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In vitro effects of extract of *Senna alata* (Ceasalpiniaceae) on the polyamines produced by Leukaemia cells (L1210)

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ABSTRACT
The present study reports the effects of *S. alata* (Ceasalpiniaceae) extract on the metabolism of polyamines resulting from the proliferation of leukaemia cells (L1210). The results established that the inhibition of cell proliferation was significantly increased with the concentration of extract from 28 to 32.80 % after 72 h. The percentage of cells viability changed significantly from 9.72 to 80 % when cells are treated with extract alone, in combination with DFMO or putrescine. The levels of the intracellular yield of putrescine, spermidine and spermine were also reduced by the extract compared to the control. The DFMO-extract complex enhanced the inhibition of the production polyamines up to 95 %. In opposite, the complex *S. alata*- putrescine complex stimulated significantly its biosynthesis of polyamines. A significant reduction of the level of protein after 72 h of treatment was observed. This result corroborated with the reduction of polyamines resulting from inhibition cell proliferation.
KEY WORDS: *polyamines, Inhibition, leukaemia, Senna alata*

INTRODUCTION

Natural polyamines; putrescine (NH_2-$(CH_2)_4$-NH_2), spermidine (NH_2-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2), spermine (NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2) are compounds formed in all living cells through specific biosynthetic pathways. Polyamines can be found in the free form, not complexed to high molecular weight compounds, principally spermidine and spermine which are transported by red blood cells (1). They can also be found in body fluids such as plasma, serum, call foetal serum (CFS) and urine (2). Polyamines are required for optimal growth in all known types of biological cells and they are essential for fundamental cellular processes such as growth, differentiation, transformation and apoptosis (3, 4). Recent studies indicated the molecular linkages between polyamines and apoptosis (5, 4). The range of cell polyamines is determined at the lower limit by their absolute requirement for cell proliferation, and at their upper limit by their toxicity (3). Due to their essential role of polyamines in important cellular functions and multiple pathways such as biosynthesis, catabolism, uptake, and excretion greatly regulate their intracellular concentration. It is widely accepted that under most circumstances the major sources for cell polyamines come from their synthesis from amino acid precursors (3,4). In this pathway ornithine is decarboxylated to form putrescine by the action of ornithine decarboxylase. An aminopropyl group generated by the action of S-adenosylmethionine decarboxylase on S-adenosylmethionine is then

attached to putrescine and spermidine to form spermidine and spermine respectively. Both enzymes are highly regulated and are subjected to feedback control by cellular polyamines (6). While these two highly regulated enzymes constitute the control points of biosynthesis, the catabolic pathway is controlled predominantly by the action of spermidine/spermine N1-acetyltransferase (SSAT) (3). The biological importance of polyamine metabolism stems mainly from the observation that this seems to be closely associated with cell proliferation and polyamines and some of their derivatives, also various drugs which affect polyamine metabolism can influence cell division and sometimes also cell differentiation (6). From a clinical view point, the metabolism of polyamines has an obvious diagnostic especially in cancerology (7, 8). L-α-Difluoromethylornithine (DFMO) is an enzyme-activated,irreversible inhibitor of ODC and causes a depletion in the intracellular concentrations of putrescine and its derivative, spermidine (9, 10)

The treatment of cells with α-2-difluoromethylornithine (DFMO) and related ornithine decarboxylase (ODC) inhibitors causes the depletion of putrescine and spermidine concentrations in cells and tissues, and the arrest of cell growth in culture as well as reduction of tumour growth (11, 12). Many polyamine studies now focus on reduction of total body polyamines for cancer prevention, or reduction of total body organ polyamines for organ cancer prevention (4). However a combination of drugs to deplete cancer cells of polyamines and additional drugs to interfere with the "downstream" polyamine regulatory events will allow for more successful cancer therapy. This is why drugs interfering with polyamine biosynthesis possess considerable potential as therapeutic agents (3). *Senna alata* (L.) Roxb. is one a plant from Caesalpipiaceae family. It is found in different areas of Africa. The plant is used in Cameroon for the treatment of constipation, gastroenteritis, jaundice, intestinal helminthiasis, Eczemia, thyphoenteritis, ringwoun and food poison (13, 14). Its leaf is also credited for the treatment of haemorrhoids, constipation, inguinal hernia, intestinal parasitosis, blennorrhagia, skin infections, syphilis and diabetes in Nigeria (15, 16). Many healers in different part of Africa have been using the leaves of *S. alata* for a long time for the treatment of tinea versicolor and ringworm infections and other diseases (17, 18). The EtOH extract of *S. alata* have also been reported to inhibit the growth of *D.congolensis* while its aqueous-MeOH extract have shown higher antifungal activity on (*Microsporum canis, Blastomyces dermatitidis, Trichophyton mentagrophyte,, Candida albicans, Aspergillus flavus)* and weak inhibition property on bacteria (*Dermatophilus congolensis, Proteus vulgaris, Staphylococcus aureus, Corynebacterium parvum, Actinomycesbovis, Nocardia asteroids,Clostridium septicum, Bacillus pumilus* (19, 20). Some studies also reported weak antifungal activity of aqueous-EtoH, HCl, EtOH, lyophilized and aqueous extracts of leaves of *S. alata* on dermatophytes and *C. albicans*. The aqueous-EtOH extract of *S. alata* have been shown to exhibit non toxic effect when administered to rats up to 16 g kg^{-1} of body (21**)**. Recently our study demonstrated the potential antitumor activity of aqeous-EtOH extract of the leaves *S. alata* using *Agrobacterium tumefaciens* (*A. tumefaciens*) in the potato disc tumor bioassay method (22). The method based on the evaluation of an antimitotic activity can help for the detection of a broad range of known and novel antitumor effects (23). Since the tumors produced by *A. tumefaciens* are histologically similar to those found in humans and animals (24, 25), *S. alata* extract can inhibit the proliferation of human cancer cells. Hence, the objective of the present study was to evaluate the *in vitro* effects of extract of S. *alata* on the proliferation of leukaemia L1210 cells.

MATERIAL AND METHODS

Plant materials

Fresh leaves of *S. alata* were collected near the Eloundem mountain in Yaounde, the capital city of Cameroon. The sample was identified at the National Herbarium and voucher specimens were deposited there with the number 1871/YA.

Extract preparation

The leaves of plant were dried at room temperature (30 ± 3° C) and pulverized. The powder obtained (250g) was macerated in a mixture (4v:1v) of ethanol/water for 72 h. The extract solution was filtered using Whatman filter paper N° 1 and concentrated in an air circulating oven at 54°C until total dryness. The extraction was repeated twice and 30 g of *S. alata* extract obtained and stored at 5°C. A range of concentrations were prepared (40 µg/ml-80 µg/ml) for the evaluation of biological activities.

Cell culture

Leukaemia cells (L1210) were obtained from the Cell Biology Laboratory of the *Centre Hospitalier Universitaire* of Rennes (France). The cells were cultured and maintained in RPMI 1640 medium supplemented with 2 mM of L-glutamine, Penicillin

(100 U/ml), Streptomycin (50 µg/ml) and 10% heat-inactivated foetal calf serum (supplemented medium) at 37°C under a 5% CO_2 atmosphere. After growth, 10^6 cells/ml were inoculated in the presence of plant extract alone or combined with 20 µl of DFMO (165 mM) or 10 µl of putrescine (1 M). The mixture was evaluated for cell viability under the light microscope after 72h.

Evaluation of biological activities

The cytotoxicity of *S. alata* extract was assessed according to the method described (26, 27). The cells viability was measured by evaluating the reduction of thiazonium salts catalysed by mitochondrial enzymes of viable cells (27). The percentage of the viability of cells was then calculated assuming that this value is 100 % for the control. The intracellular polyamines levels were determined using High Performance Liquid Chromatography (28, 6) and the protein concentration (29).

Phytochemical analysis

The qualitative phytochemical analysis was carried out to check up the bioactive components present in the extract such as polyphenols, flavonoids, tannins saponins and alkaloids (30, 31, 32).

Statistical analysis

The values were expressed as mean ± standard deviation (SD). Each value is a mean of a three replications test. One-way analysis of variance (ANOVA) was used to determine the significant differences between parameters and the Student-Newman Keuls test was used to locate these differences. p <0.05 was considered as the level of statistical significant.

RESULTS AND DISCUSSION

The results indicated that after 72 h of treatment of the leukaemia cells (L1210) with the extract, the inhibition of the proliferation of cells increased from 28 to 38 %. However its viability remained around 80 % (Table 1). The viability of cells increased significantly (p<0.05) when the extract was combined with putrescine and decreased when cells are treated with the complex extract-DFMO compared to their control respectively (Tables 1). The results demonstrated that the viability of the treated leukaemia cells was lower than non treated cells. The effect of *S. alata* extract and DFMO on the intracellular concentration of polyamines illustrated in Table 2 established that the levels of polyamines were significantly decreased when cells are treated with extract alone. The presence of DFMO alone significantly increased the reduction of the level of intracellular production of polyamines compare to the control and extract alone. The combination of the extract of *S. alata* with DFMO enhanced the reduction of the polyamines concentrations from 52 to 95 % (Table 3). When exogenous putrescine is adding in the cell culture (Table 3), the reduction of putrescine, cadaverine, spermidine and spermine altered from 9.84 to 73.28 % compared to the control. However, when the cells were treated with the complex extract-exogenous putrescine, the production of cadaverine, spermidine and spermine increased significantly with the exception of putrescine. The effect of extract of *S. alata*, DFMO, putrescine alone or in combination on protein synthesis demonstrated that the extract of *S. alata* alone inhibits the production of protein (table 4). When the extract is mixed with DFMO or exogenous putrescine the protein synthesis is increased compare to the control. The phytochemical analysis of *S. alata* extract demonstrated the presence of flavonoids, polyphenols, tannins, steroids and glycosides.

DISCUSSION

Polyamines have been shown to play a role in cells proliferation and cancer disease. Since spermidine, spermine, and putrescine appear to be reliable indices of cells proliferation, all drugs and molecules which inhibit their metabolism are potential targets for cancer therapy (33). The DFMO drastically reduced the concentration of putrescine and significantly inhibited the proliferation of neoplasm cells *in vitro* (34). The first step of the production of polyamine is catalysed by the enzyme, ornithine decarboxylase, which leads to the production of putrescine. This study shows that extract of *S. alata* can inhibit the putrescine production at certain level. Hence, this inhibition of putrescine synthesis can be explained either by the inactivation or the inhibition of the ornitine decarboxylase activity or the S-adenosylmethionine decarboxylase. The combination of putrescine with extract affects otherwise the yield of putrescine. This complex significantly reduced the production of polyamines. Similar observation was noted when cells were treated with the extract only (Table 2). The results indicated that the presence of exogenous putrescine in the cell culture promotes the production of putrescine through the action of several enzymes especially polyamine oxidase (or N-acetyltransferase) and ornithine decarboxylase (8). It has become extremely difficult to deplete 100 % of the polyamines from cancer tissue when that cancer tissue can obtain polyamines from the surrounding normal tissues (9).

Table 1: Effects of S. alata extract on the viability and the inhibition of the proliferation of the leukaemia L1012 cells.

Plant extract	Treatment	% of Cells viability	Inhibition of proliferation (%)
S. alata	40 µg/ml	80.00 ± 0.00 ^{d}	28.00 ± 0.40 ^{b}
	80 µg/ml	80.00 ± 0.00 ^{d}	32.80 ± 0.47 ^{c}
	80 µg/ml + Pu (1 M)	68.14 ± 0.85 ^{c}	
	Ctrl + Pu (1 M)	94.75 ± 0.35 ^{e}	
	80 µg/ml + DFMO (165 mM)	23.36 ± 0.36 ^{b}	
	Ctrl + DFMO (165 mM)	9.72 ± 1.12 ^{a}	
	Ctrl	100.00 ± 0.00 ^{e}	0,00

Pu: Putrescine; DFMO: Alpha difluoromethylornithine; Ctrl: Control; Values are expressed as mean ± SD; (n = 3). Means with different letter superscript within each column are significantly different (p <0.05).

Table 2: Effects of extract and DFMO on the intracellular concentration of polyamines.

Plant extract	Treatment	Pu (µM)	Ca (µM)	Sm (µM)	Sd (µM)
S. alata	80 µg/ml	5.68 ± 0.02 ^{d}	1.25 ± 0.08 ^{a}	1.92 ± 0.01 ^{c}	3.67 ± 0.01 ^{c}
	80 µg/ml + DFMO (165 mM)	0.73 ± 0.01 ^{a}	2.81 ± 0.04 ^{b}	0.87 ± 0.02 ^{b}	1.91 ± 0.01 ^{b}
	Ctrl + DFMO (165 mM)	1.92 ± 0.02 ^{b}	5.31 ± 0.02 ^{c}	0.56 ± 0.02 ^{a}	0.86 ± 0.01 ^{a}
	Ctrl	6.31 ± 0.07 ^{c}	7.46 ± 0.01 ^{d}	6.45 ± 0.04 ^{c}	11.02 ± 0.00 ^{c}

Ca: Cadeverine ; Sp : Spermime; Sd : Spermidine; DFMO: alpha difluoromethylornithine; Ctrl: Control; Values are expressed as mean ± SD; (n = 3). Means with different superscript letter within each column are significantly different (p<0.05).

Table 3: Effects of extract and exogenous putrescine on the intracellular concentration of polyamines.

Plant extract	Treatment	Pu (µM)	Ca (µM)	Sp (µM)	Sd (µM)
S. alata	80 µg/ml d'extrait	5.87 ± 0.04 ^{d}	1.66 ± 0.01 ^{a}	1.92 ± 0.01 ^{b}	3.66 ± 0.02 ^{b}
	80 µ/ml + Pu (1 M)	1.05 ± 0.02 ^{a}	4.04 ± 0.04 ^{c}	2.57 ± 0.02 ^{c}	7.19 ± 0.01 ^{c}
	Ctrl + Pu (1 M)	4.57 ± 0.05 ^{b}	4.18 ± 0.04 ^{b}	1.80 ± 0.02 ^{a}	3.87 ± 0.00 ^{a}
	Ctrl	6.24 ± 0.01 ^{c}	7.48 ± 0.02 ^{d}	18.29 ± 0.02 ^{d}	12.11 ± 1.02 ^{d}

Ca :Cadeverine; Sp : Spermime ;Sd : Spermidine; Pu: Putrescine; Ctrl: Control; Values are expressed as mean ± SD; (n = 3). Means with different superscript letter within each column are significantly different (p <0.05).

Table 4: Effects of extract, DFMO and exogenous putrescine on the level of protein.

Plant extract	Treatment	Protein (mg/ml)	Percentage of reduction / Control	Percentage of reduction / Control+ DFMO	Percentage of reduction /Control+ PU
S. alata	80 µg/ml	0.452 ± 0.005 ^{d}	11	-	84
	80 µ/ml + DFMO (165 mM)	0.180 ± 0.006 ^{a}	64	58	93
	80 µg/ml + Pu (1 M)	0.580 ± 0.0115 ^{e}		-	79
Controls	Ctrl	0.513 ± 0.007 ^{ce}			
	Ctrl +	0.430 ± 0.010 ^{bd}			

DFMO (165 mM)		
Ctrl + Pu (1 M)	2.843 ± 0.005 [f]	

Pu: Putrescine; Ctrl: Control; DFMO: Alpha difluoromethylornithine; Values are expressed as mean ± SD; (n = 3). Means with different superscript letters within each column are significantly different (p <0.05).

On the other hand, the combination of *S. alata* - putrescine contributes to the reduction of the intracellular putrescine by a feedback effect. These processes have been demonstrated in several studies (6). It was also shown that the putrescine played an important role as a growth factor during embryogenesis and proliferation of normal and cancer cells (8). The diminution of the level of polyamines cause the reduction in oncogenesis by most organic toxic chemicals when carcinogenesis studies were performed in tissue culture, intact animal or human studies (3). The extract of *S. alata*, DFMO or putrescine alone or in combination affects in different manner the intracellular synthesis of cells protein. The addition of the putrescine to the extract of *S. alata* significantly increased the production of protein confirming that the exogenous putrescine stimulates the proliferation of leukaemia cells via the production of polyamines.

A wide variety of natural compounds appear to possess significant cytotoxic, antiproliferative and chemopreventive activities. Several extracts from plants used in traditional medicine also may have similar properties. Flavonoids, flavone, flavonol, flavonone and isoflavonone classes found in many plant extract have antiproliferative effects in different cancer cell lines (35). Depending on their structural features, the antiproliferative potential of flavonoids varied significantly (35).

The presence of tannins, glycosides, polyphenols and steroids in the extract contribute to enhance its antiproliferative effects. Several studies have shown the cytotoxic effect and the highly inhibitory proliferative action of these bioactive classes of compounds on different cell lines. These occurred either through apoptosis or by induction of DNA fragmentation or cell circle (34). Today, chemoprevention of cancer diseases employing anti-polyamine drugs can be applied to high risk population groups as an attempt to decrease their cancer risk. Thus the extract of *S. alata* can be prospect for the purpose.

In conclusion, the extract of *S. alata* can potentially inhibit the proliferation of leukaemia cells. Further research needs to be done on the mechanism of inhibition by which the extract acts and also to find out different bioactive components inducing its activity.

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