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In vitro antiproliferative potential of crude extracts from *Carica papaya L.* (Caricaceae) black seeds against prostate cancer cell lines'

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Black seeds from papaya plants are utilized as traditional medicine in African and Asian cultures to improve the functioning of the male reproductive system and management of prostate cancer. This study analyzed the phytochemical composition, antiproliferative activity and cytotoxicity of *Carica papaya* black seeds against the prostate cancer cells and Vero cells. Phytochemical screening was performed by standard procedures. Methyl tetrazolium bromide (MTT) cell viability assay was used to evaluate the antiproliferative effect of crude extracts in prostate cancer cells against the control. Phytocompounds, namely flavonoids, alkaloids, tannins, terpenoids, glycosides and saponins were found in the crude extracts. All papaya seeds' extracts had antiproliferative activity towards prostate cancer cells. Ethyl acetate extract was found with higher antiproliferative activity with IC_{50} of 3.64 µg/ml. The crude extracts were not toxic towards Vero cells as compared to the control. Isolation and characterization of active compounds from herein crude extracts and papaya black seeds are therefore recommended for future studies.

Key words: Prostate cancer**,** *Carica papaya*, extracts, phytochemical analysis, antiproliferative.

INTRODUCTION

Prostatic carcinoma is a cancerous disease of the man reproductive system called the prostate gland, which can be life-threatening when it spreads to significant parts of the body such as bones and lymph nodes (Alotaibi et al., 2017). The World Health Organization (WHO) report which was published in September 2018 indicated about 1.3 million cases of prostate cancer and 358,989 deaths worldwide. It is anticipated that, by 2035 the cancer incidences will rise to 24 million and in that case, the number of deaths from cancer will increase in the future (Bray et al., 2018). The second most frequent malignancy in males (after lung cancer) was cancer of the prostate and it contributed up to 3.8% of all men worldwide deaths from cancers in 2018 (Rawla, 2019). Prostate cancer

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is the most common carcinoma in Tanzania, contributing to about 22.9% of all cancers in men (Olson et al., 2020). Some of the major contributing factors to prostate cancer include age; men who are 60 years old and above are prone to prostate cancer and males at the age of 40 years and below are less prone to the same disease. Race is another contributing factor, for instance, people of African-American origin have a higher probability of suffering from prostate carcinoma as compared to whites. It is reported that the fourth most frequent cause of death in African-American males is prostate cancer; about 19% of black men (1 in 5) are reported with the cancer of the prostate and 5% of black men diagnosed with the same disease lose their life from it. Genetics is another contributing factor. A male who has a family member with prostate malignancy is more likely to get it in comparison to another person whose family members are free from prostate cancer (Alotaibi et al., 2017).

Moreover, a male who acquired the defective breast cancer gene from his parents is more presumably to develop the incurable prostatic carcinoma (Drudge-Coates et al., 2018). Obesity and sex hormones also contribute to growth of prostate tumors. Reduced levels of testosterone are associated with metabolic syndrome, benign prostatic hyperplasia and obesity and may result into prostate cancer (Alotaibi et al., 2017). Carica papaya L. (Caricaceae family) plant was selected for this study due to the reported phytochemical composition, including saponins, terpenoids, carbohydrates, alkaloids, glycosides and flavonoids which attribute to anticancer effect. Furthermore, the plant species exhibit a combination of alkaline with potassium carbonate or borax; which produces potential results in treatment of cutaneous tubercles warts, eczema, sinuses corns and other skin hardness and also administered into tumors of indolent glandular to enhance their absorption. Papaya green fruits are useful in treatment of hypertension, constipation, dyspepsia, general debility, amenorrhea, stimulate reproductive organs and expelling of worms (Aravind et al., 2013).

Anticancer activities of various parts of papaya plant were reported in some *in vitro* laboratory experiments. The extract from papaya fruit juice induced cell growth inhibition in liver cancer cells. Papaya seeds have also been used in some Asian culture as a traditional medicine to improve male fertility, indicating its medicinal function in male reproductive system. Papaya seeds are used to improve the functions of male reproductive system and it is assumed that they may have an anti-cancer effect on prostate cancer (Alotaibi et al., 2017). Moreover, papaya leaf extract is known to mediate type 1 T-helper (Th1) cell changes in the human immune system to treat and prevent prostate cancer, allergic reactions and can further be applied as the immunoadjuvant in vaccine therapy (Otsuki et al., 2010). The aim of this study was to assess the cytotoxicity and antiproliferative activity of crude extracts of *C. papaya* black seeds. To evaluate the potential effects of *C. papaya* seeds in normal cells and cancer cells, Vero cells and prostate cancer cells were selected respectively.

MATERIALS AND METHODS

Plant materials

The ripe papaya fruits were harvested from a garden situated at Karangai village in Arusha, Tanzania, Arumeru District on 1st July, 2020. Prior to papaya fruits collection, the intended plant species was identified by Mr. Simon Laizer, a botanist. The voucher specimen No.01 was well prepared and then laid out in the national herbarium at Tanzania Pesticide Research Institute (TPRI), Arusha on 5th July, 2020.

Solvents for extraction

Methanol, distilled water, ethanol, n-hexane and ethyl acetate were provided by NM-AIST Laboratory in Arusha, Tanzania.

Positive control

Doxorubicin hydrochloride 50 mg USP was obtained from KEMRI in Nairobi, Kenya and was used as a positive control.

Culture media

10% fetal bovine serum (FBS), streptomycin 1%, Dulbecco Modified Eagle Medium (DMEM) and 1% amino acids were purchased from Keuta Technologies Ltd in Nairobi, Kenya.

Cell lines

The KEMRI in Nairobi, Kenya provided isolated prostate cancer cell lines (22VR1) and normal Vero cell lines (CCL81) which were procured from American Type Culture Collection, Rockwile, USA.

Reagents for phytochemical screening

Distilled water, ferric chloride**,** 1% of hydrochloric acid, Mayer's reagent, ammonia solution, concentrated sulfuric acid and chloroform were provided by NM-AIST Laboratory in Arusha, Tanzania.

Methyl tetrazolium bromide (MTT) assay reagents and equipment

Multi-well plates, MTT dye, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO) and automated microplate photometer were purchased from Keuta Technologies Ltd in Nairobi, Kenya.

Approach

Preparation of plant materials

The papaya fruits were washed with distilled water, dried at room temperature and thereafter were cut into half to reach the seeds. The seeds were scrapped out of papaya fruits and they were scattered over a plastic tray and left to dry in a screen house to

obtain 1 kg as a constant weight. The grinding of seeds was achieved by using a grinder machine to obtain a fine powder.

Preparation of crude extracts

Serial exhaustive extraction method was used in preparation of solvent extracts based on polarity of solvents. 150 g of powdered papaya black seeds was weighed by using beam balance and was then placed in a flat-bottomed conical flask. The successive extraction was carried out at room temperature; starting with hexane, ethyl acetate, ethanol and then methanol in the ratio of 1:10 for 72 h with frequent agitation. Thereafter, the filtrate was obtained by filtering the mixture using Butcher funnel with Whatman No.1 filter paper. The rotary evaporator was employed in drying the filtrate at low pressure to obtain a concentrated sample. The weight of extract was ascertained and stored in the refrigerator at 4°C waiting for use (Nawaz et al., 2020). 150 g of the powder of papaya seeds was soaked into 500 ml of distilled water for preparation of aqueous extract. The mixture was then heated for 6 h in a water bath at 60°C. It was then kept aside at room temperature and allowed to cool. Filtration of the content was performed by means of muslin gauze and the filtrate was frozen for 24 h. Lyophilization of the filtrate was achieved using Modulyo Edwards freeze drying machine. The weight of lyophilized extract was verified and then kept at -20°C in air tight bottle waiting for use.

Phytochemical analysis

Standard procedures were used to test the five (5) solvent fractions of papaya seeds for secondary metabolites (Neethu et al., 2018). Flavonoids, alkaloids, tannins, terpenoids, glycosides and saponins were among the secondary metabolites studied as follows:

Analysis of saponins: Analysis of saponins was done by adding 2 ml of distilled water into 2 ml of crude extract in a test tube; thereafter the mixture was vigorously agitated. The test tube was then kept aside for a while. The formation of a persistent foam layer indicated the presence of saponins.

Analysis of tannins: 5 ml of distilled water was introduced into a test tube containing 2 ml of crude extract, boiled and then 2% $FeCl_3$ was added. The formation of green precipitates indicated the presence of tannins.

Analysis of alkaloids: On a watch glass, 2 ml of crude extract was poured, followed by the addition of 3 drops of Mayer's reagent and 1% hydrochloric acid. The presence of alkaloids was shown by the formation of white precipitate.

Analysis of flavonoids: 5 ml of aqueous ammonia was added into a test tube containing 2 ml of crude extract, followed by addition of 2 ml of concentrated sulfuric acid and the whole mixture was shaken. Presence of flavonoids was indicated by intense yellow color formation.

Analysis of terpenoids: 2 ml of chloroform was added into a test tube containing 2 ml of crude extract, which was vigorously shaken before being left to stand for 1 min. 2 ml of concentrated sulfuric acid was added into the mixture; thereafter the mixture was heated for about 2 min and it was then set aside. Grey color formation suggested the presence of terpenoids.

Analysis of glycosides: 2 ml of chloroform was added into a test tube having 2 ml of the crude extract; thereafter 2 ml of sulfuric acid was added. The presence of glycosides was shown by formation of brown color.

Cell culture: Prostate cancer cells and Vero cells were provided by KEMRI. Thawing of cells was done in a water bath at 37°C with Dulbecco Modified Essential Medium (DMEM) supplemented with 100 µg/ml of streptomycin and 10% fetal bovine serum (FBS) which was then incubated at 37°C, 5% CO_2 and 95% humidity to attain confluence.

Preparation of test samples

Analytical balance was used to weigh 10 mg of the crude extracts and doxorubicin and placed in a 1.5 ml Eppendorf tube; thereafter 100 µl of the solution of dimethyl sulfoxide (DMSO) was added and the whole content was swirled. Another 1.5 ml Eppendorf tube was used whereby a concentration of 1,000 µg/ml was achieved by transferring into a tube 100 µl of the prepared mixture and then followed by adding 900 µl of PBS. The test samples were stored at -20°C until experimentation.

MTT bioassay

Selected cells were washed with saline phosphate after reaching the required confluence and then harvested by trypsinization. Trypan blue dye exclusion method (cell density counting) was employed to measure the cell viability with the help of a hemocytometer. Seeding of cells was done in 96-multi well plates by adding aliquot of 100 µl at a density of 2 x 10^5 cells/well and incubated for 24 h in an environment of 95% humidity and 5% CO_2 at 37°C. After incubation for 24 h, 15 µl of samples to be tested from serial dilution of seven different concentrations; 1000, 333.33, 11.11, 37.04, 12.35, 4.12 and 1.37 µg/ml was added respectively starting from Row H to B. Row A containing media and cells served as a negative control (Pers et al., 2019). A positive control was doxorubicin, which is a commonly used anticancer drug (Tietbohl et al., 2017). It was placed in wells 10, 11 and 12 in 96-multi well plate. Further incubation was carried out for 48 h at 37°C and 5% CO_2. The potential effect of tested samples was measured by the capacity of viable cells to reduce a yellow MTT dye to formazan which is a purple product. 100 µl of the medium was drawn after 48 h and to the remaining medium 10 µl of the MTT solution was added in each well and incubated for 4 h at 37°C in 5% CO_2. The surface media was then taken out from the plates, and using 50 µl of 100% DMSO, the formazan crystals were dissolved. The contents on the wells were shaken thoroughly followed by reading of the absorbance at 540 nm with the wavelength of 720 nm as a reference using enzyme-linked immunoassay (ELISA) reader (Reilly et al., 1998). The percentage reduction of cell growth was established via the formula indicated hereunder (Bézivin et al., 2003):

% cell growth reduction = $100-(At-Ab)/(Ac-Ab) \times 100$

Where; Ab= value of absorbance for blank (media only), At= value of absorbance for sample tested (cells + extracts) and Ac= value of absorbance of negative control (cells plus media). CC_{50} and IC_{50} values were calculated via R Software Version 3.4.1 and were used to express the effect of test samples on cells. CC_{50} and IC_{50} are concentration of test sample which killed 50% of treated Vero cells and concentration of test sample which inhibited the growth of cancer cells by 50% respectively. Classification of antiproliferative activity was as follows: >1000 µg/ml (inactive), >100–1000 µg/ml (weakly active), >20–100 µg/ml (moderately active) and ≤20 µg/ml (active) (Baharum et al., 2014).

Data management and analysis

All experimental raw data were recorded in the Microsoft Excel

Table 1. Percentage yield of *C. papaya* seeds extraction.

Extraction solvent	Sample weight (g)	Extract weight (g)	%Yield
Methanol	150	9.3	6.2
Ethyl acetate	150	6.0	4.0
n-hexane	150	4.4	2.9
Ethanol	150	10.4	6.9
Aqueous	150	13.6	9.1

Table 2. Phytocompounds present in solvent fractions of *C. papaya* seeds.

Row	Concentration (µg/ml)	Cell counts (µg/ml) for CCL81					
		Aqueous	Ethyl acetate	n-hexane	Ethanol	Methanol	Doxorubicin
A	0.00	0.95	0.92	0.95	0.92	0.89	0.84
B	1.37	0.80	0.74	0.78	0.83	0.71	0.72
C	4.12	0.63	0.63	0.71	0.73	0.48	0.58
D	12.35	0.54	0.59	0.57	0.58	0.48	0.34
E	37.04	0.47	0.41	0.41	0.47	0.38	0.27
F	111.11	0.36	0.33	0.30	0.25	0.32	0.23
G	333.33	0.25	0.23	0.25	0.12	0.27	0.18
H	1000.00	0.13	0.10	0.14	0.05	0.25	0.07

Sheets. Data transformation and analysis was performed by means of statistical packages in R Software Version 3.4.1 and Excel data sheets. The statistical difference between treatments and controls was tested by One-way Analysis of Variance (ANOVA) at $p \leq 0.05$. The percentage cancer cell growth inhibition and the percentage toxicity in Vero cells were calculated in terms of SEM (Mean ± Standard Error of Mean).

Ethics consideration

Papaya fruits were harvested on garden owner's permission at Karangai village in Arumeru district. All standards and safety laboratory procedures were observed prior study commencement. There was no human or animal involved in this study.

RESULTS AND DISCUSSION

Table 1 shows the percentage yield of each solvent extract. Dependent upon the solvent nature, the range of yield of extracts was from 2.9 to 9.1%. Aqueous extraction produced more yield (9.1%) as compared to other solvents. This could be due to high solubility of various phytocompounds in water (Dhanani et al., 2013). All solvent fractions of papaya seeds were analyzed and found to contain various phytochemicals including flavonoids, saponins, glycosides, terpenoids, tannins and alkaloids as shown in Table 2. Flavonoids were present in all screened crude extracts; these phytocompounds were reported to possess antioxidant activity which attributes to antiproliferative effects toward many cancer diseases by inducing apoptosis of cells followed by cell death (Tietbohl et al., 2017; Widyawati et al., 2020). Presence of phytocompounds in plant extracts is determined by the polarity nature of particular compounds that are selectively soluble in solvents used (Ngo et al., 2017). Ethyl acetate extract was in this study found with all the tested compounds (Table 2), and may be compared with other studies which had reported that it has the ability of dissolving both non-polar and polar components due to its semi polarity nature (Ajuru et al., 2017). Therefore, ethyl acetate may be considered as a suitable extraction solvent for phytocompounds from papaya black seeds. Moreover, phytoconstituents such as flavonoids, alkaloids, tannins, terpenoids, glycosides and saponins have been reported to be effective antiproliferative compounds which are potential in the synthesis of convectional drugs (Ulbricht et al., 2010). Results in Tables 3 and 4 and Figure 1 are for toxicity evaluation of crude extracts and doxorubicin in Vero cells. Our findings showed that the crude extracts of papaya seeds were not toxic towards Vero cells (CC_{50} >23 µg/ml) as compared to doxorubicin, the positive control. This is the first study to the best of our knowledge that reports on the toxicity of crude exacts of papaya black seeds in normal cells. The toxic effects of doxorubicin on normal cells were also reported in previous studies (Wang et al., 2004).

Tables 5 and 6 and Figure 2 are the results obtained from antiproliferative studies on prostate cancer cells. The plant extracts seemed to possess strong anticancer potential as compared to the control. Ethyl acetate extract

Table 3. Vero cell counts after treatment with papaya seeds' extracts and doxorubicin.

Extract	Alkaloids	Saponins	Flavonoids	Glycosides	Terpenoids	Tannins
n-hexane	+	-	+	_	+	+
Ethanol	+	+	+	+	-	-
Ethyl acetate	+	+	+	+	+	+
Methanol	+	+	+	+	-	+
Aqueous	+	+	+	+	-	+

+,-: Indicates presence and absence, respectively.

Table 4. CC_{50} of papaya seeds' extracts and doxorubicin in Vero cells.

Variable	Aqueous	Ethyl acetate	n-hexane	Ethanol	Methanol	Doxorubicin
%Toxicity (mean ± SE)	53.6 ± 9.5	54.5± 9.8	53.5 ± 9.7	53.5± 12.8	58.1±7.5	59.4 ± 10.3
CC_{50} (CI 95%)	25.3 (17.7-35.6)	23.2 (16.4-32.3)	25.8 (18.3-36.1)	27.1 (21.2-34.6)	12.7 (3.09-33.1)	15.0 (6.56-29.4)
χ^2	10.34*	11.75*	19.1**	12.62*	12.6*	13.5*

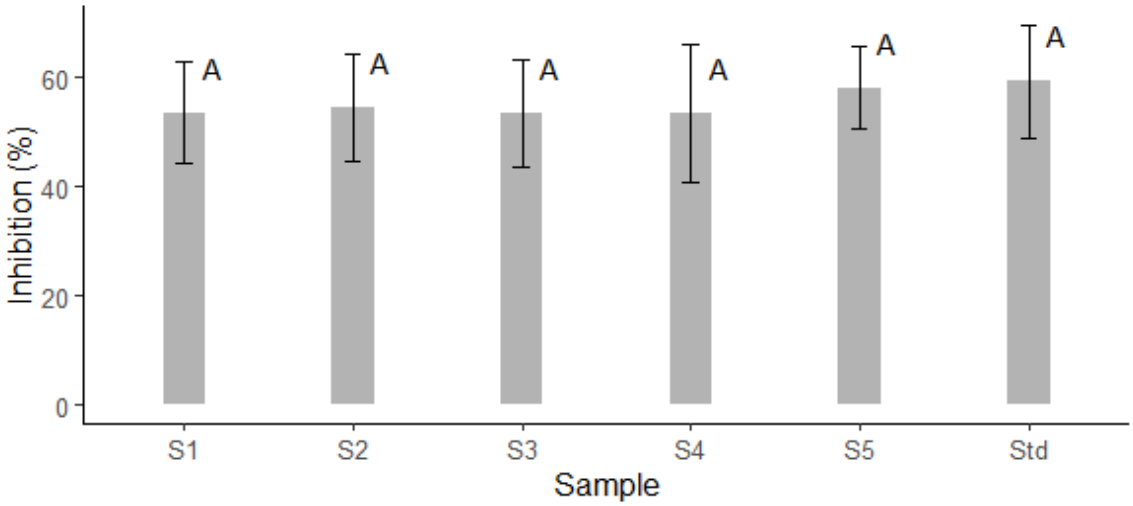

Figure 1. Percentage of toxicities of crude extracts and doxorubicin in Vero cells.

had the IC_{50} value of 3.64 µg/ml, which had induced higher inhibitory growth effect in cancer cells and could be attributed to the phytocompounds available in the respective solvent extract (Makurdi et al., 2015). Aqueous extract was also found to possess relatively higher antiproliferative activity towards cancer cells as compared to the control with the IC50 value of 19.9 µg/ml. However, water is the most commonly used solvent by herbalists for the extraction of medicinal plants (Mekonnen and Abebe, 2017). Our study findings have shown that water could also be a suitable extraction solvent for anticancer phytocompounds from black seeds of *C. papaya*. Cell growth inhibition was found to be dependent on sample concentration; thus the change in sample concentration from 1000 to 1.37 µg/ml caused low antiproliferation of cancer cells. The rate of cell proliferation was higher at minimum sample concentration and lower at maximum sample concentration from Row B towards H respectively. Our current results are supported by the previous study findings of anticancer activity of 24 plants which indicated that anticancer potency of the tested plant extracts was dose-dependent (Fadeyi et al., 2013).

CONCLUSION AND RECOMMENDATION

The study authenticated the antiproliferative activity of crude extracts from papaya black seeds towards the

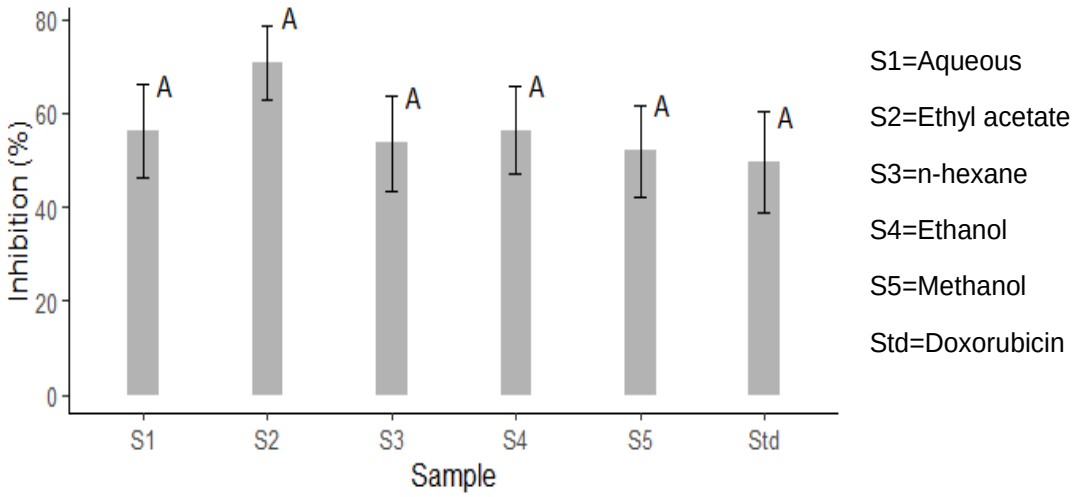

Figure 2. Percentage of cell growth inhibitions of crude extracts and doxorubicin in prostate cancer cells.

Table 5. Prostate cancer cell counts after treatment with crude extracts and doxorubicin.

Row	Concentration (µg/ml)	Cell Counts (µg/ml) for 22VR1					
		Aqueous	Ethyl acetate	n-hexane	Ethanol	Methanol	Doxorubicin
A	0.00	0.94	0.87	0.91	1.13	0.93	1.12
B	1.37	0.77	0.57	0.76	0.87	0.76	0.93
C	4.12	0.62	0.41	0.65	0.75	0.65	0.62
D	12.35	0.51	0.30	0.55	0.60	0.55	0.58
E	37.04	0.41	0.20	0.40	0.50	0.46	0.42
F	111.11	0.30	0.14	0.29	0.40	0.39	0.35
G	333.33	0.20	0.12	0.24	0.25	0.23	0.24
H	1000.00	0.06	0.05	0.08	0.08	0.09	0.13

Table 6. IC_{50} of crude extracts and doxorubicin in prostate cancer cells.

Variable	Aqueous	Ethyl acetate	n-hexane	Ethanol	Methanol	Doxorubicin
% Inhibition (Mean ± SE)	56.2^{A} ± 9.9	70.5±7.9	53.5^{A}± 10.1	56.3±9.3	51.8^{A} ± 9.6	49.6^{A} ± 10.7
IC_{50}	19.9 (14.2-27.3)	3.64 (2.06-5.69)	25.89 (18.6-35.4)	18.9 (13.0-26.6)	30.1 (21.4-42.1)	37.4 (7.94-179.0)
χ^2	12.26*	7.93*	9.14*	14.56*	12.6*	13.5*

prostate cancer cells. The observed cell growth inhibitory effect may be attributed to the presence of active phytocompounds in plant extracts (Zhou et al., 2011). Papaya seeds' crude extracts also selectively inhibited cancer cells growth. This may validate the traditional use of *C. papaya* L. black seeds' extracts in the prostate cancer management and treatment. More research involving isolation and characterization of active compounds from crude extracts is therefore required. Conduction of animal studies that could better predict the anticancer potential of papaya seeds' extracts is also important. It is also suggested that the mechanism of action for papaya seeds and phytocompounds be investigated.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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