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ORIGINAL ARTICLE

Antioxidant Activity and Enhanced Cytotoxicity of Aqueous *Mucuna pruriens* L. Leaf Extract by Doxorubicin on Different Human Cancer Cell Lines

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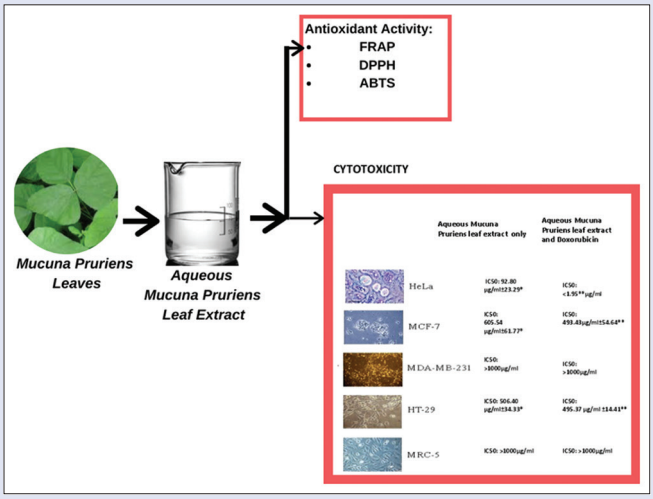
ABSTRACT

Background: Conventional cancer drugs have the disadvantage of severe side effects and resistance. Therefore, research targeted toward developing novel therapeutic strategies is needed. *Mucuna pruriens* (MP) leaf extracts have been suggested to be useful for the management of several diseases including cancer. **Objective:** The aim of this study was to evaluate the antioxidant and cytotoxic effect of an aqueous leaf extract of MP in different human cancer cell lines. This study also evaluated the enhanced cytotoxic effect of an aqueous leaf extract of MP with Doxorubicin (Dox) in the selected human cancer cell lines. **Materials and Methods:** In this study, the breast cancer cell lines (MCF-7 and MDA-MB-231), cervix carcinoma cell line (HeLa), and colon cancer cell line (HT-29) were used. As a control, the non-cancer lung cell line (MRC-5) was used. Cytotoxicity was assessed using the sulforhodamine B method. Antioxidant activity was measured with 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 2,2-diphenyl-1-picrylhydrazyl, and ferric reducing antioxidant power assay. **Results:** Aqueous MP leaf exhibited cytotoxicity in all the cell lines. The highest cytotoxic activity of the test extract was observed in HeLa cells at half-maximal inhibitory concentration (IC_{50}) = 92.8 µg/ml. Furthermore, the IC_{50} value of the test extract when combined with Dox was significantly reduced. Specifically, in HeLa cells, the IC_{50} was reduced by approximately 40 fold. **Conclusion:** This study demonstrates that aqueous MP leaf extracts could be useful as a source of antioxidants and compounds for cancer therapy. Further research is required to evaluate the chemical constituents of the leaf extracts and potential benefits for cancer therapy.

Key words: Antioxidant, cytotoxicity, doxorubicin, HeLa, *Mucuna pruriens*

SUMMARY

- Aqueous *Mucuna pruriens* (MP) leaf extract showed antioxidant activity
- Aqueous MP leaf extract (AMPLE) showed cytotoxic activity against cervical (HeLa), colon (HT-29), and breast (MCF-7) cancer cell lines compared to normal lung (MRC-5) cell line
- AMPLE cytotoxic effect was greatest against HeLa cells and the observed effect was enhanced by doxorubicin.



Abbreviations used: ABTS⁺: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric reducing antioxidant power assay; L-DOPA: L-3,4-dihydroxyphenylalanine; AMPLE: Aqueous *Mucuna pruriens* leaf extract; DOX: Doxorubicin

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INTRODUCTION

There is a prevalent practice of combining herbal medicine and conventional therapy, especially in cancer patients in different parts of the world.[1,2] These often result in herbal-drug interactions, which are suggested to have both detrimental and beneficial effects for cancer therapy.[3,4] There is evidence that plant-derived antioxidants may potentiate the cytotoxicity of conventional drugs and also mitigate their toxic effects.[5] However, research showing herb-drug interactions of crude plant extracts, which represents the more accessible and realistic patient context are needful.[2-4] This study aims to contribute to the understanding of potential herb-drug interactions using a common medicinal plant.

Mucuna pruriens belongs to the family Fabaceae and is commonly known as cowage plant.[6] It is a tropical legume that mostly grows in the

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parts of Africa and Asia.[7] In traditional Ayurvedic medicine, they were used for treating Parkinson's disease.[8] Conventionally, the seeds are also used as prophylaxis against snakebites in Northern Nigeria.[9] Its seeds contain about 3.1%–6.1% L-DOPA,[7] with trace amounts of serotonin, nicotine, and bufotenine.[10]

Conventionally, the leaves of MP are used for anemia, which is one of the complications of conventional cancer therapy. Indeed, experimental evidence in rats suggests that hydroethanolic extracts of MP leaves may ameliorate anemia.[11] Furthermore, research evidence shows that MP leaves ethanol extracts have been reported to elicit the antioxidant effects in rat-induced liver damage.[6] Taken together, this suggests MP leaves could potentially be employed in adjunct therapy during cancer therapy. However, there are few studies that report potential anticancer activity of MP leaf extracts.[12]

Considering that drug screening in a large panel of cell lines[13] and selective toxicity to cancer cells are crucial parameters for indicating potential anti-cancer drugs,[14] we showed in this study the antioxidant and selective cytotoxic activity of MP aqueous leaf extracts in various cancer cell lines. Furthermore, we showed that the selective cytotoxic effect of MP aqueous extract is enhanced by doxorubicin (Dox), especially in HeLa cells, suggesting a potentially useful herb-drug interaction.

MATERIALS AND METHODS

Materials and chemicals

DMEM AQmedia™, antibiotic antimycotic solution, EDTA, sulforhodamine B, and tris (hydroxymethyl) aminomethane were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Fetal bovine serum (advanced) (FBS) and Dulbecco's phosphate buffered saline were from Capricorn Scientific GmbH (Ebsdorfergrund, Germany) and Trypsin from Serva (Heidelberg, Germany). Dox (Sindroxocin®) was from Actavis d. o. o. (Belgrade, Serbia).

Cell lines

Cell growth activity was evaluated *in vitro* in the following human cancer and non-cancer cell lines: HeLa (cervix epithelioid carcinoma, ECACC No. 93021013), MCF7 (breast adenocarcinoma, estrogen receptor [ER+], ECACC No. 86012803), ECACC No. 86012803), MDA-MB-231 (breast adenocarcinoma, [ER-], HT-29 (colon adenocarcinoma, ECACC No 91072201), and MRC-5 (human fetal lung, ECACC 84101801). Cell lines were grown in DMEM medium with 45 mg/ml glucose, supplemented with 10% heat-inactivated FBS and antibiotic/antimycotic solution (10,000 U/ml of penicillin, 10 mg/ml of streptomycin, and 25 μg/ml of amphotericin B). Cells were cultured in 25 cm^2 flasks (Nunc, Roskilde, Denmark) at 37°C in the atmosphere of 5% CO_2 and 90% humidity and sub-cultured twice a week.

Collection, identification, and extraction of *Mucuna pruriens* leaves

Fresh leaves of MP were collected from Anambra State in Nigeria, West Africa. The samples were identified by Mr. Alfred Ozioko of Bio-resources Development and Energy Conservation Center, Nsukka, Enugu Nigeria and were stored at the International Center for Ethnomedicine and Drug Development, Enugu State, Nigeria. The specimen identification number is InterCEDD-16018.

Furthermore, the leaf samples were air-dried, powdered, and extracted by decoction in distilled water. Using a minimum plant: solvent ratio of 1:10 w/v,[15] 37 ml of distilled water was poured onto 2.5 g of powdered MP leaves. A water bath was preset at 85°C. At this set temperature, the mixture was extracted for 15 min. The extract was then filtered with Whatman filter No. 1 and lyophilized using Christ Alpha 1–2 LD plus −55°C Freeze Dryer −2 kg. Lyophilized extracts were stored at −80°C until further use.

Preparation of extract concentrations

Freeze-dried aqueous MP leaf extract (AMPLE) was redissolved in medium containing 5% FBS to obtain 10 mg/ml stock. The stock solution was diluted serially with cell culture medium containing 5% FBS in sterile conditions to the final concentrations within the range of 1.95–1000 μg/ml.

Similarly, a stock (1 mM) of Dox (Sindroxocin®; DOX) was prepared and diluted to a final concentration range of 0.0058–58 μg/ml (10 nM–0.1 mM) in cell culture medium containing 5% FBS.

Evaluation of cytotoxicity *in vitro*

Cytotoxic activity of the aqueous extract was determined as follows: the cell lines were subcultured into 96-well microplates (Nunc, Roskilde, Denmark) at a seeding density of 4–8 × 10^3 cells/well and preincubated in complete medium supplemented with 5% FBS at 37°C for 24 h. The cells were then treated with the prepared dilutions of extract (in the range of 1.95–1000 μg/mL) and DOX (in the range of 0.0058–58 μg/ml) as standard. Wells containing medium with 5% FBS only served as control wells. The microplates were then incubated at 37°C for 48 h. Afterward, the cell growth was evaluated by the colorimetric sulforhodamine B (SRB) assay of[16] with slight modifications.[17] Absorbance was measured using Multiskan Ascent (Labsystems, Helsinki, Finland) photometer at 540 nm against 620 nm as the background.

To evaluate the effect of combining DOX and test extracts, due to increase cell death of HeLa cells, lesser concentrations of both DOX and aqueous leaf extracts were used for the HeLa cells only. Briefly, MP aqueous leaf extracts were mixed with predetermined sub-half maximal inhibitory concentration (IC_{50}) of DOX 0.2 μM for all cell lines and 0.1 μM for HeLa cells only (these concentrations resulted in ~40% of cell growth inhibitions in the previous experiment). The mixtures were prepared to achieve chosen final mixture concentration, which contained 1.95–1000 μg/ml of aqueous leaf extract for HeLa and 62.5–1000 μg/ml of aqueous leaf extract for other cell lines. The setup included untreated wells as controls. Microplates were then incubated at 37°C for 48 h. The cell growth inhibition was evaluated using absorbance after performing the colorimetric SRB assay.[16]

For all cytotoxicity experiments, the IC_{50} values were calculated as 100× (*AT*/*AC*) (%), where *AT* is the absorbance of the test sample and *AC* of the control. The selectivity of the aqueous leaf extracts for cancer cells was derived by dividing IC_{50} values in non-cancer cell lines (NT) by IC_{50} values in the cancer cell lines (T). High NT/T ratio represents the high selectivity of the tested extracts cytotoxic effect on the cancer cell.

Evaluation of antioxidant activity

2,2-diphenyl-1-picrylhydrazyl radical scavenging assay

Free-radical scavenging effect of the aqueous leaf extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured spectrophotometrically using the method described by Gironés-Vilaplana *et al.*[18] The ability to scavenge DPPH radicals, i.e., SA_{DPPH}, was calculated using the following equation:

$$SA_{DPPH}\ (\%) = ([A_C - A_S]/A_C) \times 100$$

Where, A_C is the absorbance of the control and A_S is the absorbance in the presence of the aqueous leaf extract.

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging assay

Scavenging activity was also evaluated employing the modified 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium

salt ($ABTS^+$) method according to Mena *et al.*[19] by measuring the variation in absorbance at 414 nm after 35 min. The SA_{ABTS} value for the aqueous leaf extracts was calculated using the following equation:

SA_{ABTS} (%) = 100 × (A_0 – A_X)/A_0

where, A_0 and A_X are the absorbance of the blank and the sample, respectively.

Ferric reducing antioxidant power assay

Reducing power was determined by the method adopted from Oyaizu,[20] measuring the reduction of the Fe^{3+}/ferricyanide complexes to ferrous (Fe^{2+}) form. The absorbances were read at 700 nm against the control.

Statistical analysis

The results of cytotoxicity were obtained in two independent experiments, each performed in quadruplicate (n = 8). Antioxidant activity results are represented as means ± standard deviation (n = 3). A comparison of IC_{50} values was performed by the Student's t-test. For antioxidant activity, comparison between concentrations was performed using the one-way ANOVA. Both analyses were performed using OriginPro 8 SRO (OriginLab Corporation, Northampton, USA). Statistical significance was determined at $P < 0.05$ unless stated otherwise.

RESULTS

Cytotoxicity of aqueous *Mucuna pruriens* leaf extract

To determine the cytotoxic effect of AMPLEs, human cancer cell lines were used. Table 1 shows IC_{50} for the AMPLE against four different cell lines. At concentrations below 1000 µg/ml, the tested extract had significant cytotoxicity in HeLa, MCF-7, and HT-29 cells except in MDA-MB-231. Based on the IC_{50} values, the highest cytotoxic effect was observed in HeLa cells [Table 1].

Additive cytotoxic effect of *Mucuna pruriens* leaf extracts and doxorubicin

To evaluate the additive effect, the combined treatment of cells with sub-IC_{50} concentrations of Dox and a range of test leaf extract concentrations were performed. The results show that IC_{50} values for the test leaf extract were significantly reduced in HeLa, MCF-7, and HT-29 cells. This suggests an additive effect of the test extract and Dox on the cancer cell lines [Table 1]. Again, the observed combined cytotoxic effect of Dox and test leaf extract (DOX + AMPLE) was the highest in HeLa cells [Table 1]. In the other cell lines, the reduction was slight but significant.

Selective cytotoxicity of aqueous *Mucuna pruriens* leaf extract

To evaluate selective cytotoxicity, ratios of IC_{50} in normal MRC-5 cells in the selected human cancer cell lines to the IC_{50} of the leaf extract were calculated. A ratio above 1 indicated selective cytotoxicity for cancer cells. The resulting ratio (NT/T) indicates high cytotoxic selectivity of the test extract in HeLa, MCF-7, and HT-29 cells [Table 1]. A similar ratio calculated for Dox showed lesser values. Furthermore, an evaluation of the derived NT/T for combined treatment of test extract and Dox showed an increase in the ratio for HeLa, MCF-7, and HT-29 cells [Table 1]. Specifically, for HeLa cells, NT/T ratio for combined treatment was above 40 fold compared to test extracts alone and 100 fold compared to Dox alone [Table 1]. Taken together, the data suggest enhanced selective cytotoxicity of the test extract when combined with Dox.

Antioxidant activity of aqueous *Mucuna pruriens* leaf extract

Antioxidant activity was measured with free-radical generating assays. The DPPH [Figure 1] and the ferric reducing antioxidant power assay (FRAP) [Figure 2] showed that similar concentrations are required for antioxidant activity. For the $ABTS^+$ assay [Figure 3], lower concentrations showed antioxidant activity. All antioxidant activity observed was concentration dependent.

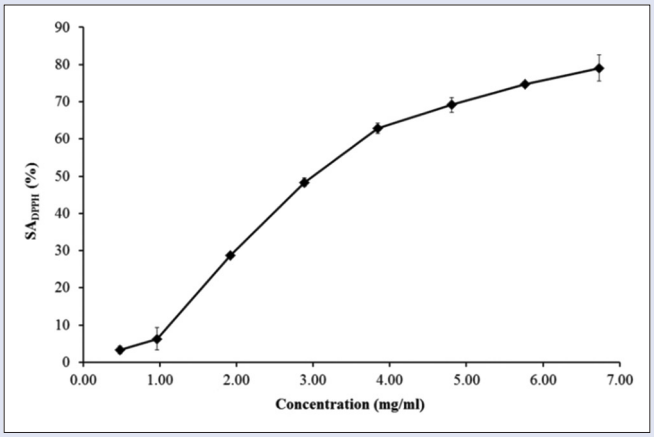

Figure 1: Concentration-dependent inhibition of 2,2-diphenyl-1-picrylhydrazyl. By aqueous *Mucuna pruriens* leaf extract. Data are represented as mean ± standard deviation (n = 3). Percentage inhibition was significantly different when concentrations were compared using the one-way ANOVA

Table 1: The half-maximal inhibitory concentration values for cytotoxic effect and calculated non-cancer cell line/cancer cell line values of doxorubicin, aqueous *Mucuna pruriens* leaf extract and doxorubicin + aqueous *Mucuna pruriens* leaf extract treatments on selected human cancer cell lines

Treatment	Cell line									
	HeLa		MCF-7		MDA-MB-231		HT-29		MRC-5	
	IC_{50}	NT/T	IC_{50}	NT/T	IC_{50}	NT/T	IC_{50}	NT/T	IC_{50}	NT/T
AMPLE	92.80±23.29*	>10.78	605.54±61.77*	>1.65	>1000	1	506.40±34.33*	>1.98	>1000	-
DOX	0.12±0.03	4.42	0.56±0.06	0.95	0.60±0.04	0.88	0.53±0.05	1	0.53±0.03	-
AMPLE + DOX	<1.95**	>512.82	493.43±54.64**	>2.03	>1000	1	495.37±14.41**	>2.02	>1000	-

Data are represented as mean±SD (n=8). *Significant cell growth inhibition as indicated by IC_{50} below 1000 µg/ml using the one-way ANOVA for comparison within individual cell lines; **A significant difference in IC_{50} values of AMPLE versus IC_{50} values of DOX + AMPLE when compared within individual cell lines using Student's t-test. IC_{50}: Half-maximal inhibitory concentration; NT/T: Non-cancer/cancer IC_{50} ratio; DOX: Doxorubicin; AMPLE: Aqueous *Mucuna pruriens* leaf extract; SD: Standard deviation; HeLa: Cervix epithelioid carcinoma cell line; MCF7: Breast adenocarcinoma cell line; MDA-MB-231: Breast adenocarcinoma cell line; HT-29: Colon adenocarcinoma cell line; MRC-5: Human fetal lung cell line

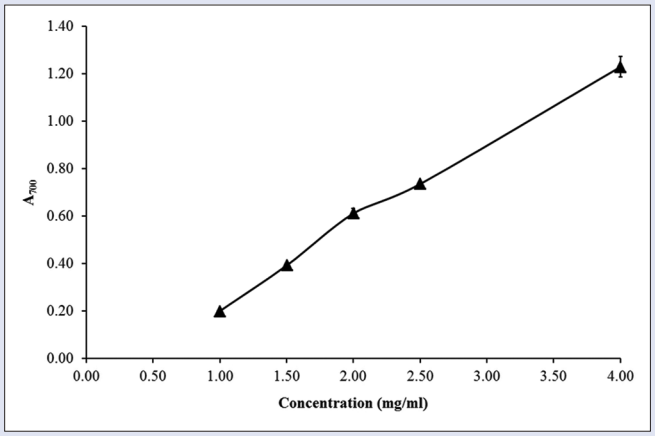

Figure 2: Concentration-dependent reduction of ferric iron in ferric reducing antioxidant power assay by aqueous *Mucuna pruriens* leaf extract. Data are represented as mean ± standard deviation (n = 3). Percentage inhibition was significantly different when concentrations were compared using the one-way ANOVA

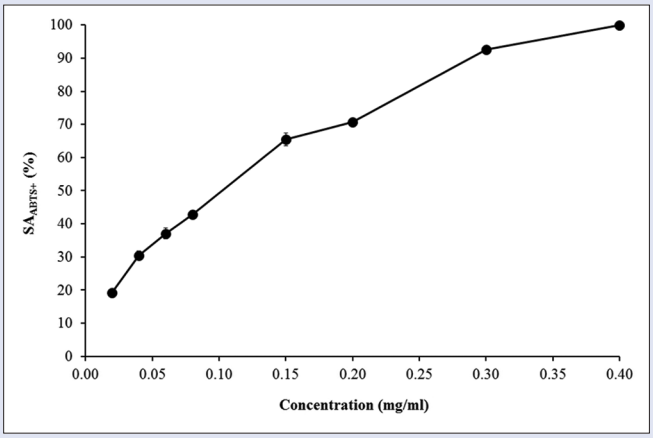

Figure 3: Concentration-dependent inhibition of $ABTS^+$ by aqueous *Mucuna pruriens* leaf extract. Data are represented as mean ± standard deviation (n = 3). Percentage inhibition was significantly different when concentrations were compared using the one-way ANOVA

DISCUSSION

The data from this study show that AMPLE possesses selective cytotoxic activity against HeLa, MCF-7, and HT-29 cells [Table 1]. Considering that conventional cancer drugs such as Dox are known to have a wide range of side effects and are prone to resistance, research into phytochemical/drug combinatory therapy for cancer is relevant. For example, curcumin, a polyphenol obtained from *Curcuma longa,* has been reported to show enhanced cytotoxic effect in prostate cancer cells *in vitro*, when combined with subclinical doses of Dox.[21] Furthermore, with plant and herbal extracts/paclitaxel combination therapy in cancer cells, a reduction in the effective doses of paclitaxel was observed.[22]

A similar effect was observed with AMPLE and Dox in this study. From the data, a combination of sub-IC_{50} values of Dox with AMPLE caused reduced IC_{50} of MP leaf extract, suggesting enhanced toxicity [Table 1]. Although the enhanced toxicity of the test extract by Dox was varied for the different cell lines. Specifically, in HT-29 and MCF-7 cell lines, the effect was slight but significant, while in HeLa cells, the effect was synergistic. However, in MDA-MD-231 cells, no difference was observed. The difference in sensitivity for the breast cancer cell lines MCF-7 and MDA-MD-231 could be due to the presence of ERs in MCF-7 cell lines.[23] The latter has been shown to be sensitive to the cytotoxic effect of polyphenols.[24]

MP leaf extract has previously been reported to contain polyphenols.[6] Phytochemical analysis of an aqueous leaf extract of MP reported the presence of flavonoids, in addition to other phytochemicals such as saponins and tannins.[6] These compounds were associated with antioxidant activity of the extract.[6] In line with the authors, the data from this study suggest aqueous leaf extract of MP possess good antioxidant activity.

From the data, good antioxidant activity was MP aqueous leaf extract in FRAP and DPPH assays [Figures 1 and 2] and the $ABTS^+$ [Figure 3] assay. In comparison to a previous study by Agbafor and Nwachukwu,[6] similar concentrations of MP aqueous leaf extract elicited higher percentages of DPPH inhibition in this study. This suggests that the extract used in this study possess better antioxidant activity. The difference may be due to different test conditions or extraction procedures. Therefore, further study is required to ascertain what extraction procedure maximizes the antioxidant activity of the leaf extracts.

Furthermore, the FRAP and DPPH assays used in this study have been correlated with the phenolic content of plant extracts.[25,26] Besides, polyphenols are widely distributed in plants and are associated with cytotoxic and antioxidant activity of medicinal plants. Therefore, further research is required to determine the phytochemisty and the mechanism of action of MP aqueous leaf extract.

CONCLUSION

The results of this study suggest that MP aqueous leaf extract exhibits good antioxidant activity. Furthermore, the results suggest that MP aqueous leaf extract possesses cytotoxic effect against a range of human cancer cell lines, especially HeLa cells. This observed cytotoxicity is enhanced when combined with Dox. Therefore, the data from this study suggest that MP aqueous leaf extract may be a useful source of antioxidants and phytochemicals, which may be useful for more effective strategies in cancer therapy.

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Conflicts of interest

There are no conflicts of interest.

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