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Evaluation of Anticarcinogenic and Antimutagenic Effects of Triphala Extract

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Abstract- The results of the present investigation showed that topical single application of DMBA. followed by 1 % croton oil produced skin papillomas. The incidence of tumours reached 100% and the value of cumulative number of papillomas in these animals were recorded 40 and the average number of papillomas per mouse (tumour yield) was found 6.6. The mice which received *Triphala extract* additionally showed a significant decrease in the number and incidence of tumour as compared with DMBA + croton oil group. When *triphala* extract was topically applied at the dose of 40 mg /kg body weight for 16 weeks to the skin of animals the tumor incidence was found 60% and the value of cumulative number of papillomas in these animals were recorded 6 and the average number of papillomas per mouse in this group was found 1.5. The differences in the values of results of the experiment was statistically analyzed and found significant in comparison to the control group at ($p< 0.05$). In another study the effect of triphala extract on B16F10 melanoma tumour bearing mice was also evaluated. The inhibition rate was 69 % in triphala extract groups group as compared to control group. The life span was also increased by *52 %* as compared to control group. The tumour volume of triphala treated mice was significantly reduced as compared to control. The result was found significant in comparison to the control group ($p< 0.05$).

In antimutagenicity studies, single application of *triphala extract* at the dose of 40, 80 and 120 mg/kg body weight, 24 hours prior the intraperitonial administration of Cyclophosphamide (at the dose of 50 mg/kg) have significantly prevented the micronucleus formations and chromosomal aberrations in dose dependent manner in bone marrow cells of mice as compared to Cyclophosphamide group. It may be concluded that triphala extract exert anticarcinogenic and antimutagenic effect in the present set of experiments.

I. Introduction

Triphala is a combination of the dried fruits of *Terminalia chebula, Terminalia belerica,* and *Emblica officinalis* in equal proportions. It is therefore making it useful as an internal cleansing, detoxifying formula (Jagetia et al,2002). This compound and its individual ingredients are highly valued in Ayurveda, and being compared to a "good manager of the house," aiding digestion, nutrient absorption and body metabolism. In a study it has been reported that Triphala possessed the ability to induce cytotoxicity in tumor cells but spared the normal cells (Sandhya et al, 2005 and 2006). Another report found that "Triphala" showed a significant cytotoxic effect on cancer cell-lines. It has been concluded that the action was due to gallic acid-a major polyphenol found in "Triphala". (Kaur et al ,2005) It was also effective in reducing tumor incidences and increasing the antioxidant status of animals. It was observed that Triphala was more effective in reducing tumor incidences as compared to its individual constituents (Deep et al,2005). Antioxidant studies conducted revealed that all three constituents of triphala are active and they exhibit slightly different activities under different conditions and the mixture, triphala, is expected to be more efficient due to the combined activity of the individual components (Naik et al, 2005). Similar results were also reported that "Triphala, an ayurvedic rasayana drug, protects mice against radiation-induced lethality by free-radical scavenging."(Jagetia et al, 2002) Triphala, may be potent and novel therapeutic agents for scavenging of Nitrous Oxide (NO) and thereby inhibited the pathological conditions caused by excessive generation of NO and its oxidation product, per oxynitrite. Since there is a paucity of information regarding anticarcinogenicity and antimutagenicity of triphala extract, we have therefore undertaken to study these parameter in the experimental animals.

II. Materials and Methods

Animals: Random bred male Swiss Albino and C57 Bl hybrid mice of (6-8 weeks old) of 15-20 gms body weight were obtained from the animal colony of our research centre. They were kept on controlled temperature (22 ^{0}C) and 12 : 12 hours light and dark cycle and were given synthetic pellet diet and water *ad libitum.* The experiment was approved by the institutional animal ethic committee before conduction of the experiments.

Chemicals: Cyclophosphamide was purchased from Sigma chemical Co., U.S.A. and other chemical were reagents grade and were procured locally for the study.

III. Preparation of Triphala extract.

Triphala was purchased from Dabur chemical co. The 50 gm powder was mixed in 50 % methanol and kept in separating funnel for 6 hours. The supernatant was collected and this process was repeated until clear solution of supernatant was obtained. All supernatant was pooled together and dried to the powder at 40 ^{0} C water bath. The final powder was soluble in water therefore it was dissolved in double distilled water before the each treatment at required concentrations.

(A) Experimental design

Skin Bioassay Protocol

Procedure

Experiments were performed as per the method reported by Berenblum (1975) and Sukumaran and Kuttan R. (1991). A group of 6 mice (Shaved on dorsal skin two days earlier) single application of 104 µg of DMBA in acetone (100 µl), was began 1 week after initiation Triphala extract (100 µl) were applied 1 hr. before each croton oil treatment. The extract was applied to the shaved area using the micropiptette. The experiment was continued for 16 weeks. Skin tumour formation was recorded weekly and the tumours greater than 1 mm in diameter were included in counting of total number of papillomas / mouse, tumour incidence and tumour yield was recorded if they persisted for two weeks or more.

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

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

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

Group 4 (Croton oil alone) 1 % Croton oil was applied on skin 2 times a week up to 16 weeks.

Group 5 (DMBA + Croton Oil) The animals were treated with a single dose of DMBA (104 µg/100 µl of acetone) over the shaven area of the skin of the mice. One week later, croton oil (1% in 100 µl of acetone) was applied as a promoter 3 times per week until the end of the experiment (i.e. 16 weeks).

Group 6 (DMBA + Triphala extract. **+ Croton Oil)** The animals were treated *with* Triphala extract (40 mg /kg) topically one week after DMBA (104 µg/100 µl of acetone) application, followed by the application of croton oil (1% in 100 µl of acetone thrice a week) until the end of the experiment (i.e. 16 weeks).

Group 7 (Triphala extract alone): - The animals were treated Triphala extract (40 mg /kg) without DMBA and Croton oil was given 3 times a week up to 16 weeks.

Each group's consists of 6 animals for skin model assay and the animals of all groups were kept under observation for gross and microscopic changes in skin.

(B) Melanoma model

Melanoma cell line was obtained from National Cell Science Research Centre, Pune and 5 lacks cells / animal were injected. After implantation of the melanoma cell line, animal were kept under observation and experiment was started after 10 days when the tumours were seen. The treatment was given orally for 30 days and tumour volume and survival time of each animal was recorded. The following groups were maintained.

Control Group: This group consisted of four mice. The melanoma cell line (B6F10) were injected subcutaneously (S.C.) in all four mice.

*Test Group***:** This group was divided into two sub groups. Each group consisted of four animals. The melanoma cell line was injected by S.C. route. The tumour bearing mice were orally given dose of 40 mg/ Kg body weight in 50 % methonolic extract of *Triphala.* as standardized by us in earlier experiments (Agrawal et al, 2009).

Antimutagenic study: The cytogenetic damage in the bone marrow cells were studied by chromosomal aberrations and micronuclei induction.

(i) Chromosomal aberrations analysis: For the chromosomal aberrations assay, the *Triphala extract* at different dose levels i.e. 40 ,80 and 120 mg/kg body weight in the volume of 0.2 ml was injected 24 hours before the treatment of cyclophosphamide. The positive control group received single i.p. injection of 50 mg/kg cyclophosphamide in 0.9% saline. Colchicine (4 mg/kg b.wt) was administered intraperitoneally 2 hours before the harvest of the cells. Animals were sacrificed by cervical dislocation and bone marrow cells were harvested. The slides were prepared essentially as per modified method of Preston *et al* (1987) for chromosomal aberrations and method of Schmid (1975) and standardized by us (Agrawal et al ,1998, 1999) for micronucleus evaluations. The femur was excised and the bone marrow was extracted in 0.56 % KCl. The harvested cells were incubated at 37^0C for 20 minutes and then centrifuged for 10 minutes at 1000 rpm. Cells were fixed in Carney's fixative (Methanol: Acetic acid, 3:1) and burst opened on a clean slide to release the chromosomes. The slides were stained with 5 % Giemsa solution for 15 minutes and then put in xylene and mounted with DPX. A total of 100 well spread metaphase plates were scored for chromosomal aberrations at a magnification of 1000 X (100 x 10 X) for each group. Different types of chromosomal aberrations such as chromatid breaks, gaps, pulverization, polyploidy, centromeric association etc. were scored and expressed as % chromosomal aberrations.

(ii) Micronuceus assay: The femur of mice was dissected out and the bone marrow was flushed out in HBBS solution as described by us earlier (Agrawal et al,1998) . The smear was made in precleaned slides, air dried and fixed in absolute methanol. The slides were stained with Maygrunwald and Giemsa stain. About 2000 cells were counted and number of micronucleated polychromatid and Normochromatid erythrocytes cells were scored. PCE/NCE ratio was also calculated. The data are presented in MNPCE+SE. The statistical significance was evaluated using Student's `t' test.

IV. RESULTS

The results of the present investigation have been summarized in Tables 1 and 2. Topical single application of DMBA at the dose of 104 mg/kg b. wt. followed by 1 % croton oil produced skin papillomas. The incidence of tumours reached 100% and the value of cumulative number of papillomas in these animals were recorded 40 and the average number of papillomas per mouse (tumour yield) was found 6.6.

The mice which received Triphala extract showed a significant decrease in the number and incidence of tumour as compared with that of the DMBA + croton oil group. When triphala extract was topically applied at the dose of 40 mg /kg b. wt. for 16 weeks to the skin of animals the tumor incidence was found 60% and the value of cumulative number of papillomas in these animals were recorded 6 and the average number of papillomas per mouse in this group was found 1.5 . The differences in the values of the results of experimental groups were statistically analyzed and found to be significant in comparison to the control group at ($p< 0.05$).

Histopathology of skin tumour

The animals which received the treatment of DMBA + Croton oil for 16 weeks showed the infiltration nests of neoplastic squamous epithelium . The tumour cells exhibited a high nuclear cytoplasmic ratio. Moderate cytoplasm and dense clumped chromatin were also seen. Adjacent epithelium showed marked hyperkeratosis. This is suggestive of kerating squamous cell carcinoma grade II. When the triphala extract was applied along with the DMBA and Croton oil malignant tumours were not seen as compared to DMBA + Croton oil group. In the case of this group only four mice had tumours and the histopathology report suggest in these animals were having papillomatous hyperplasia, papilloma, extracellular keratin and epithelial hyperplasia with mild displasia was reported and the remaining animals showed the normal skin.

The study showed the effect of triphala extract on B16F10 melanoma tumour bearing mice. The preventive effect of triphala extract was calculated using the parameter of inhibition rate (IR), Increase in the life span (ILS), and Volume of tumour . The inhibition rate was 69 % in triphala extract treated group as compared to control group. The life span was also increased in 52 % as compared to control group. The volume of triphala treated mice was significantly reduced as compared to control. The differences in the values of the results of experimental groups were statistically analyzed and found significant in comparison to the control groups ($p< 0.05$).

In cytogenetic studies, single application of triphala extract at the dose of 40, 80 and 120 mg/kg dry weight, 24 hours prior the i.p. administration of Cyclophosphamide (at the dose of 50 mg/kg) have significantly prevented the micronucleus formations in dose dependent manner in bone marrow cells of mice as compared to Cyclophosphamide group (Table 4). The dose dependent protection was also observed in chromosomal aberrations assay in bone marrow cells of mice in triphala extract treated mice as compared to known mutagen, Cyclophosphamide treated groups (Table 5)

V. DISCUSSION

The present study demonstrated that when triphala extract was given one hour before the each application of croton oil, the incidence and the number of skin papillomas was significantly decreased. The reduction in tumour count may be due to effect in the promotional phase of tumourgenesis which prevent the reduction of free radicals (Huachen and Krystyn, 1991). Triphala becomes one of the highly potential herbal medicines in cancer treatment and prevention because all three compositions of Triphala have been found to possess notable anticancer properties (Sandhya et al., 2006a).Although very little is known about the mechanism by which these plants act against cancer cells. The mechanism of in vitro cytotoxicity and tumor growth reduction in vivo induced by Triphala seems to involve apoptosis induction. In addition, the components of Triphala may exert synergistic cytotoxic action on tumor reduction. Gallic acid is one of the major components of Triphala and capable of inhibiting cancer cell proliferation suggesting the key factor responsible for antimutagenic and cytotoxic effects of Triphala (Kaur et al., 2005). Polyphenols such as tannins and gallic acid, a component unit of hydrolysable tannins, are well known inducers of apoptosis in tumor cells (Inoue et al., 2000). Topical application of TPA (active constituent of croton oil) has been reported to increase production of free radicals (Huachen, and Krystyna, 1991). This is perhaps due to the free radical oxidative stress that has been implicated in the pathogenesis of a wide variety of clinical disorders (Das, 2002). Many antioxidants and anticarcinogenic compounds appear to have major effect on the detoxification of the carcinogens by the induction of Phase II detoxification enzymes since these enzymes divert carcinogens to react with critical cellular macromolecules (Prochaska et al., 1992). The anticarcinogenic effect of triphala extract suggests its role in chemoprevention of skin cancer. These results are important because this drug is used globally as a bowl cleaning agent. It may also be an important drug for chemotherapeutic treatment of cancer.

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Table 1: Cumulative no, of Papilloma on Triphala extract treated Swiss mice.

GROUPS	DOSE	TIME OF 1st APPEARANCE OF PAPILLOMA	CUMULATIVE NO. OF PAPILLOMA	MEAN NO. OF PAPILLOMA
DMBA* + Croton Oil**	104 µg/ animal + 1 %	On 29th day	40	6.6
DMBA* alone	104 µg/ animal	-	0	-
Croton oil**	1 %	-	0	-
DMBA* + Croton Oil** + Triphala ***extract	104 µg/ animal + 1 % + 40 mg/kg b wt	On 69th day	6	1.5
Triphala ***alone	40 mg/kg b wt	-	0	-

***** Single application of DMBA was given at the dose of 104 µg/animal (4 mg/kg b.wt.)
******1 % croton oil was given one hour before each application of Triphala extract.
*** Triphala extract at the dose of 40 mg/kg body weight was given one hour before the each application of croton oil.

Table2: Tumour incidence in mice treated with Triphala extract

GROUPS	NO.OF WEEKS			
	4th WEEK	8TH WEEK	12TH WEEK	16TH WEEK
DMBA* + Croton Oil**	1/6 (16 %)	3/6 (50%)	5/6 (83%)	6/6 (100%)
DMBA alone*	0/6	0/6	0/6	0/6
Croton oil**	0/6	0/6	0/6	0/6
DMBA* + Croton Oil** +Triphala extract ***	0/6 (0 %)	1/6 (16 %)	3/6 (50%)	4/6 (60%)
Triphala extract ***	0/6	0/6	0/6	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 one hour before each application of Triphala extract.
*** Triphala extract at the dose of 40 mg/kg body weight was given one hour before the each application of croton oil.

Table 3: Malenoma Skin bio Assay (Triphala extract treatment)

GROUP	DOSE (mg/kg b wt)	TUMOUR VOLUME (in mm)	MEAN SURVIVAL (in days)	ILS(%)	IR (%)
Untreated Group	-	1638 ± 345.5	17.5	-	-
Triphala ext	40	505± 17*	26.6	52	69.2

- * denotes stastical significance at p<0.05 when compared with untreated control group.
- Each group contains 4 mice

ILS = Increase in Life span , IR= Tumour Growth Inhibition Rate

Table No 4: Effect of Triphala extract on micronucleus formation in mouse bone marrow cells

Group	MNPCE+SE	PCE/NCE RATIO
Cyclophosphamide (50 mg/kg)	2.0 ± 0.816	0.69 ± 0.10
Triphala Ext. + CP (40 mg/kg + 50)	1.25 ± 0.50	0.79 ± 0.22
Triphala Ext. +CP (80 mg/kg + 50)	0.75 ± 0.49*	0.79 ± 0.18
Triphala Ext. + CP (120 mg/kg + 50)	0.60 ± 0.50*	0.89 ± 0.06
Triphala Alone (40 mg/kg)	0.75 ± 0.28	0.50 ± 0.06
Solvent (Water)	0.45 ± 0.03	0.549 ± 0.08

* denotes statistical significance in `t' test as compared to cyclophosphamide treated group at P <0.05

Table 5: Reduction of chromosomal Aberration by Triphala extract in mouse bone marrow cells.

S.N.	Treatment	Chromosomal Aberration (%)	Chromatid Break (%)	Chromatid Fragmentation (%)	Chomatid Gap (%)	Chromatid Ring (%)	Chromtid Association
1.	Cyclophosphmide (50 mg / kg)	63.90 ± 4.80	17	13	12	11	11

2.	Triphala Ext. + CP (40 mg/kg + 50)	22.40 ± 6.70*	4	5	3	3	8
3.	Triphala Ext. + CP(80 mg/kg + 50)	17.74 ± 3.80*	3	4	2	2	7
4.	Triphala Ext. + CP(120 mg/kg + 50)	13.07 ± 3.80*	2	3	1	1	6
5.	Triphala Alone (40 mg/kg)	9.08 ± 0.80 *	2	2	Nil	Nil	5

* denotes statistical significance in `t' test as compared to cyclophosphamide treated group at $P < 0.05$