Inhibition of DMBA induced mouse skin carcinogenesis by *Centella asiatica* extract

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Summary

The present study demonstrated that topical application of the *Centella asiatica* extract at the dose of 500 and 1000 mg/kg body weight at the pre promotion phase showed a significant reduction in tumor incidence, tumor burden, tumor weight, tumor size, cumulative number of papillomas, in *Centella* extract treated groups as compare to the carcinogen control group. *Centella asiatica* significantly scavenge the hydroxyl radical generated by EDTA/H_2O_2 when compared with ascorbic acid. The percentage scavenging of *Centella asiatica* was increased in a dose dependent manner.$IC_{50\ value}$ of *Centella asiatica* was 600µg/ml and Vit.C 25µg/ml. It also indicate that *Centella* extract exhibit significant antioxidant activity.

Key words: *Centella asiatica* – skin carcinogenesis- chemoprevention- antioxidant activity

Introduction

The present study demonstrates the chemopreventive potential of *Centella asiatica* extract on DMBA-induced skin tumorigenesis in male Swiss albino mice. The skin carcinogenesis model in experimental animals has been found to be a very useful system for investigating the influence of dietary chemopreventors both mechanistically and operationally.

Literature suggests that one subminimal dose of carcinogen "initiates" tumorigenesis and the treatment with Croton oil "promotes" it to the visible tumor stage (1). Though the exact mechanism underlying the anti-inflammatory activity of *Centella asiatica*, has not been

ascertained but it may be inferred that due to presence of steroidal compounds and anti-inflammatory property of *Centella asiatica* reported might have played a synergistic role in the inhibition of tumorigenesis as observed in the present investigation. Evidence also suggests that tumor promotion may also be due to free radicals (reactive oxygen species), which play an important role in tumor initiation by enhancing or facilitating the metabolic activation and/or initiating the effects of carcinogens (2) and promotion of multistage carcinogenesis. The involvement of free radicals in tumor promotion comes from the observation:

Centella asiatica (Linn) is an ethno medical plant used in different countries by diverse ancient cultures and tribal groups. It is one of the local herbs that is claimed to possess various physiological effects and it occupies an important place in the indigenous system of medicine as a tonic in skin diseases and leprosy (3).Different uses are claimed for the plant, the more common being its use for wound healing (4)(5), memory improvement, treatment of mental fatigue, bronchitis, asthma, dysentery, kidney trouble, urethritis, allergy, leucorrhea and toxic fever (6) and it is also used as a constituent of brain tonics for the mentally retarded (7). In addition, it has been shown to promote fibroblast proliferation and collagen synthesis (8) and to have anti-ulcer activity (9) antioxidant activity (10), anticancer activity (11), anti-bacterial activity (12) and anti-inflammatory activity (13). It is also commonly used as porridge for feeding pre-school children in combating nutritional deficiencies (14).

MATERIALS AND METHODS

Animals: Male Swiss Albino mice (Mus- musculus) of 15-20 gm body weight were used in the study. They were kept on synthetic pellet diet and water *ad libitum*. The animals were randomly divided in to 8 groups. Each group comprises of 6 animals. Mice were shaved in 2cm ^{2} area with the help of hair removing cream in interscapular region initially and after every 2 weeks hair were removed with the help of scissors. The treatment was provided topically on shaved area using the following protocol. The study protocol is approved by the Departmental Animal Ethical Committee. (IAEC, Ref. no,- 670/225/2008).

Chemicals: The initiator DMBA and croton oil (used as promoter) were procured from sigma chemical Co (St Louis, MO).DMBA was dissolved at a concentration of 100µg/100µl in acetone. Croton oil was mixed in acetone to give a solution of 1%dilution.

Preparation of plant extract-

Centella asiatica was obtained from the local garden in September 2007. The identification *Centella asiatica* plants was done by botanist Dr. S.S.Khan (Voucher specimen No NR/O1/LGOB/2006) Department of Botany Safia Science College Bhopal M.P India. Whole plant of *Centella asiatica* were dried in shade without direct exposure to sun rays and it was powdered. 25g powder was taken for aqueous extraction through soxhlet apparatus and refluxed for 2-3 days at 60ºC. After the complete extraction, the extract was kept it in water bath 45ºC for removing the solvent and the dry powder was obtained.

Skin bioassay protocol: The animals were randomly divided in to 8 groups. Each group comprises of 6 animals. Mice were shaved in 2cm ^{2} area with the help of hair removing cream in interscapular region initially and after every 2 weeks hair were removed with the help of scissors. The treatment was provided topically on shaved area using the following protocol.

Group 1: (Untreated control) No treatment was given.

Group 2: (Vehicle control) 100 µl acetone 2 times /week was applied topically up to 16weeks

Group 3: (DMBA Alone) 104 µg DMBA was dissolved in 100 µl acetone and single topical application was given.

Group 4: (Croton oil Alone) 1% croton oil was applied topically 2 days / week for 16 weeks.

Group 5: (DMBA + Croton Oil) 104 µg DMBA was dissolved in 100 µl acetone and single topical application was given. Afterwards 1 % Croton oil was applied on skin 2 times a week up to 16weeks.

Group 6: (DMBA + *Centella asiatica* + Croton Oil) 104 µg DMBA was dissolved in 100 µl acetone and single topical application was given. After one week, the 100 µl of *Centella* extract, at the dose of 500 mg/kg b.wt. was given one hour before the each application of 1 % croton oil 2 times a week up to 16weeks.

Group 7: (DMBA + *Centella asiatica* + Croton Oil) 104 µg DMBA was dissolved in 100 µl acetone and single topical application was given. After one week, the 100 µl of *Centella* extract, at the dose of 1000 mg/kg b. wt. was given afterwards the each application of 1 % croton oil 2 times a week up to 16weeks.

Group 8: (*Centella asiatica***)** 100µl of *Centella* extract at a dose of 500mg/kg b.wt was given topically two times a week up to 16 weeks

Antioxidant activity: The antioxidant activity of the extracts was determined by Fenton reaction was used to generate free radicals in a test tube and the free radicals scavenging activity was determined by the degradation of deoxyribose as standardized by (Elizabeth & Rao) Fe 3+, ascorbic acid, EDTA, H_2O_2 in the system produces hydroxyl radical which react with thiobarbituric acid reactive substances (TBARS). The measurement of TBARS thus gives an index of free radical activity. Radicals scavenging by the compound resulted in the inhibition of TBARS. This will show antioxidant activity.

% of TBARS Inhibition= O.D. of sample /O.D. of blank X 100. The % inhibition was plotted against respective concentrations used and the graph IC_{50} .Was calculated by using ascorbic acid potential antioxidant, were used as a positive control.

Data analysis: The difference in the incidence of tumour among different groups were considered to be significant at 5% significance level ($p<0.05$) when evaluated by student't' test.

Results and Discussion

The results of the anticarcinogenicity study of *Centella* extract shows that Single topical application of DMBA followed by croton oil 2days/week for 16 week produced skin Papillomas which started appearing from 7 th week (53 days) onwards. The incidence of tumors reached 100% and the cumulative number and mean no. of papillomas in DMBA+ Croton oil were recorded as 14 and 2.33.respectively

The papilloma was delayed and observed after 74 days in the group which received the treatment of DMBA + Croton oil + *Centella asiatica*. When *Centella* extract was topically applied to animals along with DMBA + Croton oil, the tumor incidence was found to be 66%.and 20 % in dose of 500 and 1000 mg/kg b.wt. respectively and mean number of Papillomas were recorded as 0.83 and 0.20 respectively, these difference were observed to be

significantly decreased than DMBA + croton oil. DMBA, Croton oil, solvent (vehicle control) induced no tumour till the end of the experiment.

TABLE 1

Effect of *Centella* Extract on DMBA induced Papillomas in Swiss albino mice

	No. of Papillomas				
Groups	**4th week**	**8th week**	**12th week**	**16th week**	**Mean no. of papillomas**
Untreated	-	-	-	-	-
Vehicle control	-	-	-	-	-
Croton oil** alone	-	-	-	-	-
DMBA* alone	-	-	-	-	-
Centella *** ext. alone	-	-	-	-	-
DMBA* + Croton oil**	-	2/5 (6)	3/5 (8)	4/5 (19)	3.8
DMBA* + *Centella ext**** +croton oil**	-	1/6(1)	2/6(3)	4/6(5)	0.8
DMBA* + *Centella ext.***** + croton oil**	-	1/5	1/5(1)	1/5(1)	0.20

No in brackets denotes the cumulative no. of papillomas

* Single application of DMBA was given at the dose of 104 µg/animal (4 mg/kg b.wt.)

**1 % croton oil was given after each application of *Centella extract.*

****Centella* extract at the dose of 500 mg/kg body weight was given one hour before the each application of croton oil.

*****Centella* extract at the dose of 1000 mg/kg body weight was given one hour before the each application of croton oil

TABLE NO 2

Effect of *Centella asiatica* in skin papilloma model

Group	Dose	% of Papiloma in weeks				Total papillomas/No.of animals
		4th	8th	12th	16th	
DMBA* + Croton oil	104µg/animal+1%	0	40	60	80	19/5
DMBA* + *Centella* *** +Croton oil **	104µg/animal+1% +500mg/kg	0	16	33	66	5/6
DMBA*+*Centella* **** + Croton oil **	104µg/animal+1% +1000mg/kg	0	20	20	20	1/5
DMBA* alone	104µg/animal	0	0	0	0	0/6
Croton oil alone **	1%	0	0	0	0	0/6
Solvent (acetone)	100µl	0	0	0	0	0/6

* Single application of DMBA was given at the dose of 104 µg/animal (4 mg/kg b.wt.)

**1 % croton oil was given after each application of centella extract.

****Centella* extract at the dose of 500 mg/kg body weight was given one hour before the each application of croton oil.

Effect of *Centella* extract on antioxidant activity

The percentage of antioxidant activity of *Centella* extract and Ascorbic acid are shown in table 3. In this study *Centella* extract at different concentration i.e.100-1000 µg/ml exhibited antioxidant activity.

This result indicates that herbal extract containing higher phenolic compounds may contribute to scavenge free radical. Antioxidant compound's like phenolic acids polyphenols and flavinoids scavenge free radicals, per oxide, lipid peroxide and thus inhibit the oxidative mechanism and lead to degenerative disease Many flavanoids has been established as a strong antioxidant principle. *Centella asiatica* significantly scavenge the hydroxyl radical generated by EDTA/H_2O_2 When compared with ascorbic acid. The percentage scavenging of centella asiatica was increased in a dose dependent manner. $IC_{50\ value}$ of *Centella asiatica* was 600µg/ml and Vit.C 25µg/ml.

TABLE 3: Antioxidant activity of *Centella* asiatica extract.

Conc (µg/ml)	**OD *Centella asiatica***	**% TBARS**	**Concentration (µg/ml) Ascorbic acid**	**OD**	**% inhibition of TBARS**
100	0.002	0.6	5	0.092	35
150	0.008	2.5	10	0.104	40
200	0.078	4.5	15	0.118	45
300	0.060	19.3	20	0.128	49
400	0.074	24	25	0.130	50
450	0.078	25.1	30	0.212	68.3
500	0.098	31.6	40	0.251	81.4
600	0.168	54	50	0.375	121.7
700	0.174	56	60	0.449	145
750	0.192	62	70	0.498	159
800	0.196	63.2	80	0.558	180
900	0.220	70.9	90	0.493	183
1000	0.252	83	100	0.611	198

TABLE 4: IC_{50} Values of *Centella asiatica*

Treatment	**IC50 Value (µg/ml)**
Ascorbic acid	**25**
Centella asiatica	**600**

Discussion

Centella asiatica is traditionally a medicinal plant frequently employed in the practice of Thai folk medicine. **(**Bunpo et al.2005) examined the anti-tumor activity of the crude water extract of *C. asiatica* using human colon adenocarcinoma-derived Caco-2 cells.*C. asiatica* extract reduced the proliferation rate of Caco-2 cells significantly in a concentration- and time-dependent manner.

Moreover, previous report found that *C. asiatica* extract inhibited the formation of AOM-induced ACF in rat colons and had a chemopreventive effect on colon tumorigenesis. **(**15).

We examined single topical application of DMBA followed by 2days/week for 16 week croton oil produced skin papilloma which started appearing from 7 th week (53 days) onwards. The incidence of tumors reached 100% and the cumulative number and mean no. of papillomas in DMBA + Croton oil were recorded as 14 and 2.33.respectively.The papilloma was delayed and observed after 74 days in the group which received the treatment of DMBA+ Croton oil + *Centella asiatica*. When *Centella* extract was topically applied to animals along with DMBA + Croton oil, the tumor incidence was found to be 66%.and 20 % in dose of 500 and 1000 mg/kg b.wt. respectively and mean number of papillomas, were recorded as 0.83 and 0.20 respectively, these difference were observed to be significantly decreased than DMBA + croton oil.

evaluated protective effects of CA on antioxidant tissue defense system against adriamycin against cardiomyopathy in rats. (16)

The phenolic (Folin-Dennis) and flavonoid (colorimetric assay) constituents, antioxidant [2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) assay] and cytotoxic activities of aqueous extract (50 g/L) was obtained by infusion followed by cold maceration for 24 h(17). The levels of phenolic and flavonoid compounds were 2.86 g/100 g and 0.361 g/100 g, respectively. The AE showed elevated DPPH scavenging activity, with an IC_{50} value of 31.25 μg/mL. The AE had a promising activity against mouse melanoma ($B_{16}F_1$), human breast cancer (MDA MB-231) and rat glioma (C_6) cell lines, with IC_{50} values of 698.0, 648.0 and 1000.0 μg/mL, respectively. A positive correlation was established between the level of flavonoids, antioxidant and antitumor activities.

This result indicates that herbal extract containing higher phenolic compounds may contribute to scavenge free radical. Antioxidant compound's like phenolic acids polyphenols

and flavinoids scavenge free radicals , per oxide, lipid peroxide and thus inhibit the oxidative mechanism and lead to degenerative disease Many flavanoids has been established as a strong antioxidant principle. *Centella asiatica* significantly scavenge the hydroxyl radical generated by EDTA/H_2O_2 When compared with ascorbic acid. The percentage scavenging of centella asiatica was increased in a dose dependent manner.$IC_{50\ value}$ of *Centella asiatica* was 600µg/ml and Vit.C 25µg/ml.

Conclusion

Such protection was shown to be dose dependent as ascertained by the reduction of papilloma as well as the antioxidant potential was most prominent at a dose of 400 mg/kg centella extract. Further studies are required to determine the active ingredients responsible for the mechanism of antitumour activity of *C. asiatica* extracts.

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