J Genet Resour2020;6(1): 20-33 *Homepage: http://sc.journals.umz.ac.ir/*

RESEARCH ARTICLE *DOI: 10.22080/jgr.2020.2538*

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Comparing of Effects of Hydro-alcoholic, Ethanolic, and Methanolic Extracts of the *Frangula alnus*: Chemical Composition, Antimicrobial, and Synergism

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ARTICLE INFO

Article history:
Received 11 December 2019
Accepted 12 January 2020
Available online 24 January 2020

Keywords:
Hydro-alcoholic
Erythromycin
Chemical composition
Ciprofloxacin
Gallic acid

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p-ISSN 2423-4257
e-ISSN 2588-2589

ABSTRACT

This study was conducted to evaluate the effect of different extraction solvents, including hydro-alcoholic (H-A), ethanolic (Et-OH), and methanolic (Me-OH), on chemical composition and synergistic antibacterial activity of *Frangula alnus* extract against *Staphylococcus aureus* and *Escherichia coli*. The chemical composition of the *F. alnus* bark extract was evaluated using FTIR, UV-Vis spectroscopy, and GC/MS analysis. The synergistic antimicrobial effect of the extracts with Ciprofloxacin (CIP) and Erythromycin (ERY) was evaluated using minimum inhibitory concentrations, combined disc, and checkerboard titration method. *F. alnus* contained alkanes, alkenes, phenols, alcohols, esters, terpenes, fatty acid, tetrazole, halo-alkane, anthraquinone as well aromatic, and plasticizer compounds in the ethanolic, methanolic, and hydro-alcoholic extracts. Total phenolic compounds for ethanolic, hydro-alcoholic and methanolic extracts were recorded 110.92±0.01, 95.27±0.01 and 126.6±0.02 g/L Gallic acid, respectively. Et-OH and H-A extracts in both combined and synergistic forms significantly inhibited bacterial growth. Concerning the antibacterial activity of the extracts, it was assumed that the compounds present in the plant extracts would destroy the bacterial cell membranes and consequently cause leakage of intracellular contents. Therefore these antibiotics in combination with the extracts would be able to affect their target sites (CIP; DNA gyrase and ERY; 50S subunit) more effectively. According to the results, H-A, Me-OH, and Et-OH extract efficiently in the synergistic state with erythromycin and ciprofloxacin can be a promising candidate to be used against bacterial pathogens.

Please cite this paper as: Nejabatdoust A, Baghaei Daemi H, Salehzadeh A, Azimi SC, Darafkan S, Fallah Digsara F, Pourebrahim M, Seighalani R. 2020. Comparing of effects of hydro-alcoholic, ethanolic, and methanolic extracts of the *Frangula alnus*: Chemical composition, antimicrobial, and synergism. *J Genet Resour* 6(1): 20-33. doi: 10.22080/jgr.2020.2538

Introduction

Nowadays, the limitation of effective antibiotics for drug-resistant microorganisms has become a global health challenge (Elisha *et al.*, 2017). Emergence of drug resistance is largely associated with the overuse of antibiotics and the subsequent development of microbial resistance to some antibiotics (Mukherjee *et al.*, 2002). At present, a variety of antibacterial, antiviral, antifungal and even anti-cancer drugs are derived from plant sources (Tchakam *et al.*, 2012). Plants have certain defense mechanisms and endogenous antimicrobial compounds which make them a potential source of antimicrobial agents (Nascimento *et al.*, 2000).

Several secondary metabolites, such as saponins, glycosides, alkaloids, terpenoids, steroids, tannins, and flavonoids as well as coumarins and quinones could be derived from plants (Elisha *et al.,* 2017). In addition, plant derivatives may show synergistic antibacterial potential with different antibiotics through several complex mechanisms (Radulovic *et al.,* 2013; Cowan 1999; Daglia 2012; Coppo *et al.,* 2014). Glossy buckthorn (*Frangula alnus*) is a plant with fleshy fruits from the family *Rhamnaceae* with several multi-stemmed shrubs whose single-stemmed forms can reach a height of 7 meters (Farrar 1995; Gleason 1991). It has been established that some genera of *Frangula* are used in traditional medicine in European and American regions due to their anthraquinone content. Indeed, anthraquinone or anthracenedione have antibacterial, antifungal, antioxidant and anticancer as well as antiviral activity (Kremer *et al.,* 2012; Roudbaraki and Nori-Shargh 2016).

The chemical nature and the polarity of the solvents used in the extraction process determine the extraction yield and composition of bioactive compounds and thus, biological activity of the extract. Therefore, the current study was conducted to evaluate: 1) the probable antimicrobial activity of *F. alnus* extracts against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterial strains; 2) the effect of extraction solvents, including Hydro-alcoholic 70%, Ethanol 100%, and Methanol 100% on the contents of *Frangula alnus* extracts; and 3) synergistic antibacterial potential of different extracts with erythromycin and ciprofloxacin.

In fluoroquinolone drug class ciprofloxacin (CIP) is a well-known antibiotic, which prevents bacterial DNA replication by inhibiting bacterial DNA topoisomerase type II and IV as well DNA-gyrase. In fact, ciprofloxacin is widespread in antibiotics and covers a wide range of infections caused by Gram-negative and Gram-positive bacteria (Campoli-Richards *et al.,* 1988). In this regard, erythromycin (ERY) is a macrolide antibiotic initially discovered in 1952 and traditionally, its use has been for various infections. From a structural point of view, erythromycin classified as an active agent against Gram-positive and Gram-negative bacteria. ERY is a bacteriostatic antibiotic, which acts occurs by inhibiting protein synthesis. This antimicrobial drug binds to the 23S ribosomal RNA molecule in the 50S subunit of the bacterial ribosome; this causes a blockage in the exiting of the peptide chain that is growing (Amsden *et al.,* 1996; Kanoh *et al.,* 2010; Tenson *et al.,* 2003).

Materials and Methods

Samples collection and extract preparation

The samples were collected from the Caspian Hyrcanian mixed forests, Gilan province, Iran, between July and August 2018. The collected samples (bark) were transferred to the laboratory under sterile conditions and dried at 55°C for 24 h. Then, the samples were turned into powder using a blender (Beko®). After that, 20 g of the samples were soaked in 200 mL of solvents (hydro-alcoholic 70%, ethanol 100%, and methanol 100%) and gently shaken for 72 h. Afterward, the extracts were passed through a Whatman No.1 papers and then 0.45 µm filter. Finally, the crude plant extracts were dried and stored at 2-8°C for further investigation.

Culture of Microorganisms

Clinical isolates of *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) were used in this study. The strains were identified using morphological and biochemical conventional screening assays. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as the standard strains.

UV–Vis spectrum and FT-IR spectroscopic analysis

To evaluate the composition of each extract, Et-OH, H-A, and Me-OH extracts diluted (1:10) in deionized water and the optical density of the extracts was measured using a UV-Vis (Lambda 25, Perkin-Elmer, USA) in a range of 250 to 700 nm. In addition, the extracts were fed into Fourier-transform infrared spectroscopy (Model 8900, Shimadzu, Japan) to identify the functional groups of the active components ranging from 4000-400 cm^{-1} (Sandosh *et al.,* 2013; Kalaichelvi and Dhivya 2017).

Gas chromatography-mass spectroscopy (GC/MS)

The gas chromatography-mass spectrometry (GC-mass) method is used to characterize the compounds with antibacterial properties

(Salehzadeh *et al.*, 2018). Various compounds were analyzed in hydro-alcoholic H-A, Et-OH and Me-OH extracts of *F. alnus* using a thermo GC/MS system (model Agilent 7890B) combined with mass spectrometry (HP 6890). The column was 280 (W) ×310 (H) ×160 (D) mm when helium (2 mL/min) was used as the carrier gas.

The temperature schedule of GC columns was adjusted to the initial temperature of 60°C for 10 min, then, a gradual increase at the rate of 8.2°C/min was given until reaching 200 °C, and the column was held at 200°C for 60 minutes. Finally, the installed instrument of the Wiley 275 library on the mass spectra was used to evaluate the compounds.

Total phenol content

The total phenol content of F. alnus bark extracts was determined using the Folin-Ciocalteu method (Ordonez *et al.,* 2006). Briefly, 100 µl of each extract at a concentration of 1 mg/mL was added to 2.5 mL of the Folin reagent (diluted 1:10). After a minute, 2.0 mL of 75 g/L Na2CO3 was added to each respective tube. The final mixture was shaken thoroughly for 10 min and incubated at room temperature (25°C) for 120 min. Optical density (OD) of all samples was determined at 760 nm with a Lambda 2 Ultraviolet-visible spectroscopy (UV/Vis spectrophotometer) (Lambda 25, Perkin-Elmer, USA).

Antimicrobial activity of the extracts

Leakage of cellular contents

At first, a fresh bacterial culture was suspended in 10 mL of Mueller Hinton Broth (MHB) (OD_{600}= 0.2). Then, 100 µL of bacterial suspension was added to H-A, Me-OH and Et-OH extracts of *F. alnus* with a final concentration of 8 mg/mL. Sterile distilled water was used as control. The tubes were incubated at 37°C with shaking at 250 rpm and in one-hour intervals (0, 1, 2, 3 and 4 h), the samples were centrifuged at 3000 rpm for 5 min, supernatant was passed through a 0.22 µm filter, and their optical density (OD) was measured at 260 and 280 nm. (Huang *et al.,* 2017) (14).

Leakage of potassium ions

The integrity of bacterial membrane in the presence of H-A, Me-OH and Et-OH extracts of F. alnus was investigated by determining the release of potassium ions (K^+), according to the method described by Miao et al (2016) with minor modifications. Briefly, a fresh culture of each bacterial strain was prepared in nutrient broth (NB), the cells were harvested and washed with sterilized water. The optical density of each bacterial strain was adjusted to 0.2 at 600 nm in deionized water and 100 µL of bacterial suspensions were re-suspended in different extracts (H-A, Me-OH, and Et-OH) with a final concentration of 8 mg/mL. The tubes were incubated for 4 h at 37°C with shaking at 150 rpm.

Distilled water was added to the control tube. At one-hour intervals, the bacterial suspensions were centrifuged (5000 rpm for 10 min) and the concentration of potassium ion in the supernatants was measured using an atomic absorption spectrometer (Varian AA240FS, USA).

MIC and MBC determination

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of all plant extracts and the antibiotics ciprofloxacin and erythromycin against bacterial strains, were determined using the broth micro-dilution method according to the Clinical Laboratories Standard Institution (CLSI) guideline (CLSI 2012). Initially, a concentration gradient of each agent (concentration of 10,240 µg mL^{-1} (10-fold) for antibiotics and 400 mg/mL for extracts) was prepared in the 96-well plates and inoculated with 100 µL of the bacterial suspension with a population of 1.5×10^6 CFU/mL. The plates were incubated at 37°C for 18 h.

The minimum concentration of each agent that inhibited visible growth of bacteria was considered as the MIC value. In order to determine the MBC value of each extract against bacteria, the wells without bacterial growth were inoculated on a Muller Hinton agar and incubated at 37°C for 24 h and monitored for bacterial growth.

Combined disk assay

The synergistic antibacterial activity of herbal extracts with antibiotics against bacterial strains was screened by a combined disc diffusion method. A suspension of fresh bacterial cells

with the approximate populations of 1.5×10^8 CFU/mL was prepared in distilled water and inoculated uniformly onto the surface of Mueller Hinton agar. Then, standard discs of erythromycin (15 µg/disc) and ciprofloxacin (5 µg/disc) were impregnated with 40 µL of *F. alnus* extracts (8 mg/mL).

The discs containing antibiotics or plant extracts alone were regarded as controls. The plates were incubated at 37°C for 24 h and the antimicrobial activity of the extract was evaluated by measuring the zone of inhibition (ZOI) around the discs. The fold increase area of different antibiotics was calculated by the following equation [Increase in fold area = $(a^2 - e^2) / a^2$], Where (a) and (e) is the zone of inhibition for antibiotic (alone) and antibiotic in combination with extracts, respectively (Nejabatdoust *et al.*, 2019).

Checkerboard titration method

The synergistic antimicrobial activity (SAA) of antibiotics with extracts was confirmed using the checkboard titration method (Orhan *et al.*, 2005). Briefly, two-fold dilutions of the extracts and antibiotics were prepared in 96-well microtiter plates. Then, combinations of the extract and the antibiotic in a range of 1/32 MIC to 4 MIC were prepared. Each well was inoculated with 50 µL of bacterial suspension 1.5×10^6 CFU/mL and the plates were incubated at 37 °C for 24 h**.** The plates were monitored for visible bacterial growth in each well. The possible synergistic and antagonistic effect of each agent was evaluated with fractional inhibitory concentration (FICs) [ΣFICs = FICsA + FICsB], Where FICs A is the MIC of agent A in the combination/MIC of the agent (A) alone, and FICs B is the MIC of agent B in the combination/MIC of agent B alone. The combination is considered synergistic when the fractional inhibitory concentration (ΣFICs) index is ≤0.5. Indifference was indicated by a FICs index >0.5 to ≤4 while antagonism when the ΣFIC is >4 (Verma, 2007).

Statistical analyzes

The assays were performed in triplicates and the numerical data were evaluated with *one-way ANOVA* Tukey in SPSS (16.0) software. *P* values of <0.01** and <0.05* were considered to be statistically significant.

Results

The chemical constituents derived from *Frangula alnus* are presented in Table 1. As is evident, 35 components were identified as active compounds.

Spectrophotometric analysis

The UV-VIS qualitative spectrum result of different extracts of *F. alnus* was evaluated from 250 to 700 nm (Fig. 1). The main peaks were detected in the area of 271, 316, 391, and 442 nm with the absorption of 2.202, 1.158, 0.467, and 0.209 in Et-OH extract. The strong peaks in 271, 316, 391, and 442 nm were assigned to the absorption of 2.408, 1.066, 0.436, and 0.317 in Me-OH extract. Also, the absorption of 2.765, 2.765, 0.484, and 0.343 were the main peaks in H-A extract (Sandosh *et al.*, 2013).

FT-IR analysis

The FT-IR spectrum of different extracts of *F. alnus* was presented in figure 2 and table 2. The strong bands at 3417.07, 3414.57 and 3412.90 cm^{-1} can be assigned to the vibrational stretching frequencies of (O-H) groups in alcoholic compounds. The bands at 3005.96-2858.83 cm^{-1} are attributed to the stretching vibration (CH_2) and (CH_3) groups of alkanes (Nallasamy *et al.*, 2002). The band at 1728.23-1635.10 cm^{-1} are due to (C=C) stretching associated with the alkenes or aromatic frameworks of the extracts (Karpagasundari et al., 2014) (20). The (C-H) bending vibration of alkanes are shown at 1436.91-1120.16 cm^{-1} (Nallasamy *et al.*, 2002). The (C-O) stretching vibrations of alcohol occurred in 1021.97-1021.07 cm^{-1} (Karpagasundari et al., 2014; Oliveira et al., 2016). The bands at around 953.79-902.17 cm^{-1} can be assigned to the aromatic compound stretching (Muthuselvi *et al.*, 2018). The bands at 708.81-561.46 cm^{-1} are related to stretching vibration of Halogen compounds (Karpagasundari *et al.*, 2014).

Table 1. GC/MS analysis of *F. alnus* bark extracts.

No.	Compounds	Molecular formula	Nature of Compound	Extracts		
				H-A	Me-OH	Et-OH
1	Dimethyl malonic acid, 4-chlorophenyl pentadecyl ester	$C_{15}H_{19}C_lO_4$	Ester		✓	
2	Fumaric acid, nonyl pentadecyl ester	$C_{28}H_{52}O_4$	Ester		✓	
3	6,6-Diethylhoctadecane	$C_{22}H_{46}$	Alkane		✓	
4	Linalool	$C_{10}H_{18}O$	Oxygenated Monoterpene		✓	✓
5	Eicosane	$C_{20}H_{42}$	Alkane		✓	
6	Eicosyl isopropyl ether	$C_{23}H_{48}O$	Ether		✓	
7	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	Phenol		✓	
8	Butylated Hydroxytoluene	$C_{15}H_{24}O$	Phenol		✓	
9	4-{2-[4-(Dimethylamino)phenyl]-5-phenyl-1H-imidazol-4-yl}phenol	$C_{23}H_{21}N_3O$	Phenol		✓	
10	Octadecamethylcyclononasiloxane	$C_{18}H_{54}O_9Si_9$	Polysiloxane		✓	
11	Diethyl phthalate	$C_{12}H_{14}O_4$	Plasticizer compound		✓	
12	p-Xylene	C_8H_{10}	Aromatic			✓
13	Dodecane	$C_{12}H_{26}$	Alkane			✓
14	5-Aminotetrazole	CH_3N_5	Tetrazole			✓
15	Oxalic acid, allyl octyl ester	$C_{13}H_{22}O_4$	Ester			✓
16	Oxalic acid, isohexyl neopentyl ester	$C_{13}H_{24}O_4$	Ester			✓
17	Oleic acid	$C_{18}H_{34}O_2$	Fatty acid	✓		✓
18	Tetradecane	$C_{14}H_{30}$	Alkane			✓
19	Heptacosane	$C_{27}H_{56}$	Alkane			✓
20	Caryophyllene oxide	$C_{15}H_{24}O$	Oxygenated Terpenoid		✓	✓
21	4-Methyl-5-propylnonane	$C_{13}H_{28}$	Alkane			✓
22	2,5-Di-tert-butylphenol	$C_{14}H_{22}O$	Phenol			✓
23	Butylated hydroxytoluene	$C_{15}H_{24}O$	Phenol			✓
24	(3Z)-2-Methyl-3-octen-2-ol	$C_9H_{18}O$	Alcohol			✓
25	Hexadecane	$C_{16}H_{34}$	Alkane			✓
26	Hentriacontane	$C_{31}H_{64}$	Alkane			✓
27	Octadecane	$C_{18}H_{38}$	Alkane			✓
28	Nonadecane	$C_{19}H_{40}$	Alkane			✓
29	1-Bromoeicosane	$C_{20}H_{41}Br$	Haloalkane			✓
30	Chrysophanic acid	$C_{15}H_{10}O_4$	Anthraquinone	✓		✓
31	2-Methyloctacosane	$C_{29}H_{60}$	Alkane	✓		
32	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	Ester	✓		
33	Ethyl palmitate	$C_{18}H_{36}O_2$	Ester	✓		
34	Hexadecamethyloctasiloxane	$C_{16}H_{50}O_7Si_8$	Polysiloxane	✓		
35	Butyl 2-Ethylhexyl Phthalate	$C_{20}H_{30}O_4$	Plasticizer compound	✓		

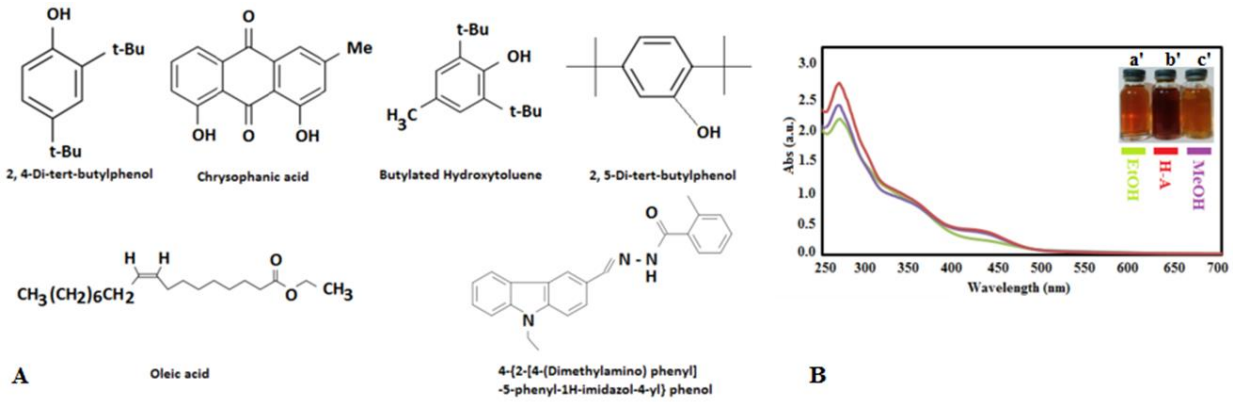

Fig. 1. Schematic of Chemical structures compounds and UV-Visible spectrum analysis: A) of some compounds isolated from plant extracts; B) UV-Visible spectrum analysis (a'= Et-OH; b'= H-A; c'= Me-OH extracts).

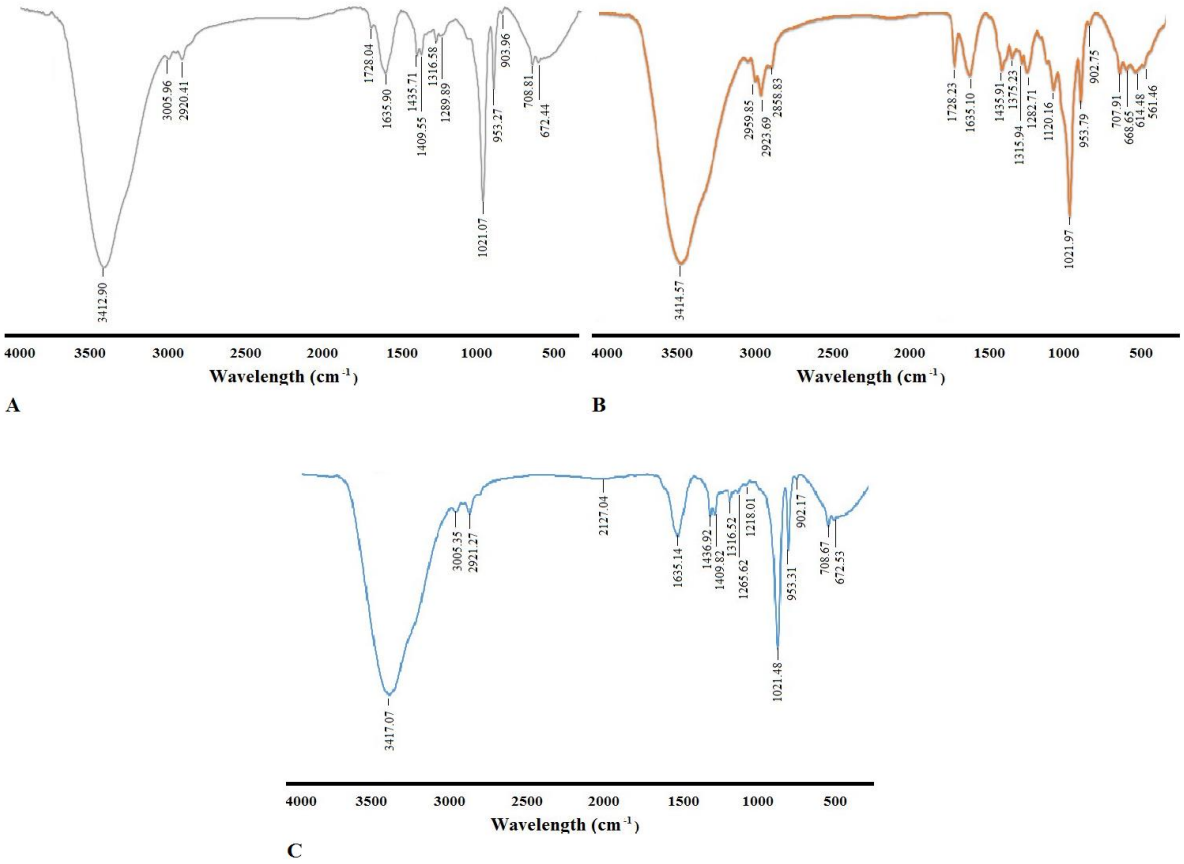

Fig. 2. FT-IR spectrum: A) Et-OH extracts; B) H-A extracts; C) Me-OH extracts**.**

Table 2. The FT-IR spectrum of H-A, Me-OH, and Et-OH extracts from *F. alnus*.

H-A extract			Me-OH extract		Et-OH extract	
S.No	**Peak Value**	**Functional Group**	**Peak Value**	**Functional Group**	**Peak Value**	**Functional Group**
1	3414.57	Alcohol	3417.07	Alcohol	3412.90	Alcohol
2	2959.85	Alkane	3005.36	Alkane	3005.96	Alkane
3	2923.69	Alkane	2921.27	Alkane	2920.41	Alkane
4	2858.83	Alkane	2127.08	Alkane	1726.04	Alkane
5	1728.23	Alkene	1635.14	Alkene	1635.90	Alkene
6	1635.10	Alkane	1436.92	Alkane	1436.71	Alkane
7	1435.91	Alkane	1409.82	Alkane	1409.55	Alkane
8	1375.23	Alkane	1316.52	Alcohol	1316.58	Alcohol
9	1315.94	Alcohol	1256.62	Alkane	1289.89	Alkane
10	1282.71	Alkane	1218.01	Alkane	1021.07	Alcohol
11	1120.16	Alkane	1021.48	Alcohol	953.27	Aromatic compound
12	1021.97	Alcohol	953.31	Aromatic compound	903.96	Aromatic compound
13	953.79	Aromatic compound	903.96	Aromatic compound	708.81	Halogen compound
14	902.75	Aromatic compound	902.17	Aromatic compound	672.44	Halogen compound
15	707.91	Halogen compound	708.67	Halogen compound	—	—
16	668.65	Halogen compound	672.53	Halogen compound	—	—
17	614.48	Halogen compound	—	—	—	—
18	561.46	Halogen compound	—	—	—	—

Phenolic content

According to the results, total phenolic content (TPC) of ethanol, hydro-alcoholic, and methanol extracts were 110.92±0.01, 95.27±0.01 and 126.6±0.02 g GAE/L solvents, respectively. In general, methanol extract had the highest yield and the hydro-alcoholic extraction method showed the lowest efficiency in the extraction of total phenol.

Leakage of cellular contents at 260–280 nm

Antimicrobial activity of different extracts was evaluated against bacterial strains by evaluation of the leakage of intracellular contents like nucleic acids and proteins at 260 and 280 nm (Fig. 3). The results showed that Et-OH and H-A extracts had the strongest effect on the leakage of intracellular contents against both Gram-positive and Gram-negative strains. Meanwhile, the absorption rate of nucleic acids and proteins in the time range of 2 to 4 h was higher in Gram-positive bacteria than Gram-negative strains. After 2 h of incubation, nucleic acid (OD_{260}) and protein (OD_{280}) adsorption of *S. aureus* exposed to H-A extract were 0.31 and 0.96, respectively whereas the adsorption at 260 and 280 nm versus Me-OH extract compounds was measured 0.26 and 0.66, respectively. The highest leakage of nucleic acid and protein was observed for Et-OH extract with OD_{260} and OD_{280} of 0.32 and 1.01, respectively. On the other hand, the highest absorbance was observed for the nucleic acids and proteins contents against H-A extract in *E. coli* with OD_{260} and OD_{280} 0.31 and 0.91, respectively. A similar trend of results was observed for both bacteria after 4 h of incubation with the extracts.

Leakage of potassium ions

The leakage rate of potassium ions (K^+) from bacterial cells exposed to various extracts (H-A, Me-OH, and Et-OH), which corresponds to the cell wall damage, was determined with an atomic absorption spectrometer (Fig.4).

The amount of potassium ion released into extracellular from *E. coli* exposed to H-A, Me-OH, and Et-OH extracts were measured 19.28, 18.20 and 22.54 ppm after 4 h of incubation, respectively, while for *S. aureus*, it was measured 21.45, 19.22 and 23.64 ppm for H-A, Me-OH, and Et-OH extracts, respectively. The control samples for both strains showed a slight increase in the concentration of potassium ions during 4 h and reached 3.25 and 3.14 ppm for *E. coli* and *S. aureus*, respectively.

MIC and MBC of extracts

The antimicrobial activity of H-A, Me-OH and Et-OH extracts of *F. alnus* was evaluated by measuring MIC.

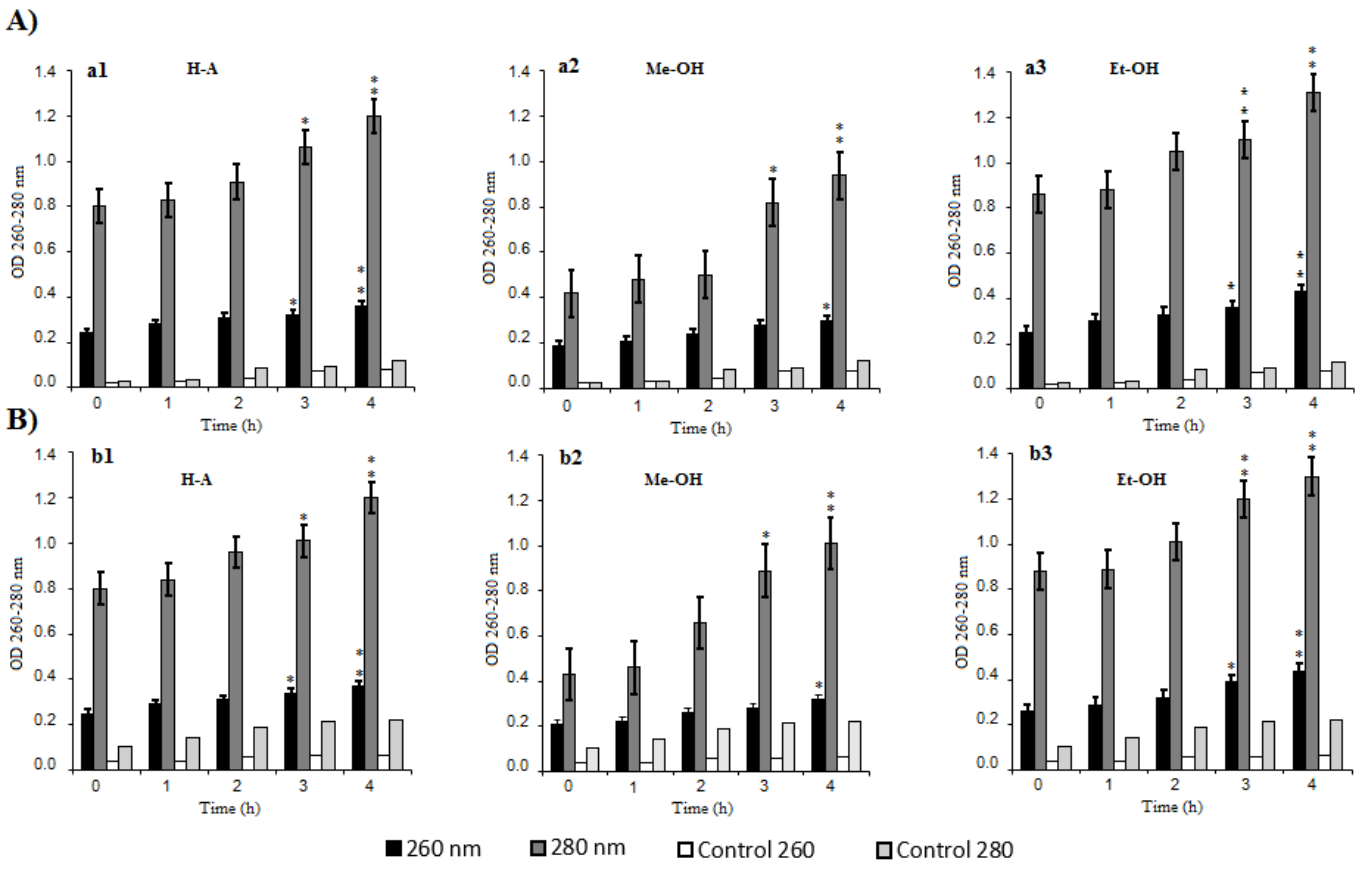

Fig. 3. Effect of H-A, Me-OH and Et-OH extracts (8 mg/mL) on the leakage of cellular contents: A; a1-a3) *E. coli*; B; b1-b3) *S. aureus*; Note: Absorptions at 260 and 280 nm correspond to the released nucleic acids and proteins, respectively.

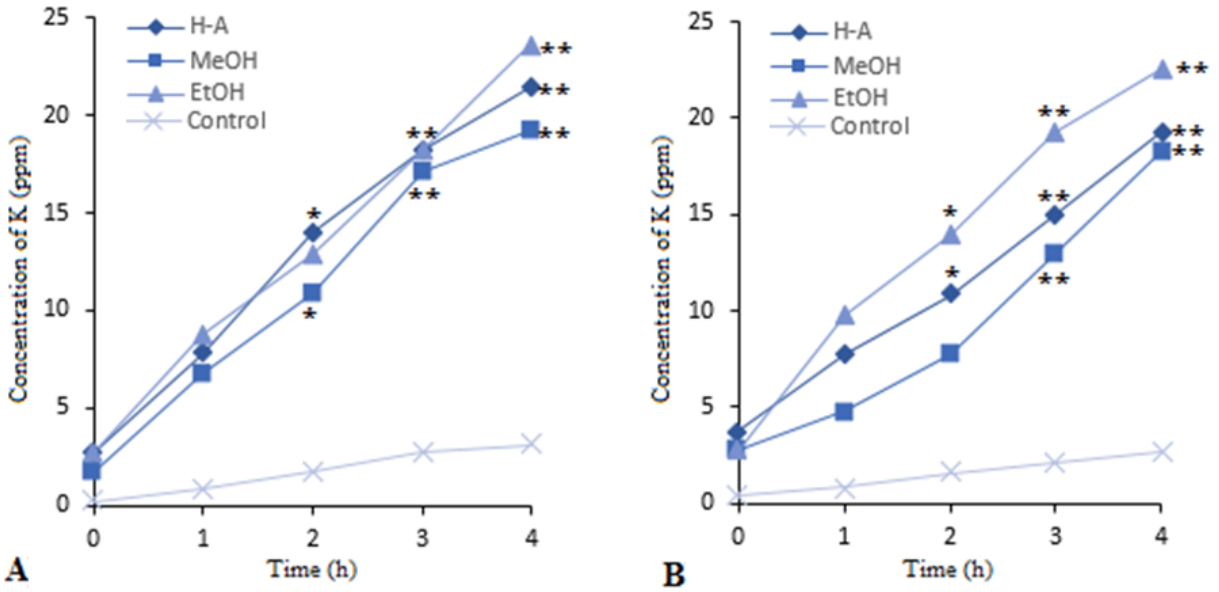

Fig. 4. Release of potassium (K^+) ions during incubation of bacteria with different extracts: A) *S. aureus*; B) *E. coli*. H-A= hydro-alcoholic; Me-OH= methanol; Et-OH= ethanol extracts.

The result indicated that *S .aureus* was more susceptible than *E. coli* to the extracts. The MICs of H-A, Me-OH and Et-OH extracts against *E. coli* were recorded 6.25, 12.5 and 6.25 mg/mL, respectively.

The MIC values of H-A, Me-OH and Et-OH extract against *S. aureus* were determined 3.25, 12.5 and 3.25 mg/ml. The MBC of all extracts against *E. coli* was found in a range of 12.5 to 25 mg/mL. On the other hand, the MBC value of the extracts against *S. aureus* was observed in a range of 6.25 to 25.0 mg/mL.

Synergistic activity

The potential antimicrobial activity of each extract in combination with antibiotics was evaluated using combined disk and checkerboard titration methods. The average ZOI of H-A extract combined with erythromycin (ERY) and ciprofloxacin (CIP) against *S. aureus* was observed to be 27.00 and 25.00 mm, respectively (Table 3, Fig. 5). On the other hand, the combination of H-A extract with ERY and CIP against *E. coli* showed a ZOI of 18.00 and 16.00 mm, respectively.

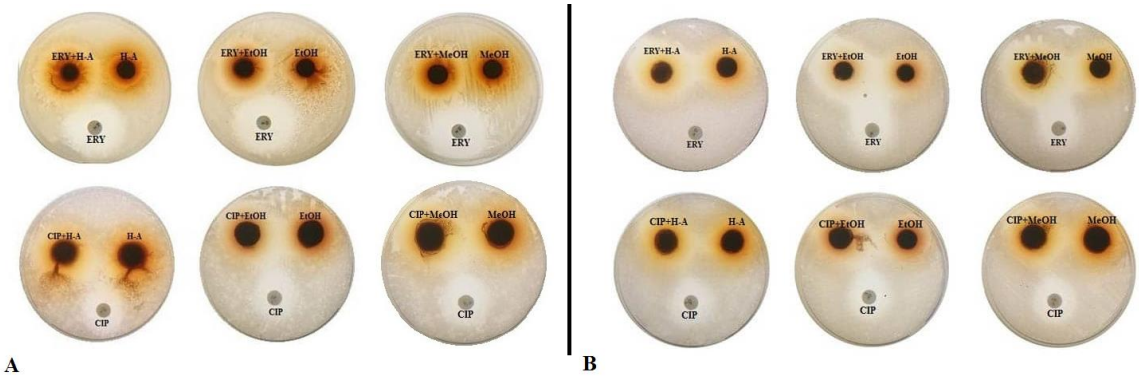

Fig. 5. Antimicrobial activity of the extract: A) Antimicrobial activity alone and combined with antibiotics against *S. aureus*; B) Antimicrobial activity alone and combined with antibiotics against *E. coli*, strains. ERY=Erythromycin; CIP=Ciprofloxacin; H-A=Hydro alcoholic; Me-OH=Methanolic; Et-OH=Ethanolic.

The combination of Me-OH extract with CIP and ERY caused ZOI of 16.00–20.00 and 17.00–19.00 mm for *S. aureus* and *E. coli*, respectively (Table 3). The highest antibacterial activity in combined mode was observed in the presence of Et-OH extract. The zone of inhibition for Et-OH extract in combination with ERY and CIP was observed 23.00 and 19.00 mm, respectively against *S. aureus*, and 23.00 and19.00 mm, respectively, against *E. coli*. In fact, the antibacterial activity of H-A and Et-OH extract ($p < 0.05$) of *F. alnus* extract was statistically higher than the activity of the Me-OH extract. The association between the ethanolic extract and antibiotics against *S. aureus* and *E. coli* increased the antibacterial effects of antibiotics by around 2-folds. In addition, erythromycin combined with extracts exhibited stronger antimicrobial effects against both strains compared to ciprofloxacin.

The synergistic action of H-A, Me-OH and Et-OH extracts of *F. alnus* and antibiotics (erythromycin and ciprofloxacin) were confirmed by measuring FICs. For *E. coli*, the H-A extract exhibited an appropriate synergistic capacity with CIP with FIC of 0.32 ($FIC < 0.5$). The FICs of Me-OH extract with CIP and ERY against *E. coli* were 0.62 and 0.75, respectively, which were considered indifferent. The synergism was noticed for Et-OH extract combined with CIP and ERY with FIC values of 0.32 and 0.25, respectively. On the other hand, a synergistic effect of *F. alnus* extracts with antibiotics against *S. aureus* was observed only for methanol extract in combination with CIP (FIC= 0.25) and H-A and Et-OH extracts combined with ERY with FIC values of 0.25 and 0.28, respectively (Table 3).

Table 3. Antimicrobial potential of extracts in combined mode with antibiotics and individual forms against tested strains.

Agents	*S. aureus*				*E. coli*			
	ZOI (mm)				ZOI (mm)			
	CI	ATCC 25923	FIC	Rank*	CI	ATCC 25922	FIC	Rank*
Erythromycin (ERY), (15µg/disc)	14.00±1.0	20.00±0.6		B	13.00±0.2	12.00±0.6		B
H-A extracts (8 mg/mL)	11.00±0.6	15.00±1.2		C	10.00±0.2	11.00±0.6		C
ERY + H-A	16.00±0.6	27.00±01	0.25	A	16.00±0.6	21.00±0.6	0.50	A
Increase in fold area	0.30	0.82		-	0.51	2.0		-
Ciprofloxacin (5 µg/disc)	13.00±0.2	13.00±01		B	12.00±1.0	10.00±0.6		B
H-A extracts (8 mg/mL)	11.00±0.6	15.00±0.6		B	10.00±1.0	11.00±1.0		C
CIP + H-A	14.00±1.0	25.00±1.0	0.50	A	15.00±0.6	17.00±1.0	0.32	A
Increase in the fold area	0.15	2.6		-	0.56	1.8		-
Erythromycin (ERY), (15µg/disc)	14.00±1.0	20.00±0.6		B	13.00±0.2	12.00±0.6		B
Me-OH extracts (8 mg/mL)	10.00±1.0	12.00±1.5		C	11.00±0.6	14.00±0.2		B
ERY + Me-OH	17.00±0.2	24.00±0.0	0.62	A	15.00±0.6	24.00±1.0	0.75	A
Increase in fold area	0.47	0.21		-	0.33	3		-
Ciprofloxacin (5 µg/disc)	13.00±0.2	13.00±1.0		B	12.00±1.0	10.00±0.6		C
Me-OH extracts (8 mg/mL)	10.00±0.0	12.00±1.0		C	11.00±0.0	14.00±0.2		B
CIP + Me-OH	13.00±1.0	20.00±0.2	0.25	A	16.00±1.0	19.00±0.2	0.62	A
Increase in the fold area	0	1.3		-	0.77	2.6		-
Erythromycin (ERY), (15µg/disc)	14.0±1.0	20.00±0.6		B	13.00±0.2	12.00±0.6		B
Et-OH extracts (8 mg/mL)	12.0±0.2	15.00±0.0		C	11.00±0.0	14.00±1.0		B
ERY + Et-OH	22.0±0.0	25.00±0.0	0.28	A	21.00±1.0	25.00±1.0	0.25	A
Increase in the fold area	1.4	0.56		-	1.6	3.3		-
Ciprofloxacin (5 µg/disc)	13.00±0.2	13.00±1.0		C	12.00±1.0	10.00±0.6		C
Et-OH extracts (8 mg/mL)	12.00±0.0	15.00± 0.0		B	11.00±0.0	14.00±0.6		B
CIP + Et-OH	17.00±0.2	21.00±0.6	0.56	A	19.00±0.0	20.00±0.2	0.32	A
Increase in fold area	0.71	1.6	-	-	1.5	3	-	-

CI= Clinical isolates; *=Alphabets indicate the rank order ($p < 0.05$*); vertically, ranking associated with antibiotic combinations against bacterial strains. ZOI= zone of inhibition.

Discussion

This study was conducted to investigate and establish the antimicrobial potential of *F. alnus* extracts alone and in combination with current antimicrobial drugs (ciprofloxacin, erythromycin) against *S. aureus* and *E. coli* bacterial strains. The chemical constituents derived from *F. alnus* have been evaluated using GC/MS, UV-VIS, and FT-IR. The GC/MS analysis of *F. alnus* revealed the presence of alkanes, alkenes, phenols, alcohols, esters, terpenes, fatty acid, tetrazole, haloalkane, anthraquinone, aromatic, and plasticizer compounds in the ethanol, methanol, and hydro-alcoholic extracts. The FT-IR spectrum of *F. alnus* with different extracts showed functional groups according to the GC/MS analysis of *F. alnus*. Based on the results obtained in this study, the highest yield of the TPC was observed in ethanol and methanol extracts. In fact, polar solvents including ethanol, methanol, ethyl acetate, and acetone have been recognized as efficient solvents for phenol extraction (Do *et al.*, 2014) (10). Ethanol is also regarded as a safe solvent for pharmaceutical products.

Our study demonstrated that the extracts from *F. alnus*, could inhibit and kill bacterial cells at low concentrations. The antimicrobial potential of extracts was investigated by evaluation of the leakage of cellular contents (nucleic acid-protein) and the release of K^+ ions. The H-A, Me-OH and Et-OH extracts showed an appropriate antimicrobial effect due to release intracellular contents. From a structural point of view, the bacterial cytoplasmic membrane has selective permeability which controls the entry and exit of important compounds throughout the cell membrane and is vitally important for cellular normal function and viability (Cox et al. 2000). The phenolic compounds in the plant extracts have been reported to be able to damage the bacterial cytoplasmic membrane, which interrupts membrane selective permeability and causes leakage of intracellular contents which results in cell death. It is likely that the detected phenolic compounds in this study including, 2, 4-Di-tert-butylphenol, Butylated Hydroxyl-toluene, 4-{2-[4-(Dimethylamino) phenyl]-5-phenyl-1H-imidazol-4-yl} phenol from Me-OH extracts and 2, 5-Di-tert-butylphenol, Butylated

hydroxyl-toluene from Et-OH extracts could lead to the leakage of cellular content and release of K^+ ions. In general, the effects of phenolic compounds can be attributed to the destruction of bacterial outer membrane (in Gram-negative bacteria) and cytoplasmic membrane which interfere cellular energy production, and membrane electrical charge and membrane dependent physicochemical properties which cause harmful effects on the other cellular functions and results in cell death (Calo *et al.*, 2015; Carson *et al.*, 2002).

The results showed that there was an association between incubation with the extracts and leakage of cellular contents. In other words, as incubation time was prolonged, a higher rate of cell destruction and leakage of cell contents was observed which could be associated with higher penetration of antimicrobial molecules into the bacterial cells.

Based on our study, *F. alnus* extracts had a board range of antimicrobial activity against *E. coli* and *S. aureus* strains. In this regard, some studies have demonstrated that this effect could be attributed to the hydrophobic compounds that are found in the essential oils of plants (Chouhan *et al.*, 2017). It is possible that the derived oleic acid by ethanol and hydro-alcoholic extract in this study could cause antimicrobial effects on the bacterial cell. Oleic acid is a fatty acid (FFA) which was detected in Et-OH and hydro-alcoholic extracts of *F. alnus* from GC/MS analysis in this study. The permanent or transient pores with variable sizes are created by interacting with fatty acids in the cell membrane. The membrane can be solubilized at higher concentrations of antimicrobial lipid compounds. Dilika et al. (2000) demonstrated that the extraction of oleic acid by dichloromethane from the leaves of *Helichrysum pedunculatum* had appropriate antimicrobial activity against several Gram-positive bacterial strains. In another research which was conducted by Stenz *et al.* (2008), showed that oleic acid could inhibit bacterial viability and biofilm production by *S. aureus*. Actually, essential oils have the ability to attach cell membranes and induce changes in bacterial cell membranes. With such an action, the transfer of antimicrobial agents throughout the cytoplasmic membrane is accelerated and bacteria cannot transfer them from the intracellular space to the extracellular space (Souza *et al.*, 2010; Ultee *et al.*, 2001). However, *E. coli* exhibited greater resistance to these compounds which could be associated with its outer membrane which is found in Gram-negative bacteria and can inhibit the penetration of the variety of antimicrobial compounds (Calo *et al.*, 2015).

The association between the Et-OH bark extract with ERY and CIP against Gram-positive and Gram-negative strains increased the antibacterial effects of the antibiotic by around 2-folds in the combined mode of action. Actually, these combinations were beneficial as they provided enhanced antimicrobial efficacy and increased inhibitory potential of the combination compared to the extracts and the antibiotics alone.

The FICs of extracts was also evaluated in the presence of antibiotics (CIP and ERY), and the result indicated that Et-OH and H-A extracts had suitable synergistic capacity with antibiotics. Actually, no antagonistic behavior was found through the interaction of antibiotics and extracts. Concerning the synergistic effect of antibiotics and various extracts, it can be concluded that the active compounds present in the extracts can damage the bacterial cytoplasmic membrane and cell wall, and then the antimicrobial drugs will be able to enter simply into the bacterial cell and cause adverse effects on their targeted sites (Souza *et al.*, 2010; Hutchings and Cock 2018).

In addition, chrysophanic acid as an anthraquinone derivative was also observed in Et-OH and hydro-alcoholic extract of *F. alnus* by GC/MS analysis. Anthraquinones, anthracenediones or dioxoanthracenes, are the main members of the quinone family with antibacterial activity. Up to date, most of the isolated anthraquinones from diverse plant sources indicated the antibacterial activity (Daly *et al.*, 2015). For example, the antibacterial activity of several anthraquinone derivatives such as 2-acetyl-3, 8-dihydroxy-6-methoxyanthraquinone, glucofrangulin A, 1, 8-dihydroxy-2-[(z)-4-methylpenta-1, 3-dien-1-yl] anthraquinone, and emodin isolated from the methanol extract of *Rhamnus cathartica* was studied. The results were shown that emodin and 1, 8-dihydroxy-2-[(z)-4-methylpenta-1, 3-dien-1-yl] anthraquinone have activity against *S. aureus*

and *E. coli*. The 2-acetyl-3, 8-dihydroxy-6-methoxyanthraquinone only displayed activity against *E. coli*. The negative effect against *Aspergillus niger* was exhibited by all compounds together with the methanol extract (Hamed *et al.*, 2015). The mechanisms of antibacterial activity in anthraquinones are diverse. Most of them are involved with alteration of metabolic pathways, simple destabilization of the cell wall, or DNA inclusions via oxidative stress. The polarity of group substituents, steric effect, and pH are the main factors in the efficacy of these mechanisms (Malmir *et al.*, 2015).

Conclusion

In conclusion, the different extracts of *F. alnus* showed good antibacterial activity against *E. coli* and *S. aureus*. In the synergism mode of action with ciprofloxacin and erythromycin, the efficacy of antibiotic was enhanced by around 2-fold when compared to the drugs alone. Their roles on bacterial cell membrane integrity showed that active compounds in the *F. alnus* extracts can penetrate into the cell membrane and destroy cell wall structure and subsequently, induce the leakage of intracellular contents and potassium ions. Such features make *F. alnus* and consequently its derivatives appropriate candidates for some specific applications including therapeutic, food and/or sanitary industry. However, further studies must be conducted to determine toxicity of the extracts in *in vivo* condition.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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