EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 8
Issue: 6
573-578
Year: 2021

ANTIBACTERIAL ACTIVITY AND MODE OF ACTION OF SELECTED PHENOLIC ACIDS AGAINST SOME PATHOGENIC BACTERIA

***Yasser M. Kamel**

Department of Microbiology, Desert Research Center, Cairo 11753, Egypt.

***Corresponding Author: Yasser M. Kamel**
Department of Microbiology, Desert Research Center, Cairo 11753, Egypt.

Article Received on 21/04/2021 **Article Revised on 11/05/2021** **Article Accepted on 01/06/2021**

ABSTRACT
There has been growing concern about the emergence and spread of antibiotic-resistant bacteria worldwide, so it is important to explore alternative effective antimicrobial compounds. This work was designed to evaluate the antibacterial activities of gallic and caffeic acids against gram-positive and gram-negative bacteria with the attempt to elucidate their mode of action through estimation of various bacterial physiological indices as minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), releasing of intracellular potassium ions (K+) and membrane permeabilization. The results show that both acids inhibited the growth of both types of bacteria with various degrees and their effect on gram-negative bacteria was greater than that on gram positive. Moreover, caffeic acid had an antibacterial effect more potent than Gallic acid. The current study emphasizes the potential use of caffeic and gallic acids as a good source of new antibacterial products, particularly against gram-negative strains.

KEYWORDS: MIC, MBC.

INTRODUCTION
In recent years, there is a big concern about the emerge of antibiotic resistance among bacteria.[1,3] Resistance to antibiotics used over decades for the treatment of bacterial infections resulted from different ways including adaptation of the pathogen to extensively used agents.[4,6] The antibiotic-resistant bacteria can colonize various sites in the animal or human body causing many types of infections for which non-therapeutic agents are available, and therefore, there is greater potential for prevention and/or treatment failure. In this context, there is growing interest in the discovery of novel antibiotics with new mechanisms of action.

Plants produce amazing diversity of low molecular weight compounds known as secondary metabolites which considered the first line of defense where they have antimicrobial actions against viruses, bacteria, and fungi,[7,9]

especially bioactive phytochemicals compounds containing phenolic ring as caffeic acid (CA) and gallic acid (GA), their chemical structure is shown in figure (1).

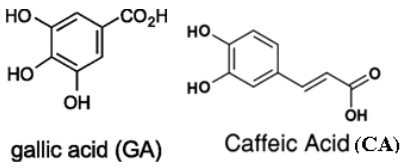

Fig. 1: Chemical structure of GA.

The current study is targeted to assess the antimicrobial efficacy and antibacterial mechanism of action of caffeic and gallic acids against the common pathogenic gram-negative strains, *Escherichia (E.) coli*, *Klebsiella (K.) pneumoniae* and *Pseudomonas (P.) aeruginosa* as well as *Staphylococcus (S.) aureus* as instance of gram-positive bacteria aiming to discover a new generation of antibiotic agents against the clinical drug resistant pathogens.

1. MATERIAL AND METHODS
1.1 Bacterial cultures and chemical reagents
In the current study, four strains were tested: *E. coli* (ATCC, 25922), *K. pneumoniae* (ATCC, 4352), *P. aeruginosa* (ATCC, 7221) and *S. aureus* (ATCC, 25923). Before use, all strains were streaked onto Muller Hinton (MH, Himedia, Mumbai) agar plates, incubated overnight, and inoculated into MH broth with agitation (150 rpm) at 37^{o}C for 14 hrs. The phenolic acids: caffeic acid and gallic acid were purchased from Sigma-Aldrich, USA.

1.2 Determination of Minimum inhibitory concentration (MIC)

The antibacterial activity of polyphenols was determined using MIC assay by applying microdilution method according to the instructions of CLSI.[10] Briefly, the bacterial cells were picked in the exponential phase of growth, and the bacterial densities were adjusted to be $1x10^6$ cells/mL. Phenolic acids (CA and GA) were dissolved in 5% DMSO (dimethyl sulfoxide, Merck, Darmstadt, Germany) and transferred to sterile 96-well microplate wells (Orange Scientific, Belgium) to get a twofold serial dilution ranging from 0.156 to 10 mg/mL. The plates were incubated at 37°C for 24 hrs. Bacterial growth was evaluated by adding 10 µL of 0.01% resazurin sodium salt (Sigma-Aldrich, USA) to each well. The lowest concentration of phenolic acids that had no visible growth was reported as MIC. The assay was conducted in triplicate.

1.3 Determination of Minimum Bactericidal concentration (MBC)

The estimation of MBC was performed according to the method designated by Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[11] Accordingly, 10 µL of each well without visible growth was picked and inoculated onto MH agar plates. MBC values were defined as the lowest concentration of phenolic acids at which 99% of tested bacteria were killed.

1.4 Crystal Violet Uptake Assay

Alteration of membrane permeability was determined by crystal violet assay according to Devi et al.[12] with minor modification. Tested bacteria were adjusted to the concentration of 5×10^5 CFU/mL, then centrifuged using 4500xg for 5 min at 4°C. Bacterial pellets were washed twice and resuspended in phosphate buffer saline (PBS) solutions with pH 7.4. For treatment reaction, 1xMIC of tested phenolic acid was added to each bacterial suspension and incubated at 37°C for 2 hrs. Bacterial suspensions without phenolic acids were considered as negative control. After incubation, the samples were centrifuged at 9300xg for 5 min at 4°C and the pellet was resuspended again in the PBS solution with 5 µg/mL of Crystal Violet (Merck, USA). Subsequently, the samples were incubated for 10 min at 37°C and centrifuged at 13,400xg for 15 min. Spectrophotometric measurements of all samples using optical density (OD) 590 nm at 0, 4 and 8 h intervals were applied. The OD reading of Crystal violet (CV) stock solution was assumed as 100% excluded, Crystal violet uptake by the sample cells. The percentage of Crystal violate uptake was calculated according to the following formula:

% CV uptake =100 - [(OD590 of the sample/OD590 value of CV solution) x 100].

1.5 Determination of intracellular K+ efflux

The intracellular K^+ efflux was evaluated from the tested microorganisms using atomic absorption spectrophotometry according to the method conducted previously.[13] In brief, the tested bacterial cells were subjected to culture and incubation at 37°C for 14 hrs. The obtained cells were washed three times and resuspended in PBS with pH 7.2 at a concentration $1x10^7$ CFU/mL. One milliliter of the previous suspensions was pipetted and incubated with MIC of each tested phenolic acid at 37°C for 30 minutes. Then, the suspensions were centrifuged, and the amount of released K^+ was determined in the supernatants. Bacterial strains were suspended in PBS and used as control.

1.6 Nucleotide leakage

The potential effect of phenolic acids causing nucleotide leakage was evaluated according to the previously mentioned method.[14] with some modifications. The overnight cultures of the bacterial strains at 37°C were washed and resuspended in 10 mM phosphate buffer saline (PBS) at pH 7.4, reaching the final concentration of 1×10^7 CFU/mL. Strains were incubated with the tested phenolic acids at the density of MIC for different time periods: 4, 8, 12, 16, 20 and 24 hrs; the strains were incubated with 10 mM PBS (pH 7.4) and used as control. The bacterial cells were removed from the mixtures via filtration through 0.22 µm pore size filter. The absorbance of the filtrates was detected at OD 260 nm using spectrophotometry at room temperature (25°C).

1.7 Detection of DNA fragmentation

Bacterial DNAs were isolated following the protocol of DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany, 69504). The bacterial cells were harvested, and the pellets placed in 1.5 mL tube, suspended, and vortexed in 180 µL of buffer ALT then 20 µL proteinase K was added. Overnight incubation was done in a hot block at 55 °C. Next, the mixture was vortexed and 200 µL buffer AL was added and mixed by vortex. The tube was incubated in heat block at 70 °C for 10 minutes and 200 µL absolute ethanol was added and the entire volume was transferred onto spin column with successive addition of 500 µL of buffer AW1, 500ul of buffer AW2 and 200 µL buffer AE, in each step the volume was centrifuged at 8000 rpm for 1 minute and flow-through was discarded. Ten micrograms of isolated DNA per lane were mixed after heating to 68 °C with the same volume of a worm 1% low melting-point agarose solution. Samples were loaded in the agarose slots and allowed to harden. Next, running buffer was overlaid. Electrophoresis was carried out in a 1% agarose gradient gel at 100 V for 3 hrs. DNA was visualized by ethidium bromide staining under ultra-violet lamp.[15]

1.8 Detection of morphological alteration in the cell membrane

The pathological changes and damage that occurred in the bacterial cells were investigated by using TEM following the method established previously.[16] Bacterial strains were cultured in MHB up to the exponential phase and then treated with tested phenolic acids at 1xMIC and incubated for 90 minutes at 37°C. The

bacterial broth was centrifuged, and the bacterial pellets were treated according the previous work.[17] Succinctly, the growing bacterial cells were harvested and fixed in glutaraldehyde (2.5% in 0.1 M PBS) at 4°C for two hrs. Subsequent, the bacterial cells were washed and fixed in osmium tetroxide (1% M PBS at pH 7.2). The bacterial cells were dehydrated after washing twice, infiltered and finally, embedded in Spur`s stain. The prepared bacterial cells were cut to ultrathin sections and mounted on copper grids and examined with the electron microscope under standard operating conditions.

2. RESULTS

2.1 Inhibitory and bactericidal concentration of phenolic acids

The MIC is the lowest concentration (mg/mL) that inhibits visible bacterial growth while the MBC is the lowest concentration that kill the bacteria. The MIC, MBC and MBC/MIC ratios for GA and CA used against the four bacterial strains are illustrated in **table (1).** The ratio had narrow range between 1 and 1.5 for all the tested bacteria.

Table 1: The minimum inhibitory and bactericidal concentrations of phenolic acids on different bacterial strains.

Tested bacteria	Gram +/-	Phenolic acids					
		(GA)			(CA)		
		MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
S. aureus	+	1.925	2.250	1.29	1.850	2.0	1
E. coli	-	1.500	1.500	1	1.0	1.500	1.5
K. pneumoniae	-	1.650	1.850	1.14	1.5	1.50	1.33
P. aeruginosa	-	0.500	0.500	1	0.250	0.250	1

2.2 The effect of phenolic acids on K+ ion leakage from tested bacterial cells

As shown in **table 2**, the concentration of the K^+ ions that had released from the tested bacteria, *S. aureus, E. coli, K. pneumoniae and P. aeruginosa* treated with 1xMIC by GA after 30 minutes incubation was 0.35, 0.40, 0.65 and 0.82 µg/ml, respectively, while the amount of K^+ ions leakage concentration when treated with CA at the same incubation time were 0.38, 0.51, 0.77 and 0.91 µg/ml, respectively.

Table 2: The concentration of K^+ ions after 30 minutes incubation of tested bacteria with MIC concentration of GA and CA.

	K^+ ions concentration (µg/ml)			
	S. aureus	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
Control	0.33±0.01	0.24±0.00	0.39±0.00	0.41±0.01
(GA)	0.35±0.01	0.40±0.00	0.65±0.00	0.82±0.01
(CA)	0.38±0.00	0.51±0.02	0.77±0.00	0.91±0.00

The mean ±SD for 5 replicates is shown.

2.3 Effect of isolated antibacterial compounds on nucleotide leakage

The extent of nucleic acids leakage from the tested bacterial cells done after exposure to 1xMIC by GA and CA was recorded as absorbance values. According to the results observed in figure (2, A and B), treatment of tested gram-negative bacteria and partially the *S. aureus* with 1xMIC of GA and CA had produced steady leakage of intracellular nucleotide content with time. Both acids showed substantial release of cellular material of all tested bacteria. Treatment with GA yield OD values 0.352, 0.504, 0.548 and 0.604 nm while CA treatment gave OD values 0.500, 0.740, 0.820 and 0.670 nm with *S. aureus, P. aeruginosa, E. coli and K. pneumoniae*, respectively.

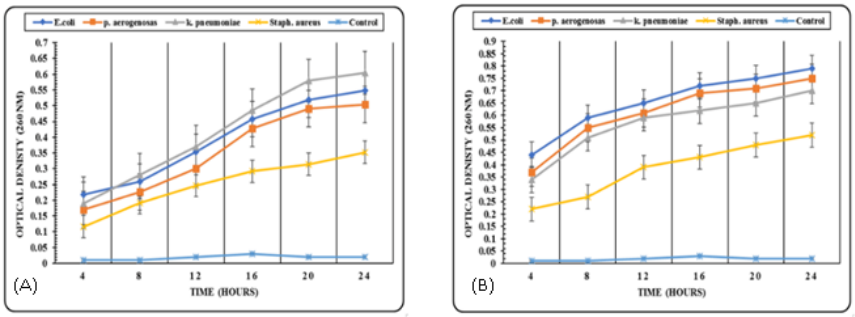

Fig. 2: The effect of GA (A) and CA (B) on 260 nm absorbing materials leakage from tested bacteria (♦) *E. coli,* (■) *P. aeruginosa*, (▲) *K. pneumoniae,* (×) *S. aureus* and (x) control.

2.4 Effect of phenolic acids treatment on bacterial membrane permeability

The effect of phenolic acids treatment on the permeability of bacterial cells to crystal violet was investigated. The crystal violet dye uptake was evaluated by quantifying the remaining amount in the supernatant. The crystal violet uptake of the control untreated bacterial cells has not exceeded 5% of the input; but it increased dramatically in case of *S. aureus* in a time reliant matter, reaching 36% and 65% uptake after 4hrs and 8h of GA treatment respectively (figure 3, A). The same trend was observed when CA was used wherein the uptake reached 40% and 73%, after 4h and 8h of CA treatment, respectively. Regarding Gram negative bacteria, *E. coli, K. pneumoniae and P. aeruginosa*, there was a significant increase in the dye uptake with time (figure 3, A), where the percentage was 13, 30 and 8, 18 and 11, 24 after 4h and 8h of treatment with GA, respectively. Concerning CA (figure 3, B) the percentage of the dye uptake by *E. coli, K. pneumoniae and P. aeruginosa* was 14.5, 33 and 10, 21 and 13, 27, respectively. The increase in dye uptake in case of gram-negative bacteria was lower when compared with that of *S. aureus*.

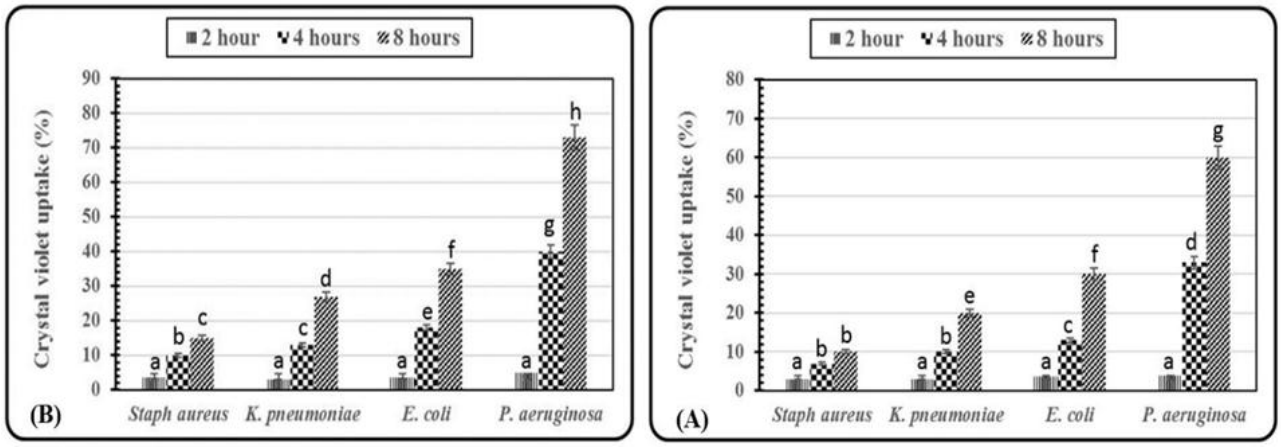

Fig. 3: Effect of GA (A) and CA (B) treatment on DNA fragmentation. A = GA, B = CA, and C = control. 1 = *E. coli*, 2 = *K. pneumoniae*, *P. aeruginosa* and 4 = *S. aureus*.

2.5 Effect of phenolic acids treatment on genomic DNA of the bacteria

The tested bacterial cells were exposed to phenolic acids to verify if GA and CA could induce DNA fragmentation, hence, apoptosis occurred. All treated and untreated bacterial cells were subjected to DNA fragmentation assay by agarose gel electrophoresis (figure 4). The result revealed that all tested bacterial cells showed apoptotic DNA fragmentation profiles when incubated with GA (figure 4, A) and CA (figure 4, B). On the other hand, no fragmentation was detected in negative controls represented by untreated bacterial cells.

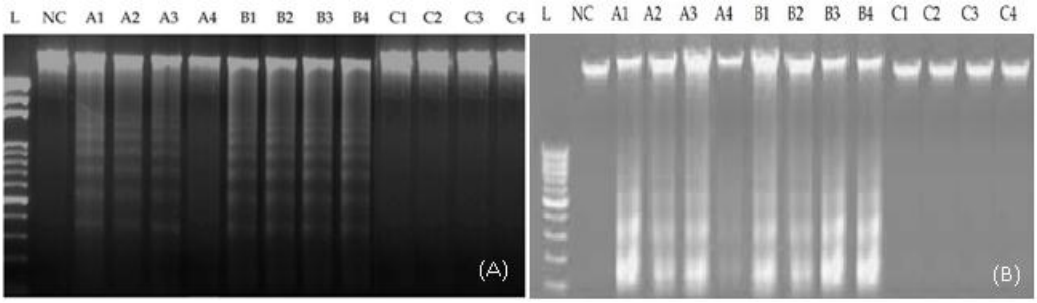

Fig. 4: Effect of GA (A) and CA (B) treatment on crystal violet uptake by tested Gram (+) and Gram (-) bacteria at particular time intervals.

2.6 Phenolic acids causing morphological changes.

The transmission electron micrographs examination of cells from the exponential phase of growth of tested bacteria in the presence of GA or CA are presented in figure (5). The morphology of control cells seemed normal with intact peptidoglycan and cytoplasmic membranes layers. While GA treated bacterial cells displayed only some disruption to both peptidoglycan and cytoplasmic membrane (Fig. 5: B, C), CA treated ones showed clear damage resulting in leakage of intracellular materials and overall morphological changes (Fig. 5: E, F).

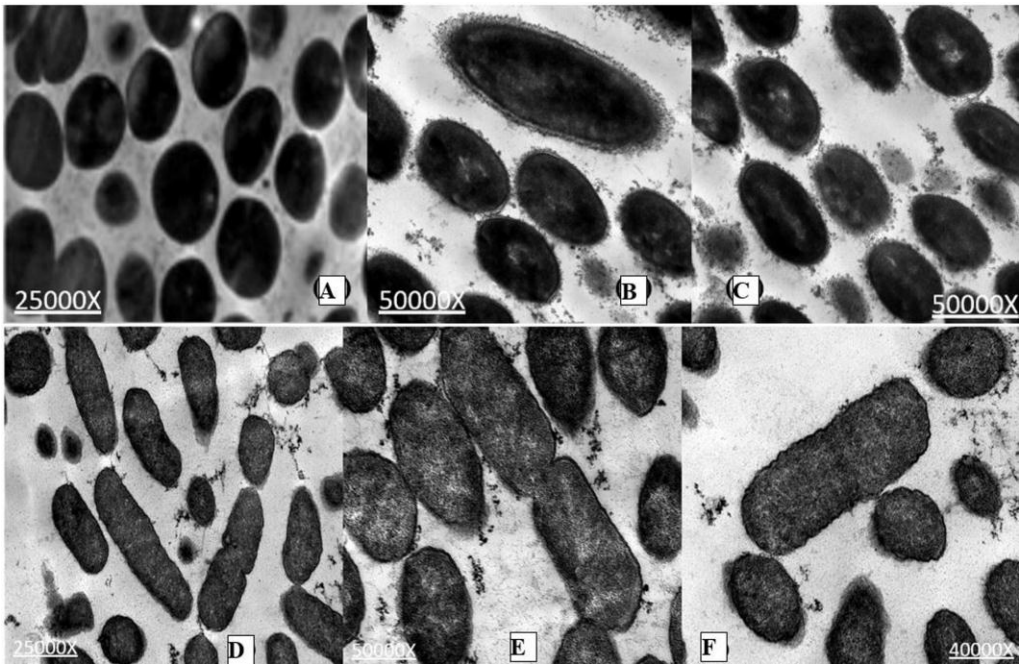

Fig. (5): Effect of phenolic acids on the ultrastructure of *S. aureus* and gram-negative bacteria as demonstrated by TEM. (A) Control *S. aureus* (D) Control gram-negative bacteria from exponential growth phase. (B, C) *S. aureus* treated with GA and CA. (E, F) gram negative bacteria treated with GA and CA, respectively.

DISCUSSION

The emergence and incidence of drug resistance of human pathogenic and opportunistic bacteria extensively documented in the recent years, are causing troubles for treatment and infection control.[18-21] Therefore, serious attempts were made to find new and efficient antimicrobial agents. Bioactive compounds extracted from plants are excellent candidates for antibiotics chemotherapy.[22-25] Antimicrobial activities of GA and CA, (hydroxybenzoic acid and hydroxycinnamic acid) can exhibit various mechanism of action, namely permeabilization of cytoplasmic membrane, enzyme inhibition, nonspecific interactions with the proteins and/or inhibition of nucleic acids synthesis of both gram-negative and gram-positive bacteria.[26]

In the current study, the activity and mechanism of phenolic acids action against four selected bacterial strains, commonly infect human and animals, *E. coli, K. pneumoniae, P. aeruginosa* and *S. aureus* were investigated. In this respect, morphological changes and different bacterial physiological properties were studied as MIC, MBC, crystal violet uptake and releasing of intracellular K^+ ions.

Both phenolic acids (GA and CA) have powerful and effective antibacterial activity against Gram negative strains, *E. coli*, *K. pneumoniae* and *P. aeruginosa* while they have less potent activity against Gram-positive *S. aureus* as shown in table (1). *P. aeruginosa* was more susceptible to both GA and CA with MIC at 0.500 mg/mL and 0.250 mg/mL, respectively. The gram-positive bacterium (*S. aureus*) was less sensitive to both phenolic acids with MIC of 1.925 mg/mL for GA and 1.850 mg/mL for CA. Previous studies showed different results, a study recorded MIC against *E. coli* strains was 0.490 mg/mL for GA and 0.160 mg/mL for CA.[27] In another study, MIC of 0.533 mg/mL for GA against *P. aeruginosa* and *S. aureus* has been reported.[28] In a third study the MIC result was 0.200 mg/mL with GA for five strains of *P. aeruginosa* including clinical isolates. In another study, the MIC of CA obtained for Gram negative and Gram-positive bacteria was 0.8 and 1.0 mg/mL, respectively.[29] The difference in our MIC of GA and CA values with those observed in the previous studies may be contributed to the difference and discrepancy in methods for determination of antibacterial action combined with the use of different bacterial species and strains. Moreover, it is well known that no strain can represent the behavior of a species.[30]

Based on that most reports of research work in the field of natural products and extracts consider MIC values over 1000 µg/mL has little relevance for clinical application,[31] the results obtained with CA against *S. aureus* (1 mg/mL), *E. coli* (1 mg/mL) and *P. aeruginosa* (0.250 mg/mL) are considered relevant while those of GA revealed were only relevant regarding *P. aeruginosa* (0.5 mg/mL). In this context, the active principles derived from phytochemicals are organized as antimicrobials depending on the susceptibility tests that yield inhibitory concentration within the range of 0.1–1 mg/mL and these chemicals could be classified therefore as antimicrobial.[32,33]

Concerning the MBC, CA appears to be more effective than GA particularly against *P. aeruginosa* with 0.25 mg/mL and 0.500 mg/mL, respectively. On the other hand, *S. aureus* with both phenolic acids (GA, 2.25 mg/mL; CA, 2.0 mg/mL) were the most resistant bacteria

while the MBC regarding *E. coli* and *K. pneumoniae* was nearly the same with concentration around 1.5 mg/ml of both phenolic acids. The values of MBC obtained in our study are in accordance with those recorded in the other previous studies. The MBC of GA was more than 3.2 mg/mL when tested against methicillin-resistant *S. aureus* strains.[34,35] In another study, the MBC of phenolic acid fractions against *S. aureus*, *E. coli*, *P. aeruginosa*, and was 5.4 mg/mL, 4.86 mg/mL and 3.78 mg/mL, respectively.[36]

The current study shows that the values of MBC and MIC of CA for the tested bacteria were lower than those of GA. This result is in accordance with those of other authors2.[23,37,39] Regarding the concerned ratio of MBC to MIC, bacteriostatic action was determined as MBC/MIC ratio >4, while bactericidal effect was verified as MBC/MIC ≤ 4[36]. Based on that, both phenolic acids exhibited bactericidal activity against all the four pathogens tested. The MIC/MBC values obtained are in the range of those described in other studies.[23,36,40,44]

The main reactivity difference between the bacteria by tested phenolic acids may be attributed to the type of microorganisms and its cell membrane structure and composition.[33] as increasing the cell wall thickness of the gram-positive bacteria and the presence of the outer cell membrane in the gram-negative species only where the path through it is reliant mainly on the chemical nature of the antimicrobial product and is controlled by the hydrophilic channels, known as porins (pore-forming proteins) which normally prevent the entry of hydrophobic substances. The outer membrane can be permeabilized by phenolic acids that disintegrate the lipopolysaccharides (LPS) layer.[14,43,45] leading to release of LPS and increasing the permeability of cytoplasmic adenosine triphosphate (ATP).[45] On the other hand, peptidoglycan is considered the major component of the cell wall of gram-positive bacteria being responsible for maintaining the integrity of the cell. Hence, any related damage either through mutations, internal or external stresses will lead to cell lysis.[41,46] Depending on the results of the referred studies, we can explain the higher sensitivity of gram-negative bacteria to GA and CA than *S.* aureus.[47]

Efficacy of any antibacterial compound depends on its ability to penetrate or disrupt the bacterial plasma membrane. In our study, phenolic acids disrupted the membrane of all tested bacteria through membrane disintegration and generation of reactive oxygen species (ROS) resulting in release of cellular contents thus showing indices of bacterial cell damage, pore formation and most likely cell lysis.[48] as established basing on the absorbance values measured at 260 nm. As seen in figure (4, B), treatment of *S. aureus* with phenolic acids caused a less leakage of intracellular components including nucleic acids mainly the DNA compared to a high leakage from the treated gram-negative bacteria.

Uptake of the crystal violet (CV) dye had been used to study the effect of tested phenolic acids on the bacterial outer membrane permeability. Permeabilization was detected in uptake of CV, a stain poorly penetrates the intact outer bacterial membrane, therefore, it rapidly accesses and enter the bacteria when its membrane suffering from any defect or damage.[49] The obtained results demonstrate that phenolic acids have a bad effect on the integrity of the bacterial membranes, depending on the time of exposure, represented in stunning uptake of crystal violet in *S. aureus* were reaching over 60% and 70% after 8h when the bacteria incubated with GA and CA, respectively (figure 2, A and B) when compared to the effect at zero time. Concerning gram negative bacteria, they followed the same trend in increasing the crystal violet uptake with increasing the time of exposure. However, this increase was far lower compared to *S. aureus*. This displays that phenolic acids boosted crystal violet uptake which means that there was an increasing level of outer membrane leakage, so altered the membrane permeability in a manner renders the bacterial cells hyperpermeable to solutes and, hence causing the bacterial death. This result may explain the higher bactericidal activity of tested phenolic acids against gram-negative bacteria than those of gram-positive.

It is well known that the cytoplasmic membrane is the barrier between extracellular and intracellular medium. The intracellular cytoplasm is rich in K^+, therefore, its leakage has been used as primary indicator for gross and/or irreversible membranolytic effects in bacteria.[50] Phenolic acids cross the cell membrane by passive diffusion due to their lipophilic property. Dependent on their concentration, they produce structural cell membrane deformities in targeted bacteria (figure 5). TEM examination revealed degeneration of the treated bacterial cell membranes, conceivably causing protein denaturation and K^+ efflux because of cytoplasm acidification leading to irreversible changes leading to bacterial cell death.[51]

Based on all obtained results we can conclude that GA and CA have antimicrobial activities through several mechanisms of action. These phenolic acids led to significant irreversible degeneration in the bacterial cell membranes, cytoplasmic acidification causing protein denaturation and K^+ ions leakage, the species, composition and structure of the bacterial cell membrane play an essential and important role in the antibacterial susceptibility.[49] Finally, phenolic acids can be considered as new active products qualified to combat the bacterial resistance problem and can be represented as members of what is named resistance-modifying agents (RMAs) that act as a gorgeous strategy to diminish the spread of drug resistance bacteria where it could modify the well-established antibiotics and render them more active against resistant bacteria, in addition, they are less in pricey and toxicity than new discovered antibiotic drugs.

3. ACKNOWLEDGMENT

The Deanship of Scientific Research, (DSR), King Abdulaziz University, Jeddah, funded this project under grant number (438/828/224). The authors, therefore, acknowledge with thanks, DSR technical and financial support.

4. CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Beovic B. The issue of antimicrobial resistance in human medicine. *International journal of food microbiology*, 2006; 112: 280-7.
2. Ventola C. L. The antibiotic resistance crisis: part 1: causes and threats. *P & T: a peer-reviewed journal for formulary management*, 2015; *40*(4): 277–283.
3. Aslam B, Wang W, Arshad MI, Khurshid M, Muzammil S, Rasool MH, Nisar MA, Alvi RF, Aslam MA, Qamar MU, Salamat MKF, Baloch Z. Antibiotic resistance: a rundown of a global crisis. *Infect Drug Resist.*, Oct 10 2018; 11: 1645-1658.
4. Nohynek, L.J., H.L. Alakomi, M.P. Ka¨hko¨ nen, M. Heinonen, I.M. Helander, K.M. Oksman-Caldentey, and R.H. Puupponen-Pimia¨. Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr.* Cancer, 2006; 54: 18–32.
5. Friedman ND, Temkin E, Carmeli Y. The negative impact of antibiotic resistance. *Clin Microbiol Infect.*, 2016 May; 22(5): 416-22.
6. Bottery, M.J., Pitchford, J.W. & Friman, VP. Ecology and evolution of antimicrobial resistance in bacterial communities. *ISME J*, 2021; 15: 939–948.
7. Gorlenko, C. L., Kiselev, H. Y., Budanova, E. V., Zamyatnin, A. A., Jr, & Ikryannikova, L. N. Plant Secondary Metabolites in the Battle of Drugs and Drug-Resistant Bacteria: New Heroes or Worse Clones of Antibiotics?. *Antibiotics* (Basel, Switzerland), 2020; 9(4): 170.
8. Othman, L., Sleiman, A., & Abdel-Massih, R. M. Antimicrobial Activity of Polyphenols and Alkaloids in Middle Eastern Plants. *Frontiers in microbiology*, 2019; 10: 911.
9. Godstime, O., Felix, E., Augustina, J. and Christopher, E. Mechanisms of Antimicrobial actions of phytochemicals against enteric pathogens–A Review. J. *Pharmaceut. Chem. Biol. Sci.*, 2014; 2(2): 77-85.
10. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. Wayne, PA, USA: M100, 27th Edition, 2017.
11. The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters, version 10, 2020.
12. Devi KP, Nisha SA, Sakthivel R, Pandian SK. Eugenol (an essential oil of clove) acts as an antibacterial agent against Salmonella typhi by disrupting the cellular membrane, *J Ethnopharmacol*., 2010; 130: 107–115.
13. Hao G, Shi Y-H, Tang Y-L, Le G-W. The membrane action mechanism of analogs of the antimicrobial peptide Buforin 2. *Peptides*., 2009; 30(8): 1421–7. (11)
14. Meyuhas S, Assali M, Huleihil M and Huleihel M. Antimicrobial activities of caffeic acid phenethyl ester. *J. Mol. Biochem*., 2015; 4: 21–31.
15. Yang, W.; Gong, X.; Zhao, X.; An, W.; Wang, X.; Wang, M. Capsaicin induces apoptosis in HeLa cells via Bax/Bcl-2 and caspase-3 pathways. *Asian Pac. J. Trop. Med*., 2006; 1: 3–4.
16. Eumkeb G, Sakdarat S, Siriwong S. Reversing beta-lactam antibiotic resistance of Staphylococcus aureus with galangin from Alpinia officinarum Hance and synergism with ceftazidime. *Phytomedicine*., 2010; 18: 40–5
17. Hao G, Shi Y-H, Tang Y-L, Le G-W. The membrane action mechanism of analogs of the antimicrobial peptide Buforin 2. *Peptides*, 2009; 30(8): 1421–7.
18. Talebi Bezmin Abadi, A., Rizvanov, A.A., Haertlé, T. et al. World Health Organization Report: Current Crisis of Antibiotic Resistance. *BioNanoSci.,* 2019; 9: 778–788.
19. Serwecińska L. Antimicrobials and Antibiotic-Resistant Bacteria: A Risk to the Environment and to Public Health. *Water*, 2020; 12(12): 3313.
20. Zahran WA, Zein-Eldeen AA, Hamam SS, Sabal MSE. Surgical site infections: problem of multidrug-resistant bacteria. *Menoufia Medical J*., 2017; 30(4): 1005.
21. Serra-Burriel M, Keys M, Campillo-Artero C, Agodi A, Barchitta M, Gikas A, et al. Impact of multi-drug resistant bacteria on economic and clinical outcomes of healthcare-associated infections in adults: Systematic review and meta-analysis. *PLoS ONE*, 2020; 15(1): e0227139.
22. Biswas, D.; Roymon, M.G. Validation of antibacterial activity of saponin against diarreagenic E. coli isolated from leaves and bark of Acacia arabica. *J. Phytol.*, 2012; 4: 21–23.
23. Saleem, M.; Nazir, M.; Ali, S.; Hussain, H.; Lee, S.; Riaz, N.; Jabbar, A. Antimicrobial natural products: An update on future antibiotic drug candidates. *Nat. Prod. Rep.*, 2010; 27: 238–254.
24. Teo SP, Bhakta S, Stapleton P, Gibbons S. Bioactive Compounds from the Bornean Endemic Plant Goniothalamus longistipetes. *Antibiotics*, 2020; 9(12): 913.
25. Cheesman, M. J., Ilanko, A., Blonk, B., & Cock, I. E. Developing New Antimicrobial Therapies: Are Synergistic Combinations of Plant Extracts/Compounds with Conventional Antibiotics the Solution?. *Pharmacognosy reviews*, 2017; 11(22): 57–72.
26. Cushnie, T.P.T., and A.J. Lamb. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents*, 2005; 26: 343–356.

27. Eumkeb G, Sakdarat S, Siriwong S. Reversing beta-lactam antibiotic resistance of Staphylococcus aureus with galangin from Alpinia officinarum Hance and synergism with ceftazidime. *Phytomedicine*, 2010; 18: 40–5.
28. Mokwala, P. W. Antibacterial activity of plants that are used in the treatment of heartwater in livestock and the isolation and identification of bioactive compounds from Petalidium Oblongifolium and Ipomoea adenioides, 2007; *Thesis (PhD (Botany) University of Pretoria*.
29. Kubo, I., H. Muroi, and A. Kubo. Structural functions of antimicrobial long-chain alcohols and phenols. *Bioorg. Med. Chem*., 1995; 3: 873–880.
30. Ahmad, I., and A.Z. Beg. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *J. Ethnopharmacol*., 2001; 74: 113–123.
31. Davies, J., and D. Davies. Origins and evolution of antibiotic resistance. Microbiol. *Mol. Biol. Rev*., 2010; 74: 417–433.
32. Godstime, O., Felix, E., Augustina, J. and Christopher, E. Mechanisms of Antimicrobial actions of phytochemicals against enteric pathogens–A Review. *J. Pharmaceut. Chem. Biol. Sci.*, 2014; 2(2): 77-85.
33. Rogers, H., Perkins, H. R. & Ward, J. B. (1980). Microbial Cell Walls, and Membranes. (Springer Netherlands, Dordrecht; 1980).
34. Borges, A.; Saavedra, M.J.; Simões, M. The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling*, 2012; 28: 755–767.
35. Tegos, G.; Stermitz, F.R.; Lomovskaya, O.; Lewis, K. Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimicrob. Agents Chemother., 2002; 46: 3133–3141.
36. Oloyede, G.K. Toxicity, antimicrobial and antioxidant activities of methyl salicylate dominated essential oils of Laportea aestuans (Gaud). *Arab. J. Chem*., 2011.
37. Fux, C.A., M. Shirtliff, P. Stoodley, and J.W. Costerton. Can laboratory reference strains mirror 'real-world' pathogenesis? *Trends Microbiol*., 2005; 13: 58–63.
38. Gibbons, S. Anti-staphylococcal plant natural products. Nat. Prod. Rep., 2004; 21: 263–277.
39. Szaufer-Hajdrych, M., and O. Gos´lin´ ska. The quantitative determination of phenolic acids and antimicrobial activity of Symphoricarpos albus (L.) Blake. *Acta Pol. Pharm.*, 2004; 61: 69–74.
40. Biswas, D.; Roymon, M.G. Validation of antibacterial activity of saponin against diarreagenic E. coli isolated from leaves and bark of Acacia arabica. *J. Phytol*., 2012; 4: 21–23.
41. Farshori, N.N.; Banday, M.R.; Ahmad, A.; Khan, A.U.; Rauf, A. 7-Hydroxy-coumarin derivatives: Synthesis, characterization, and preliminary antimicrobial activities. *Med. Chem. Res*., 2011; 20: 535–541.
42. Hassan, S.M.; Byrd, J.A.; Cartwright, A.L.; Bailey, C.A. Hemolytic and antimicrobial activities differ among saponin-rich extracts from guar, quillaja, yucca, and soybean. *Appl. Biochem. Biotechnol*., 2010; 162: 1008–1017.
43. Souza, S.M.; Monache, F.D.; Smânia, A. Antibacterial activity of coumarins. *Zeitschrift für Naturforsch. C.*, 2005; 60c: 693–700.
44. Vollmer, W., Blanot, D. & De Pedro, M. A. Peptidoglycan structure and architecture. *FEMS microbiology revie*w, 2008; 32: 149–167.
45. A. J. O'Neill and I. Chopra, Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. *Expert Opinion on Investigational Drugs*, 2004; 13(8): 1045–1063.
46. Zhou K, Zhou W, Li P, Liu G, Zhang J & Dai Y, Mode of action of pentocin 31-1: an antilisteria bacteriocin produced by Lactobacillus pentosus from Chinese traditional ham. *Food Control*, 2008; 19: 817-822.
47. Kubo, I., K. Fujita, K. Nihei, and A. Kubo. Anti-Salmonella activity of (2E)-alkenals. *J. Appl. Microbiol*., 2004; 96: 693–699.
48. Campos, F.M., J.A. Couto, A.R. Figueiredo, I.V. To´ th, A.O.S.S. Rangel, and T.A. Hogg. Cell membrane damage induced by phenolic acids on wine lactic acid bacteria. *Int. J. Food Microbiol*., 2009; 135: 144–151.
49. Jayaraman, P., M.K. Sakharkar, C.S. Lim, T.H. Tang, and K.R. Sakharkar. Activity and interactions of antibiotic and phytochemical combinations against Pseudomonas aeruginosa in vitro. *Int. J. Biol. Sci*., 2010; 6: 556–568.
50. Kubo, I., K.I. Fujita, and K.I. Nihei. Molecular design of multifunctional antibacterial agents against methicillin resistant Staphylococcus aureus (MRSA). *Bioorg. Med. Chem.*, 2003; 11: 4255–4262.
51. Fattouch, S.; Caboni, P.; Coroneo, V.; Tuberoso, C. I. G.; Angioni, A.; Dessi, S.; Marzouk, N. & Cabras, P. Antimicrobial activity of Tunisian quince (Cydonia oblonga Miller) pulp and peel polyphenolic extracts. *J. Agric. Food Chem*., 2007; 55: 963-9.