

Anti tumor activity of mercaptopurine in combination with trikatu and gomutra on 20-Methylcholantrene induced Carcinogenesis

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ABSTRACT

Cancer is one of the most life threatening diseases and serious health problem in both developing and developed countries. Many synthetic and chemotherapeutic agents used in cancer therapy are having low bioavailability and involve the risk of life threatening host toxicity. Modern researchers are increasingly showing interest toward the improvement of bioavailability of a large number of drugs by addition of various herbs with bioenhancing properties. In oral drug delivery system, the co-administration of therapeutic agents with natural compounds possessing absorption improving activities, has also garnered great interest. Hence the present study was conducted to evaluate the anti-tumor activity of Mercaptopurine in combination with Trikatu and Gomutra. 20-Methylcholanthrene a Polycyclic aromatic hydrocarbons was used to induce tumor in albino mice. Haematological and endogenous antioxidant parameters were evaluated in the study. Individual treatment with Mercaptopurine (5mg/kg) and trikatu (100mg/kg) significantly restored the altered haematological and antioxidant parameters to normal values. Even Mercaptopurine (2.5mg/kg) at its sub therapeutic dose showed equivalent effects as that of therapeutic dose of Mercaptopurine (5mg/kg) when it was co administered along with trikatu compared to the positive tumor control group.

INTRODUCTION

Cancer is one of the major public health problems worldwide and accounts for an estimated 2.5 million cases in India alone (Devi, 2009). In the wake of resistance to chemotherapy and the escalating toxic effect of synthetic drugs/compounds, all possible avenues are being explored to develop new and novel anticancer drugs that will overcome these limitations. One of the avenues is Phytotherapy, which is a recognized complementary and alternative therapeutic modality. Many cancer patients, who are already crippled with this disease, and further burdened by drug-induced toxic side effects, now turn to complementary and alternative medicines hoping for a better cure or at least palliation (Kang *et al*, 2005). Mercaptopurine is one of the antimetabolite antineoplastic agents with immunosuppressant properties. It has been widely used in the treatment of certain types of cancer, human leukaemia and inflammatory bowel disease. The bioavailability of oral Mercaptopurine at standard doses is very low, largely as a result of extensive first-pass metabolism by xanthine oxidase.

Treatment with Mercaptopurine is associated with many of the severe adverse side effects like alopecia, skin eruptions, reduced immunity, hepatotoxicity etc which are dose related (Goodman and Gillman, 2008). Natural compounds have been expected to play an important role either as chemo preventive or chemotherapeutic agents to fight against cancer. Therefore, the identification of new anti-cancer drug with low side effects on the immune system has become an essential goal in many studies of immunopharmacology. In oral drug delivery system, the co-administration of therapeutic agents with natural compounds possessing absorption improving activities, has garnered great interest. Various active components from natural compounds with bioenhancing properties are being isolated for their possible use along with modern medicines. Imperative organic compounds present in plants could exaggerate to diminish the toxicity caused due to chemotherapy. Trikatu is an Ayurvedic preparation containing an equal ratio of long pepper (*Piper longum*), black pepper (*Piper nigrum*) and ginger (*Zingiber officinale*). Piperine an amide alkaloid, from a different species of pepper is mainly responsible for enhancing the bioavailability of concurrently administered drugs. Literature has revealed a number of pharmacological activities of piperine (Gurpreet *et al*, 2011).

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Gomutra has been elaborately explained in Ayurveda as an effective medicinal substance with innumerable therapeutic properties particularly as a bioenhancer along with antibiotics, antifungal and anticancer drugs (Kekunda *et al.*, 2010). As a bioenhancer Gomutra has increased the potency of "Taxol" (Paclitaxel) against MCF-7, a human breast cancer cell line, in *in-vitro* assays. These milestone achievements highlight the potential role of cow urine, Trikatu in chemotherapy and enhance the efficacy and potency of other drugs.

MATERIAL AND METHODS

Animals

Healthy Swiss albino mice (28-32g) of either sex were used for the experiments. They were maintained under standard conditions (temperature $22 \pm 2^\circ\text{C}$, relative humidity $60 \pm 5\%$ and 12 h light/dark cycle). The animals were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellet diet and water *ad libitum*. Experiments were conducted between 10:00 to 15:00h. The Institutional Animal Ethics Committee approved the experimental protocol (SCP/CPCSEA/P14/F150/2011). All the animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the "National Academy of Sciences" and published by the "National Institute of Health".

Chemicals

All the chemicals and reagents used were of analytical grade and were purchased from M/S Sigma chemicals, USA.

Collection of the plant material

Dried fruits of Black Pepper (*Piper nigrum*), Long Pepper (*Piper longum*) and Ginger (*Zingiber officinalis*) were collected from local suppliers of Mangalore. Trikatu was prepared by mixing equal quantities of the powder of the dried fruits of *Piper nigrum*, *Piper longum* and rhizomes of *Zingiber officinale* and then sieved through muslin cloth. This was stored in airtight container for further processing (Rakesh and Sushil, 2003).

Preparation of extract

The aqueous extract was prepared by adding 40g of Trikatu churna in 200ml of distilled water and heated at 60°C for 2 hrs. It was then filtered through cloth and the filtrate was evaporated on sand bath. Dried extracts were stored in labeled sterile screw capped bottles. The extract was weighed in a required dose and dissolved in known volume of gomutra, separately for further treatment.

20-METHYLCHOLANTHRENE INDUCED UTERINE CARCINOGENESIS

Induction of Uterine cancer

Carcinoma was induced by delivering 20-methylcholanthrene (20-MC) into the uterine cervix through the

vaginal opening for 45 days. Cotton balls of small diameter moistened with 0.1ml of 0.5% 20-methylcholanthrene (20-MC) dissolved in acetone was inserted intravaginally until firm resistance indicated contact with the cervix and vaginal vault (Gagandeep *et al.*, 2005; Randolph and Charles *et al.*, 1965)

Experimental protocol

Virgin female Swiss albino mice of 5 to 6 week old weighing 28-30g were chosen for the study and divided into five groups of six each. Group I was served as normal control, Group II were uterine carcinoma bearing animals which served as the Tumor control.

Group III were the uterine cancer bearing animals treated with therapeutic dose of Mercaptopurine (5mg/kg) alone. Group IV were the tumor induced animals treated with Trikatu alone at the dose of 100mg/kg. Group V were the tumor induced animals treated with sub therapeutic dose of Mercaptopurine (2.5mg/kg) in combination with trikatu (100mg/kg) in gomutra. The treatment was started orally 24 hrs after the induction and continued for the duration of 45 days except tumor control group. The animals had free access to feed and water *ad libitum*.

On 45th day, after administration of the last dose, food and water was withheld. On 46th day the animals in each cage were anaesthetized and blood samples were collected by sino orbital puncture in sterilized tubes for the estimation of various haematological parameters. Then the animals were sacrificed by cervical dislocation for the estimation of liver biochemical parameters.

CHEMICALLY INDUCED SOLID FIBROSARCOMA TUMOR MODEL

Induction of fibrosarcoma

Fibrosarcoma was induced in Swiss albino mice by subcutaneous injection of the 5% suspension of 20-MC in paraffin oil. The animals were examined twice weekly for palpable tumors or other manifestations of malignancy.

Tumors which appeared in about four weeks after implantation were highly localized and were maintained by serial transplantation. The tumor was minced and suspended in normal saline. A suspension of about 1×10^6 cells in 0.5ml of saline were injected subcutaneously into the dorsal region of the animals. The transplanted tumor became palpable in 4-6 days time (Pallab *et al.*, 2011; JaiPrakash *et al.*, 2001).

Experimental Protocol

Inbred strain of Swiss albino mice of either sex weighing 28-32g were used to carry out the experimental trials. The animals were divided into 5 groups of 6 each.

Group I was served as normal control, Group II were Fibrosarcoma bearing animals after incubation period which served as the Tumor control. Group III were Fibrosarcoma uterine cancer bearing animals treated with therapeutic dose of Mercaptopurine (5mg/kg) alone. Group IV were the Fibrosarcoma

induced animals treated with Trikatu alone at the dose of 100mg/kg. Group V were the tumor induced animals treated with sub therapeutic dose of Mercaptopurine (2.5mg/kg) in combination with trikatu (100mg/kg) in gomutra. All the treatments were given orally 24 hrs after tumor induction and continued once daily for 30 days. On 30th day, after the administration of the last dose, food and water was withheld. On the 31st day the animals in each group were anaesthetized and blood samples were collected by sino orbital puncture in sterilized tubes for the estimation of various haematological parameters. Then the animals were sacrificed by cervical dislocation for the estimation of liver biochemical parameters.

Estimation of Haematological parameters

Blood was withdrawn from animals by retro orbital plexus method for the estimation of Haemoglobin content, Red Blood cell count, White blood cell count and differential leucocyte count of WBC from the blood smears of normal, Tumor control and treated groups. (Mohan H.2005).

Estimation of endogenous Antioxidant parameters

The liver was excised, rinsed in ice-cold normal saline solution. A 10% w/v homogenate was prepared and a portion utilized for the estimation of lipid peroxidation (LPO) (Niehaus and Samuelson *et al.*, 1968) and other portion of the same after precipitating proteins with TCA was used for the estimation of glutathione (GSH) (Ellaman *et al.*, 1958).

The remaining homogenate was centrifuged at 1500 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of superoxide dismutase (SOD), (Beauchamp and Fridovich *et al.*, 1971) catalase (CAT) (Sinha 1972) respectively.

Statistical analysis

All data were expressed as Mean \pm SEM. The statistical significance between groups were compared using One way ANOVA, followed by Dunnett's multiple comparison test.

RESULT

Effect on Haematological parameters

Haematological parameters in 20-MC induced tumor group animals were found to be significantly altered as compared to that of normal group in both the models. Hb content and RBC count in the tumor control group were significantly decreased whereas total WBC count was significantly increased in 20-MC control group compared with the normal group. In a differential count of WBC, the presence of Neutrophils increased, while the lymphocyte count and Monocytes decreased in the 20-MC control group. Individual treatment with Mercaptopurine (5mg/kg) restored these altered parameters approximately to the normal values. In the same way Trikatu at the dose of 100mg/kg also significantly restored these altered parameters approximately to the normal values. Combination therapy with sub therapeutic dose of Mercaptopurine (2.5mg/kg) along with trikatu showed very significant effects which is comparable to effect of Mercaptopurine (5mg/kg) at its therapeutic dose. (Table: 1 and Table 2).

Effect on antioxidant parameters

Lipid peroxidation levels indicated by TBARS were considerably higher in animals treated with 20-methylcholanthrene as compared to the normal animals. A significant decrease in the activities of glutathione and enzymatic antioxidants (SOD and CAT) were also noted after the administration of 20-methylcholanthrene.

Treatment with Mercaptopurine (5mg/kg) alone resulted in a significant decrease in levels of TBARS to near normal values. The activities of glutathione and enzymatic antioxidants were also significantly reversed to near normal values. In the same way Trikatu at the dose of 100mg/kg alone was found to be less effective in normalising the altered parameters than Mercaptopurine (5mg/kg). Mercaptopurine even at its sub therapeutic dose (2.5mg/kg) showed equivalent effects as that of therapeutic dose of Mercaptopurine (5mg/kg) when it was co administered along with trikatu. (Table: 3 and Table 4).

Table. 1: Effect of Mercaptopurine in combination with Trikatu and gomutra on Haematological parameters of 20-MC induced uterine carcinogenesis.

Parameters	Normal control	Tumor control	Tumor control +6-MP (5mg/kg)	Tumor control +Trikatu(100mg/kg)	Tumor control+6-MP (2.5mg/kg) + Trikatu (100mg/kg)
Hb content (g/dl)	12.28 \pm 0.14	6.18 \pm 0.36	11.97 \pm 0.14***	7.93 \pm 0.39**	10.47 \pm 0.19***
Total RBC count 10 ⁶ cells/mm ³	8.11 \pm 0.29	3.46 \pm 0.31	7.86 \pm 0.29***	5.20 \pm 0.26**	7.27 \pm 0.22***
Total WBC count 10 ³ cells/mm ³	6.44 \pm 0.09	18.29 \pm 0.12	7.01 \pm 0.08***	13.36 \pm 0.22**	9.03 \pm 0.14***
Monocytes (%)	2.01 \pm 0.06	1.16 \pm 0.09	1.87 \pm 0.06**	1.47 \pm 0.06*	1.57 \pm 0.08**
Neutrophils (%)	17.79 \pm 0.39	70.81 \pm 0.33	24.47 \pm 0.21***	60.91 \pm 0.30**	43.79 \pm 0.47**
Lymphocytes (%)	84.8 \pm 0.18	33.17 \pm 0.16	83.86 \pm 0.38***	46.50 \pm 0.34*	67.44 \pm 0.30**

Values are expressed as Mean \pm SEM. n = 6 for each group. * p <0.05, ** p<0.01, *** p<0.001. One way ANOVA followed by Dunnett's test compared to Tumor control.

Table. 2:Effect of Mercaptopurine in combination with Trikatu and gomutra on Haematological parameters of Fibrosarcoma bearing animals.

Parameters	Normal control	Tumor control	Tumor control +6-MP (5mg/kg)	Tumor control +Trikatu(100mg/kg)	Tumor control+6-MP (2.5mg/kg) +Trikatu (100mg/kg)
Hb content (g/dl)	12.30 \pm 0.14	8.43 \pm 0.14	11.63 \pm 0.40***	10.01 \pm 0.31**	11.13 \pm 0.24***
Total RBC count 10 ⁶ cells/mm ³	8.01 \pm 0.28	4.02 \pm 0.42	7.04 \pm 0.10***	5.89 \pm 0.16**	6.39 \pm 0.31***
Total WBC count 10 ³ cells/mm ³	6.59 \pm 0.10	17.02 \pm 0.27	7.18 \pm 0.13***	14.13 \pm 0.17**	9.11 \pm 0.05***
Monocytes (%)	2.02 \pm 0.05	1.08 \pm 0.05	1.84 \pm 0.04***	1.30 \pm 0.05*	1.45 \pm 0.03***
Neutrophils (%)	14.17 \pm 1.3	67.88 \pm 0.74	15.83 \pm 1.77***	23.82 \pm 1.62**	19.82 \pm 0.64***
Lymphocytes (%)	87.17 \pm 0.87	35.83 \pm 2.0	85.17 \pm 1.49***	68.83 \pm 1.88**	80.33 \pm 2.80***

Values are expressed as Mean \pm SEM. n = 6 for each group. * p <0.05, ** p<0.01, *** p<0.001. One way ANOVA followed by Dunnett's test compared to Tumor control.

Table. 3: Effect of Mercaptopurine in combination with Trikatu and gomutra on liver LPO, GSH, CAT, SOD levels of 20-MC induced uterine carcinogenesis.

Parameters	Normal control	Tumor control	Tumor control +6-MP (5mg/kg)	Tumor control +Trikatu (100mg/kg)	Tumor control+6-MP (2.5mg/kg) + Trikatu (100mg/kg)
LPO	0.058±0.01	0.378±0.02	0.112±0.01***	0.233±0.03**	0.148±0.02***
Abs at 535nm			(-70.35)	(-38.37)	(-60.88)
GSH	0.382±0.01	0.173±0.01	0.30±0.01***	0.25±0.02*	0.28±0.01**
Abs at 412nm			(+73.17)	(+46.15)	(+61.63)
SOD	1.52±0.07	0.083±0.01	0.505±0.04***	0.243±0.02**	0.356±0.02***
Abs at 560nm			(+83.49)	(+65.75)	(+76.63)
CAT	0.501±0.05	0.223±0.01	0.425±0.03***	0.338±0.01*	0.391±0.04**
Abs at 620nm			(+47.45)	(+33.99)	(+42.97)

Values are expressed as Mean ± SEM. n = 6 for each group. % Increase and % Decrease is shown in Parentheses. *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test compared to Tumor control.

Table. 4: Effect of Mercaptopurine in combination with Trikatu and gomutra on liver LPO, GSH, CAT, SOD levels of Fibrosarcoma bearing animals.

Parameters	Normal control	Tumor control	Tumor control +6-MP(5mg/kg)	Tumor control +Trikatu(100mg/kg)	Tumor control+6-MP (2.5mg/kg) + Trikatu(100mg/kg)
LPO	0.063±0.01	0.413±0.08	0.167±0.01**	0.243±0.01*	0.180±0.01**
Abs at 535nm			(-59.47)	(-41.12)	(-56.37)
GSH	0.372±0.03	0.190±0.01	0.348±0.01**	0.279±0.03*	0.313±0.02**
Abs at 412nm			(+78.63)	(+43.07)	(+60.59)
SOD	1.647±0.02	0.146±0.01	0.653±0.02***	0.255±0.02**	0.481±0.01***
Abs at 560nm			(+77.55)	(+42.48)	(+69.55)
CAT	0.526±0.05	0.25±0.01	0.445±0.02***	0.323±0.02*	0.370±0.03***
Abs at 620nm			(+74.50)	(+26.79)	(+48.82)

Values are expressed as Mean ± SEM. n = 6 for each group. % Increase and % Decrease is shown in Parentheses. *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test compared to Tumor control.

DISCUSSION

The reliable criteria for judging the value of any anticancer drug are prolongation of lifespan and disappearance of leukaemic cells from the blood (Oberling and Guerin *et al.*, 1954) Treatment with Mercaptopurine at their sub therapeutic dose of 2.5mg/kg in combination with Trikatu (100mg/kg) in gomutra demonstrated very significant reduction in WBC from blood of tumor bearing mice which is comparable to the effect of Mercaptopurine (5mg/kg) at its therapeutic dose It can therefore be inferred that sub therapeutic dose of Mercaptopurine (2.5mg/kg) in combination with trikatu (100mg/kg) in gomutra increased the life span of tumor bearing mice by preventing tumor progression. Usually the major problems in cancer chemotherapy are Myelosuppression and anaemia. The anaemia encountered in tumor bearing mice is mainly due to reduction in RBC or haemoglobin percentage and this may occur either due to iron deficiency or due to haemolytic or myelopathic conditions (Hoagland, 1982). Present study indicates that combination therapy with sub therapeutic dose of Mercaptopurine (2.5mg/kg) along with trikatu brought back the haemoglobin content and RBC count more or less to normal levels which is comparable to effect of Mercaptopurine (5mg/kg) at its therapeutic dose. This indicates that the test compounds possess protective action on haemopoietic system. These observations assume great significance as anaemia is a common complication in cancer and the situation aggravates further during chemotherapy since a majority of antineoplastic agents exert suppressive effects on erythropoiesis and thereby limiting the use of drugs. Free radicals and reactive oxygen species (ROS) are continuously produced in the human body. These oxygen species are the cause of cell damage and the progression of tumour cells to cancer cells (Niki *et al.*, 1991).

Increase in MDA levels and decrease in SOD, GSH and CAT activities described in tumors is regarded as markers of malignant transformation. Similar findings were observed in the tumor induced animals. Treatment with Mercaptopurine at the sub therapeutic dose in combination with trikatu in gomutra significantly elevated the reduced hepatic SOD, CAT and GSH levels and inhibited hepatic lipid peroxidation in tumor bearing mice which is comparable to the effect of Mercaptopurine (5mg/kg) at its therapeutic dose.

The lowering of lipid peroxidation, and increases in levels of SOD, catalase and GSH in treated groups indicates its potential as an inhibitor of tumor induced intracellular oxidative stress.

CONCLUSION

The findings indicate even sub therapeutic dose of Mercaptopurine (2.5mg/kg) in combination with Trikatu (100mg/kg) in gomutra showed antitumor activity which is comparable to antitumor activity of Mercaptopurine alone at its therapeutic dose (5mg/kg) in all tumor induced animal models. Adverse effects of Mercaptopurine are usually dose related. Trikatu in Gomutra co-administered with Mercaptopurine will augment the bioavailability; hence low dose of Mercaptopurine is required.

Thus, reduced dose of Mercaptopurine with Trikatu in gomutra can give possible protection against chemotherapy induced toxicity and it can also shorten the duration of therapy. Thus treatment also becomes cost effective, minimizing drug toxicity and adverse reactions. The study provides scientific basis for use of Trikatu in gomutra to enhance the therapeutic efficacy of the concurrently administered drugs.

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