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RESEARCH ARTICLE

ANTICANCER, ANTIOXIDANT AND ANALGESIC PROPERTIES OF *CROTON CAUDATUS* GEISEL LEAF EXTRACTS

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ABSTRACT

Croton caudatus Geisel is a traditional anticancer medicinal herb of the people of Mizoram, India. In the present study, the *in vivo* and *in vitro* anticancer potentials, antioxidant and analgesic properties of *C. caudatus* leaf extracts were evaluated. Phytochemical constituents of its methanol and aqueous extracts were also analyzed. Methanol (CC-Meth) and aqueous (CC-Aq) extracts exhibited potent anticancer activity *in vivo* (%ILS~92.5%) while *in vitro* cytotoxic activity was noted only with the aqueous extract. CC-Aq extract exhibited an IC_{50} value 28.36 µg/ml *in vitro*. The two extracts also significantly reduce acetic acid and formalin-induced pains in mice. The analgesic activity in both the first and second phases of formalin-induced pain test suggested that the extracts have both analgesic and anti-inflammatory properties. The aqueous extract showed significant DPPH and nitric oxide radical scavenging potentials. Results of preliminary phytochemical screening of aqueous extract of *C. caudatus* revealed the presence of alkaloids in larger amount and flavonoids, saponins, tannins and cardiac glycosides in lesser amount. The phytochemical constituents and antioxidant properties of the plant extract may be an important contributory factors involved in the anticancer and analgesic activities of aqueous extract of *C. caudatus* leaf.

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INTRODUCTION

Researchers have been realized the role of oxidative stress in many human diseases. Oxidative stress results from an imbalance between formation and neutralization of pro-oxidants. It is initiated by free radicals including hydroxyl, peroxyl and superoxide radicals, which become stable through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. Oxidative stress-induced damages have been implicated as a potential contributor to the pathogenesis of many diseases (Braca *et al.,* 2002; Maxwell, 1995). Biological systems have antioxidant defense mechanism to protect them from oxidative damages. In some cases, this natural antioxidant defense mechanism can be inefficient; hence dietary intake of antioxidant is required. Antioxidants are substances that prevent cells from free radical attacks. Consumption of fruits and vegetables rich in fibers and vitamins is known to help protect cells from the risk of several diseases including cancers caused by oxidative stress (Willett, 2002 and Steinmetz and Potter, 1996).

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Plant species are steadily being investigated for the identification of novel therapeutic agents based on traditional system of medicine. *Croton caudatus* Geisel (Euphorbiaceae) is a large scandent shrub. It is widely distributed in South East Asia (Shantabi *et al.,* 2014). It usually grows in deciduous and evergreen forests. Sometimes, it grows near marginal areas along river or stream tracts. In Mizoram, they are usually available at an altitude between 500m to 9000m.

It has been traditionally used for the treatment of several human diseases such as cancer, malaria, sprains, liver diseases, convulsions, stomach problems, rheumatic arthritis, etc. (Kritikar and Basu, 1996 and Yusuf *et al.,* 2007). Our preliminary investigation through consultation of local herbal practitioners and elders revealed that the local people of Mizoram have been used *C. caudatus* leaf as a traditional medicine for the treatment of diseases particularly cancer-suspected diseases. Hot water extract of *C. caudatus* leaf is being sold by some villagers as a remedy for diseases. One bottle (one liter) cost hundred Indian rupees. The plant is locally called *Sai ek-hlo* (Mizo). Therefore, considering its traditional uses for various medicinal purposes the present study was undertaken to investigate the anticancer, antioxidative and analgesic properties of *Croton caudatus* leaf extracts *in vitro* and *in vivo* using ascites Dalton's lymphoma cells.

MATERIALS AND METHODS

Animals and tumor model

Inbred Swiss albino mice were maintained under conventional laboratory conditions at room temperature (20 ± 2°C) with free access to standard food pellets and water *ad libitum*. Ascites Dalton's lymphoma (DL) tumor is being maintained *in vivo* in 10-12 weeks old mice by serial intraperitoneal (i.p.) transplantations of $1x10^6$ viable tumor cells per animal. Tumor-transplanted hosts usually survived for 20 days. The use of animals in the present study is as per the ethical norms and has been cleared by institutional ethical committee of Mizoram University, Aizawl.

Plant materials and extract preparation

C. caudatus Geisel was authenticated by Dr. R. Lalfakzuala, Department of Botany, Mizoram University, Aizawl and the herbarium specimen (Voucher No. GRS-SKH 001) was deposited in the Department of Zoology, Mizoram University, Aizawl, India. The leaves were washed with tap water, shade dried and then blended into powder with an electric-grinder. The powdered material was subjected to sequential extractions with hexane, chloroform and methanol using a Soxhlet apparatus until the solvents became clear. The remaining residues were processed for hot water extraction. Solvents from each extract were evaporated under reduced pressure at 45°C to dryness. Hexane extract was discarded. Chloroform and methanol extracts were dissolved in dimethylsulfoxide (DMSO) and diluted with minimum essential medium (MEM) to get the required concentrations for the study. Aqueous extract was dissolved in MEM.

In vivo antitumor activity studies

In vivo antitumor activity of *C. caudatus* leaf extracts was studied in mice using Dalton's lymphoma (DL) tumor model (Sakagami *et al.,* 1987). One million viable DL cells (in 0.25ml PBS, 7.4 pH) were transplanted intraperitoneally in 10-12 weeks old Swiss albino mice. Tumor transplantation day was designated as day 0. Different doses of the extract (in 0.25ml) were administered intraperitoneally for seven consecutive days starting from day one of tumor transplantation. Control group of mice received 0.25ml extract vehicle alone. Control and treated mice (10 to 400mg/kg/day) consisted of 10 mice each. The antitumor efficacy was reported in percentage of average increase in life span (%ILS) calculated using the formula (T/C x 100) – 100, where, T and C are the mean survival days of treated and control groups of mice respectively. The pattern of changes in body weight of experimental mice from normal, control and treated at a dose of 150mg/kg/day showing maximum %ILS were also recorded.

In vitro cytotoxicity study by MTT assay

DL cells are plated on to 96 well plates at a cell density of $1x10^4$ per well in 200 µl of MEM supplemented with gentamycin and allowed to grow in CO_2 incubator for 24 h (37°C, 5% CO_2). Thereafter, 10 µl of different concentrations of plant extracts (5 – 150 µg/ml) were added and incubated for an additional 48 h. 20µl MTT ([3- (4, 5-dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide]) stock solution (5mg/ml in PBS) was added to each well and incubated for 5 h. The formazan produced by the viable cells was solubilized by addition of 20µl DMSO and incubated for 2 h. The absorbance was recorded at 560 nm using microplate reader (iMark Microplate Reader). The percentage cytotoxicity was calculated with respect to vehicle control using the formula:

% cytotoxicity = {(Control absorbance – Test absorbance)/ Control absorbance} x 100.

Analgesic activity

Acetic acid-induced writhing test

Acetic acid-induced abdominal writhing test was performed according to Nguelefack *et al.*, 2004. Mice (six in each group) were injected intraperitoneally with 0.5% acetic acid at a dose of 10 ml/kg. Different concentrations of the extracts (100 to 1200 mg/kg) dissolved in double distilled water were administered 1 hour prior to acetic acid treatment. The writhing induced by the acid, consisting of abdominal constrictions and hind limbs stretching, were counted for 15 minutes after acetic acid treatment. The percentage analgesic activity was calculated as follows:

Percentage analgesic activity = (C – T)/C x 100

Where C is the average number of writhing of control and T is the average number of writhing of treated.

Formalin-induced pain test

The formalin-induced pain test was done according to Gaertner *et al*., 1999. Pain was induced by injecting 20 µl of 2.5% formalin (40% formaldehyde) in distilled water in the subplantar of the right hindpaw. Mice were given intraperitoneal extract treatment 1 hour prior to injecting formalin. The amount of time spent licking the injected paw was indicative of pain. The number of lickings from 0 to 5 min (first phase) and 15–30 min (second phase) were counted after injection of formalin. These phases represented neurogenic and inflammatory pain responses, respectively (Hunskaar and Hole, 1987). The percentage of analgesic activity (% inhibition) at each phase was calculated using the following formula:

% inhibition = (C - T)/C x 100.

Where C is the average number of licking of control and T is the average number of licking of treated.

Phytochemical analysis

Phytochemical analyses of methanol and aqueous extracts were performed using standard procedures (Brinda *et al.,* 1981; Trease and Evans, 1989 and Harborne, 1998). The tests for phytomchemical screening include: tests for alkaloids, flavonoids, terpenoids, phenols, saponins, tannins, carbohydrates, cardiac glycosides and triterpenoids.

Free radical scavenging activity

In vitro DPPH radical scavenging assay

The radical scavenging activity of the most potent plant extract (CC-Aq) against 2,2-Diphenyl-1-picryl hydrazyl (DPPH)

radical was determined using the method described by Ayoola *et al.,* 2008. Briefly, different concentrations of CC-Aq extract (10 to 5000 µg/ml) were prepared. Ascorbic acid was used as the antioxidant standard at concentrations of 50, 100, 500 and 1000 µg/ml. 1 ml of the extract was placed in a test tube, and 3 ml of methanol was added followed by 0.5 ml of 1 mM DPPH in methanol. After 20 min, absorbance was recorded at 517 nm. The radical scavenging activity was calculated using the following formula:

$$\% \text{ scavenging} = \{[A_b - A_a]/A_b\} \times 100$$

where, A_b and A_a are the absorbance of the blank sample and test/extract sample respectively.

In vitro nitric oxide radical scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates Nitrite oxide which interacts with oxygen to produce Nitrite ions, which can be measured at 546 nm by spectrophotometer in the presence of Griess reagent (Kumar *et al.,* 2008). Sodium nitroprusside 5 mM was prepared in phosphate buffer pH 7.4. To 1 ml of various concentrations (10 to 5000 µg/ml) of CC-Aq extract, 0.3 ml of sodium nitroprusside was added. The mixture was incubated at 25^{o}C for 5 hours and then 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylene dihydrochloride) was added. The absorbance was read at 546 nm. The same reaction of equal volume but without the Griess reagent served as control. The experiment was performed in triplicate. Same procedure was done with ascorbic acid which was standard in comparison to the extract.

Statistical analysis

All statistical analysis was done using statistical software 'OriginPro 8 SRO v8.0724 (B724), Northampton, MA, USA'.

RESULTS

In the antitumor activity study of drugs, ascites Dalton's lymphoma has been used as an important murine experimental tumor model (Prasad and Giri, 1994 and Rosangkima and Jagetia, 2015). For the *in vivo* antitumor activity studies, chloroform (CC-Chl), methanol (CC-Meth) and aqueous (CC-Aq) extracts of *C. caudatus* leaf were used. Different doses of extracts and their effects on the survivability of the hosts have been described in Figure 1. The deaths of mice, if any, were recorded daily and the survival pattern of mice in different treatment groups was also determined. Out of three different extracts studied, only methanol and aqueous extracts (CC-Meth, CC-Aq) exhibited potent antitumor activity in a dose-dependent manner. Among different doses of the extracts, 150 mg/kg/day of CC-Aq showed comparatively better antitumor activity (%ILS~ 92.5%) against ascites Dalton's lymphoma. The comparative survival patterns of tumor-bearing mice treated with different doses of CC-Aq extract were shown in Figure 2. Hundred percent survivors were noted till 34 days with 150 mg/kg/day dose. During tumor growth progression, there was a rapid increase in the body weight of tumor-bearing control mice reaching 32g on the 18th day of tumor growth. Treatment with CC-Aq extract at 150 mg/kg/day significantly decreases the body weight of tumor-bearing mice after 12th day of tumor growth (Figure 3).

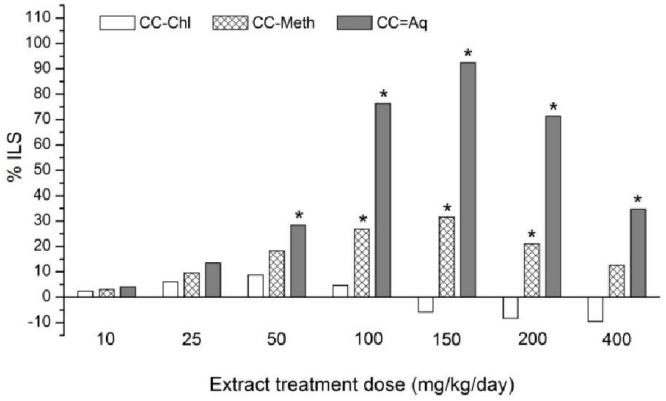

Fig. 1. Graph showing percentage increase in life span (%ILS) of tumor-bearing mice after treatment with different doses of chloroform (CC-Chl), methanol (CC-Meth) and aqueous (CC-Aq) extracts of *C. caudatus* leaf. Results are mean ± S.D. Student's *t*-test, N = 10. *p < 0.05 represents the statistically significant difference between treated and control groups.

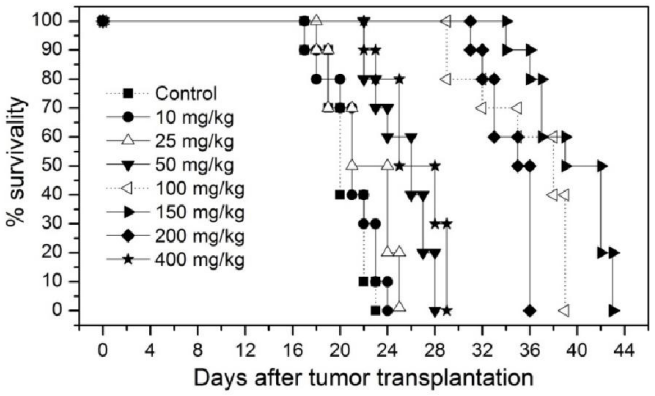

Fig. 2. Graph showing percentage survival of tumor-bearing mice after treatment with different doses of aqueous extract (CC-Aq) of *C. caudatus*

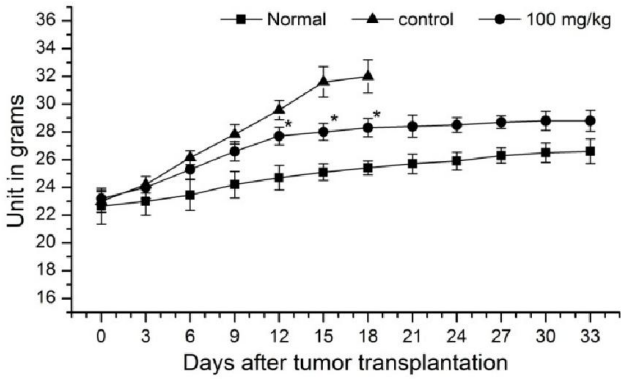

Fig. 3. Graph showing the pattern of changes in the body weight of mice during tumor growth after treatment with aqueous extract of (CC-Aq) of *C. caudatus* at a dose of 150 mg/kg/day. Student's *t*-test, N = 10. p < 0.05 represents the statistically significant difference between treated and control groups.

Cytotoxicity studies using MTT assay shows a concentration-dependent cytotoxic activity of extracts of *C. caudatus* leaf against DL cells *in vitro* at a dose range of 5 – 150 µg/ml during 48 h of treatment (Figure 4). However, out of three

different extracts studied *in vitro*, only CC-Aq extract exhibited potent cytotoxicity on DL cells showing maximum percentage cytotoxicity at 150 µg/ml as well as IC_{50} value 28.36 µg/ml. The present results revealed a significantly higher *in vitro* cytotoxic potential of aqueous extract of *C. caudatus* leaf (CC-Aq) as compared to chloroform and methanol extracts.

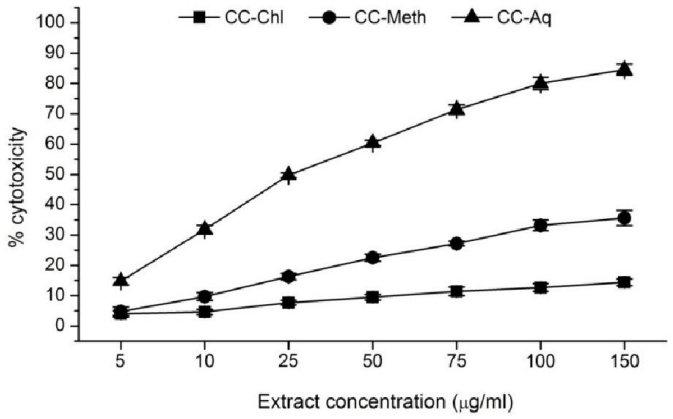

Fig. 4. Graph showing the *in vitro* cytotoxic activity of *C. caudatus* leaf extracts on DL cells after 48 hours of incubation by MTT assay. Results are mean ± S.D

Intraperitoneal injection of acetic acid in mice induced a pain in the region of abdomen causing abdominal contractions. Intraperitoneal administration of CC-Meth and CC-Aq extracts resulted to cause a dose-dependent significant reduction in the number of abdominal contractions when compared with the control animals (Table 1). CC-Aq extract exhibited better antinociceptive activity showing 37.59 and 49.81 % of inhibition at a dose of 800 and 1200 mg/kg respectively. Aspirin, a reference drug showed 54.96 % of inhibition at 100 mg/kg dose. Table 2 shows the effects of CC-Meth and CC-Aq extracts of *C. caudatus* leaf on the pain induced by formalin.

The result shows that the animals of the control group licked the leg for 116.3 seconds during the first five minutes and 97.3 seconds during the 15 – 30 minutes. Administration of the two extracts dose-dependently inhibited the pain induced by formalin during the two phases (0-5 and 15-30 min). CC-Aq exhibited better inhibition of formalin-induced pain. It shows 36.19, 43.42 and 49.01 % inhibition during the first five minutes and 38.54, 50.56 and 57.24 % inhibition during the second phase at the dose of 400, 800 and 1200 mg/kg respectively.

Table 1. Effects of methanol and aqueous extracts of *Croton caudatus* leaf on acetic acid-induced writhing in mice

Treatment Group	Doses (mg/kg)	No. of writhing (per 15 min)	% Inhibition
Control	-	52.4±4.6	-
CC-Meth	100	51.4±3.5	01.91
	200	48.2±3.1	08.01
	400	45.6±2.7*	12.97
	800	40.3±3.4*	23.09
	1200	36.8±2.9*	29.77
CC-Aq	100	48.6±2.7	07.25
	200	44.8±3.3*	14.51
	400	41.2±2.6*	21.37
	800	32.7±3.1*	37.59
	1200	26.3±2.4*	49.81
Aspirin	100	23.6±3.2*	54.96

Values are mean ± S.D. *P < 0.05, significantly different from control, Student's *t*-test, N = 6.

Preliminary phytochemical analysis of methanol and aqueous extracts of *C. caudatus* leaf revealed that alkaloids and saponins were present in larger quantities in CC-Aq extract while flavonoids, tannins and cardiac glycosides were present in small amount (Table 3). CC-Meth extract contains alkaloids, saponins, tannins and cardiac glycosides in small quantities. However, terpenoids, phenols, carbohydrates and triterpenoids tested negative in both CC-Aq and CC-Meth extracts.

Table 2. Effects of methanol and aqueous extracts of *Croton caudatus* leaf on formalin-induced pain in mice

Treatment Group	Doses (mg/kg)	First phase (0 – 5 min)		Second phase (15 – 30 min)	
		Licking times	% Inhibition	Licking times	% Inhibition
Control	-	116.3±2.2	-	97.3±2.4	-
CC-Meth	100	113.5±2.7	2.41	92.6±2.2	4.83
	200	109.3±3.1	6.02	86.2±3.2*	11.41
	400	101.4±1.8*	12.81	71.7±1.7*	26.31
	800	92.4±2.3*	20.55	66.8±2.6*	31.34
	1200	81.6±2.1*	29.83	62.5±1.9*	35.76
CC-Aq	100	104.7±1.7	9.97	81.7±2.5*	16.03
	200	96.7±2.5*	16.85	73.3±2.1*	24.66
	400	74.2±2.2*	36.19	59.8±2.8*	38.54
	800	65.8±3.2*	43.42	48.1±3.1*	50.56
	1200	59.3±2.5*	49.01	41.6±2.2*	57.24
Aspirin	100	62.2±2.2*	46.51	43.5±1.8*	55.29

Values are mean ± S.D. *P < 0.05, significantly different from control, Student's *t*-test, N = 6.

Table 3. Preliminary phytochemical analysis of *Croton caudatus* leaf extracts

Test	Alkaloids	Flavonoids	Terpenoids	Phenols	Saponins	Tannins	Carbohydrates	Cardiac glycoside	Triterpenoids
CC-Meth	+	-	-	-	+	+	-	+	-
CC-Aq	++	+	-	-	++	+	-	+	-

DPPH radical is commonly used as a substrate to evaluate antioxidant activity. It is a stable free radical that can accept an electron to become a stable molecule. Figure 5 shows the DPPH and nitric oxide radical scavenging activity of CC-Aq extract of *C. caudatus* leaf. The result shows potent radical scavenging activity of CC-Aq extract with higher activity on DPPH than that of nitric oxide. At a concentration of 4000 µg/ml, the percentage scavenging activity of CC-Aq extract on DPPH reached 72.3%, while at the same concentration its scavenging activity on nitric oxide reached 44.2%. Though the free radical scavenging activity of the extract is less than that of ascorbic acid (83.7%) at1000 µg/ml, the study suggested that the extract have the proton-donating ability and hence it may serve as free radical inhibitor or scavengers.

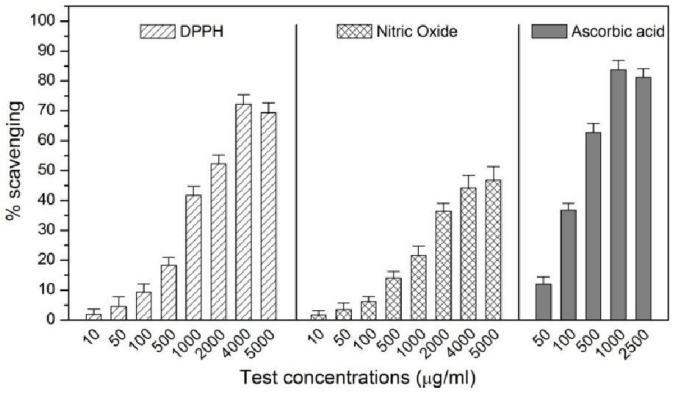

Fig. 5. Graph showing DPPH and nitric oxide scavenging activity of the aqueous extract (CC-Aq) of *C. caudatus* leaf. Results are mean ± S.D. N = 6

DISCUSSION

Cancer is the uncontrolled growth and spread of cells. Cancer often invades the surrounding tissues and organs and can metastasize to distant areas in the body. Chemotherapy is regarded as one of the most effective cancer treatment approach. However, therapeutic efficacy of most of them is limited due to the development of various side effects in the host (Black and Livingston, 1990 and Kartalou and Essigmann, 2001). In an attempt to avoid these side effects, many cancer patients seek alternative and complementary methods of treatment such as usage of phytomedicine (Sridharan *et al.,* 2012). Therefore, researchers are focusing towards the anticancer drug discovery from natural plant products resulting in the discovery of a variety of agents such as paclitaxel, vincristine, camptothecin, podophyllotoxin etc.

Ascites Dalton's lymphoma has been used as murine experimental tumor model in the investigation of anticancer activity (Nicol and Prasad, 2002). The same tumor model was used in the present study. The host survival data indicate a significant increase in the survivability of Dalton's lymphoma tumor-bearing mice (%ILS≥20%) treated with CC-Meth and CC-Aq estracts of *C. caudatus* at a dose of 50, 100, 150, 200 and 400 mg/kg/day as compared to the control tumor-bearing mice suggesting its anticancer potentials with the most potent anticancer activity (%ILS~ 92.5) with CC-Aq extract at a dose of 150 mg/kg/day. In the present study, *in vitro* cytotoxicity of plant extracts were determined by MTT assay. The principle of MTT assay is based on the reduction of a soluble tetrazolium salt by mitochondrial dehydrogenase activity of viable cancer cells into a soluble colored formazan product which can be measured spectrophotometrically (Edrini *et al,* 2002). The IC_{50} value has been used as a parameter of cytotoxicity. The criterion for cytotoxicity for the plant crude extracts, as established by the US National Cancer Institute (NCI), is an IC_{50} value less than 30 µg/ml (Suffness and Pezzuto, 1990). Based on this NCI criterion, the results of preliminary *in vitro* anticancer screening indicated that only aqueous extract of *C. caudatus* leaf (CC-Aq) showed potent cytotoxic activity on Dalton's lymphoma cells. The results of present study showed that the intraperitoneal administration of CC-Aq significantly inhibits the pains induced by the acetic acid and formalin.

The results also revealed that the extract decreases the noxious answers of the chemical stimuli in the abdominal contractions induced by acetic acid. The acetic acid produces pain by activation of chemo-sensitive nociceptors (Stai *et al.,* 1995) or by irritation of visceral surface, which leads to the release of histamine, serotonin, bradykinines and the prostaglandins (Garcia *et al.,* 2004). In order to find out the mechanism of action of the CC-Aq extract, it was tested on two phases of the pain induced by the formalin. The under-plantar injection of formalin causes pain which appears in two phases: the first or neurogenic phase during which there are activation of the fibers C and release of the substance P, and the second or inflammatory phase during which there is release of serotonin, histamine, bradykinin and prostaglandins (Murray *et al.,* 1994 and Tjolsen *et al.,* 1992). The peripheral analgesics usually inhibit only the second phase while the central analgesics inhibit the two phases (Stai *et al.,* 1995). The aqueous extract (CC-Aq) of *C. caudatus* leaf exhibited analgesic activity in both the phases with a slightly higher activity during the second phase suggesting that the extract exerts its action at the central level. Its higher inhibitory activity during the second phase also suggests that it has an anti-inflammatory activity.

Plants are a rich source of phytochemicals with various bioactivities such as antioxidant, anti-inflammatory and anticancer properties. Fruits, vegetables and medicinal herbs possess positive effects against cancer compared with antineoplastic drugs (Wu *et al,* 2002). The biological activities of the medicinal plants are usually the result of secondary metabolites including alkaloids, flavonoids, terpenoids, phlobatannins and reducing sugars (Balakrishnan and Sharma, 2013). The result of present studies also shows the presence of alkaloids and flavonoids in the aqueous extract of *C. caudatus* leaf (CC-Aq) which could be an important contributory factor involved in its anticancer potentials. Reactive oxygen species are considered to be implicated in tumor initiation and progression (Sugan *et al.,* 2006). Low level of antioxidant enzymes coupled with increased free radicals are well acknowledged in carcinogenesis (Szatrowski and Nathan, 1991). Many tumor cells have pro-oxidant status and promote oxidative stress thereby increasing the surviving potentials of the cancer cells by inducing mutations, activating redox signaling and stimulating pro-survival factors such as NF-kB and AP-1 (Seeram *et al.,* 2005). Antioxidants can alter the intracellular redox state by which it enhances the effects of cytotoxic therapy. The significant DPPH and nitric oxide

scavenging potentials of CC-Aq extract observed in the present study demonstrate its antioxidant activity. This antioxidant principle of the extract may also be a contributory factor involved in its cytotoxicity toward tumor cells and antitumor activity in experimental animals.

Conclusion

Based on the results of present studies, it may be concluded that aqueous extract of *C. caudatus* possess potent anticancer activity on Dalton's lymphoma with an IC_{50} value 28.36 µg/ml. The extract also acquires analgesic and anti-inflammatory activities in mice. It may be suggested that the phytochemical constituents and the antioxidant property of *C. caudatus* may be an important contributory factors involved in its anticancer potential.

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