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Research Article

Antimicrobial Properties and Phytochemical Analysis of Mustard Leaves (*Brassica juncea*)

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Abstract

Introduction: The aim of this study was to investigate the antibacterial and antifungal properties of the leaves from the mustard plant (*Brassica juncea*) against various strains of *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans and Cryptococcus neoformans*. Different concentrations of three solvents were used, and we also sought to identify some of the phytochemicals present.

Materials and Methods: The extracts from the leaves were obtained using ethanol, hexane, and ethyl acetate and concentrated using a rotary evaporator. Serial dilution was used to obtain varying concentrations of the extracts (100-0.78 mg/ml) and sterile filter paper discs were placed in the extracts. The Kirby Bauer disc diffusion method was done, using Mueller Hinton agar for the bacteria, and Sabouraud Dextrose agar for the fungi. Discs were placed in triplicate. Discs soaked in pure solvent, were used as the negative control. Ciprofloxacin, ceftazidime and tetracycline constituted the positive controls for the bacteria, and fluconazole and ketoconazole, for the fungi. After incubation, zones of inhibition around the discs were measured in millimeters and the results expressed as mean ± Standard Deviation. Screening for six phytochemicals was done using standard techniques.

Results: The most effective solvent was ethyl acetate (EA) at 100 mg/ml concentration. The MIC exhibited by EA extracts against *P. aeruginosa* ATCC 27853 was 6 mg/ml; against *K. pneumoniae* 700603, it was 1.6 mg/ml; and against *K. pneumoniae* ESBL in-house strain, it was 0.8 mg/ml. The zone diameters for 100 mg/ml EA for *P aeruginosa; K. pneumoniae* 700603; and *K. pneumoniae* ESBL were 13.3 ± 1.5mm; 20.7 ± 5mm and 13.0mm respectively; and they showed resistance to tetracycline; ceftazidime; and ciprofloxacin and tetracycline, respectively. Two of the *C. albicans* strains were resistant to fluconazole but susceptible to EA extracts. The MIC of EA extracts against *C. neoformans* was 0.8 mg/ml with a zone diameter of 15.7 ± 4.0mm at 12.5 mg/ml concentration. Terpenoids and steroids were found in all of the extracts but the only unique parameter in the EA extracts was alkaloids.

Conclusions: Mustard leaves (*B. juncea*) clearly possess promising antimicrobial properties. Further investigations should focus on EA extracts and their antimicrobial effects on clinical isolates and a comprehensive analysis of their phytochemical constituents.

Keywords: Antibacterial; Antifungal; *Brassica juncea*; Solvents; Phytochemicals

Abbreviations

AFAs: Antifungal Agents; AFP1: Antifungal Protein 1; AMR: Antimicrobial Resistance; ATCC: American Type Culture Collection; CLSI: Clinical and Laboratory Standards Institute; ESBL: Extended Spectrum Beta Lactamases; GPHC: Georgetown Public Hospital Corporation; MHA: Mueller Hinton Agar; MIC: Minimum Inhibitory Concentration; SDA: Sabouraud Dextrose Agar

Introduction

Antimicrobial resistance (AMR) is a global problem since the emergence and transmission of drug resistant pathogens with new resistance mechanisms, continue to hamper the successful treatment of communicable diseases. A review of the literature indicates that there are only a few, modern antibiotics available to effectively treat Gram positive and Gram negative bacteria [1] and even fewer against multi-drug resistant strains such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and the Extended Spectrum Beta Lactamases (ESBLs). In addition, ESBL strains such as *Klebsiella pneumoniae* ATCC 700603 are inherently resistant to ceftazidime [2]. Therefore, there is an urgent need for novel strategies to address this issue [3].

Furthermore, many clinical infections, some of which may be dangerous and life-threatening, are caused by fungi. These include *Cryptococcus neoformans, Candida albicans, Candida auris, Rhizopus oryzae* and *Aspergillus fumigatus.* Due to acquired fungal resistance to the existing antifungal drugs, treatment options are now more limited. Fluconazole, amphotericin B and echinocandin are some antifungal agents (AFAs) used to treat fungi-related diseases [4]. Hospitalised patients, especially those who are immunocompromised, face serious challenges if they become infected with resistant strains of fungal species.

Resistance to AFAs can occur intrinsically for some fungal species even without previous exposure to the agent. Resistance by *Candida sp.* to the azoles, and *Cryptococcus sp.* to echinocandins, has been observed [5]. Furthermore, there has been an increased prevalence of nosocomial fungal infections, especially in the ICU and community acquired fungal infections, for COVID-19 patients with pneumonia [6]. Indeed, the emergence of COVID-19 has presented new challenges with the emergence or reappearance of fungal infections which are unaffected by standard antifungal treatment [7].

Medicinal plants contain a variety of secondary metabolites, some of which have demonstrated antibacterial and antifungal activities, in vitro and therefore there is considerable interest in this form of alternative therapy [8]. Plants such as mustard (*Brassica juncea*) which is commonly known as Chinese mustard, Indian mustard, Oriental mustard or mustard greens, are widely used in Oriental dishes worldwide and have been studied for their antioxidant and antihyperglycemic activities [9]. However, there is a paucity of information about the antimicrobial properties of the leaves of *B. juncea*.

The extraction of medicinal plants is a complex process and solvents frequently used include polar solvents (such as water and alcohols), non-polar solvents (for example hexane and chloroform) and semi-polar solvents such as acetone and ethyl acetate [10,11]. The most common methods used to determine antimicrobial activity are disc diffusion and broth dilution and these also allow an estimate of the most effective concentration of the agent [12].

Mustard leaves are prized for both their unique flavor and possible health benefits and seem to be rich in glucosinolates and carotenoids. Antioxidants, vitamins, and minerals are among the phytochemicals that give these leaves their nutritional profile [13]. Plant extracts contain a wide range of bioactive compounds including alkaloids, tannins and saponins; and numerous investigations have shown that these chemicals have beneficial properties. Phytochemical analysis has grown in importance as a means of identifying and isolating the compounds which contribute to the properties of plant extracts. Phytochemical screening of various plants has been carried out extensively and the techniques are well documented [14,15].

The main objective of this study was to investigate the antibacterial and antifungal properties of the leaves from the mustard plant (*B. juncea*) against various strains of *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans and Cryptococcus neoformans*, using three solvents: ethanol, hexane and ethyl acetate. We also sought to identify some of the phytochemicals present in *B. juncea.*

Materials and Methods

This was an experimental study which was conducted primarily at the Main Laboratory of the College of Medical Sciences at the

University of Guyana. *B. juncea* seeds were purchased and then grown for approximately four weeks. All the plants were healthy, and the leaves (approximately 3kg) were washed with distilled water and dried at room temperature for about two weeks.

Preparation of the extract

The dried plants were ground in a food processor, then sieved to remove the unwanted stems, weighed (approximately 90g) and stored in an airtight container. The ground leaves were soaked in a polar solvent (ethanol), a non-polar solvent (hexane) and a semi-polar solvent (ethyl acetate). Three (3) large, dark bottles were sterilised. Thirty grams (30g) of leaves each, were soaked in each solvent of 300mls of ethanol, 300mls of ethyl acetate and 450mls of hexane for 24 hours at room temperature with occasional shaking. The extraction was repeated, and the extracts obtained were filtered using sterilised Whatman No. 1 filter paper and funnel. Subsequently, the extracts were concentrated to dryness under reduced pressure using a rotary evaporator at 45 °C. The concentrated (crude) extracts were stored in the dark until use.

Sterile containers were used, and serial dilution was carried out to obtain the different concentrations (conc.) The crude extract represented 100% concentration or 100 mg/ml conc. For 50 mg/ml conc., 1 ml of the crude extract was added to 9mls of the solvent, that is, 1:10 dilution (10^{-1}). For 25 mg/ml conc., 1 ml of the 50 mg/ml (10^{-1}) was added to 9mls of solvent to give 1:100 dilution (10^{-2}). The process continued until 0.78 mg/ml (10^{-7}) was achieved. The extracts were then used for antimicrobial investigation and phytochemical screening.

Preparation of the discs

Whatman No. 1 filter paper was perforated with a paper punch to make 6 mm discs. The discs were stored in a sealed petri dish and sterilised in an autoclave. Three (3) mls each, of the various concentrations (100 – 0.78 mg/ml), were placed in sterile vials and approximately 150 discs were placed into each vial. The discs were allowed to soak overnight and then used for antimicrobial susceptibility testing.

Test organisms

The test organisms used were *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 700603 all obtained from KWIK-STIK™ and ESBL *K. pneumoniae* (in-house strain) obtained from the Microbiology Department of Georgetown Public Hospital Corporation (GPHC). The following fungi were used: *Candida albicans* – three strains: *C. albicans* ATCC 24058; and two in-house strains (A and B). An in-house strain of *Cryptococcus neoformans* was also used. The in-house strains were obtained from Eureka Medical Laboratory and GPHC.

Antimicrobial susceptibility testing

For the bacteria, the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA) was carried out using comparison with the 0.5 McFarland standard. Clinical Laboratory Standards Institutes (CLSI) guidelines were followed for the inoculation of the agar and the placement of the discs.

The antibiotics, ciprofloxacin, ceftazidime and tetracycline, were also placed on separate MHA plates along with the test bacteria, and these plates were used as the positive control. Discs which had been soaked in the pure solvent (with no extract) were used as the negative control. MHA plates were incubated at 37°C for 18-24 hours, following which, any zones of inhibition around the discs were measured in millimeters (mm) and recorded. Zones were validated by two microbiologists.

The antifungal susceptibility testing was done using colonies of the fungi, growing on Sabouraud Dextrose Agar (SDA), which were placed in dextrose broth, and compared with the turbidity of a 0.5 McFarland standard. Discs were placed, after the SDA was seeded with the appropriate fungus. Positive and negative controls were also used. Fluconazole and ketoconazole suspensions were made up to act as the positive controls using 0.1g to 10mLs of sterile water (1%) and the pure solvents were the negative controls. Plates were incubated at 37°C for 48-72 hours to facilitate the longer incubation time of fungi. Zones of inhibition were measured in mm and validated. Extra precautions were taken when handling the fungi, to include the wearing of KN95 masks, in addition to gloves.

The discs were placed in triplicate on each MHA or SDA plate and each disc measured approximately 6mm in diameter. For the plant extracts, a zone diameter of 6mm was considered as resistant. For the positive controls (ceftazidime, ciprofloxacin and tetracycline), the performance standards for determining susceptibility versus resistance were used after zone sizes were measured (Table 1). The information on the table was extracted from CLSI 2020 [16].

				Zone diameters (mm)		
Antimicrobial Agent	**Disc Code**	**Potency**	**Bacteria**	**S**	**I**	**R**
Ceftazidime	CAZ	30µg	*Enterobacteriaceae*	≥21	18-20	≤17
			P. aeruginosa, Acinetobacter, Staphylococcus	≥18	15-17	≤14
Ciprofloxacin	CIP	5µg	Enterobacteriaceae	≥31	21-30	≤20
			Staphylococcus, Acinetobacter	≥21	16-20	≤15
			P. aeruginosa	≥25	19-24	≤18
Tetracycline	TE	30µg	*Enterobacteriaceae* and *Acinetobacter*	≥15	12-14	≤11
			Staphylococcus	≥19	15-18	≤14

Table 1: Performance standards for Antimicrobial Susceptibility Testing – Zone diameters for Ceftazidime, Ciprofloxacin and Tetracycline.

S – Susceptible, I – Intermediate, R-Resistant.

Information extracted from CLSI Performance Standards for Antimicrobial Susceptibility Testing 2020 [16].

Calculation of MIC

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism (bacterium/fungus). To determine the MIC of the various plant extracts, we adopted the method outlined by Faujdar., *et al*. [17].

Phytochemical screening

The methods described by Wadood., *et al*. [18], were used to screen for alkaloids, tannins, flavonoids and saponins, steroids and terpenoids from the ethanol, ethyl acetate and hexane crude extracts.

Data analysis

The independent or exposure variables were the different concentrations of the *B. juncea* plant extract with the various solvents and the dependent or outcome variables were the zones of inhibition measured in mm which indicate susceptibility. The controlled variables, which were fixed and did not change throughout the experiment, were the different strains of the bacteria and fungi; the solvents and the extraction and preparation of the crude extract.

We used descriptive statistics in the form of graphs and tables. Illustrations were made using the *R* package 'ggpubr' [19] on *R version 4.1.2* [20] and Microsoft Excel 2019 (Microsoft Corporation, 2019).

Results and Discussion

Our results showed that the extracts from *B. juncea* leaves showed both antibacterial and antifungal properties (Tables 2-10). If the bacterium or fungus grew up to the disc, then it was considered resistant, and the value was recorded as 6mm. The most effective solvent was ethyl acetate at a concentration of 100 mg/ml (Figure 1,2). Table 3 shows that antibacterial activity was also observed with ethanol and hexane extracts against *K. pneumoniae* ATCC 700603 (MIC 12.5 mg/ml and 3.1 mg/ml respectively). Ethanol extracts also worked against the in-house ESBL *K. pneumoniae; P. aeruginosa, C. albicans and C. neoformans* (Tables 4,5,7-10). Hexane extracts were not effective against any of the microorganisms except *K. pneumoniae* ATCC 700603 and *C. albicans* in-house strain A (Tables 3,7).

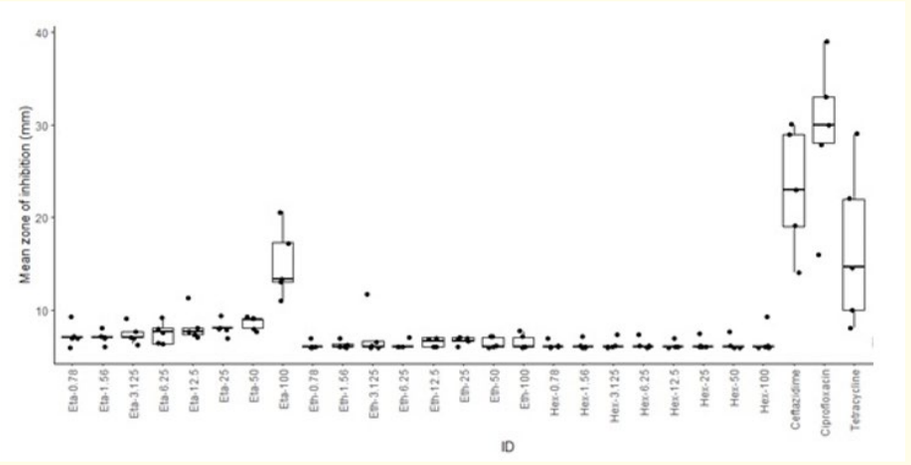

Figure 1: Distribution of mean zone of inhibition (mm) for each solvent at differing concentrations (mg/ml) to compare the antibacterial effects of *Brassica juncea* extract against positive controls (Ciprofloxacin, Ciprofloxacin, Tetracycline).

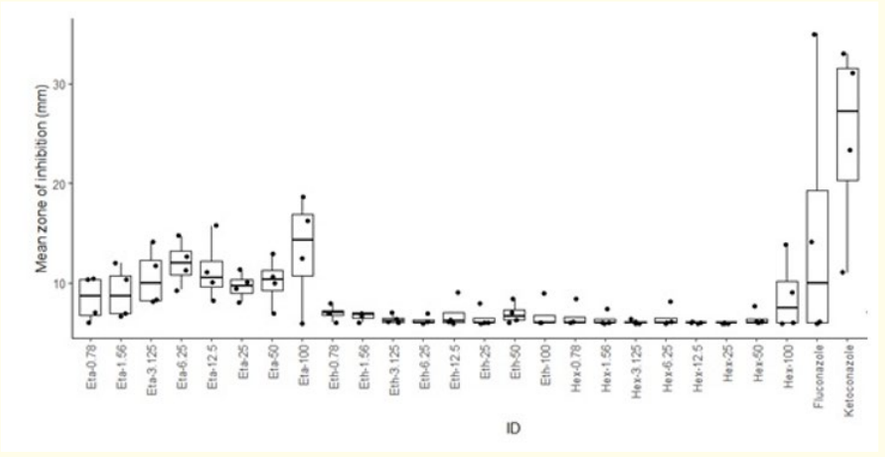

Figure 2: Distribution of mean zone of inhibition (mm) for each solvent at differing concentrations (mg/ml) comparing the antifungal effects of *Brassica juncea* extract against positive controls (Fluconazole and Ketoconazole).

Solvent		Zone of inhibition (mean ± SD) in mm at different concentrations (mg/ml)						Negative Control (mg/ml)	Positive Control (mm)		
	MIC	3.125	6.25	12.5	25	50	100	Pure Solvent	CAZ	CIP	TE
Ethanol	12.5	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.7 ± 0.6	6.0 ± 0.0	6.0 ± 0.0	6.7 ± 0.6	30.0	39.0	22.0
Ethyl Acetate	3.2	7.0 ± 0.0	8.0 ± 2.6	8.0 ± 2.6	8.0 ± 1.7	9.3 ± 1.2	11.0 ± 1.0	6.7 ± 0.6			
Hexane	--	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0			

Table 2: Activity of *Brassica juncea* against *Escherichia coli* ATCC 25922.

6.0 mm zone diameter indicates that the bacterium grew right up to the disc.

CAZ - Ceftazidime; CIP – Ciprofloxacin; TE- Tetracycline. -- indicates that the organism did not show any susceptibility to the extract.

Solvent		Zone of inhibition (mean ± SD) in mm at different concentrations (mg/ml)							Negative Control (mg/ml)	Positive Control (mm)		
	MIC	1.56	3.125	6.25	12.5	25	50	100	Pure Solvent	CAZ	CIP	TE
Ethanol	12.5	6.0 ± 0.0	11.7 ± 4.5	6.0 ± 0.0	7.0 ± 00	7.0 ± 00	7.0 ± 00	7.7 ± 1.5	6.0 ± 0.0	14.0	30.0	18.0
Ethyl Acetate	1.6	7.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0	11.3 ± 1.5	8.0 ± 1.7	9.0 ± 1.7	20.7 ± 5.0	6.3 ± 0.6			
Hexane	3.1	7.0 ± 0.0	7.3 ± 0.6	7.3 ± 0.6	7.0 ± 0.0	7.3 ± 0.6	7.7 ± 1.2	9.3 ± 1.5	7.0 ± 0.0			

Table 3*:* Activity of *Brassica juncea* against *Klebsiella pneumoniae* ATCC 700603.

6.0 mm zone diameter indicates that the bacterium grew right up to the disc.

CAZ - Ceftazidime; CIP - Ciprofloxacin; TE - Tetracycline. -- indicates that the organism did not show any susceptibility to the extract.

***Klebsiella pneumoniae* ESBL**		Zone of inhibition (mean ± SD) in mm at different concentrations (mg/ml)						Negative Control (mg/ml)	Positive Control (mm)		
Solvent	MIC	3.125	6.25	12.5	25	50	100	Pure Solvent	CAZ	CIP	TE
Ethanol	1.6	6.7 ± 0.6	6.0 ± 0.0	7.0 ± 0.0	7.0 ± 1.7	6.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0	23.0	16.0	8.0
Ethyl Acetate	0.8	9.0 ± 0.6	9.3 ± 0.0	7.7 ± 0.0	9.3 ± 1.7	9.0 ± 0.0	13.0 ± 0.0	7.0 ± 0.0			
Hexane	--	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0			

Table 4*:* Activity of *Brassica juncea* against *Klebsiella pneumoniae* ESBL – in house strain.

6.0 mm zone diameter indicates that the bacterium grew right up to the disc.

CAZ - Ceftazidime; CIP - Ciprofloxacin; TE - Tetracycline. -- indicates that the organism did not show any susceptibility to the extract.

Solvent		Zone of inhibition (mean ± SD) in mm at different concentrations (mg/ml)						Negative Control (mg/ml)	Positive Control (mm)		
	MIC	3.125	6.25	12.5	25	50	100	Pure Solvent	CAZ	CIP	TE
Ethanol	12.5	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.7 ± 0.6	7.0 ± 0.0	7.0 ± 0.0	6.0 ± 0.0	29.0	33.0	10.0
Ethyl Acetate	3.1	6.3 ± 0.6	6.3 ± 0.6	7.0 ± 0.0	7.0 ± 0.0	8.0 ± 1.0	13.3 ± 1.5	6.0 ± 0.0			
Hexane	--	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0			

Table 5*:* Activity of *Brassica juncea* against *Pseudomonas aeruginosa* ATCC 27853.

6.0 mm zone diameter indicates that the bacterium grew right up to the disc.

CAZ - Ceftazidime; CIP - Ciprofloxacin; TE - Tetracycline. -- indicates that the organism did not show any susceptibility to the extract.

Solvent		Zone of inhibition (mean ± SD) in mm at different concentrations (mg/ml)						Negative Control (mg/ml)	Positive Control (mm)		
	MIC	3.125	6.25	12.5	25	50	100	Pure Solvent	CAZ	CIP	TE
Ethanol	12.5	6.0 ± 0.0	7.0 ± 0.0	6.7 ± 0.6	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	7.0 ± 0.0	19.0	28.0	29.0
Ethyl Acetate	0.8	7.7 ± 1.2	7.7 ± 0.6	7.3 ± 0.6	8.0 ± 0.0	7.7 ± 2.1	17.3 ± 6.8	7.7 ± 1.2			
Hexane	--	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0			

Table 6: Activity of *Brassica juncea* against *Staphylococcus aureus* ATCC 25923.

6.0 mm zone diameter indicates that the bacterium grew right up to the disc.

CAZ - Ceftazidime; CIP - Ciprofloxacin; TE - Tetracycline. -- indicates that the organism did not show any susceptibility to the extract.

Solvent		Zone of inhibition (mean ± SD) in mm at different concentrations (mg/ml)							Negative Control (mg/ml)	Positive Control (mm)	
	MIC	1.56	3.125	6.25	12.5	25	50	100	Pure Solvent	FLU	KETO
Ethanol	--	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.3 ± 0.6	6.0 ± 0.0	6.3 ± 0.6	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	11.0 ± 0.9
Ethyl Acetate	0.8	10.3 ± 1.5	11.7 ± 1.5	14.7 ± 1.5	9.3 ± 1.5	9.0 ± 0.0	10.0 ± 1.0	18.7 ± 3.1	7.0 ± 0.0		
Hexane	--	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0		

Table 7*:* Activity of *Brassica juncea* against *Candida albicans* ATCC 24058*.*

6.0 mm zone diameter indicates that the fungus grew right up to the disc. FLU – Fluconazole; KETO – Ketoconazole. -- indicates that the organism did not show any susceptibility to the extract.

Solvent		Zone of inhibition (mean ± SD) in mm at different concentrations (mg/ml)							Negative Control (mg/ml)	Positive Control (mm)	
	MIC	1.56	3.125	6.25	12.5	25	50	100	Pure Solvent	FLU	KETO
Ethanol	12.5	7.0 ± 1.0	6.3 ± 0.6	6.0 ± 0.0	9.0 ± 0.0	8.0 ± 1.0	6.0 ± 0.0	6.0 ± 0.0	7.3 ± 0.6	14.0 ± 4.0	31. 0 ± 0.9
Ethyl Acetate	0.8	12.0 ± 1.0	14.0 ± 0.0	9.3 ± 3.2	10.0 ± 0.0	9.3 ± 2.5	10.0 ± 1.0	12.3 ± 0.6	7.0 ± 0.0		
Hexane	3.1	7.3 ± 1.5	8.3 ± 0.6	8.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.1	7.7 ± 0.0	13.7 ± 1.5	7.0 ± 0.0		

Table 8*:* Activity of *Brassica juncea* against *Candida albicans* in-house strain A*.*

6.0 mm zone diameter indicates that the fungus grew right up to the disc. FLU – Fluconazole; KETO – Ketoconazole.

Solvent		Zone of inhibition (mean ± SD) in mm at different concentrations (mg/ml)							Negative Control (mg/ml)	Positive Control (mm)	
	MIC	1.56	3.125	6.25	12.5	25	50	100	Pure Solvent	FLU	KETO
Ethanol	50	6.3 ± 1.2	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	8.3 ± 0.6	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	23.3 ± 1.3
Ethyl Acetate	1.6	6.7 ± 0.6	8.0 ± 1.7	12.7 ± 1.2	11.0 ± 3.0	11.3 ± 1.2	13.0 ± 2.6	16.3 ± 1.5	7.0 ± 0.0		
Hexane	--	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0		

Table 9*:* Activity of *Brassica juncea* against *Candida albicans* in-house strain B*.*

6.0 mm zone diameter indicates that the fungus grew right up to the disc. FLU – Fluconazole; KETO – Ketoconazole. -- indicates that the organism did not show any susceptibility to the extract.

Solvent		Zone of inhibition (mean ± SD) in mm at different concentrations (mg/ml)							Negative Control (mg/ml)	Positive Control	
	MIC	1.56	3.125	6.25	12.5	25	50	100	Pure Solvent	FLU	KETO
Ethanol	12.5	7.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	7.0 ± 0.0	9.0 ± 2.6	7.0 ± 0.0	35 ± 0.0	33.0 ± 1.5
Ethyl Acetate	0.8	7.0 ± 0.0	8.3 ± 2.3	11.3 + 0.6	15.7 ± 4.0	10.0 ± 0.0	10.7 ± 0.6	10.0 ± 0.0	7.0 ± 0.0		
Hexane	--	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0		

Table 10*:* Activity of Brassica juncea against *Cryptococcus neoformans* in-house strain*.*

6.0 mm zone diameter indicates that the fungus grew right up to the disc. FLU – Fluconazole; KETO – Ketoconazole. -- indicates that the organism did not show any susceptibility to the extract.

A study in Turkey on sweet basil (*Ocimum basillicum* Labiatae), also found that hexane and ethanolic extracts have limited antibacterial and anticandidal activities. However, the authors conceded that their findings did not support earlier research, that claimed that hexane was a superior solvent over water and the alcohols, for a more reliable investigation of antimicrobial activities. The authors concluded that other solvents may be needed to extract antimicrobial components more effectively [21].

The MIC is the lowest concentration of the extract at which no visible growth is seen. In some cases, the extract had no effect at any concentration and the MIC was not recorded. This was seen especially with hexane. The maximum zones of inhibition were observed for 100 mg/ml for ethyl acetate for all of the bacteria (Tables 2-10). Early research had shown that the MIC values of ceftazidime, especially as it relates to activity against *Pseudomonas aeruginosa*, were MICs of ≤8 mg/l (>17 mm zone diameter) for susceptibility; and for resistance ≥32 mg/l (≤13mm) [22]. Our results showed an MIC of 3.1 mg/ml with a maximum zone diameter of 13.3 ± 1.5mm with the 100 mg/ml ethyl acetate extract (Figure 3) as compared to the ceftazidime (positive control) which yielded a zone diameter of 29mm. The 3.1 mg/ml is equivalent to approximately 3000 mg/l which therefore indicated that the ethyl acetate has a weaker antipseudomonal activity than ceftazidime. However, our results also showed that the ethyl acetate (100 mg/ml) had a larger zone diameter than tetracycline (Table 5). This is similar to study done in China on dried and pickled mustard leaves. However, the authors observed larger zone diameters for

ethyl acetate (10-30 mg/ml) against *Pseudomonas fluorescens*; probably because the leaves were pickled and then dried and therefore had a more pronounced antibacterial effect [23]. This is interesting, as *P. aeruginosa* is intrinsically resistant to tetracycline which is a bacteriostatic antibiotic [24]. Perhaps further research can be conducted to determine if there is a synergistic effect when tetracycline is used in conjunction with ethyl acetate extracts.

Figure 3: Zones of susceptibility for *Pseudomonas aeruginosa* with ethyl acetate 100 mg/ml.

Based on Table 1 and comparing our results, the only bacteria which were found to be resistant to the positive controls were *K. pneumoniae* ATCC 700603 which was resistant to ceftazidime; *K. pneumoniae* ESBL in-house strain which was resistant to ciprofloxacin and tetracycline and *P. aeruginosa* ATCC 27853 which was resistant to tetracycline as discussed previously. Our findings, in terms of susceptibility of these *K. pneumoniae* strains, are very interesting and significant as *K. pneumoniae* 700603 has been found to be resistant to a wide variety of antibiotics [2]. Cross resistance with ciprofloxacin and tetracycline for *K. pneumoniae* has been well documented [25] so it is not surprising that the ESBL in-house strain which we obtained, also showed cross resistance.

In a study published in 1996, on the MIC of *K. pneumoniae*, the researchers used MICs of ≤8µg/ml as indicative of susceptibility and ≥ 32µg/ml as resistance [26]. Current studies to determine MIC involve the combination of ceftazidime and avibactam which is useful for the carbapenem resistant Enterobacteriaceae; however, resistance to even this combination is becoming more evident [27]. Carbapenems, such as imipenem and meropenem, have the broadest spectrum of activity and are often used as the 'last line' antibiotics [28]. Therefore our research is even more remarkable. Our findings show a zone diameter of 14mm with ceftazidime in comparison to a zone diameter of 20.7 ± 5.0mm with 100 mg/ml ethyl acetate and an MIC of 1.6 mg/ml for *K. pneumoniae* ATCC 700603 (Table 3), so although this strain is resistant to ceftazidime, it is very susceptible to the ethyl acetate extract. In addition, for the in-house *K. pneumoniae* ESBL strain which was resistant to tetracycline (8 mm zone diameter), the zone diameter was 13 ± 0mm for ethyl acetate, with an MIC of 0.8 mg/ml (Table 4).

Interestingly, our study shows that the ethyl acetate extracts have a broad spectrum activity, that is, they were effective against both Gram positive and Gram negative bacteria. A previous study with ethyl acetate and *Acacia nilotica* (gum arabic tree or Egyptian acacia) which is native to the Middle East, India and Africa, found that the MIC (mg/ml) against *E. coli, S. aureus, K. pneumoniae and P. aeruginosa* were 0.78,1.56.1.56 and 0.39 (mg/ml) respectively [29]. This is comparable to our study where we found 3.2, 0.8, 0.8, and 3.1 (mg/ml) respectively. We believe that further studies on other ESBL strains of other Enterobacteriaceae such as *E. coli* and also on Methicillin Resistant *Staphylococcus aureus* (MRSA), should be carried out with the ethyl acetate extracts of *B. juncea*.

We also investigated the antifungal effects of the extracts. The ethanolic extracts seemed to have some effect on some of the fungal strains (Tables 7-10) however, because the negative control also showed some antifungal activity, this was not considered significant. A study was done using 30% ethanolic extracts against several microbes and the researchers observed that *C. albicans* showed susceptibility with a zone diameter of 19.3 mm [30]. Table 8 indicates some anticandidal effects with the ethanolic extracts so this may be an area for future research.

The hexane extracts showed no antifungal activities, and it was difficult to find similar studies using hexane and mustard leaves. Most of the studies with hexane were about the effects on fungal pathogens of plants but we did find one study which investigated the hexane extract against *C. albicans* using the *Cassia alata* plant. The researchers recorded antifungal effects for hexane extracts and for ethyl acetate extracts [31]. Our investigations revealed significant antifungal activity against all four of the fungi, with

nearly all ethyl acetate doses exhibiting activity against the various fungi in comparison to the positive control (Figure 2). Of note, two of the *C. albicans* strains were resistant to fluconazole but were susceptible to ethyl acetate extracts with MICs of 0.8 mg/ml (*C. albicans* ATCC 24058) and 1.6 mg/ml (*C. albicans* in-house B) (Tables 7,9). Researchers have found that an antifungal defensin (Antifungal protein 1 -AFP1) in mustard leaves prevents the growth of *C. albicans* [32].

Although we were unable to locate any studies on the effects of *B. juncea* on *Cryptococcus neoformans*, our results indicate that ethyl acetate extracts, in particular, have strong effects against this fungus (Figure 4). Table 10 shows an MIC of 0.8 mg/ml with a maximum zone diameter of 15.7 ± 4.0mm with 12.5 mg/ml EA. Treatment of cryptococcosis, especially cryptococcal meningitis, frequently involves the use of highly toxic medications that have trouble passing across the blood-brain barrier. Some of the drugs include amphotericin B, fluconazole and 5-flucytosine and researchers have indicated that newer strategies for HIV patients with cryptococcosis are urgently needed [33]. Our findings are therefore timely and important since ethyl acetate extracts are effective even at small doses.

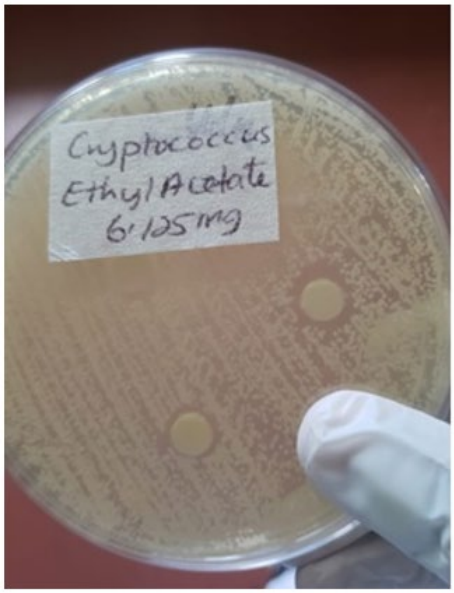

Figure 4: Zones of susceptibility for *Cryptococcus neoformans* with ethyl acetate 6 mg/ml.

Further research is warranted to explore the usefulness of the ethyl acetate extracts of the mustard leaves against fungi from clinical samples. Additionally, investigating their efficacy against moulds would also be important since we have established that they are effective against yeasts. Fungal infections are a major global health concern; despite the effectiveness of some conventional antifungal medications, some of the drawbacks include drug resistance and adverse side effects. Our study paves the way for similar investigations on isolates from hospitalised patients and on moulds such as *Aspergillus* and *Rhizopus* and dimorphic fungi such as *Histoplasma* and *Coccidioides.*

Phytochemical screening is a valuable technique in elucidating and maximising the potential of plant extracts. Table 11 shows that the only unique parameter in the ethyl acetate extracts was the presence of alkaloids. Alkaloids in plants, control growth and shield them from plant predators [34]. Alkaloids are soluble in water in acidic environments and soluble in lipids at neutral and alkaline pH. This attribute is one that can be scrutinised further, to determine if this allows the bioactive antifungal compounds in the ethyl acetate extracts, to cross the blood-brain barrier in the treatment of diseases such as cryptococcal meningitis.

Phytochemicals	Ethanol	Ethyl acetate	Hexane
Alkaloids	-	+	-
Tannins	+	-	-
Saponins	-	-	+
Flavonoids	+	-	-
Terpenoids	+	+	+
Steroids	+	+	+

Table 11: Phytochemical Screening of extracts from *B. juncea.*

We also found terpenoids and steroids in all of the extracts (Table 11). These are naturally occurring substances with long-established anti-inflammatory and anti-cancer capabilities; and originate from the same isoprenoid precursor [35]. Previous studies have shown that terpenoids, alkaloids and flavonoids, contribute to the antimicrobial activity of metabolites obtained from plants. For example, terpenoids inhibit protein synthesis in fungi and break down cell membranes in bacteria and alkaloids interfere with nucleic acid synthesis and act as efflux pump inhibitors [36]. Many

bacteria become resistant to efflux pumps which physically remove the antibiotic agents out of the cell. Therefore, further research should be carried out to determine the specific types of alkaloids and terpenoids which *Brassica juncea* possesses.

Conclusion

As far as we are aware, this is the first study which evaluated mustard leaves against several bacteria and fungi using the three solvents, hexane, ethyl acetate and ethanol, together with the phytochemical screening. In addition to their culinary value, mustard leaves (*Brassica juncea*) clearly possess promising antibacterial and antifungal qualities. Our study establishes the groundwork for future research into the possible applications of mustard leaves in health care. We strongly recommend that further investigations focus on the antimicrobial effects on clinical isolates and a comprehensive phytochemical analysis of ethyl acetate extracts.

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Conflict of Interest

No financial interest nor any conflict of interest exists.

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