ORIGINAL ARTICLE

Synergistic effect of ampicillin and dihydrobenzofuran neolignans (myticaganal C) identified from the seeds of *Myristica fragrans* Houtt. against *Escherichia coli*

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ABSTRACT

The present study was designed to enhance the antibacterial activity of ampicillin against *Escherichia coli* by combining it with myticaganal C. Antibacterial activity of ampicillin combined with myticaganal C against *E. coli* was assessed by agar well diffusion. Minimum inhibitory concentrations (MICs) and synergy by checkerboard assay of ampicillin and myticaganal C were assessed by resazurin-based 96-well microdilution. Bacterial responses were assessed by flow cytometry. Ampicillin in combination with myticaganal C showed better zone of inhibition (31.67 ± 0.58 mm) than myticaganal C or ampicillin alone. MIC of ampicillin was found to be 12.5 μg/mL, but myticaganal C was ineffective against *E. coli*. Myticaganal C (8000 μg/mL) with ampicillin (0.0975 μg/mL) exhibited strong synergy, so the need for ampicillin was reduced 128-fold. Combination inhibited *E. coli* by acting on cell membrane and by granularity disruptions. These findings indicate that myticaganal C enhances the potential of ampicillin against *E. coli*, thus providing an effective alternative to deal with the problem of bacterial resistance.

Key words: Ampicillin, checkerboard assay, flow cytometry, myticaganal C, synergistic effect

INTRODUCTION

Resistant bacterial strains are known to evolve into antibiotic-resistant forms, with modified hereditary materials.[1] This is becoming the main cause of failures in the treatment of diseases. Developing novel approaches is to complement known antibiotics with secondary metabolites. Several studies have reported that secondary metabolites decreased the amount of antibiotics needed while increasing the antibacterial activity of the treatment.[2,3]

Plant secondary metabolites could enhance antibacterial activity when combined with antibiotics.[4] Some secondary metabolites, namely dihydrobenzofuran-type neolignans, have been associated with various activities.[5-7] These compounds have been reported as antibacterial agents against pathogens.[8,9] *Myristica fragrans* Houtt. is an important plant source of neolignans used for preventing cancer and leishmaniasis.[10-12] The activity-guided

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fractionation of crude extract from *M. fragrans* seeds has led to the isolation of myticaganal C.[13,14] The effects of this compound on pathogenic bacteria have not been reported. There is no information regarding synergy of the compound extracted from *M. fragrans* seeds, with antibiotics against pathogenic bacteria. Gram-negative *E. coli* bacteria are most commonly commensal and can also be pathogenic in humans.[15] Resistant *E. coli* strains to antibiotics are risky as they are the most common Gram-negative bacteria infecting humans, and there are strains with extended-spectrum β-lactamases.[16,17] This study was aimed at determining the antibacterial activities of ampicillin and myticaganal C alone and as combinations against *E. coli*.

MATERIALS AND METHODS

Chemicals and bacterial strain

Myticaganal C [Figure 1] was purified from ethyl acetate–hexane (1:4) extract of *M. fragrans* seeds using the method previously described by Chumkaew and Srisawat.[14] Ampicillin was obtained from HiMedia (HiMedia Laboratories Pvt. Ltd., Mumbai, India). The tested organism was *E. coli* ATCC 25922.

Agar well diffusion

Briefly, the antibacterial activity was tested on Mueller–Hinton agar by agar well diffusion method. After the culture of 10^8 CFU/mL concentration of *E. coli* suspension was swabbed on the plates, wells (6 mm diameter) were punched in each plate using a cork borer. Then, each well was filled with 50 µL of myticaganal C (125–4000 µg/well), or ampicillin (3.125 and 50 µg/well), or mixture of myticaganal C (125–4000 µg/well) with ampicillin (3.125 and 50 µg/well). Inoculated plates were incubated at 37°C for 24 h. The antibacterial activity is expressed as mean of the growth inhibition zone in millimeters.

Determination of minimum inhibitory concentration

280 µL of mixed solution of Mueller–Hinton Broth (MHB) and two-fold myticaganal C (8000 µg/mL) or ampicillin (200 µg/mL) was filled in the first well of 96-well plate. Wells 2–8 had 140 µL MHB. To prepare myticaganal C concentrations of 62.5–8000 µg/mL or ampicillin concentrations of 1.56–200 µg/mL, 140 µL aliquot from the first well was pipetted and filled into the next well to make a two-fold serial microdilution in the 96-well plate. 50 µL of *E. coli* suspension was added into each well. The 96-well plate was then incubated at 37°C for 24 h. Then, 0.015% resazurin (10 µL) was added into each well, and it was further incubated before measuring for a color change. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the tested compound that prevented oxidation of the blue dye to a pink resorufin product.[18]

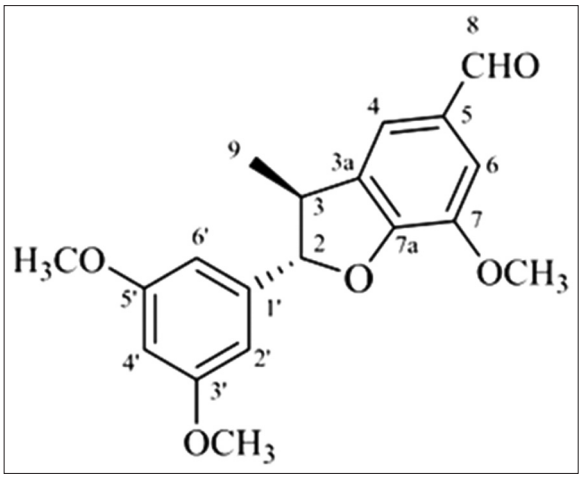

Figure 1: Chemical structure of myticaganal C from the seeds of *Myristica fragrans*[14]

Checkerboard 96-well plate assay

Two-fold serial dilutions of myticaganal C and ampicillin were performed, until reducing concentration of each substance 128-fold. Columns 1–8 and rows A–H were used to test mixtures of myticaganal C (62.5–8000 µg/mL) and ampicillin (0.0975–12.5 µg/mL), respectively. Columns 9A–9H were used for ampicillin alone (1.56–200 µg/mL). Columns 10A–10H were used to test myticaganal C alone (62.5–8000 µg/mL). 50 µL of bacterial solution was added into each well. Then, the final volume was 190 µL. The plates were incubated for 24 h at 37°C before adding 0.015% resazurin. The plate was then determined for color changes, as described above.

The synergy between ampicillin and myticaganal C was estimated as a fractional inhibitory concentration index following the method described by Mohammadi *et al*.[19]

Flow cytometric determination of cell membrane permeability and granular integrity of *Escherichia coli*

The final concentrations that corresponded to 0.5 MIC (0.049 µg/mL of ampicillin: 8000 µg/mL of myticaganal C), MIC (0.098 µg/mL of ampicillin: 8000 µg/mL of myticaganal C), and 2 MIC (0.196 µg/mL of ampicillin: 8000 µg/mL of myticaganal C) were added to wells containing 5×10^6 CFU/mL and incubated at 37°C for 0–24 h. After incubation, the cells were washed and re-suspended in 950 µL of phosphate-buffered saline. Samples were then incubated with 50 µg/mL propidium iodide in the dark for 15 min. The samples were analyzed on BD FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). The populations of each sample were analyzed on density plot diagrams generated by WinMDI version 2.9 software (Scripps Institute, La Jolla, CA, USA).

Data analysis

One-way ANOVA was used for antibacterial zones of inhibition by myticaganal C in combination with ampicillin. A $P < 0.05$ was considered statistically significant. Results are reported as

mean ± standard deviation. All calculations were done using SPSS version 11.0 software (IBM Corp., Armonk, NY, USA).

RESULTS

Antibacterial activity

The antibacterial activity of ampicillin (3.125 and 50 µg/well) and myticaganal C (125–4000 µg/well) singly and synergy of these substances against *E. coli* ATCC 25922 were evaluated by measuring the zone of inhibition of bacterial growth around the hole. The results showed that ampicillin had significant activity against *E. coli*, with 27.33 ± 0.58 mm inhibition zone. However, the tested organisms did not show any zone of inhibition with any tested concentration of myticaganal C singly. Interestingly, the growth of tested organisms was significantly inhibited synergistically with high inhibition by 50 µg/well of ampicillin combined with any tested concentration of myticaganal C, giving 31.67 ± 0.58 mm inhibition zone [Table 1].

Table 1: Antibacterial activity of ampicillin, myticaganal C, and the combination of myticaganal C with ampicillin against *Escherichia coli* ATCC 25922

Extract concentration (µg/well)	Zone of inhibition (mm)
Alone	
Ampicillin	
50	27.33±0.58^{c}
3.125	13.33±0.58^{e}
Myticaganal C	
4000	0
2000	0
1000	0
500	0
250	0
125	0
Combination	
Ampicillin with Myticaganal C	
50:4000	31.67±0.58^{a}
50:2000	31.67±0.58^{a}
50:1000	31.67±0.58^{a}
50:500	31.33±0.58a^{b}
50:250	30.67±0.58^{b}
50:125	30.67±0.58^{b}
3.125:4000	20.67±0.58^{d}
3.125:2000	20.67±0.58^{d}
3.125:1000	20.33±0.58^{d}
3.125:500	20.67±0.58^{d}
3.125:250	20.33±0.58^{d}
3.125:125	20.00±0.00^{d}
5% DMSO	0

Values are mean inhibition zone diameter (mm)±SD from three replicates. Statistically significant differences between dose levels are shown by different superscripts, based on DMRT (P=0.05). SD: Standard deviation, DMRT: Duncan's multiple range test, DMSO: Dimethyl sulfoxide

Minimal inhibitory concentration of ampicillin and myticaganal C alone and their combinations

After 24 h of incubation, unchanged resazurin blue color was observed for the wells with 12.5–200 µg/mL of ampicillin, whereas the wells with myticaganal C did not show any inhibition (resazurin changed from blue to pink) [Figure 2]. The wells containing 0.0975 and 0.195 µg/mL of ampicillin combined with 8000 µg/mL myticaganal C had the resazurin blue color. Therefore, the MICs of ampicillin alone and ampicillin and myticaganal C in combination were 12.5 µg/mL and 0.0975 and 8000 µg/mL, respectively. Myticaganal C at 8000 µg/mL reduced the MIC of ampicillin 128-fold from that of ampicillin alone, against *E. coli*.

Effects of myticaganal C combined with ampicillin on membrane permeability and granular integrity of *Escherichia coli* ATCC 25922

An investigation of the mortality rates and the response patterns of *E. coli* when treated with 0.5 MIC, MIC, and 2 MIC of ampicillin combined with myticaganal C for 12 h involved the identification of four populations [Figure 3]. The synergy of ampicillin with myticaganal C was observed to be dose dependent, with 12 h of incubation, as the rates of dead cells when treated at 0.5 MIC, MIC, and 2 MIC of the synergistic plant–antibiotic mix were 2.4%, 4.7%, and 9.3%, respectively [Table 2]. The response pattern of the bacterial cells to ampicillin with myticaganal C inhibited *E. coli* by both cell membrane damage and granularity disruption.

DISCUSSION

Antibiotic-resistant bacterial strains are an emerging threat to the health of people worldwide.[20] There is an urgent need to discover novel alternative treatments complementing the use of antibiotics. The synergy of medicinal plant

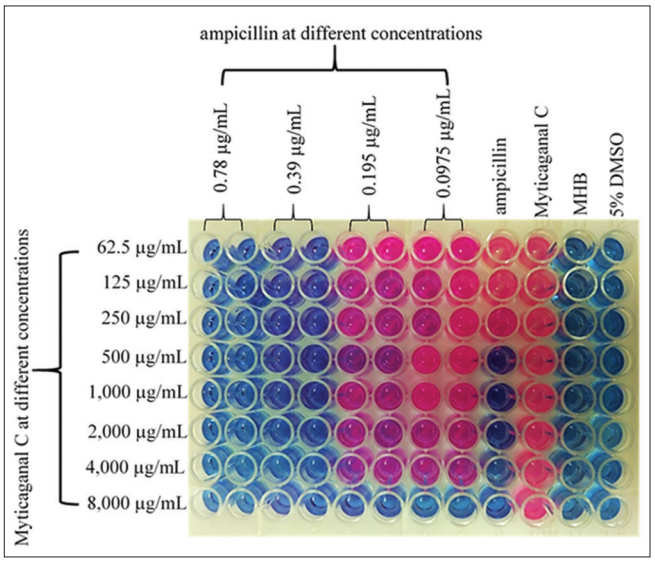

Figure 2: Synergistic effect of Myticaganal C with ampicillin against *Escherichia coli* tested by the checkerboard assay

Table 2: The combination of myticaganal C with ampicillin at 0.5 minimum inhibitory concentration, minimum inhibitory concentration, and 2 minimum inhibitory concentration (12 h treatment with each) caused responses in bacterial cells

Treatment	Response pattern of *Escherichia coli* ATCC 25922				
	Cell viability	Damaged cell membranes	Disrupted cell granularity	Cell death	Other than viable
5% DMSO	97.7	0.3	1.2	0.8	2.3
Myticaganal C					
8000 μg/mL	88.0	0.6	5.8	5.5	11.9
Ampicillin (μg/mL)					
12.5	90.8	0.3	8.1	0.8	9.2
0.0975	95.8	1.7	1.5	1.0	4.2
Combination treatment					
0.5 MIC					
0.049:8000 μg/mL	95.2	1.9	0.5	2.4	4.8
MIC					
0.0975:8000 μg/mL	91.9	2.6	0.8	4.7	8.1
2 MIC					
0.195:8000 μg/mL	80.1	9.7	0.9	9.3	19.9

MIC: Minimum inhibitory concentration, DMSO: Dimethyl sulfoxide

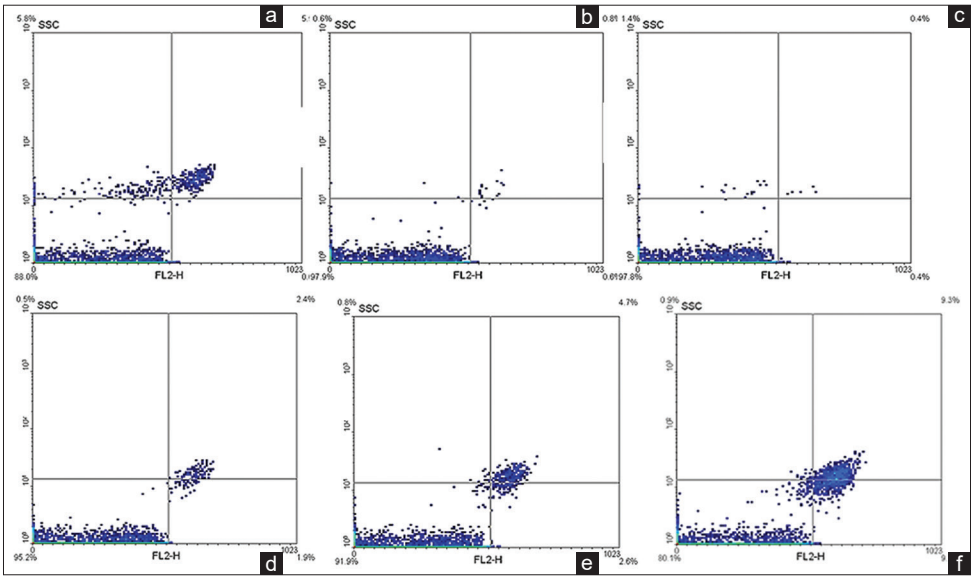

Figure 3: Flow cytometry dot plots for *Escherichia coli* treated with 8000 μg/mL of myticaganal C (a), 12.5 μg/mL of ampicillin (b), 0.0975 μg/mL of ampicillin (c), 0.5 minimum inhibitory concentration (d), minimum inhibitory concentration (e), and 2 minimum inhibitory concentration (f), for 12 h. *The regions divided by the lines were interpreted as: lower left for viable cells, lower right for membrane-damaged cells, upper left for injured cells, and upper right for dead cells

compounds with antibiotics is of interest in this context. In many previous studies, plant compounds combined with antibiotics have delayed the emergence of resistant bacteria.[3,21] This study aimed at finding antibacterial synergy between a compound from a medicinal plant and an antibiotic, to inhibit the growth of *E. coli*.

In experiments, ampicillin combined with myticaganal C showed a good inhibition zone against *E. coli*, better than myticaganal C or ampicillin alone. Dihydrobenzofuran neolignans are secondary metabolites in a plant extract such that they have bioactive activities.[11,12] On the other hand, antibacterial action of dihydrobenzofuran neolignans against an agent panel of cariogenic bacteria has been reported by Fukui *et al*.[7] Ampicillin is a β-lactam antibiotic that inhibits cell wall peptidoglycan synthesis of bacteria.[22] Then, the bacterial cell wall becomes mechanically weak and the cell dies.[23] After cell wall disruption, lipophilicity of the dihydrobenzofuran neolignans might allow these compounds to diffuse across the cell membrane.[7] After passing the cell membrane, the compound could affect bacterial metabolism and organelles causing death to the

bacteria. The MIC of ampicillin combined with myticaganal C indicates that myticaganal C reduced the MIC of ampicillin 128-fold to 0.097 μg/mL from the 12.5 μg/mL MIC of ampicillin alone. These results resemble those obtained by Navrátilová *et al*.,[24] who reported on efficacy of a medicinal plant that can increase the antibacterial activity of antibiotics. Similarly, trihydroxyflavone from *M. fragrans* could increase the activity of tetracycline against Gram-negative bacteria.[25]

The results of flow cytometry indicate that ampicillin combined with myticaganal C inhibited *E. coli* both by acting on cell membrane and by granularity disruptions. This synergy decreased viability and increased mortality of the bacteria in a dose-dependent manner.

CONCLUSION

The results of this study indicate improved antibacterial efficacy against *E. coli* ATCC 25922 of ampicillin when supplemented with myticaganal C. The supplementation could reduce 128-fold the effective dose of ampicillin, while the supplement alone had no activity against these bacteria. Further studies are needed to evaluate the *in vivo* effects, as well as possible additional mechanisms underlying the antibacterial activities.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Chandar B, Poovitha S, Ilango K, MohanKumar R, Parani M. Inhibition of New Delhi metallo-β-lactamase 1 (NDM-1) producing *Escherichia coli* IR-6 by selected plant extracts and their synergistic actions with antibiotics. Front Microbiol 2017;8:1580.
2. Chowdhury N, Ashrafuzzaman M, Ali H, Liza LN, Zinnah MA. Antimicrobial activity of some medicinal plants against multi drug resistant human pathogens. Adv Biosci Bioeng 2013;1:1-24.
3. Masoumian M, Zandi M. Antimicrobial activity of some medicinal plant extracts against multidrug resistant bacteria. Zahedan J Res Med Sci 2017;19:e10080.
4. Semeniuc CA, Pop CR, Rotar AM. Antibacterial activity and interactions of plant essential oil combinations against Gram-positive and Gram-negative bacteria. J Food Drug Anal 2017;25:403-8.
5. Teponno RB, Kusari S, Spiteller M. Recent advances in research on lignans and neolignans. Nat Prod Rep 2016;33:1044-92.
6. Castro O, Gomes L, Brito LM, Alves MM, Amorim LV, Sobrinho-Júnior EP, *et al*. *In vitro* effects of the neolignan 2,3-dihydrobenzofuran against *Leishmania amazonensis*. Basic Clin Pharmacol Toxicol 2017;120:52-8.
7. Fukui MJ, Dias HJ, Severiano ME, Souza GM, Oliveira PF, Ambrósio SR, *et al*. Antimicrobial and cytotoxic activity of dihydrobenzofuran neolignans. ChemistrySelect 2018;3:1836-9.
8. Sun ZL, He JM, Wang SY, Ma R, Khondkar P, Kaatz GW, *et al*. Benzocyclohexane oxide derivatives and neolignans from: Piper betle inhibit efflux-related resistance in *Staphylococcus aureus*. R Soc Chem Adv 2016;6:43518-25.
9. de La Cruz-Sánchez NG, Gómez-Rivera A, Alvarez-Fitz P, Ventura-Zapata E, Pérez-García MD, Avilés-Flores M, *et al*. Antibacterial activity of *Morinda citrifolia* Linneo seeds against methicillin-resistant *Staphylococcus* spp. Microb Pathog 2019;128:347-53.
10. Ranaware AM, Banik K, Deshpande V, Padmavathi G, Roy NK, Sethi G, *et al*. Magnolol: A neolignan from the Magnolia family for the prevention and treatment of cancer. Int J Mol Sci 2018;19:1-21.
11. Zhou L, Yao GD, Lu LW, Song XY, Lin B, Wang XB, *et al*. Neolignans from red raspberry (*Rubus idaeus* L.) exhibit enantioselective neuroprotective effects against H_2O_2-induced oxidative injury in SH-SY5Y cells. J Agric Food Chem 2018;66:11390-7.
12. Amaral M, de Sousa FS, Silva TA, Junior AJ, Taniwaki NN, Johns DM, *et al*. A semi-synthetic neolignan derivative from dihydrodieugenol B selectively affects the bioenergetic system of *Leishmania infantum* and inhibits cell division. Sci Rep 2019;9:6114.
13. Abourashed EA, El-Alfy AT. Chemical diversity and pharmacological significance of the secondary metabolites of nutmeg (*Myristica fragrans* Houtt.). Phytochem Rev 2016;15:1035-56.
14. Chumkaew P, Srisawat T. New neolignans from the seeds of *Myristica fragrans* and their cytotoxic activities. J Nat Med 2019;73:273-7.
15. Amenu D. Antimicrobial activity of medicinal plant extracts and their synergistic effect on some selected pathogens. Am J Ethnomed 2014;1:18-29.
16. Fair RJ, Tor Y. Antibiotics and bacterial resistance in the 21st century. Perspect Medicin Chem 2014;6:25-64.
17. Dhillon RH, Clark J. ESBLs: A clear and present danger? Crit Care Res Pract 2012;2012:625170.
18. Elshikh M, Ahmed S, Funston S, Dunlop P, McGaw M, Marchant R, *et al*. Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. Biotechnol Lett 2016;38:1015-9.
19. Mohammadi M, Khayat H, Sayehmiri K, Soroush S, Sayehmiri F, Delfani S, *et al*. Synergistic effect of colistin and rifampin against multidrug resistant *Acinetobacter baumannii*: A systematic review and meta-analysis. Open Microbiol J 2017;11:63-71.
20. World Health Organization. Antibiotic Resistance; 2018. Available from: https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance. [Last accessed on 2020 Jan 03].
21. Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol 2009;7:629-41.
22. Akinyele T, Igbinosa E, Akinpelu D, Okoh A. *In vitro* assessment of the synergism between extracts of *Cocos nucifera* husk and some standard antibiotics. Asian Pac J Trop Biomed 2017;7:306-13.
23. Uzair B, Hameed A, Nazir S, Khan BA, Fasim F, Khan S, *et al*. Synergism between *Nigella sativa* seeds extract and synthetic antibiotics against Mec A gene positive human strains of *Staphylococcus aureus*. Int J Pharmacol 2017;13:958-68.
24. Navrátilová A, Nešuta O, Vančatová I, Čížek A, Varela-M RE, López-Abán J, *et al*. C-Geranylated flavonoids from *Paulownia tomentosa* fruits with antimicrobial potential and synergistic activity with antibiotics. Pharm Biol 2016;54:1398-407.
25. Dzotam JK, Simo IK, Bitchagno G, Celik I, Sandjo LP, Tane P, *et al*. *In vitro* antibacterial and antibiotic modifying activity of crude extract, fractions and 3',4',7-trihydroxyflavone from *Myristica fragrans* Houtt against MDR Gram-negative enteric bacteria. BMC Complement Altern Med 2018;18:15.