidrug transport protein	2.09
marR	transcriptional repressor of marRAB operon,	4.28
ybhS	putative ABC superfamily transport protein	3.14
Molecular chaperone		
grpE	molecular chaperone heat shock protein	-8.21

SprB, a transcriptional regulator, is encoded within the SPI1 and regulates the expression of *sipC, sptP, avrA, sopB* and *sopE* (269). Naringenin repressed *sprB* as well as *sipC,*

sptP, *avrA, sopB* and *sopE*. In addition, naringenin also significantly repressed SPI1 encoded *pipC* (5.5 fold).

9.4.4 Flagellar Genes Are Repressed by Naringenin

Another important system repressed by the naringenin was the bacterial flagellar apparatus. In *S.* Typhimurium, flagellar genes have been classified in three classes. The first class, termed as the master operon, consists of *flhCD* and is controlled by various factors including GrpE. In present study, both the *grpE* (8.2 fold) and *flhCD* (1.4 fold) were repressed (Table 9.3). The FlhDC complex is a positive transcriptional activator of σ^{70}-dependent transcription from the class 2 promoters (283) and regulates Class 2 and 3 flagellar genes. Consistent with the down-regulation of *flhDC*, the genes encoded by class 2 promoters in the operons *fliEFGHIJKLMOPQR* and *flgNMABEFGHIJ* were repressed by 1.3 to 1.5 fold (Table 9.3). This observation suggests that naringenin affects the flagellar genes by down-regulating the *flhDC* possibly via *grpE*. In addition, cell motility genes *fimZ* and *fimA* were also repressed in the study.

9.4.5 Genes Involved in Defense Mechanism Influenced by Naringenin

The third important system influenced by naringenin treatment was multi-drug transporter efflux pump of the *Salmonella*. The AcrAB system provides low level of resistance to basic dyes, detergents and antibiotics (286). In present study, *acrA*, *acrB* and *acrF* were induced (Table 9.3) by 3.4, 3.2 and 2.1 fold, respectively. In addition, transcriptional repressor for marRAB operon, *marR* was also induced by the treatment of naringenin. Another member of ABC-type multidrug transport system, *ybhS*, a permease, was also induced by 3.14 fold.

9.4.6 qRT-PCR of Specific Naringenin Regulated Genes

To confirm the microarray expression data, the levels of specific *Salmonella* mRNAs by qRT-PCR in naringenin and control samples were assessed. Oligonucleotide primers (Table 9.1) were designed for a subset of genes identified by the microarrays as differentially expressed in response to naringenin. A total of seven genes were chosen based upon the interest in SPI and efflux pump. The gene expression pattern of these selected genes was studied at three different growth stages. The relative transcript levels of the selected genes were also determined at exponential phase (OD_{600} =0.5) and stationary phase (OD_{600} =2.0), in addition to the early stationary phase (OD_{600} =1.0) used for microarray study. The qRT-PCR results obtained at early stationary phase were in agreement with those obtained by using the microarrays and confirm the data obtained by using the whole-genome analysis (Table 9.4).

The expression level of *hilD, hilC, orgA* and *invA* in exponential phase (OD_{600} =0.5) was similar to control however, at OD_{600} ≈1.0 these genes were repressed. Similar pattern was observed with the *fimZ*. The efflux pump genes *acrA, acrB, acrF* and the repressor *marR* were induced in the microarray study. The mRNA level measurements by qRT-PCR demonstrated that the expression level of *acrA* was induced at OD_{600} 0.5 and 1.0 (≈ 2.9 fold) but was not different from control at OD_{600} 2.0 (Table 9.4). The repressor *marR* also demonstrated similar behavior.

TABLE 9.4 Comparison of gene expression changes by microarray and qRT-PCR

Gene	Function	Fold Change in microarray	Fold change in qRT-PCR		
			A	B	C
acrA	acridine efflux pump	3.40	2.94	2.93	1.31
hilD	regulatory helix-turn-helix proteins, araC family	-6.48	-1.15	-6.7	-9.87
hilC	bacterial regulatory helix-turn-helix proteins, araC family	-5.47	-0.99	-4.94	-25
hilA	invasion protein regulator	-	-0.52	-5.5	-22.63
orgA	putative inner membrane protein	-12.35	-0.96	-9.09	-25
invA	invasion protein	-8.22	-0.72	-10	-50
fimZ	fimbrial protein Z, putative transcriptional regulator (LuxR/UhpA)	-3.03	-1.0	-2.8	-10
marR	transcriptional repressor of marRAB operon, multiple antibiotic	3.88	3.27	4.10	1.64

A – at OD_{600} of 0.5, B - at OD_{600} of 1.0, C- at OD_{600} of 2.0

9.4.7 Naringenin Represses *hilA* Expression

We did not observe a differential expression of *hilA* in microarray experiment. However, based on differential expression of SPI1, it was speculated that *hilA* is also differentially regulated by naringenin. Therefore, *hilA* transcript levels were measured by qRT-PCR. The results demonstrated that *hilA* was repressed by 5.5 and 22.63 fold at OD_{600} ≈1.0 and 2.0, respectively (Table 9.4) in presence of naringenin. In addition, *S.* Typhimurium reporter strain EE658 (214) was employed to independently confirm the differential expression of *hilA*. Naringenin (100 µg/ml) significantly ($p<0.05$) repressed the expression of *hilA* in reporter strain EE658 (Fig. 9.4).

9.4.8 Naringenin Attenuates *Salmonella* Adhesion/Invasive Ability to Colon Epithelial Cells and Motility

Colon adenocarcinoma SW480 cells were grown in DMEM and infected with *S.* Typhimurium LT2 in presence of 100 µg/ml naringenin. Adhesion of *Salmonella* to SW480 cells was tested at moi 10 and 100. The data show that naringenin treatment reduced the ability of *Salmonella* to attach and internalize the SW480 cells, which supported the observations made in the microarray experiment. Naringenin reduced the number of attached CFUs by ≈ 1 log unit ($p < 0.05$) (Fig. 9.3A, B). This lesser number of cell-adhesion also translated into lower number of *Salmonella* cells being internalized. Mean number of internalized *Salmonella* cells/SW480 cell, in presence of naringenin was reduced by ≈0.5 ($p < 0.05$) (Fig. 9.3C).

Furthermore, *S.* Typhimurium demonstrated significantly ($p < 0.01$) reduced motility on plates containing 100 µg/ml naringenin (Fig. 3D). The mean zone diameter for naringenin treatment was 2.96 cm, significantly ($p < 0.01$) less than DMSO (4.30 cm) (Fig. 9.3E).

9.4.9 Naringenin Regulates *hilA* Expression via *hilD*

The microarray data suggested that naringenin regulates *hilA* and consequently SPI1 by modulating *hilD* and *hilC* expression. To further test our hypothesis, effect of naringenin on *hilA* expression was measured in a series of mutants containing disruptions in *hilD, fadD, fliA, pstS* and *envZ* (214). We reasoned that *hilA* expression will not be affected in a mutant by naringenin, if naringenin is exerting its effect through the particular gene. Exposure of naringenin resulted in suppression of *hilA* expression in

mutants with disruptions in *fadD* (RL21), *fliA* (RL119) and *envZ* (EE711). However, *hilA* was not differentially regulated in *hilD* mutant LM401 and *pstS* (RL147) (Fig. 9.4). Taken together, the data indicate that naringenin exerts its effect through *pstS* and *hilD*.

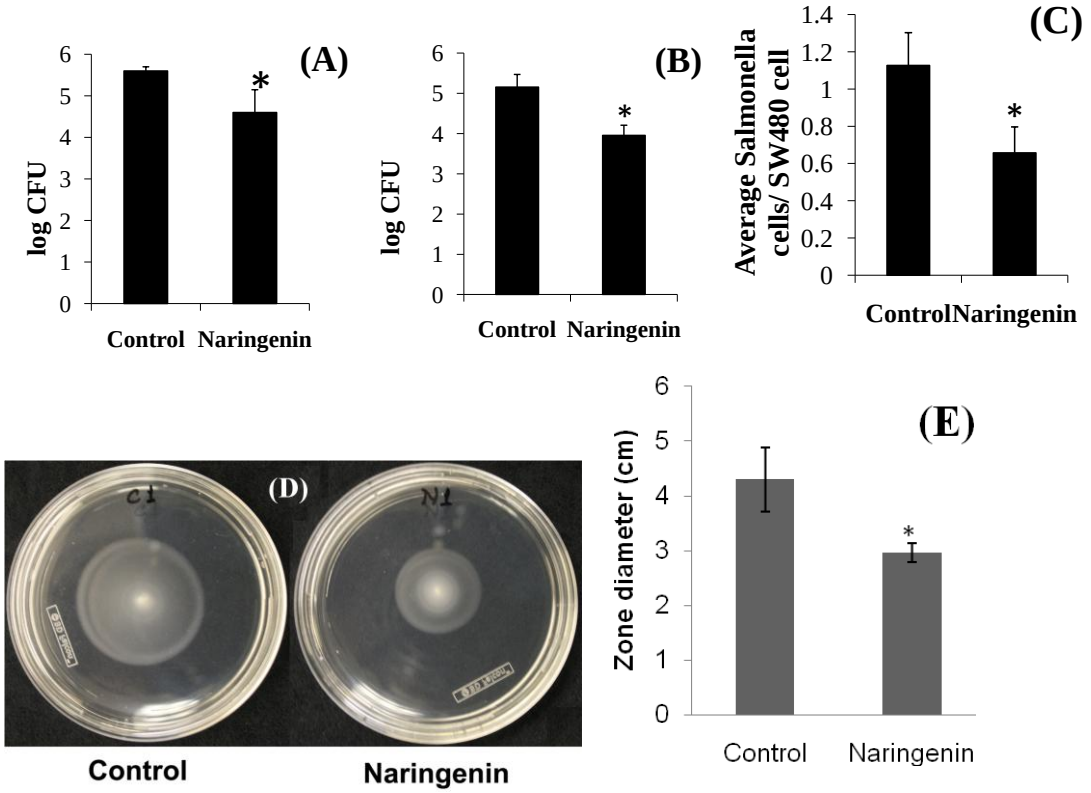

FIG. 9.3 Adhesion of *Salmonella* Typhimurium LT2 on SW480 cells in presence of 100 µg/ml naringenin at multiplicity of infection (A) 10 and (B) 100. (C) Invasion of SW480 cells by S. Typhimurium in presence of naringenin as measured by gentamicin protection assay. (D) Inhibition of *S.* Typhimurium motility by 100 µg/ml naringenin in comparison with equivalent volume of DMSO (Control) (E) Mean zone diameters calculated for motility from five replicates. Star (∗) denotes significant difference from control

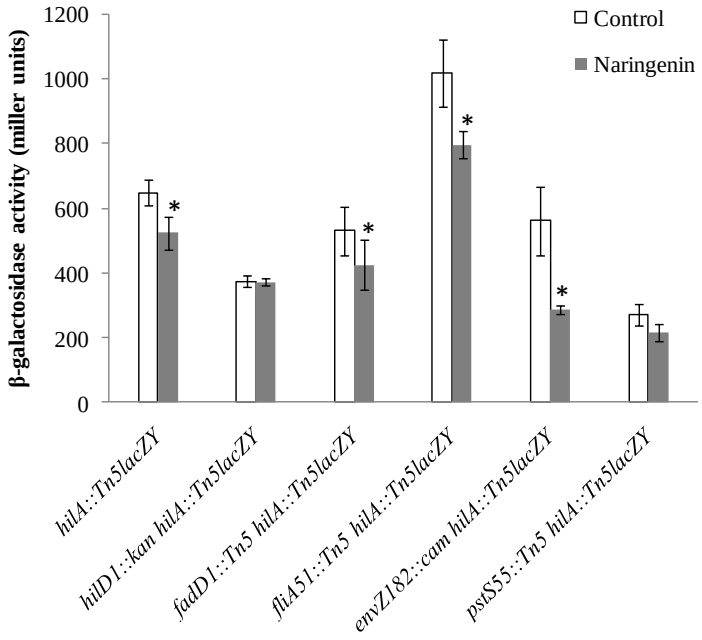

FIG. 9.4 Effect of 100 µg/ml naringenin exposure upon *hilA* expression in *hilA* reporter (EE658) and *hilD* (LM401), *fadD* (RL21), *fliA* (RL119), *envZ* (EE711) and *pstS* (RL147) mutants

9.5. Discussion

Naringenin, a flavonone, is present in grapefruit and related species. Naringenin did not affect the growth of *S.* Typhimurium as measured by OD_{600}. Since certain flavonoids are demonstrated to uncouple the energy transducing cytoplasmic membrane (344), we employed AlamarBlue to study the effect of naringenin on activity of the respiratory chain. The results indicate that naringenin did not affect respiratory chain in *S.* Typhimurium at the tested concentrations. Taken together, the data indicate that naringenin did not influence *S.* Typhimurium growth. Further, the luxS/Autoinducer-2 mediated cell-cell signaling was suggested to regulate the biofilm formation in *S.* Typhimurium (176). Since naringenin demonstrated inhibition of AI-2 signaling and

biofilm formation, (371), we wondered if naringenin has inhibitory effect on *S.* Typhimurium biofilm. Therefore, the effect of naringenin on the *S.* Typhimurium biofilm formation was investigated. Naringenin did not seem to inhibit the *S.* Typhimurium biofilm. However, an increase in biofilm formation was observed in the presence of 200 µg/ml naringenin, suggesting that naringenin influence *S.* Typhimurium cells differently than *E. coli* O157:H7 and *V. harveyi* (371). Biofilm formation in S. Typhimurium is negatively correlated. Specifically, inactivation of *hilA* and *flhDC*, central regulators of SPI1 and flagellar operon resulted in increased biofilm formation by *S.* Typhimurium (358). In our studies, we observed a repression of SPI1 and *flhDC* by naringenin, which may be the possible reason for the increased biofilm at higher concentration of naringenin.

In order to further understand the effect of naringenin on *Salmonella*, transcriptional profile was studied. The microarray data suggested that the naringenin differentially regulated SPI1, flagellar operons, chemotaxis genes and molecular chaperon *grpE* and AcrAB efflux pump. A <2 fold repression of flagellar genes under experimental conditions was recorded. However, inhibition of *Salmonella* motility by naringenin suggests that this result is biologically important.

SPI1 is essential for initial attachment and entry into the host cells. Furthermore, SPI1 encoded effectors influence post-entry early and late events (46, 139, 167). Regulation of SPI1 is very complex. Recently, a model for the regulation of SPI1 has been proposed (96), suggesting regulation of SPI1 is mediated through the regulation of HilA by HilD, HilC and RtsA, with HilD playing a predominant role (96). A differential

expression of *invF*, *hilA*, *hilC* and *hilD* by naringenin was observed. Furthermore, naringenin repressed *invF* dependent genes as well as *hilA* dependent *prg/org* operon. HilA belongs to OmpR/ToxR family of transcriptional regulators and regulates *prg/org* and *inv* operons by directly binding to HilA box (211). In turn, *hilA* is regulated by HilD and HilC (321). In addition, HilD and HilC directly activate *invF*, *sipA* and *sipC* in non-HilA dependent manner (7). Our data indicate that naringenin exerts its effect on SPI1 plausibly by regulating expression of *hilD/hilC*, and differential expression of *invF* was possibly mediated by *hilC/hilD/hilA*. However, a direct effect of naringenin on *invF* cannot be negated. Furthermore, repression of SPI1 indicated a possibility of reduced attachment and internalization of *Salmonella* to host cells in presence of naringenin. Indeed, in vitro analysis employing colon epithelial adenocarcinoma cells demonstrated that naringenin reduced the invasive ability of the *S.* Typhimurium. However, these results must be interpreted with caution since the influence of naringenin in mixed microbial species and in vivo conditions were not investigated.

Cell motility is an important contributor to pathogenicity of *Salmonella* (185). The downregulation of flagellar operon indicated that naringenin may reduce *Salmonella* motility. The hypothesis was tested by measuring the motility of *S.* Typhimurium in presence of 100 µg/ml naringenin. The results demonstrated that naringenin significantly impedes the *Salmonella* motility (Fig. 3D and E). Altogether, our data suggest that naringenin influences *S.* Typhimurium motility and invasiveness without affecting the growth.

A differential expression of *hilA* was not observed in microarray data. To determine the modulation of *hilA* by naringenin, the expression of *hilA* was measured using qRT-PCR (Table 9.4) and *hilA* reporter EE658 (Fig. 4). The results indicated that *hilA* was repressed by naringenin. Further, modulation of *hilD* suggested that SPI1 suppression by naringenin is plausibly *hilD* dependent. In order to further elucidate the regulation of *hilA* in naringenin mediated suppression of SPI1, expression of *hilA* using β-galactosidase reporter was measured in different mutants. We hypothesized that if the effect of naringenin is dependent on a particular gene, the differential expression of *hilA* will not be observed in a strain carrying disruption in the target gene. The expression analysis of *hilA* in different mutant backgrounds supported the hypothesis that the modulation of SPI1 by naringenin is *hilD* dependent (Fig. 4). Further, *hilD* is regulated by several factors and results of the analysis seem to suggest that naringenin represses *hilD*/*hilA* in *pstS* dependent manner (Fig. 4). Regulation of HilC and HilD is complex and both are capable of inducing expression of one another as well as their own expression in response to various environmental stimuli (96). Further studies are needed to elucidate the mode of repression of *hilD* and *pstS* by naringenin.

Coordinate expression of SPI1 and flagellar operon in *Salmonella* was reported (181). In our study, down-regulation of SPI1 and class 1 and 2 genes of flagellar operons was observed. The results are in agreement with class 1 and 2 flagellar genes and SPI1 being similarly regulated (95). Furthermore, FliA (σ^{28}) is proposed to regulate the expression of SPI1 via FliZ (171) in HilC/HilD dependent fashion (181). To test the hypothesis, that naringenin suppresses the *hilD* via *fliA*, *hilA* expression was measured in

fliA mutant. However, differential regulation of *hilA* in *fliA* mutant suggested that naringenin do not act via *fliA*. Furthermore, a differential regulation of *fliA/fliZ* was not observed in microarray analysis. Altogether the data suggests a model, where naringenin modulates the expression of SPI1 in *pstS*/*hilD* dependent manner (Fig. 9.5). However, negative regulation of SPI1 and flagellar operon in presence of naringenin may be attributed to different factors as the level of observed regulation was different. FimYZ regulates the *fimA*, and were reported to control virulence by regulating *hilE* (28). It is possible that naringenin influences different targets for SPI1 and flagellar genes.

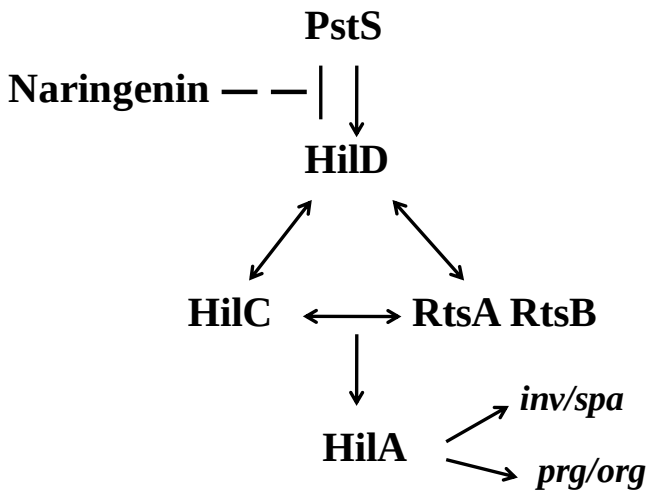

FIG. 9.5 Suggested model of SPI1 regulation by naringenin. The model for SPI1 regulation is adopted from Ellermeier et al (96) and modified to demonstrate the possible action of naringenin on SPI1. (Broken line indicates unknown direct or indirect effect)

Multidrug efflux pump AcrAB-TolC plays a major role in anti-biotic resistance in *Salmonella* (27). Induction of AcrAB-TolC by naringenin suggests activation of drug resistance phenotype in order to shuttle the naringenin out of the cell. The induction of

marR may also be a response to the activated *acrAB*, to prevent over accumulation of the efflux pump. At OD_{600} = 2.0 (Table 9.4), the expression of *acrA* and *marR* in naringenin treated samples were similar to control, which is consistent with the idea that *marR* was possibly activated in response to induced *acrAB* operon. However, the actual mechanism of *acrA* dynamic behavior requires further study. At the same time (OD_{600} = 2.0), when *acrA* mRNA levels were similar to control, SPI1 was repressed more strongly, indicating naringenin stimulates different responses on SPI1 and *acrAB* operon.

Altogether results of the study support an anti-pathogenic effect of naringenin on *S.* Typhimurium LT2. Furthermore, naringenin was found to attenuate virulence and flagellar operons in *S.* Typhimurium. TTSS encoded by SPI1 is required for the invasion of non-phagocytic cells in the intestine. Naringenin, by repressing the SPI1 and flagellar operon may help in preventing or delaying the onset of infection. Moreover, naringenin plausibly exert its effect on SPI1 in *pstS*/*hilD* dependent manner. Although several factors regulating *hilD* were analyzed in the present study, only *pstS* seems to be probable target candidate of naringenin. However, as *hilD* and consequently *hilA* are regulated by several other factors, it remains possible that factor/s other than those investigated in the present study may also contribute to regulation of SPI1 by naringenin. In addition, a role of HilC mediated repression of *hilA* was not investigated. It is possible that HilC mediated repression of SPI1 may also contribute to the overall activity of naringenin.

In conclusion, naringenin seems to possess anti-pathogenic activity against *S.* Typhimurium. Since naringenin is prominent constituent of grapefruit along with other

quorum sensing inhibitors present in Citrus (130, 374-375), consumption of citrus fruits and juices may help in maintaining colon health.

CHAPTER X

SUMMARY AND CONCLUSIONS

Limonoids and flavonoids from Citrus constitute important dietary ingredients. Since certain limonoids are not commercially available, their isolation is necessary for elucidation of biological activity. Being good source for certain limonoids, seeds were chosen as raw material for isolation and purification. The seeds were extracted with organic solvents using soxhlet technique. Soxhlet is apparently an old but simple technique. While several new extraction methods were developed in recent years. most of them require small sample size- a limitation for large scale purification. The use of small sample size coupled with shorter extraction time is advantageous for analytical procedures, where a number different analysis can be performed with small sample. However, for isolation and purification, the starting sample is often considerably bigger- few grams to kilograms, therefore restricting the use of such techniques. In contrast, a large sample can be extracted in batch fashion using Soxhlet apparatus. For the purposes of current research, a 2 Kg sample was extracted with different solvents in Soxhlet apparatus. The extracted material was then used for further isolation procedure.

The limonoid aglycones were purified on silica gel columns using DCM and EtOAc. Previous studies have used a variety of stationary phases such as alumina and silica. In the current study, a combination of silica gel as stationary phase and DCM+EtOAc resulted in adequate separation of seven aglycones. The purification of limonoid glucosides is more challenging compared to aglycones. Several factors influence the separation of the glucosides including presence of flavonoid glucosides. A

technique of purifying glucosides was previously developed in our laboratory. The technique uses a unique combination of cation exchange and adsorbant resins to eliminate the flavonoids by virtue of their weak ionization in water (150). The flavonoids are adsorbed on the ion-exchange resin, while limonoids pass through the ion-exchange resin and retained on the adsorbant resin. The adsorbant resin column is then separated and eluted with ACN/water mobile phase to obtain the purified limonoid glucoside. Using this technique, three limonoid glucosides were purified from grapefruit and sour orange seeds. The purified compounds were identified using mass spectrometric methods.

During the purification process, the limonoids were analyzed using TLC and HPLC. Analytical methods are the backbones for determination of food quality and elucidating the nutrient composition of fruits/vegetables/other edibles. HPLC has gained a prominent place as analytical method of choice for determination of phytochemicals in fruits and vegetables. Previous HPLC methods measured aglycones and glucosides separately; thereby two different methods were required for analyzing aglycones and glucosides. The two methods often required different columns and mobile phase forcing the machine to switch between two methods. In order to overcome this shortcoming, a method to analyze aglycones and glucosides simultaneously was developed. The developed method facilitated analysis of both aglycones and glucosides in the same run, on a C_{18} column with a gradient of 0.03 M phosphoric acid in acetonitrile.

The limonoids constitute and significant proportion of Citrus secondary metabolite profile along with flavonoids. One serving of orange juice may provide upto

24 mg of limonoids and 81 mg of flavonoids. The ingested limonoids and flavonoids are speculated to provide the health benefits (222, 229). The flavonoids are absorbed in the small intestine, however, a large part of flavonoids pass to the colon. In addition, appearance of intact limonin suggested that, some amount of limonoids may travel through GI –tract with little modifications (229), and therefore may be available to interact with the intestinal microflora. Furthermore, the limonin is more stable at acidic pH with optimal stability being observed at pH 5.0 (177). Since the pH of GI tract varies from acidic in stomach to neutral in intestine, the loss in ingested limonoids due to variation in pH is expected to minimal. The GI tract is a highly complex ecosystem, where numerous interactions among the resident microflora and with the host take place (311). The GI tract microflora competes for the available nutrients and significantly modifies the dietary components. In turn, dietary components are also speculated to profoundly influence the resident microbial communities as well as any pathogens present in the GI tract. The secondary metabolites, which escape the digestive processes, reach to the colon and interact with the colonic microflora. It was speculated that these secondary metabolites may alter the behavior of colonic microflora. Food-borne pathogens such as EHEC and *S.* Typhimurium typically colonize the distal ileum and colon and cause diarrhea. It is possible that the ingested limonoids and flavonoids may influence the virulence of these pathogens. Once inside the host system, several environmental, bacterial and host signals regulate the expression of virulence in EHEC and *S.* Typhimurium. Bacterial cell-cell signaling involving autoinducer molecules and host hormones - epinephrine/norepinephrine was shown to regulate virulence in both

EHEC and *S.* Typhimurium. Furthermore, presence of autoinducer molecules in GI-tract and regulation of EHEC virulence by host factors indicates a role of bacterial cell-cell signaling in complex three way interactions among bacterial population and host.

The current work was focused on elucidating the anti-QS and anti-virulence activity of citrus limonoids and flavonoids. The studies were conducted in three bacterial species – *V. harveyi*, *E. coli* and *S.* Typhimurium LT2. Four limonoids and a flavonoid were identified as potent inhibitor of autoinducer mediated cell-cell signaling in *V. harveyi*. Genetic studies demonstrated that two limonoids- isolimonic acid and ichangin interfere with *V. harveyi* QS pathway by up-regulating the response regulator *luxO*. Furthermore, naringenin, a flavonoid was demonstrated to be a potent inhibitor of *V. harveyi* cell-cell signaling. In addition, these compounds also inhibited the QS-regulated processes including biofilm.

The studies with *E. coli*, particularly EHEC demonstrated that limonoids and flavonoids possess the inhibitory potential against biofilm and TTSS. Further investigations revealed that isolimonic acid inhibits the EHEC biofilm in QseC dependent fashion, while repressed the expression of EHEC TTSS in QseA dependent manner. To further investigate the effect of structural modifications of limonoids on the QS inhibitory ability, limonin analogues were prepared. The modification at 7^{th} position (Fig. 6.1) resulted in loss of QS inhibitory activity in *V. harveyi*. At the same time, modified limonoids showed increased inhibition of *E. coli* biofilm. Interestingly, limonin-7-methoxime was found to inhibit the attachment of *E. coli* cells to the surface, possibly by interacting with type I pili and Ag43.

The studies with *S.* Typhimurium LT2, demonstrated the anti-virulence potential of naringenin and obacunone. Both naringenin and obacunone significantly repressed the expression of SPI1, which is required for establishment and intracellular survival of the pathogen. Genetic studies revealed the probable mode of action of these compounds. Naringenin seems to modulate the expression of SPI1 in PstS and HilD dependent fashion. In addition, naringenin significantly reduced the flagellar motility and attachment of *Salmonella* cells to colon epithelial cells. Obacunone demonstrated a different mode of action for repression of SPI1 than naringenin- an EnvZ dependent mechanism. Furthermore, reduction in attachment and invasion of colon epithelial cells by *Salmonella* in presence of obacunone validated the repression of SPI1. In addition, obacunone repressed the hydrogenase operon and maltose transporters, which are regulated by EnvZ/OmpR, further indicating a central role of EnvZ/OmpR in modulation of *Salmonella* pathogenicity by obacunone. Altogether, the evidence suggests significant anti-virulence potential of naringenin and obacunone.

QS pathways regulate the early events in the pathogenesis of EHEC, specifically, attachment of EHEC to epithelial cells in TTSS mediated fashion. It was suggested that targeting early events in pathogenesis presents a viable option for chemotherapy and intervention with the infection processes (8). The limonoids and flavonoids identified in the current project seems to target the early events in the pathogenesis of EHEC and *S.* Typhimurium LT2 and may serve as the lead compounds for search of better strategies to control the infection processes of these two enteric pathogens. Furthermore, these dietary components are expected to attenuate the cell-cell signaling in pathogens as well

as in the commensal bacteria. However, our studies demonstrate that isolimonic acid, ichangin, obacunone, nomilin and naringenin specifically target the EHEC Pathogenicity traits such as TTSS and biofilm. The TTSS is widely distributed in gram negative pathogens, while being generally absent from non-pathogenic bacteria. Therefore, targeting TTSS is likely to affect the pathogens selectively, while having minimal effect on commensal bacterium. Secondly, as the citrus bioactive compounds are not growth inhibitory, they are not likely to perturb the distribution of colonic microflora population. At the same time, the host immune system can detect the pathogens by their pathogen associated molecular patters (PAMPs) and initiate the immune response, resulting in clearing of pathogens from the host-system. EHEC and *S.* Typhimurium also respond to host hormones epinephrine/epinephrine. We demonstrated that isolimonic acid inhibits the epinephrine induced response in EHEC, therefore, blinding the pathogen to the presence of hormone. However, such speculations require further investigations to provide proof of concept. The current project provides a foundation for further studies in the field of dietary components-pathogen interaction. In addition, elucidation and identification of secondary metabolites with potential to specifically target pathogenic traits has application in functional foods as well as in medicine. On one hand, identification and dissemination of such knowledge will benefit the society by enhancing the awareness about the bioactive components of their food. On the other hand, the information may provide a better tool to develop novel compounds for antimicrobials.

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SELECTED PUBLICATIONS

- Vikram, A., P. R. Jesudhasan, G. K. Jayaprakasha, S. D. Pillai, and B. S. Patil. (2011). Citrus limonoids interfere with *Vibrio harveyi* cell-cell signaling and biofilm formation by modulating response regulator *luxO***.** Microbiology 157:99-110
- Vikram, A., G. K. Jayaprakasha, P. R. Jesudhasan, S. D. Pillai, A. Jayaraman and B. S. Patil. (2011). Attenuation of *Salmonella enterica* Typhimurium virulence by naringenin. Int. J. Food Microbiol. 145: 28-36
- Vikram A., Jayaprakasha G. K., Jesudhasan P. R., Pillai S. D, & Patil B. S. (2010) Suppression of bacterial cell-cell signaling, biofilm formation and type III secretion system by citrus flavonoids. J Appl Microbio 109: 515-527
- Vikram, A., P. R. Jesudhasan, G. K. Jayaprakasha, S. D. Pillai, and B. S. Patil. (2010). Grapefruit bioactive limonoids modulate *E. coli* O157:H7 TTSS and biofilm. Int. J. Food Microbiol. 140, 109-116

AWARDS AND HONORS

- 2011 Association of Former Students Distinguished Graduate Student Award for Excellence in Doctoral Research, TAMU
- 2008 Withycombe-Charalambous Graduate Student Prize, 235th ACS National Meeting
- 2008 First Prize, Warren S. Barham Ph.D. Graduate Student Paper Competition, 68th Annual Meeting of Southern Region ASHS
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