Original Article

Aesculus hippocastanum-Derived Extract β-Aescin and *In vitro* Antibacterial Activity

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Abstract

Objectives: The objective of this study was to investigate the antibacterial activity of β-aescin against common Gram-negative and Gram-positive bacteria. **Materials and Methods:** Agar well diffusion assay was used to determine the antibacterial activity of β-aescin against common Gram-negative and Gram-positive bacteria including *Klebsiella pneumoniae, Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus,* and *Pseudomonas aeruginosa*. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of β-aescin were evaluated by serial dilution method. **Results:** β-aescin led to significant antibacterial effects on the tested Gram-negative and Gram-positive bacteria compared to the negative control, $P < 0.05$ for *K. pneumoniae* and *P. aeruginosa* and $P < 0.01$ for *E. coli*, *S. epidermidis,* and *S. aureus*. On the other hand, β-aescin produced a comparable less antibacterial effect on *K. pneumoniae*, *E. coli,* and *P. aeruginosa* compared to the positive control, $P < 0.01$, whereas β-aescin illustrated a comparable effect with that of the positive control on Gram-positive *S. epidermidis*, $P = 0.05$. Furthermore, β-aescin illustrated a concentration-dependent antibacterial effect against Gram-positive *S. epidermidis* and *S. aureus* compared to the different concentrations, $P < 0.01$. MIC and MBC of β-aescin were high for Gram-negative bacteria and low for Gram-positive bacteria compared to MIC of the positive control. **Conclusions:** β-aescin is an effective antibacterial herb mainly against Gram-positive *S. epidermidis* and *S. aureus* in a concentration-dependent manner.

Keywords: Agar well diffusion, antibacterial activity, minimal bactericidal concentration, minimal inhibitory concentration, *β-aescin*

Introduction

Resistance to available antibiotics in pathogenic bacteria is currently a global challenge since the number of strains that are resistant to multiple types of antibiotics has increased dramatically each year and has spread worldwide.[1]

Therefore, an urgent need for new antibiotics and other antimicrobial agents is recommended to compensate for drastic deficiency of the anti-infective arsenal. Consequently, a search for alternative agents against different microbial species is mandatory to overcome existence and future microbial resistances. It has been reported that different plants and herbal medicine have noteworthy antimicrobial activity, but only 1%–10% of these agents are used by human.[2] Nevertheless, many antimicrobial agents were developed in a period of 1981–2006; among these, 69% are originated from plants and herbs.[3] There are three generations of herbal medicines, first-generation compounds are botanical used on empirical evidence, second-generation compounds depend on scientific isolation of their active constituents, and third-generation compounds are phytotherapeutic agents which depend on biochemical and pharmacological studies. Furthermore, the antimicrobial activity of natural products depends mainly on their resin, active constituents, and secondary metabolites.[4] *Aesculus hippocastanum* L. from Hippocastanaceae family, known as horse chestnut, is commonly native to Western Asia used in the treatment of different medical conditions including leg ulcer, varicose veins, hemorrhoids, cellulitis, hematoma, and edema.[5]

There are different active constituents of *A. hippocastanum* which are rutin, quercetin, kaempferol, essential oils(oleic acid and linoleic acid), and amino acids (adenine, adenosine, and

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guanine). The main active constituent of *A. hippocastanum* is aescin which is a mixture of triterpene saponins. β-aescin is the active component of this mixture which has a specific chemical structure [Figure 1].[6]

Indeed, *A. hippocastanum* has some antibacterial activity against *Proteus vulgaris* and *Listeria monocytogenes* without antifungal activity.[7]

The main mechanisms of β-aescin are related to increase the sensitivity of vascular endothelium to the calcium ion, serotonin antagonist, and release of nitric oxide and reduce the catabolism of mucopolysaccharides that enhance the vascular tone which explains the potential effect of β-aescin in the management of edema, inflammation, and varicose veins.[8] β-aescin is a safe herbal agent. Only 0.9%–3.0% of patients treated with β-aescin reported adverse effects such as headache, dizziness, and gastrointestinal symptoms.[9]

The antibacterial activity of β-aescin is little known that necessitate the need for more in vivo and *in vitro* experimental studies to confirm the antibacterial activity of β-aescin. Therefore, the aim of the present study was to investigate the antibacterial activity of β-aescin against common Gram-negative and Gram-positive bacteria.

Materials and Methods

This *in vitro* experimental study was done at the Department of Clinical Pharmacology and Therapeutics in cooperation with the Department of Medical Microbiology, College of Medicine, Al-Mustansiriyia University, from March to July 2019, Bagdad, Iraq.

The antibacterial activity of β-aescin was screened against common Gram-negative and Gram-positive bacteria including *Klebsiella pneumoniae, Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus,* and *Pseudomonas aeruginosa* using agar well diffusion assay.[10] These bacterial isolates were obtained from medical culture collection of Bacteriology Laboratory, Department of Medical Microbiology, College of Sciences, Baghdad University. Bacterial isolates were maintained in a nutrient agar for 24 h at 37°Ċ, and one colony from each stock culture was inoculated in a 4-mL nutrient broth.

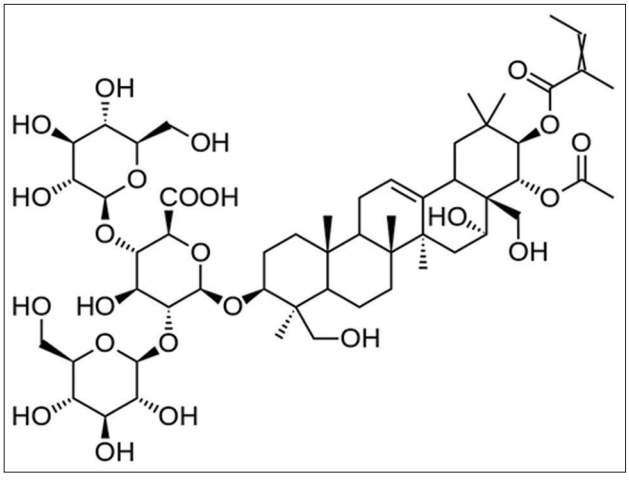

Figure 1: Chemical structure of β-aescin

Drugs and chemicals

β-aescin tablet (reparil 40 mg, MADAUS Ltd., GmbH 51101, Koln, Germany), and dimethyl sulfoxide (DMSO) were used in this experimental study. However, DMSO was used as solvent and regarded as negative control, while ciprofloxacin drug was used as a positive control. All drugs and chemicals were purchased from a private pharmaceutical and laboratory industrial company. To get stock solution, β-aescin tablet (40 mg) was dissolved in 10 mL DMSO, so this stock solution had 4 mg/mL. From these stocks, four different concentrations were prepared which were 25 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL which finally yielded disc potencies of 25 μg/disc, 50 μg/disc, 100 μg/disc, and 200 μg/disc, respectively, after their impregnation in each concentration.[11]

Determination of antibacterial activity

Disc diffusion method was done using Mueller-Hinton agar which was poured in a sterile dish (9-mm diameter). Agar discs were placed at room temperature for 30 min and then at 37Ċ for 24 h at a digital incubator. The antibacterial activity of tested agents was manifested as an inhibition zone which was measured by graduated ruler in millimeters.[12]

Determination of minimal inhibitory concentration

Minimal inhibitory concentration (MIC) represents the lowest concentration of tested agent at which no visible growth was detected. Serial dilution method was used to obtain the final concentration of β-aescin and controls which were dissolved in 1 mL of DMSO, and then, final serial dilutions were prepared and added on Mueller-Hinton gold agar. A Steer's replicator containing 5×10^5 CFU/drop of tested bacteria was added on each test plate and then incubated at 37Ċ for 18 h. MICs of tested agents were determined according to the resistance and susceptibility in the breakpoint tables of the Clinical and Laboratory Standards Institute.[13]

Determination of minimal bactericidal concentration

Minimal bactericidal concentration (MBC) is the lowest concentration of tested agent that kills the bacteria. MBC test

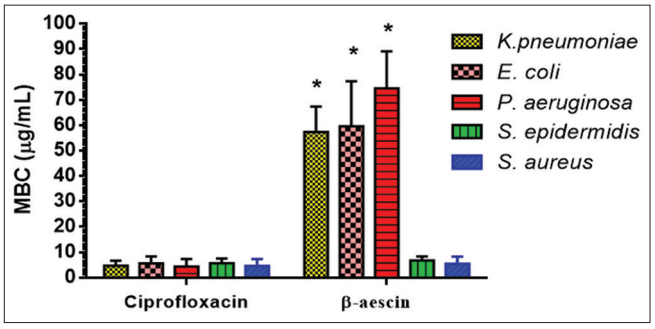

Figure 2: Minimal inhibitory concentration of β-aescin against Gram-positive and Gram-negative bacteria compared to the positive control. *$P < 0.01$

was done following the results of MIC in order to confirm if β-aescin is actually killing the bacteria or inhibiting their growth. Ten microliters from each MIC tube was dropped on the nutrient agar and spread using a sterile rod and then incubated at 37Ċ for 24 h.[14]

Statistical analysis

Data analysis was done using SPSS (IBM SPSS Statistics for Windows version 20.0, 2014 Armonk, NY, IBM, Corp.). The data were presented as mean ± standard deviation, and unpaired Student's *t*-test was used to determine the level of differences. Analysis of variance followed by Bonferroni *post hoc* test was used to compare the results of study variables among different groups. The level of significance was regarded when $P < 0.05$.

Results

Zone of inhibition of β-aescin

In the present study, β-aescin led to significant antibacterial effects as revealed by zone of inhibition on the tested Gram-negative and Gram-positive bacteria compared to the negative control (DMSO), $P < 0.05$ for *K. pneumoniae* and *P. aeruginosa* and $P < 0.01$ for *E. coli*, *S. epidermidis,* and *S. aureus*. On the other hand, β-aescin produced a comparable less antibacterial effect on *K. pneumoniae*, *E. coli,* and *P. aeruginosa* compared to the positive control, $P < 0.01$, whereas β-aescin illustrated a comparable effect with that of the positive control on Gram-positive *S. epidermidis* ($P = 0.05$) [Table 1].

β-aescin illustrated a concentration-dependent antibacterial effect against Gram-positive *S. epidermidis* and *S. aureus* compared to the different concentrations, $P < 0.01$. There were insignificant differences in the concentration-dependent antibacterial effect against Gram negative ($P > 0.05$) [Table 2].

Minimal inhibitory concentration of β-aescin

MIC of β-aescin was high for Gram-negative bacteria and low for Gram-positive bacteria compared to MIC of the positive control. MIC of β-aescin against *K. pneumoniae* was 52.11 ± 11.05 μg/mL compared to 2.69 ± 1.81 μg/mL of the positive control ($P = 0.0001$), MIC of β-aescin against *E. coli* was 44.63 ± 12.05 μg/mL compared to 3.62 ± 1.69 μg/mL of the positive control ($P = 0.0001$), MIC of β-aescin against *P. aeruginosa* was 64.63 ± 14.85 μg/mL compared to 3.69 ± 1.65 μg/mL of the positive control ($P = 0.0001$), MIC of β-aescin against *S. epidermidis* was 4.85 ± 1.63 μg/mL compared to 3.73 ± 1.79 μg/mL of the positive control ($P = 0.33$), and MIC of β-aescin against *S. aureus* was 3.84 ± 1.12 μg/mL compared to 2.59 ± 1.05 μg/mL of the positive control ($P = 0.10$) [Figure 2].

Minimal bactericidal concentration of β-aescin

MBC of β-aescin was high for Gram-negative bacteria and low for Gram-positive bacteria compared to MBC of the positive control. MBC of β-aescin against *K. pneumoniae* was 57.34 ± 10.05 μg/mL compared to 4.69 ± 1.99 μg/mL of the positive control ($P = 0.0001$), MBC of β-aescin against *E. coli* was 59.66 ± 17.74 μg/mL compared to 5.62 ± 2.77 μg/mL of the positive control ($P = 0.0001$), MBC of β-aescin against *P. aeruginosa* was 74.55 ± 13.61 μg/mL compared to 4.41 ± 2.99 μg/mL of the positive control ($P = 0.0001$), MBC of β-aescin against *S. epidermidis* was 6.72 ± 1.79 μg/mL compared to 5.72 ± 1.76 μg/mL of the positive control ($P = 0.39$), and MBC of β-aescin against *S. aureus* was 5.45 ± 2.82 μg/mL compared to 4.59 ± 2.78 μg/mL of the positive control ($P = 0.60$) [Figure 3].

Discussion

There is an urgent and incessant need to find and discover new antimicrobial drugs and agents with their novel mechanisms to overcome the alarm and challenge of bacterial resistances. Therefore, the finding of new antibiotics or alternative herbal medicine is recommended to fight against these resistance microorganisms.[15]

The present study illustrated that β-aescin had a significant antibacterial activity mainly for Gram-positive bacteria (*S. epidermidis* and *S. aureus*) and less activity against Gram-negative bacteria as documented by Anitha *et al*.'s study that confirmed a significant *in vitro* antibacterial activity of β-aescin against Gram-positive oral microbes.[16]

Besides, in the present study, a concentration-dependent antibacterial effect of β-aescin revealed that its antibacterial following serial doubling of concentration was significant only for Gram-positive bacteria. The previous study showed that β-aescin is an effective herbal medicine against Gram-positive and Gram-negative bacteria.[17]

Table 1: Antibacterial zone of inhibition (millimeter, in diameter) of β-aescin (25 μg/mL) compared to positive and negative controls

Bacterial types	DMSO	Ciprofloxacin	β-aescin	*Post hoc* test			ANOVA
				A	B	C	
Klebsiella pneumoniae	2.69±0.73	18.31±0.82	8.79±0.78	0.0001*	0.01×	0.0001*	0.0001
Escherichia coli	1.64±0.48	21.82±0.63	9.89±0.49	0.0001*	0.001*	0.001*	0.0001
Pseudomonas aeruginosa	1.82±0.61	14.68±0.81	5.72±0.61	0.001*	0.03×	0.001*	0.001
Staphylococcus epidermidis	1.69±0.99	14.72±0.99	13.11±0.96	0.001*	0.003*	0.05	0.001
Staphylococcus aureus	1.07±0.31	16.02±0.12	14.92±0.97	0.001*	0.001*	0.02×	0.001

*$P<0.01$, ×$P<0.05$. Data are presented as mean±SD, One-way ANOVA test and *post hoc* test. DMSO: Dimethyl sulfoxide, A: DMSO versus ciprofloxacin, B: DMSO versus β-aescin, C: Ciprofloxacin versus β-aescin, SD: Standard deviation, ANOVA: Analysis of variance

Table 2: Concentration-dependent antibacterial effect (zone of inhibition, in millimeter) of β-aescin

Bacterial isolates	25 μg/mL	50 μg/mL	100 μg/mL	200 μg/mL	*P*
Klebsiella pneumonia	8.79±0.78	8.81±0.33	8.73±0.64	9.72±0.76	0.08
Escherichia coli	9.89±0.49	9.94±0.78	10.61±0.83	10.64±0.89	0.26
Pseudomonas aeruginosa	5.72±0.61	5.71±0.55	5.73±0.82	6.03±0.74	0.85
Staphylococcus epidermidis	13.11±0.96	14.94±0.84	16.71±0.93	17.87±0.67	0.001*
Staphylococcus aureus	14.92±0.97	15.86±0.92	17.45±0.89	18.67±0.81	0.001*

Data are presented as mean±SD, One-way ANOVA test and *post hoc* test, *P<0.01. SD: Standard deviation

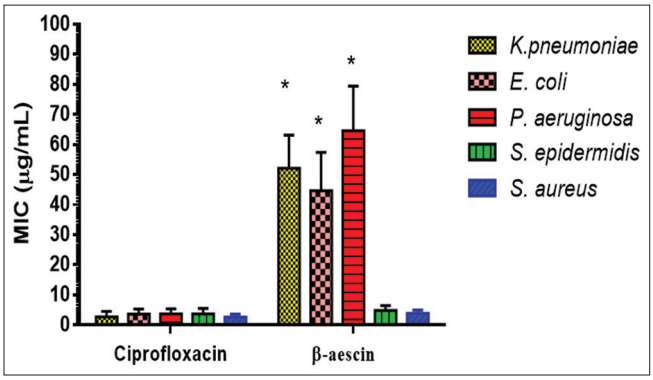

Figure 3: Minimal bactericidal concentration of β-aescin against Gram-positive and Gram-negative bacteria compared to the positive control. *P < 0.01

Our findings as well demonstrated that β-aescin had significant MIC and MBC mainly against Gram-positive bacteria compared to ciprofloxacin as a positive control. Parekh and Chanda reported that β-aescin and other medicinal plants possess a significant antibacterial activity mainly against Gram-positive *S. epidermidis* and *S. aureus*.[18] In contrast, Schmidt *et al*. showed that β-aescin alone or in combination with antibiotics reveals a minimal antibacterial activity compared to other medicinal plants.[19]

In spite of divers' antibacterial activity of β-aescin, it is mainly effective against *S. epidermidis* and *S. aureus* which are common Gram-positive bacteria implicated in the infective complications following surgical vascular graft and implantations of prosthetic devices. *Staphylococcus* species are highly vulnerable for antibiotic resistances and causing permanent and relapsing infection.[20]

The mechanism of antibacterial activity of β-aescin is not known and was not discussed previously as it is not used mainly for their antibacterial effects. In reference to their composition, *A. hippocastanum* contains saponins, known as aescin. Therefore, β-aescin saponin is the main active constituents responsible for their antibacterial effects.[21]

Saponins are glycosides with hydrophilic sugar residue connected with lipophilic triterpenes and steroidal alkaloids. Saponins are found in different plants in a concentration up to 30% and play a potential role against plant infections.[22] Saponins have significant antibacterial effects and may synergize the antibiotic effect against resistant bacteria such as Methicillin-resistant *S. aureus*. Recently, Sun *et al*. confirmed that less polar saponins lead to bacterial damage through the inhibition of membrane potential and induction of pore formations in bacterial cell membrane and wall leading to the leakage of the cytoplasmic contents and cell death.[23,24] In addition, triterpene of β-aescin may inhibit bacterial cell since Cunha *et al*. reported that hydroxy and carboxy groups of triterpenes are involved in the bactericidal effect of plant containing triterpenes.[25]

Therefore, β-aescin has a bactericidal effect through the inhibition of bacterial cell membrane and wall, as documented in our study.

The limitations of the present study were small sample size of bacterial isolates, concentrations of saponins and triterpenes, and synergistic effect of β-aescin with other antibiotics were not estimated. In spite of these limitations, this study is regarded as a novel study that demonstrated the antibacterial activity of β-aescin against *Staphylococcus* species.

Conclusions

β-aescin is an effective antibacterial herb mainly against Gram-positive *S. epidermidis* and *S. aureus* in a concentration-dependent manner.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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