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In vitro cytotoxic effect of *Cleistanthus collinus* extracts and fractions on mouse cell line

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ABSTRACT

Aqueous, methanol and ethyl acetate crude extracts and fractions were prepared using *Cleistanthus collinus* Roxb. dry leaf powder. All the extracts and fractions were subjected to *in vitro* cytotoxic analysis using mouse 3T3- L1 pre-adipocytes cell line. Rate of cell proliferation was calculated to determine the anti-proliferative activity. Aqueous, methanol and ethyl acetate extracts (≥100 µg/ml) significantly control cell proliferation at 48 hr incubation. However, fractions exhibited higher level of toxicity and affect cell growth even at 50 µg/ml concentration within 28 hr incubation. Fractions obtained from methanol extract showed cytotoxic effect about 43-76 % at 50-250 µg/ml at 48 hr incubation (Concentration necessary to inhibit cell growth at 50 % is ~75 µg/ml). Followed by, ethyl acetate fraction exhibited 23-59 % of anti-proliferative activity (Concentration necessary to inhibit cell growth at 50 % is ~180µg/ml). It may be concluded that promising fractions of *C. collinus* with higher toxicity level could be exploited for pharmacological purposes.

INTRODUCTION

It is obvious that many pharmacological research groups have focused into natural products as source of new drug with pharmacological potential; these drugs were mainly used to cure diseases and syndromes with less toxic in human and animals. *Cleistanthus collinus* a member of Euphorbiaceae family is harboring pharmacologically potential and interesting property (Maji *et al.,* 2010; Suman *et al.,* 2013: Arivoli *et al.,* 2011). *C. collinus* were normally used as a source of dangerous poisons (suicidal and homicidal) in Southeast Asian countries. All parts of the plant are toxic to the human and veterinary animals and principal toxins extracted from various parts of the plants are lignan lactones, diphyllin and glycosides such as Cleistanthin A and B (Eswarappa *et al.,* 2000; Annapoorani *et al.,* 1984). *C. collinus* leaves contain saponin, tannin and oduvin and the poisonous effect is attributed to oduvin usually occurs after drinking the decoction of the leaves of *C. collinus* leading to death within 1-3 days. Main clinical features of poisoning with *C. collinus* are hypokalemia, acidosis, hypotension and respiratory failure (Thomas *et al.,* 1991; Subrahmanyam *et al.,* 2003; Nandakumar *et al.,* 1989). Perusal of the literature revealed that most of the works have been done on effect of the *C. collinus* extract on controlling the cancer cells either *in vitro* or *in vivo* level. The present study was carried out to investigate the effect or response of the normal cell line to the *C. collinus* extract and selected fraction.

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MATERIALS AND METHODS

Plant collection, extraction and fractionation

The *C. collinus* plant leaves were collected from Virallimalai, Tamilnadu, India. The plant leaves were shade dried at room temperature and powdered to precede extraction. 2000g of plant sample was subjected to the hot crude extract preparation using soxhlet apparatus. Distilled aqueous, methanol and ethyl acetate (Merck, Germany)) were used as solvents, crude extracts were concentrated and used for further analysis. Both methanol and ethyl acetate were subjected to column chromatography using silica gel (230–400 mesh, Merck, Germany)

and eluted various fractions with chloroform, methanol, toluene and ethyl acetate as mobile phase. Fractions were subjected to develop TLC (Silica gel 60 F254 (Merck, Germany). Selected spot on the TLC plates were scrubbed and separated partially purified botanicals.

Cytotoxicity assay

The cytotoxicity assay was performed for extracts and fractions using EZ-CYTOX Cell Viability Assay Kit (ITSBIO, Korea). The water soluble tetrazolium [WST; 2(2-Methoxy-4-nitrophenyl) - 3(4-Nitrophenyl)-5-(2, 4- disulfophenyl)-2-H-tetrazolium monosodium] salt was used for analysis of the cell proliferation. Mouse 3T3- L1 pre-adipocytes cells were seeded in the 96 well at a density of $1x10^5$ cells/ well. The cells were treated with different concentrations (100-500 µg for methanol and ethyl acetate extracts, 50-250 µg/ml for each fraction) of extracts and fractions. One well was kept as control without any addition of extracts and fractions. The setup was incubated at the 37^{o}C in 5 % CO_2incubator for 24 hr and 48 hr and then the culture was treated with WST reagent and incubated for 2 to 4 hr. The living cells absorbed the WST then it was converted into an orange color product. The intensity of colour was measured at 450 nm using spectra count ELISA reader and ratio of inhibition of proliferation was calculated.

RESULTS

Aqueous, methanol and ethyl acetate solvent based *C. collinus* leaf extracts were prepared and further fractionated the methanol extract with chloroform: methanol (4:1 ratio) solvents to get 27 mg (dry weight) of residue. Similarly 175 mg of residue as fraction was also obtained from ethyl acetate extract when using toluene: ethyl acetate (3:2 ratio) as eluent. Methanol fraction was yellow in color, partially soluble in methanol but highly soluble in chloroform. Final residue obtained after complete evaporation of methanol was amorphous in nature and yellow in colour. TLC analysis of the fractionated compound revealed that the single spot obtained with Rf value 0.55 in chloroform: methanol (1:1 ratio) as mobile phase. Second fraction that is ethyl acetate fraction was crystal in nature and dark green in color, soluble in all organic solvents. The Rf value of this spot is 0.37 in toluene:ethyl acetate (4:1 ratio) as mobile phase. In this present study, crude extracts as well as partially purified fractions were subjected to determine the antiproliferation property. Cell proliferation of 3T3- L1 pre-adipocytes cells was moderately affected when incubated along with crude extracts (50 - 250 µg/ml) rather than the acute impact observed when incubated along with the partially purified fractions (50 - 250 µg/ml) (Table 1 & 2). Compared with control the antiproliferation activity was propositionally increased along with the augment of toxicant concentration. From this study it is obvious that higher concentration of toxicants destroy the cells within short time of incubation, however, lower concentration also effective in controlling the growth of the cells provided the extension of the incubation period. All the three *C. collinus* leaf extracts (each 250 µg/ml) namely aqueous, ethyl acetate and methanol exhibited 37, 44 and 38 % of antiproliferative activity at 48 hr incubation respectively as compared with control. However, partially purified fractions that are ethyl acetate and methanol fractions (each 250 µg/ml) significantly affect the cell proliferation and it could be represented as 59 and 76 % of antiproliferative activity at 48 hr incubation with normal 3T3- L1 pre-adipocytes cells. Concentration of toxicant necessary to inhibit cell growth at 50 % level was also calculated for three kinds of extracts and two partially purified fractions. All the five test materials showed significant variations in the antiproliferative effect between the short (28 hr) and long (48 hr) time incubation periods. Of the three extracts, ethyl acetate extract exhibited the lowest GI_{50} at 48 hr incubations than methanol and aqueous extracts which are having almost similar GI_{50} (Table 1). However, in the case of the fractions, methanol solvent derived one was highly effective (GI_{50} 75 µg/ml) in ceasing the cell growth rather than ethyl acetate solvent derived fraction (GI_{50} 180 µg/ml) (Table 2).

Table 1: Anti-proliferative effect of various crude extracts of *C. collinus* on mouse 3T3- L1 pre-adipocytes normal cell line

Toxicant Conc. (µg/ml)	Anti-Proliferative Activity (%) ± S.D*					
	Aqueous extract		Ethyl acetate extract		Methanol extract	
	28hr	48hr	28hr	48hr	28hr	48hr
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50	12 ± 1.2	14 ± 1.5	8 ± 1.1	19 ± 0.9	14 ± 0.8	16 ± 1.0
100	16 ± 2.3	22 ± 2.1	13 ± 2.1	25 ± 1.4	19 ± 1.6	22 ± 1.3
150	19 ± 2.8	26 ± 1.3	16 ± 0.8	31 ± 1.7	23 ± 1.4	27 ± 0.9
200	23 ± 1.5	33 ± 1.2	20 ± 1.3	33 ± 1.5	28 ± 1.1	33 ± 1.5
250	27 ± 1.7	37 ± 1.6	23 ± 0.7	44 ± 1.8	32 ± 1.7	38 ± 2.0
**GI_{50}	470	325	550	290	390	320

*****Standard Deviation; ******Concentration (µg/ml) necessary to inhibit the growth at 50 %.

Table 2: Anti-proliferative effect of various fractions of *C. collinus* on mouse 3T3- L1 pre-adipocytes normal cell line

Toxicant Conc. (µg/ml)	Anti-Proliferative Activity (%) ± SD*			
	Ethyl acetate fraction		Methanol fraction	
	28hr	48hr	28hr	48hr
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50	2 ± 0.6	23 ± 1.8	35 ± 1.6	43 ± 1.8
100	4 ± 0.8	32 ± 2.1	42 ± 2.0	60 ± 2.3
150	9 ± 1.4	39 ± 2.0	47 ± 2.1	69 ± 2.7
200	27 ± 2.3	56 ± 1.7	48 ± 1.6	70 ± 1.8
250	41 ± 2.6	59 ± 2.2	58 ± 2.3	76 ± 2.4
**GI_{50}	350	180	210	75

*****Standard Deviation; ******Concentration (µg/ml) necessary to inhibit the growth at 50 %.

DISCUSSION

Cleistanthus collinus (Euphorbiaceae) is a naturally distributed in the dry forests of the southern and central parts of India. Many parts of the plants were reported as toxic however it has been exploited as drug since it harbours many valuable pharmacological properties. Many researchers have paid attention to this plant for isolating new phytochemical molecule for developing drugs against human cause (Govindachari *et al.,* 1969; Ramar *et al.,* 2014; Nagarajan *et al.,* 2014). The present study has

brought out some important observations in the context of toxic effect of *C. collinus*. Here the aqueous, methanol and ethyl acetate extracts from plant leaf showed higher level of toxicity at lowest concentration. Likewise the alcoholic extract of the whole plant of *C. collinus* was tested for anticancer activity in human epidermoid carcinoma of nasopharynx in culture, Walker carcinoma sarcoma 256 in rats and L-1210 lymphoid leukaemia in mice. The extract showed significant anticancer activity against human epidermoid carcinoma of the nasopharynx. The aqueous extract of *C. collinus* showed the cytotoxicity level at 10 µg/ml concentration in 28 hr incubation itself (Bhakuni *et al.,* 1969). It is further confirmed that *C. collinus* extracts and fractions have shown cytotoxicity effect on normal cell line of mouse (Pinho *et al.,* 2007; Lakshmi *et al.,* 1970). Similarly, the percent survival values of Chinese hamster ovary CHO cells, cervical carcinoma (Si Ha) cells and *p53* deficient cell line K562 treated with 20, 40, 60 and 80 mg/ml of Cleistanthin A were 78 %, 52 %, 30 % and 12 % respectively. The results indicated a progressive decrease in the survival of cells in response to increasing doses of Cleistanthin A. The IC_{50} value of Cleistanthin A ranged from 0.4×10^{-6} to 0.9×10^{-6} M. Higher concentrations and long exposure to Cleistanthin A stimulate DNA strand breaks and apoptosis in Chinese hamster ovary (CHO) cells, in cervical carcinoma (Si Ha) cells and in a p53 deficient cell line K562 (Pradheep Kumar *et al.,* 2000). *In-vitro* studies carried out by Pradheep Kumar *et al.,* (1996) revealed that Cleistanthin B, isolated from the poisonous plant *C. collinus* showed potent anticancer properties in normal and tumor cells. Cleistanthin B exhibited 50 % growth inhibition (GI_{50}) to normal cell lines at 2×10^{-5} to 4.7×10^{-4} M and for tumor cells the values ranged from 1.6×10^{-6} to 4×10^{-5} M.

CONCLUSION

The present study is a primary analysis of *C. collinus* extracts and its fractions to ensure the toxicity property against mouse 3T3- L1 pre-adipocytes cell proliferation. Cell type cytotoxic specificity has to be done further for those promising fractions. In general, these results indicates that there was a significant antiproliferative effect even to the normal mouse cell line. As per the traditional knowledge, the plant *C. collinus* is used up for the antitumor treatment, so, cautions has to be taken to optimize the method of fractionation of the promising compounds having anticancer property and also fixing the effective concentration of the impure or pure phyto-compounds when used as drug. Moreover, fractions that have pronounced cytoxic activity should be evaluated further for the possible isolation and characterization of the phytocompounds and validation of the same to prove the specific biological properties.

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