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In vitro antiproliferative potential of *Annona senegalensis* Pers. and *Allophylus africanus* P Beauv. plant extracts against selected cancer cell lines

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The medicinal plants *Annona senegalensis* Pers. and *Allophylus africanus* P Beauv. are used in Tanzania traditional medicine for the treatment of cancer. However, there is no scientific documentation on their therapeutic effectiveness. The present study evaluated antiproliferative potential as an indicator of anticancer activity of *A. senegalensis* and *A. africanus* plant species from Tanzania. *A. senegalensis* and *A. africanus* were collected from Ugweno village at Kilimanjaro, Tanzania. Different types of extracts were prepared in dichloromethane/methanol (DCM:MeOH), petroleum ether, DCM, ethyl acetate (EtOAc), MeOH and water respectively. Antiproliferative activity against HCC 1396 (breast), HEp-2 (throat) and CT 26 (colon) cancer cell lines was assessed by the MTT cell viability assay. The results of the present study showed that the antiproliferative activity varied between plant extracts and the cancer cell lines. The highest antiproliferative activity was achieved with petroleum ether extract of *A. senegalensis* against HEp-2 with an IC_{50} value of 0.42 ± 0.09 µg/ml. This also depicted the highest selectivity to cancerous cells (SI value 94.19) compared to the other extracts. *A. africanus* also depicted good antiproliferative activity against HEp-2 with IC_{50} values of 1.00 ± 0.41 and 2.37 ± 1.45 µg/ml for DCM:MeOH and petroleum ether extracts, respectively. The findings validate the traditional use of *A. senegalensis* and *A. africanus* in the treatment of cancer. Results also support previous studies which demonstrated the effect of extraction solvent used in extraction of bioactive agents from medicinal plants. Further studies involving the isolation of pure antiproliferative compounds against cancer cells from the two plants are recommended to elucidate bioactive molecules.

Key words: *Annona senegalensis*, *Allophylus africanus*, antiproliferative, cancer cells, plant extracts.

INTRODUCTION

Cancer is a leading cause of death worldwide Max Parkin et al., 2005). Globally, the number of people dying of cancer is expected to increase from 8.2 million in 2012 to 14.6 million by 2035 (WHO, 2014; Siegel et al., 2018). Cancer is a threat to the economy as a lot of money has been invested in cancer treatment but survival rate remains unchanged (Kaur et al., 2009). Cancer is associated with several factors such as environmental,

lifestyle, social, cultural, hormonal and gender factors (Aslam et al., 2014). Among these factors, tobacco use, lack of physical exercise, unhealthy diet, alcohol consumption, automobile exhaust pollutant, Solar UV radiation and bacterial or viral infections are included (Prakash et al., 2013).

Cancer treatments approach involve chemotherapy, surgery, radiation therapy, immunotherapy, targeted therapy, and hormonal therapy, of which have side effects (Baskar et al., 2012; WHO, 2014). Of all treatments, chemotherapy is the most effective but due to high dose requirements, it kills normal cells, hence causing side effects such as fatigue, nausea, hair loss, vomiting, loss of appetite, constipation, anaemia and diarrhoea (Conklin, 2004; Prakash et al., 2013). Herbal medicines have been used as alternative sources of treatment since the early ages (Kooti et al., 2017). It has been used by our ancestors to treat various diseases (Elansary et al., 2018). According to the World Health Organization (WHO), almost 75% of the world's population use herbal medicines (Yasser, 2016; Napagoda et al., 2018). Plants produce secondary metabolites which have been reported to possess therapeutic effects which are non-toxic to normal cells, hence not harmful to the body (Greenwell and Rahman, 2015; Priya et al., 2015). The metabolites are tannins, carbohydrates, alkaloids, saponins, terpenoids, phenolic compounds, steroids, glycosides and flavonoids (Khalid et al., 2018). Study of Al-asady et al. (2014) indicated that the glycoside Fraction I from *Convolvulus arvensis* had more cytotoxic inhibition at 10 mg/ml against rhabdomyosarcoma (RD) tumour cell line *in vitro* after 72 h, compared with other extracts (aqueous and methanol) crude extracts of the leaves, stems and roots. Glycoside FI had cytotoxicity concentration 50% (CC 50%) 1.775, 0.870 and 0.706 mg/ml after 24, 48, and 72 h, respectively.

In Tanzania, medicinal plants play an important role in providing primary health care to rural and urban communities (Kisangau et al., 2007). Traditional health practices also provide a source of income to traditional healers within the country (Kitula, 2007). However, there is lack of scientific documentation on the therapeutic effect of most of the medicinal plants. In an attempt to fill the gap, we selected *Annona senegalensis* and *Allophylus africanus* to validate traditional use for cancer treatment. *A. senegalensis* popularly known as the African custard apple or wild custard apple is a shrub or small tree of about 2-7 m or more belonging to the family Annonaceae (Okoli et al., 2010; Mustapha, 2013). It is native and widely distributed in Africa (Okoye et al., 2012). Ethnomedically, it has been reported to exhibit antimicrobial, antioxidant, antiparasitic, anti-inflammatory, anticonvulsant, antimalarial, trypanocidal, anti-snake venom, anti-nociceptive and anthelmintic activities (Ajaiyeoba et al., 2006; Awa et al., 2012; Mustapha, 2013). It has been reported to be effective against cervical, skin and pancreatic cancers (Graham et al., 2000; Okoye et al., 2014). *A. africanus* belongs to the family Sapindaceae (Balogun et al., 2016). It is widely spread in tropical and subtropical regions of the America, Africa, Asia and Indian archipelago (Chavan and Gaikwad, 2016). Scientifically, it is reported to have strong antimalarial, antibacterial and antioxidant activities (Sofidiya et al., 2012; Balogun et al., 2016). One of the species from the same genus, *Allophylus cobbe*, was confirmed to have anticancer activity against human prostate cancer cell lines (Ghagane et al., 2017).

This study evaluate the *in vitro* antiproliferative potential of *A. senegalensis* and *A. africanus* that are used in Tanzania traditional medicine as anticancer remedies by using three cancer cell lines namely; HCC 1396 (breast), HEp- 2 (throat) and CT 26 (colon).

MATERIALS AND METHODS

Plants collection

The fresh stem bark of each plant was sustainably collected from Ugweno village in Kilimanjaro region of Tanzania during the dry season in the month of December, 2017. Traditional healers guided plants collection which were then identified by a taxonomist at the Tropical Pesticides Research Institute (TPRI) Herbarium located in Tanzania. Voucher specimens assigned numbers EB.01 and EB.02 for *A. senegalensis* and *A. africanus* respectively were then deposited at the herbarium

Plant processing and extraction

Plant materials (stem bark) were chopped into small pieces, air dried and ground to a fine powder using an electric blender then stored at room temperature until used. Extraction was done using six solvents for each plant making a total of 12 extracts. The solvent used was dichloromethane/methanol (DCM:MeOH) at a ratio of 1:1 (Fouche et al., 2008). Briefly, 500 g of each plant powder was soaked completely into a mixture of 1 L of DCM and 1 L of MeOH for 72 h. The extract solutions were filtered and concentrated using a rotary evaporator. Extraction was also done sequentially with petroleum ether, DCM, ethyl acetate (EtOAc) and MeOH starting from least polar to most polar solvent respectively. For sequential extraction, 500 g of each plant powder was soaked in 1 L of petroleum ether, and then the filtrate re-soaked in the rest of solvents sequentially. All solvents were filtered after every 48 h and extracts concentrated by rotary evaporator (Bandar et al., 2013). The remaining powder material was further extracted in aqueous medium by soaking 500 g of fine powder of each plant material in 1 L of water at 60°C for 1 h. The filtrate was then freeze-dried to free

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powder (Rukunga et al., 2009).

Phytochemical analysis

The 12 plant extracts were screened for secondary metabolites using standard methods (Sowmya and Lakshmidevi, 2013; Ajuru et al., 2017). Secondary metabolites tested were alkaloids, tannins, glycosides, flavonoids, saponins, and terpenoids.

***In vitro* antiproliferative screening**

Cell lines culturing

CT 26: Colon carcinoma (colon cancer), HEp- 2: Human larynx carcinoma (throat cancer), and HCC 1396: Human breast carcinoma (breast cancer) were used as the cancer cell lines. VERO P23 (African green monkey kidney) was used as the normal cells for reference purpose. The cell lines were originally obtained from the American Type Culture Collection (ATCC) and sub-cultured at the Center for Traditional Medicine and Drug Research (CTMDR), Kenya Medical Research Institute (KEMRI). The cell lines were cultured in Dulbecco Modified Essential Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine and 100 µg/ml of streptomycin at 37°C in a 5% CO_2 and 95% humidity.

Antiproliferative assay

Upon attainment of confluence, cells were washed with saline phosphate buffer and harvested by trypsinization. The number of viable cells was determined using the trypan blue exclusion method (cell density count) using a hemocytometer. They were then seeded in 96 well plates at a concentration of 2 x 10^5 cell/ml in 100 µl per well and incubated for 24 h at 37°C in a 5% CO_2 and 95% humidity to let cells adhere onto to the surface of the wells. Three columns of each plate were left without cells. These were filled with 100 µl per well of media only to serve as a blank. Each extract was then added onto row H of the plate at a concentration of 100 µg/ml. This was followed by three folds serial dilution to get different concentrations from 100, 33.33, 11.11, 4.0, 1.33, 0.44 and 0.146 µg/ml from row H to B respectively. Row A was left as a negative control. Doxorubicin, a standard drug for cancer treatment was used as positive control (Wang et al., 2004). All concentrations were replicated three times for each plant extract and then incubated for 48 h at 37°C in a 5% CO_2 and 95% humidity. The extracts were also added to the columns filled with media only so as to evaluate the effect of extract concentrations without the cells and to allow evaluating whether extracts alone would cause MTT quenching. After 48 h incubation, 10 µl of MTT dye (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) was added to each well and incubated for 2 h to allow interaction with the living cells to form formazan (Twentyman and Luscombe, 1987). The blue insoluble formazan product which is directly proportional to the number of living cells present during MTT exposure was then dissolved by 50 µl DMSO. Absorbance was then read at a wavelength of 540 nm and a reference wavelength of 720 nm using ELISA Reader (MULTSKAN GO Thermo scientific, USA). The effect of the plant extracts on the cells was expressed as IC_{50} values, (drug concentration inhibiting cell growth by 50% compared to untreated cells). Antiproliferative activity was classified according to the standards of the National Cancer Institute (NCI) as follows; high antiproliferative when an IC_{50} <20 µg/ml, antiproliferative for an IC_{50} between 20 µg/ml to 30 µg/ml, moderately for IC_{50} between 30 µg/ml to 100 µg/ml and inactive with IC_{50} >100 µg/ml (Boik, 2001). The percentage growth inhibition was calculated using the following formula below (Bézivin et al., 2003).

$$\text{Percentage inhibition} = 100 - (At - Ab) / (Ac - Ab) \times 100$$

Where;At=Absorbance value of test compound (cells plus extracts)
Ab=Absorbance value of blank (media only)
Ac=Absorbance value of negative control (cells plus media)

Statistical analysis

Data were analyzed using one way analysis of variance (ANOVA, MiniTab Version 18) to determine differences ($p \leq 0.05$) among plant extracts IC_{50}. Multiple comparisons of IC_{50} were done by Tukey test. Experimental results are expressed as mean ± SEM and all measurements were in triplicate. Selectivity index (SI) was determined by the equation below;

$SI = CC_{50}$ (VERO cells)/IC_{50} (cancer cell)

RESULTS AND DISCUSSION

The use of herbal medicines in the treatment of various diseases has received increasing attention due to their varied phytochemical contents with multiple biological activities. *A. senegalensis* and *A. africanus* were collected from Ugweno village of Kilimanjaro region in Tanzania and evaluated for antiproliferative activity. A total of 12 extracts were made by using six solvents for each plant species. The percentage yield of each extract is shown in Table 1. Extraction yields varied from 1 to 6.8% depending on the type of solvent used for extraction. Highest yields were obtained with aqueous extractions. This may be due to the high solubility of different plant compounds in this solvent (Senguttuvan et al., 2014). The stem bark of *A. senegalensis* and *A. africanus* was screened for secondary metabolites and results presented in Tables 2 and 3, respectively. Presence of flavonoids was observed in all extracts. Alkaloids, saponins, glycosides and tannins were absent in petroleum ether extracts of both plants. All tested metabolites were detected in ethyl acetate extract of *A. senegalensis*. The presence or absence of phytochemicals in extracts used can be explained by the different polarities of compounds which were selectively more soluble in different solvents (Ngo et al., 2017; Thouri et al., 2017; Snehlata et al., 2018).

The concentration of plant extracts that inhibited cell growth by 50% (IC_{50}) for the twelve plant extracts was calculated and the results displayed in Table 4. Tabulated results show that antiproliferative activity varied between plant extracts and the cancer cell lines. This variation may be due to the type of bioactive compounds present in the different extraction solvents used (Gberikon et al., 2015). The highest antiproliferative activity was achieved with petroleum ether extract of *A. senegalensis* against HEp-2 with an IC_{50} value of 0.42 ± 0.09 µg/ml. This demonstrated the efficiency of petroleum ether over the other extraction solvents for extracting antiproliferative

Table 1. Extraction yield (%) of *A. senegalensis* and *A. africanus.*

Plant sample (stem bark)	Sample weight (g)	Extracted weight (g)	Yield (%*W/W*)
AS Pet ether	500	5	1.0
AA Pet ether	500	6	1.2
AS DCM:MeOH	500	25	5.0
AA DCM:MeOH	500	10	2.0
AS DCM	500	6	1.2
AA DCM	500	8	1.6
AS Ethyl acetate	500	9	1.8
AA Ethyl acetate	500	11	2.2
AS MeOH	500	15	3.0
AA MeOH	500	26	5.2
AS Aqueous	500	34	6.8
AA Aqueous	500	32	6.4

AS: *Annona senegalensis*, AA: *Allophylus africanus*

Table 2. Phytochemical analysis of different solvent extracts of *A. senegalensis.*

Solvent	Alkaloids	Saponins	Flavonoids	Glycosides	Terpenoids	Tannins
Pet ether	-	-	+	-	+	-
DCM:MeOH	+	+	+	+	+	+
DCM	+	-	+	-	+	+
Ethyl acetate	+	+	+	+	+	+
MeOH	+	+	+	+	-	+
Aqueous	+	+	+	+	-	+

Present phytochemicals denoted by (+) sign; absent phytochemicals denoted by (-) sign.

Table 3. Phytochemical analysis of different solvent extracts of *A. africanus.*

Solvent	Alkaloids	Saponins	Flavonoids	Glycosides	Terpenoids	Tannins
Pet ether	-	-	+	-	+	-
DCM:MeOH	+	-	+	+	+	+
DCM	-	-	+	-	+	-
Ethyl acetate	+	+	+	+	-	+
MeOH	+	+	+	+	-	+
Aqueous	+	+	+	+	-	-

Presence of phytochemicals denoted by (+) sign; absence of phytochemicals denoted by (-) sign.

compounds against HEp-2 from *A. senegalensis* stem bark. Among all plant extracts, the following exhibited high activity; DCM extract of *A. senegalensis*: IC_{50} 10.41 ± 2.07 µg/ml and MeOH extract of *A. africanus*: IC_{50} 7.33 ± 0.43 µg/ml against HCC 1396; petroleum ether extract of *A. senegalensis*: IC_{50} 0.42 ± 0.09 µg/ml and DCM:MeOH extract of *A. africanus*: IC_{50} 1.00 ± 0.4 µg/ml against HEp-2 cancer cells; petroleum ether extract of *A. senegalensis*: IC_{50} 9.19 ± 0.81 µg/ml and MeOH extracts of *A. africanus*: IC_{50} 9.04 ± 1.05 µg/ml against CT 26 cancer cells. Water is the common solvent used by traditional healers for extraction of medicinal plants due to its availability (Mekonnen and Abebe, 2017). In our study, the aqueous extracts exhibited antiproliferative activity ranging from moderate to none. This signified the inefficiency of an aqueous medium as an extraction solvent for antiproliferative compounds from these plant species. Four extracts were observed to be inactive (IC_{50}>100 µg/ml) while the rest were moderately active with IC_{50} ranging between 30 and 100 µg/ml.

Table 4. Mean IC_{50} of the plant extracts on HCC 1396, HEp-2, CT 26 and mean CC_{50} on VERO cell line.

Plant	Solvent	IC_{50}(µg/ml) HCC 1396	IC_{50} (µg/ml) HEp- 2	IC_{50} (µg/ml) CT 26	CC_{50}(µg/ml) VERO
A. senegalensis	Pet Ether	21.88±2.18^{cd}	0.42±0.09^{a}	11.59±2.58^{b}	39.56±1.73^{b}
A. africanus	Pet Ether	41.10±1.42^{e}	2.37±1.45^{a}	9.19±0.81^{b}	62.70±2.04^{bc}
A. senegalensis	DCM:MeOH	27.41±2.28^{d}	4.50±0.72^{a}	54.02±4.13^{f}	>100
A. africanus	DCM:MeOH	12.61±1.67^{bc}	1.00±0.41^{a}	21.52±0.06^{c}	78.20±1.47^{cd}
A. senegalensis	DCM	10.41±2.07^{b}	12.36±3.20^{b}	12.19±2.70^{b}	52.21±1.95^{b}
A. africanus	DCM	8.76±0.43^{b}	5.02±0.71^{a}	19.04±0.78^{c}	57.73±1.05^{b}
A. senegalensis	Ethyl acetate	17.19±0.19^{c}	12.00±1.11^{b}	26.08±0.04^{d}	93.33±0.67^{d}
A. africanus	Ethyl acetate	18.60±0.28^{c}	9.48±0.42^{b}	27.61±4.57^{d}	68.33±3.79^{c}
A. senegalensis	MeOH	47.98±4.52^{f}	97.12±2.88^{f}	36.52±3.23^{e}	>100
A. africanus	MeOH	7.33±0.43^{b}	25.38±2.57^{c}	9.04±1.05^{b}	55.72±1.00^{b}
A. senegalensis	Aqueous	76.31±1.22^{g}	76.20±2.38^{e}	65.03±0.04^{g}	>100
A. africanus	Aqueous	28.58±0.71^{d}	65.10±3.49^{d}	>100	>100
Doxorubicin	-	1.14±0.01^{a}	0.21±0.04^{a}	2.94±0.05^{a}	10.94±0.06^{a}
$LSD_{(0.05)}$	-	6.07	3.73	4.95	25.43

Values are expressed as Mean±SEM. Doxorubicin was used as a positive control. The IC_{50} values of the plant extracts were compared with the doxorubicin for each cell line. Values that do not share a letter are significantly different ($p \leq 0.05$). LSD is least significance difference between two mean.

Table 5. Selectivity index of *A. senegalensis* and *A. africanus* plant extracts.

Plant	Solvent	HCC 1396	HEp-2	CT 26
A. senegalensis	Pet Ether	1.81	94.19	3.41
A. africanus	Pet Ether	1.53	26.46	6.82
A. senegalensis	DCM:MeOH	3.65	22.22	1.85
A. africanus	DCM:MeOH	6.2	78.2	3.63
A. senegalensis	DCM	5.02	4.22	6.42
A. africanus	DCM	6.6	9.92	2.74
A. senegalensis	Ethyl acetate	5.43	7.78	3.58
A. africanus	Ethyl acetate	3.67	7.21	2.47
A. senegalensis	MeOH	2.08	1.03	2.74
A. africanus	MeOH	7.6	2.2	6.16
A. senegalensis	Aqueous	1.31	1.31	1.54
A. africanus	Aqueous	3.49	1.54	N/A*
Doxorubicin	-	9.6	52.1	3.8

N/A*: Not applicable because the test extract did not inhibit growth of the cells.

The previous study by Okoye et al. (2014) showed root bark of *A. senegalensis* has anticancer activity against pancreatic and cervical cancer cells. This study, therefore, revealed that the stem bark of the same plant species has antiproliferative activity against colon, breast and throat cancer cells. Likewise, the study support a previous study conducted by Sofidiya et al. (2012) which showed that *A. africanus* had the best antioxidant activity which could be related to anticancer activity.

All the plant extracts were not cytotoxic to VERO (normal) cells (CC_{50} >39 µg/ml). The plant extracts were found to have higher CC_{50} compared to the positive control drug, doxorubicin whose CC_{50} was 10.94 µg/ml. There was a variation of selectivity among plant extracts and cancer cell lines tested as indicated in Table 5. SI value >3 were considered selective for cancer cell line while SI values <3 were considered non-selective to specific cancer cell line (Bézivin et al., 2003). Some extracts showed selectivity against one cancer cell line but not against the others. However, at least one extract for each plant species showed selectivity to all cancer cell lines. These are DCM:MeOH for *A. africanus* and DCM for *A. senegalensis.* Generally, aqueous extract of *A. senegalensis* was found not to be selective (SI<3) to any

cancer cell line. This could be due to the presence of large quantities of polar based compounds that dilutes concentration of the active compounds (Bézivin et al., 2003). Petroleum ether extract of *A. senegalensis* depicted the highest selectivity on HEp-2 cancer cell lines with SI value of 94.19.

Doxorubicin, a standard drug for cancer treatment was used as positive control. The results showed that doxorubicin was more potent than all the plant extracts with an IC_{50} value of 1.14 ± 0.01 µg/ml for HCC 1396, 0.21± 0.04 µg/ml for HEp-2, and 2.94 ± 0.05 µg/ml for CT 26. This was expected as the drug is purified as opposed to the extracts which were in crude form. This result agrees with that obtained by Al-asady et al. (2014) who found that the glycoside Fraction I from Convolvulus arvensis had more cytotoxic inhibition at 10 mg/ml against rhabdomyosarcoma (RD) tumour cell line *in vitro* after 72 h, compared with other extracts (aqueous and methanol) crude extracts of the leaves, stems and roots . Glycoside FI had cytotoxicity concentration 50% (CC 50%) 1.775, 0.870 and 0.706 mg/ml after 24, 48, and 72 h, respectively. The CC_{50} of doxorubicin on VERO cells was also low (10.94 ± 0.06 µg/ml), which gave more evidences that doxorubicin provides side effect against normal tissue (Wang et al., 2004). Of particular interest, petroleum ether extract of *A. senegalensis* depicted high activity against HEp-2 at an IC_{50} value of 0.42 ± 0.09 µg/ml comparing well to the reference standard doxorubicin. Selectivity index for the same was also high (SI = 94.19). This implied its high potential for the development of a safe anticancer agent. The potency of plant extracts varied with plant species and the screened cancer cell lines. High potency (IC_{50} <20 µg/ml) coupled with high selectivity (SI>3) was observed on extracts of *A. senegalensis* extracted using DCM against HCC 1396, petroleum ether on HEp-2 and CT 26. For *A. africanus,* this was observed on DCM:MeOH against HEp-2, MeOH against HCC 1396 and CT 26 as depicted in Tables 4 and 5. This indicated that the aforementioned are suitable extraction solvents for antiproliferative compounds from these plants respectively. Extracts from both polar and non-polar solvents showed varied levels of activity (Bandar et al., 2013). This signified the possibility of *A. senegalensis* and *A. africanus* to possess both polar and non-polar compounds with antiproliferative activity as indicated in Tables 4 and 5. Regarding the variation on the performance of plant extracts shown by solvent used for extraction, the results supported study conducted by Koffi et al. (2010) and Dhawan and Gupta (2016), which showed that the solvent type used in extraction has an effect to the potency of medicinal plants.

Conclusion

This study indicated that, *A. senegalensis* Pers. and *A. africanus* P Beauv. have potential antiproliferative activity on throat, breast and colon cancer cells. The different solvent used for extraction showed varied activity and selectivity against the cancer cells. Petroleum ether extract of *A. senegalensis* was in particular found to have a high potential for the development of an anticancer agent against throat cancer. These findings validate the traditional use of *A. senegalensis* and *A. africanus* in the treatment of cancer. Current findings also support previous studies which indicated the effect of extraction solvents used on the extraction of bioactive molecules from medicinal plants. Further studies involving the isolation of pure antiproliferative compounds against cancer cells from the two plants are recommended to elucidate bioactive molecules.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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CONSENT FOR PUBLICATION

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