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SYNERGISTIC AND ANTAGONISTIC EFFECTS ON ANTIMICROBIAL PROPERTIES OF ORGANIC AND AQUEOUS EXTRACTS OF *BACILLUS CLAUSII* KP10 IN COMBINATION WITH CONVENTIONAL ANTIBIOTICS

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ABSTRACT

Due to emergence of multidrug resistance, alternative approaches are practiced and needed in conventional antimicrobial therapies. Drug combination therapies have been used like synergism for better results with decreased drug dosage to avoid toxicity than monotherapy, but results can be additive and antagonistic depending on their combined effect. The present project was therefore designed to assess the effects of already reported bioactive fractions of *B. clausii* KP10 with each other and with antibiotics in synergistic manner to combat microbes. Different solvents were used for the extraction and fractionations. Antimicrobial activities were performed against different available bacterial and fungal strains. In our experiments crude methanolic extract (CME), n-hexane fraction (n-HF), chloroform (CLF), ethyl acetate (EAF), Methanol Soluble (MSF) and water soluble fractions (WSF) were obtained and then combinations of each were prepared with (1:1, v/v) each other and with reported antibacterial and antifungal drugs streptomycin (STM) and Terbinafine (TER) separately (1:1:1). Single extract with drug combination was also prepared (1:1). Maximum zone of inhibition was shown by combination of n-HEF:MSF (24 mm) against *S. aureus*. CLF:WSF:STM showed maximum ZOI (37.2 mm) against *E. coli*. WSF: STM showed maximum ZOI (33 mm) against *S. aureus*. Maximum antifungal activity was shown by CME:WSF:TER (26 mm) and EAF:TER with maximum ZOI (17 mm) against *Fusarium solani.* n-HEF:EAF:TER showed maximum ZOI (18 mm) against *Aspergillus niger*. Combination of extracts with each other usually showed antagonistic behavior but extracts with standard drugs have shown synergy which could be a better approach in the development of antimicrobials therapies.

Keywords: Antagonistic, Antimicrobial, *Bacillus clausii* KP10, Bioactive compounds, Synergistic.
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INTRODUCTION

Infectious diseases caused by bacteria and fungi affect millions of people worldwide. The condition is getting worst day-by-day due to rapid evolution of multidrug-resistant microbes, bacteria have a multitude of mechanisms by which they can rapidly acquire resistance (Buroni *et al*., 2019). Therefore, new approaches are needed to combat infective microbes and overcome their microbial resistance (Martins *et al*., 2020). Gram positive bacteria such as *Staphylococcus aureus* are mainly responsible for toxic shock syndrome, post-operative wound infections, endocarditis, pneumonia, food poisoning and osteomyelitis (Benayache *et al*., 2001). *Bacillus subtilis* cause anthrax infections to human (La Jeon *et al*., 2012). Gram negative bacteria such as *Pasteurella multocida* are often associated with chronic as well as acute infections in both animals and humans (Harper *et al*., 2006). In intestine of human *Escherichia coli* is present and causes the infection of lower urinary tract, septicaemia or coleocystis. (Benhassaini *et al*., 2003). Fungi can also cause many diseases to plants and humans (Panackal *et al*., 2006). For example, *A. niger* cause invasive diseases associated with otomycosis and other infections to human (Araiza *et al.*, 2006), *A. niger* also caused Black mold disease on certain fruits and vegetables such as onions, grapes and peanuts (Moghtader, 2013). *Fusarium solani* is the main causative agent in 37%–50% of fungal keratitis cases. Onychomycosis is another human infection with a high mortality rate caused by *Fusarium* fungi (Monod and Mehul, 2019), also caused localized infections in skin and other parts of body (Gupta *et al*., 2000).

Resistance of multidrug in the human pathogenic microbes has been developed as a result of indiscriminate usage of antimicrobial drugs that are mostly used to treat infectious diseases. Resistance of antibiotic is caused by a multi-factorial reasons, including the specific nature of relationship of the microbes to antibiotics & also usage of antimicrobial agent, host characteristics and environmental factors (Xie *et al*., 2009). This alarming situation has convinced scientists to find out new antimicrobial agents from different sources as novel antimicrobial chemotherapeutic agents. One of the leading approach is to control these infectious diseases without side effects is use of some specific types

of bacteria which are capable of producing bioactive compounds that can help to cure diseases such as probiotics (Xie *et al*., 2009). Bacteria produce biologically active compounds against other bacteria and fungi that acts against the specific physiological conditions of a diseased body as antimicrobial agents. *Bacillus* genus is well known for producing the bioactive compounds that work as antimicrobial agents. Compounds like bacteriocins and bacteriocin like inhibitory compounds are synthesized ribosomally antimicrobial peptides which are formed by different bacteria which are mostly effective against species that are closely related too (Riley and Wertz 2002; Cherif *et al*., 2003; Abriouel *et al*., 2011).

In clinical practice, antimicrobial combination therapy is one of the leading novel advances to combat resistance of microbes (Lambert, 2000; Hemaiswarya *et al*., 2008; Van Vuuren *et al*., 2009). Combination therapy has stimulated renewed interest in recent years with major safety concerns. For example, combinations of gentamicin and chloramphenicol could be enhanced by use of the plant materials against Methicillin resistant *Staphylococcus aureus* (MRSA) (Darwish *et al*., 2002; Lu and Di, 2020). The most common reasons of combination therapy usage are to reduce the emergence of resistance in strains to minimize toxicity by lowering the dose of toxic drug, treat poly-microbial infection and also increase killing or inhibition of resistance in organisms to appropriate doses of single antimicrobial compound (Rahal, 1978; King *et al*., 1981).

Therefore, the purpose of present work was to determine the antimicrobial effects of already reported bioactive fractions of *B. clausii* KP10 with each other and in combination with standard drugs to find out the possible synergistic or antagonistic effects of the combination for the development an improved antimicrobial therapy.

MATERIALS AND METHODS

Experimental work was conducted in August 2019 in Bioactive Molecules Research Laboratory (BMRL), Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.

Culturing of bacteria and extraction: *Bacillus clausii* KP10 isolated previously by our research group (Erum *et al*., 2017), that was used in this experiment as source of bioactive extracts. Nutrient agar and broth (MERCK) were used for the growth of bacterial cultures (Muller *et al*., 2016). The medium of the pH was maintained at 10 with 0.1 N HCl / NaOH before sterilization. The medium was autoclaved at 121 °C for 20 minutes at 15psi pressure. Bacterial culture was grown at the 40 °C for 24 hours for sporulation (Erum *et al*., 2017). Then cell mass of bacteria was obtained when centrifuged at 11963 ×g for 10 minutes. Bioactive fractions were obtained. For extraction of bioactive compounds different organic (n-hexane, chloroform, ethyl acetate and methanol) and aqueous solvents were used as described in literature (Nighat and Mushtaq, 2019; Nisa, 2011). Finally, carefully layers were separated then dried weighed and dissolved in Dimethyl sulfoxide and stored at 4 °C for bioactivities.

Working and stock solutions preparation: In distilled water streptomycin was dissolved and terbinafine was solubilized in Dimethyl sulfoxide to make 10 mg/mL final concentration and then stored at the 4 °C until use (Liu *et al*., 2015). For positive control streptomycin and terbinafine was also used with concentration of 50 mg/mL for antimicrobial activities. Combinations of extracts were prepared as follows 1:1 and 1:1:1 v/v as shown in the table 1.

Antimicrobial assay: Antimicrobial assays of extracts of each combination were checked against certain gram-negative bacteria (*Pasteurella multocida* and *Escherichia coli*), gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and also two fungal strains (*Fusarium solani* and *Aspergillus niger*) using well-diffusion method (Zaidan *et al*., 2005; Ahmad and Aqil, 2007).

Statistical analysis: The data was analyzed by one-way analysis of variance (ANOVA) and Tukey HSD's multiple range test ($p \leq 0.05$) using the SPSS software to test significance of differences among mean values of samples for different combinations regarding bacterial and fungal strains.

Table 1. Combinations for the Evaluation of Synergistic and Antagonistic interaction of selected antibiotics with *Bacillus clausii* KP10 extracts/fractions.

Sample #	Combination I (1:1)	Combination II (1:1:1)	Combination III (1:1)
1	CME:n-HEF	CME:n-HEF:+ve drugs	CME:+ve drugs
2	CME:CLF	CME:CLF:+ve drugs	n-HEF:+ve drugs
3	CME:EAF	CME:EAF:+ve drugs	CLF:+ve drugs
4	CME:MSF	CME:MSF:+ve drugs	EAF:+ve drugs
5	CME:WSF	CME:WSF:+ve drugs	MSF:+ve drugs
6	n-HEF:CLF	n-HEF:CLF:+ve drugs	WSF:+ve drugs

7	n-HEF:EAF	n-HEF:EAF:+ve drugs
8	n-HEF:MSF	n-HEF:MSF:+ve drugs
9	n-HEF:WSF	n-HEF:WSF:+ve drugs
10	CLF:EAF	CLF:EAF:+ve drugs
11	CLF:MSF	CLF:MSF:+ve drugs
12	CLF:WSF	CLF:WSF:+ve drugs
13	EAF:MSF	EAF:MSF:+ve drugs
14	EAF:WSF	EAF:WSF:+ve drugs
15	MSF:WSF	MSF:WSF:+ve drugs

Note: CME:Crude Methanolic Extract, n-HF:n-Hexane Fraction, CF:Chloroform Fraction, EAF:Ethyl acetate Fraction, MSF:Methanol Soluble Fraction, WSF:Water Soluble Fraction, +ve drugs= streptomycin (STM), Terbinafine (TER)

Antimicrobial activities were performed by well diffusion method with the help of using above mentioned combinations (table1).

RESULTS AND DISCUSSION

Combination drug therapy has been shown to delay the emergence of microbial resistance and could also produce beneficial effects in treatment of microbial infections. Synergism of drug with bioactive microbial extracts and with known antibiotics is a modern concept and it could be advantageous (additive or synergistic interaction) or may be deleterious (toxic or antagonistic outcome) (Gibbon, 2004). Bioactive compounds can be good option when used concurrently with the standard drugs, where they enhance the activity of the drug (Aiyegoro and Okoh, 2009). Pharmacological benefits were recorded in way when one drug is involved to clear infection from one part of the body while other drug clear it from different site (Williamson, 2001).

Drug interactions with microbial extracts have gained remarkable scientific interest (Ocampo *et al*., 2014; Yilancioglu *et al*., 2014). Therefore, antimicrobial assays of bioactive fractions of *B. clausii* KP10 with each other and with standard drugs were analyzed. Following fractions were obtained from the *Bacillus clausii* KP10 by using solvents such as methanol extract, n-hexane fraction, chloroform fraction, ethyl acetate fraction, methanol soluble fraction and water soluble fraction. Yields were 50, 100, 110, 113, 100 and 90 mg. All obtained extracts were then further diluted in the DMSO to a specific concentration.

According to our previous data published bioactive extracts of *Bacillus clausii* KP10 were bioactive in nature and effective against *Escherichia coli, Pasteurella multocida, Bacillus subtilis* and *Staphylococcus aureus*, but most effective against *P. multocida*. So they can be used as antibacterial and antifungal agents (Nighat and Mushtaq, 2019; Nighat *et al*., 2020). *Bacillus clausii* KP10 bioactive fractions have great potential as antimicrobials against different tested microorganisms. The results obtained from combination I as mentioned in materials and methods are summarized in Table 2 and Figure 1. CME:CLF showed maximum ZOI (11 mm) against *P. multocida*, CME:EAF showed maximum ZOI (22 mm) against *E. coli* and n-HEF:MSF showed highest antibacterial assay against the *S. aureus* with maximum ZOI (24 mm) recorded. Results of combination I shows that the interactions between organic and aqueous extracts were mostly antagonistic, when used with each other against selected bacterial and fungus strains. Antagonism occurs when one drug hinders another drug's effect (Johansen *et al*., 2000). Similar studies in literature showed that the combination of Ethyl acetate:Chloroform (1:1), Water:Methanol (1:1) and Methanol:Acetone (1:1) extracts of *Trichophyton*, *Microsporum* and *Epidermophyton* genera exhibited no synergistic activity with each other and showed antagonistic behavior in combination against all tested microorganisms (Koroishi *et al*., 2008). The results give clear evidence that antimicrobial assay of organic and aqueous extracts of *B. clausii* KP10 is reduced when used in combination, but when the same extract of *B. clausii* KP10 used concurrently with standard drug streptomycin (STM) they enhance the activity of the drug.

Antibacterial activities of combination II and III: In our experiments we checked both combinations single extract of *B. clausii* with antibiotic and also combined extract of *B. clausii* KP10 with standard antibiotic streptomycin as shown in combination II and III. We conclude that the combined effects with streptomycin was almost equal in general behavior in both combinations. Combinations showed promising synergistic results and antagonistic interactions were not observed with streptomycin. These results were further confirmed by measuring the zone of inhibition (Figure: 2a, 2b and 3) which showed that the highest synergism was observed in the combination of CLF:WSF:STM (1:1:1) against *E. coli* maximum inhibition zone were recorded (37 mm). CLF:STM showed maximum ZOI (34 mm) against *E. coli*, WSF:STM showed maximum ZOI (33 mm), CLF:MSF:STM maximum ZOI (37 mm) against *B. subtilis*, WSF:STM showed maximum ZOI (34 mm) against *S. aureus*, CLF:STM showed maximum ZOI (31 mm), EAF:MSF:STM showed maximum ZOI (31 mm) against *P. multocida*.

Oliveira *et al*., (2011) studied synergistic effect of tetracycline, norfloxacin and erythromycin with ethanol extracts of *Mangifera indica L*. peel against the selected *Staphylococcus aureus* strains. Separate extracts did not have any beneficial antibacterial activities but when these extracts combined with antibiotics, significant synergistic effect was observed.

Table 2. Antimicrobial activities of combination I.

Sample #	Sample Name	*S. aureus* Mean ZOI (mm)±S.D	*B. subtilis* Mean ZOI (mm)±S.D	*P. multocida* Mean ZOI (mm)±S.D	*E. coli* Mean ZOI (mm)±S.D	*F. solani* Mean ZOI (mm)±S.D	*A. niger* Mean ZOI (mm)±S.D
1	CME:n-HEF	-	-	-	-	-	-
2	CME:CLF	-	-	11.76±1.1	-	-	-
3	CME:EAF	-	-	-	22.6±0.31	-	-
4	CME:MSF	-	-	-	16.2±0.26	-	-
5	CME:WSF	-	-	-	-	-	-
6	n-HEF:CLF	-	-	10.4±0.58	-	-	-
7	n-HEF:EAF	-	-	-	-	-	-
8	n-HEF:MSF	24.2±0.21	-	-	-	-	-
9	n-HEF:WSF	-	-	10.3±0.10	-	-	-
10	CLF:EAF	-	21±0.81	10.3±0.2	-	-	-
11	CLF:MSF	-	-	-	-	-	-
12	CLF:WSF	-	-	-	-	-	-
13	EAF:MSF	-	-	-	-	-	-
14	EAF:WSF	-	18.0±0	-	-	-	-
15	MSF:WSF	17.7±0.3	-	-	-	-	-
16	+ve	48±0.29	48±0.29	48±0.29	48±0.29	28±0.18	27±0.01

*Results are expressed as mean (ZOI)±S.D.

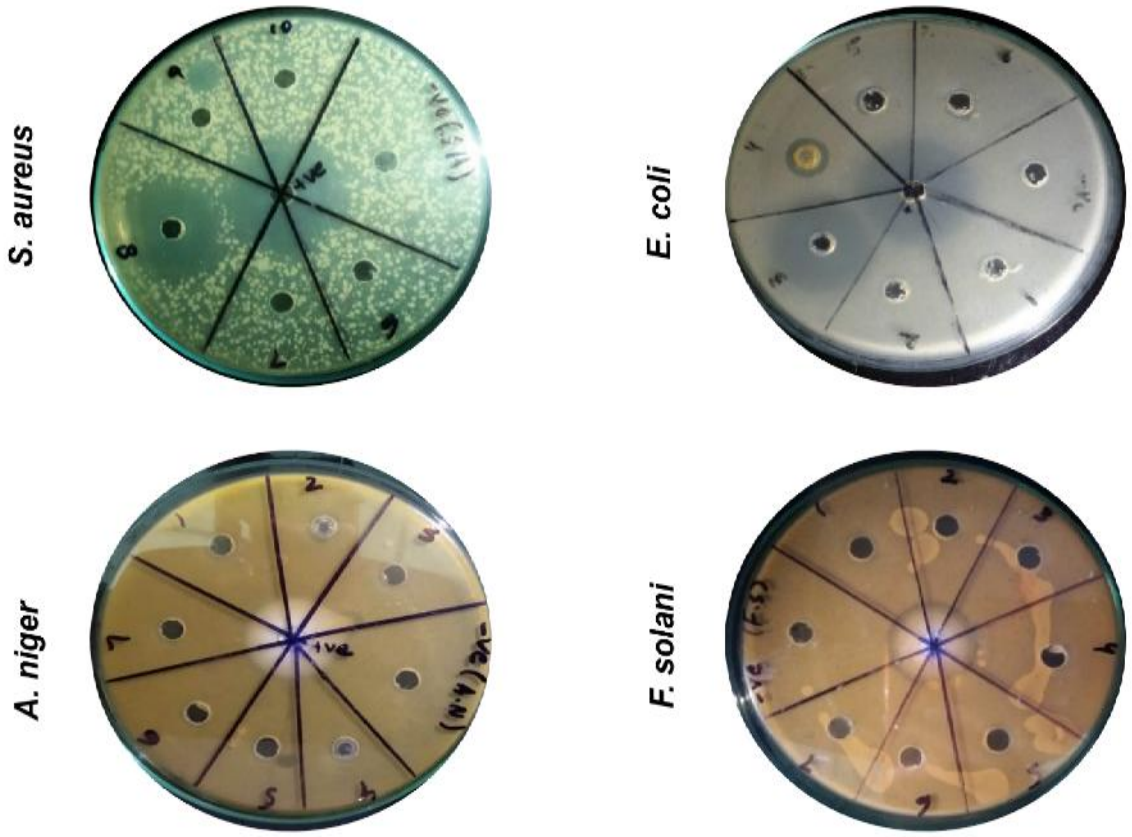


Fig. 1. Antimicrobial activities of combination I (1:1) by well diffusion method. Sample 3 showed maximum inhibition zone 22 mm against *E. coli* and sample 8 showed 24 mm against *S. aureus.* For positive control streptomycin was used that showed 48 mm ZOI. No antifungal activities was observed against *Aspergillus niger* and *Fusarium solani* except +ve control. Each experiment performed in triplicate.

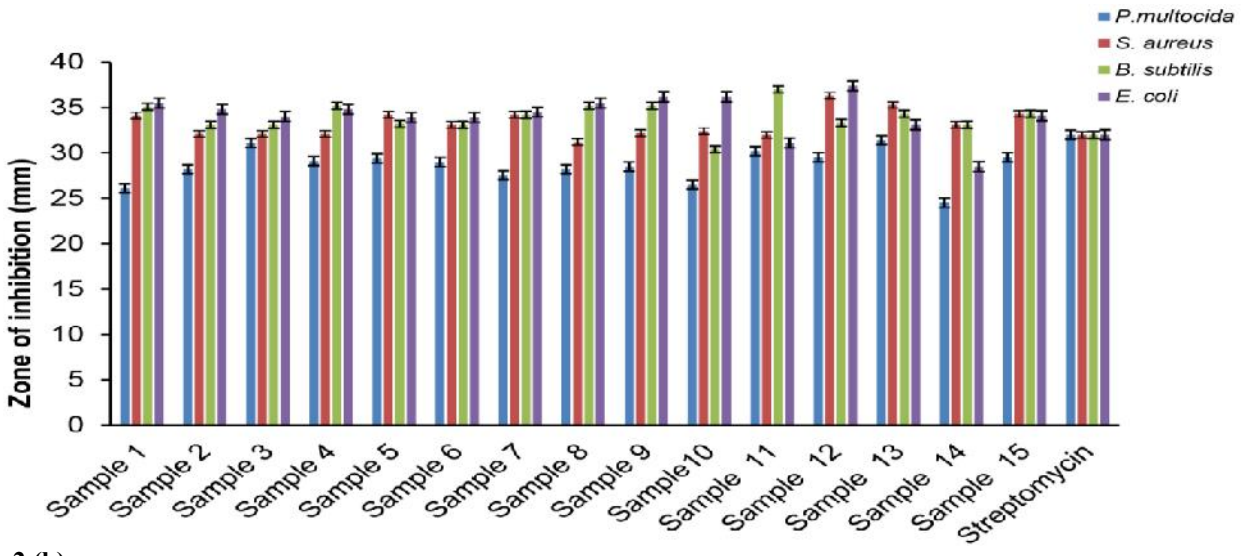


Fig 2 (b)

Fig 2 (a)

Fig. 2. Antibacterial activities of combination II (1:1:1). (a) Graphical representation of results: Each bar indicating data from three independent experimental replicates with standard error. Maximum antibacterial activity was shown by (CLF:WSF:STM) 37 mm against *E. coli*. **(b) Well diffusion method's Agar plates:** Sample 13 showed maximum ZOI 31 mm against *P. multocida*, sample 11 showed 37 mm against *B. subtilis* and sample 12 showed 37 mm against *E. coli*. For positive control streptomycin was used that showed 32 mm ZOI averagely.

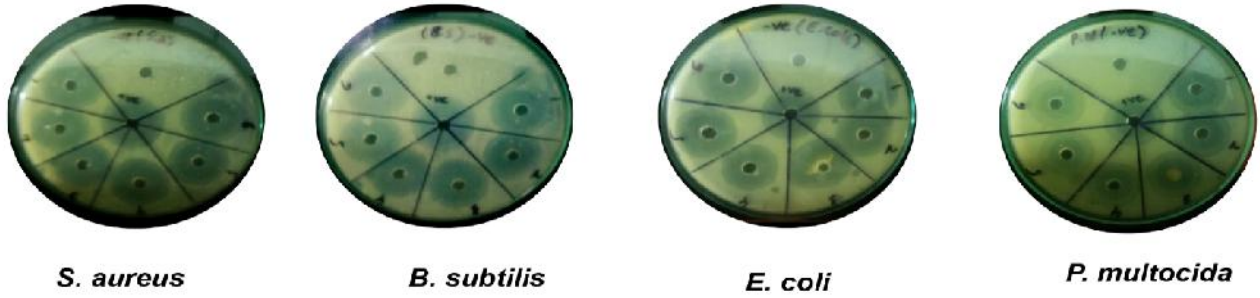
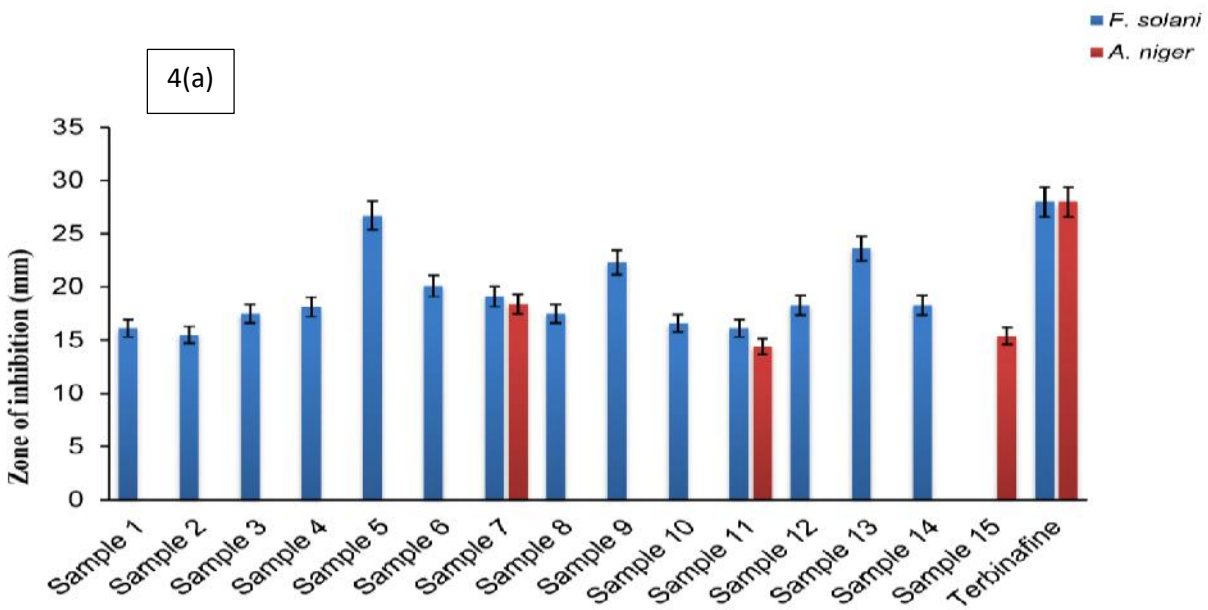


Fig. 3. Antibacterial activities of combination III (1:1). Sample 6 maximum ZOI 34 mm against *S. aureus*, sample 3 showed 34 mm against *E. coli* and sample 6 showed 33 mm ZOI against *B. subtilis* and Sample 3 showed maximum ZOI 31.6 mm against *P. multocida*.

Antifungal activities of combination II and III: Results obtained from single extract of *B. clausii* against *Fusarium solani* no antifungal activity was observed and also combination I exhibited no interaction against tested fungi aresummarized in table 2. But when the same combinations were used with standard Antifungal drug (1:1:1) in combination II and (1:1) in combination III as mentioned in materials and methods the activity was enhanced and showed synergistic effects in (1:1:1) mostly against *Fusarium solani* (CME:WSF:TER) showed maximum inhibition zone (26 mm) EAF:TER showed maximum ZOI (17 mm) against *Fusarium solani*. In some combinations we observed both results minor synergy in (CLF:TER) showed maximum ZOI (14 mm) but mostly additive/antagonistic effects against *A. niger* (Table 3) and (Fig 4a and Fig 4b). Advantages over monotherapy include increased fungal killing potency decreased resistant strains and reduced dose-related toxicity of the antifungal drugs (Vitale *et al*., 2005). Overall, our results proved that drug combinations that inhibit growth of one fungus but it may not be always suitable for other due to the potential differences towards drug combination and type of strains too.

Literature revealed that the combination of fluconazole with crude extracts of green tea showed no significant difference in zones of inhibition. Whereas crude extracts showed diminished activity with combination of fluconazole than with fluconazole alone activity. Result shows combination of crude extracts of green tea with conventional antifungal fluconazole showed antagonism along with minor synergy (Erolls *et al*., 2015).



4(b)

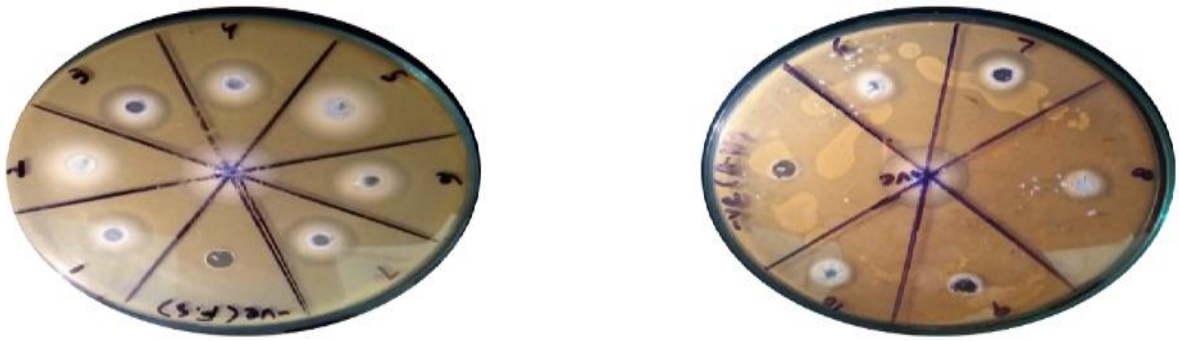


Fig. 4. Antifungal activities of combination II (1:1:1). (a) Graphical representation of results: Each bar indicating data from three independent experimental replicates with standard error. Maximum activity was shown by sample 5 CME:WSF:TER showed maximum ZOI 26 mm against *F. solani*. **(b) Well diffusion method's Agar plates:** Sample 5(1:1:1) showed maximum ZOI 26 mm against *Fusarium solani* and sample 7 showed 18 mm maximum ZOI against *Aspergillus niger*. Terbinafine was also used as positive control.

Table 3. Antimicrobial activities of combination III.

Sr#	Sample Name	Bacterial strains				Fungal strains	
		S. aureus	*B. subtilis*	*P. multocida*	*E. coli*	*F. solani*	*A. niger*
		Mean ZOI (mm)±S.D	Mean ZOI (mm)±S.D	Mean ZOI (mm)±S.D	Mean ZOI (mm)±S.D	Mean ZOI (mm)±S.D	Mean ZOI (mm)±S.D
1	CME:+ve drug	32.5±0.98	32.3±0.94	30.9±0.82	31.3±0.43	15.4±0.7	-
2	n-HEF:+ve drug	31.6±0.95	31±1.6	31.0±0.8	30.0±0.82	14.3±0.3	-
3	CLF:+ve drugs	31.3±0.86	30.6±0.94	31.6±0.94	34.5±1.59	16.3 ± 0.2	14.3±0.3
4	EAF:+ve drug	32.2±0.82	32.3±0.4	31±0.93	29.6±0.57	17.3 ±0.4	-
5	MSF:+ve drug	32.5±0.62	32±0.81	30.3±1.24	33.3±0.32	11.2 ±0.1	-
6	WSF:+ve drug	34.4±0.93	33.6±0.94	30.6±0.94	30.9±.0.07	10.5 ±0.58	-
7	STM/TER	32.3±0.22	32.6±0.94	32.6±0.94	32.0±0.61.	28±0.28	28±0.28

*Results are expressed as mean (ZOI)±S.D, +ve control streptomycin (STM) for bacteria , Terbinafine (TER) for fungus.

Table 4. Analysis of variance (mean squares) for different strains regarding significance of different samples.

Source of variation	Bacterial strains								Fungal strains			
	P. multocida		*E. coli*		*S. aureus*		*B. subtilis*		*F. solani*		*A. niger*	
	df	MS	df	MS	df	MS	df	MS	df	MS	df	MS
Samples	27	193.485**	25	87.388**	26	66.540**	25	78.582**	22	76.476**	6	129.944**
Error	56	0.477	52	0.305	51	0.328	52	0.458	46	0.083	14	0.023
Total					83		77		77		68	20

NS = Non-significant (P>0.05); * = Significant (P<0.05); ** = Highly significant (P<0.01)
df = Degree of freedom; MS = Mean squares

Table 5. Comparison of mean±SE.

Combination	Sample	Bacterial strains				Fungal strains	
		P. multocida	*E. coli*	*S. aureus*	*B. subtilis*	*F. solani*	*A. niger*
I	**S2**	11.76±0.83j	-	-	-	-	-
	S3	-	22.66±0.29l	-	-	-	-
	S4	-	16.27±0.15m	-	-	-	-
	S6	10.40±0.35j	-	-	-	-	-
	S7	-	-	24.50±0.00h	-	-	-
	S8	-	-	24.22±0.22h	-	-	-
	S9	10.39±0.08j	-	-	-	-	-
	S10	10.31±0.16j	-	-	21.33±0.61j	-	-

	S14	-	-	-	18.00±0.00k	-	-
	S15	-	-	17.70±0.26i	-	-	-
	S16	48.31±0.16a	48.31±0.16a	48.31±0.16a	48.31±0.16a	28.13±0.09a	27.03±0.03b
II	**S1**	26.03±0.03hi	34.70±0.35cde	34.10±0.06cde	35.10±0.00bcd	16.10±0.00jkl	-
	S2	28.07±0.07fgh	34.13±0.07de	32.10±0.06fg	33.10±0.00c-g	15.50±0.00kl	-
	S3	31.03±0.03bcd	33.83±0.44de	32.33±0.33efg	33.10±0.00c-g	17.50±0.00ghi	-
	S4	29.03±0.03d-g	34.27±0.07de	32.07±0.03fg	35.20±0.00bc	18.10±0.00gh	-
	S5	29.13±0.07d-g	33.33±0.15ef	34.10±0.06cde	33.20±0.00c-f	26.70±0.00b	-
	S6	29.00±0.00d-g	33.33±0.15ef	33.10±0.06def	33.10±0.00c-g	20.10±0.00e	-
	S7	27.18±0.18gh	34.17±0.09de	34.10±0.06cde	34.20±0.00cde	19.10±0.00f	18.40±0.00c
	S8	28.07±0.07fgh	35.17±0.09cd	31.07±0.07g	35.20±0.00bc	17.50±0.00ghi	-
	S9	28.17±0.17e-h	36.10±0.06bc	32.10±0.06fg	35.20±0.00bc	22.30±0.00d	-
	S10	26.17±0.09hi	36.10±0.06bc	32.17±0.12fg	30.13±0.03i	16.60±0.00ij	-
	S11	30.07±0.03c-f	31.03±0.03hij	32.33±0.33efg	37.00±0.00b	16.10±0.00jkl	14.40±0.00e
	S12	29.17±0.07d-g	37.23±0.03b	36.20±0.10b	33.10±0.06c-g	18.30±0.00fg	-
	S13	31.13±0.13bcd	33.07±0.03efg	35.17±0.09bc	34.10±0.10c-f	23.60±0.00c	-
	S14	24.13±0.09i	28.17±0.09k	33.03±0.03def	33.03±0.03d-g	18.30±0.00fg	-
	S15	29.17±0.09d-g	34.07±0.03de	34.10±0.10cde	34.10±0.06c-f	-	15.40±0.00d
	S16	32.00±0.00bc	32.00±0.00fgh	32.00±0.00fg	32.00±0.00f-i	28.00±0.00a	28.00±0.00a
III	**S1**	30.93±0.58bcd	31.39±0.31ghi	32.59±0.70efg	32.33±0.67e-h	15.44±0.54l	-
	S2	31.00±1.00bcd	30.07±0.58ij	31.65±0.68fg	31.00±1.15ghi	14.36±0.23m	-
	S3	31.67±0.67bc	34.52±1.13cde	31.33±0.61fg	30.67±0.67hi	16.37±0.19jk	14.36±0.23e
	S4	31.00±0.00bcd	29.63±0.40jk	32.20±0.58fg	32.33±0.33e-h	17.39±0.31hi	-
	S5	30.33±0.88cde	33.36±0.23ef	32.50±0.44efg	32.00±0.58f-i	11.24±0.14n	-
	S6	30.67±0.67bcd	30.96±0.06hij	34.48±0.72bcd	33.67±0.67c-f	10.50±0.33n	-
	S7	32.67±0.67b	32.07±0.43fgh	32.31±0.16efg	32.67±0.67e-h	28.31±0.16a	28.00±0.00a

Means sharing similar letter in a column are statistically non-significant (P>0.05).

Table 6. Analysis of variance (mean squares) for different strains regarding significance of different combinations.

Source of variation	Bacterial strains								Fungal strains			
	P. multocida		*E. coli*		*S. aureus*		*B. subtilis*		*F. solani*		*A. niger*	
	Df	**MS**	**Df**	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**	**df**	**MS**
Comb.	2	814.415**	2	97.915*	2	34.925NS	2	87.296*	2	213.298**	2	77.090NS
Error	81	44.715	75	26.73	75	22.359	75	24.184	66	19.086	18	37.767
Total	83		77		77		77		68		20	

NS = Non-significant (P>0.05); * = Significant (P<0.05); ** = Highly significant (P<0.01)
Comb. = Combinations; df = Degree of freedom; MS = Mean squares.

Table 7. Comparison of mean±SE of each combination results.

Combination	Bacterial strains				Fungal strains	
	P. multocida	*E. coli*	*S. aureus*	*B. subtilis*	*F. solani*	*A. niger*
I	18.24±4.03b	29.08±4.90b	30.11±4.65a	29.21±4.80b	28.13±0.09a	27.03±0.03a
II	28.60±0.29a	33.79±0.31a	33.13±0.20a	33.80±0.22a	19.59±0.56b	19.05±1.62a
III	31.18±0.27a	31.71±0.40ab	32.44±0.28a	32.10±0.31ab	16.23±1.23c	21.18±3.05a

*Means sharing similar letter in a column are statistically non-significant (P>0.05).

Approach of Synergistic effects is highly applicable for antimicrobial combinational therapies also more beneficial against those antibiotics which are not effective for microbial treatment because of resistance. In several conditions antimicrobial synergism can take place. A microbial metabolic mechanism may be sequentially blocked by two drugs. For example, drug like a cell wall inhibitor (cephalosporin or penicillin) can increase entry of aminoglycoside in the bacteria and therefore generates synergistic effect. One drug may affect membrane and to make the second drug easier to enter. Combined effect can be greater than individual. One drug can also inhibit the second drug inactivation through some microbial enzymes. In such condition synergism takes place even in lesser concentrations of drugs used. For these reasons, multidrug treatment needs to be continuously explored (Brooks *et al*., 1995; Vazquez-Muñoz *et al*., 2019). Combination results can be

synergistic and antagonistic. In our experiment both antagonistic and synergistic effects were seen against generally all tested microorganisms.

Conclusions: Our results reveal that combination extracts or fractions of the *Bacillus clausii* KP10 with each other often showed antagonistic behavior but when we used same extracts with standard drugs they enhanced the activity of drugs and their antibacterial activities were encouraging the real efficiency with low toxic effects. Therefore, they may be used for the treatment of infectious diseases that are caused by microbial resistance to the existing antimicrobial agents and may also serve as the potential candidate of medicinal importance.

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