Seriphidium kurramense (Qazilb.) Y. R. Ling reforms antimicrobial resistance: A study of Synergistic evaluation

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ABSTRACT

Antimicrobial resistance anchors for treatment failures of resistant microbes. To combat such treatment challenges, *Seriphidium kurramense* can be a prospective solution, therefore, this study aims at antimicrobial profiling of *S. kurramense* with emphasis on its combinational interactions with contemporary antimicrobial drugs. An extract library of n-hexane (SK-nH), ethyl acetate (SK-EA), methanol (SK-M) and aqueous (SK-Aq), extracts was subjected to phytochemical analysis by RP-HPLC to quantify therapeutically significant polyphenols. Antimicrobial potential (broth dilution) against resistant bacterial and fungal isolates, synergistic interactions (checkerboard method) with contemporary antimicrobials (both bacterial and fungal), time-kill kinetics, and protein estimation (bradford method) were performed to evaluate the potential of extracts against microbial infections. RP-HPLC quantified a significant amount of syringic acid, caffeic acid, gentisic acid, and quercetin in Sk-Aq, while maximum amounts of thymoquinone and luteolin along with apigenin were present in Sk-M and highest quantities of ferulic acid, myricetin and kaempferol were found in Sk-EA. Extracts presented prominent antimicrobial activity against both the resistant bacterial and fungal isolates (MIC, 25-62.5 µg/ml). The checkerboard method showed synergism with 4- and 8-fold reductions in the MICs of extracts (SK-M and SK-nH) and doses of antimicrobial drugs. Even after 9 and 12 hours of treatment, the synergistic therapy demonstrated a time-dependent reduction in fungal growth. Along with the cell membrane damage caused by reference drugs, it was also shown that the inhibition of fungal proteins was surged with the use of synergistic combinations than with the treatment of extracts alone thus preventing the resistant fungus from surviving. The extracts of *S. kurramense* could be promising alternatives to combat resistance and other challenges of microbial infections.

Keywords: Antimicrobial resistance, Synergistic studies, HPLC, *Seriphidium kurramense,* time-kill studies

INTRODUCTION

Globally, infections brought on by microorganisms (bacteria and viruses) constitute the main cause of illnesses and fatalities in people. At least 65% of recent outbreaks of common human infectious diseases have been caused by viruses that are zoonotic in nature (Lisi et al., 2021). Around 100 million individuals worldwide suffer from microbial diseases each year (Alwan, 2011). The only treatment for bacterially induced microbial illnesses has been antibiotics. However, unjust and inappropriate use has caused bacteria and other microbes to develop resistance againstdrugs, making the task of treating illnesses difficult (Cantas et al., 2013, Van Boeckel et al., 2015). As a result, trails to find alternate treatments to manage microbial infections in people have increased. Traditional medicinal plants have a wide range of secondary metabolites with bioactive qualities, including alkaloids, coumarins, flavones, flavonols, phenolic acids and terpenoids, etc (Elisha et al., 2017). Therapeutics created from plants with antibacterial action come from these substances. Additionally, herbal treatments display a clear antimicrobial effect when combined with prescription medications because plant phytochemicals could resensitize microbes that have developed a resistance to the available antibiotics (Thakur and Kumar, 2019; Shriram et al., 2008; Shriram et al., 2010). (Shriram et al., 2013). Seriphidium Genus, a member of Compositae family, is effective in the treatment of variety of illnesses like hyperglycemia, hypertension, and different diseases related to gastrointestinal system in the Middle East (Shafiq *et al.*, 2017). A strong medicinally important plant innate to Upper Kurram Agency, near the Pakistani-Afghanistan border, is *Seriphidium kurramense* (Qazilb.) Y. R. Ling (Gilani et al., 2010). The local population uses this plant as an anthelmintic, an anti-diabetic, and a treatment for

infections and stomach issues. Despite being of such importance, it is underexplored in many spheres of modern medicine, therefore in this study, appraisal of antimicrobial properties of the crude extracts of *Seriphidium kurramense* with emphasis on resistance reversal in case of bacterial and fungal infections has been thoroughly studied. To the best of our knowledge, in this article for the very first time, the effect of combinational therapy of its extracts and contemporary antimicrobials (both antibacterial and antifungals) has been evaluated. Kinetics study via time-kill analysis & the estimation of protein has further provided an insight into the possible mechanism of action.

MATERIALS AND METHODS

Chemicals and reagents

Dimethyl sulfoxide (DMSO), n-hexane (nH), Distilled water (DW), ethyl acetate (EA),methanol (MeOH), fetal bovine serum, typical antibacterial and antifungal drugs, agar nutrient and phosphate buffer were purchased from Riedel-de Haen in Germany. Tween-80, Sabouraud Dextrose agar (SDA), and RPMI-1640 were acquired from Merck-Schuchardt in the United States, Oxoid in England, and United Traders in Rawalpindi, Pakistan, respectively.

Cultures and strains

Bacterial cultures

Four resistant bacterial clinical isolates that were preserved in the laboratory were used to evaluate the antimicrobial potential of tested extracts. These included resistant (R.) *klebsiella pneumoniae* (Kb-K), R. *Escherichia coli* (MIC-102), R. *Acinetobacter* (AB-29), and methicillin-resistant *S. aureus* (MRSA; MIC-104).

Fungal cultures

The current investigation comprised *Aspergillus flavus* (FCBP 0064), *Aspergillus niger* (FCBP-0198), and Mucor spp. as non-dermatophyte fungal strains (FCBP 0300). Armed Forces Institute of Pathology (AFIP), Combined Military Hospital (CMH), Rawalpindi gave resistant clinical isolates of *Fusarium dimerum*, *Rhizopus arrhizus*, *Aspergillus terreus*, as non-dermatophytes and *Trichophyton rubrum Alternaria alternate* as dermatophytes.

Extract preparation

In the order of inclining polarity, four types of solvents were used to prepare crude extracts i.e., n-hexane (nH), ethyl acetate (EA), methanol (M) and distilled water (DW) respectively. Successive series of extraction together with maceration assisted with ultra-sonication was employed. The biomass of the plants to the solvent was 1:4 (w/v). 5 kgs of powdered plant material were initially macerated in respective solvent(s), for three days in 1000 ml Erlenmeyer flasks before being regularly sonicated at 25 kHz. First, muslin cloth was used for filtration, and then filter paper was employed for fine filtering. The solvent was extracted from the filtrate using a rotary evaporator at 40°C under a vacuum. The crude extracts obtained from plants were held at 4°C by placing in containers which were properly lablled once they were dried completely (Ali *et al.*, 2022).

RP-HPLC quantitative analysis

High-performance liquid chromatography system along with DAD (diode array detector) was employed as reported earlier in the previous publication whereby polyphenolic characterization of *S. kurramense* was documented (Ali *et al.*, 2022). This is in continuation with the previously reported article to establish, extend and appraise the biological attributes.

Antimicrobial evaluation

Preliminary resistant profiling of antibiotics Initially, the disc diffusion method was used to evaluate antibiotics against clinical isolates. Cefixime, ciprofloxacin, doxycycline, lincomycin, and clarithromycin stock solutions (4 mg/ml) were made in DMSO. Sterile discs loaded with 5 µl of antibiotics were placed on agar media-coated plates, which were then put in the incubation at temperature of 37°C for a period of 24 hours. With the help of a vernier calipers, zone of inhibition around each disc was measured. The assay was done three times. The antibiotic with minimal to no ZOI was chosen for additional research. Microbroth dilution previously described (Zahra et al., 2017) was employed to assess MIC of antibiotics.

Antibacterial assay

To determine the MIC of *S. kurramense* extracts micro broth dilution method as formerly described was adapted (Zahra *et al.*, 2017). Bacterial inoculum was created by setting the seeding density to $5x10^4$ CFU/ml. In nutrient broth, each test extract was prepared to get the final concentrations of 300, 250, 150, 125, 100, 75, 62.5, 50, 37.5, 25 ,and 12.5 µg/ml and antibiotic was prepared at varied concentrations as well (10, 3.33, 1.11, and 0.334 µg/ml). In each well of a 96-well plate, 5 µl of test sample and 195 µl of inoculum were mixed. The plate was then incubated at 37°C for 24 hours, and absorbance at 600 nm was measured in a microplate reader (BioTek, USA) after 24 hours of incubation.

Preliminary resistance profiling of antifungals

Broth microdilution method was used to test antifungal medications against clinical isolates of dermatophytes and non-dermatophytes (Wayne, 2008). Terbinafine and Amphotericin-B stock solutions (1 and 1.6 mg/ml) were created in DMSO. RPMI-1640 was utilized as the culture media. 5 µl

from each antifungal stock and 95 µl of RPMI were transferred into the corresponding wells of the microtiter plate, and then 100 µl of the respective fungal suspension were added in each well. The turbidity of the inoculum (in sterile saline 0.85%) was adjusted in accordance with McFarland standard 0.5. Terbinafine was utilized as a positive control for dermatophytes, amphotericin B for non-dermatophytes, and DMSO dissolved in RPMI-1640 (less than 1%) was used as a growth control. The plates were put for incubation at temperature of 35°C for a variety of times, depending on the strains, or until growth was visible in the growth control well. The findings were noted via visual assistance with the help of a magnifying glass. The last well, which had no signs of fungal growth, was expressed as MIC. Three runs of the assay were completed. The antifungal drug resistant to a specific strain was utilized further in combination studies.

Antifungal susceptibility testing (AST)

Agar well diffusion and microbroth dilution were used for predicting the antifungal nature of plant crude extract *in-vitro*. It was done in accordance with the protocol outlined in Clinical and Laboratory Standards Institute (CLSI) document M38-A2 (Wayne, 2008) also reported in our previous publication (Ali *et al.*, 2022) where primary biological evaluation of *S. kurramense* was outlined.

Determination of synergy

The checkerboard method as previously reported (Bidaud et al., 2021) in compliance with CLSI recommendations (document M38-A2) was used to estimate interactions between plant extracts and contemporary antifungal drugs (terbinafine for non-dermatophytes and amphotericin B for dermatophytes) as well as with antibiotic (cefixime) (Fadli *et al.*, 2012)(Wayne, 2008). While the respective drug was applied vertically in 96-well plates at varied doses, sample extracts were added horizontally. For evaluation of interactions, each sample extract was first concentrated to a concentration of 500 µg/ml RPMI (2MIC), from which two-fold serial dilutions were made (250, 125, 62.5, 31.25 and 15.75 µg/ml RPMI). The assay used terbinafine at final doses of 0.01 (2MIC), 0.005, 0.0025, 0.00125, and 0.000625 µg/ml RPMI. Different concentrations of amphotericin-B were utilized in the experiment, including 6.4 (2MIC), 3.2, 1.6, 0.8, and 0.4 µg/ml RPMI. The first concentrations of both extracts and drugs were put into the respective wells and then 100 µl of inoculum (density 4×10^4 CFU/ml) was added in each. The next concentrations were applied using the same method. Ciprofloxacin was used as a positive control for bacterial assay and terbinafine served as the assay's positive control for dermatophytes, whereas amphotericin B for non-dermatophytes. The bacterial plates were then incubated at 37°C for 24 h and fungal plates at 35°C for varied times. The absorbance at 600 nm was measured in a microplate reader (BioTek, USA) after 0 and 24 hours of incubation. The entire process was carried out three times. The fractional inhibitory index (FIC) was determined using the formula below:FIC of antimicrobial = MIC Of antimicrobial in combination/ MIC of antimicrobial alone……Eq 1FIC of extract (FICextract)=

MIC of extract in combination/ MIC of extract alone. Eq 2While FIC index was the sum of FIC of both antifungal and plant extractFIC Index (FICI) = FICantimicrobial + FICextract……Eq3FICI values are interpreted as indicated in table 1.

Table 1 Interpretation of FICI values.

FICI Values	Interpretation
≤ 0.5	Synergistic
> 4	Antagonistic
> 0.5-1	Additive
>1 & <4	In-different

(Cuenca-Estrella, 2004), (Iten *et al.*, 2009).

Time-kill kinetics

Bacteria & Fungi were cultured to grow to the mid-logarithmic phase, and the diluted suspension (10^4 CFU/ml) was then incubated at 35°C for 12 hours with MIC, 2MIC, FICI, and 2FICI of extracts and their various combinations. At 600 nm, absorbance was measured at 0, 3, 6, 9 and 12 hours. By establishing a graph between absorbance and time, bacterial and fungal growth was observed (Selestino Neta et al., 2017). The experiment was performed thrice.

Protein estimation assay

The Bradford method, as previously reported, was used to estimate the protein level in untreated bacteria and fungus, extracts treated microbes, and extracts as well as antimicrobial treated microbes. (Nouroozi et al., 2015). Bovine serum albumin (BSA, A8806 Sigma), prepared in phosphate buffer (0–50µg/ml), was used as the positive protein standard. 5 µl of each test sample were used in the assay, later to which 195 microliters Bradford reagent was added to to microplate wells. For 60 seconds, the content was continuously mixed. For the measurement of absorbance values, the plates were put in the incubation at 595nm with a microplate reader at for a period of 5 minutes at normal room conditions in terms of temperature. (Biotek, USA). The samples colours, subsequently on addition of reagent was noticed, and if protein content was present in the solution, a shift in colour purple-blue or blue color was seen. When the absorbanve values of standard dilutions were plotted

against their corresponding concentrations, it resulted in standard curve and calibration equation. The sample's protein concentration was determined using the formula.

Table 2 Reverse phase high performance liquid chromatography-based quantification of polyphenols in S. kurramense extracts

Sr.no.	Polyphenol standards	Signal (nm)	Concentration (µg/mg of Extract) (n=3)		
			Sk-EA	Sk-M	Sk-Aq
1	Plumbagin	257	Nd	Nd	Nd
2	Vanillic acid	257	Nd	Nd	Nd
3	Thymoquinone	257	Nd	0.21 ± 0.02	Nd
4	Gallic acid	279	Nd	Nd	Nd
5	Catechin	279	Nd	Nd	Nd
6	Syringic acid	279	1.23 ± 0.03	Nd	1.43 ± 0.05
7	Coumaric acid	279	Nd	Nd	Nd
8	Caffeic acid	325	Nd	Nd	0.48 ± 0.02
9	Luteolin	325	3.82 ± 0.11	3.90 ± 0.03	Nd
10	Gentisic acid	325	Nd	Nd	6.44 ± 0.01
11	Ferulic acid	325	3.05 ± 0.03	0.42 ± 0.02	2.98 ± 0.01
12	Apigenin	325	2.23 ± 0.01	3.72 ± 0.03	3.01 ± 0.04
13	Quercetin	368	Nd	2.07 ± 0.03	4.39 ± 0.01
14	Myricetin	368	1.04 ± 0.02	Nd	Nd
15	Kaempferol	368	1.23 ± 0.01	0.78 ± 0.03	0.78 ± 0.02

Nd= Not detected, Sk-EA= ethyl acetate extract, Sk-M= methanol extract, Sk-Aq= aqueous extract, Data values shown represent Mean ± SD (n=3)

Resistance profiling of Antibiotics

By disc diffusion method, the antibiotic susceptibility of selected bacterial clinical isolates was evaluated (Table 3). Any antibiotic with a ZOI ≤ 14 mm is regarded as resistant at the standard Clinical and Laboratory Standard Institute (CLSI) fixed dose, in accordance with criteria from the CLSI (Humphries *et al.*, 2021). Results demonstrated that ciprofloxacin, doxycycline, clarithromycin, and lincomycin reduced

Table 3: Antibacterial susceptibility testing of antibiotics.

Antibiotics (20µg/disc)	Antibacterial activity (ZOI mm ± SD)			
	R.E. coli	***R. Acinetobacter***	***R.K. pneumoniae***	***MRSA***
Ciprofloxacin	17±0.6	16±0.1	24±0.4	20±0.12
Doxycycline	30±0.1	25±0.76	24±0.01	28±0.11
Cefixime	-	-	-	-
Clarithromycin	30±0.3	35±0.23	37±0.1	35±0.10
Lincomycin	21±0.3	25±0.5	24±0.1	20±0.01

ZOI= Zone of Inhibition, Data values shown represent Mean ± SD (Standard deviation) (n=3). "-" = no activity

Antibacterial Assay

The findings of antibacterial susceptibility have been summarized in Table 4. It is evident that SK-Aq and SK-M showed the least value of MIC i.e., 25 µg/ml and 100 µg/ml for Acinetobacter. SK-nH showed 50 µg/ml for all resistant isolates except MRSA for which the values of MIC was 300 µg/ml. SK-EA also showed 50 µg/ml for all except MRSA where the value of minim inhibitory concentration was MIC was 250 µg/ml.

The improper and improper use of antibacterial medicines promotes the development of germ resistance, making it difficult for medical personnel to treat bacterial illnesses. Additionally, bacteria have developed other processes that add to their resistance to antibiotics. These involve enzymatic or chemical changes (Todar, 2011). Due to their high level of safety and little side effects, plants have been frequently used in traditional medicinal systems for their effectiveness against infectious diseases. Recent studies on plant extracts' antibacterial activity show how they work and how plants have defence mechanisms against resistance (Ruddaraju et al., 2020).

S. kurramense Extracts have demonstrated a large below:

$y = mx + b$……Eq 4

Where x is considered as unknown protein concentration, y being the absorbance, b as the intercept and m is considered as standard curve slop

Table 4 Antibacterial activity of *S. kurramense* extracts against resistant bacterial strains.

Extracts	***MRSA***		***R. Acinetobacter***		***R. Klebsiella pneumoniae***		***R. Escherichia coli***	
	Activity	**MIC (µg/ml)**	**Activity**	**MIC (µg/ml)**	**Activity**	**MIC (µg/ml)**	**Activity**	**MIC (µg/ml)**
SK-Aq	Active	300	Active	100	Active	25	Active	300
SK-M	Active	300	Active	100	Active	25	Active	100
SK-nH	Active	300	Active	50	Active	50	Active	100
SK-EA	Active	250	Active	50	Active	50	Active	50
Ciprofloxacin	Active	3.33	Active	1.11	Active	1.11	Active	3.33

DMSO	---	NA	---	NA	---	NA	---	NA

Sk-nH= n-hexane extract, Sk-EA= ethyl acetate extract, Sk-M= methanol extract, Sk-Aq= aqueous extract, Positive control= Ciprofloxacin (10 µg/ml), Negative control= DMSO; MRSA= Methicillin resistant Staphylococcus aureus. R= Resistant.

Statistical Analysis

Results were shown in the form of mean and standard error of three replicates. For graphical representations Origin 2D software was used.

RESULTS AND DISCUSSION

RP-HPLC analysis

The results of HPLC analysis have already been published and discussed in the previous publication of this series (Ali *et al.*, 2022). The findings have been tabulated in Table 2 for reference.

the growth of a few clinical isolates. They had ZOIs that varied from 17 to 30 mm for *E. coli*, 16 to 35 mm for *Acinetobacter*, 24 to 37 mm for *K. pneumoniae*, and 20 to 35 mm for MRSA, respectively. Interestingly, all clinical isolates were resistant to cefixime (10 g). According to the CLSI recommendations, the resistance ZOI value for cefixime is set at 15 mm or less at 5 µg/disc (Humphries *et al.*, 2021). Therefore, for combination studies against clinical isolates resistant to cefixime, cefixime was combined with *S. kurramense* extracts.

amount of efficacy against clinical isolates that have proven resistant to modern antibiotics, demonstrating their exceptional activity.The outcomes of antimicrobial action of *S. kurramense* might be associated with different phenolic acids like ferulic acid, luteolin, quercetin & apigenin which were identified in various plant extracts. A noticeable antimicrobial property is known to be possessed by Phenolic acids. (Cetin-Karaca, 2011). Different efficacy levels regarding the activities of apigenin against parasites, bacteria & fungi have been documented with the mode of action concerning with disruption of cell membrane. (Wang *et al.*, 2019) (Kim *et al.*, 2020). Besides, a rapid & continuous inhibitory effect on *Coronobacter sakazakii* was noticed by ferulic acid via alteration across the permeability of cell membrane. (Shi *et al.*, 2016). Luteolin affects membrane permeability and inhibits DNA topoisomerases I and II activity to produce antibacterial effects (Wang and Xie, 2010). Quercetin is a different polyphenol that has been quantified. It has antibacterial properties via modifying cell permeability, damaging bacterial cell walls, lowering enzyme activity, affecting the expression & synthesis of protein also preventing the nucleic acids formation (Yang et al., 2020). It is clear from the antibacterial assay results that *S. kurramense* extracts and/or its separated phytochemicals may be important in the development.

Preliminary resistance profiling of antifungals

Selective clinical isolates of dermatophytes (*T. rubrum and A. alternata)* and non-dermatophytes (*A. niger, A. flavus, F. dimerum, R. arrhizus, and A. terreus*) were tested using the microbroth dilution method for susceptibility to modern antifungals (terbinafine and amphotericin-B) (Table 5). The findings demonstrated that terbinafine suppressed the

Table 5 Resistance profiling of selected antifungals against dermatophyte and non-dermatophyte clinical isolates.

Selected antifungals	Antifungal Activity						
	T. R	Al. A	A. T	Rh	A. F	A. N	F. D
Terbinafine	**S**	**S**	**R**	**R**	**R**	**R**	**R**
Amphotericin-B	**R**	**R**	**S**	**S**	**S**	**S**	**S**

T.R = *Trichophyton rubrum*, Al. A = *Alternaria alternata*, Rh= *Rhizopus arrhizus*, A. F = *Aspergillus flavus*, A. N= *Aspergillus niger*, F. D = *Fusarium dimerum*, *S*= sensitive, R=resistant

Antifungal Susceptibility testing (AST)

The AST as already been performed and reported in

Table 6 Antifungal susceptibility testing of *S. kurramense* extracts against dermatophytes and non-dermatophytes.

Fungal	Minimum Inhibitory Concentration (µg/mL) (n=3)

strains	Sk-EA	Sk-nH	Sk-M	Sk-Aq	Terbinafine	Amphotericin-B	DMSO
T. rubrum	250	500	125	125	0.005	---	---
A. alternata	250	500	125	250	0.005	---	---
A. terreus	250	500	125	250	---	3.2	---
R. arrhizus	250	500	250	---	---	3.2	---
A. flavus	---	250	500	---	---	3.2	---
F. dimerum	62.5	250	62.5	125	---	3.2	---

---= no activity, DMSO= dimethyl sulfoxide, Sk-nH= n-hexane extract, Sk-EA= ethyl acetate extract, Sk-M= methanol extract, Sk-Aq= aqueous extract, Data values were represented as Mean ± SD (n=3), A. fumigatus= Aspergillus fumigatus, A. solani= Alternaria solani, A. niger= Aspergillus niger, A. alternata= Alternaria alternata, A. terreus= Aspergillus terreus, T. rubrum=Trichophyton rubrum, R. arrhizus= Rhizopus arrhizus, A. flavus= Aspergillus flavus, F. dimerum= Fusarium dimerum.

Evaluation of Synergy

The interactions between Cefixime (antibiotic) and test extracts has been displayed in Table 7 while Table 8 shows the interpretation of interactions between contemporary antifungals and test extracts. SK-Aq showed synergistic interactions against MRSA, Sk-M against R. *E. coli* while against *Acinetobacter*, SK-EA showed synergistic interactions. In the case of non-dermatophytes, SK-M showed synergistic synergy against *A. terreus* and *A. flavus* while SK-nH against *R. arrhizus*. Additive interactions were observed against dermatophytes. Earlier studies have reported that that flavonoids have considerable antibacterial and antifungal potentials and when used in combination with antimicrobials may show synergism and therefore uplift the total effect to counter the infectious diseases (Al Aboody and Mickymaray, 2020) (Castillo-Reyes et al., 2015) (Hossein and Maldonado, 1982) (Arif et al., 2009). As the metabolites obtained from plant sources do not lie under the umbrella of standard medication

Table 7 Interpretation of synergistic interactions of *S. Kurramense* extracts with Cefixime.

Extracts	MIC alone (µg/ml)	MIC combination (µg/ml)	Fold reduction	FICI	Interpretation
R. *E. coli*					
SK -Aq	300	37.5	8		
cef	20	10	2	0.625	Additive
SK-M	300	75	4		
cef	20	5	4	0.5	Synergistic
SK-nH	300	150	2		
cef	20	10	2	1	Additive
SK-EA	250	31.25	8		
cef	20	10	2	0.625	Additive
MRSA					
SK -Aq	300	75	4		
cef	40	5	8	0.37	Synergistic
SK-M	100	50	2		
cef	40	5	8	0.62	Additive
SK-nH	100	50	2		

cef	40	20	2	1	Additive
SK-EA	50	12.5	4		
cef	40	10	4	0.5	Additive
R. *Acinetobacter*					
SK -Aq	100	50	2		
cef	40	40	1	1.5	Indifference
SK-M	100	25	4		
cef	40	20	2	0.75	Additive
SK-nH	50	50	1		
cef	40	80	0.5	3	Indifference
SK-EA	50	6.25	8		
cef	40	10	4	0.375	Synergistic
R. *K. pneumoniae*					
SK -Aq	25	3.125	8		
cef	40	40	1	1.125	Indifference
SK-M	25	25	1		
cef	40	40	1	2	Indifference
SK-nH	50	6.25	8		
cef	40	40	1	1.125	Indifference
SK-EA	50	25	2		
cef	40	5	8	0.625	Additive

Sk-nH= n-hexane extract, Sk-EA= ethyl acetate extract, Sk-M= methanol extract, Sk-Aq= aqueous extract, FICI ≤0.5= Synergistic ,0.5>FICI≤1=Additive ,1>FICI<4=Indifferent, FICI≥4=Antagonist, R. E.coli= Resistant Escherichia coli, MRSA= Methicillin resistant Staphylococcus aureus.

Table 8 Interpretation of synergistic interactions of *S. Kurramense* extracts with Terbinafine and Amphotericin-B.

Extracts	MIC alone (µg/ml)	MIC combination (µg/ml)	Fold reduction	FICI	Interpretation
Aspergillus terreus					
SK-EA	250	250	1	2	Indifference
TBN	0.005	0.005	1		
SK-nH	500	250	2	1.5	Indifference
TBN	0.005	0.005	1		
SK-M	125	31.25	4	0.5	Synergistic
TBN	0.005	0.00125	4		
SK-Aq	250	62.5	4	1.25	Indifference
TBN	0.005	0.005	1		
Rhizopus arrhizus					

SK-EA TBN	250 0.005	125 0.005	2 1	1.5	Indifference
SK-nH TBN	500 0.005	125 0.00125	4 4	0.5	Synergistic
SK-M TBN	250 0.005	62.5 0.005	4 1	1.25	Indifference
SK-Aq TBN	500 0.005	500 0.005	1 1	2	Indifference
Aspergillus flavus					
SK-EA TBN	500 0.005	250 0.005	2 1	1.5	Indifference
SK-nH TBN	250 0.005	62.5 0.0025	4 2	0.75	Additive
SK-M TBN	500 0.005	62.5 0.00125	8 4	0.375	Synergistic
SK-Aq TBN	500 0.005	250 0.005	2 1	1.5	Indifference
Fusarium dimerum					
SK-EA TBN	62.5 0.005	31.25 0.0025	2 2	1	Additive
SK-nH TBN	250 0.005	250 0.005	1 1	2	Indifference
SK-M TBN	62.5 0.005	15.625 0.0025	4 2	0.75	Additive
SK-Aq TBN	125 0.005	125 0.0025	1 2	1.5	Indifference
Trichophyton rubrum					
SK-EA Amp-B	250 3.2	125 1.6	2 2	1	Additive
SK-nH Amp-B	500 3.2	500 3.2	1 1	2	Indifference
SK-M Amp-B	125 3.2	31.25 1.6	4 2	0.75	Additive
SK-Aq Amp-B	125 3.2	125 3.2	1 1	2	Indifference
Alternaria alternata					
SK-EA Amp-B	250 3.2	62.5 1.6	4 2	0.75	Additive
SK-nH	500	250	2	1.5	Indifference

Amp-B	3.2	3.2	1		
SK-M	125	31.25	4	0.75	Additive
Amp-B	3.2	1.6	2		
SK-Aq	250	125	2	1.5	Indifference
Amp-B	3.2	3.2	1		

Sk-nH= n-hexane extract, Sk-EA= ethyl acetate extract, Sk-M= methanol extract, Sk-Aq= aqueous extract, FICI ≤0.5= Synergistic ,0.5>FICI≤1=Additive ,1>FICI<4=Indifferent, FICI≥4=Antagonist, TBN Terbinafine, Amp-B Amphotericin-B

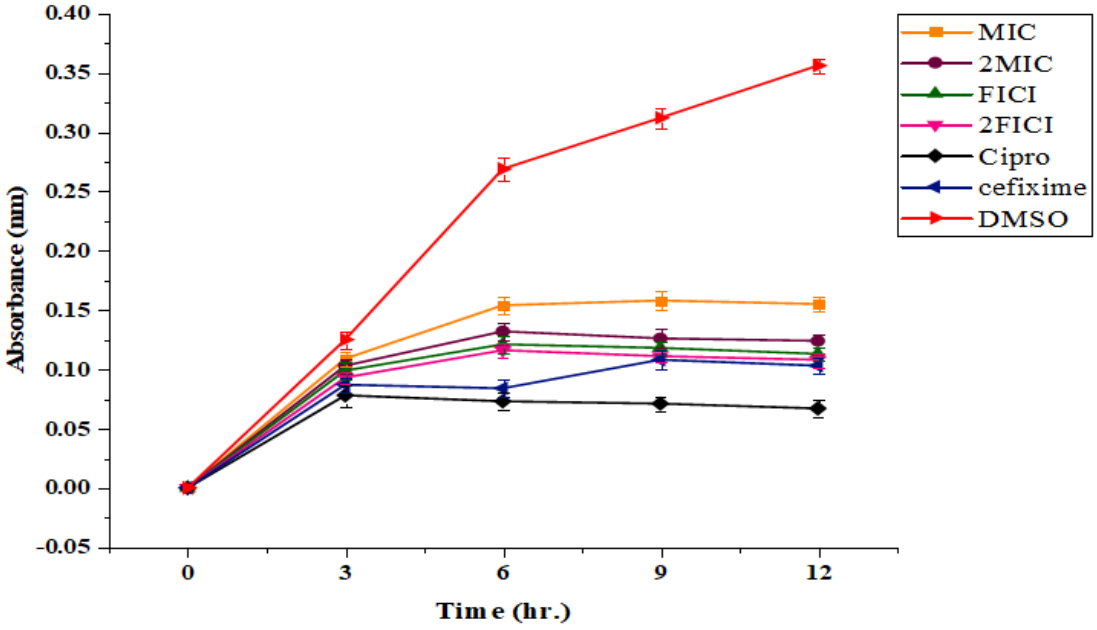
Time-kill Kinetics

The curves exhibiting time-kill nature of extracts with synergistic interactions have been displayed in Figures 1 and 2. Against bacterial isolates extracts have shown a similar trend of increase in the growth of bacteria from 0-9 hours and then the bacteria have shown themselves to be in the stationary phase for 9-12th hours. While in case of combinational therapy the extracts and Cefixime have shown to work in synergy by a decline in the bacterial growth. Similarly in the case of fungi, except against *A. flavus*, the constant decline in fungal growth in case of combinational treatment, is portraying the synergistic work of extracts with terbinafine. Time of leads to combat antibacterial medication resistance.

growth of clinical isolates of dermatophytes while amphotericin-B had no impact at all. While all clinical isolates of non-dermatophytes were terbinafine-resistant and suppressed by amphotericin-B.

Therefore, to combat dermatophytes, AMB was combined with tested extracts, and Terbinafine was utilized in combination trials against clinical isolates of non-dermatophytes. the previous publication of this study (Ali *et al.*, 2022), whereby the results were as follows; therapy therefore it can be thought of them as a sole therapeutical agent or otherwise can also be used in they can be contemplated as monotherapy or in combination therapies to fight against diseases caused by microbes (Amin *et al.*, 2015). The polyphenols found in the *S. kurramense* extracts, including quercetin from SK-M and luteolin from both SK-M & SK-EA, may be responsible for the antifungal activity demonstrated by the different extracts of S. kurramense. By controlling quorum sensing and inducing mitochondrial failure, quercetin enhances fluconazole-resistant *C. albicans* induced apoptosis (Yang et al., 2020). (Rocha et al., 2019). Like this, luteolin causes morphological and ultrastructural alterations in fungi (Báidez et al., 2006). The phenolics' structure enables them to diffuse across microbial membranes and enter cells, where they interfere with the metabolic pathways by intervening in ergosterol synthesis, proteins, chitin and glucosamine of fungi.

kill analysis delivers significant evidence such as idea about anti-fungal properties which are dependent on concentration and time. Against four kinds of concentrations (MIC, FIC, 2MIC and 2FICI), all the samples have been targeted. Due to the fact that the measurements are made over time, the time-kill analysis generates more reliable results on the impact of the combinations (Adusei *et al.*, 2019) (Doern, 2014). The curves exhibiting time-kill nature of extracts from this study conducted provide us with a hint of possibility regarding mode of action of the extracts being treated solely as well as on combining with other agents, the continuous decline of activity by fungi against inclining time shows that the extracts being tested along with drugs used as reference have shown action in synergistic fashion.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Absorbance (nm)
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Time (hr.)
MIC
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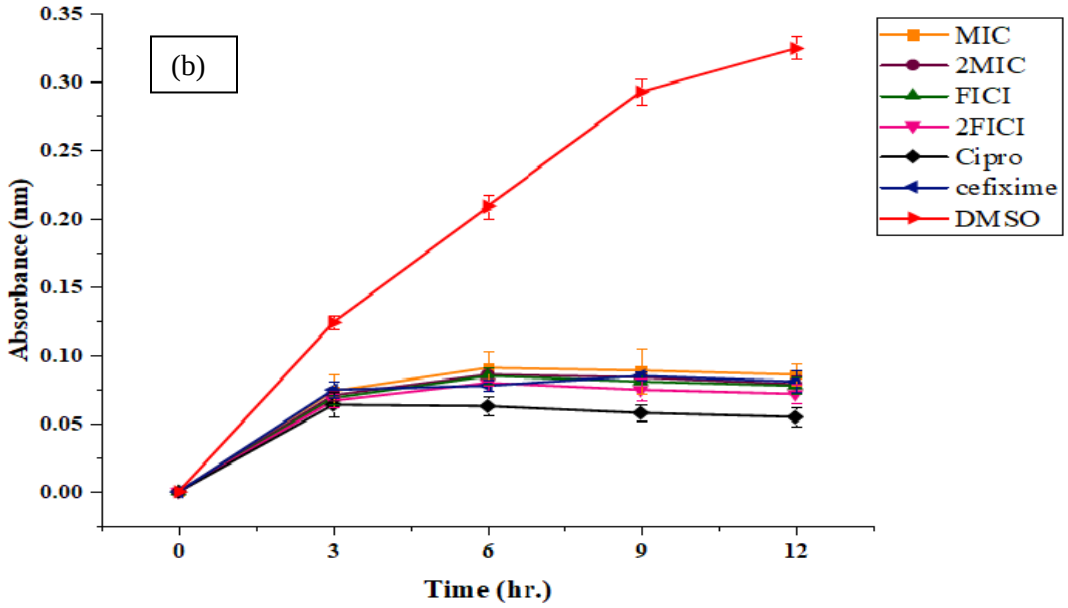
FICI
2FICI
Cipro
cefixime
DMSO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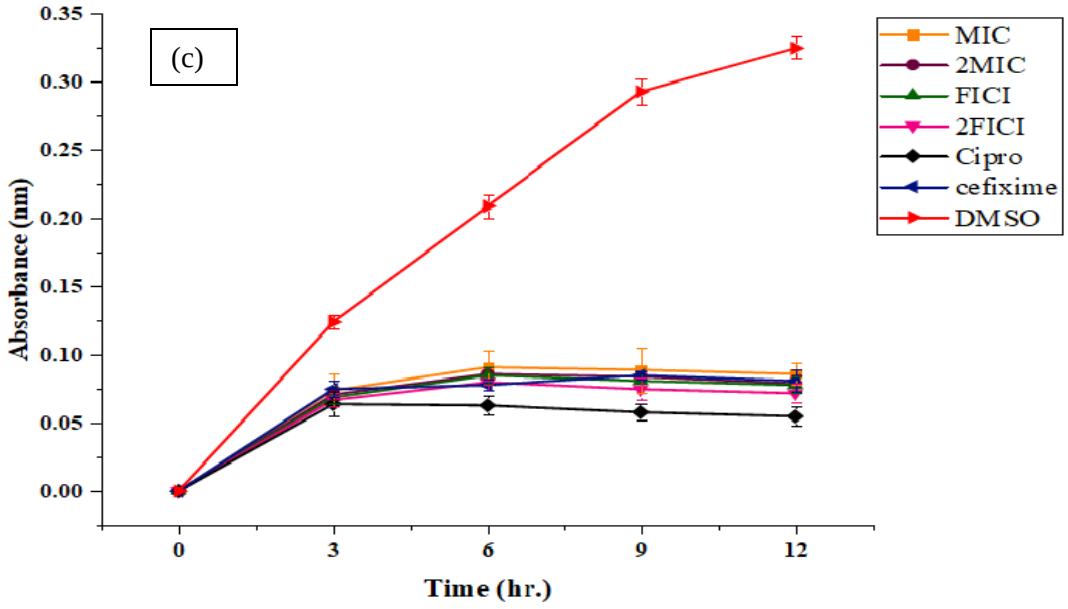
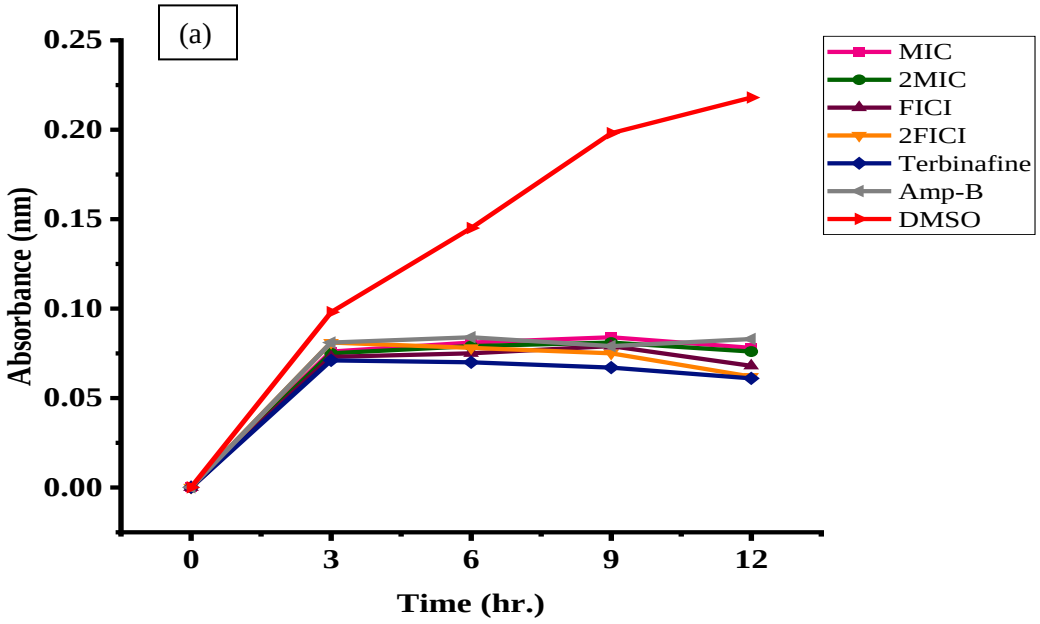


Figure 1 Time-kill curves of (a) SK-Aq against MRSA, (b) SK-M against R. *E. coli* and (c) SK-EA against *Acinetobacter*



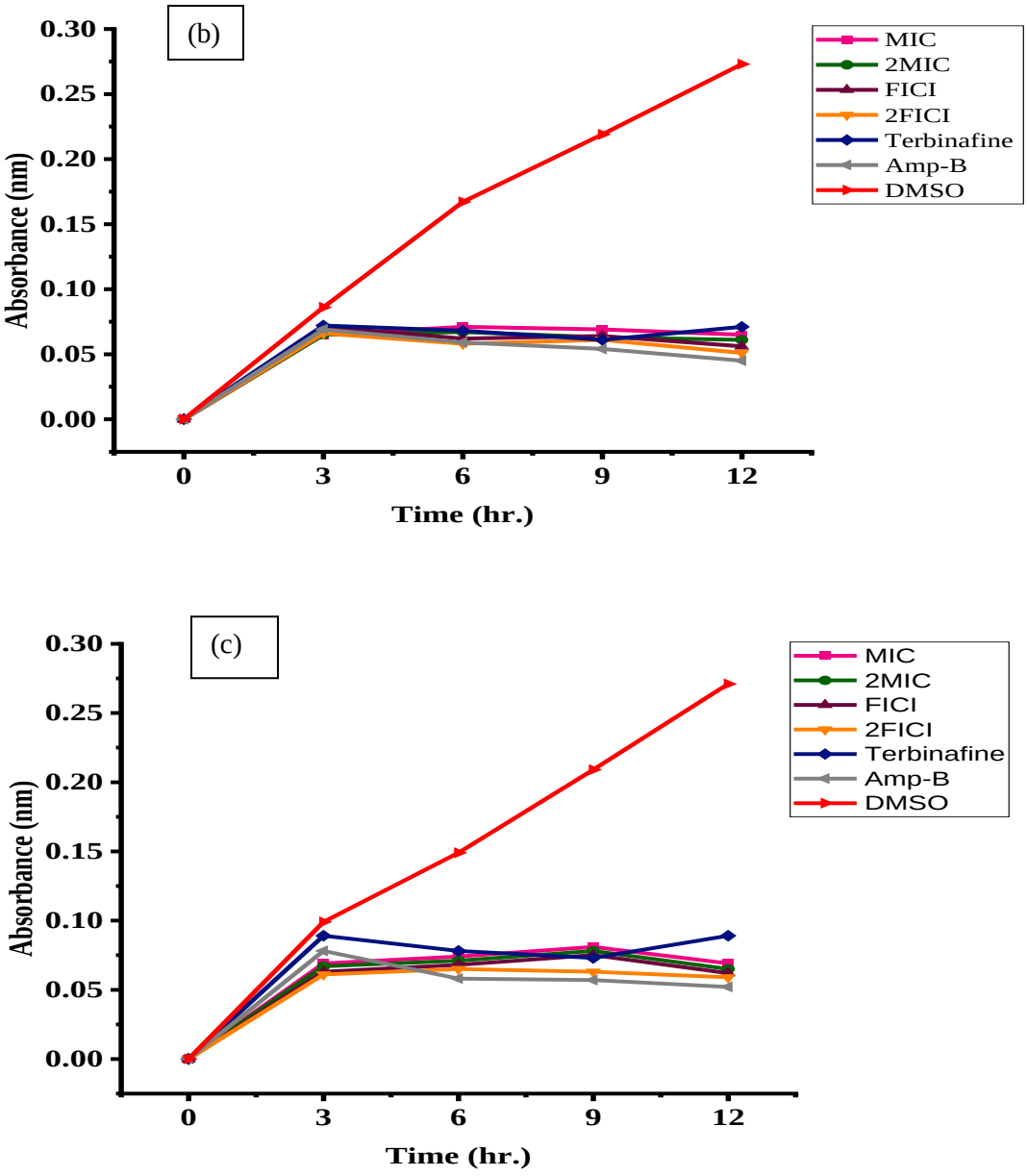


Figure 2 Time-kill curves of (a) SK-M against *A. terreus,* (b) SK-nH against *R. arrhizus* and (c) SK-M against *A. flavus.*

Protein Estimation

To calculate the unknown protein concentration in samples treated with microbes at their MIC and fractional inhibitory concentration and minimum inhibitory concentration, a BSA standard curve was created with absorbance against various BSA concentrations. The concentration of protein & the percentage of inhibition of protein shown by the chosen extracts either alone or given together with other market available drugs against selected microbial strains. It is very evident from the findings that the percentage of inhibition of protein augmented when the extracts were combined with contemporary drugs. SK-Aq against MRSA showed 84.70% inhibition in combination while alone it displayed 65.12% inhibition. Similarly against fungal proteins, SK-M showed 79.92% inhibition in synergy against *A. flavus* while when given alone it only inhibited 43.38% proteins.By calculating the amount of cellular protein leakage resulting from cell death, the disintegration of cell membrane was quantified. To identify the root cause

Table 9: Estimation of protein content in *S. kurramense* extracts and combinations.

Extracts and combinations	MIC or FICI (µg/ml)	Concentration of protein (µg/ml)	Percent protein inhibition
Methicillin resistant *Staphylococcus aureus* (MRSA)			
SK-Aq	300	34.88	65.12
SK-Aq + cef	37.5+10	85.30	84.70
Cefixime	20	71.78	28.22
control	---	100.04	0.00
R. Acinetobacter			
SK-EA	50	65.39	34.61
SK-EA + cef	6.25+10	58.10	41.90
Cefixime	40	58.56	41.44
control	---	100.00	0.00
Aspergillus terreus			
SK-M	125	22.54	38.53
SK-M+TBN	31.62+0.00125	23.28	40.53
TBN	0.005	18.96	48.29
Control	---	36.67	0.00
Rhizopus arrhizus			

SK-nH	500	20.16	51.47
SK-nH+TBN	125+0.00125	23.46	73.51
TBN	0.005	18.87	54.56
Control	---	41.53	0.00
Aspergillus Flavus			
SK-M	500	25.02	43.38
SK-M+TBN	62.5+0.00125	35.39	79.92
TBN	0.005	25.75	41.72
Control	---	44.19	0.00

Sk-nH= n-hexane extract, Sk-M= methanol extract, "--" = not observed, control= untreated fungal protein concentration, MIC= minimum inhibitory concentration, FICI= Fractional inhibitory concentration index, TBN= Terbinafine

CONCLUSION

The results of this study imply that the extracts obtained from *S. kurramense* display antifungal & antibacterial and activity which is indicative of their potential as a significant antimicrobial agent. When given in combination with other modern antibiotics, the extracts of *S. kurramense* work in a synergistic fashion which results in enhanced antimicrobial effect of either. The findings obtained from the time-dependent kinetics provided insight into the potential mode of action via the death of microbes dependent on time by these extracts and their concomitant use with modern antifungal agents. The outcomes resulting from estimating proteins gave a clue which indicated that among possible causes of the antimicrobial effect include the breakdown of microbial cell wall. All these findings suggest that these extracts might be employed as complementary treatments against microbial infections.

Author's Contribution

We corroborate that each author contributed significantly to the effort and gave their seal of approval for the final published description. The study was conceptualized by HF the study along with the supervising the undertaking of experiments, and critical revision of the manuscript. The whole experimentation was carried out by NA, who also wrote and revised the text as well as analyzed and of antimicrobial effect, the protein content in extracellular media of untreated as well as treated microbial strains were put under examination. Both reference drugs used i.e, cefixime and AMB have detrimental action on the cell wall of fungi (Ashraf *et al.*, 2015) (Mesa-Arango *et al.*, 2016). The results have shown decline in the protein content when treated with the combination of extracts and drugs, therefore, it would be correct to claim that both agents, the drugs and the extract exhibit an interference with the cell wall of fungi and may work via distressing fungi's proteins, though an exact mechanism by which they do so is unknown. The witnessed synergism can be accredited to the existence of phytochemicals with recognized antifungal property quantified through HPLC. (Ali *et al., 2022)*. The polyphenols have the ability to interact with microbial (both bacterial and fungal) cell walls And cause permanent changes in them thus rendering them inactive or unable to survive (Papuc et al., 2017) (Mohsin et al., 2022) interpreted the results. STBK and SAM contributed to the data curation, writing down the manuscript and its critical evaluation. HA helped in software usage and analysis. The validation of data & manuscript revision were contributed by SF.

Declaration of Competing Interest

It has been declared by the authors that they find neither any personal nor financial interests that may have interfered with the work being reported in this paper.

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