In vitro antimicrobial, antihemolytic potential of gallic acid and its combined effect with metronidazole against pathogenic bacteria

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of
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Declaration

It is hereby declared that

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3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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Abstract

Multidrug-resistant (MDR) or extensive drug-resistant (XDR) pathogenic bacteria pose a grave threat to human and animal health on a global scale. Plant-derived phytochemicals including alkaloids, flavonoids, and terpenoids reported having antimicrobial activity against UTI, dysentery, and diarrhea-causing pathogenic bacteria. More importantly, natural compounds (e.g. gallic acid) alone or in a combination with FDA-approved antibiotics can be used to target resistant pathogenic bacteria. In our study, Gallic acid's minimum inhibitory concentration was measured at 1600±28.87, 650±28.87, 675±14.53, 187.5±17.24, 295±24.27, 737.5±20.21, 1500±28.87, 1000±28.87, 550±14.529 μg/mL against *E. coli* ATCC 25922, *Staphylococcus aureus, Enterococcus faecalis, Proteus vulgaris, Shigella flexneri, Shigella dysenteriae, Enteropathogenic E. Coli, Hafnia alvei,* and *Bacillus Cereus* respectively. Co-administration of metronidazole and GA exhibited additivity in *E. Coli*, *Enteropathogenic E. Coli*, *Bacillus cereus, Shigella flexneri*, *Shigella dysenteriae*, *Staphylococcus aureus*, and *Hafnia Alvei* (FIC index at 0.84, 0.65, 0.76, 0.98, 0.76, 0.89, 0.76) while *Enterococcus faecalis*, *Proteus vulgaris* was indifferent. Furthermore, we have explored the *in vitro* hemocompatibility of various concentrations of GA in all the human blood groups (male and female) that demonstrated exceptional hemocompatibility. Our results suggest that GA has significant potentiating activity in combination with metronidazole. GA decreases metronidazole's MIC significantly when applied together, which can be a promising factor for AMR control.

Keywords: Antimicrobial Resistance (AMR), Gallic Acid, Minimum Inhibitory Concentration (MIC), Fractional Inhibitory Concentration (FIC), Synergy, RBC Hemocompatibility.

Dedication

We dedicate our thesis to our parents and elder sisters for their unconditional love, support, and encouragement.

Acknowledgment

We would like to begin by thanking the Almighty for allowing us to complete this research and for all of the other blessings bestowed upon us. Our sincerest gratitude and appreciation goes to **Md. Salman Shakil**, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Mohakhali, Dhaka, our supervisor on this project. We want to thank him for being such a wealth of information and helping us immensely in our studies. His encouragement and boundless energy were constant sources of motivation, and his watchful eye and helpful input were instrumental in our success.

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List of Acronyms

AMR	Antimicrobial Resistance
MDR	Multi-drug Resistant
XDR	Extensive-drug Resistant
PS	Phytosterol
OSC	Organosulfur
GA	Gallic Acid
NF-kB	Nuclear factor kappa- light chain enhancer
iNOS	Nitric oxide synthase
COX-2	Cyclooxygenase-2
`8TNF	Tumor necrosis factor
ICAM-1	Intercellular cell Adhesion Molecule 1
VCAM-1	Vascular cell adhesion Molecule 1
IL- 1β	Interleukin -1 beta
ROS	Reactive oxygen species
TACE	Tumor necrosis factor-α- converting enzyme
ADAM17	A disintegrant and metalloprotease 17
PKC	Protein kinase C
ERK	Extracellular signal-regulated kinase
JNK	c-JUN N-terminal Kinase
Akt	Protein Kinase B
MCF7	Breast Cancer Cell Line
PC	Prostate cancer
MIC	Minimum Inhibitory Concentration

MBC	Minimum Bactericidal Concentration
NA	Nutrient Agar
MHA	Muller Hinton Agar
LB	Luria Bertani Broth
PBS	Phosphate Buffered Saline
DMSO	Dimethyl Sulfoxide
OD	Optical Density
RBC	Red blood cells
RPM	Revolutions per minute
FIC	Fractional Inhibitory Concentration
NR	Not reported

Chapter 1

Introduction

1.1Antimicrobial resistance

Over the past few decades, numerous microbes, particularly antibiotic-resistant bacteria have spread worldwide. Some clinical isolates, including *Klebsiella pneumonia*, *E. coli*, and Proteus sp., as well as methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-Resistant Enterococci (VRE), and other members of the family Enterobacteriaceae, quickly develop antibiotic resistance and spread in the environment. These MDR and XDR pathogens are responsible for causing UTI, Diarrhea, and other deadly infectious diseases (Basak, Singh, & Rajurkar, 2016; Lima et al., 2016; Mishra et al., 2017). Infectious diseases continue to be a significant cause of morbidity and mortality in developing and industrialized countries. Resistance to antimicrobial agents is increasing the mortality and morbidity associated with infectious diseases. Antibiotic resistance might exacerbate infectious illnesses in low- and middle-income countries (O'Neill, 2016). In 2019, the total worldwide burden of AMR was 4.95 million fatalities, with bacterial AMR alone accounting for 1.27 million deaths (Murray et al., 2022). AMR appears to be the most common cause of death in tropical countries, surprisingly accounting for more than half of all deaths in such tropical countries (Cowan, 1999). Failure to address the global burden of AMR threatens to return mankind to a time when even minor infections could be fatal. There has been a significant investment in the hunt for novel antimicrobials to combat the growth of resistant microbes. In recent years, it has been clear that some of the vast range of secondary metabolites (phytochemicals) produced by plants have positive impacts on human health, including antibacterial capabilities (Anabela Borges, Carla Ferreira, Maria J Saavedra, & Manuel Simões, 2013).

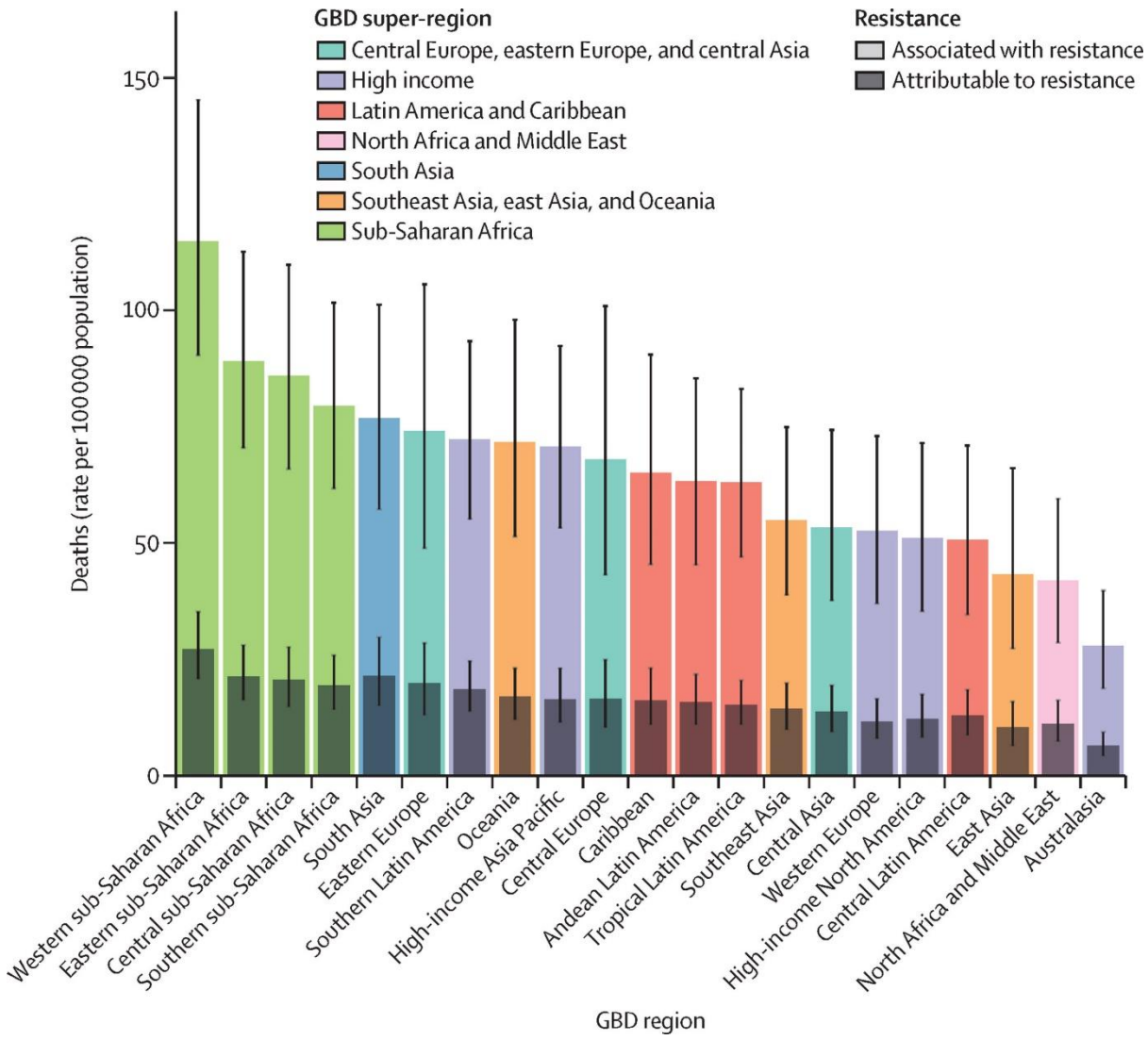
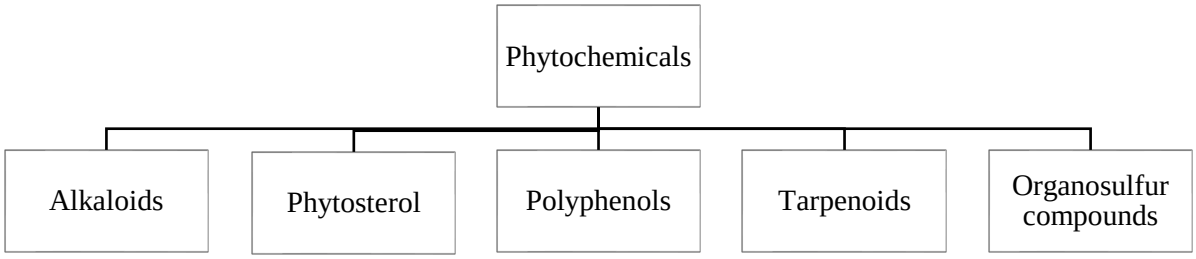


Figure 1: All-age mortality rate due to linked by antimicrobial resistance in 2019- Adopted from Antibiotic Resistance Collaborators, The Lancet, 2022.

1.2 Plant phytocompound

In response to the growing antimicrobial resistance, natural substances like phytocompounds that can serve as an alternative to antibiotics are now being investigated (AMR). Plant phytocompounds like polyphenols, alkaloids, and terpenoids have a promising future for

application as antibacterial or antimicrobial resistance modifiers because of their remarkable capacity to combat bacterial infections (AlSheikh et al., 2020). In recent years, it has been clear that some of the vast range of secondary metabolites (phytochemicals) produced by plants have positive impacts on human health, including antibacterial capabilities (Anabela Borges et al., 2013). Evidence shows that phenolic compounds possess antimicrobial effects against a plethora of microorganisms. Phenolic compounds show antimicrobial activities by degrading the cell membrane of bacteria (Jorge Dávila-Aviña, Carolina Gil-Solís, Jose Merino-Mascorro, Santos García, & Norma Heredia, 2020; Li Fu, WenQing Lu, & XiaoMin Zhou, 2016a; Miklasińska-Majdanik, Kępa, Wojtyczka, Idzik, & Wąsik, 2018). The phenolic compounds obstruct some virulence factors of microbes, such as enzymes and toxins. Moreover, these natural phenolic compounds show synergistic effects if they are alloyed with antibiotics, nanoparticles, and other therapeutic agents (Jorge Dávila-Aviña et al., 2020).



Combining phytochemical molecules with failing antibiotics seems to restore the desired antibacterial activity, so this has been suggested as a strategy to restore antimicrobial activity (Brown, 2015). Phytochemicals have exerted potential antibacterial activities against sensitive and resistant pathogens via different mechanisms of action.

Alkaloids

Alkaloids are plant-derived basic nitrogenous heterocycle molecules with high physiological activity. They form appropriate salts with acids considering that they are basic in nature. To date, about 6500 alkaloids have been identified. The presence of alkaloids is not limited to specific plant compounds. They are, nevertheless, founds in numerous parts of plants. A few examples are as follows: In the seeds (Strychnic), the leaves (Belladonna), the roots (Rauwolfia), the corns (Colchicum), and the bark (Cinchona) (M. Lu et al., 2017; Robinson, 1974).

Phytosterol

All plant-based foods contain phytosterols (PS), which are plant compounds with a chemical similar structure to cholesterol; vegetable oils have the highest concentration (Yoshida & Niki, 2003). Beta-sitosterol, campestral, and stigmasterol are the most prevalent dietary PSs. PS has been demonstrated to potentially have additional beneficial properties, such as anti-inflammatory, antiatherogenic, antioxidant, and anticancer effects (Ostlund Jr, Racette, & Stenson, 2003).

Polyphenols

These substances are referred to collectively as phytochemicals (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011). They are abundant in plant-based foods such as vegetables and fruits (Williamson, 2017). GA is a significant polyphenol (Halliwell, 2008).

Terpenoids

Isoprene, a five - carbon substance, and terpenes, as these are isoprene polymers, are the building blocks of terpenoids, also referred to as isoprenoids, a large and diverse class of natural occurring organic compounds (Eran Pichersky & Robert A Raguso, 2018). Depending on how many carbon atoms they contain, terpenoids are classified into 5 more categories (Huang et al.,

2012; Eran Pichersky & Robert A Raguso, 2018). In the battle against numerous different infectious diseases, a number of terpenoids that are biologically active are used (Huang et al., 2012; Thoppil & Bishayee, 2011).

Organosulfur

Organosulfur compounds (OSCs) are bioactive or nutraceuticals derived from plant and animal sources (Gonzalez, Soto, Sance, Camargo, & Galmarini, 2009; Miękus et al., 2020; Polshettiwar & Kaushik, 2006). Various OSCs are claimed to have strong antioxidant potential (Gonzalez et al., 2009).

1.3 Gallic Acid

GA is a trihydroxy benzoic acid with hydroxy groups located in positions 3, 4, and 5. It is an astringent, cyclooxygenase 2 inhibitor, plant metabolite, antioxidant, anticancer agent, human xenobiotic metabolite, EC 1.13.11.33 (arachidonate 15-lipoxygenase) inhibitor, and apoptosis inducer. It is a gallate conjugate acid (Information, 2022). GA is also termed called trihydroxy benzoic acid) (Sarjit, Wang, & Dykes, 2015). It is a crucial oxidant. GA has also shown antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Acinetobacter baumannii* (Chanwitheesuk, Teerawutgulrag, Kilburn, & Rakariyatham, 2007); (Sarjit et al., 2015).

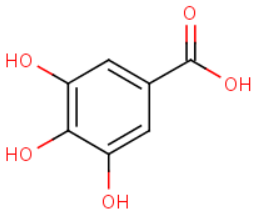


Figure 2: Schematic structure of GA

1.4 Physiochemical properties of GA

It is a colorless or slightly yellow crystalline compound with a melting point of 210 °C. It decomposes between 235 °C and 240 °C, generating carbon dioxide and carbon monoxide. At 20 °C, its density is 1.69 kg/L, pKa is 4.40, and log P is 0.70. It is soluble in water, alcohol, ether, and glycerol but insoluble in benzene, chloroform, and petroleum ether. Ester and catechin derivatives are two different types of GA derivatives. In contrast, the most common ester derivatives of GA are alkyl esters, which are primarily composed of propyl gallate (PG), dodecyl gallate (DG), methyl gallate (MG), and octyl gallate (OG) (Choubey, Varughese, Kumar, & Beniwal, 2015; Kim, Quon, & Kim, 2014; Peluso & Serafini, 2017).

1.5 GA as a therapeutic agent

GA has antioxidant, anticancer, anti-inflammatory, and antimicrobial properties (Lima et al., 2016; Locatelli, Filippin-Monteiro, Centa, & Creczinsky-Pasa, 2013). As GA is an antioxidant, it neutralizes free radicals by providing the extra electron needed to make the pair or breaking down the free radical molecule to render it harmless (Khan, Hassan, & Khan, 2019; D.-S. Lee & J.-Y. Je, 2013). Its products, such as lauryl gallate, propyl gallate, octyl gallate, tetradecyl gallate, and hexadecyl gallate, can inhibit oxidative stress (Locatelli et al., 2013).

1.6 Anti-inflammatory activity of GA

NF-kB, or nuclear factor kappa-light chain enhancer, is a protein group that controls all process in a cell, including activation of B cells, DNA transcript, cytokine generation, and cell survival. Furthermore, NF-kB regulates the expression of various Enzymes involved in immunological and inflammatory responses, such as iNOS (Nitric oxide synthase), COX-2 (Cyclooxygenase -2,) and TNF (Tumor necrosis factor) (Locatelli et al., 2013; Malinin, Boldin, Kovalenko, & Wallach, 1997). NF-kB is a target for the treatment of several inflammatory disorders due to its involvement in inflammatory gene expression, and most anti-inflammatory medications

have been demonstrated to limit the expression of inflammatory cytokines by decreasing activation via NF-kB (Yamamoto & Gaynor, 2001). GA and its derivatives are phenolic compounds, which are exceptional due to their anti-inflammatory properties. GA suppresses pro-inflammatory cytokines, enzymes, and chemokines such as COX-2 (which is an enzyme implicated in cancer inflammation that is vital in abnormal cell division and tumor growth) via lowering NF-KB activation (Dolcet, Llobet, Pallares, & Matias-Guiu, 2005; Locatelli et al., 2013).

1.7 Anti-tumoral activity of GA

Locatelli, C., et al found that GA possesses antitumoral activity in addition to anti inflammatory activity. GA and its derivatives were found to be active against a variety of cancerous cell lines, including leukemia, melanoma, lung cancers, and breast cancer cell lines (Locatelli et al., 2013). As common cues, intracellular Ca^{2+} concentrations were necessary. The elevated level of ROS and Ca^{2+} from natural compounds such as GA and propyl gallate enhances tumor cell apoptosis. Cells that are susceptible to GA also produce a lot of catalases, which are known as Caspase 3 and Caspase 9 protease enzymes. This enzyme is significant in apoptosis and anticancer therapy. GA increases the production of caspase enzymes (Isuzugawa, Inoue, & Ogihara, 2001). TACE (Tumor necrosis factor-α- converting enzyme) is a membrane of disintegrating and metalloprotease-17 (ADAM17). It has been suggested that ADAM17 imbalance leads to the pathophysiology of many malignancies. GA inhibits ADAM17 expression and activity (Y. Lu et al., 2010; Weng & Yen, 2012).

GA exhibits several antimetastatic actions and has the potential to be transformed into a prostate cancer antimetastatic agent. GA may reduce proliferation and invasion in PC-3 (Human prostate cancer) cells by downregulation of PKC, ERK, JNK, and p13K/ AKT signaling pathways, as well as NF-KB resulting in a reduction of MMP-2 and MMP-9

(Locatelli et al., 2013). GA and its derivatives also can block the drug efflux pump via P-glycoprotein 1(P-GP). Alkyl gallates were not released from the cells, although this was depending on the length of their alkyl chain (Kitagawa et al., 2005).

Cell survival and cell cycle changes in MCF7 (human breast cancer), MCFADF7 (human breast cancer multidrug-resistant), and MDA-MB-231 cells (Mutant p53 breast cancer). The upregulation of p21(a potent cyclin-dependent kinase inhibitor that functions as a cell cycle regulator) was detected in all the breast cancer cell lines, followed by apoptotic cell death (Kitagawa et al., 2005). Dodecyl gallate-induced cell survival and cell cycle changes in MCF7 (human breast cancer), MCF7 ADR (Human breast cancer multidrug-resistant), and MDB-MB- 231 cells (mutant p53 breast cancer). The Upregulation of p21 (a potent cyclin-8 dependent kinase inhibitor that functions as a cell cycle regulator) was detected in all three breast cancer cell lines, followed by apoptotic cell death (Kitagawa et al., 2005; Locatelli et al., 2013).

1.8 Antioxidant activity of GA

GA has been shown to have both pro-oxidant and antioxidant. Properties surprisingly a phenolic molecule such as GA has lately been linked to cell death caused by oxidative stress generated by ROS and mitochondrial malfunction (Inoue, Sakaguchi, Isuzugawa, TANI, & OGIHARA, 2000; Sakagami, Satoh, & Kochi, 1997). It was discovered that GA boosted intracellular ROS formation using a particular probe. In the presence of GA, the levels of a well-known ROS superoxide anion fell from 100 M to 400 M thus, GA and its derivatives can prevent lipid peroxidation in the cell membrane (Inoue et al., 2000).

1.9 Antibacterial mode of action of GA

ROS scavenger: Oxidative stress develops when there is a bacterial infection. When there is oxidative stress, the balance between reactive oxygen species and the regular biological system

is disturbed. The imbalanced concentrations of ROS inside cells, harm the host cell's lipids, proteins, and DNA. GA is a versatile scavenger, that might neutralize reactive oxygen species (ROS) (K. Asokkumar, S. Sen, M. Umamaheswari, A. Sivashanmugam, & V. Subhadradevi, 2014). GA acts as an antioxidant in scavenging to stop an oxidative chain reaction by directly transferring an H (hydrogen) atom to the free radical through the 0-H bond cleavage (Badhani, Sharma, & Kakkar, 2015). Metal chelation is also an inhibitory action of GA. GA inhibits metal-induced aggregation by acting as a metal chelator and forming an Mg^{2+}-GA complex. GA can be further developed in metal-based therapy against neurodegenerative disease. GA can disintegrate the outer layer of gram-negative bacteria through the chelation of divalent cations (Badhani et al., 2015; Khan et al., 2019). GA is responsible for damaging cell membrane integrity in both gram-positive and gram-negative bacteria. Thus, changing the charge of bacteria and decreasing the permeability of the cell membrane. Moreover, GA can increase the permeability of cell membranes in different classes of bacteria, such as *Campylobacter jejuni*, and as a result, upraises the antibiotic intake in the microorganism that interfere with cell signaling pathways and induce apoptosis (Sarjit et al., 2015).

Table 1: antimicrobial activity of GA against different bacteria

Bacterial species	Gram stain	MIC (µg/mL)	MBC (µg/mL)	ZOI (mm)	Reference
E. coli	-	2500	5000	25	(Li Fu, WenQing Lu, & XiaoMin Zhou, 2016b)
P. aeruginosa	-	500	NR	NR	(A. Borges, C. Ferreira, M. J. Saavedra, & M. Simões, 2013)
E. coli ATCC 25922	-	1500	NR	NR	(Li Fu et al., 2016b)
Carbapenems-resistant P. aeruginosa	-	2500	2500	NR	(Li Fu et al., 2016b)
Salmonella sp	-	250	NR	NR	(Ammar, Heneter, El-Khateib, Abd-El-Malek, & Abo Markeb, 2021)
Proteus Vulgaris	-	80	200	NR	(Li Fu et al., 2016b)
Shigella flexneri	-	250	800	NR	(Kang, Liu, Liu, Wu, & Li, 2018)
Shigella dysenteriaee	-	300	800	NR	(D. S. Lee & J. Y. Je, 2013)
Klebsiella pneumonia	-	400	NR	NR	(D. S. Lee & J. Y. Je, 2013)
Enteropathogenic E.Coli	-	750	1250	NR	(J. Dávila-Aviña, C. Gil-Solís, J. Merino-Mascorro, S. García, & N. Heredia, 2020)
Salmonella typhimurium	-	10,000	NR	NR	(Ammar et al., 2021)
Chromobacterium violaceum	-	2000		NR	(Dusane, O'May, & Tufenkji, 2015)
Pasteurella multocida	-	500	NR	NR	(K. Rajamanickam, Yang, & Sakharkar, 2018)

Multidrug-resistant Acinetobacter baumannii	+	2500	2500	NR	(Kubo, Xiao, & Fujita, 2002)
Methicillin-resistant Staphylococcus aureus MRSA ATCC 33591	+	1067	>3200	NR	(Kubo et al., 2002)
Staphylococcus aureus	+	1750	5250	22	(A. Borges et al., 2013)
Listeria monocytogenes	+	2000	5500	NR	(Li Fu et al., 2016b)
Staphylococcus xylosus	+	NR	NR	24	(Bouaziz et al., 2014)
Mannheimia haemolytica	+	250	NR	NR	(K. Rajamanickam et al., 2018)
Bacillus cereus	+	2500	3000	NR	(L. Fu, W. Lu, & X. Zhou, 2016)
Vancomycin resistant Staphylococccus aureus (VISA)	+	0.007	NR	NR	(Basri, Zin, Bakar, Rahmat, & Mohtar, 2008)
Methicillin resistant Staphylococcus aureus (MRSA)	+	63-125	NR	NR	(Chusri & Voravuthikunchai, 2009)
Staphylococcus aureus (ATCC- 25923)	+	125	NR	NR	(Chusri & Voravuthikunchai, 2009)
Staphylococcus aureus (ATCC- 6538)	+	1500	NR	NR	(Sanhueza et al., 2017)
Staphylococcus aureus (ATCC- 8275)	+	1500	NR	NR	(Sanhueza et al., 2017)
Methicillin resistant Staphylococcus aureus (MRSA)	+	3000-2000	NR	NR	(Sanhueza et al., 2017)
E.coli (ATCC- 25922)	-	2000	NR	NR	(Sanhueza et al., 2017)
Methicillin resistant Staphylococcus aureus (MRSA)	+	750	1500	NR	(Hossan et al., 2018)

Enterococcus faecalis	+	1500	1500	NR	(Hossan et al., 2018)
Escherichia coli	-	750	750	NR	(Hossan et al., 2018)
Pseudomonas aeruginosa	-	750	750	NR	(Hossan et al., 2018)
Klebsiella pneumoniae	-	750	750	NR	(Hossan et al., 2018)
Acinetobacter baumannii	-	1500	1500	NR	(Hossan et al., 2018)
Mannheimia haemolytica (ATCC- 29702	-	250	NR	NR	(Karthic Rajamanickam, Yang, & Sakharkar, 2019)
Pasteurella multocida (ATCC-43137)	-	500	NR	NR	(Karthic Rajamanickam et al., 2019)
Arcobacter butzleri	-	1024	NR	NR	(Sousa, Luís, Oleastro, Domingues, & Ferreira, 2019)
Escherichia coli	-	≥600	NR	NR	(Farrag, Abdallah, Shehata, & Awad, 2019)
Acenetobacter baumannii	-	≥600	NR	NR	(Farrag et al., 2019)
Pseudomonas spp.	-	≥600	NR	NR	(Farrag et al., 2019)
Klebsiella pneumoniae	-	≥600	NR	NR	(Farrag et al., 2019)
Enterobacter spp.	-	≥600	NR	NR	(Farrag et al., 2019)
Escherichia coli (ATCC-25922)	-	NR	NR	NR	(Ng, Sit, Ooi, Ee, & Lim, 2020)

Aeromonas hydrophilia	-	>1500	NR	NR	(Santos, Lima, Franco, & Pinto, 2021)
Chromobacterium violaceum (ATCC-12472)	-	1500	NR	NR	(Santos et al., 2021)
Chromobacterium violaceum (O26)	-	1500	NR	NR	(Santos et al., 2021)
Salmonella Montevideo	-	>1500	NR	NR	(Santos et al., 2021)
Serratia marcescens	-	>1500	NR	NR	(Santos et al., 2021)
Streptococcus pyogenes	+	25-1000	NR	Sensitive	(Neyestani, Khalaji, & Gharavi, 2007a)
Escherichia coli	-	50-1000	NR	Sensitive	(Neyestani, Khalaji, & Gharavi, 2007b)

Note: MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; ZOI: Zone of Inhibition; NR: Not Reported

1.10 Mechanism of action of conventional antibiotics

Inhibition of cell synthesis

Antibiotics that interfere with forming of bacterial cell walls are the most efficient therapeutic antibiotics. These drugs have a high therapeutic efficacy since bacterial cell walls have a distinctive structure that neither eukaryotic nor mammalian cells have. Bacterial cells are protected with peptidoglycan cell walls (Hoerr et al., 2016; Kapoor, Saigal, & Elongavan, 2017). Antibiotics that disrupt the bacterial cell wall operate at various phases of peptidoglycan production and cell wall synthesis. Such as Beta Lactams, Bacitracin, Vancomycin, Penicillin, Cephalosporin, Ampicillin, and Methicillin. Since mammalian cells lack cell walls, this class of antibiotics is quite selective. They are designed to kill bacteria with little impact on host cells.

Disruptors of cell wall

Certain antibiotics destroy the permeability of the cell membrane by adhering to membrane phospholipids. As human cells have cell membranes, some antibiotics are detrimental to host cells while delivered comprehensively. As a result, their clinical utility is restricted to topical applications (Galizzi, Cacco, Siccardi, & Mazza, 1975). Polymyxins are such significant clinical medications. E.g., Polymyxin B and Polymyxin E (colistin) (Dowling, O'Dwyer, & Adley, 2017; Galizzi et al., 1975; Kapoor, Saigal, & Elongavan, 2017).

Inhibitors of protein synthesis

Antibiotics that suppress bacterial protein synthesis might affect various phases. These medicines have high therapeutic efficacy but are not as potent as cell wall synthesis inhibitors. Several antibiotics also disrupt eukaryotic human counterparts, but their effect on bacterial ribosomes is much stronger. On the other hand, some of these drugs are also therapeutically beneficial and practical research tools because they inhibit certain phases of protein synthesis (Hoerr et al., 2016).

The following steps are involved in this process: synthesis of the 30S initiation complex, assembly of the 50S ribosomal subunit, formation of the 70s ribosome from the 30s and 50s complexes, and elongation (Chopra & Roberts, 2001; Kapoor et al., 2017). Examples are Streptomycin, Chloramphenicol, Tetracycline Erythromycin aminoglycosides, and macrolides. Most antibiotics in this group are specific in that they do not react with human analogs of these enzymes, while the others do. Certain antibiotics disrupt DNA synthesis by attaching to bacterial topoisomerase II, an enzyme that relaxes supercoil DNA during replication (Dowling et al., 2017; Nelson & Levy, 2011).

Inhibitors of nucleic acid synthesis

Several antimicrobials and antibiotics prevent the production of nucleic acids. These aren't as specifically harmful as other medications. Certain antibiotics disrupt DNA synthesis by attaching to bacterial topoisomerase II, an enzyme that relaxes supercoil DNA during replication. Others prevent RNA synthesis by blocking RNA polymerase (Hoerr et al., 2016). Quinolone antibiotics, which mainly suppress bacterial topoisomerase II, are utilized to synthesize DNA in this case (Kapoor et al., 2017). Rifampicin, on the contrary, inhibits bacterial RNA polymerase, which decreases RNA synthesis. Antibiotics that affect human cells for cancer treatment and antibiotics that affect human cells include irinotecan, etoposide, doxorubicin, and actinomycin D (Dowling et al., 2017).

Inhibitors of folic acid synthesis or blocking metabolic pathways

Numerous therapeutic drugs act as antimetabolites and block the functioning of metabolic pathways (Hoerr et al., 2016). They hinder the major enzymes in the metabolic process of bacteria. Sulfonamides and trimethoprim are examples. The bacterium produces its folic acid, unlike humans, who obtain it from food (Seydel, 1968). Therefore, antibiotics that specifically inhibit enzymes involved in folic acid production are used (Dowling et al., 2017; Seydel, 1968).

1.11 Minimum Inhibitory Concentration (MIC)

The MIC of an antibacterial agent is the lowest quantity indicated in mg/L (g/**mL**) that, under precisely regulated in vitro conditions, entirely stops observable growth of the tested strain of an organism (Kowalska-Krochmal & Dudek-Wicher, 2021). To identify whether the strain is resistant or sensitive to the antibacterial, the calculated MIC value must be matched to MIC clinical breakpoints. Antimicrobial susceptibility evaluation based on MIC value does not imply the determination of the resistance mechanism. Nonetheless, for epidemiological reasons, classifying such a strain as resistant based on the MIC value may be a trigger to conduct additional studies on the identification of mechanisms of resistance (Ellner & Neu, 1981; Kowalska-Krochmal & Dudek-Wicher, 2021).

1.12 Synergistic effects of GA

Synergistic effects are the nonlinear cumulative effects of two active chemicals that produce comparable related results from their actions. Taking advantage of the synergistic effect is a viable remedy for the limited therapeutic efficacy of natural antioxidants. Alternatively, active antibiotics with the sequential or supplementary activity of natural compounds limit the dosage of antibiotics, which helps to combat AMR (Badhani et al., 2015; Teixeira et al., 2013).

Multiple studies have shown that combining GA with many antibiotics and other antibacterial drugs has a big effect on bacteria that have become resistant. Specifically, a study found GA with a MIC value of 2000 μg/mL in combination with some major antibiotics with various MIC values against *P.aeruginosa* (Teixeira et al., 2013). Ciprofloxacin, Sulfamethoxazole, Tetracycline, Ceftazidime, Trimethoprim, Polymyxin, and Piperacillin have MIC values of 0.125μg/mL, 128μg/mL, 32μg/mL, 2μg/mL, 32μg/mL, and 2μg/mL, respectively. Only two interactions exhibited synergistic effects: GA with sulfamethoxazole and GA with tetracycline. Aside from these two pairings, the others provide indifferent or additive outcomes (Teixeira et al., 2013). Another study discovered a

combination of GA and Gentamycin again *P.aeruginosa* (Basak, Singh, & Rajurkar, 2016). This interaction is also observed using MIC values of GA1024 μg/mL and Gentamycin 156 μg /mL, which showed an indifferent result (Basak, Singh, & Rajurkar, 2016).

In other research, GA was used with various antibiotics to combat *Escherichia coli*. GA with a MIC of 1024 μg/mL. When used in conjunction with Amoxicillin, Ceftiofur, Penicillin, Cefotaxime, Thiamphenicol, and Marbofloxacin. Only interactions between GA and Thiamphenicol had a synergistic impact against *Escherichia coli* (Thoppil & Bishayee, 2011). In comparison, the same MIC value (1024 g/mL) was found for gentamycin, which had a MIC of 156 g/mL, and norfloxacin, which had a MIC of 49.21 g/mL (Basak, Singh, & Rajurkar, 2016). Both combinations have an antagonistic effect on *Escherichia coli* (Ammar et. al.,2021).

A. baumannii and *K. pneumonia* are antibiotic-resistant pathogens. Antimicrobials have been mixed with GA to make these two pathogens less dangerous, according to research (Yoshida & Niki, 2003). In one investigation, Ampicillin, Tetracycline, Chloramphenicol, Gentamicin, Cefotaxime, and Ciprofloxacin with MIC values of 256 μg/mL, 256 μg/mL, 256 μg/mL, 128 μg/mL, and 8 g/mL was used in conjunction with GA with a MIC of 400 g/mL.

The exception of one interaction, GA with Ciprofloxacin, demonstrated an antagonistic effect in the case of *A. baumannii* (Yoshida & Niki, 2003). In *K. pneumonia*, the combination of GA with Tetracycline showed a negative interaction. Aside from these two combinations of GA and antibiotics against *A.baumannii* and *K. Pneumonia*, all other interactions yielded indifferent results (Yoshida & Niki, 2003). GA is effective against both *Enterococcus faecalis and Staphylococcus aureu*s (K Asokkumar et al., 2014). GA with a MIC value of 3150 μg/mL was combined with two different antimicrobial agents. Thymol with a MIC value of 1200 μg/mL and Carvacrol with a MIC value of 800 μg/mL against *Enterococcus faecalis* (Basak, Singh, & Rajurkar, 2016). Both combinations demonstrated synergy. On the contrary, GA with a MIC of 1024 μg/mL was combined

with Norfloxacin (49.21 µg/mL) and Gentamycin (156 µg/mL), and both combinations demonstrated synergism against Staphylococcus aureus (Yoshida & Niki, 2003).

Table 2: Combined efficiency of GA in combination with different antibiotic

Bacterial species	Pair combination with GA (µg/mL)	Interactions	Methods	Ref.
P. aeruginosa	GA (2000) +Ciprofloxacin (0.125)	Indifferent	CBA, FIC	(Jayaraman, Sakharkar, Lim, Tang, & Sakharkar, 2010)
	GA (2000)+ Sulfamethoxazole	Synergy		
	GA (2000) + Tetracycline (32)	Synergy		
	GA (2000) + Ceftazidime (2)	Additive		
	GA (2000) + Trimethoprim (32)	Additive		
	GA (2000) + Polymyxin B (2)	Indifferent		
	GA (2000) + Piperacillin (2)	Additive		
Chromobacterium violaceum	GA (2000) + Tetracycline (15)	Antagonist	CBA	(Dusane et al., 2015)
	GA + Carbenicillin (12000)	Antagonist		
***E.coli*(ATCC-25922)**	GA (1024) + Ampicillin (2)	Additive	FIC	(Hossain, Park, Park, et al., 2020)
	GA (1024) + Amoxicillin (1)	Indifferent		
	GA (1024) + Ceftiofur (1)	Indifferent		
	GA (102) + Penicillin G (16)	Indifferent		
	GA (1024) + Cefotaxime (0.125)	Additive		
	GA (1024) + Thiamphenicol (256)	Synergy		
	GA (1024) + Marbofloxacin (0.25)	Additive		
***Staphylococcus Aureus* (MRSA)**	GA-g-Chitosan (16) + Ampicillin (128-512)	Synergy	CBA	(Lee et al., 2014)
	GA-g-Chitosan (16) + Penicillin (128-512)	Synergy		
	GA-g-Chitosan (16)+ Oxacillin (64-512)	Additive		
	GA-g-Chitosan (16)+ Chloramphenicol (64-512)	Indifferent		

Campylobactor jejune	GA (512) + Ciprofloxacin (10)	Synergy	CTA	(Oh & Jeon, 2015)
	GA (512) + Erythromycin (10)	Synergy		
Enterococcus faecalis	GA (3150) + Thymol (1200)	Synergy	MIC, FIC	(Gutiérrez-Fernández et al., 2013)
	GA (3150)+ Carvacrol (800)	Synergy		
Staphylococcus aureus	GA (1024) + Norfloxacin (49.21)	Synergy	MIC, FIC	(Lima et al., 2016)
	GA (1024) + Gentamycin (156)	Synergy		
P. aeruginosa	GA (1024) + Gentamycin (156)	Indifferent	MIC, FIC	(Lima et al., 2016)
Escherichia coli	GA (1024)+ Gentamycin (156)	Antagonist	MIC, FIC	(Lima et al., 2016)
	GA (1024) + Norfloxacin (49.21)	Antagonist		
A.baumannii **(ATCC 19606)**	GA (400) + Ampicillin (256)	Indifferent	CBA	(Buchmann et al., 2022)
	GA (400) + Tetracycline (256)	Indifferent		
	GA (400) + Chloramphenicol	Indifferent		
	GA (400) + Gentamicin (256)	Indifferent		
	GA (400) + Cefotaxime (128)	Indifferent		
	GA (400) + Ciprofloxacin (8)	Antagonist		
K. Pneumonia **(ATCC 700603)**	GA (400) + Ampicillin (256)	Indifferent	CBA	(Buchmann et al., 2022)
	GA (400) + Tetracycline (256)	Indifferent		
	GA (400) + Chloramphenicol (256)	Antagonist		
	GA (400) + Gentamycin (256)	Indifferent		
	GA (400) + Cefotaxime (128)	Indifferent		
	GA (400) + Ciprofloxacin (8)	Indifferent		

Staphylococcus Aureus (MRSA)	GA(63-125) +Amoxicillin (32-512)	Additive	CBA	(Chusri & Voravuthikunchai, 2009)
	GA(63-125)+ Oxacillin (160-1280)	Additive		
	GA(63-125)+ Penicillin G (16-256)	Additive		
S. mutans	GA-s-Gold Nanoparticle	Synergy	MIC	(Moreno-Álvarez et al., 2010)
Pseudomonas aeruginosa	GA (11.8)+ Sulfamethoxazole (0.5)	Synergy	CBA, FIC	(Jayaraman, Sakharkar, Sing, Chow, & Sakharkar, 2011)
	GA (11.8) + Trimethoprime (0.11)	Indifferent		
Pseudomonas aeruginosa (ATCC- 15729)	GA (8-8192)+ Tobramycin (0.0125-128)	Synergy	MIC, FIC	(Kyaw, Arora, & Lim, 2011)
	GA (8-8192) + Streptomycin (0.0125- 128)	Additive		
Staphylococcus Aureus (MRSA)	GA (100-5000) + Fusidic Acid(0.031-512)	Additive	MIC, FIC	(Kyaw & Lim, 2012)
	GA (100-5000) + Cefotaxime Sodium (0.031-512)	Additive		
	GA (100-5000)+ Minocycline (0.031-512)	Additive		
Staphylococcus Aureus (MRSA)	GA (100-5000) + Vancomycin (0.031-512)	Indifference	MIC, FIC	(Kyaw & Lim, 2012)
	GA(100-5000)+ Ofloxacin (0.031-512)	Additive		
	GA (100-5000) + Rifampicin (0.031-512)	Additive		
Staphylococcus aureus (ATCC-6538)	GA (600-1500)+ Nalidixic acid (60)	Synergy	FICI	(Sanhueza et al., 2017)
	GA (600-1500)+ Ciprofloxacin (1.5)	Synergy		

Staphylococcus aureus (ATCC-6538)	GA (600-1500) + Norfloxacin (1.5)	Synergy	FICI	(Sanhueza et al., 2017)
	GA (600-1500) + Levofloxacin (1.5)	Synergy		
	GA (600-1500) + Oxacillin (3)	Synergy		
	GA (600-1500) + Tetracycline (1.5)	Synergy		
	GA (600-1500) + Chloramphenicol (16)	Synergy		
Staphylococcus aureus (ATCC-8275)	GA (1500) + Nalidixic acid (300)	Synergy	FICI	(Sanhueza et al., 2017)
	GA (1500) + Ciprofloxacin (30)	Synergy		
	GA(1500) +Norfloxacin (25)	Synergy		
	GA (1500) +Levofloxacin (10)	Synergy		
	GA (1500) +Oxacillin (50)	Synergy		
	GA (1500) + Tetracycline (5)	Synergy		
	GA (1500) + Chloramphenicol (75)	Synergy		
Methicillin resistant Staphylococcus aureus (MRSA)	GA (3000) + Nalidixic acid (300)	Synergy	FICI	(Sanhueza et al., 2017)
	GA (3000) + Ciprofloxacin (15)	Synergy		
	GA (3000) + Norfloxacin (30)	Synergy		
	GA (3000) + Levofloxacin (3)	Synergy		

Methicillin resistant Staphylococcus aureus (MRSA)	GA (3000) + Oxacillin (150)	Synergy		
	GA (3000) + Tetracycline (750)	Synergy		
	GA (3000) + Chloramphenicol (1)	Synergy		
E.coli (ATCC-25922)	GA (300-2000) + Nalidixic Acid (16)	Synergy	FICI	(Sanhueza et al., 2017)
	GA (300-2000) + Ciprofloxacin (1)	Synergy		
	GA (300-2000) + Norfloxacin (1.5)	Synergy		
	GA (300-2000) + Levofloxacin (0.75)	Synergy		
	GA (300-2000) +Ampicillin (15)	Synergy		
	GA (300-2000) +Tetracycline (3)	Synergy		
	GA ((300-2000) + Chloramphenicol (8)	Synergy		
Mannheimia haemolytica (ATCC- 29702)	GA (3.91-500) + Tulathromycin (0.04-0.31)	Synergy	CBA, MIC	(Karthic Rajamanickam et al., 2019)
Pasteurella multocida (ATCC-43137)	GA (3.9-500) + Tulathromycin (0.04-5)	Synergy	CBA, MIC	(Karthic Rajamanickam et al., 2019)
Arcobacter butzleri	GA (1024) + Tetracycline (4)	Additive	CBA, MIC	(Sousa et al., 2019)
	GA (1024) + Chloramphenicol (16)	Indifferent		
	GA (1024) + Erythromycin (4)	Indifferent		

Arcobactor butzleri	GA (1024) + Ciprofloxacin (0.0625)	Indifferent	CBA, MIC	(Hossain, Park, Lee, et al., 2020)
Escherichia coli	GA-Ag-NPs	Synergy	NR	(Liu et al., 2020)
Staphylococcus aureus	GA-Ag-NPs	Synergy	NR	(Liu et al., 2020)
Salmonella enterica serovar Typhimurium (ATCC-14028)	GA (256)+ Ampicillin (1)	Additive	MIC, FIC	(Hossain, Park, Lee, et al., 2020)
	GA (256) + Amoxocillin (0.5)	Indifferent		
	GA (256)+ Ceftiofur (1)	Additive		
	GA (256) + Penicillin G (8)	Indifferent		
	GA (256) + Cefotaxime (2)	Indifferent		
	GA (256)+ Erythromycin(128)	Indifferent		
	GA (256)+ Thiamphenicol (128)	Additive		
	GA (256)+ Marbofloxacin (0.062)	Indifferent		
Staphylococcus Aureus (VISA)	GA+ Oxacillin	Additive	FICI	(Basri et al., 2008)

Note: MIC: Minimum Inhibitory Concentration; CBA: Checkerboard Assay; CTA: Check-board Titration Assay; FIC: Fractional Inhibitory Concentration, GA: Gallic Acid; NR: Not Reported.

Chapter 2

Methods and materials

2.1 Place of conducted experiment

This research was done at Biochemistry and Environmental Microbiology (BEM) lab, Department of Mathematical and Natural Sciences Department, BRAC University.

2.2 Materials

Nutrient Agar, Nutrient Broth, Mueller Hilton Agar (MHA), Casein Enzyme Hydrolysate Type-1, Tryptase, and McFarland Standard Set was purchased from Himedia (India). Agar for bacteriological use was purchased from Liofilchem Italy. Dimethyl sulfoxide was purchased from Roth (Germany). Analysis grade Sodium Chloride (NaCl), Paraffin was purchased from Merck (Germany). GA was purchased from QualiChem's (India). EDTA, Ethanol, and spirit was purchased from Sigma-Aldrich.

2.3 Bacterial strain

The following bacterial strains were used: *E. coli* (ATCC- 25922), *Staphylococcus aureus* (ATCC- 25923), *Enterococcus faecalis* (ATCC- 29212), *Pseudomonas aeruginosa* (ATCC- 27853), *Proteus vulgaris, Shigella flexneri, Shigella dysenteriae,* Enteropathogenic *E. coli, Hafnia alvei, Bacillus cereus*. These bacterial samples were collected from the lab's microorganism inventory. All microbial strains were preserved at -20°C in a cryovial containing T1N1 media submerged on very liquid sterile paraffin, and they were sub-cultured in Nutrient Agar (NA) before testing.

2.4 Apparatus and instruments

The important equipment used in the study is listed below:

Table 3: List of apparatus and instruments

Number	Apparatus/ Instruments	Brand/ Model
1.	General incubator	Incucell
2.	Shaking incubator	JSR
3.	Ultra-centrifuge machine	TOMY MX-307
4.	Autoclave machine	TOMY ES - 315
5.	Laminar	Haier Biomedical
6.	Refrigerator	Samsung
7.	ELISA machine	Thermofisher multiskan ex
8.	Vortex	DIGISYTEM VM- 2000
9.	Spirit lamp	N.A.
10.	Micropipette	Eppendorf
11.	Micropipette tips	NEST
12.	96- well ELISA microplates	N.A.
13.	Petri dishes	N.A.
14.	Conical Flask	SCHOTT Duran®
15.	Screw- Capped bottles	SCHOTT Duran®
16.	Polypropylene screw-capped tubes	Falcon
17.	Glass test tubes	Pyrex
18.	Borosilicate vials with Cap	Pconlab
19.	Microcentrifuge tubes (1.5mL & 2.0mL)	Eppendorf
20.	Inoculation loop	N.A.
21.	Inoculation needle	N.A.
22.	Cotton Swab	Dearon
23.	Paraffin tape	Bemis Company, INC
24.	0.22µ filter	Pconlab
25.	Syringe (5mL and 10 mL)	JMI
26.	Magnetic Stirrer	JSR

2.4.1. List of used media, reagents, and chemicals

Table 4: List of used media, reagents, and chemicals

Number	Media, Reagents, chemicals	Brand
1.	Nutrient Agar	Himedia
2.	Muller Hinton Agar	Himedia
3.	Luria Bertani Broth	Himedia
4.	NaCl	Himedia
5.	PBS	N.A.
6.	DMSO	N.A.
7.	Hydrogen peroxide (H_2O_2)	N.A.
8.	EDTA	N.A.
9.	Ethanol	Sigma-Aldrich
10.	Spirit	Sigma-Aldrich

2.5 Preparation of media and reagents

2.5.1 Preparing Nutrient Agar (NA)

The organisms were first grown on nutrient agar. By heating the agar until it melted, 28 grams of nutrient agar powder was dissolved in 1 liter of purified water. The dissolved agar was then autoclaved for 15 minutes at 121°C. As the autoclaved is done, the media then quietened down and the medium was then put into petri dishes. These poured petri dishes were placed in the laminar to solidify. Finally, the solidified dishes were placed in the new media refrigerator.

2.5.2 Preparing Muller-Hinton Agar (MHA)

A non-selective, non-differential media for bacterial growth is Mueller-Hinton agar. This means any kind of bacterial species can grow on MHA. Beef extract, acid casein hydrolysate, and agar are MHA's ingredients. It also has starch in it. Toxins generated by bacteria are known

to be absorbed by starch, preventing them from interfering with antibiotics. Additionally, it controls how quickly the medicines diffuse through the agar. Casein acid hydrolysate and beef extract contain nitrogen, vitamins, carbon, amino acids, sulfur, and other vital nutrients. A standardized solid medium called MHA is advised to research how well bacteria respond to antibiotic or antimicrobial drugs using the diffusion method (Kirby-Bauer method). A more appropriate zone of inhibition results from improved diffusion.

To prepare MHA, 38 grams of the MHA media should be dissolved in 1 liter of purified water. For the medium to fully dissolve, heat it while stirring often and bring it to a boil for five to ten minutes. Then autoclaved it for 15 minutes at 121°C.

MHA that has cooled down should be added to sterile Petri dishes, on a horizontal surface to ensure equal depth. Every petri dish should have 35mL of MHA.

2.5.3 Preparation of Luria Bertani broth (LB)

In this experiment, all MIC and combination methods were conducted using LB. To prepare LB, 25.0 grams of LB powder were dissolved in 1 liter of purified water. The dissolved LB was then poured into vials and autoclaved for 15 minutes at 121°C. As the autoclaved is done, the media then quietened down. Finally, the vials containing LB were kept in the new media refrigerator for further usage.

2.5.4. Physiological saline preparation

To make bacterial suspensions, physiological saline was used. The saline should not include more than 0.9% NaCl, all of the bacteria would die due to excessive alkaline conditions, 0.9 grams of NaCl was dissolved in 100 mL of distilled water. Then, using a glass pipette, 10 mL of saline was pipetted into each test tube. The test tubes were then autoclaved at 121°C for 15 minutes before being stored at room temperature.

2.5.5 Preparation of GA working solution

To determine the MIC, a stock solution of GA was prepared. 1.0 grams of GA was completely dissolved in 100 mL of distilled water. Thus, per mL of water contains 10 milligrams or 10,000 micrograms of GA. The prepared stock solution was stored in an Eppendorf tube at -20°C. A magnetic stirrer was used so that GA can be completely dissolved into the water.

2.5.6 Preparation of metronidazole working solution

Filmet 200mg/5mL by Beximco Pharmaceuticals was ordered and collected from a local pharmacy. Different concentration of metronidazole was prepared from it by diluting it in distilled water.

2.5.7 Preparation of PBS buffer

PBS (phosphate-buffered saline) is a pH-adjusted mixture of ultrapure-grade-phosphate buffers and saline. This is an isotonic solution used to keep the pH of blood type-dependent red blood cells (RBC) hemolysis test consistent.

To prepare 1litre of PBS (pH 7.4) the required components are mentioned below-

Table 5: Preparation of PBS

Component	Amount	Concentration
Sodium Chloride	8g	0.137M
Potassium Chloride	0.2g	0.0027M
Sodium Phosphate	1.44g	0.01M
Potassium Phosphate	0.245g	0.0018M

In a suitable beaker, 800 mL of distilled water was prepared. Then 8g of NaCl was added to the solution. After that 0.2g of potassium chloride was added to the solution. Thirdly 1.44g of sodium phosphate dibasic was added to the solution. Lastly, 0.245g of Potassium Phosphate

Monobasic was added to the solution. After adding all the reagents, the pH of the solution was adjusted to 7.4. Then distilled water was added until the volume is 1l.

2.6 Preparation of bacterial suspension

For each bacterial strain, 3 test tubes were prepared to contain 9 mL saline. In total, 30 test tubes were designed for three repeats for all bacterial samples. Afterward, bacterial samples were inoculated into the saline solution. This was accomplished by first choosing one or two colonies from the cultural plate using inoculating loops. Later, a vortex was used to create a smooth suspension in the test tubes for two to five seconds.

2.6.1 Comparing with McFarland solution

When adjusting the turbidity of the liquid or bacterial suspension in the vial or tube in the microbiology laboratory, McFarland Standards are employed as the Reference standard for microbial testing that helps to maintain and guarantee that the number of bacteria will be within a specified range. The cell count density varies based on the concentration of the McFarland standard, which can be generated in concentrations ranging from 0.5 to 4. However, the McFarland Standard of 0.5 is typically employed in microbiological laboratories for antibiotic susceptibility testing and culture media performance testing.

Table 6: MacFarland Standard Suspension to achieve bacterial suspension

MacFarland Standard	Approximate Bacterial Suspension /mL
0.5	1.5×10^8
1.0	3.0×10^8
2.0	6.0×10^8
3.0	9.0×10^8
4.0	1.2×10^8

According to the McFarland standard, we have taken 1.5×10^8CFU/mL to achieve the 0.5 McFarland standard.

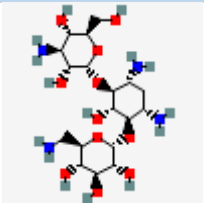
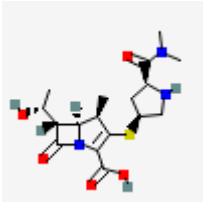
2.6.2 Creating the bacterial suspension lawn culture on MHA media

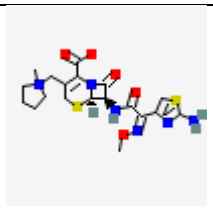
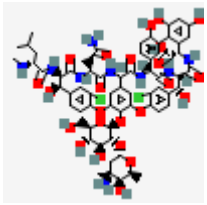
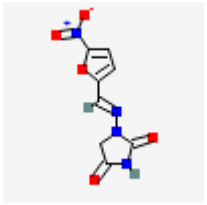
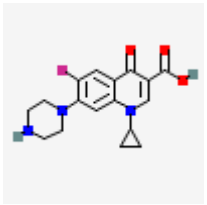
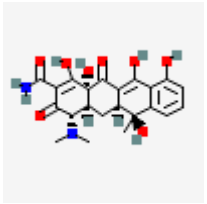
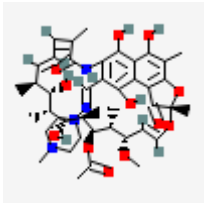
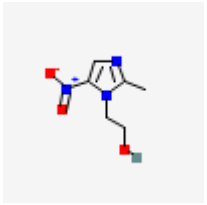
Bacterial samples must be grown in NA media for 24 hours prior antibiogram test. From that fresh culture media, one-two single colonies are taken and suspended into 9 mL saline solution using a sterile loop and mix it by vertexing until we got 0.5 MacFarland standard. A sterile cotton swab is dipped into the bacteria-containing saline solution. Excess water was removed from the swab. The next step was to repeatedly swipe the swab at various angles to ensure that the bacteria were distributed evenly over the MHA surface. Before adding the antibiotic, the plate was given time to soak in the suspension with the lid slightly open for 2.5–4 minutes**.**

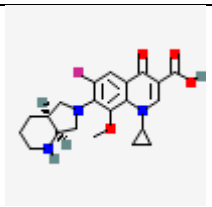
2.7 Experimented antibiotics for Zone of Inhibition test

Ten antibiotics were selected from ten antibiotic classes for the design of the antibiogram test to observe antibiotic resistance and susceptibility. As follows:

Table 5: List of antibiotics used in this study

Name of antibiotic Groups	Used antibiotics	Manufacturer	Structure	CAS ID
Aminoglycosides	Kanamycin	HiMedia		8063-07-8
Carbapenem	Meropenem	HiMedia		119478-56-7

Cephalosporin	Cefepime	HiMedia		88040-23-7
Glycopeptides	Vancomycin	HiMedia		1404-90-6
Monobactams	Nitrofurantoin	HiMedia		67-20-9
Quinolones/ Fluroquinolones	Ciprofloxacin	HiMedia		85721-33-1
Tetracyclines	Tetracycline	HiMedia		60-54-8
Ansamycin	Rifampicin	HiMedia		13292-46-1
Nitromidazole	metronidazole	HiMedia		443-48-1

Fluoroquinolones	Moxifloxacin	HiMedia		354812-41-2

2.7.1 Placing the antibiotic disc on the bacterial lawn

Sterile tweezers were used to put the antibiotic disc on the lawn. The lawn was completely contacted by the discs by being softly placed on it and then pressed against it. For each bacterial sample, the antibiotic disc diffusion orientation was the same for three repeats. After arranging all the discs on the lawn properly, the lid was closed. After that, the plates were transported for a 24 hours incubation period at 37°C.

2.8 Measuring the Zone of Inhibition (ZOI)

The zone of inhibition of each antibiotic was measured two times after incubation. Such as after 12 hours and 24 hours for each bacterial sample. A measuring centimeter ruler was used to take the measurements. When the zone was visible, the ruler was pressed up against the rear of the plate to measure its diameter. The results of the measurements were noted and converted into millimeters, then they were compared to an antibiotic susceptibility chart.

2.9 Measuring Minimum Inhibitory Concentration (MIC)

MIC was measured for both GA and metronidazole using an ELISA microplate reader. For all the bacterial species triplicate tests were run using distinct working solutions of antimicrobials. 1.0 g of GA will be added and completely dissolved in 100 mL of distilled water. Per mL contains 10 mg or 10,000 micrograms (stock solution). Firstly, a working solution was prepared of desired concentration by the V1S1=V2S2 equation. With this equation, various concentrations of GA were prepared i.e.- 50, 100, 150, 200, 250, 300, 500, 650, 750, 1000,

1500, 1750 μg/mL, etc. The dilution must be continued until it reaches different test concentrations of GA. Previously, the bacterial strains were grown in a Nutrient agar (NA) media overnight at 37°C. The single colonies were taken to make a saline solution to compare with the McFarland solution. Afterward, 890 μl of LB media was taken into each vial. 10 microliters of 0.5 MacFarlane Standard bacterial suspension will be added to each vial. The bacterial sample was incubated in the shaker incubator for 4 hours at 120 RPM/ 37°C. Then previously mentioned concentrations of GA were added with the media to make a total 1000μl or 1mL concentration. To maintain the proper 1000μl concentration water was added. As a positive control, we used 200 μl DMSO and as a negative control, we used only 200 μl water in Luria Bertani broth containing bacterial samples. Then GA incubated the cultures overnight at 37°C and 120 RPM. To ensure the MIC values, the optical density (OD) of the various bacterial strains was evaluated using 96 wells of microtiter ELISA plates at 600 nm.

metronidazole's MIC was measured following the same methodology.

2.10 Blood group-dependent red blood Cell (RBC) hemolysis assay

The hemocompatibility of GA in different blood groups from both genders was investigated against human RBCs by following this protocol (Ranjan Sarker et al., 2019). However, there were some modifications considered here and there. Briefly, from a 20–30-year-old healthy individual, 10 mL of human blood was collected through the venipuncture method and preserved in a tube containing 10% EDTA as an anticoagulant. The blood samples were centrifuged at 5000 RPM for 5 min at room temperature to separate RBCs from serum. The serum was aspirated, followed by the resuspension of precipitated RBCs in 5 mL phosphate-buffered saline (PBS, pH 7.4), and centrifuged at 5000 RPM for 5 min. Then the RBCs were washed twice with 5 mL phosphate-buffered saline (PBS, pH 7.4) solution at 5000 RPM for 5

min. The RBC suspension was then produced in 35 mL of phosphate-buffered saline (PBS, pH 7.4). The RBC suspension (1 mL) was then combined with 200 µL of various GA concentrations (100, 300, 500, 700, 1000, 1200, 1500, and 1700 µg/mL) and incubated for 1 hour at 37°C with moderate shaking. RBC and GA combinations were centrifuged at 5000 RPM for 5 minutes. The supernatant was collected, and the absorbance at 570 nm was measured. RBCs treated with PBS and 30% Hydrogen Peroxide were used as negative and positive control respectively in this experiment. The entire experiment was performed three times, and the degree of lysis was assessed for each replicate.

2.11 Measuring Fractional Inhibitory Concentration (FIC) Index

The FIC index is a statistical technique for the evaluation of the effectiveness of combination drugs. FIC index values range from 0.5 to 4 and between this ranges, four distinct levels represent the effectiveness of the combination.

Table 6: FIC index value ranges.

FIC index range	Effectiveness of the combination
FIC value < 0.5	Synergism
FIC value > 0.5 – 1	Additive
FIC value >1-4	Indifference
FIC value >4	Antagonism

The FIC index was calculated by the formula-

FIC index = ∑ **(MIC of the agents in combination / MIC of the agent alone).**

These formulas were used to calculate the MIC values of GA and metronidazole and their combined effect on different bacterial species.

Chapter 3

Results

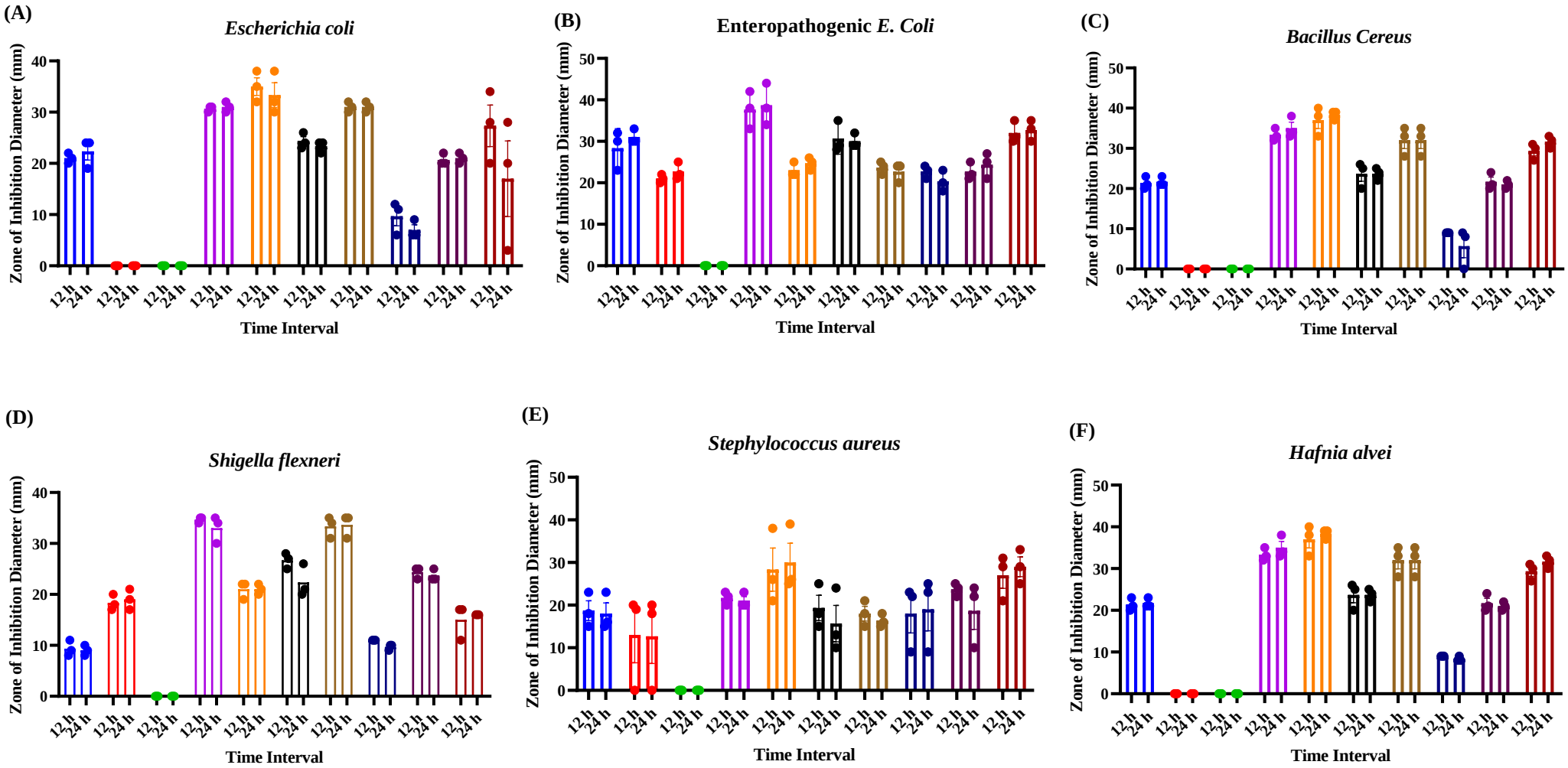
3.1 Antibiotic susceptibility testing of pathogenic species

Antibiotic susceptibility testing was carried out on nine bacteria using ten distinct antibiotic classes. Eight of the ten bacteria tested positive for MDR, while one tested positive for XDR. This implies that these microorganisms were resistant to at least one to three medications. Examples include *Staphylococcus aureus*, *Proteus vulgaris*, *E. coli*, *Enterococcus faecalis*, *Shigella dysentery*, and *Hafnia alvei*. The Nitroimidazole antibiotic group was found to be resistant to all nine microorganisms.

Table 7: Antibiotic susceptibility pattern of the pathogenic species

Bacterial Species	Antibiotic Resistance	Resistance
E.coli ATCC 25922	Vancomycin, Rifampicin, metronidazole	MDR
Enteropathogenic	metronidazole	MDR
Bacillus cereus	Tetracycline, metronidazole	MDR
Shigella flexneri	Kanamycin, metronidazole	MDR
Staphylococcus aureus	Nitrofurantoin, metronidazole	MDR
Hafnia alvei	Vancomycin, Rifampicin, metronidazole	MDR
Enterococcus faecalis	Vancomycin, Rifampicin, metronidazole	MDR
Shigella dysenteriae	Vancomycin, metronidazole, Tetracycline	MDR
Proteus vulgaris	Vancomycin, Tetracycline, Rifampicin, metronidazole	XDR

Note: MDR: Multi-drug Resistance; XDR: Extensive drug Resistance.


(A)
Escherichia coli
(B)
Enteropathogenic E. Coli
(C)
Bacillus Cereus
(D)
Shigella flexneri
(E)
Stephylococcus aureus
(F)
Hafnia alvei
Zone of Inhibition Diameter (mm)
Time Interval
12 h
24 h

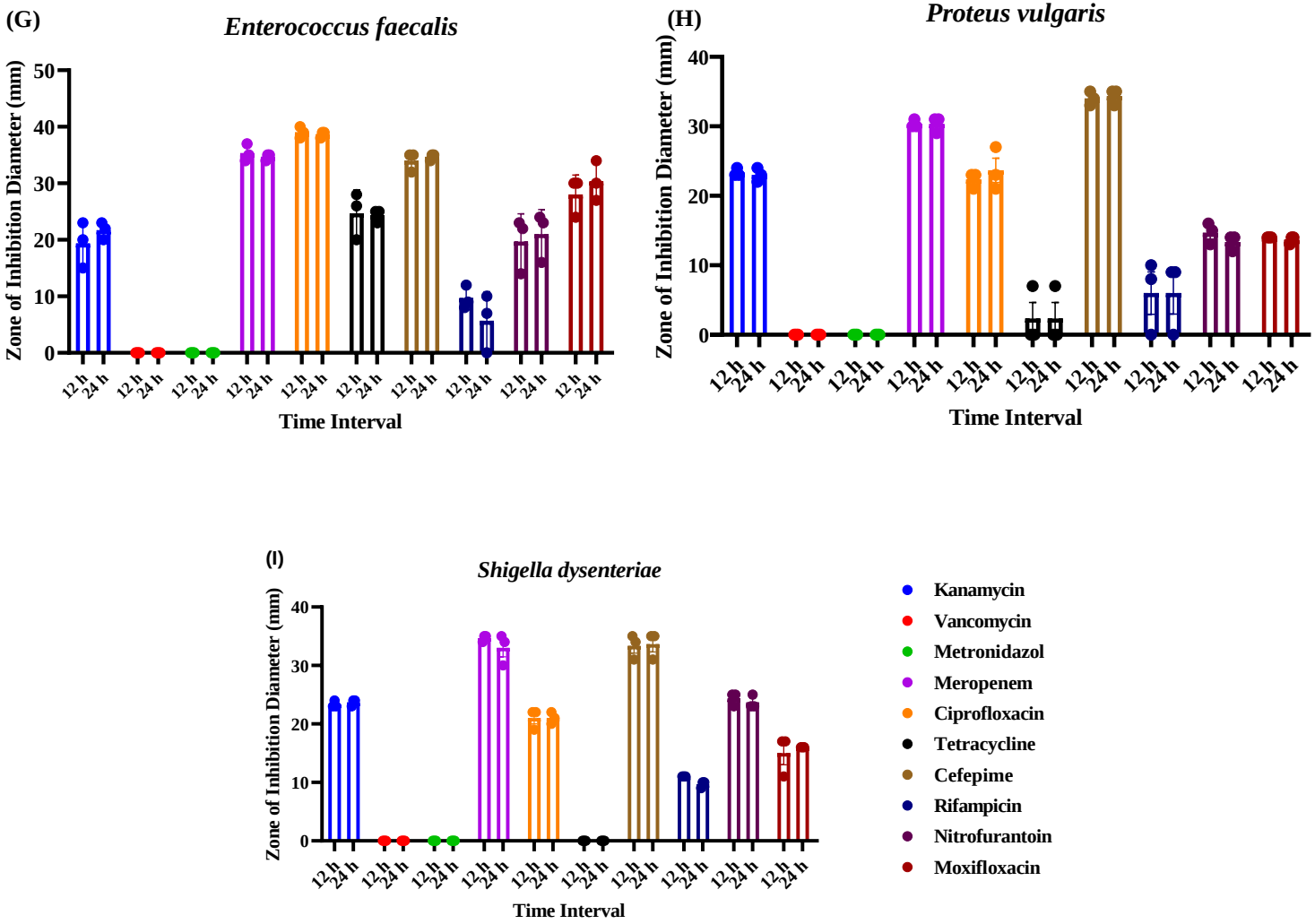


Figure 3: Time-dependent (12-hour and 24-hour) bacterial susceptibility pattern of 10 antibiotic classes. The antibiotic susceptibility pattern of (A*) E.coli* ATCC 25922, (B) Enteropathogenic *E.coli*, (C) *Bacillus cereus*, (D) *Shigella flexneri*, (E) *Staphylococcus aureus*, (F) *Hafnia alvei*, (G) *Enterococcus faecalis*, (H) *Proteus vulgaris*, and (I) *Shigella dysenteriae* in 12 hours and 24 hour time intervals-plotted ZOI (Y-axis) and different antibiotics were indicated by different columns (X-axis)

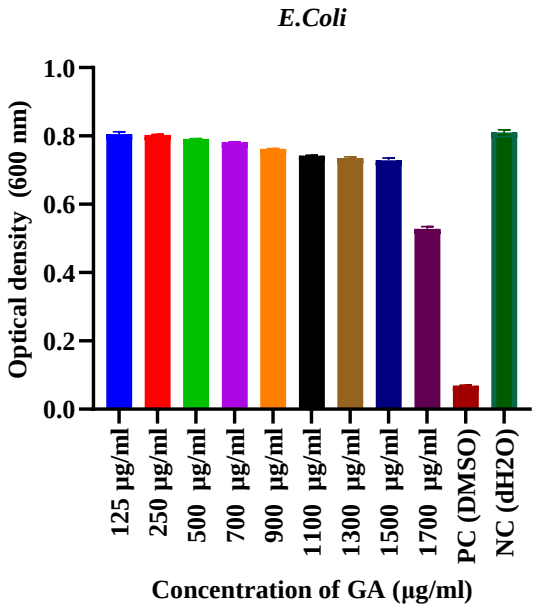
3.2 Determination of Minimum Inhibitory Concentration (MIC) of GA against bacterial strains

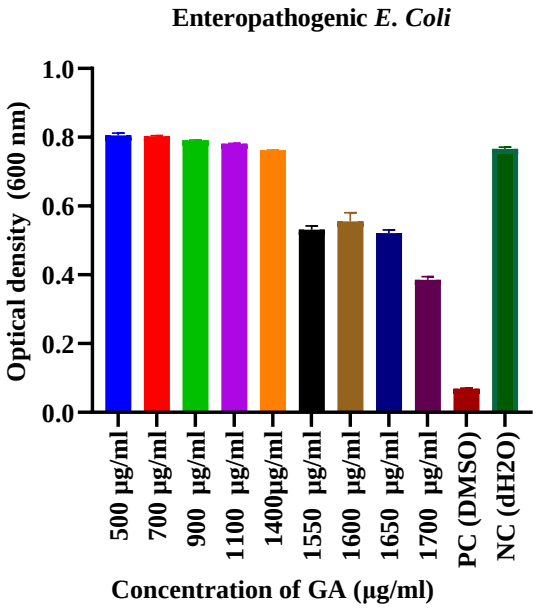
The antibacterial activity of GA consistently increased against all tested bacterial strains as the GA dose progressed. The MIC value of GA against all tested microorganisms has been presented below.

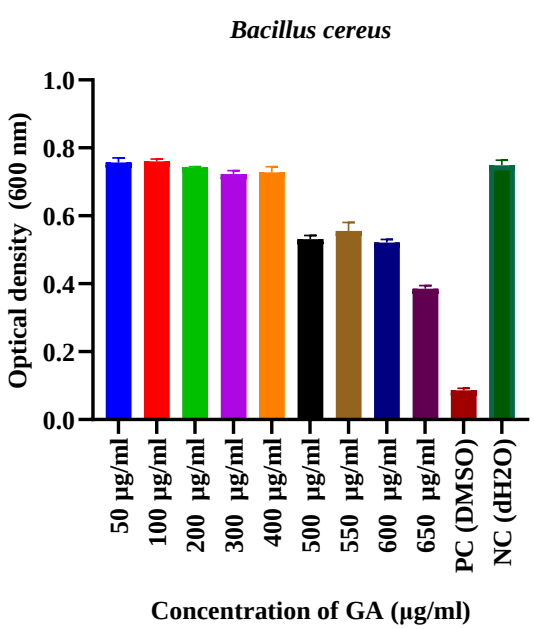
Table 8: Antibacterial activity (Minimum Inhibitory Concentration) of GA

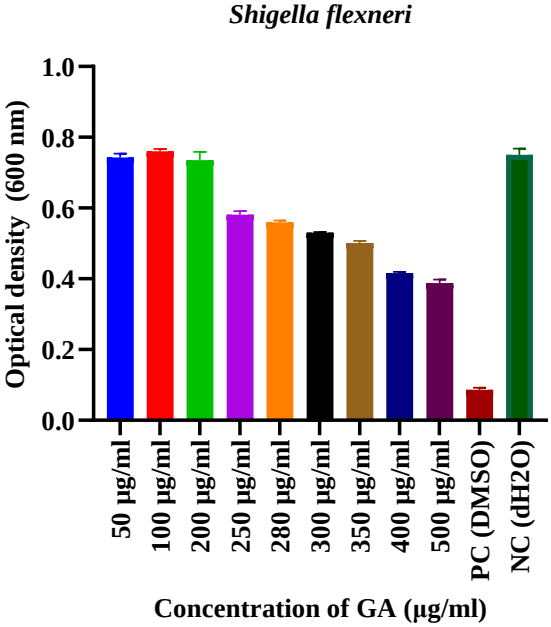
Microorganisms	**MIC (GA)/ µg/mL**
E. Coli ATCC 25922	1600±28.87
Staphylococcus aureus	650±28.87
Enteropathogenic E.coli	1500±28.87
Shigella dysenteriae	737.5±20.21
Shigella flexneri	295±24.27
Proteus Vulgaris	187.5±17.24
Bacillus cereus	550±14.529
Enterococcus faecalis	675±14.529

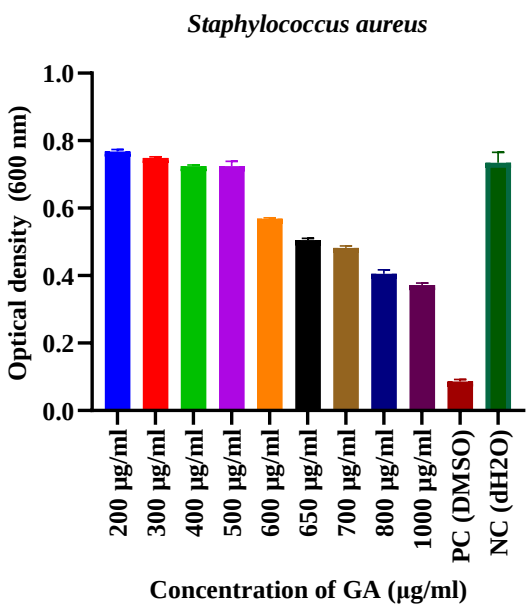
Note: Minimum Inhibitory Concentration (MIC) was calculated as (Mean ± Standard Error of the Mean). We performed the experiment using GA as test sample. Variation between each concentration was 30 µg/mL or 50 µg/mL.

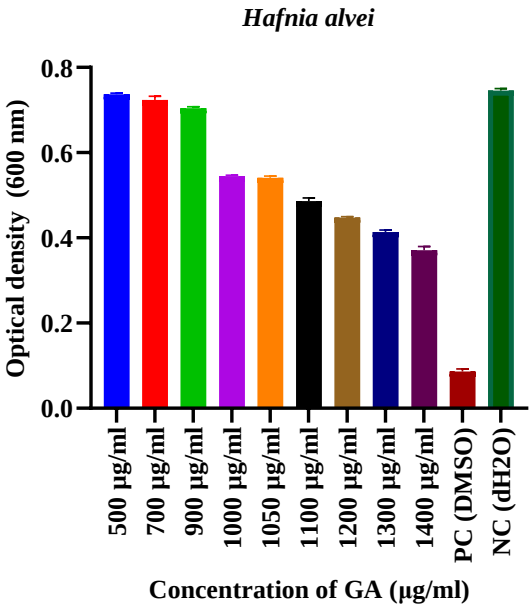

E.Coli
Optical density (600 nm)
1.0
0.8
0.6
0.4
0.2
0.0
125 µg/ml
250 µg/ml
500 µg/ml
700 µg/ml
900 µg/ml
1100 µg/ml
1300 µg/ml
1500 µg/ml
1700 µg/ml
PC (DMSO)
NC (dH2O)
Concentration of GA (µg/ml)

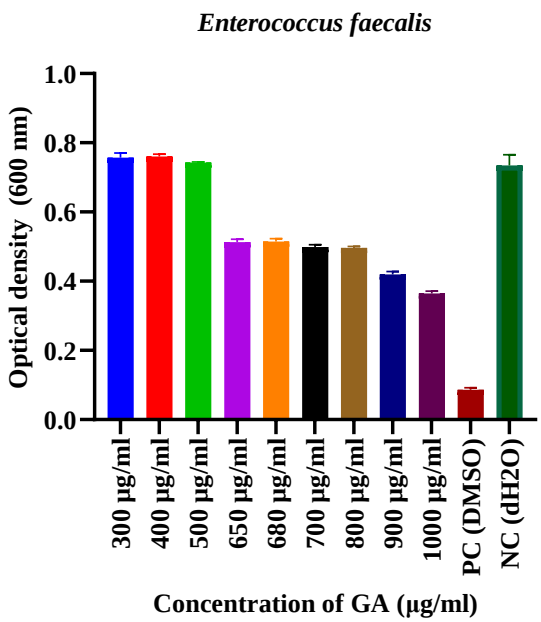

Enteropathogenic E. Coli
Optical density (600 nm)
1.0
0.8
0.6
0.4
0.2
0.0
500 µg/ml
700 µg/ml
900 µg/ml
1100 µg/ml
1400µg/ml
1550 µg/ml
1600 µg/ml
1650 µg/ml
1700 µg/ml
PC (DMSO)
NC (dH2O)
Concentration of GA (µg/ml)

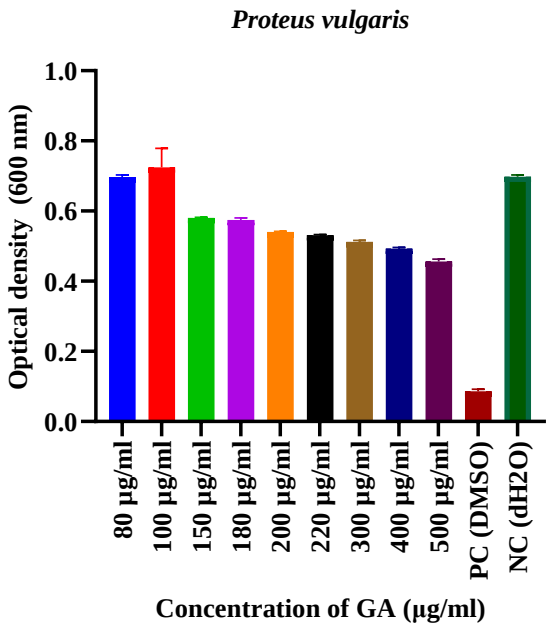

Bacillus cereus
Optical density (600 nm)
1.0
0.8
0.6
0.4
0.2
0.0
50 µg/ml
100 µg/ml
200 µg/ml
300 µg/ml
400 µg/ml
500 µg/ml
550 µg/ml
600 µg/ml
650 µg/ml
PC (DMSO)
NC (dH2O)
Concentration of GA (µg/ml)


Shigella flexneri
Optical density (600 nm)
1.0
0.8
0.6
0.4
0.2
0.0
50 µg/ml
100 µg/ml
200 µg/ml
250 µg/ml
280 µg/ml
300 µg/ml
350 µg/ml
400 µg/ml
500 µg/ml
PC (DMSO)
NC (dH2O)
Concentration of GA (µg/ml)


Staphylococcus aureus
Optical density (600 nm)
1.0
0.8
0.6
0.4
0.2
0.0
200 µg/ml
300 µg/ml
400 µg/ml
500 µg/ml
600 µg/ml
650 µg/ml
700 µg/ml
800 µg/ml
1000 µg/ml
PC (DMSO)
NC (dH2O)
Concentration of GA (µg/ml)


Hafnia alvei
Optical density (600 nm)
0.8
0.6
0.4
0.2
0.0
500 µg/ml
700 µg/ml
900 µg/ml
1000 µg/ml
1050 µg/ml
1100 µg/ml
1200 µg/ml
1300 µg/ml
1400 µg/ml
PC (DMSO)
NC (dH2O)
Concentration of GA (µg/ml)


Enterococcus faecalis
Optical density (600 nm)
1.0
0.8
0.6
0.4
0.2
0.0
300 µg/ml
400 µg/ml
500 µg/ml
650 µg/ml
680 µg/ml
700 µg/ml
800 µg/ml
900 µg/ml
1000 µg/ml
PC (DMSO)
NC (dH2O)
Concentration of GA (µg/ml)


Proteus vulgaris
Optical density (600 nm)
1.0
0.8
0.6
0.4
0.2
0.0
80 µg/ml
100 µg/ml
150 µg/ml
180 µg/ml
200 µg/ml
220 µg/ml
300 µg/ml
400 µg/ml
500 µg/ml
PC (DMSO)
NC (dH2O)
Concentration of GA (µg/ml)

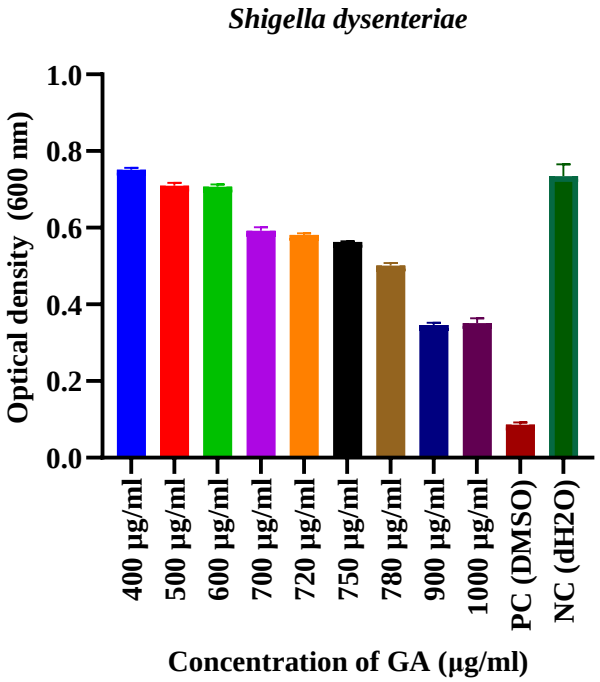


Figure 4: Illustration of MIC determination for GA against the bacterial strains- The MIC is defined as the lowest antimicrobial concentration resulting in no obvious growth compared to the background. Error bars represent the standard error of 3 replicate measurements. PC stands for positive control; NC stands for negative control, DMSO-Dimethyl sulfoxide, and dH_2O - deionized distilled water.

3.3 Fractional Inhibitory Concentration of GA in combination with metronidazole

The Fractional Inhibitory Concentration (FIC) index assay was conducted to assess whether the co-administration of metronidazole and GA could have a synergistic or additive impact.

According to equation 1, the fractional inhibitory concentration (FIC) index of the combination of tulathromycin and GA was computed. In this equation, FICA and FICB represent the FIC indices of A and B in combination, A and B represent the MICs of A and B in combination, and MICA and MICB represent the individual MICs of A and B, respectively. The interpretation of the FIC index calculated by the checkerboard approach is as follows: FIC 0.5 indicates synergy; 0.5 FIC 4.0 indicates additivity, and FIC > 4.0 indicates antagonism. The FICs were validated by three independent test replications.

FIC index = FICA+FICB = A/MICA+B/MICB (1)

The results of the FIC index analysis for the two self-drug combinations utilized in this investigation are summarized in the following table-

Table 8: Combination of GA and metronidazole and their antimicrobial interactions against pathogenic strains

Bacterial Species	MIC GA		Mic metronidazole		FIC Index	Outcome
	Alone (µg/mL)	With metronidazole (µg/mL)	Alone (µg/mL)	With GA(µg/mL)		
E.coli	1600	350	400	250	0.84	Additive
Enteropathogenic *E.coli*	1500	250	313.5	150	0.65	Additive
Bacillus cereus	550	200	500	200	0.76	Additive
Shigella flexneri	295	200	530	150	0.96	Additive
Shigella dysenteriae	737.5	200	350	250	0.98	Additive
Staphylococcus aureus	650	350	700	250	0.89	Additive
Hafnia alvei	1000	400	829.5	300	0.76	Additive
Enterococcus faecalis	587.5	350	675	300	1.04	Indifference
Proteus vulgaris	187.5	200	262	150	1.64	Indifference

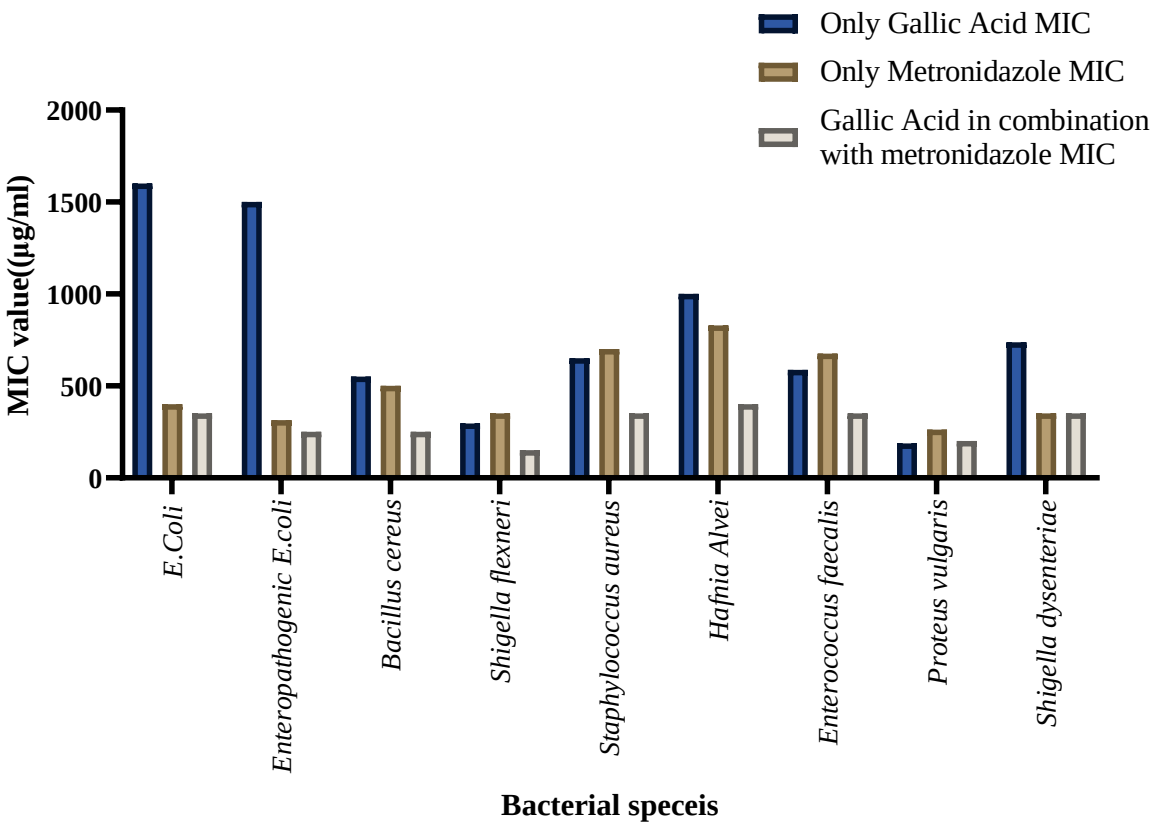
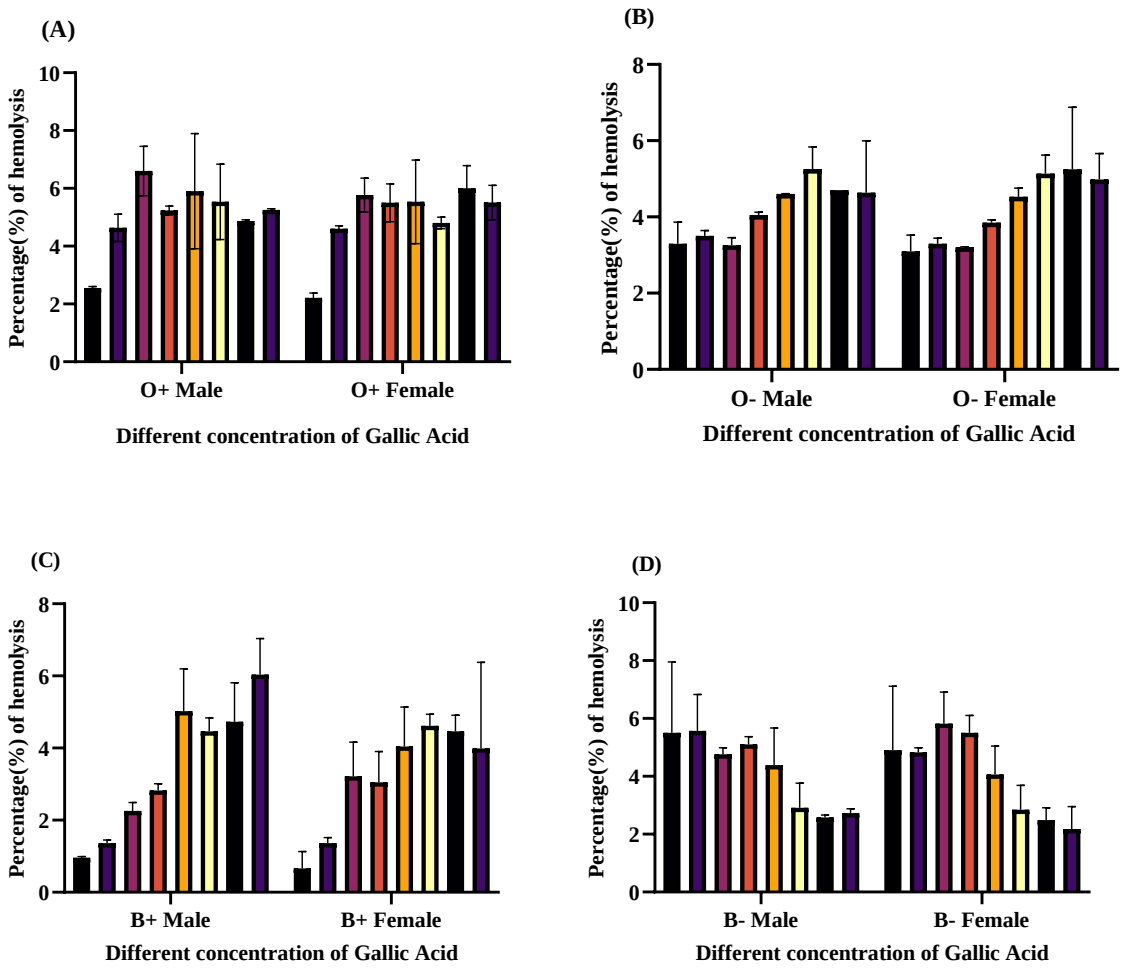


Figure 5: Mean MIC value of GA and metronidazole combination with individual MIC of the drug alone

When assessing the capacity to adjust metronidazole's MIC value in conjunction with GA, a potentiating effect of antibacterial activity was discovered, as indicated by a decrease in MIC when GA and metronidazole were employed together.

3.4 Hemocompatibility of different concentrations of GA at different

Biocompatibility has a significant impact on the biomedical applications of GA. Therefore, we examined it's *in vitro* blood group-dependent hemocompatibility using human RBC. Hemolysis is the most frequently used initial toxicity assessment, and human erythrocytes are the most commonly used *in vitro* testing material for the hemolytic interaction of antimicrobial drugs. Human RBCs were used to test the in vitro hemocompatibility of GA solutions prepared at various concentrations. The highest concentration of GA was 1,700 µg/mL, while the lowest concentration was 100 µg/mL. In addition to that different other concentrations of 300 µg/mL, 500 µg/mL, 700 µg/mL, 1000 µg/mL, 1200 µg/mL, and 1500 µg/mL were utilized to determine the hemolytic potential. It has been found that all the blood group both male and female has shown around 6% hemolysis at the highest concentration of GA (1,700 µg/mL).



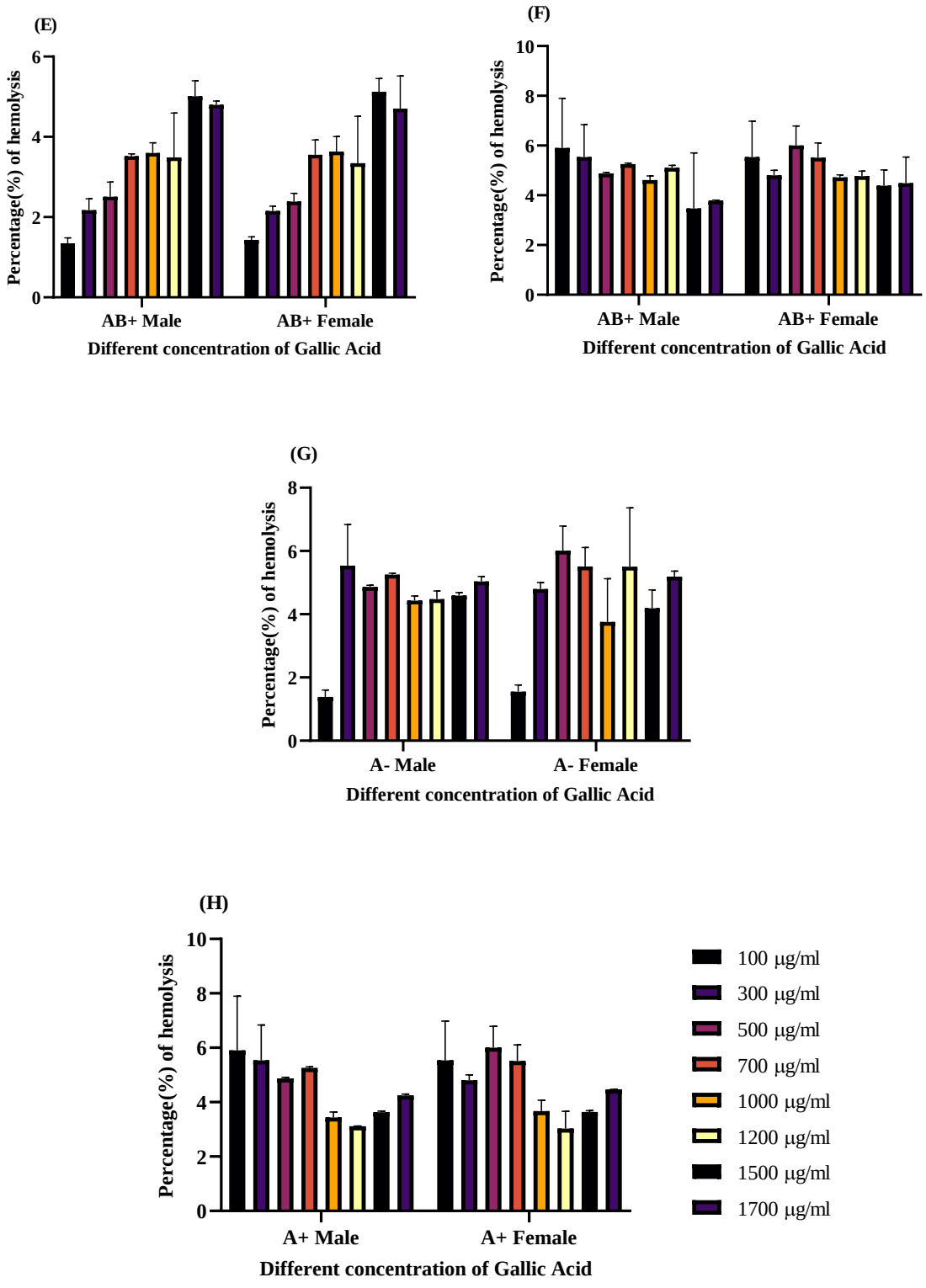


Figure 6: Hemocompatibility of GA at different concentrations against ABO blood groups (male and female) The percentage of hemolysis presented on the Y-axis and X-axis represents different concentrations of GA that have been checked on the same blood group (male and female) human RBC. (A) A+ male and female, (B) A- male and female, (C) B+ male and female, (D) B- male and female, (E) AB+ male and female, (F) AB- male and female, (G) A- male and female, and (H) A+ male and female. For the control group, 3% hydrogen peroxide is considered the positive control (100% hemolysis), and dH^2O was considered a negative control.

Chapter 4

Discussion

Antimicrobial resistance in bacterial pathogens is a huge challenge with substantial morbidity and death. Multidrug resistance patterns in Gram-positive and Gram-negative bacteria are difficult to cure and may be challenging to treat with conventional antibiotic drugs. There is now a scarcity of effective medicines. Ineffective prevention strategies, and only a few new antibiotics, necessitate the development of novel treatment alternatives.

Antibiotic-resistant microorganisms are on the rise in underdeveloped countries such as Bangladesh (Ahmed, Rabbi, & Sultana, 2019). Unfortunately, the development of effective antibacterial agents has been accompanied by the emergence of drug-resistant organisms due to irrational and misuse of antibiotics, inability to follow a course of therapy, genetic adaptability of microbial species, and horizontal transfer of resistant genes within and between species of bacteria which later on, impedes the effectiveness of antibiotics (Hasan et al., 2011).

This study discovered the *in vitro* antimicrobial activities of GA, combination therapy of GA with metronidazole, and GA's hemolytic activity against extremely drug-resistant *E. coli* (ATCC-25922), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC-29212), *Proteus vulgaris*, *Shigella flexneri*, *Shigella dysenteriae*, Enterococcus pathogenic *E. Coli* (EPEC), *Hafnia alvei*, and *Bacillus cereus*.

In this study, the initial susceptibility screening was done by measuring the zone of inhibition via antibiotic susceptibility assay. Through this experiment, it was determined that among the ten classes of antibiotics, Nitromidazole (metronidazole) was the least effective antibiotic among all the bacterial strains.

To increase the efficacy of metronidazole, it was combined with GA. By scavenging, GA demonstrates its antibacterial mechanism of action. GA has the potential to neutralize reactive oxygen species (ROS) (K Asokkumar et al., 2014). GA also causes bacterial cell membrane destruction. By destroying the cell membrane, they reduce bacterial cell permeability, inhibiting bacterial growth (Kim et al., 2014). Before seeing the combined effect of GA and metronidazole, their MIC value was measured against all nine bacterial species. And their combined effect was then measured by the FIC index. It was found that for different bacterial species, the MIC values for both GA and metronidazole were different.

For *E. coli* (ATCC-25922) the MIC value of GA is 1600 μg/mL, which was decreased to 350μg/mL while combined with metronidazole. Metronidazole's MIC decreased from 400μg/mL to 100μg/mL in the combination therapy. FIC index scored 0.47 which is less than 0.5 demonstrating a synergistic effect between GA and metronidazole against *E. coli*.

In the case of Enteropathogenic *E. coli* (EPEC), the MIC value of GA reduced from 1500 μg/mL to 350μg/mL in the combination with metronidazole. The MIC of metronidazole was reduced from 313.5 μg/mL to 100 μg/mL in the combined effect. The FIC index for Enteropathogenic *E. coli* is 0.48, which is also a synergistic combined effect.

On the Contrary, for *Bacillus cereus,* the MIC value for both GA and metronidazole lessened from 500μg/mL to 250μg/mL in the combination. This combination showed an additive FIC index with a result of 0.95. On the other hand, GA and metronidazole also showed an additive result on the FIC index against *Shigella flexneri*. The MIC value lowered from 295 μg/mL (alone) to 150 μg/mL in the combination which is not a significant change compared to *E. coli* and EPEC. For metronidazole, MIC decreased from 350 μg/mL (alone) to 150 μg/mL in combination.

For *Staphylococcus aureus* (ATCC-25923) the MIC value of GA decreased from 650 μg/mL to 350μg/mL while combined with metronidazole. For metronidazole, the MIC decreased from 700 μg/mL to 350 μg/mL in the combination with GA, which is an additive result with 0.96 in the FIC index.

For *Hafnia alvei*, the FIC index shows an additive result with 0.76 in the combination. Here GA showed a significant change in the MIC value. The MIC value of GA in combination lowered to 400μg/mL from 1000 μg/mL alone. The MIC of metronidazole also decreased from 829.5 μg/mL alone to 300 μg/mL in combination.

In favor of *Enterococcus faecalis* (ATCC-29212), *Proteus vulgaris*, and *Shigella dysenteriae* the FIC index showed an indifferent result and their FIC results are accordingly 1.04, 1.64, 1.33. For these pathogens, the MIC value for both GA and MT decreased in the combination but did not show a remarkable FIC result compared to the other six pathogens.

This research has found a notable finding in the combination effect of GA and metronidazole for MDR *E. coli* (ATCC-25922) and Enteropathogenic *E. coli*, compared to another combination study of GA with other antimicrobials or antibiotics. In a research GA interacted with Ampicillin, Amoxicillin, Ceftiofur, Penicillin G, Cefotaxime, Thiamphenicol, and Marbofloxacin to treat *E. coli* (ATCC-25922) (Eran Pichersky & Robert A. Raguso, 2018). Among these seven interactions of GA, only one combination effect showed synergism, which is GA with Thiamphenicol) (Eran Pichersky & Robert A. Raguso, 2018). On the contrary, another study showed the interaction of GA with Gentamycin and Norfloxacin and both of the combined effects were antagonists for *E. coli* (K. Asokkumar, S. Sen, M. Umamaheswari, A. T. Sivashanmugam, & V. Subhadradevi, 2014). This means after the combination the MIC value for both GA and antibiotics was increased.

A combination of GA and metronidazole showed an additive interaction for *Staphylococcus aureus* in our study and another study showed two significant combinations of GA with Norfloxacin and Gentamycin. Both combinations were synergistic (K. Asokkumar, S. Sen, M. Umamaheswari, A. T. Sivashanmugam, & V. Subhadradevi, 2014). Compared to this study the amount of GA was higher (1024μg/mL). Here it can be demonstrated that if the amount of GA is increased for the interaction with metronidazole, the combined effect can change into synergism from the additive.

It shows that the combination of GA with metronidazole is consistent against MDR pathogens. Since this combination did not show any antagonistic result against any pathogenic bacteria. Moreover, it showed either synergism, additive, or indifference compared to other analyses.

To provide GA as a compatible and safe medication with antibiotics, any other antibacterial, or alone, it is necessary to see how GA interacts with human RBC. One study discovered that different doses of GA in the 25 μM-1 mM range caused approximately 8% hemolysis (Suwalsky et al., 2016).

Therefore, an in vitro blood group-dependent hemocompatibility or hemolysis effect was done using different concentrations of GA (i.e.- 100 μg/mL, 300 μg/mL, 500 μg/mL 700 μg/mL, 1000 μg/mL, 1200 μg/mL, 1500 μg/mL, 1700 μg/mL). Among all the blood groups none of them showed greater than 6% hemolysis in the highest concentration, which displayed remarkable in vitro hemocompatibility in human RBC.

Conclusions

The findings in this study demonstrate the possibility of combining an antibiotic with a phytochemical to alleviate infections caused by multiple drug-resistant bacteria. Nowadays conventional antibiotics are not always the best and most effective options to treat bacterial infection. To prevail over these MDR and XDR pathogens, a combined interaction between a plant phytochemical (GA) and an antibiotic (metronidazole) was observed. Our study found GA to be an effective antibacterial agent. To increase metronidazole's antibacterial activity, it was combined with GA and their incorporation was measured against nine bacterial species. It was seen that after combining with GA, the antibacterial activity of metronidazole increased. FIC index assay showed combinations of GA and metronidazole were additive against seven bacterial species and against two bacteria the combinations were indifferent. Finally, GA's RBC hemolysis activity was observed to determine if GA can be used for further biomedical applications or human trials in the future. It was seen that GA has the most negligible hemolytic activity over any type of blood group and it has remarkable RBC hemocompatibility. Further study is recommended to assure the findings of the current research.

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