Antioxidant and Antiproliferative Activity of *Azadirachta indica* A. Juss Extracts against Cancer Cell Lines: An experimental study

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Summary

BACKGROUND

Management of cancer using medicinal plants is a common practice in Kenya. The rationale for use of these plants is not supported by empirical evidence.

Objective: This study evaluated the antioxidant and antiproliferative activity of *Azadirachta indica.*

MATERIALS AND METHODS

Antiproliferative activity was determined against cervical (Hela) and prostate (DU145) cancer cells lines using MTT assay method. Combined active extracts were assayed *in vitro* using the checkerboard method. The *in vitro* antioxidant activity was assayed using 2, 2' diphenylpicrylhydrazyl (DPPH) free radical scavenging assay.

RESULTS

Methanol RB and SB extracts reported high IC_{50} values of 1.85 ± 0.01µg/ml and 2.59 ± 0.29µg/ml respectively, against Hela cancer cell line and IC_{50} values of 1.53 ± 0.07µg/ml and 3.26 ± 0.28µg/ml respectively, against DU145 cancer cell line. SB methanol extract exhibited antioxidant activity with an IC_{50} value of 69.31µg/ml. Synergy was observed.

CONCLUSION

The results show the potential of *A. indica* in the management of cervical and prostate cancer.

Keywords: *Medicinal plants, Azadirachta indica; MTT Assay; DPPH Assay; Checkerboard method.*

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Introduction

Cancer is a class of diseases in which a group of cells display uncontrolled growth, with intrusion and destruction of adjacent tissues. Cancer sometimes spreads to other locations in the body via lymph or blood [1]. Cancer of the cervix is the second most common cancer among women worldwide. Incidence of cervical cancer is highest in developing countries where it is the leading cause of cancer deaths. In 2008, 529,409 new cases occurred globally, about 274,883 women died in the same year. About 86% of new cases occur in developing countries, where 80-90% of deaths occur within the same year [2]. Prostate cancer is the most common cancer among elderly male population all over the world, with a slight preponderance in blacks [3].Treatment of prostate cancer is also determined to a great extent by the cancer

stage. The management options can be low risk, intermediate risk and high risk [4]. These treatment options have certain drawbacks that cannot be overlooked. The most outstanding drawback for chemotherapy and radiotherapy is that they cause undesirable effects to the patients. The post-operative survival rates for surgery on the other hand are low hence patients always tend to have poor prognosis due to late diagnosis when on these treatment options [5]. Medicinal herbs and their derivative phytocompounds are being increasingly recognized as useful complementary treatments for cancer. A large volume of clinical studies have reported the beneficial effects of herbal medicines on the survival, immune modulation, and quality of life (QOL) of cancer patients, when these herbal medicines are used in combination with conventional therapeutics [6].

Materials and Methods

Collection of the plant materials

The plant materials of *Azadirachta indica*, that is stem bark (SB) and root bark (RB) were collected separately form shimba hills then, air-dried in mesh bags and deposited at the University of Nairobi Herbarium with the voucher specimen number, PGK/2014/10. The raw materials were stored separately at the Centre for Traditional Medicine and Drug Research, (CTMDR), KEMRI. A plant taxonomist was engaged during plant collection and identification.

Aqueous extraction

One hundred grams dried plant materials was accurately weighed and soaked in 200ml distilled water and placed in a water bath at 70°C for 1 hour. The extract was then filtered in a 500ml conical flask. A two layer of sterile gauze filtration was achieved and the filtered extract was freeze dried using a Freeze Dryer (Edwards freeze drier Modulyo). A powder-like extract was collected after 72 hours. The extracts were then weighed, labelled and stored in an airtight container at 4°C prior to use.

Organic extraction

One hundred grams of dried plant materials were weighed and soaked in 200ml flat bottomed conical flask and covered tightly by cotton gauze at room temperature for 24-hours. The extract was filtered through Whatman filter paper no.1 and concentrated under reduced pressure using a rotary evaporator (Buchi Rotavapor R-114). The resultant extract was then weighed, labeled and stored in an airtight container at 4°C prior to use. The % yield was calculated as follows:

$$\%\ yield = \frac{total\ weight\ of\ the\ extract\ obtained}{total\ weight\ of\ the\ dried\ plant\ material} \times 100$$

Determination of antioxidant (free radical scavenging) activity of the plant extracts

The antioxidant activity of the extracts was measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical with slight modifications [7]. Briefly, 1ml of 0.1mM of DPPH solution in methanol was mixed with 1ml of the plant extract solution of varying concentrations (100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/ml). A corresponding blank sample was prepared and L-Ascorbic acid was used as reference standard. The decrease in absorbance was measured at 517nm after 30minutes in darkness using a spectrophotometer (UV-VIS Shimadzu). Lower absorbance of the reaction mixture indicated higher free radical activity. The % DPPH scavenging effect was calculated using the following equation:

% Inhibition = [1 – OD (DPPH + sample)/OD (DPPH)] x 100%

A dose response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of a

maximum scavenging capacity. All tests and analyses were done in triplicate and averaged.

Determination of antiproliferative activity of the plant extracts

The assay was carried out [8] on Vero, prostate and cervical cancer cell lines were cultured and maintained in DMEM supplemented with 10% FBS at 37^{o}C in 5% CO_2. The cells were harvested by trypsinization, pooled in a 50 ml vial and 100µl cell suspension (2 x 10^5 cell/ml) put into 3 wells of rows A-H in a 96-well micro-titer plate for each sample. The cells were incubated at 37^{o}C in 5% CO_2 for 24 hours to attach, the medium aspirated off from row H and 150µl. The highest concentration of each of the test samples (serial dilutions in MEM) were added into the same row and a serial dilution was carried out up to row B. The experimental plates were incubated further at 37^{o}C with 5% CO_2 for 48 hours. The controls used were cells with no drugs, medium alone (no drugs and no cells) and 5-fluorouracil as positive control. MTT assay was used to determine cell viability.

The MTT assay is a laboratory test which measures changes in colour for measuring the activity of the enzyme that reduces MTT to insoluble formazan, giving a purple colour. Yellow MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole] reduce to purple formazan in living cells [9]. At the end of the incubation time, 10µl of the MTT dye [(5mg of MTT, dissolved in 1ml serum free medium (phosphate buffered saline (PBS)] was added into each well and the cells incubated for 2-4 hours until a purple precipitate was clearly visible under a microscope. The medium together with MTT was aspirated off from the wells and 100µl of 100% DMSO added and the plates were shaken for 5 minutes to solubilize the formazan crystals. The absorbance was measured for each well at 562 nm and 690 nm as reference using a micro-titer plate reader [10].

To determine the activity of blend extracts, the checkerboard method was applied in this combination experiment and the degree of synergy was evaluated [11]. Briefly, the *in vitro* combination studies involved two extracts (a) and (b). One extract (component a) with a predetermined concentration that needed to inhibit cell growth by 50% (IC_{50}) was kept constant while the other extract also with a predetermined IC_{50} (component b) was added at different concentrations to determine anticancer effect of the two extracts in combination.

The same experiment was repeated with component b as a constant while component (a) was varied. Fractional inhibition concentration (FIC) was calculated using the formula: $A_c/A_a + B_c/B_a$ = FIC where A_c and B_c represents equally effective concentrations (IC_{50}) when used in combinations, and A_a and B_a was the equally effective concentrations (IC_{50}) when used alone. Cell viability (%) was calculated using the formula; [12].

$$\text{Proliferation rate} = \frac{\text{At} - \text{Ab}}{\text{Ac} - \text{Ab}}$$

$$\text{Percentage viability} = \frac{\text{At} - \text{Ab}}{\text{Ac} - \text{Ab}} \text{X } 100$$

$$\text{Percentage inhibition} = 100 - \frac{\text{At} - \text{Ab}}{\text{Ac} - \text{Ab}} \text{X}100$$

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of negative control (cells plus media)

In vitro cytotoxicity assay

The cytotoxicity assay for extracts singly and when combined was carried out using a modified method described by Mosmann [8]. The cell survival for each cell

was determined using a microplate reader at 562 nm wavelength. The IC_{50} values were given as a mean of three independent experiments. Extract cytotoxicity was evaluated by this formula used to determine the sum of fractional inhibitory concentrations (FIC).

$$FICA = \frac{IC50\ (\mu g/ml)\ of\ extract\ (a)\ in\ combination}{IC50\ (\mu g/ml)\ of\ extract\ (a)\ alone}$$

$$FICB = \frac{IC50\ (\mu g/ml)\ of\ extract\ (b)\ in\ combination}{IC50\ (\mu g/ml)\ of\ extract\ (b)\ alone}$$

Data was interpreted as follows: FIC < 1, synergism; FIC = 1, additive effect and FIC > 1, antagonism [13, 14].

Data management and statistical analysis

The data collected was both qualitative and quantitative. Data generated from antioxidant activity, *in vitro* antiproliferative and *in vivo* acute toxicity assays of the plant extracts was quantitative data. This data was saved in excel spreadsheets, descriptive statistics done and values recorded as Mean ± SEM. Means among treatment were compared with One-way ANOVA and tukey's post-hoc test carried out for pairwise separation and comparison of means. Values were considered significantly different where $_P \leq 0.05$.Dose response curve was plotted and used to determined IC_{50}, CC_{50} and LD_{50}. Analysis of the data was done using Minitab statistical software version 17.0. The data was presented in graphs and tables.

Ethical considerations

The study was approved by KEMRI Scientific and Ethical Review committee (DPC.KEMRI/RES/7/3/1) and also by the University Board.

Results

IC_{50} Values for antioxidant activity of the plant extracts.

In this assay, the following criteria was used to rank the activity, ≤ 10 µg/ml is highly active, 10-150 µg/ml active, 150-500 µg/ml moderately active and > 500 µg/ml inactive. The IC_{50} value represents the concentration of test extracts that reduced 50% of DPPH solution. Ascorbic acid was used as a reference drug and it exhibited an IC_{50} value of 42.74 µg/ml. Methanolic extract of stem bark (SB), aqueous root bark (RB) and ethyl acetate extract of SB exhibit antioxidant activity with IC_{50} values of 69.31 ± 1.22 µg/ml, 102.87 ± 0.45 µg/ml and 134.9 ± 0.39 µg/ml respectively. While methanolic extract of root bark (RB), aqueous Stem bark (SB) and ethyl acetate extract of root bark (RB) exhibited antioxidant activity with IC_{50} values of 152.46 ± 0.84 µg/ml, 295.11 ± 1.34 µg/ml and 313.53 ± 1.12 µg/ml, respectively.

Determination of antiproliferative activity of the plant extracts

The six plant extracts that were initially screened for antioxidant activity were further tested for their antiproliferative activity against Hela and DU145 cell lines.

Table 1: Results of the Plant Extracts with HELA, DU 145 and Vero Cells Lines.

	HELA		DU 145		VERO
Plant extracts	**IC_{50}(µg/ml)**	**SI**	**IC_{50}(µg/ml)**	**SI**	**CC_{50}(µg/ml)**
A. indica rootbark aqueous	24.3± 0.14	5.6	51.39 ± 0.42	19.46	135.34 ±0.47
A. indica rootbark ethylacetate	328.87±1.32	1.26	483.65± 0.58	1.21	480.11±0.28
A. indica rootbark methanol	2.59±0.29	135.14	3.26 ± 0.28	39.6	350.02 ±0.13
A. indica stembark methanol	1.85±0.10	436.52	1.53 ± 0.07	35.94	807.56 ± 0.76
A. indica stembark ethyl acetate	39.00±0.52	12.72	24.75 ± 0.61	8.31	496.38±0.12
A. indica stembark aqueous	125.45±1.35	4.8	287.7 ± 0.47	1.49	605.52±0.24
5-Fluorouracil	2.04 ± 0.87	7.35	5.06± 0.28	3	15 ± 0.12

Table 2: Fractional Inhibitory Combination and Indexes of the Selected Plant Extracts

Combination a+b	IC_{50} of (b) in combination (µg/ml)	IC_{50} of (a) in combination (µg/ml)	FIC (A_c/A_a) (µg/ml)	FIC (B_c/B_a) (µg/ml)
SB+RB	2.50	4.55	0.89	1.23
SB+SD	1.81	2 .83	0.55	0.88
SD+SB	1.29	2.24	0.403	0.534

Where SB= ethylacetate stem bark RB= Methanol root bark SD= Standard drug (5-fluorouracil)

An extract is defined to be highly active if it has IC_{50} < 10 µg/ml, active when the IC_{50} is between 10 µg/ml and 100 µg/ml, moderately active if the IC_{50} is between 100µg/ml and 500µg/ml and low activity if the IC_{50} is > 500 µg/ml [14]. Methanol extracts of RB and SB extracts exhibited high activity with IC_{50} values of 1.85 ± 0.01 µg/ml, 2.59 ± 0.29 µg/ml respectively while aqueous RB had IC_{50} value of 24.3 ± 0.14 µg/ml against Hela cells. The reference drug, 5-fluorouracil, had an IC_{50} value of 2.04 ± 0.87 µg/ml against HeLa cell line (Table 1).

Against prostrate (DU145) cell line, methanol extracts of SB and RB also exhibited high activity with IC_{50} values of 1.53 ± 0.07 µg/ml and 3.26 ± 0.28 µg/ml respectively while ethylacetate SB extract had a moderate activity with an IC_{50} value of 24.75 ± 0.61 µg/ml. The reference drug, 5-fluorouracil, had an IC_{50} value of 5.06 ± 0.28 µg/ml. (Table 1).

Combination study on the antiproliferative Activity of Stem bark and Root bark extracts and 5-fluorouracil.

A. indica ethylacetate SB extract and *A. indica* methanol RB extract that exhibited mono-antiproliferative activity were investigated for antiproliferative activity when in combination or with a standard drug, 5-fluorouracil (5-FU).From the 6 combined extracts that were analysed, 3 extracts that exhibited good activity were further treated with Vero cell lines and there cytotoxic concentration (CC_{50}) and Selectivity index calculated. SD + SB had the lowest CC_{50} value of 190.24 µg/ml while SB + RB had a high CC_{50} value of 245.09 µg/ml. Further, fractional inhibitory concentration was calculated, SB+SD and SD+SB exhibited antagonistic effect in both cell lines while SB+RB was synergistic in Hela and antagonist in DU145.

Discussion

In the current study methanol SB extract had the best antioxidant activity while ethylacetate root bark extract exhibited the least antioxidant activity. The standard drug, ascorbic acid had an antioxidant activity that was higher than that of the test extracts. Results from the previous studies revealed that the antioxidant activity of *A. indica* has been carried out and observations reported that both the barks, methanolic leaves, and aqueous leaf extract of *A. indica* have antioxidant activity [15, 16]

The activity of SB and RB methanolic extracts exhibited strong antiproliferative activity on both cell lines. It has been reported before that the anti-carcinogenic activity of the extracts from the leaves of *A. indica* is due to its major production of an antioxidant, glutathione that is a carcinogen detoxifying enzyme and other valuable active compounds such as azadirachtin [16]. In a recent study, methanol and aqueous neem leaf extracts were determined to inhibit growth of HeLa cell lines with IC_{50} values of 7 µg/ml and 22 µg/ml respectively in [17]. The methanolic extracts from the SB and RB of the Kenyan species have demonstrated 2-6 fold improved activity against prostate and cervical cancer cell lines,

a panacea for development of locally affordable and effective alternative to synthetic drugs.

In combination assay, ethyl acetate SB extract and methanol RB extract that exhibited mono-antiproliferative activity were investigated for antiproliferative activity when in combination or with a standard drug, 5-fluorouracil (5-FU). Some combinations such as those in RB + SB, RB + SD and SD + RB did not show any significant difference in their activity. However significant enhancement of activity was observed between SB + RB, SB + SD and SD + SB. This shows that some of these components may have acted synergistically [11].In most cases, increased activity was noted whenever ethylacetate SB extract was one of the component used in combination. The strongest activity was recorded in combinations of the SD and SB ethylacetate extract with IC_{50} values of 1.09 ± 0.24 µg/ml against Hela and 2.04 ± 0.07 µg/ml against DU-145 cell lines. These extracts also reported high selectivity index of 37.59 and 102.83 in Hela and DU-145 cell lines, respectively. Three combinations that showed promising anticancer activity were selected and tested against vero cell lines (L6). There was no significant difference and the combinations were not cytotoxic to L6 cell lines at the concentrations tested. In order to ascertain the relationships between the selected combinations, their fractional inhibitory concentrations (FIC) were assessed (Table 2) as described by Berenbaum [10]. The combinations between the SD and SB ethylacetate extracts, whose activity was significantly enhanced in the experiment, showed an FIC value less than 1 against both Hela and DU-145 cell lines (Table 2) an indication of synergy [18]. In this study, all the combinations showed a synergistic effect expect combination between SB ethylacetate extract and RB ethylacetate extract on DU145 cell line showed antagonist effect (Table 2).

Conclusion and Recommendations

This study was aimed at validating the use of *Azadirachta indica* in management of prostate and cervical cancer in Kenya. Our results demonstrates a very strong case for the use of the methanolic extract from the RB of *A. indica* in management of the two types of cancer. This extract has an activity that is slightly better than that of 5-FU, the standard drug, has high SI and is safe in murine model even at a concentration of 2000 mg/kg body weight.

This study therefore scientifically validates and supports the claim for traditional use of this plant in the management of cancer. It would be interesting to carry out this experiment using the murine models of prostate and cervical cancer.

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References

1. **Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E. & Forman, D.** (2011): Global cancer statistics. *A Cancer Journal for Clinicians*, 61(2), 69-90.
2. **Ministry of Health** (2013): *National Guidelines for Cancer Management Kenya.* Office of the Director of Medical Services.
3. **Ministry for Public Health and Sanitation and Ministry of Medical Services** (2016): *National Cancer Control Strategy.*

4. **Stangelberger, A., Waldert, M., & Djavan, B** (2008): Prostate Cancer in Elderly Men. *Reviews in Urology*, *10*(2), 111–119.
5. **Ministry for Public Health and Sanitation and Ministry of Medical Services** (2016): *National Cancer Control Strategy.*
6. **Molassiotis, A., Fernadez-Ortega, P., Pud, D., Ozden,G., Scott, J. A., Panteli, V., Margulies, A.** (2004): Use of complementary and alternative medicine in cancer patients: A European Survey. *Annals of Oncology*, (16), 655-663.
7. **Nelson, P. S. and Montgomery, B** (2003): Unconventional therapy for prostate cancer: good, bad or questionable? *Nature Reviews Cancer* 3(11):845-858,
8. **Mosmann, T** (1983): Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, *65*(1-2), 55-63.
9. **Wang, X., Ge, J., Wang, K., Qian, J., & Zou, Y** (2006): Evaluation of MTT assay for measurement of Emodin induced cytotoxicity. *Assay and drug development technonogies* 4(2), 203-207,
10. **Brand-Williams W, Cuvelier ME, Berset C** (1995) Use of free radical method to evaluate antioxidant activity. *Lebensm Wiss Technology*. 28, 25-30.
11. **Hall, M. J., Middleton, R. F., & Westmacott, D** (1983): The fractional inhibitory concentration (FIC) index as a measure of synergy. *Journal of antimicrobial chemotherapy*, 11(5), 427-433.
12. **Berenbaum, M. C** (1978): A method for testing for synergy with any number of agents. *Journal of Infectious Diseases*, *137*(2), 122-130
13. **Hall, M. J., Middleton, R. F., & Westmacott, D** (1983): The fractional inhibitory concentration (FIC) index as a measure of synergy. *Journal of antimicrobial chemotherapy*, 11(5), 427-433.
14. **Njagi, S. M., Lagat, R. C., Mawia, A. M., Arika, W. M., & Wambua, F. K** (2016): In Vitro Antiproliferative Activity of Aqueous Root Bark Extract of *Cassia abbreviata* (Holmes) Brenan. *Journal of Cancer Science and Therapy*, *8*,114-121.
15. **Vasenwala, S. M., Seth, R., Haider, N., Islam, N., Khan, T., Maheshwari, V., & Rehman, S** (2012): A study on antioxidant and apoptotic effect of *Azadirachta indica* (neem) in cases of cervical cancer. *Archives of gynecology and obstetrics*, *286*(5), 1255-1259,
16. **Nabende, P. N., & Namukhosi, P**. Safety and anti-proliferative activity of *Prunus africana*, *Warburgia stuhlmannii* and *Maytenus senegalensis* extracts in breast and colon *cancer cell lines* (Doctoral dissertation, JKUAT), 2015.
17. **Adenyeye, A. A., Ajagonna O, P., Adeleke, T. L., Bello S. & O** (2006): Preliminary toxicity and phytochemical studies of the stembark aqueous extract of *Musanga cecropioides* in rats. *Journal of ethnopharmacol* 105:374-37.
18. **Webb, M. S., Johnstone, S., Morris, T. J., Kennedy, A., Gallagher, R., Harasym, N., & Mayer, L. D** (2007): *In vitro* and *in vivo* characterization of a combination chemotherapy formulation consisting of vinorelbine and phosphatidylserine. *European journal of pharmaceutics and biopharmaceutics*, *65*(3), 289-299,