Archives of Ecotoxicology, Vol. 4, No. 3, pp. 77-83, 2022

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Archives of Ecotoxicology

Journal homepage: https://office.scicell.org/index.php/AE

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Enzymatic Characterization, Antioxidant and Antimicrobial Activities of Protease from Chickpea (*Cicer arietinum L.*) Seed and Leaf Extracts

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Article info
Received 30 March 2022
Revised 05 October 2022
Accepted 08 October 2022
Published online 11 December 2022

Regular article

Keywords:
Enzyme extract,
Leaf supernatant,
MIC, MBC, MFC,
Zone of inhibition diameter.

Abstract

Chickpea (*Cicer arietinum L.*) proteins are appreciated due to their high biological values, well balanced amino acid content and low content of anti-nutritional factors. The present study was undertaken to investigate enzymatic characterization, antioxidant and antimicrobial activities of protease extract from chickpea (*Cicer arietinum L.*). The chickpea seed and leaf samples collected from farmer's field were used for protease enzyme extraction. The protein concentration was determined by Lowry method. Protease activity was assayed by using casein as a substrate. Optimization of the enzyme activity was made based on pH and temperature. The antioxidant activity was tested based on hydrogen peroxide, and DPPH free radical scavenging activities. The antimicrobial experiment was conducted based on disc diffusion and broth dilution methods. The result of crude protein extracts and protease activity has demonstrated that significantly the highest protein concentration (74.53%) and protease activity (22.20U/ml) in seed supernatant solution. The effect pH on protease activity demonstrated the optimum pH for the protease extract was ≥ pH6. The effect of temperature on protease activity demonstrated that the optimum temperature for protease activity was around 40°C. Significantly the highest free radical scavenging activity of diphenyl-1-picrylhydrazyl DPPH (40.65%) and HPSA (57.95%) were recorded for seed supernatant enzyme extract. The strongest antibacterial activity with maximum zone of inhibition (22.35mm), minimum inhibitory concentration MIC (1.5µg/ml) and corresponding minimum bactericidal concentration MBC (2.5µg/ml) against S. aureus were recorded for leaf supernatant enzyme extract against Staphylococcus *aureus*. The highest dose of the enzyme extract (30µg/ml) revealed maximum antifungal activity with the highest zone of inhibition (18.25mm), MIC (1.5µg/ml) and corresponding minimum fungicidal concentration MFC (2µg/ml) was recorded for leaf supernatant against *Candida albicans.* The finding of the present study demonstrated that the supernatant enzyme extract has got higher biological activities including antioxidant and antimicrobial potentials.

1. Introduction

Pulse consumption has been associated with positive health outcomes across multiple populations **(Viguiliouk *et al.,* 2017).** Consumption of chickpeas, which are commonly available pulses worldwide, has been proposed to be beneficial in the managing of multiple chronic diseases such as cardiovascular diseases and obesity **(Gupta *et al.,* 2017; Padhi and Ramdath, 2017).** Although the exact mechanisms of how compounds present in chickpea can help alleviate symptoms associated with various chronic illnesses are still relatively unknown, bioactive peptides produced from chickpea proteins could play a role **(Sanchez-Chino *et al.,* 2018)**. Bioactive peptides are amino acid polymers produced from the hydrolysis of proteins that can exert beneficial health effects through interactions with biological molecules **(Erdmann *et al.,* 2008)**. Bioactive chickpea peptides are not currently added as ingredients to food products, but given the rise in popularity of foods containing chickpea ingredients in recent years, chickpea peptides may be sought off after as potential bioactive ingredients in the future **(Siegner, 2018).**

Humans have been consuming chickpeas for at least the past 10,000 years as indicated by the presence of chickpea grains in archaeological sites in northern Syria **(Willcox *et al.,* 2008)**. India, Australia, and Myanmar were the top chickpea producing countries, with 9,075,000, 2,004,000, and 526,772 metric tons of chickpea, respectively **(FAOSTAT, 2019)**. Although chickpea is widely grown in Ethiopia, the major producing areas are concentrated in the two regional states - Amhara and Oromia. These two regions cover more than 90% of the entire chickpea area and constitute about 92% of the total chickpea production **(Mitiku, 2011)**.

Globally, the industrial production of proteases accounts for 60% of the enzyme sales economy **(Kim *et al.*, 2016)**. Protease enzyme has been utilized widely in industries to engender a wide range of products such as, detergent, leather, waste management, brewing, meat softening, milk-clotting, food, pharmaceutical, cancer treatment, diagnostics, digestion, viral

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https://doi.org/10.36547/ae.2022.4.3.77-83

disorders and silver recovery **(Kuddus, 2015)**. Proteases belong to a class of enzymes that can be classified based on their physical and biological property **(Gupta *et al.*, 2012)**. Apart from this, the plant proteases have many biological roles, such as anti-cancer activity, avert edema, avails in the digestive process, procoagulant activity, meat tenderization and many more. **(Van der Hoorn, 2008; Gonzalez-Rabade *et al.*, 2011)**. However, the molecular mechanism defining the biological activity of the enzyme remains obscure **(Shivaprasad *et al.*, 2012)**. The present study was undertaken to investigate enzymatic characterization, antioxidant and antimicrobial activities of protease extract from chickpea (*Cicer arietinum*).

2. Material and methods

2.1 Sample collection, Preparation and Enzyme Extraction

The study was conducted at Biotechnology lab in School of Biological Sciences and Biotechnology, Haramaya University. The fresh chickpea samples of seeds, and leaf were collected from Erer district, Harari Regional State, Ethiopia. The seed and leaf samples were separated and washed with distilled water intensively, wiped with sterile cotton. The seeds were kept overnight for germination. Fresh seed samples (60 g) were taken and ground using 100ml acetone to remove fat content, using mortar and pestle. Air dried leaf samples (60g) were mixed using a mechanical mixer along with acetone. Both seed and leaf samples were mixed with chilled 10mM Tris-Hydrochloride (Tris-HCl) buffer at pH 8.0 and 2MNaCl for three hours on an orbital shaker. The samples were then centrifuged at 10,000 rpm for 10 minutes at 4°C and filtered through Whatmann filter paper.

2.2. Ammonium sulphate precipitation

Ammonium sulphate precipitation was performed for the seed, and leaf samples, in a stirrer maintained at 4°C. Overnight ammonium sulphate precipitation were performed at 6 saturation percentages consecutively 30%, 40%, 50%, 60%, 70% and 80% to determine the saturation percentage. After precipitation, centrifugation at 10,000 rpm for 30 minutes at 4°C were done. Both, the supernatant and pellet was collected after each saturation percentage, and the pellet was resuspended in phosphate buffer (pH 7.0)**.** Measurement of protein total protein concentration was done by the method of **Lowry et al (1951)** using bovine serum albumin as a standard and the concentration was expressed in mg/ml.

2.3. Protease assay

In protease assay, a standard curve was plotted, and protease activity using casein as a substrate. For this purpose, a 5ml of casein was added to four test tubes labeled – Test 1, Test 2, Test 3 and Blank. It was incubated in a water bath at 37°C for 5 minutes. 1ml of enzyme solution (supernatant and pellet samples obtained after ammonium sulphate precipitation was diluted with sodium acetate buffer), was added to the three test samples and then mixed and incubated at 37°C for 10 minutes. A 5ml of trichloroacetic acid is added to all test tubes, and 1ml of enzyme solution were added to the test tube labeled blank. After mixing and incubating at 37°C for 30 minutes, the samples were centrifuged at 10,000 rpm for 10 minutes at 4°C. To 2 ml of the supernatant, 5ml of sodium carbonate and 1ml of Folin-Ciocalteu reagent was added. Spectrophotometric readings was determined at 660 nm. Enzyme activity was calculated for each saturation percentage, to measure the amount of active enzyme present. It was determined at what saturation percentage highest protease activity is detected for seed supernatant, seed pellet, leaf supernatant and leaf pellet. After protease assay performed for samples, enzyme activity values for each sample were calculated using the standard formula **(Cupp-Enyard, 2008)**.

$$\text{Casein concentration (mg/ml)} = \frac{\text{Test OD} - \text{BlandkOD}}{\text{Slope of std curve}} \text{x}100\%$$

$$\text{EA (U/ml)} = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank})(\text{df})}}{(\text{ODstd} - \text{ODblank})(\text{t})(\text{V}_{\text{enzyme}})}$$

$$\text{df} = \frac{\text{final dilution volume}}{\text{volume of enzyme sample}}$$

Where, EA: enzyme activity; U: activity unit; t: time of incubation, (min); df: dilution factor.

2.4. Optimization of Protease Activity

Optimization experiments were done based on effect of PH and temperature as follows.

2.4.1. Effect of pH on protease activity

Effect of pH on protease activity was measured using a Tris-HCl buffer. The pH was adjusted to 4, 6, and 8 respectively. Each of the six samples was mixed with Tris-HCl buffer in 1:1 ratio. After an incubation period of 24 hours at room temperature, protease assay was performed with each of the six samples at the pH mentioned above, and enzyme activity values were calculated.

2.4.2. Effect of incubation temperature on protease activity

The effect of temperature on protease activity was determined testing enzyme activity at different temperatures (20, 30, 40 and 50^0C with an interval of 10^0C) with the pH and time of incubation remains constant at 11 and 24hr respectively. The protease assay was carried out to determine the concentration of the enzyme.

2.5 Antioxidant activity test

2.5.1. DPPH Radical Scavenging Activity

The radical scavenging activity (RSA) of the enzyme extract was adopted to measure antioxidant activity using the DPPH method **(AOAC, 1990; Loizzo *et al.*, 2016).** Briefly, 2 mL of the enzyme extract solution (1–100µg/mL) in methanol was added to 2mL of DPPH (0.1 mM) solution. The mixtures were kept aside in a dark area for 30 min and absorbance was measured at λmax 517 nm against an equal amount of DPPH and methanol as a blank. The percentage of DPPH radical scavenging activity (RSA %) was estimated using the equation:

$$\text{DPPH radical scanveging activity}(\%) = \frac{(\text{A}_{\text{o}} - \text{A}_{\text{sample}})}{\text{A}_{\text{o}}} \text{x}100$$

where **A**0 is the absorbance of the control and A**1** is the absorbance in the presence of the sample. Ascorbic acid was used as positive control.

2.5.2. Hydrogen Peroxide Scavenging Activity

The radical scavenging activity of individual enzyme extracts was determined using the H_2O_2 method **(Bozin *et al.*, 2008)**. Briefly, 2 mL of the extract solution (10–100 _g/mL) in methanol was added to 4.0 mL of H_2O_2 (20 mM) solution in phosphate buffer (pH 7.4). After 10 min, the absorbance was measured at λ_{max} 230 nm against the phosphate buffer blank solution. The

percentage scavenging of H_2O_2 was calculated using the equation:

% scavenging of H_2O_2 = [(A0 - A1)/A0] x100,

where A0 = absorbance of the control (phosphate buffer with H_2O_2) and A1 = absorbance of the test extracts.

2.6. Test Pathogens

Four test pathogens including two bacteria (*E. coli, and S. aureus*), *and* two fungal spp *(Candida albicans* and *A. niger)* were obtained from Ethiopian Public Health Institute (EPHI). The fungal and bacterial pathogens were subcultured and maintained on Potato Dextrose Agar (PDA) and Nutrient Agar, respectively. In addition, the fungal and bacterial cultures were incubated for 72 h at 27 ºC and for 18-24 h at 37 ºC, respectively.

2.6.1. Media Preparation and Standardization of Inoculum

Nutrient Agar (NA), Potato Dextrose Agar (PDA), and Muller Hinton agar (MHA) were used for sub-culturing of bacterial test organism, fungal test organism, and determination of antimicrobial activities, respectively. These media were prepared and sterilized using an autoclave according to the manufacturers' instructions. Two to three bacterial colonies on the plate were picked up with a sterile inoculating loop and transferred into a test tube containing sterile normal saline solution and vortexed thoroughly. The spores of the test fungi were harvested by washing the surface of the fungal colony using 5mL of sterile saline solution (0.85%). This procedure was repeated until the turbidity of each bacterial and fungal spore suspension matched the turbidity of 0.5 McFarland Standards as described by the Clinical Laboratory Standards Institute **(CLSI, 2012).** The resulting suspension was used as inoculum for the test pathogen in the antimicrobial susceptibility test.

2.6.2. Disc diffusion method

Thr discs of 6 mm diameter were prepared from sterile filter paper cut into small, circular pieces of equal size by a perforator and then they were impregnated with 0.1 ml of the prepared test extract solution and buffer solution. Within 15 minutes after adjusting the turbidity of the suspension of inoculum, a sterile cotton swab was dipped into adjusted suspension and rotated several times by pressing firmly on the inside wall of the tube above the fluid level. This removes excess fluid from the swab. Then, the dried surface of MHA plate was inoculated by streaking using the swab three times over the entire surface and rotating the MHA plates approximately 60° each time to ensure an even distribution of the inoculum. Then, the MHA plates were left open for three to five minutes to allow for any excess surface moisture to be absorbed **(Hudzicki, 2009).**
Following this step, the impregnated discs were dispensed onto the surface of the inoculated agar plates using sterile forceps. Each disc was pressed down to ensure complete contact with the agar surface. The discs were distributed evenly so they were not closer than 24 mm from center to center **(CLSI, 2012).** Discs of commercial amoxicillin (30µg/disc) and fluconazole (30µg/disc) were used as positive controls for bacterial and fungal pathogens, respectively and the buffer impregnated discs were used as negative controls.
Then the MHA plates were sealed with parafilm and incubated at 37°C for 24 hrs and 27°C for 72 hrs for bacterial and fungal pathogens, respectively. After incubation, the diameters of the zone of inhibition around each disc was measured to the nearest millimeter along two axes (i.e. 90° to each other) using a transparent ruler and the means of the two readings were recorded. For each selected pathogen the experiment was carried out with three replications.

2.6.3. Determination of Minimum Inhibitory Concentration

The enzyme extracts that showed significant antimicrobial activity in the antimicrobial activity tests were selected for determination of MIC. The MIC of the extracts was determined by broth dilution method **(Andrews, 2001).** Two mL of nutrient broth and potato dextrose broth for bacteria and fungi were added into all test tubes and 0. 1 ml of the prepared concentration of each extract was mixed with the nutrient broth and potato dextrose. Thereafter, standardized inoculums of 0.1 ml of the respective test pathogens were dispensed into the test tubes containing the suspensions of the broth and the extract. Then, all test tubes were properly corked and incubated at 37°C for 24 hrs for bacteria and 27°C for 72 hr for fungi. After which, they were observed for absence or presence of visible growth. The lowest concentration at which no visible growth of organisms were regarded as the MIC. The experiment was carried out for each organism in duplicates.

2.6.4. Determination of minimum bactericidal (MBC) and fungicidal concentrations (MFC)

For the determination of the MBC and MFC, fresh nutrient agar and potato dextrose agar plates were inoculated with one loopful of culture taken from each of the broth cultures that showed no growth in the MIC tubes. While MBC assay plates were incubated for 48 h, MFC assay plates were incubated for 3 days. After the incubation periods, the lowest concentration of the extract that did not allow any bacterial or fungal growth on solid medium was regarded as MBC and MFC for the extract **(CLSI, 2012).** This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation for bacteria or spore germination after 3 days of incubation for fungi.

2.7. Data Analysis

All data were entered into Microsoft excel. Mean comparison and Analysis of variance (ANOVA) were carried out using SAS version 20 software package. Statistically significant difference was indicated by $p<0.05$.

3. Results and Discussion

3.1. Crude protein extract and protease activities of chickpea

The result of crude protein extracts and protease activity (Table 1) has demonstrated that significantly the highest protein concentration (74.53%) and protease activity (22. 20 U/ml) in seed supernatant solution. As the least protein concentration (34.55 mg/ml) and protease activity (10.40 U/ml) were recorded for leaf pellet extract. Generally the supernatant solution has demonstrated higher protein content and enzyme activity as compared to the pellet which is in agreement with the previous reports **(Aloweidat, 2014)** that the majority of chickpea proteins (>60%) are soluble globulin proteins. Like other legumes, chickpea's albumins and globulins represent the two major protein fractions. The albumin fraction constitutes up to 15-25% whereas the globulins, represented mainly by vicin and legumin, reach up to 60-80% of the extractable proteins. Albumins display a higher nutritive value due to their high lysine content and sulfur amino acids. Chickpea proteins are appreciated due to their high biological values, well balanced amino acid content and low content of antinutritional factors. However, there have been concerns about chickpea protein

isolates due to their low-fat content among other reasons **(Aloweidat, 2014).**

3.2. Optimization of chickpea protease activity

The effect pH on protease activity (Fig 1) demonstrated that the enzyme activity generally increased as pH increased from 4 to 6; however, the enzyme activity was declined at pH 8. Thus, the optimum pH for the protease extract was ≥ pH6.

3.3. Antioxidant activities of protease extract from chickpea seeds and leaves

The antioxidant activities were measure as free radical scavenging activities of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide scavenging activities (HPSA) (Table 2). Significantly the highest DPPH (40.65%) and HPSA (57.95%) was recorded by seed supernatant enzyme extract while significantly the least antioxidant activity DPPH (26.45%) and HPSA (43.75%) were recorded by leaf pellet enzyme extract. It was found that the supernatant solution presented better antioxidant activities as compared to the pellet enzyme extract. Antioxidants inhibit oxidation of food also quench dreaded free radicals produced due to environmental and physiological stress which leads to aging, atherosclerosis and cancer. Antioxidation and oxidation processes in plants are complex and therefore it is difficult to measure each antioxidant component separately **(Jeya et al., 2019).** DPPH is often used to test how far compounds can act as free radical scavengers or hydrogen donors, and to quantify antioxidants in complex systems **(Antolovich *et al.*, 2002).** Antioxidants may be reductants and inactivation of oxidants by reductants are redox reactions in which one reaction species is reduced when the other is oxidized **(Apak *et al.*, 2004).**

Table 1 Crude protein extract and protease activities of chickpea seed and leaf supernatant and pellet

Enzyme extract	Protein concentration (mg/ml)	Protease activity (U/ml)
Seed supernatant	74.53a	22.20a
Seed pellet	48.87b	14.63b
Leaf supernatant	41.67bc	12.50bc
Leaf pellet	34.55c	10.40c

Means followed by the same letter within a column were not significantly different at 0.05 probability level based on Least Significance difference (LSD) test. Small letters: significance within a column.

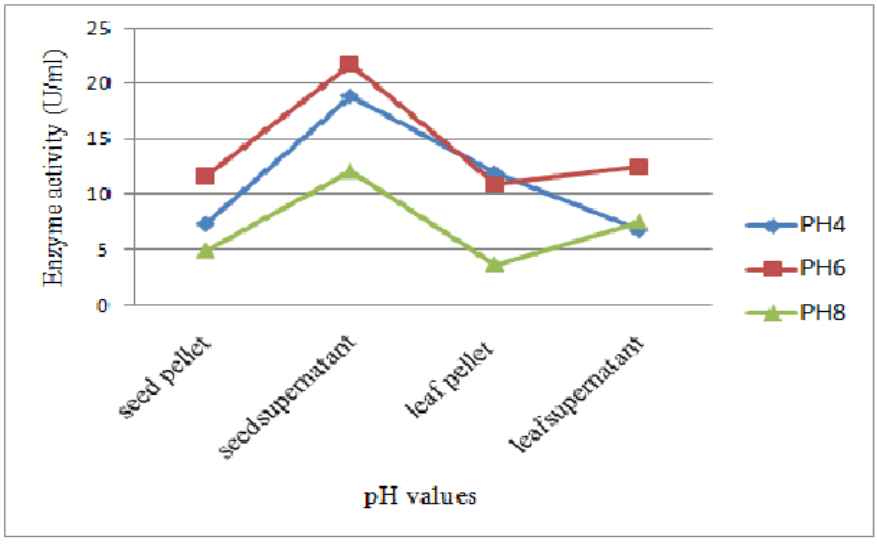

Figure 1 The effect of pH on enzyme activity

Table 2 Free radical scavenging activities of chickpea protease

Protease extract	DPPH	HPSA
Seed supernatant	40.65a	57.95a
Seed pellet	34.90b	52.20b
Leaf supernatant	31.15c	48.45c
Leaf pellet	26.45d	43.75d

Means followed by the same letter within a column were not significantly different at 0.05 probability level based on Least Significance difference (LSD) test. Small letters: significance within a column. DPPH: 2, 2-diphenyl-1-picrylhydrazyl; HPSA: hydrogen peroxide scavenging activity.

Table 3 Antimicrobial activities of protease extract from seeds and leaves of chickpea

Test pathogens	enzyme extract	Conc of enzyme extract			Amoxicillin (30µg/ml)
		10µg/ml	20µg/ml	30µg/ml	
E.coli	seed pellet	8.45eC	10.20eBC	12.90eB	19.90aA
	leaf pellet	10.10dC	11.85dC	15.65cdB	18.90aA
	seed supernatant	9.70dC	11.55dC	14.60dB	18.90aA
	Leaf supernatant	11.30cBC	13.20cB	16.60bcAB	18.65aA
S. aureus	seed pellet	11.65cC	14.70bB	17.05bcA	18.65aA
	leaf pellet	12.05bcB	17.30aA	18.90bA	19.90aA
	seed supernatant	12.90bB	13.30cB	21.15aA	19.90aA
	Leaf supernatant	14.30aC	14.28bcC	22.35aA	18.90aB
					Fluconazole (30µg/ml)
C. albicans	seed pellet	12.55bC	13.80cBC	15.75cB	20.50aA
	leaf pellet	12.75bC	14.80bBC	16.45bcB	20.40aA
	seed supernatant	11.65bC	15.05bB	17.30abA	19.90aA
	Leaf supernatant	14.30aB	17.30aAB	18.25aA	19.90aA
A. niger	seed pellet	8.80cdD	10.90eC	12.25eB	19.40aA
	leaf pellet	7.50dD	9.30fC	13.75dB	19.90aA
	seed supernatant	10.05cD	11.55dC	13.70dB	18.90aA
	Leaf supernatant	9.85cC	10.75eC	15.75cB	19.95aA

Means followed by the same letter within a column were not significantly different at 0.05 probability level based on Least Significance difference (LSD) test. Small letters: significance within a column, capital letter significance within row.

Table 4 MIC, MBC or MFC of protease enzyme extracts

Test pathogens	Enzyme extract	MIC(µg/ml)	MBC/MFC (µg/ml)
E.coli	seed pellet	10	30
	leaf pellet	5	10
	seed supernatant	5	10
	Leaf supernatant	5	10
S. aureus	seed pellet	2.5	5
	leaf pellet	2.5	5
	seed supernatant	1.5	2.5
	Leaf supernatant	1.5	2.5
C. albicans	seed pellet	5	20
	leaf pellet	2.5	5
	seed supernatant	2.5	5
	Leaf supernatant	1.5	2
A. niger	seed pellet	10	0
	leaf pellet	10	0
	seed supernatant	5	20
	Leaf supernatant	8	10

3.4. Antimicrobial activities of chickpea protease

The antimicrobial activities of the enzyme extracts from chickpea seed and leaf was determined based on disc diffusion method (Table 3). The zone of inhibition has revealed considerable antimicrobial activities. Antibacterial activity of oil extracts with colony growth inhibitory effect at the highest dose (30µg.ml) showed a mean zone of inhibition ranged from 12.90 to 22.35mm.

The antimicrobial activities of the enzyme extract against *S. aureus* and *C. albicans* has demonstrated insignificant differences from amoxicillin (used as positive control) for antibacterial activity and fluconazole (as positive control) for antifungal activity test respectively. The superiority of antibiotics might be due to the method of extraction of the enzyme. For most of the test extracts, the highest concentration (30µg/mL) exhibited a significantly higher ($P<0.05$) zone of inhibition as compared to the respective lowest concentration (10µg/mL). The highest concentration of the enzyme extract (30µg/ml) presented the strongest antibacterial activity with maximum zone of inhibition (22.35mm) recorded for leaf supernatant enzyme extract against *S. aureus* (gram positive) indicating that *S. aureus* is more susceptible to the enzyme extract than *E.coli* (negative control) On the Other hand, the weakest antibacterial activity with minimum inhibition zone (12.90mm) was observed for seed pellet enzyme extract against *E. coli* indicating that *E. coli* is more resistant to antimicrobial protease extract. It can be observed from the result of zone of inhibition diameter (Table 3) that leaf supernatant was the best antibacterial potential while seed pellet was the least antibacterial potential.

The highest dose of the enzyme extract (30µg/ml) revealed maximum antifungal activity with the highest zone of inhibition (18.25mm) was recorded for leaf supernatant against *C. albicans* showing that *C. albicans* was more susceptible to the enzyme extract than *A. niger* whereas the weakest antifungal activity with minimum zone of inhibition (12.25mm), with the highest concentration of the extract, was recorded for seed pellet enzyme extract against *A. niger*. Thus, this result reveals leaf supernatant enzyme extract had strongest antimicrobial potential while the seed pellet enzyme extract has exhibited the least antimicrobial potential.

The antimicrobial activity was further tested by determining minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) for the enzyme extracts from chickpea seeds and leaves against test pathogenic microbes as in Table 4.

The enzyme extracts from leaf and seed supernatants have presented the strongest antibacterial activity with MIC (1.5µg/ml) and corresponding MBC (2.5µg/ml) against *S. aureus* while the weakest antibacterial activity with MIC (10µg/ml) and corresponding MBC (30µg/ml) was observed for seed pellet enzyme extract against *E. coli* showing that supernatant enzyme extracts could have strongest antibacterial potential and *S.aureus* was more susceptible to the enzyme extracts. On the other hand, the strongest antifungal activity with MIC (1.5µg/ml) and corresponding MFC (2µg/ml) was recorded for leaf supernatant against *C. albicans* whereas no effect antifungal activity was recorded for seed and leaf pellet against *A. niger* indicating that the pellet enzyme extract didn't exhibit effective antifungal potential.

4. Conclusion

The finding of the present study indicated that the supernatant enzyme extract has got higher biological activities including antioxidant and antimicrobial potentials. However, studies need to be undertaken to test the chemical groups that are responsible for biological activities of the chickpea enzyme extracts. The present study has contributed for better use of chickpea nutritionally as well as pharmaceutically. Further study is required to test with a number of microbes and drug development tests using model animal experiment. The biological activities of phytochemicals can be affected both internal genetic factor of the plant and external environmental conditions including geographical location, growing seasons, and method of extractions. Thus, studies are required to identify the major factors contributing for variations in antioxidant and antimicrobial activities of the plant extracts.

Conflict of Interest

The authors report no conflicts of interest. The authors are responsible for the content of the paper.

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