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Synthesis and Evaluation of Antimicrobial Properties of Some Chalcones

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Authors' contributions

This work was carried out in collaboration between all the authors. Author NB participated in protocol writing, supervision of chalcone synthesis and manuscript writing. Author FNC designed the study, developed the protocol, data mining, wrote and review of the manuscript. Author NNA wrote the protocol and laboratory synthesis of chalcone. Author TFE gave the concept and designed of antibacterial studies, data mining and review of manuscript. Author FKA participated in laboratory bacteria testing, drafting of protocol and review of manuscript data analysis. Authors AP, GKH, TAR and AKO did chalcone synthesis, reviewed of literature and bacteria testing. Author BTN supervised the chalcone synthesis and reviewed the manuscript. All the authors read and approved the final manuscript.

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ABSTRACT

Introduction: Recently there has been a great deal of interest in the health benefits of phytochemicals, particularly prenylated and allylated flavonoids. Chalcones (1, 3-diaryl-2-propen-1-ones) and their derivatives are important intermediates of the flavonoid synthetic pathway.
Aims: To synthesis chalcones, and investigate their antimicrobial properties by determining their diameters of inhibition; minimum inhibitory concentration (MIC); and their minimum bactericidal and fungicidal concentrations (MBC), using the Kirby-Bauer diffusion and microdilution method.

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Study Design: Experimental analytical study.
Place and Duration of Study: The study was done in the Laboratory of Organic Chemistry of the Faculty of Science, University of Yaounde1 and the Bacteriology Laboratory of the Yaoundé University Teaching Hospital.
The study was done in a period of 6 months, from the 15th of December 2014 to the 22nd of May 2015.
Methodology: The chalcones (**3a-c**) were synthesized by the Claisen Schmidt condensation reaction of vanillin (1) with different acetophenone derivatives (**2a-c**).
Results: The structures of the compounds were confirmed by spectral data (^{1}H and ^{13}C Nuclear Magnetic Resonance). Compound 3c is a new compound while the compounds 3a and 3b have already been reported [26,27]. The synthesized compounds were tested for their antimicrobial activities (agar disc-diffusion and microdilution methods). All the synthesized chalcones were active against the test microbes: The most potent compound was **3c**; bactericidal on 50% of strains with a MIC between 62.5 and 250 µg/mL, the most sensitive strains being *S. aureus* and *C. albicans*, and the least sensitive being *E. faecalis*.
Conclusion: Synthesized chalcones showed antimicrobial properties and are very promising as lead compounds for drug development.
We demonstrated that all the test microbes were susceptible to the 3 synthesized chalcones at low concentrations. The halogenated chalcone (**3c**) was the most potent of the synthesized chalcones against reference strains of Gram negative and Gram positive bacteria, and the clinical strain of *C. albicans.* Therefore the presence of halogens in chalcones increases their microbial susceptibility.

Keywords: Synthesis; chalcones; antimicrobial; structure- activity relationship.

1. INTRODUCTION

Recently there has been a great deal of interest in the health benefits of phytochemicals, particularly prenylated and allylated flavonoids. Chalcones (1, 3-diaryl-2-propen-1-ones) and their derivatives are important intermediates of the flavonoid synthetic pathway. Chalcones, one of the major classes of natural products with a widespread distribution in fruits, vegetables, spices, tea and soy based food stuff have also been the subject of great interest for their interesting pharmacological activities especially against infectious diseases [1,2].

Till date infectious diseases remain key public health problems. Globally, pneumonia, meningitis, and nosocomial infections remain serious and costly health problems. Global travel and migration increases everyone's susceptibility to epidemic and emerging diseases, from both natural (e.g., bird flu) and deliberate (terrorism) causes [3]. In 2008, a total of 8.8 million children died before their fifth birthday – half of them in Sub-Saharan Africa. Pneumonia, diarrhoea, malaria, HIV/AIDS and measles caused 44 percent of the deaths in children under five years. New born deaths from sepsis and tetanus in the first four weeks of life accounted for a further 7 percent [4].

Antibiotics and similar drugs, together called antimicrobial agents, have been used for almost a century to treat patients who have infectious diseases. Since the 1940s, these drugs have greatly reduced illness and death from infectious diseases. They are obtained from microorganisms, plants and animals; some are Semi-synthesized from natural products while others are purely synthetic products. However, these drugs have been used so widely and for so long that the infectious organisms they are designed to kill have developed resistance against them [5].

The emergence and dissemination of drug resistant microorganisms represent an expanding threat, "antibiotic resistance is rising for many different pathogens that are threats to health, if we don't act now, our medicine cabinets will be empty and we won't have the antibiotics we need to save lives," said the Centre for Diseases Control and prevention (CDC) Director Tom Frieden. Our abilities to develop and deliver new drugs and vaccines to control these diseases have also increased [4,6].

The current strategies put in place by organizations to overcome this global problem of antimicrobial resistance include the research and development of new molecules with antimicrobial potentials [6]. Plants possess bioactive metabolites like alkaloids, saponins, tannins, phenolic compounds and flavonoids which are potential antimicrobial compounds. Their use in the treatment of infectious diseases is known

from antiquity [7]. Chalcones, a group of flavonoid compounds with various substitution patterns on its two aromatic rings, constitute an important class of natural products isolated from plants. They have been reported to possess a wide spectrum of biological activities, including anti-bacterial, anti-fungal, anti-inflammatory, anti-tumor, insect anti-feedant and anti-mutagenic activities [1,8-17]. Additionally, some chalcone derivatives have been found to inhibit several important enzymes in cellular systems, such as xanthine oxidase and protein tyrosine kinase. Chalcones are also key precursors in the synthesis of many biologically important heterocycles such as benzothiazepine, pyrazolines, 1, 4-diketones and flavones [18]. Hence, the synthesis of chalcones has generated vast interest among organic as well as medicinal chemists, and they are very promising agents for the fight against resistant microbes.

This has prompted us to carry out the synthesis of substituted Chalcones and to evaluate their antimicrobial properties. These compounds have been reported by several authors to have antimicrobial properties [10,14,15], but several substituted chalcones, their susceptibility on common species of microbes and their structure activity relationship are still to be elucidated. Hence we have conducted the synthesis of some chalcones, and have investigated their antimicrobial properties by determining their diameters of inhibition; minimum inhibitory concentration (MIC); and their minimum bactericidal and fungicidal concentrations (MBC), using the Kirby-Bauer diffusion and microdilution methods.

2. MATERIALS AND METHODS

2.1 Chemistry

All of the compounds were characterized by ^{1}H and ^{13}C NMR spectra recorded with Bruker WM-300 in MeOD at 400 and 100 MHz, respectively using tetramethyl sylane (TMS) as the internal standard. All chemical shifts are reported on δ scale.Tin layer chromatography (TLC) was carried out using Merck silica gel 60 F-254 plates (layer thickness: 0.25 mm) and UV lamp; Silica gel for column chromatography, and all solvents were distilled before using.

2.2 Synthesis

The chalcones **3a-c** were obtained by the Claisen-Schmidt condensation of respective quantities of vanillin (**1**) and acetophenones **2a-c** in the presence of potassium hydroxide, by a known literature method [1].

2.3 Biology

In order to determine the diameters of inhibition of our test compounds, the inoculums were used to prepare bacteria solutions to the Mc Farland standard. The test compounds were diluted in test tubes, with a serial two fold dilution. A sterile swab was dipped into the microbial suspension and the excess moisture was expressed by pressing the swab against the side of the tube. The surface of the agar was completely swabbed. Swabbing was done to ensure that no area was left on the plate unswabbed. After completely swabbing the plate, it was turned on 90° and the swabbing process repeated. The swabbing was done around the circumference of the plate before discarding the swab in the discard bag. The surface of the plate was allowed for 5 minutes to get dry, before the test compounds were placed on the agar. A pair of forceps was used to remove each antibiotic disc or sterilized spherical filter paper from the dispenser. The spherical filter papers were placed in the disc at equidistant from each other, around the periphery, with the reference antibiotic (Norfloxacine or Amphotericin B) placed at the centre—not too close to the edge of the plate. The forceps were sterilised on the Bunsen burner flame. The spherical papers were numbered from 1 to 7. Different concentrations of each test compound from the numbered test tubes were placed on the respective papers in the medium. The disk were labelled, turned upside down and incubated at 37℃ for 18-24 hrs. The diameter of inhibition was read using a calliper and reported in millimetres, the results were interpreted as S (sensitive), if there was inhibition or R (resistant) if no inhibition.

The different microbial inoculum were used to prepare Mc Farland standard bacteria suspensions. Serial two fold dilutions of the test compounds were prepared in test tubes, beginning from 2000 ug/mL to 62.5 ug/mL. 50 ul of the test compound solution was placed in the wells of the microtitre trays except in the positive control well, and labelled. 50 uL of broth was added to each well. 1 uL of microbial suspension of each microbe was inoculated into the wells, except in the negative control well. The trays were kept in the oven at 37℃ for 18 hours. The minimal inhibitory concentration was read by observing the opacity of the wells. The inhibition of microbial growth by the test compounds left some wells clear (not turbid), except in the well

that had no microbe added to it, (negative control). Beginning from the least concentrated well, the first well in which no turbidity was observed was considered the minimum inhibitory concentration of the test compound.

Culture media were prepared, segmented and labelled with the corresponding microbe name and well number, that is, the wells with no turbidity observed in the microtitre trays; the positive; and the negative control wells. Using the inoculating loop, each segment of the culture media was inoculated with the solution taken from the corresponding well of the microtitration tray. The culture media were incubated at 37ºC for 18 hours and the results were read by observing if there was growth or inhibition of growth. The segments showing no microbial growth had the microbes killed by the test compound. This is confirmed by the fact that growth of microbes was observed in the positive control segment (segment that contained only broth and microbes, with no test compound). The segment containing the lowest concentration of test compound in which growth was inhibited was considered the minimal bactericidal concentration of the compound.

3. EXPERIMENTAL

3.1 4'-Hydroxychalcone (3a)

1.118 g (being 0.00734 mols) of vanillin (**1**) was weighed and placed in a mortar, 1.000 g (being 0.00734 mols) of 4'-hydroxyacetophenone (**2a**) was added and mixed thoroughly until a homogenous mixture was obtained. To the mixture we added 0.8241 g (being 0.01468 mols) of KOH and grinded with a pestle. Samples of the reaction mixture were collected every two minutes and analysed by TLC till the end of the reaction. The pale yellow solid mixture obtained at the end of the reaction was washed with cold distilled water and product **3a** was obtained (1252 mg yield 163.1%). 1H NMR (400MHz; MeOD) see Table 1.

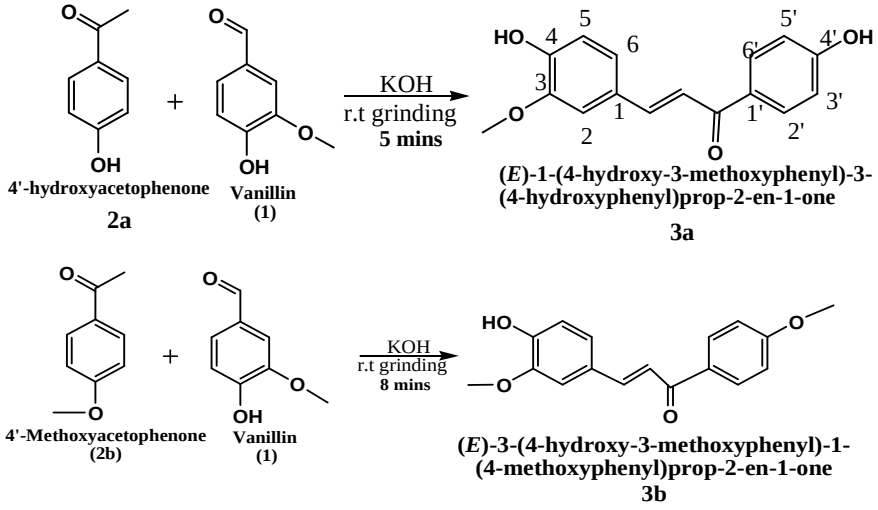
3.2 4'-Methoxychalcone (3b)

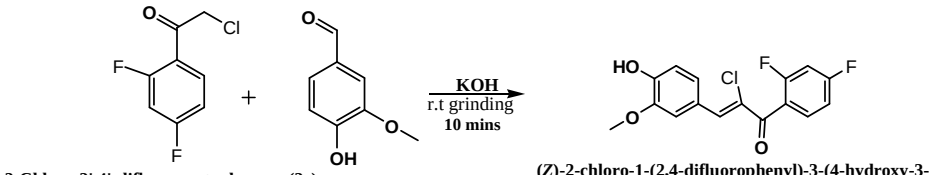
1.013 g (being 0.006659 mols) of vanillin (**1**) was weighed and placed in a mortar, 1.000 g (being 0.006659 mols) of 4'-methoxyacetophenone (**2b**) was added and mixed thoroughly until a homogenous mixture was obtained. To the mixture we added 0.747 g (being 0.006659 mols) of KOH and grinded with a pestle until the mixture became a clear colourless liquid which gradually changed into a yellowish solid.

The yellowish solid later changed to a white pasty solid substance. Samples of the reaction mixture were collected every two minutes and analysed by TLC till the end of the reaction.

3.3 2-Chloro-2',4'-difluorochalcone (3c)

0.798 g (being 0. 00525 mols) of vanillin (**1**) was weighed and placed in a mortar. 1.000 g (being 0. 00525 mols) of 2-chloro-2', 4'-difluoroacetophenone (**2c**) was added and mixed thoroughly until a homogenous mixture was obtained. To the mixture we added 0.588 g (being 0.01048 mols) of KOH and grinded with a pestle. The reaction mechanism is presented below. The reaction mixture was collected every two minutes and analysed by TLC till the end of the reaction was observed.





2-Chloro-2',4'-difluoroacetophenone (2c) + Vanillin (1)

(*Z*)-2-chloro-1-(2,4-difluorophenyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one

(3c)

4. RESULTS AND DISCUSSION

We carried out the synthesis of several substituted chalcones, and from the results obtained, synthesis by grinding was found to be the most convenient, eco-friendly and affordable method, which also required less apparatus, as compared to synthesis by solvent dissolution. The yields of the reaction carried out by grinding had no significant difference to that of the reaction carried out in a solvent. This is in conformity with the works published by several authors who carried out the synthesis of chalcones by the grinding method [19-21].

The grinding method is more convenient, requiring less cumbersome apparatus, and hence reducing the dangers associated with the use of such apparatus.

This method is eco-friendly in the sense that no solvent is used in the course of the chemical reaction, which can act as a pollutant in the atmosphere.

The method is more affordable than using a solvent, because the cost of production is greatly reduced: cost of solvent is eliminated; cost of cumbersome apparatus eliminated; and the cost of technical knowledge on the manipulation of such apparatus is also eliminated. This reaction can actually be carried out in any average chemistry laboratory, which is an advantage to those living in under developed countries.

The grinding method also requires far lesser time than synthesis in a solvent. Therefore the number of products one can obtain per unit time is far greater using this method, making it a better method for the research and development of chalcones with medicinal potentials.

Pure products were isolated in quantitative yield by washing with water to remove excess of base, and by column chromatography when there was a need. The reaction furnished chalcones (**3a**, **3b**, **3c**) in 60-75 % yield. From the results, we can deduce that the presence of electron releasing and withdrawing substituents have no significant effect in the formation of products, this is in conformity with the work of Zangade et al. [19].

In the reported synthesis, all the reactions were proceeded in the same manner and there was no significant difference in yield, purity or reaction time when different substituents were present on ketone.

All the products obtained were characterized on the basis of their analytical and spectral NMR data, which in modern laboratories, is the first choice method for gaining structural information according to the work published by Bruice PY in 2011 [22].

Tables 1-3 give the ^{1}H and ^{13}C data of the three compounds. A compound **3c** is reported here for the first time. These values are in conformity with values obtained by Ngameni et al. [11].

The synthesis of compound **3c** gave the main product in a high yield, with several secondary products at the end of the reaction. This is probably due to the fact that the reaction was carried out for a longer period of time than necessary (the other reactions yielding little or no secondary products were carried out during a shorter period of time), this is in conformity with the work published by Zangade et al. [19].

4.1 Microbial Susceptibility Testing

All the chalcones synthesized showed antibacterial activity with varying minimum inhibitory and minimum bactericidal concentration (MIC and MBC) values.

The Kirby-Bauer diffusion method was used to determine the diameters of inhibition of the test compounds, compared to that of standard antibiotics. Most of the test microbes were susceptible to the test compounds at 1000 µg/m (Fig. 2), but owing to the fact that this method has so many shortcomings, and having in mind that this method is only used to determine

microbial susceptibility or resistance to an antimicrobial we proceeded with the dilution (microdilution) method, being the most reliable standard test for the determination of the MIC and the MBC of an antimicrobial, as published by Rene Hendriksen in 2009 [23,24].

All the compounds inhibited the growth of bacteria in a concentration dependent mode. A minimal inhibitory concentrations (MIC) of 62.5 µg/mL was recorded with compound 3c, though this was the lowest diluted concentration on which we carried out the determination of MIC and MBC/MFC on the test microbes. This implies that the dilution of compound 3c could be increased to concentrations less than 62.5 µg/mL and re-evaluated. Similar results were obtained for the MBC of **3c** on Gram negative (*H. influenza*), Gram positive (*S. aureus*) and for the MFC on *C. albicans* strains with a value of 62.5 µg/mL.

Compound **3a** was found to be active against most of the microbes, with a MIC of 62.5 µg/mL and an MFC of 500 µg/mL recorded against *C. albicans,* and 125 µg/mL recorded against *S. aureus* being the lowest recorded against all the bacterial strains on which this compound was tested. The compound showed no activity against *H. influzae, E. coli and N. gonorrheae*.

Table 1. ^{1}H NMR (400MHz; MeOD)δ_{ppm}(m, J(Hz), and ^{13}C NMR (100MHz; MeOD)δ_{ppm} of compound 3a

Position of atom	^{1}H NMR(400MHz; MeOD)δ_{ppm}(m, J(Hz)	^{13}C NMR (100MHz; MeOD)δ_{ppm}:
1	-	127.9
2	7.33(1H; d; J=2.0 Hz)	116.4
3	-	149.7
4	-	148.7
5	6.83(1H; d; J=7.2 Hz)	116.2
6	7.31(1H; dd ; J=7.2 and 2.0 Hz)	132.1
C=O	-	192.9
α	7.77(1H; d; J=12.0 Hz)	120.8
β	7.90(1H; d; J=12.0 Hz)	147.9
1’	-	131.1
2’	7.32(1H; d; J=8.4 Hz)	130.7
3’	6.75(1H; d; J=7,6 Hz)	115.9
4’	-	154.7
5’	6.75(1H; d; J=7.6 Hz)	115.8
6’	7.32(1H; d; J=8.4 Hz)	130.7
CH_3O-	3.74(1H; s)	56.5

Table 2. ^{1}H NMR (400MHz; MeOD) δ_{ppm}(m, J(Hz), and ^{13}C NMR (100MHz; MeOD)δ_{ppm} of compound 3b

Position of atom	^{1}H NMR (400MHz; MeOD)δ_{ppm}(m, J(Hz)	^{13}C NMR (100MHz; MeOD)δ_{ppm}:
1	-	131.1
2	7.31(1H; d; 1.6 Hz)	115.8
3	-	148.9
4		149,7
5	-6.87(1H; d; J=2.0 Hz)-	114,8
6	7.33(1H; dd; J=8 and 2.0 Hz)	131.1
C=O	-	192.9
α	7.51(1H; d; J= 15,6 Hz)	120.8
β	7.90(1H; d; J=15,6 Hz)	147.9
1’	-	130.7
2’	6.99(1H; dd; J=10 and 2.0 Hz)	127.9
3’	-	148.9
4’	-	154.7
5’	6.78(1H; dd; J=8.4 and 1.4 Hz)	111.2
6’	6.97(1H; dd; J=10.0 and 2.0 Hz)	130.4
CH_3O-	3.93(3H; s)	56.5
CH_3O-	3.93(3H; s)	56.4

Table 3. ^{1}H NMR (400MHz; MeOD)δ_{ppm}(m, J(Hz), and ^{13}C NMR (100MHz; MeOD)δ_{ppm} of compound 3c

Position of atom	^{1}H NMR (400MHz; MeOD)δ_{ppm}(m, J(Hz)	^{13}C NMR (100MHz; MeOD)δ_{ppm}:
1	-	131,1
2	7.33(1H; d; 2.0 Hz)	115.8
3	-	149.7
4	-	147,9
5	6.66(1H; d; J=2.0 Hz)	116,4
6	7.31(1H; dd; J=8.0 and 2.0 Hz)	120,8
C=O	-	192.9
α	-	120,7
β	7.34(1H; s)	128,0
1’	-	116,3
2’	-	148,9
3’	6.68(1H; d; J=1.6 Hz)	115,7
4’	-	154.7
5’	6.85(1H; d; J=8.0 Hz)	111.2
6’	6.78(1H; dd; J=8.0 and 1.6 Hz)	130,72
CH_3O-	3.73(3H; s)	56.5

Table 4. Elemental analysis of products

Entry N$^{0.}$	Codes	%C	%H	%O	%Cl	%F	Expected yield (g)	Actual yield (g)	Percentage yield (%)
1	**3a**	65.44	5.47	23.68	0	0	1.9851	1.2521	63.1
2	**3b**	62.40	5.22	22.51	0	0	1.8932	1.4231	75.2
3	**3c**	58.86	5.16	14.78	10.92	11.7	1.7039	1.0251	60.2

Table 5. Physico-chemical properties of products

Compound	Chemical Name	Molecular Formula	Molecular weight (g)	Melting Point (ºC)	Reaction Time (minutes)
3a	4’-Hydroxychalcone	$C_{16}H_{14}O_4$	270.28	79.8	5
3b	4’-Methoxychalcone	$C_{17}H_{16}O_4$	284.3065	81.0	8
3c	2-Chloro-2’,4’-difluorochalcone	$C_{16}H_{11}ClF_2O_3$	324.7065	79.05	10

Compound **3b** was the least potent compound tested on the microbes, with a MIC and MBC of 250 µg/mL recorded against *H. influenzae* and a MIC and MFC of 500 µg/mL recorded against *C. albicans*. Though compound **3b** was found to be less potent than both **3a** and **3c**, yet it was active at higher concentrations (between 1000 µg/mL and 2000 µg/mL) against all the strains of the test microbes (broad spectrum) unlike **3a** which had a limited spectrum of action.

Compound **3b** was noted to be more potent on Gram negative microbes, while **3a** was more potent on the Gram positive microbe (*S. aureus*) and the fungus *C. albicans.*

4.2 Structure Activity Relationship (SAR)

We noted that all the chalcones synthesized were more potent than the reagents on the test microbes, this is partly due to the fact that the α, β-unsaturated carbonyl group present on chalcones enhances the antimicrobial activity of chalcones, as this structural feature was distinctive for all the chalcones synthesized, this finding is in line with that of Padarthi et al. [1].

The presence of halogens in the chalcones (compound **3c**), increased their potency (MIC value of 62.5 µg/mL) against Gram negative, Gram positive organisms, and *C. albicans*, this is similar to the finding of Doan et al. [10].

The substitution of a methoxy group at position 4' of a chalcone (compound **3b**) was noted to render them bactericidal with increase potency against Gram positive organisms, and reduced potency against Gram negative organisms, this is similar to the findings of Rahman in 2011 [25,26, 27].

On the other hand, the substitution of a hydroxyl group at the same position (compound **3a**) had an opposite effect: increased potency against Gram negative organisms, and decreased potency against Gram positive organisms, with bactericidal action against Gram negative bacterial and bacteriostatic and fungistatic against *S. aureus* and *C. albicans*.

Substituting the hydroxyl group with a methoxy group at position 4' showed no change in the activity on *C. albicans* (MIC: 500 µg/mL), suggesting that the antifungal activity of compound **3a** and **3b** is not due to these groups, neither is it affected by these groups, it is rather due to the presence of the α, β-unsaturated carbonyl group present in chalcones, as suggested by literature [1,20].

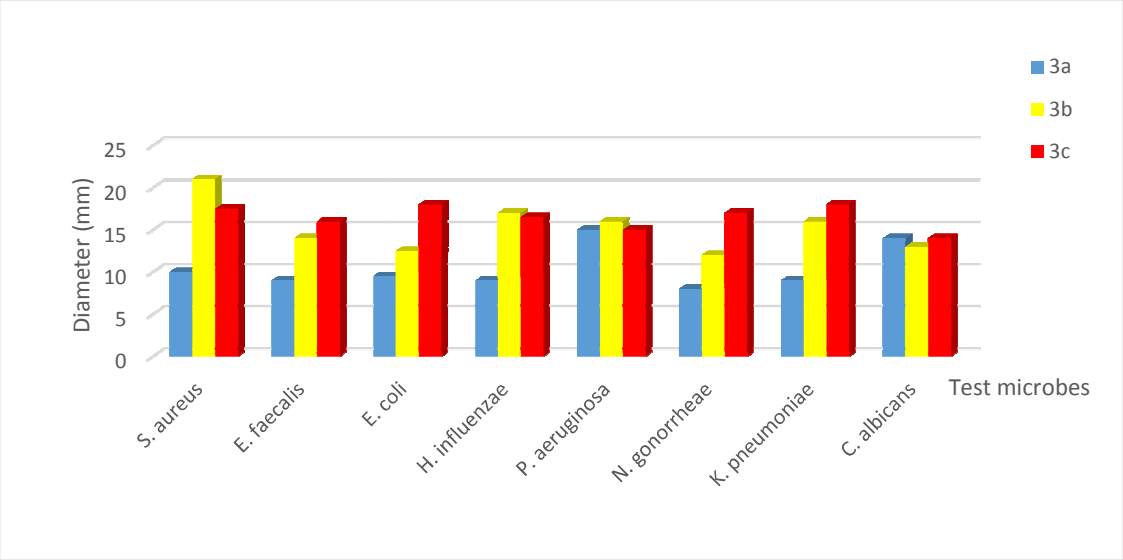


Fig. 1. Diameters of ihnibition of test Compounds on test microbes

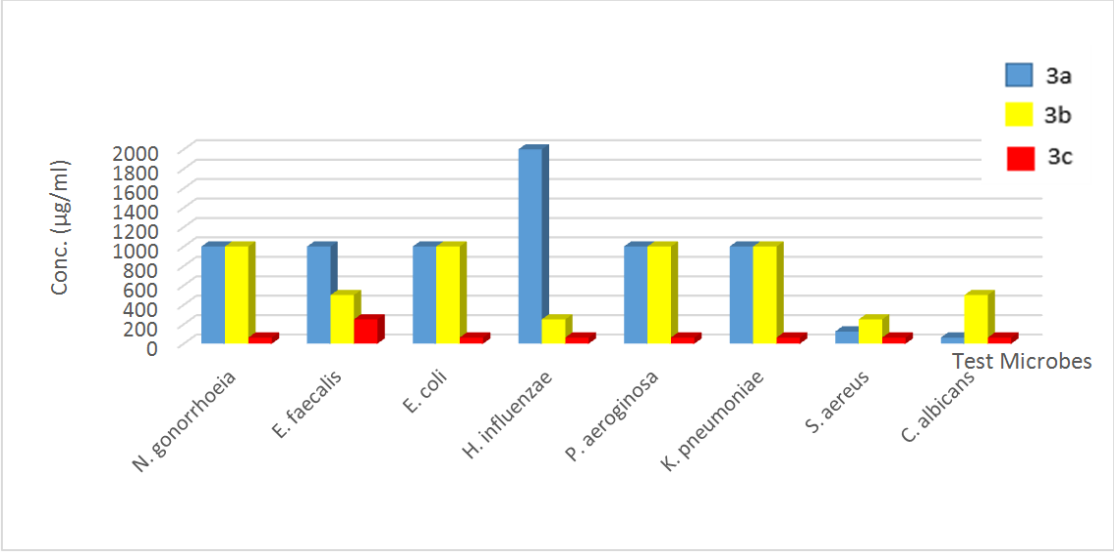


Fig. 2. MICs of compound 3a; 3b; and 3c (µg/mL)

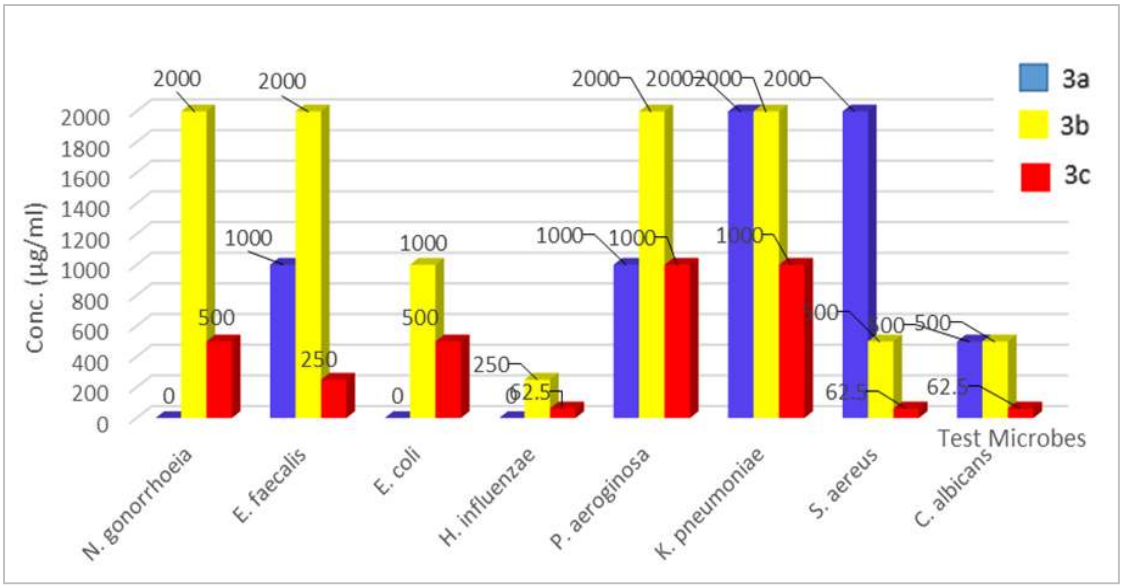


Fig. 3. MBCs of compound 3a; 3b; and 3c (µg/mL)

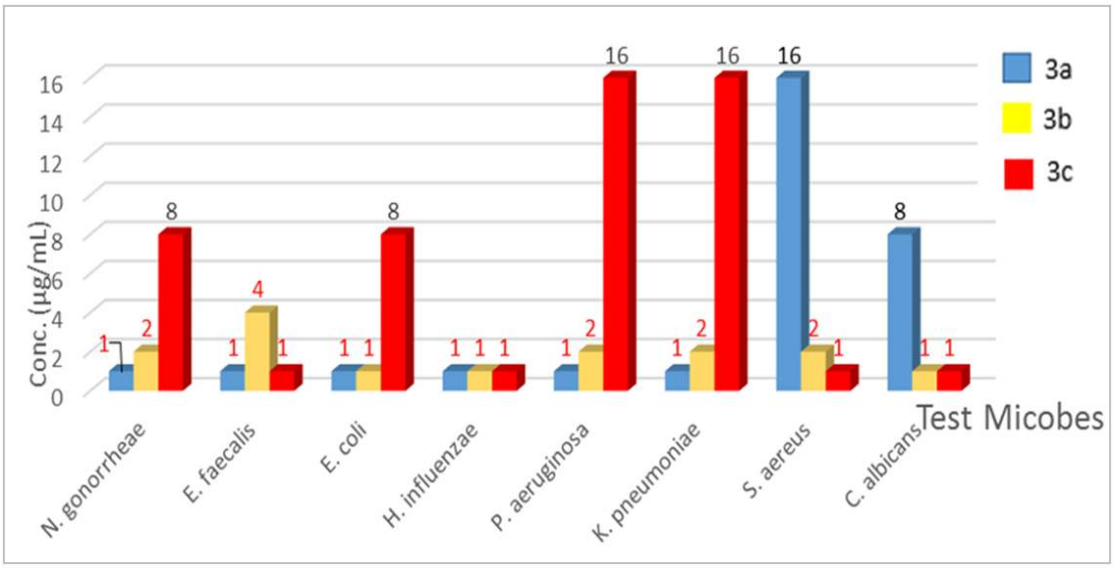


Fig. 4. The ratios of MBC to MIC of compounds 3a; 3b and 3c

5. CONCLUSION

We demonstrated that all the test microbes were susceptible to the 3 synthesized chalcones at low concentrations. Compound **3a** being bactericidal against reference Gram negative bacteria and bacteriostatic against the Gram positive reference strain *S. aureus* and *C. albicans.* Compound **3b** being bactericidal to all the strains of microbes, and compound **3c** bactericidal against 2 reference strains of Gram negative bacteria (*Haemophilus influenza* and *Enterococcus faecalis*) and against the reference Gram positive bacteria (*S. aureus*), and fungicidal against clinical strain of *C. albicans.* Bacteriostatic action of **3c** was observed against 4 reference strains of Gram negative bacteria (*Klebsiella pneumonia*; *Pseudomonas aeruginosa*; *Escherichia coli*; *Neisseria gonorheae*).

The halogenated chalcone (**3c**) was the most potent of the synthesized chalcones against reference strains of Gram negative and Gram

positive bacteria, and the clinical strain of *C. albicans.* Therefore the presence of halogens in chalcones increases their microbial susceptibility.

The presence of a hydroxyl group at position 4' increases the potency of chalcones on Gram negative organisms, while the presence of a methoxy group at position 4'increases potency against Gram positive organisms. Neither of these groups when present at position 4' have any effect on the potency of chalcones against *C. albicans*. Hence synthesized chalcones have antimicrobial properties and can be very promising lead compounds for drug development.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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