

## Phthalate derivatives from marine *Penicillium decumbens* and its synergetic effect against sepsis bacteria

Mohamed S. Amer<sup>1</sup>, Khoulood M. Barakat<sup>1,\*</sup>, Abd-Elatif A. Hassanein<sup>2</sup>

<sup>1</sup>Marine Microbiology Laboratory, Division of Marine Environment, National Institute of Oceanography and Fisheries, Alexandria, Egypt

<sup>2</sup>Marine Biotechnology Laboratory, Division of Marine Environment, National Institute of Oceanography and Fisheries, Alexandria, Egypt

\*corresponding author e-mail address: [kh2m2@yahoo.com](mailto:kh2m2@yahoo.com)

### ABSTRACT

Septicemia is a serious bloodstream bacterial infection resulting in blood poisoning and rapidly become human threats. The excessive usage of antibiotics against septicemic diseases developed the pan-drug resistant bacteria. Out of 15 marine fungal isolates, *Penicillium decumbens* was isolated from El-Shatby, Alexandria, Egypt, and identified as the potent isolate produced bioactive material(s) against some virulent septicemic pathogens. Diisooctyl phthalate (DIOP) in the butanol crude extract was determined by gas chromatography/mass spectrum. Antimicrobial activities were tested in comparison with some known antibiotics used for sepsis treatment. Five antibiotics were, in vitro, tested against septicemic resistant bacteria, either individually or in combination with butanol extract (BE) showed minimum inhibitory concentrations (MIC) 14.7 and 7.3 µg/ml against G+ve and G-ve tested strains, respectively. Combination of BE with azithromycin (AZM) reduced the MIC value by 73% for G+ve bacteria with the synergistic effect (fractional inhibitory concentration index) FICI = 0.39. Moreover, combination of BE with azithromycin (AZM), also, reduced the MIC value by 92% for G-ve bacteria with the synergistic effect FICI = 0.37. The highest antiheamolytic activity reduction percentage was detected at 88% when the combined BE-AZM interact with the supernatant of *K. pneumonia*, whereas, the lowest antiheamolytic reduction percentage of 57% against *E. coli*. The findings suggest the use of Diisooctyl phthalate as improving agent in combination with antibiotics in pharmaceutical preparations.

**Keywords:** marine fungi, diisooctyl phthalate, septicemia, combination therapy, synergy, anti-heamolytic action.

### 1. INTRODUCTION

Sepsis is the major reason of mortality amongst critically ill patients [1]. Sepsis is the host's immune response to injury or stimuli caused by bacterial, viral or fungal infection which derives from the skin, abdomen, lungs, or urinary tract [2]. Blood hemoglobin as a potent foreteller of patient's symptoms, is a crucial, hence, it is used to determine the mechanisms of sepsis-induced hemolysis with the sight of deriving the adequate therapy [3].

Many Gram positive and Gram negative pathogenic species are the leading source of severe infections in humans which represent a continuous threat to human health and welfare [4]. Antibiotics are the mean of treating infectious diseases, however microbial resistance against these drugs led to the emergence appearance of new infectious diseases [5]. Attempts have been studied to deal with this problem using combination therapy, where, synergy process depend on the working of two or more drugs together to make an effect greater than the sum of their individual effects [6]. Many researches were motivated to start sampling and screening the large collections of fungal strains that produced antibiotics and pharmacologically active agents' [7]. *Penicillium*, as a reputative

fungal species for antibiotics production, able to synthesis a much diversified array of active secondary metabolites, like antibacterial, antifungal, immunosuppressant, and cholesterol lowering agents that could to be used by the pharmaceutical industry [8].

Phthalates are a family of man-made chemicals, however, they naturally found in fatty foods such as milk, butter, and meats. Phthalates have also been studied as a bioactive compound produced from many bacterial species [9-11]. Besides, many bio-sources of di-n-butyl phthalate were produced by different filamentous fungi [12].

Thus, the goal of the present study was to evaluate the synergistic effect of marine *Penicillium decumbens* bioactive materials with some antibiotics used in sepsis therapy against some bacterial strains. Furthermore, GC/MS autogram of the fungal extract was utilized for preliminary detection of active constituents. Hence the analysis of this extract was led to the isolation of the active diisooctyl phthalate compound showed antimicrobial, synergistic and anti-hemolytic activities.

### 2. MATERIALS AND METHODS

#### Fungal cultivation conditions.

Marine *Penicillium decumbens* was isolated from El-Shatby, Alexandria, Egypt, cultured on potato dextrose medium and kindly identified by Assiut University, Mycological Center, and Faculty of Science. One ml spore suspension ( $10^7$  spore ml<sup>-1</sup>) was prepared from a 7-d-old slants and inoculated into 100 ml

potato dextrose media in a 250 ml flask. The cultures were incubated at 30°C, 180 rev min<sup>-1</sup> for 15-21 d.

#### Test organisms.

Swabbed samples were kindly provided from Vascular Surgery Unit of Alexandria Main University Hospital, Faculty of Medicine, Alexandria University, then applied on nutrient agar

plats. Purified colonies were subcultured in 5 ml nutrient broth over-night and subjected for APi20E identification kits. Six strains were selected for bioassay: three G+ve: *Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus pyogens* and three G-ve: *Enterobacter faecalis*, *Escherichia coli* and *Klebsiella pneumonia*.

**Antibiotics.**

Five anti-septicemic drugs used throughout this study, were purchased from the local market of El-Amriya Pharma. Ind. Companies (Table 1). The antimicrobial action from these antibiotics was assayed against the selected strains using Müller Hinton-susceptibility test medium according to the CLSI or manufacturer's recommendations.

**Antimicrobial assay.**

Fungal bioactivities were screened using disc diffusion assay on Müller-Hinton agar. Twenty-five ml of sterilized Muller Hinton agar medium were mixed with over- night culture of the tested organism ( $10^4$  CFU/ml), followed by pouring into sterile Petri-dishes to solidify. Sterile discs were immersed in free cell filtrate of *P. decumbens*, and applied on the prepared plates followed by overnight incubation at 30°C.

**Extraction of bioactive agent(s).**

Cell free supernatant was extracted using three solvents: methanol, butanol and ethyl acetate. Fifty ml from each solvent were equally added to the fungal fermented broth medium in a 250 ml separating funnel. Minimal inhibitory concentrations (MIC) of each evaporated compound(s) were checked [13]. MIC was detected by choosing the lowest concentration of bioactive extractable agent(s), preventing the appearance of turbidity. The lower the MIC the higher the activity of the tested extracts.

**Spectroscopic analysis.**

GC-MS (Gas chromatography-mass spectrum) analysis of the purified product(s) was carried out using Agilent Technologies 7890 A GC System, 5975 C inert XL MSD Triple-Axis Mass Detector. GC- MS conditions include: 1 µL of sample was injected with an evaporation temperature of 250 °C, 1.8 bar,

2.5 mL/min, split 20:1. He, the carrier gas temperature was gradient 50 °C/1 min, 40 °C/min gradient 300 °C/min, 300 °C/5 min. The components were identified by comparing their retention times to those of authentic samples of Wiley 275 Library. The obtained product(s) was further analyzed using Fourier Transform Infrared Spectroscopy. FTIR analysis was performed in the mid IR region of 400–4000 cm<sup>-1</sup>. The sample was prepared by mixing with pure KBr, followed by sample fixation in a holder for analysis.

**Microbroth dilution assay.**

In interaction studies, antibiotic- extract combinations were examined against the most resistant strains. MICs were recorded for each combination by broth microdilution according to standards of the CLSI. Each combination was made in three replicates. Fraction inhibitory concentration index (FICI) for the combinations of three drugs was calculated according to the equation:

$$FICI = FIC_{Ab} + FIC_F = Ab / MIC_{Ab} + F/MIC_F$$

(Ab and F refer to antibiotics and fungal extract concentrations, respectively)

Where, synergism was defined as FICI ≤ 0.5, additive effect as FICI > 0.5 and ≤ 1, indifference effect as FICI > 1 and ≤ 2 and antagonism effect as FICI > 4.

**Anti-hemolytic action.**

The hemolytic activity of the used septicemic pathogens was determined by the appearance of a lytic zone on the surface of a blood agar plate. The hemolytic action of the extracted crude was measured using methods described by Chen et al [14]. Wells were punched and two application tests were done: (A) application for the butanol crude extract combination(s), (B) application of pathogens filtrate and (C) application for the interaction between both pathogens and the combined product. The data were determined within 24h. The purpose of this assay was to determine the degrees of the ability of butanol extract to inhibit the virulence effect of the pathogens.

**Table 1.** Antibiotics used against sepsis causing bacterial pathogens.

Ab group	Penicillins	Cephalosporin	Quinolones	Glycopeptide	Macrolides
Ab	Piperacillin-Tazobactam (TZP)	Ceftriaxone (CRO)	Ciprofloxacin (CIP)	Vancomycin (VAN)	Azithromycin (AZM)
Conc µg/disc	20	20	10	20	20

**3. RESULTS**

**Antimicrobial profile.**

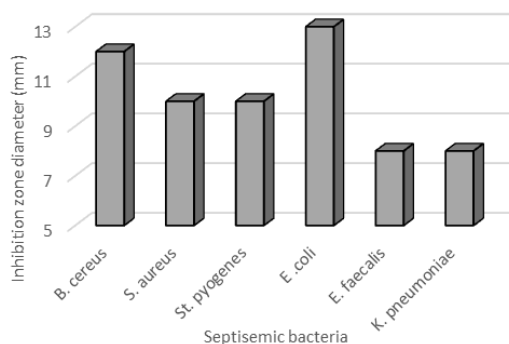
*P. decumbens* was subcultured onto potato-dextrose agar (PDA) supplemented with Rose Bengal for 5–7 days at 28–33°C. *P. decumbens* is anamorph species of *Penicillium* which mainly found in nature [15]. Analysis has shown that *Penicillium decumbens* has antimicrobial activity [16]. In the present investigation, marine *P. decumbens* gave a maximal activity 13 mm inhibition zone against *E. coli*, followed by *B. cereus* (12 mm) while, *E. faecalis*, *S aureus* and *St. poygens* showed lower activities ranged from 8-10 mm (Figure 1). The cell free culture medium of endophytic *P. chrysogenum* associated mangroves showed detectable activity against *Vibrio cholerae*, (MCM B-322) from 14–16 mm inhibition zone [17]. Diameter of inhibition zones

(mm) of tested supernatant of some *Penicillium* sp on disc diffusion assay ranged from 10-30 mm against both Grams' pathogens [18]. The antimicrobial efficacy of *P. chermesinum* TTMF3 was recorded from 10 – 12 mm against the tested strains [19].

**Antibiotics Susceptibility test.**

Using disc diffusion assay, five antibiotics were applied to test against the 3 Gram-positive and 3 Gram-negative bacteria and determine the sensitivity of these bacteria to those antibiotics resistance. Resistant strains were observed using Piperacillin-Tazobactam (TZP) especially G+ve bacteria, showing no IZ. Lower activities were represented in an average IZ range of 3- 7 mm using Ciprofloxacin and Vancomycin (Table 2). A recent

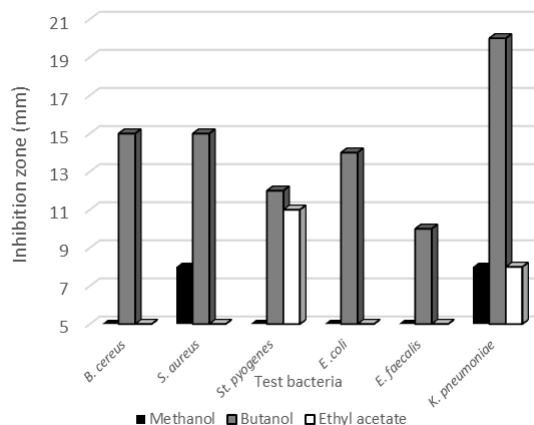
report by Thapa and Sapkota [20] studied antibiotic susceptibility test against neonatal septicemia isolates, where, the highest susceptibility rate towards amikacin clindamycin, cotrimoxazole, erythromycin, meropenem, ofloxacin, teicoplanin and vancomycin, were shown among gram-positive isolates *S. aureus*, while, gram negative Enterobacteriaceae showed the highest susceptibility towards amikacin, ampicillin/sulbactam, gentamicin, meropenem, ofloxacin and piperacillin/tazobactam. Muley et al. [21] showed that the most effective drug for both gram-negative and gram-positive septicemic isolates was amikacin. Also, gram-positive septicemic isolates *S. aureus* had 100% vancomycin sensitivity [22].



**Figure 1.** Antimicrobial profile of the produced bioactive agent(s) from *P. decumbens* against septicemic bacteria.

**Bioactive agent(s) extraction.**

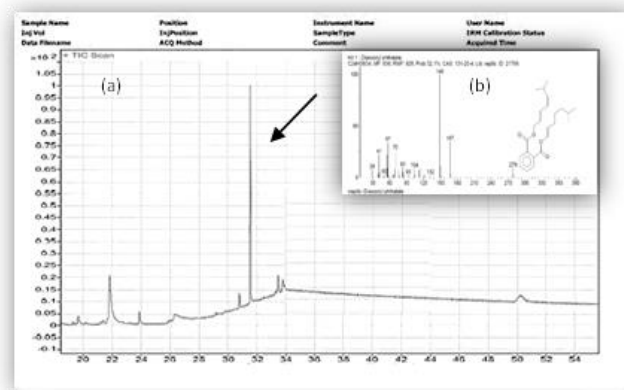
Butanol was the potent solvent for the extraction process showed the maximum values of the inhibition zone ranged from 10- 20 mm against all the tested clinical isolates. Methanol and ethyl acetate showed no or lower tendencies for the antimicrobial activity (Figure 2). Therefore, butanol solvent was selected for further testing. Similarly, the extraction and purification of active group/component from *Penicillium* sp., three liters of the culture broth were extracted with equal volume of butanol (1:1) witch exhibited antimicrobial activities from 14-35 mm [23]. Contrary, the antibacterial activity of ethyl acetate extract from marine *P. chermesinum* TTMF3 was maximum against *Proteus vulgaris* followed by *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumoniae*, *K. oxytoca*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Enterobacter aerogenes* and there was no zone of inhibition in *Escherichia coli* and *Vibrio cholera*, however, diethyl ether extracts showed 13.5 and 13.3 mm zone of inhibition against *Proteus vulgaris* and *Klebsiella pneumoniae*, respectively [19]. Correspondingly the antibacterial activities of marine fungi was reported by various workers [24 – 26].



**Figure 2.** Bioactivity pattern showing the potent solvent(s) for extraction process.

**Spectral analysis.**

In the last ten years, many novel bioactive natural products from marine fungi have been discovered that possess antibacterial, anticancer, antifungal, antiviral, or cytotoxic activities [27, 28]. Marine fungi and their antibacterial compounds have been quickly achieved since 2010 [29]. In this study, potent compound extract from *P. decumbens* was spectrally analyzed using GC–MS; GC detected compound with retention time (RT) at 31 min 54 s and with 92.5% relative abundance (Figure 3). Mass spectral analysis suggested at  $m/z$  149 of the detected compound which could be presented a molecular formula corresponds to  $C_{24}H_{38}O_4$ , diisooctyl phthalate. FTIR spectra showed broad peaks from 3416 – 2918 (C-H), 1641 (C=O), 1409 (C=C), 1115 (C–O) and 662 (C–C)  $cm^{-1}$  (Figure 4).



**Figure 3.** GC analysis showing the major peak of the active compound at specific retention time (31 min 54 s ) (a), followed by MS suggestion of diisooctyl phthalate in the crude (b).

**Table 2.** Antibiotics susceptibility test against septicemic isolates.

Antibiotic name	Abs Abv.	Inhibition zone (mm)							
		Gram positive isolates				Gram negative isolates			
		<i>B. cereus</i>	<i>S. aureus</i>	<i>St. pyogenes</i>	Avg. IZ	<i>E. faecalis</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	Avg. IZ
Ceftriaxone	CRO	20	0	9	9.7	20	9	9	11.0
Piperacillin-Tazobactam	TZP	0	0	0	0.0	9	0	0	1.3
Ciprofloxacin	CIP	10	0	0	3.3	14.5	0	0	4.0
Vancomycin	VA	20	0	0	6.7	20	8	0	7.8
Azithromycin	AZM	8	20	10	12.7	0	17	20	12.5

Many reports investigated the production of many phthalate derivatives produced by different fungal species. The Chemical characteristics of active fraction produced by marine derived fungi *Phoma herbarum* was determined based on the GC-MS spectral data as Dibutyl phthalate and Mono (2-ethylhexyl) phthalate with the molecular formula  $C_{16}H_{22}O_4$ ; molecular weight 278 [30]. Extraction, biological activity and structure elucidation of Di-(2-ethylhexyl) DEHP phthalate produced by *Penicillium janthinellum* 62 were studied depending on the potent dose for antitumor activity against Ehrlich cells *in vivo* [9]. Also, *Aspergillus fumigatus* 3T-EGY produces DEHP [31] and *Aspergillus awamori* synthesized the same compounds showing activity against *Sarcina lutea* and *Candida albicans* [32].

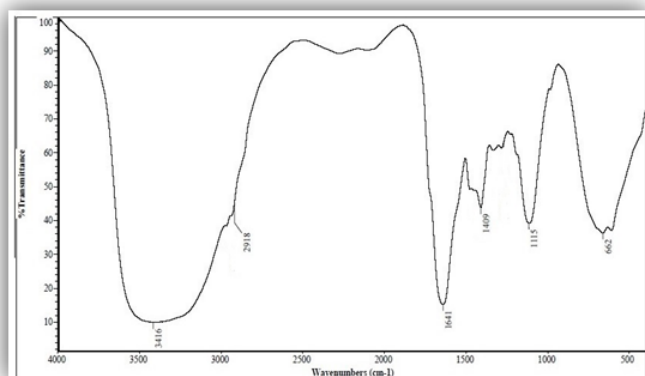


Figure 4. The IR analysis of the suggested compound.

#### Minimal Inhibitory concentrations.

The most resistant strains exhibited average minimum inhibitory concentration (MIC) values of the antibiotics ranged from 53.7- 256  $\mu\text{g/ml}$  for Gram positive and 84.7-181.3  $\mu\text{g/ml}$  for Gram negative isolates compared with diisooctyl phthalate (DIOP) showing the maximum average of MIC 32  $\mu\text{g/ml}$  against Gram positive and 54.7 $\mu\text{g/ml}$  against Gram negative. Azithromycin showed the maximum average of MIC 53.7  $\mu\text{g/ml}$  against G+ve, while ceftriaxone (84.4  $\mu\text{g/ml}$ ) against G-ve bacteria. The lowest average MICs showed by piperacillin-tazobactam for both type of bacteria (Figure 5a). Butanolic extract from *Penicillium* sp. showed significant antimicrobial activity with MIC against G+ve bacteria ranged from 100 – 700  $\mu\text{g/ml}$  and against G-ve ranged from 100 - 20000  $\mu\text{g/ml}$ , however, the minimum inhibitory concentration of the purified compound against G+ve ranged from 2-5  $\mu\text{g/ml}$  and for G-ve from 1-10  $\mu\text{g/ml}$  [23]. Lai et al. [33] described moderate activity MIC = 64  $\mu\text{g/ml}$  of the isolated compounds from *P. citrinum* extract against *S. aureus* ATCC29213. Bioactive materials extract from *P. nordicum* showed antibacterial activity > 20 mg/ml [34].

Our results reveal the importance of diisooctyl phthalate – antibiotics combinations to improve antibacterial activities against resistant bacteria, where butanol extract - azithromycin showed the maximum average of MIC 14.7  $\mu\text{g/ml}$  against G+ve and 7.3 $\mu\text{g/ml}$  against G-ve. Lower MIC averages were recorded with butanol extract - piperacillin-tazobactam at 106.7  $\mu\text{g/ml}$  against G+ve and 90.7  $\mu\text{g/ml}$  against G-ve bacteria (Figure 5b). Penicillenol A2 extracted from deep-sea fungus *P. biourgeianum* DFFSCS023 provides a novel treatment against methicillin-sensitive

*Staphylococcus* MRSA-caused infections [35]. Five *P. chrysogenum* isolates produced penicillin under different conditions gave maximum antibacterial effect in the combination of penicillin with tea extract [36]. Detection of antibacterial activity of silver nanoparticles (AgNPs) synthesized by *Penicillium* spp.in combination with some tested antibiotics was studied by Verma et al. [37].

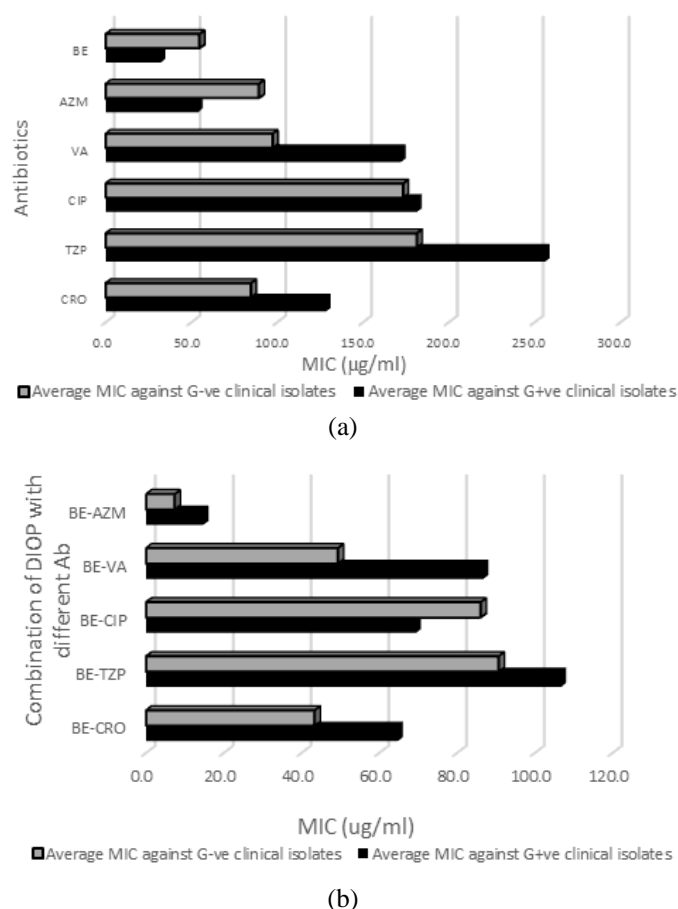


Figure 5. Averages of minimum inhibitory concentration of antibiotics against G+ve and G-ve clinical isolates (a) and Averages of minimum inhibitory concentration of antibiotics – DIOP combinations against G+ve and G-ve (b).

#### Fractional Inhibitory concentration index.

Synergistic action was observed when combined butanol extract - azithromycin at maximum  $FIC_{index}$  0.4 for both G+ve and G-ve isolates (Figure 6 a&b). As a blind preliminary chemotherapeutic dose, a combination of butanol extract with azithromycin reduced the MIC value by 73 % from 53.7 to 14.7  $\mu\text{g/ml}$  for Gram positive and 92% from 89.3 to 7.3  $\mu\text{g/ml}$  for Gram negative bacteria.

Accordingly, suggested formulae and recommended dose were:

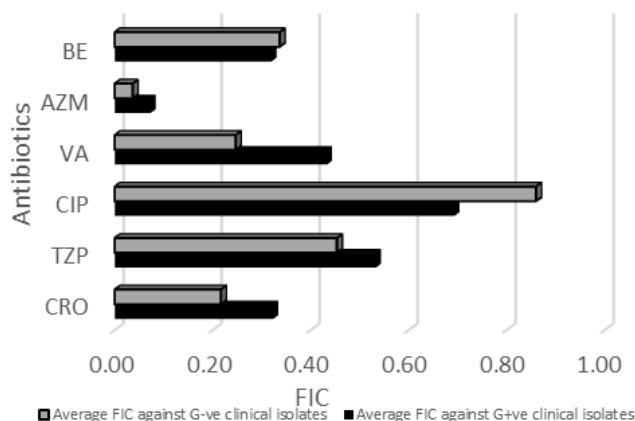
For Gram positive:

Formula 1: Butanol extract + azithromycin (3.2 + 14.7  $\mu\text{g/ml}$ )

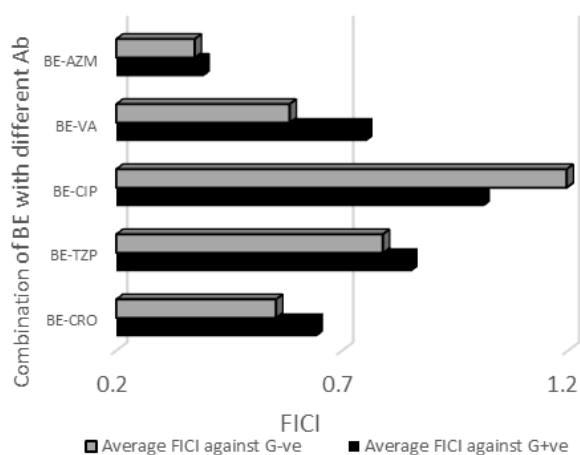
For Gram negative:

Formula 2: Butanol extract + azithromycin (2.4+ 7.3  $\mu\text{g/ml}$ ).

A small antifungal protein (PAF) isolated from *P. chrysogenum*, inhibits the growth of many pathogenic filamentous fungi. FICI values *in vitro* indicated a synergic effect when amphotericin B (AMB) was combined with PAF. The FICI value calculated from the individual and combined MICs (0.25  $\mu\text{g/ml}$  of AMB and 50  $\mu\text{g/ml}$  of PAF) of the drugs is 0.42 [38].



(a)



(b)

**Figure 6.** Averages of Fractional inhibitory concentration (FIC) of antibiotics (a) and combinations of butanol extract - antibiotics (b) against G+ve and G-ve clinical isolates.

**Antiheamolytic activity.**

The present experiment was attempted to antagonize the heamolytic virulence factor produced by the six tested organisms. The applied plats were incubated at 30°C for 24h and the inhibition zone diameter of blood removal color was measured.

**4. CONCLUSIONS**

*In vitro* diisooctyl phthalate as antibiotics synergizers could prove to be a promising alternative in the treatment of septicemic patients for whom existing antimicrobial treatment fails. The final formula against G+ve and G-ve isolates reduced the resistance of

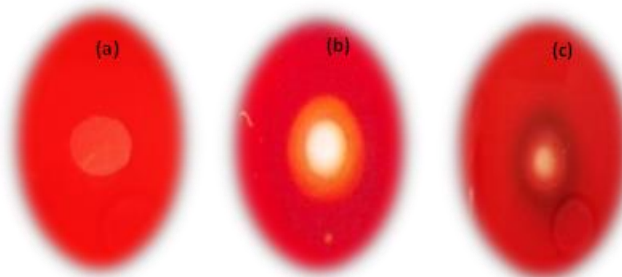
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The results represented in Table 3 showed that the highest antiheamolytic activity reduction percentage was obtained at 88% when the combined BE-AZM interact with the supernatant of *K. pneumoniae*. While, the lowest antiheamolytic reduction percentage 57% against *E. coli*. Reduction degree of the antimicrobial agent activity interaction comparing with the heamolytic zone produced by the three tested pathogens on the blood agar medium was observed in Figure 7. Red blood cells complete lysis around the colony considered as  $\beta$ -hemolytic activity while  $\alpha$ -hemolysis designate a partially lyse of hemoglobin and appear green.  $\gamma$ -hemolysis is referring to a lack of hemolytic activity. Blood hemoglobin is playing a vital role in the sepsis pathogenicity to predict patient's outcome. Hence to derive the possible therapeutic principles, it is critical for further studies on the action of sepsis-induced hemolysis [3].

**Table 3.** The antiheamolytic activity of the butanol crude extract against.

Test bacteria	Hemolytic reduction %
<i>B. cereus</i>	75
<i>S. aureus</i>	76
<i>St. pyogenes</i>	60
<i>En. faecalis</i>	65
<i>E. coli</i>	57
<i>K. pneumoniae</i>	88



**Figure 7.** Photographs show the  $\gamma$ -hemolysis action of combined BE-AZM (a) and the  $\beta$ - hemolytic action of the tested bacteria (b) and effect of combined BE-AZM against these tested pathogens represent  $\alpha$ -hemolytic (i.e. the conversion from  $\beta$ - hemolysis to  $\alpha$ -hemolysis).

strains toward antibiotics. Also, these data encourage further studies with diisooctyl phthalate and other antimicrobial classes and *in vivo* animal experiments to validate these interesting findings before clinical tests can move forward

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