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In vitro Antimicrobial Activity of *Cucurbita pepo* on the Selected Bacterial Pathogen and Ethnobotanical Knowledge of the Community at Gara Muleta, Girawa, Eastern Ethiopia

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Abstract

Background: In developing countries, 80% of the population use traditional medicine to meet their primary healthcare needs. *Cucurbita pepo* is a widely used food and medicinal plant as a natural anti-inflammatory, antiviral, and analgesic agent for treating various public health problems. This study was conducted to evaluate the *in vitro* antimicrobial activity of *Cucurbita pepo* on the selected bacterial pathogen and Ethnobotanical knowledge.

Methods: The study was conducted at Gara Muleta, Eastern Ethiopia from April 30 to May 30, 2020. Sixty experimental tests were performed to evaluate the *in vitro* antimicrobial activity of *Cucurbita pepo* seed extracts using three extraction methods (water-based, methanol-based, and chloroform) at different concentrations (higher, medium, and lower concentrations). An experimental study was carried out using three standard pathogenic bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*) using the disc diffusion and broth dilution techniques. A community-based cross-sectional study was carried out among 384 households in Girawa town to assess the ethnobotanical knowledge of the community regarding *Cucurbita pepo*.

Results: Water, methanol, and chloroform extracts of *Cucurbita pepo* seeds showed maximum inhibition zones of 9.0 mm, 12.0 mm, and 10.0 mm and minimum inhibitory concentrations of 18, 24, and 20 mg/mL, respectively, against *P. aeruginosa*. Of the 384 study participants, 376 (98.2%) of the participants had ethnobotanical knowledge of *Cucurbita pepo* seeds. However, 240 (62.5%) and 255 (66.4%) of the study participants had no information about the medical use and health benefits of *Cucurbita pepo* seeds, respectively.

Conclusion: The methanol and chloroform extracts of *Cucurbita pepo* seeds possessed notable antibacterial activity, but water extract showed no zone of inhibition against *E. coli* and *S. aureus*.

Keywords: *Cucurbita pepo*; Antimicrobial activity; Seed extraction; Ethnobotanical knowledge

Introduction

The Curcurbiteae family, also referred as cucurbits form a very large group with approximately 130 genera and 800 species and can be cultivated in warmer region of worldwide and make popular food crop plants some of these species include squashes, pumpkins, melons and gourds. *Cucurbita pepo* is one of the oldest cultivated species [1]. This family has medicinal and nutritional benefits [2]. The immature fruits are also consumed as vegetables. The mature fruit is sweet and used to produce beverages. Seeds that are rich in oil are also used with honey to prepare desserts. The flower buds and flowers are edible. Pumpkin seeds also used as antidiabetic, antihyperlipidemic, antitumor, antihypertensive, anti-inflammatory, antibacterial, and anthelmintic agents in many countries, such as India, China, Brazil, Yugoslavia, and America [3-5].

Medicinal plants constitute an important component of the pharmaceutical and primary healthcare systems at the local level. Approximately 80% of the world's population depends on ethno medicines for primary health care [6-8]. The use of crude extracts of plant parts and phytochemicals with known antimicrobial properties may be of great significance for therapeutic treatments. Recently, several studies have been conducted in various countries to demonstrate their efficiency. Many plants have been used because of their antimicrobial properties, which are attributed to the secondary metabolites synthesized by plants [9].

Determination of the antibacterial activities of different medicinal plants is of special interest because of the current global issue of increasing antibiotic resistance of microorganisms [10]. Therefore, it is imperative to identify compounds that can be used to develop novel medicines with higher antimicrobial properties [11].

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Currently, researchers are searching for novel antimicrobial molecules that have a broad spectrum of activity against both gram-negative and gram-positive bacteria, without many or any side effects [12,13]. The current study aimed to identify the antibacterial activity of *Cucurbita pepo* against selected bacterial pathogens and the endogenous knowledge of the community on this plant as a traditional healer.

Materials and Methods

Study area and period

This study was conducted in Girawa Town from April 30 to May 30, 2021. The town is located in Gara Muleta District, East Hararge Zone, Oromia National Regional State, and Eastern Ethiopia. It is located about 555.87 km east of Addis Ababa, the capital city of Ethiopia. The district is located at an altitude ranging 1187-2700 meters above sea level, with an average annual temperature of 25.6°C and an average rainfall of 950 mm.

Study design

An experimental study and community-based cross-sectional study were carried out to evaluate the antibacterial activity of *Cucurbita pepo* on the selected bacterial pathogens and ethnobotanical knowledge of the community.

Inclusion and exclusion criteria

Household members aged over 18 years in the selected households who had lived in the selected ketene for >6 months were included in the study. Individuals who were terminally or mentally ill were also excluded.

Sample size determination

Sixty tests (30 for water-based extraction and 30 for alcohol-based extraction) were performed to evaluate the antimicrobial activity of *Cucurbita pepo* against the selected bacterial pathogen (Table 1).

Table 1: Sample size for antimicrobial activity on the selected microorganisms.

Extraction methods	Microorganisms to be used	MIC	# of tests	# of control
Water based	*Escherichia coli* (ATCC 25922)	High	3	1
		Medium	3	
		Low	3	
	Staphylococcus aureus (ATCC 25923)	High	3	1
		Medium	3	
		Low	3	
	Pseudomonas aeruginosa (ATCC 27853)	High	3	1
		Medium	3	
		Low	3	
Alcohol based	*Escherichia coli* (ATCC 25922)	High	3	1
		Medium	3	
		Low	3	
	Staphylococcus aureus (ATCC 25923)	High	3	1
		Medium	3	
		Low	3	

	Pseudomonas aeruginosa (ATCC 27853)	High	3	1
		Medium	3	
		Low	3	

Note: MIC: Minimum Inhibitory Concentration

The sample size to assess the ethnobotanical knowledge of the community calculated using single population proportion formula by considering 50% of the community proportion had the ethnobotanical knowledge (P) of *Cucurbita pepo*, 95% confidence level, and 5% marginal error (d). In total, 384 households were interviewed.

Sampling techniques

First, out of five kebeles of Girawa district, one kebele (Girawa kebele) has been selected using a lottery method. Of the seven ketenas of Girawa Kebeles, two were selected using simple random sampling. Finally, the sample size allocated proportionally to each ketena and study participant was selected from every third household interval (Figure 1).

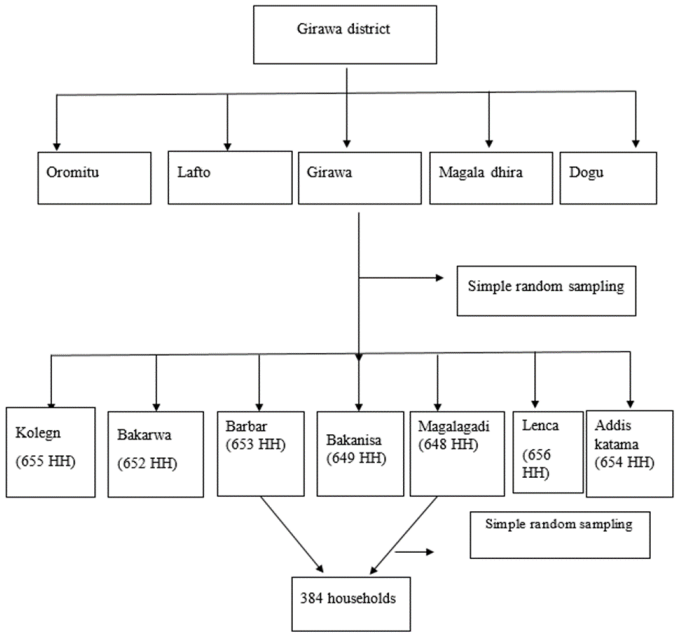

Figure 1: Schematic presentation of sampling procedure of households.

Data collection

Structured questionnaires were prepared in English based on information from the available literature. The questionnaires were translated into the local language (Afaan Oromoo) and pre-tested. During pre-testing, additional information was gathered, and some of the questionnaires were modified. Two trained BSc public health professionals collected data. The first author supervised the overall activities of the data-collection process.

Collection and identification of plant material

Mature *Cucurbita pepo* plants were obtained from local markets. *Cucurbita pepo* seeds were collected from the fruits using a clean and dry leak proof container, transported to Haramaya University, medical microbiology laboratory using a sample transport box, and kept in a dry and clean area. The collected plant specimens were identified using taxonomic keys by plant taxonomic experts. Voucher specimens (Number: 060987) were deposited in the Herbarium of the department of plant science, Haramaya University, Ethiopia.

Powder extraction from *Cucurbita pepo* seeds

The *Cucurbita pepo* seeds were washed vigorously and dried in the shade for five days. After the seeds were completely dried, they were crushed and powdered using a grinder and a blender. The seed powder was then filled in the test tube, and the extraction process was carried out sequentially with distilled water as a solvent in a Soxhlet extractor for 48 h. Finally, the seed solvent extracts were concentrated under compact pressure and stored at 5°C in airtight bottles for experimental use (Figure 2).

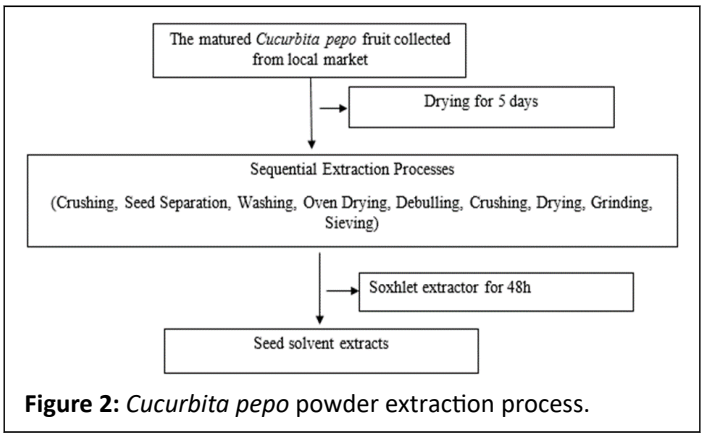

Figure 2: *Cucurbita pepo* powder extraction process.

Agar well diffusion and Minimum Inhibitory Concentration (MIC)

Media preparation and agar well diffusion assays were performed according to the manufacturer's instructions. The American type culture collection of *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922) was obtained from the microbiology laboratory of the school of medical laboratory sciences, Haramaya University. The test suspension was standardized to match the McFarland turbidity standard of 0.5, which corresponds to approximately 1.5×10^8 CFU/ml. A working solution (50 µg/ml) of streptomycin (mM EDTA, analytical

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standard) was prepared from stock solution as a positive control, and 5% Dimethyl Sulfoxide (DMSO) (ACS reagent grade) was used as a negative control.

Three concentrations (200, 100, and 50 mg/ml) of *Cucurbita pepo* seed extract were used as test materials. The standardized inoculant was swabbed uniformly onto solidified Mueller-Hinton agar, and the seeded media were allowed to dry for approximately 5 min. Five wells per plate (three wells for extracts, one for each of the positive and negative controls) were made in the seeded agar using a 6 mm cork borer. Using a micropipette, 20 µl of the test solution was poured into each well. The media were kept biosafety for 1 h for proper diffusion and then incubated at 37°C for 24 h. The experiment was repeated three times, and the results and zones of inhibition for each dose were expressed as the mean of the replicates.

The MIC was evaluated using the agar dilution method, and MHA culture media was used. Serial dilutions of the plant extracts were prepared by dissolving in 5% DMSO to obtain concentrations of 15.625-1000 µg/ml. Two milliliters aliquot of the plant extract dilution of each concentration to be tested was added to 19 ml of molten agar. The test was performed in triplicate (Table 1).

Operational definition

Sensitive (S): The inhibition zone of the isolated bacteria was greater than the inhibition zone set for the antimicrobial disk mentioned in the testing panel.

Resistant (R): The inhibition zone of the isolated bacteria was less than the inhibition zone set for the antimicrobial disk mentioned in the testing panel.

Intermediate (I): When the inhibition zone of the isolated bacteria was less than or equal to the inhibition zone set for the antimicrobial disk mentioned in the testing panel.

Ethnobotany: This is a study of the relationship between plants and people, with a particular emphasis on traditional cultures.

Ethnobotanical knowledge: Knowledge of *Cucurbita pepo* on *Cucurbita pepo*, medical use, and health benefits.

Knowledgeable: who knew all the three key knowledge on *Cucurbita pepo*.

Less knowledge: who had one out of three of the key knowledge of *Cucurbita pepo*.

Quality control

Aseptic techniques were applied at all steps of plant extraction and inoculation into the culture media to minimize contamination. All the culture media were prepared according to the manufacturer's instructions. The sterility and performance of the culture medium were tested. The pre-analytical, analytical, and post-analytical stages of quality assurance incorporated in the Standard Operating Procedures (SOPs) of the microbiology laboratory of the School of Medical Laboratory Sciences were strictly followed.

Statistical analysis

The collected data were entered into EpiData 3.1 and exported to SPSS 23.0. Data cleaning was performed by running the frequency of each categorical variable and cross-tabulation of different categorical variables. Descriptive results are summarized as percentages, means, and standard deviations.

Ethical considerations

The study protocol was reviewed and approved by the Institutional Health Research Ethics Review Committee (IHRERC) of the College of Health and Medical Sciences (reference number: C/AC/R/D/01/1085/21). An official support letter was written to the Gara Muleta Girawa Town Administration Office. Information on the study, including its objectives, procedures, potential risks, and benefits, was explained to each participant. Participants were informed of their right to refuse or withdraw from the study at any time. Participants' confidentiality was ensured by excluding names and identifiers from the questionnaire. Informed voluntary written and signed consent was obtained from all respondents throughout the study.

Results

Antimicrobial activity of seed extracts of *Cucurbita pepo* against selected bacteria

Overall, 60 experimental studies were conducted to evaluate the antimicrobial activity of *Cucurbita pepo* seed extracts using water and alcohol-based extraction. Accordingly, 30 tests were conducted to determine the antibacterial activity of the water-based seed extracts of *Cucurbita pepo* at different concentrations (high, medium, and low) against *E. coli*, *S. aureus*, and *P. aeruginosa*. No zones of inhibition were observed for *E. coli* or *S. aureus*.

Similarly, 15 tests were conducted to evaluate the antimicrobial activities of methanol against *E. coli*, *S. aureus*, and *P. aeruginosa*. Minimal zones of inhibition were observed for *E. coli* (6 mm) and *S. aureus* (9 mm). A relatively large zone of inhibition was observed for *P. aeruginosa* (22 mm). Finally, 15 tests were performed to evaluate the antimicrobial activity of different concentrations of *Cucurbita pepo* seed extract against *E. coli*, *S. aureus*, and *P. aeruginosa*. The seed extracts showed a minimum zone of inhibition (5 mm) against *E. coli*, and *S. aureus* and a relatively higher zone of inhibition (18 mm) was observed against *P. aeruginosa* (Table 2).

Table 2: Antibacterial activity of extracts of the seed of C. Pepo against selected bacteria using paper disc diffusion method at Gara Muleta, Girawa eastern Ethiopia, 2021.

Conc.	Extraction methods	Zone of inhibition			Comparative control	
		E. coli (mean and SD)	*S. aureus* (mean and SD)	*P. aeruginosa* (mean and SD)	Ciprofloxacin (mean and SD)	Gentamycin (mean and SD)
High*	Water	NA	NA	10.25 ± 1.85	9 ± 1.73	8.5 ± 1.68
	Methanol	3.5^ ± 1.08	4.5 ± 1.0	11 ± 1.92	12 ± 2.00	11 ± 1.92
	Chloroform	2.5 ± 0.91	2.5 ± 0.912	9 ± 1.73	10 ± 1.85	11.5 ± 1.95
Medium**	Water	NA	NA	8.5 ± 1.68	9 ± 1.73	8.5 ± 1.68
	Methanol	2.5 ± 0.91	3.5 ± 1.08	10 ± 1.82	12 ± 2.00	11 ± 1.92
	Chloroform	2.5 ± 0.91	2.5 ± 0.912	9 ± 1.73	10 ± 1.85	11.5 ± 1.95
Low***	Water	NA	NA	6.5 ± 1.50	9 ± 1.73	8.5 ± 1.68
	Methanol	2.5 ± 0.91	3.5 ± 1.08	10 ± 1.82	12 ± 2.00	11 ± 1.92
	Chloroform	2.0 ± 0.82	2.5 ± 0.91	9 ±1.73	10 ± 1.85	11.5 ± 1.95

Note: *: One part of extracted plant: Two part of extraction solvent; **: One part of extracted plant: Four part of extraction solvent; ***: One part of extracted plant: Eight part of extraction solvent; ^: Numbers indicate the mean diameters of inhibition of triplicate experiments; SD: Standard Deviation; NA: Not Applicable

Minimum Inhibitory Concentrations (MICs o f the different solvent extracts of *Cucurbita pepo* to the selected bacterial pathogen

The Minimum Inhibitory Concentrations (MIC) for methanol and chloroform-based extracts were determined using three serially diluted *Cucurbita pepo* extracts. The MIC of *Cucurbita pepo* extract in both extraction solvents ranges from 4.2 mg/ml to 24 mg/ml. An overview of the findings showed that a relatively lower concentration was required to inhibit ATCC *P. aeruginosa* than the other tested bacteria. The least (4.2 mg/ml) minimum inhibitor concentration was observed in the chloroform-based extraction at low concentrations against ATCC *E. coli* and methanol-based extracts against ATCC *S. aureus*. The result shows that MIC of methanol extract range from 5.0 mg/L to 24.0 mg/L for tested organisms and chloroform extract had MIC that ranges from 4.2 mg/L to 20.5 mg/L for tested bacteria (Table 3).

Table 3: Minimum Inhibitory Concentrations (MICs) of the different solvent extracts of Cucurbita pepo seed on selected pathogenic bacteria Gara Muleta, Girawa eastern Ethiopia, 2021.

ATCC bacteria	Concentrations	MIC (mg/ml)		
		Methanol	Chloroform	Positive control (Ciprofloxacin)
E. coli	High	7.2	5.3	24.0
	Medium	5.6	5.0	24 .0
	Low	6.0	4.2	24.0
S. aureus	High	9.3	6.2	22.0
	Medium	8.0	5.2	22.0
	Low	5.0	5.0	22.0
P. aeruginosa	High	24.0	20.5	20.0

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	Medium	22.5	20.0	20.0
	Low	21.5	19.0	20.0

Ethnobotanical knowledge of the community at Gara Muleta

Sociodemographic characteristics of study participants

Overall, 384 study participants included in the study, gives a 100% response rate.

The majority were male (212/384) and aged >34 years (219/384) (Table 4).

Table 4: Socio-demographic characteristic study participants who have been enrolled to assess the ethnobotanical knowledge of the community at Gara Muleta, Girawa Eastern Ethiopia, 2021 (N=384).

Variable	Category	Frequency	Percentage
Gender	Male	212	55.2
	Female	174	44.8
Age (in years)	<-34	219	57.3
	>34	165	42.7
Educational status	Illiterate	87	22.6
	Only write and read	116	30.2
	Certificate level	20	5.2
	Diploma level	90	23.5
	Degree level	70	18.5
Duration of stay	One year	34	8.8
	Two years	22	5.7
	Five	24	6.2
	Many years	304	79.3
Monthly income (in Ethiopian birr)	100-1000	121	31.5
	1001-10000	256	66.7
	10001-80000	7	1.8
Occupation	Farmer	170	44.3
	Merchant	65	17.0
	Health professional	32	8.0
	Police	12	3.1
	Others	105	27.3

Knowledge of study participants on *Cucurbita pepo*

Approximately 98.2% of the study participants had knowledge of *Cucurbita pepo* seeds with and 256 (66.8%) of them have been no knowledge regarding on the *Cucurbita pepo* seeds that killed bacteria with (Table 5).

Table 5: Knowledge of study participants on *Cucurbita pepo* seed by gender Gara Muleta, Girawa eastern Ethiopia, 2021 (n=384).

Questions	Category	Responses				P-value	95% CI
		Male		Female			
		n	%	n	%		
Do you know *Cucurbita pepo*?	Yes	209	54	167	44.2	0.94	0.39-0.36
	No	4	1	3	0.8		
Do you think that the *Cucurbita pepo* kills bacteria?	Yes	67	18	58	15.2	0.52	0.15-0.08
	No	144	37.6	112	29.2		
Which of the following nutrients do you think are in *Cucurbita pepo*?	Minerals	9	2.3	14	3.7	0.14	0.01-0.09
	Protein	86	22.5	53	13.8		
	Starch	28	7.3	11	2.9		
	Vitamins	42	11	37	9.7		
	Do not know	48	12.5	55	14.4		
Are there any medicinal uses of the in *Cucurbita pepo* (roots, fruits and seeds?)	Yes	80	20.9	63	16.4	0.83	0.11-0.14
	No	133	34.7	107	27.9		
Do you have information about health benefit of *Cucurbita pepo*?	Yes	70	18.3	58	15.1	0.75	0.15-0.11
	No	143	37.3	112	29.2		

Discussion

In this study, the antibacterial activity of *Cucurbita pepo* seeds was evaluated against three selected bacterial pathogens: One gram-positive (*S. aureus*) and two gram-negative bacteria (*E. coli* and *P. aeruginosa*). *E. coli* and *S. aureus* shared the same trend, in that the diameter of the zone of inhibition was found to parallel with increasing concentrations.

The effects of the different types of solvents used during extraction were clearly observed. The methanol extract of *Cucurbita pepo* seeds showed the best antibacterial activity, followed by the chloroform and water extracts. This result is supported by the study conducted by Yamaji, et al. which showed that water-soluble compounds have no antimicrobial significance, water-soluble phenolic compounds generally demonstrate significant antioxidant properties [14]. The trend of antibacterial susceptibility was found to be dependent on the polarity of the solvents. Parekh, et al. reported that plant extracts from organic solvents have been found to have more consistent antimicrobial activity than water extract [15]. In another study, among the 20 different solvents evaluated, chloroform was found to be the best solvent for the extraction of non-polar biologically active compounds [16].

In this study, no difference was observed between Gram-positive and gram-negative strains. This finding is in agreement with the findings of Nurmahani, et al. where no clear difference was found between the different types of bacteria on the antibacterial activity of the drug on fruit peel extracts [17]. In another study, plant extracts showed that gram-positive strains are considered more susceptible to antibacterial agents than gram-negative strains due to different cell wall structures [18]. Gram-negative strains possess an outer phospholipid membrane

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carrying structural components of lipopolysaccharides. Thus, the cell wall is impermeable to antibacterial agents. Compared with the gram-positive strain, it contains an outer peptidoglycan layer, which is not an effective barrier [19].

Minimum inhibitory concentration test was used in diagnostic laboratories to confirm unusual resistance, to give a definitive answer when a borderline result is obtained by other methods of testing, or when disc diffusion methods are not appropriate. The result shows that minimum inhibitor concentration of methanol extract range from 5.0 mg/L to 24.0 mg/L for tasted organisms and chloroform extract had minimum inhibitory concentration that ranges from 4.2 mg/L to 20.5 mg/L for tested bacteria. The findings showed that a relatively low concentration was required to inhibit or kill ATCC P. aeruginosa compared with the other tested bacteria.

Medicinal plant knowledge is shaped by the ecological diversity of the country and varies across people with different religious, linguistic, and cultural backgrounds. Out of the 384 study participants, 376 (98.2%) of the participants had knowledge of *Cucurbita pepo* seeds. However, 256 (66.8%) of them had no knowledge of the *Cucurbita pepo* seed antibacterial ability of the seed. Similarly, 240 (62.5%) and 255 (66.4) of the study participants had no information on medical use and health benefits of *Cucurbita pepo* seeds, respectively. Most respondents were above 31-45 years of age. This hints at the fact that ethnomedicinal knowledge is concentrated in the elderly members of the community and the relative difficulty in its transfer from the elderly to the younger generation. Studies in different areas have shown that medicinal plant knowledge and the transfer of knowledge to the young generation have been affected by modernization [20].

Conclusion

The methanol and chloroform extracts of *Cucurbita pepo* seeds exhibited notable antibacterial activities. *Cucurbita pepo* seed extract showed high activity against *P. aeruginosa* among the methanol and chloroform extracts. However, further investigations are required to understand the precise mechanisms of action and isolate the compound(s) responsible for such activities. Many communities were not knowledgeable about the health benefits and medical uses of *Cucurbita pepo* seeds.

Disclosure

This paper is based on the thesis of Mohammed Nasir. It has been published on the Haramaya University institutional repository. The author announced that they have no conflict of interests.

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