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Peninah Njoki Muchirah
Department of Medical Science, Faculty of Applied and Health Sciences, Technical University of Mombasa, Kenya

Rebecca Waihenya
School of biological sciences, College of Pure and Applied Sciences, Jomo Kenyatta University of Agriculture and Technology, Kenya

Shadrack Muya
School of biological sciences, College of Pure and Applied Sciences, Jomo Kenyatta University of Agriculture and Technology, Kenya

Leila Abubakar
Department of Pure and Applied Science, Faculty of Health and Applied Sciences, Technical University of Mombasa, Kenya

Hastings Ozwara
Department of Tropical and Infectious diseases, Institute of Primate Research, Kenya

Anselimo Makokha
Department of Food Science, Faculty of Agriculture, Jomo Kenyatta University of Agriculture and Technology, Kenya

Correspondence:
Peninah Njoki Muchirah
Department of Medical Science, Faculty of Applied and Health Sciences, Technical University of Mombasa, Kenya
Email: muchirahpeninah[at]tum.ac.ke

Characterization and anti-oxidant activity of *Cucurbita maxima* Duchesne pulp and seed extracts

Peninah Njoki Muchirah, Rebecca Waihenya, Shadrack Muya, Leila Abubakar, Hastings Ozwara. Anselimo Makokha

ABSTRACT

The study aimed to characterize phytochemical composition of *C. maxima* seeds and pulp. The ethanol and aqueous extract of *C. maxima* seeds and pulp were done using percolation method. The phytochemical composition of ethanol and aqueous extract were determined using methodology described by the association of official analytical chemists. Qualitative screening of both aqueous and ethanol seed and pulp extracts showed presence of alkaloids, flavanoids, phenolics, carbohydrates, tannins, saponins, terpenoids and proteins. The quantitative tests reported concentration of 0.336g/100g of phenols, 0.012g/100g flavanoids and 0.009g/100g tannins in seed aqueous extracts while 0.213g/100g, 0.175g/100g and 0.016/100g in pulp respectively. The levels of phenols, flavanoids and tannins reported in ethanol extracts were 0.277g/100g, 0.246g/100g and 0.010g/100g in pulp and 0.530g/100g, 0.252g/100g and 0.064g/100g in seed respectively. Saponins were present in ethanol extracts only. The percentage content of alkaloids was 4.9% in pulp and 5.97% in seed aqueous extracts while ethanol pulp and seed extracts was 9.49% and 9.74% respectively. All extracts showed significant levels of anti-oxidant activity at different concentration levels. The presence of these phytochemical compounds shows that *C. maxima* are a potential medicinal candidate that needs to be explored further for actual use as in pharmaceutical industry.

Keywords: *Cucurbita maxima* Duchesne, phytochemical, percolation, anti-oxidation, qualitative screening, quantitative tests.

INTRODUCTION

Pumpkin is one such plant that is frequently being used as food as well as traditional medicine for long days. The pumpkin, *Cucurbita maxima* Duchesne belongs to the family *Cucurbitaceae*. It is a large climbing herb, annual or perennial. Its aerial part consists of flexible succulent stem with trifoliate leaves [1]. *Cucurbita maxima* are widely cultivated in most warm regions of the world, for use as vegetable as well as medicine. Both of its fruits and the aerial parts are commonly consumed as vegetable. *C. maxima* plant is a nutritious plant of the *Cucurbiceae* family which has made one of the best, recommended traditional foods particularly for children. The plant has shown high component of proteins, polysaccharides, sterols, fixed oils and para amino-benzoic acids generally in all plant parts [2, 3]. In addition, carotenoids and γ-amino butyric acid have been isolated from the *C. maxima* fruits [4, 5]. Further, 11E-octadecatrienoic acid, polysaccharides, 13-hydroxy-9Z and phenolic glycosides have been isolated from seeds and leaves [6]. Previous studies have reported high protein content from pumpkin (*Curcurbita spp*) seeds particularly those germinated [7]. The presence of these phytochemicals has been found to possess various medicinal functional functions which are essential in the management of various diverse ailments. In addition, they help in developing the immune system of the young children as well as detoxifying the body. Different studies have reported conflicting results about the phytochemicals in both seed and pulp using different extraction methodologies. In addition, limited solvents have been used to isolate phytochemicals from this plant which calls for more similar studies using different solvents and extraction methodologies. The purpose of this study was to characterize phytochemical compounds in aqueous and ethanol seed and pulp extracts. It also aimed to examine the anti-oxidation activity of these extracts in order to contribute to existing knowledge that would be useful in drug development and pharmaceutical industry.

MATERIALS AND METHODS

Ethical consideration

Scientific and ethical approval of the study was acquired from Institute of Primate Research (IPR) Ethical Review Committee prior to any protocol-related procedures being conducted. This research was carried out in accordance with IPR Institutional guidelines as well as International regulations including those of AAALAC, WHO, NIH, PVEN, Good Clinical Practice and Helsinki Convention on humane treatment of animals for scientific purposes and Good Laboratory Practice (GLP). The investigator continuously informed the IPR-ERC as to the progress of the study on a regular basis per the ERC requirements, but at minimum once a year.

Collection of Plant Material

Fresh and uniformly shaped pumpkins of C. maxima Duchesne were selected from a commercial pumpkin farm in Embu County, Kenya and taxonomically identified and authenticated by a taxonomist in department of Botany JKUAT (Voucher no.4321). The fresh pumpkins approximately 3kgs each were cut by a sharp knife and peeled, approximately 2000g seeds were hand collected and washed with water, and the pulp sliced into pieces measuring 0.5 × 2.0 × 5.0 cm. The slices and the seeds were dried in hot air oven at 70 0 C for 72hrs and ground into powder using a mechanical grinder. The fine flour of the whole seeds and the pumpkin pulp were packed in a low dense polyethylene (LDPE) bag and kept in a refrigerator (40 C) until analysis.

Figure 1: Dried seeds of *Cucurbita maxima*

Preparation of plant extract

i) ***Preparation of aqueous (seed) extract*:** Pumpkin seeds powder (100 g) was mixed with the addition of 500 ml distilled water and left to stand for 2hrs. The mixture was then incubated at 40 ^{0}C for 18 to 24 hrs. The obtained mixture was filtered through centrifugation at 900rpm for 10 minutes and passed through Whatman No.1 filter paper. The filtrate was evaporated by using a freeze dryer and pumpkin seed extract was obtained as pulverized. This was stored at 4 ^{0}C for further analysis.

ii) ***Preparation of aqueous (pulp) extract*:** Pulp powder from pumpkin (100 g) was mixed with the addition of 2000 ml distilled water and left to stand for 2hrs.The mixture was then incubated at 40 ^{0}C for 18 to 24 hrs. The obtained mixture was filtered through centrifugation at 900rpm for 10 min and passed through Whatman No.1 filter paper. The filtrate was evaporated by using a freeze dryer and pumpkin seed extract was obtained as pulverized. This was stored at 4^{0}C for further analysis.

iii) ***Preparation of ethanol (seed) extract***: Pumpkin seeds (50g) was extracted by adding 500 ml ethanol. The mixture was shaken for 4hrs and left to stand at room temperature for 24hrs. The mixture was then filtered using Whatman no.1 filter paper. Ethanol from the filtrate (Liquid part) was then evaporated using vacuum evaporator at 60 ^{0}C. All the extracts was stored at 4 ^{0}C for further analysis.

iv) ***Preparation of ethanol (pulp) extract***: Pumpkin pulp (75g) was extracted by adding 750 ml of ethanol. The mixture was shaken for 4hrs and left to stand at room temperature for 24hrs.The mixture was then filtered using Whatman no.1 filter paper. Ethanol from the filtrate (Liquid part) was then evaporated using vacuum evaporator at 60 ^{0}C. All the extracts was stored at 4 ^{0}C for further analysis.

Qualitative Analysis of Phytochemicals

Qualitative analysis was carried out to ascertain the presence of the different phytochemicals before quantitative analysis was done. All chemicals used in the study were of analytical grade (Sigma and Merck).

i) ***Flavonoids*:** Five milliliters of dilute ammonia solution was added to a portion of the aqueous filtrate of the extract followed by addition of concentrated H_2S0_4. A yellow coloration observed indicated the presence of flavonoids. The yellow coloration disappears on standing. Quantity was determined according to the method of Harborne [8]. Briefly, 5 g of the sample was boiled in 50 ml of 2M HCl solution for 30min under reflux. It was then allowed to cool and filtered through Whatman No. 42 filter paper. A measured volume of the extract was treated with equal volume of ethyl acetate starting with drop. The flavonoid precipitated was recovered by filtration using Whatman filter paper. The resulting weight difference was the weight of flavonoid in the sample.

ii) ***Alkaloids***: Two grams of the extract was extracted by warming with 20 ml 1% sulphuric acid in a 50 ml conical flask on a water bath, with intermittent shaking for 2 minutes. It was then centrifuged and the supernatant pipetted off into a small conical flask. One drop of Meyer's reagent was added to 0.1 ml supernatant in a semi-micro tube. Cream precipitate indicated presence of alkaloids. Quantification of alkaloids was done by alkaline precipitation gravimetric method described by Harborne [8]. Briefly, 200 ml of 10% acetic acid in ethanol was added to 5 g of the sample in 250 ml beaker, covered and allowed to stand for 4h at 28 ^{0}C. It was later filtered via Whatman No. 42 filter paper. The filtrate was concentrated to one quarter of its original volume by evaporation. Concentrated ammonium hydroxide was added drop wise to the extract until the alkaloid precipitated. The alkaloid precipitated was received in a weighed filter paper, washed with 1% ammonia solution dried in the oven at 80 ^{0}C. Alkaloid content was calculated and expressed as a percentage of the weight of sample analyzed.

iii) ***Glycosides*** - *Keller-kiliani Test*: To 2 ml of extract, 1ml glacial acetic acid and 1-2 drops of $Fecl_3$ was added followed by 1ml of concentrated H_2SO_4. Formation of green blue color indicated the presence of cardiac glycosides.

iv) ***Terpenes:*** Five milliliters of each extract was mixed in 2 ml of chloroform, and concentrated sulphuric acid was added to form a layer. A reddish brown coloration of the inter face formed to show positive results for the presence of terpenoids.

v) ***Phenolics***: Ferric chloride test was carried out where the extract was diluted to 5 ml with distilled water. To this, a few drops of neutral 5% Ferric chloride solution was added. A dark green or a blue-black color indicated the presence of phenolic compounds. For total phenols, 2 g of the sample was defatted with 100 ml of diethyl ether using a soxhlet apparatus for 2 hours. The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. Five milliliters of the extract was pipetted out into a 50 ml flask, then 10 ml of distilled water added. Two milliliters of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to the mark and left to react for 30 minutes for color development and the intensity measured at 505 nm.

vi) ***Carbohydrates*** - *Molisch's test*: To 2ml of extract, 2 drops of alcoholic α-nephthol solution in a test tube was added. Formation of violet ring at the junction indicates the presence of carbohydrate.

vii) ***Proteins*****:** To 2 ml of extract, a few drops of concentrated nitric acid were added. Formation of yellow colour indicates the presence of proteins.

viii) ***Saponins*** - *Frothing Test*: About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. Ten milliliters of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion. Quantity was determined by the method of Obadoni and his colleague [9]. Twenty grams of the ground sample was put in a conical flask and 100 cm^3 of 20% aqueous ethanol added. It was heated over a hot water bath for 4h with continuous stirring at about temperature of 55 ^{0}C. The mixture was then filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40ml over water bath at about 90 ^{0}C. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethyl ether added and shaken vigorously. The aqueous layer was recovered while the ether layer discarded. The purification process was repeated. Sixty milliliters of n-butanol were then added and the combined n-butanol extracts washed twice with 10ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage.

ix) ***Tannins***: About 0.5g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added. A brownish green coloration indicated presence of tannins. Tannin content was determined by the Folis-Denis colorimetric method described by Kirk and another [10]. Five grams of the sample was dispensed in 50ml of distilled water and shaken. The mixture was allowed to stand for 30 min at 28 ^{0}C before it is filtered through Whatman No. 42 filter paper. Two milliliters of the extract was dispersed into a 50 ml volumetric flask. Similarly 2 ml standard tannin solution (tannic acid) and 2ml of distilled water was put in separate volumetric flasks to serve as standard and reagent was added to each of the flask and the 2.5ml of saturated Na_2C0_3 solution was added. The content of each flask was made up to 50ml with distilled water and allowed to incubate at 28 ^{0}C for 90 min. Their respective absorbance were measured in a spectrophotometer at 260nm using the reagent blank to calibrate the instrument at zero.

Determination of antioxidant activity

The radical-scavenging activity was determined using diphenyl picryl hydrazyl radical (DPPH) according to Ayoola and others [11]. The following concentrations of the extracts were prepared, 0.5, 1.0, 2.0, 5.0 and 10 mg/ml in methanol in cuvette placed in the spectrophotometer (Analar grade). Vitamins C was used as the antioxidant standard at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5 and 0.75 mg/ml. One ml of the extract was placed in a test tube, and 3 ml of methanol added followed by 0.5 ml of 1 mM DPPH in methanol. The mixture was shaken vigorously and left to stand for 5 min. A blank solution was prepared containing the same amount of methanol and DPPH. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer [12]. The radical scavenging activity was calculated using the following formula:

% inhibition =
{[Absorbance blank-Absorbance sample]/Absorbance blank} x 100

Where Ab is the absorption of the blank sample and Aa is the absorption of the extract

All tests were run in triplicate, and analyses of all samples run in duplicate and averaged.

RESULTS

Phytochemical compositions of Cucurbita maxima

Both qualitative and quantitative analysis were performed this study. Qualitative analysis involves screening tests that indicate presence or absence of particular chemical compounds while quantitative analysis involved actual determination of the amount of each component in a specific weight of the plant sample.

i. *Qualitative analysis*

Table 1: Phytochemicals composition of aqueous extracts of *Cucurbita maxima* seeds and pulp

S/No	Phytochemicals	SEEDS	PULP
1	Alkaloids	+	-
2	Flavonoids	-	+
3	Phenolics	-	-
4	Carbohydrates	+	+
5	Tannins	+	-
6	Saponins	-	-
7	Terpenoids	-	+
8	Glycosides	+	_
9	Proteins	-	+

Note: (-) Indicates Absence (+) Indicates Presence

Table 2: Phytochemicals composition of ethanol extracts of *Cucurbita maxima* seeds and pulp

S/No	Phytochemicals	SEEDS	PULP
1	Alkaloids	+	+
2	Flavonoids	-	+
3	Phenolics	-	+
4	Carbohydrates	+	+
5	Tannins	+	+
6	Saponins	-	+
7	Terpenoids	+	+
8	Glycosides	-	-
9	Proteins	-	+

Note: (-) Indicates Absence (+) Indicates Presence

Tables 1 and 2 show the phytochemical compositions of aqueous and ethanol extracts of *Cucurbita maxima* pulp and seeds. The results illustrate the presence of various phytochemicals in *Cucurbita maxima* pulp and seeds extracts. The results show that *Cucurbita maxima* pulp extracts contain alkaloids, flavanoids, proteins, carbohydrates, polyphenols, glycosides, saponins and terpenoids as phytochemical constituents. Ethanol pulp extract contained majority of the phytochemicals except glycosides and hence could be more useful for treatment of diseases. Aqeuos extract of both seed and pulp contained carbohydrates while ethanol extracts of the same contained carbohydrates, terpenoids, tannins and alkaloids. Therefore, these extract would have varying medicinal activity due to different chemical components.

Quantitative analysis

Table 3: Phytochemical composition in Cucurbita maxima aqueous extracts

Phytochemical	Pulp Initial Weight (g)	Phytochemical level in Pulp (g/100g)	Seed dry Weight Used (g)	Phytochemical level in Seed (g/100g)
Phenols	0.1101±0.001	0.213±0.014	0.2416±0.002	0.336±0.013
Flavanoids	0.1101±0.001	0.175±0.008	0.2416±0.002	0.012±0.008
Tannins	0.1060±0.004	0.016±0.005	0.1382±0.002	0.009±0.005
Saponin	-	Negative	-	Negative

Values are given as means of three replicates ±St.dev

Table 3 shows composition of phytochemicals in pulp and seed. Both seeds and pulp contained phenols, flavanoids and Tannis at varying levels. Comparing the level of phytochemicals using one sample t-test in seeds and pulp showed significant difference ($t = 4.209$, $p = 0.001$). Furthermore, One-way ANOVA Post Hoc analysis reported significant difference within groups ($F_{5, 12} = 774.16$, $p = 0.001$) in levels of phytochemical composition for both seed and pulp parts of *Cucurbita maxima* fruit. Notably, saponin was absent in both seed and pulp.

Table 4: *Phytochemical composition in Cucurbita maxima ethanol extracts*

Phytochemical	Pulp Initial Weight (g)	Phytochemical level in Pulp (g/100g)	Seed dry Weight Used (g)	Phytochemical level in Seed (g/100g)
Phenols	0.2403±0.005	0.277±0.004	0.3233±0.006	0.530±0.026
Flavanoids	0.2403±0.005	0.246±0.013	0.3233±0.006	0.252±0.004
Tannins	0.1651±0.005	0.010±0.006	0.1202±0.002	0.064±0.001
Saponins		Positive		Positive

Values are given as means of three replicates ±St.dev

Table 4 shows phytochemicals extracted *C. maxima* from using ethanol. The difference between aqueous extract is that saponons were present in both pulp and seeds. Furthermore, the phytochemical composition also varied in concentration with tannins showing little concentration compared to phenols and flavanoids. Comparing the level of concentration of the phytochemicals in pulp and seed using one sample t-test showed significant difference (t = 5.638, p = 0.001). Similarly, One-way ANOVA comparison of the levels showed significant difference ($F_{5, 12}$ = 671.05, p = 0.001). Addition, saponin was able to be extracted using ethanol even though the levels were not quantified.

Table 5: Alkaloids composition (%) in *Cucurbita maxima*

Sample	Weight Used (g)	Extracted content (g)	Content (%)
Pulp Aqua	0.4504±0.001	0.0221±0.001	4.90±0.110
Seed Aqua	0.5821±0.001	0.0347±0.002	5.97±0.330
Pulp EtOH	0.6729±0.010	0.0638±0.004	9.49±0.600
Seed EtOH	0.5403±0.004	0.0526±0.002	9.74±0.380

Values are given as means of three replicates ±St.dev

Table 5 shows the percentage content of alkaloids in *C. maxima* both in aqueous and ethanol extraction. Using One sample student t-test to compare the percentage alkaloid content extracted by water in both seed and pulp, there was a significant difference (t = 21.343, p = 0.001). Similarly, there was a significant difference (t = 50.045, p = 0.001) for both seed and pulp ethanol extraction. This indicates that the seed has higher alkaloids than the pulp and hence would have more medicinal activity than the pulp and hence more economical to use.

ii. Anti-oxidant activity

Table 6: Percentage (%) inhibition of aqueous and ethanol extracts of *Cucurbita maxima*

Conc. mg/ml	Pulp aqua	Seeds aqua	Pulp EtOH	Seeds EtOH	VitC (Standard)	F value	*p* value
0.0	0.000	0.000	0.000	0.000	0.000	-	-
0.5	0.637±0.069	0.876±0.069	5.974±0.239	7.686±0.344	71.567±0.069	3.517E4	0.001
1.0	1.115±0.182	3.186±0.453	11.947±0.239	12.465±0.345	84.468±0.483	2.065E4	0.001
2.0	7.806±0.483	7.646±0.119	19.634±0.249	22.740±2.730	98.447±0.001	2.816E3	0.001
5.0	11.470±0.239	10.315±0.552	29.271±1.346	37.435±0.301	98.686±0.001	8.659E3	0.001
10.0	15.000±0.324	14.000±0.211	39.000±0.301	49.000±0.432	98.900±0.001	1.742E4	0.001

Values are given as means of three replicates ±St.dev

Table 6 show anti-oxidant activity of seed and pulp aqueous and ethanol extracts of *Cucurbita maxima*. Both extract exhibit anti-oxidant activity with increasing concentration. However, ethanol extract has more anti-oxidant activity compared to aqueous extracts but less that the standard anti-oxidant (Vit C). This is due to presence of sapponins which were absence in aqueous extracts and therefore would show the better medicinal activity. Using One-way ANOVA to compare anti-oxidant activity of the test extracts, there was significant difference across all concentration levels. However, Post Hoc analysis by Game-Howell method showed no significant difference (p = 0.293) between pulp and seed aqueous extracts at concentration 10 mg/ml.

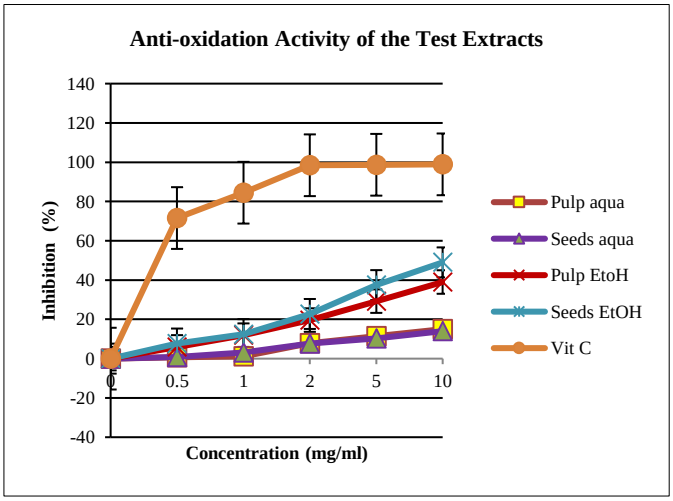

Figure 2: Anti-oxidant activity of seed, pulp extracts of *C. maxima* and Vitamin C

Figure 2 shows ethanolic extracts of the both seed and pulp are better oxidants compared to aqueous extracts. This is attributed to the fact that ethanol was able to extract saponins which were absent in aqueous extracts. This therefore shows that *C. maxima* can a good source of anti-oxidants in the body.

DISCUSSION

Phytochemical analysis of *C. maxima* seeds and pulp ethanolic and aqueous extracts were investigated in the current study. The findings showed presence of several phytochemicals including alkaloids, flavanoids, phenols, carbohydrates, tannins, saponins, terpenoids and proteins. This indicates that *C. maxima* not only being used as food in various communities across the globe, it can act as an important raw material for drug development in pharmaceutical industries. Our results are consistent to those reported by Kabbashi and colleagues [13]. In their study, they reported high anti-oxidant activity of ethanolic seed extract of *C. maxima.* The study also reported high antimicrobial activity against several microbes including *P. aeruginosa, A. niger*, *C. albicans*, *E. coli* and *B. subtilis*. The high levels of phytoconstituents show that the *C. maxima* can be used to treat various ailments in the body. As a matter of fact Caili and others [2] in their review reported anti-inflammatory activity in the seeds of *C. maxima* while anticancer activity was reported by Abou-Elella and a colleague [14]. *Cucurbita maxima* seeds are used as traditional medicine against irritable bladder and prostatic hyperplasia in the early stages I and II. These activities are linked to presence of highly hyroxylated phenolic substances (Flavonoids) which form complexes with bacterial cell wall disrupting its integrity thereby killing the microbe [15]. The presence of saponins in *C. maxima* not only contributes to its medicinal activity but also makes it applicable for industrial uses in production of detergents and service active agents [16]. The current study reported presence of tannins in *C. maxima* which corroborates with the findings of Ethiraj and another using acetone as the solvent [17]. Tannins are important phytochemicals because they have shown to influence nutritional value of various human and animal foodstuffs. Tannins' medicinal activity as reported by Salah and others include quick recovery of inflamed mucous membrane and wounds [18]. The available evidence shows that phytochemical studies on *C. maxima* using different solvents and also its medicinal activity have been explored. Currently, research on ethnobotany has doubled in the recent years but loss of biodiversity due climate change, encroachment of forest and other habitat may slow down the search for plant-based medicines [19]. In addition research has shown that drugs from plants are cost effective, safe and environmental friendly and hence the need to conserve and explore *C. maxima* for medicinal value [20]. Therefore, the current study suggests the actual use of the plant in drug development in pharmaceutical industries.

CONCLUSION

The information obtained from phytochemical and antioxidant evaluation from this study can be of great importance in relation to identification, authentication and chemical composition estimation. This may be useful for the standardization and isolation of bioactive compounds from this plant. The constituents of *Cucurbita maxima* seed and pulp extracts may have several medicinal properties that leading to opening up new avenues in the use of natural products for therapeutic purpose. Future studies should focus on cost-effective analysis of the phytochemicals levels in order to estimate the value of this plant in drug discovery and development hence its eventual use as raw material for pharmaceutical industry.

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