RESEARCH COMMUNICATION

Clastogenic Potential of *Ruta graveolens* Extract and a Homeopathic Preparation in Mouse Bone Marrow Cells

Korengath C Preethi, Cherappally KK Nair, Ramadasan Kuttan*

Abstract

***Ruta graveolens* belonging to family Rutaceae has long been traditionally used as a medicinal plant as well as a flavoring agent in food. However, very little data are available on the toxicity of the plant. This report presents evidence on the genotoxic and clastogenic potential of an extract of *Ruta graveolens* and Ruta 200C, a homeopathic preparation. Various types of chromosomal aberrations were noted in bone marrow cells after treatment. The percentage of aberrated cells in 4the 00mg/kgb.wt extract administered group was found to be 21% and with 1000mg/kg.b.wt it was 31%. The 23% for the Ruta 200C treated group was also elevated as compared to the 3%for untreated animals. In addition, bone marrow cells had higher incidence of micronuclei induction when treated with the extract (400mg and 1000mg/kg body weight) and 20µl/animal Ruta 200C for 30 days. Administration of the extract (1000mg/kg.b.wt) over a period of 30 days also resulted in damage to cellular DNA as evidenced by comet formation where the comet parameters such as percentage DNA in tail, tail length, tail moment of the bone marrow cells were increased several fold over control values. The comet tail moment of the bone marrow cells increased from 4.5 ± 2.5 to 50.2 ± 25.2 after the extract treatment. Administration of 20µl/ animal Ruta 200C for 5 consecutive days increased the tail moment to 11.7 ± 2.9. These results indicate that *Ruta graveolens* and Ruta 200C may induce genotoxicity in animals.**

Key Words: Chromosomal aberration - comet assay - homeopathy - micronuclei - *Ruta graveolens* - genotoxicity

Asian Pacific J Cancer Prev, **9**, 763-769

Introduction

Ruta graveolens (common name - rue), a folklore plant of family Rutaceae is traditional medicine for various ailments including hysteria, gastrointestinal disorders, and menstrual problems. Ancient Egyptians and early Greeks used rue to improve eyesight. The juice of fresh rue has been used to relieve toothaches and earaches. In Chinese medicine, rue is used to eliminate intestinal worms. It has been reported to possess antifungal (Oliva et al., 2003), antibacterial (Ojala et al., 2000), anti-inflammatory (Raghav et al., 2005), antitumour (Preethi et al., 2006) and cytotoxic activities (Ivanova et al., 2005). Ruta has been reported to be useful for the treatment of multiple sclerosis (Bohuslavizki et al., 1992) and also possess hypotensive activity (Chiu and Fung, 1997). Rue oil has a flavor similar to the bitter oil in orange or lemon rinds which are used in cosmetics and foods. Fresh rue leaves are sometimes added to mixed salads, used in making pickles or put in cooked dishes for a bitter taste and flavour. In Italy, rue is used to flavor grappa, a type of brandy.

Rue was reported to be toxic to goats at high concentrations (El Agraa et al., 2002). Rutin, which constitutes about 2% of the extract, is one of the major components in the plant. Rutin and its hydrolysis product quercetin were reported to be genotoxic and possess prooxidant activity (Sahu and Gray, 1996; Da Silva et al., 2002). Although there are some reports on the toxicity, no data is available on the genotoxic and clastogenic effect of this plant extract in animal models.

Alternative system of medicine is gaining more popularity among common people world wide. Homeopathic preparation of Rue has been found to be useful in several disease conditions including cancer. Ruta 6 has been reported to cure intra cranial cysticercosis (Banerji and Banerji, 2001) and Pathak et al has reported the effectiveness of Ruta 6 on regression of the growth of human glioma brain cancer cells in patients. It was found that Ruta induces severe telomere erosion in MGRI brain cancer cells (Pathak et al., 2003). Ruta 200C is a homeopathic preparation made from *Ruta graveolens* which has been potentiated (a process of repeated agitation for diluting a substance) 200 times. In our previous study Ruta 200C was found to inhibit chemically induced carcinogenesis in animal model and was also found to be effective against transplanted tumors (Harikumar et al, 2006; Sunila et al, 2007a). We also reported that Ruta 200C was capable of producing cytotoxic effect to L929 cells *in vitro* and inducing apoptosis in Dalton's lymphoma ascites tumor cells (Sunila et al, 2007b).

*Department of Biochemistry, Amala Cancer Research Centre, Amala Nagar, Thrissur-680555, Kerala, India *For correspondence: amalaresearch@rediffmail.com, amalaresearch@hotmail.com*

In order to elucidate a mechanism of action of this drug, we have evaluated genotoxic activity of the extract of Ruta graveolens and Ruta 200C invivo in mice model by cytogenetic studies on chromosomal aberration and micronuclei and genomic DNA damage by comet assay.

Materials and Methods

Chemicals

High melting point agarose, low melting agarose and propidium iodide were purchased from Sigma Aldrich Inc. USA. Histidine, biotin, nicotinamide adenine diphosphate (NADP), colchicine, glucose-6-phosphate and agar were from Sisco Research Laboratories, nutrient broth from Himedia Laboratories, Mumbai, andMay Grunwald and Geimsa from Merck, India. All other reagents and chemicals used were of analytical reagent grade.

Drug

Ruta graveolens was purchased from local market and identified by Dr.SheejaTTharakan, Botanist, Amala Cancer Research Centre, Thrissur. A voucher specimen with number Ru02 was deposited at the herbarium of Amala Cancer Research Centre, Amala Nagar, Kerala, India after authentication. Fresh leaves and tender stem (100gms) of *Ruta graveolens* were blended to a paste in a mixer and then extracted first with 1m000ml of 75% methanol by soaking overnight with occasional stirring. The supernatant was decanted and the residue was again extracted with 500ml 75% methanol. The supernatants were pooled and concentrated in a vacuum evaporator at 42˚C (yield was found to be 8.3%). The concentrated extract was resuspended in water for the study. Ruta 200C was purchased from Willmar Schwabe, Germany.

Animals

Female Swiss albino mice of 4 week old (20-25 gm) were obtained from Small Animal Breeding Station, Kerala Agriculture University, Mannuthy, Thrissur, Kerala, India. They were maintained in well-ventilated cages with normal mouse chow (Sai Durga food and feed, Bangalore, India) and water ad libitum. All the animal experiments were done as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, and implemented through the Institutional Animal Ethical Committee of the Research Centre.

Radiation

Whole body irradiation was given using Cobalt-60 Theratron Phoenix Teletherapy Unit (Atomic Energy Ltd, Ottawa, Canada). Animals were kept in specially constructed restraining boxes with a capacity of holding 7 animals at a time and exposed to whole body gamma rays in a field size of 25x 25 cm2 and at a distance of 80 cm from the source at a dose rate of 1.44 Gy/ minute.

Determination of clastogenic effect of Ruta graveolens and Ruta 200C.

For induction of chromosomal aberrations by Ruta graveolens and Ruta 200C Swiss albino mice were divided into 5 groups of 3 animals each, as follows:

Group I : Normal, without any treatment.

Group II : Positive control (whole body gamma irradiated; 3Gy).

Group III: Treated with Ruta graveolens extract orally via gavage (400mg/kgb.wt).

Group IV: Treated with Ruta graveolens extract orally via gavage (1000mg/kgb.wt).

Group V: Treated with Ruta 200C, 20µl/animal (oral).

Groups III to V animals were treated with the drug as one single dose everyday for a period of 1 month. Forty-eight hours before sacrifice the group II (positive control) animals received a dose 3Gy gama radiation in a single whole body exposure. All the animals were injected intraperitoneally with colchicine (2mg/kgb.wt, i.p) 90 minutes before sacrifice to cause mitotic arrest. The bone marrow cells were collected in phosphate buffered saline (PBS) containing 10% FCS and processed for chromosomal aberration studies (Josely and Kuttan, 1996). Four slides were prepared from each animal and stained with Geimsa and observed under oil immersion and screened for metaphase chromosomes. A minimum of 100 metaphase spreads were scored for aberrations.

Induction of micronuclei by Ruta graveolens and Ruta 200C:

Swiss albino mice were divided into 5 groups of 6 animals each. They were grouped as follows:

Group I : Normal without any treatment.

Group II : Positive control (whole body gamma irradiated; 1.5Gy).

Group III: Treated with Ruta graveolens extract orally via gavage (400mg/kgb.wt).

Group IV: Treated with Ruta graveolens extract orally via gavage (1000mg/kgb.wt).

Group V: Treated with Ruta 200C, 20µl/animal (oral).

The animals were treated with the drug as one single dose everyday for a period of 1 month. The group II positive control animals received 1.5 Gy gamma radiation in a single whole body exposure. The animals were sacrificed by cervical dislocation and bone marrow preparations were made (Schmid, 1975; Thresiamma et al., 1998). Four slides were prepared from each animal and stained with May Grunwald Geimsa (MGG). Two thousand polychromatic and corresponding normochromatic erythrocytes were scored for the presence of micronuclei from each animal.

Determination of effect of Ruta graveolens and Ruta 200C on bone marrow DNA by comet assay.

Swiss albino mice were divided into 3 groups as follows:

Group I : Normal without any treatment. (n=2)

Group II : Treated with Ruta graveolens extract orally via gavage (1000mg/kgb.wt (single dose/animal/day)). (n= 8).

Group III: Treated with Ruta 200C, 20µl/animal/day (orally) (n=4).

Two animals each were sacrificed on 5th, 10th, 20th and 30th day of extract treatment. On 5th and 30th day two animals were sacrificed from Ruta 200C treated group.

Table 1. Effect of *Ruta graveolens* and Ruta 200C on Chromosomes of Mouse Bone Marrow Cells

Group	Chromatid		Chromosome			% aberration
	Gap	Break	Gap	Break	Others	
Normal	0	0	0.0	2.0	1.0	3.0 ± 1.0
Radiation	2	3	5.0	11.3	23.3	44.7 ± 2.5*
Ruta graveolens 400mg/kg b.wt	0	0	0.0	1.3	20.3	21.0 ± 4.6*
Ruta graveolens 1000mg/kg b.wt	0	0	2.7	14.7	14.0	31.0 ± 3.6*
Ruta 200C 20µl/animal	0	0	6.7	11.3	5.3	23.3 ± 0.6*

*p<0.001

The bone amrrow was collected and the comet assay was carried out by the method described earlier (Singh, 2000) with some modification (Maurya et al., 2005). The stained images were visualized in a fluorescent microscope and were captured using a high performance camera. The images were analyzed using CASP software (Konca et al., 2003) to get percentage DNA in tail, tail moment, tail length etc. and were compared with that of normal.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Significance levels of comparison of differences were determined by one-way ANOVA followed by post-hoc Dunnett's test using Graphpad In stat 3 software.

Results

Effect of Ruta graveolens and Ruta 200C on induction of chromosomal aberration

Treatment of mice with an extract of Ruta graveolens was found to induce chromosomal aberration in the rapidly dividing mouse bone marrow cells. Normal value of 3% aberration were found to be increased to 21.0% in animals treated with 400mg/kg b.wt extract for one month and in 1000mg/kg b.wt extract treated group the percentage again increased to 31% ($p<0.001$) (Table 1). The predominant type of aberration in the extract treated group, was the formation of accentric fragments. Whole body exposure of animals to 3Gy gamma radiation resulted in an increase of chromosomal aberration to 44%.

Effect of Ruta graveolens on formation of micronuclei

There was significant increase in the number of micronucleated erythrocytes in the bone marrow cells of animals after treatment with 1000mg/kg b.wt of the extract (Table 2). The percentage of micro nucleated polychromatic erythrocytes (MNPCE) were 0.22 ± 0.10 and 2.03 ± 0.26 ($p<0.001$) for 400 and 1000mg/kg b.wt extract treated group of animals while in normals it was 0.13 ± 0.04. In the gamma-irradiated (1.5Gy) positive controls, the percentage of MNPCE was 2.5 ± 0.15. The percentage of micro nucleated normochromatic erythrocytes (MNNCE) was found to be 0.15 ± 0.06 and 0.47 ± 0.06 ($p<0.001$) for 400 and 1000mg/kg b.wt extract treated group of animals while it was 0.08 ± 0.03 for normal animals. The percentage of MNNCE in whole body gamma-irradiated positive controls was 0.33 ± 0.02. The P/N ratio was significantly decreased from normal level of 0.55 ± 0.02 to 0.37 ± 0.01 in 1000mg/kg b.wt extract treated group of animals.

There was also a significant increase in the number of micronucleated erythrocytes in the bone marrow cells of animals after treatment with Ruta 200C (Table 2). The percentage of MNPCE was 0.8 ± 0.18 ($p<0.001$) and MNNCE was found to be 0.36 ± 0.08 ($p<0.001$). The P/N ratio was significantly decreased to 0.43±0.04 in Ruta 200C treated group of animals which indicates it effect on DNA.

Effect of Ruta graveolens on bone marrow DNA evaluated through comet assay

Comet assay involving single cell gel electrophoresis, allows detection of DNA fragments resulting from a wide variety of DNA damage. It was found that the extract treatment did not induce any detectable comet formation in the bone marrow cells of mice on 5th, 10th and 20th day. But there was significant increase in all the comet parameters such as percentage DNA in tail, tail moment, tail length etc. when compared with that of the normal cells on 30th day of drug treatment indicating significant induction of DNA damage by the extract (Fig. 1 and Table 3).

In animals treated with Ruta 200C there was significant increase in all the comet parameters on day 5th itself indicating the induction of DNA damage by the Ruta 200C. There was massive degradation of DNA was seen after electrophoresis on 30th day (Table 3).

Discussion

In present study we report the potential genotoxic effect of Ruta graveolens, which is being used as flavoring agent as well as in medicine in many European countries. This drug is reported to possess several pharmacological

Table 2. Effects of *Ruta graveolens* and Ruta 200C on Micronuclei Formation in Mice Bone Marrow Cells

Group	% MNPCE	%MNNCE	%(MNPCE +MNNCE)	%PCE	P/N ratio
Normal	0.13 ± 0.04	0.08 ± 0.03	0.21 ± 0.06	35.5 ± 0.82	0.55 ± 0.02
Control (Radiation alone)	2.50 ± 0.15*	0.33 ± 0.02*	2.85 ± 0.14*	23.0 ± 0.70*	0.30 ± 0.01*
Ruta graveolens (400 mg/kg b.wt)	0.22 ± 0.10	0.15 ± 0.06	0.40 ± 0.09*	33.8 ± 2.50	0.51 ± 0.06
Ruta graveolens (1000 mg/kg b.wt)	2.03 ± 0.26*	0.47 ± 0.06*	2.50 ± 0.24*	26.9 ± 2.18*	0.37 ± 0.04*
Ruta 200C 20µl/animal	0.8 ± 0.18***	0.36 ± 0.08*	1.16 ± 0.24*	30.2 ± 1.90*	0.43± 0.04***

*p<0.001; MNPCE: Micronucleated polychromatic erythrocytes; MNNCE: Micronucleated normochromatic erythrocytes; PCE : Polychromatic erythrocytes

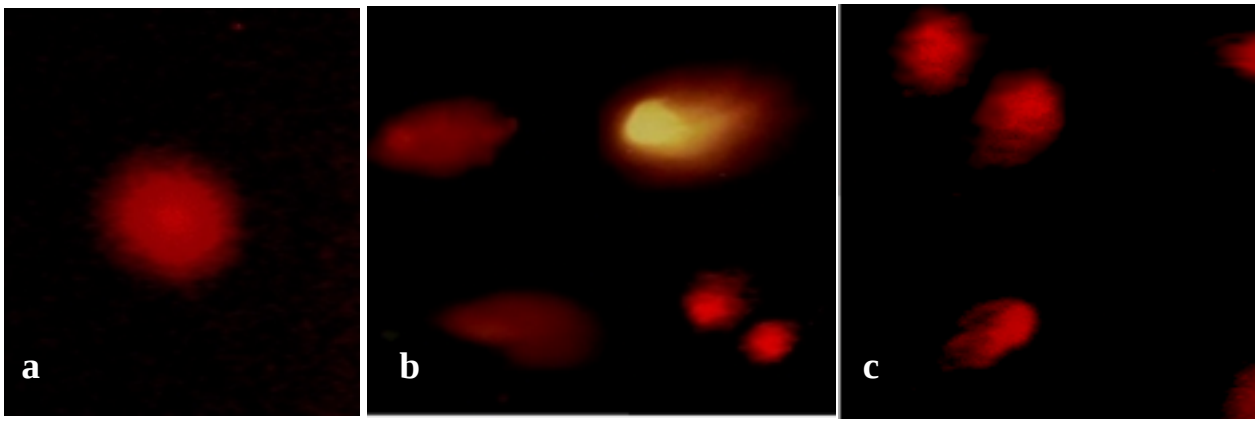

Figure 1. Effects of *Ruta graveolens* on Mouse Bone Marrow DNA as Detected by Comet Assay. a) Normal untreated cell; b) Treated with Ruta graveolens 1000mg/kg.b.wt showing comet; c) Treated with Ruta 200C (20µl/animal) showing comet

Table 3. Effect of *Ruta graveolens* and Ruta 200C on Mouse Bone Marrow Cells Detected by Comet Assay

Group	% DNA in tail	Tail length	Tail moment
Normal	22.0±7.4	19.0±6.4	4.5±2.5
Ruta graveolens (1000 mg/kg b.wt) on 30th day	73.1±16.6**	58.9±19.8**	50.2±25.2**
Ruta 200C 20µl/animal on 5th day	39.3±7.2*	29.1±2.9**	11.7± 2.9**

*p<0.01; **p<0.001

activities. The homeopathic preparation from this plant, Ruta 200C was also found to possess genotoxicity.

We have demonstrated the induction of DNA damage in bone marrow cells of animals treated with the extract at 1g/ kg.b.wt by comet assay. However 30 days of treatment was needed for comet formation indicating that long time exposure to the extract is needed to cause DNA damage. The chromosomal aberrations occur because of lesions in the DNA that lead to discontinuities in the DNA double helix. It was found that the feeding of the Ruta graveolens extract on long term lead to chromosomal aberration in the rapidly multiplying bone marrow cells and the most prominent type of aberration to be the formation of accentric fragments. The micronucleus, which is small nuclei in interphase in cells, formed from accentric fragments again confirmed its action on DNA and is consistent with the data obtained in chromosomal aberration assay.

There is one report on the toxicity of *Ruta graveolens* in goats (El Agraa et al., 2002). The major active principle is a flavonoid- rutin, which comprises 2% in the plant. The other active components present in the plant include alkaloids like coquisagenine, skimmianine and graveoline; furocoumarins (psoralens) like bergaptene, xantotoxine; essential oils include methyl-nonyl-ketone, methyl-n-octyl-ketone and methyl-heptyl-ketone. The other compounds present are limonins, dictamine, gammafagarine etc. The psoralens present in the plant are reported to be photo toxic (Wessener et al., 1999) and mutagenic (Paulini et al., 1987). They are also capable of binding to DNA to induce apoptosis (Furniss and Adam, 2007).

The flavonoid rutin and its hydrolysis product quercetin were reported to be genotoxic and possess prooxidant activity (Sahu and Gray, 1996; Da Silva et al., 2002). This prooxidant activity has been attributed to the apoptotic inducing property of these flavonoids and hence implicated in cancer chemoprevention (Chang et al., 2006; Colic and Pavelic, 2000). Rutin was also reported to possess protective effect on carcinogen induced DNA damage (Webster et al., 1996). In our study Ruta graveolens and Ruta 200C are found to produce ROS as well as to induce apoptosis in DLA cells (Unpublished data). It was also found that both the extract and Ruta 200C possess antitumor activity and could inhibit the chemically induced carcinogenesis in animal model (Preethi et al, 2006; Harikumar et al, 2007).

The free oxygen radicals generated through Fenton reaction induced by the extract may be causing DNA strand break. Many of the known anticancer agents exert their effect through their action on DNA to induce cytotoxicity (Mizutani et al., 2005; Alexandre et al., 2007).The present study indicate that Ruta graveolens and Ruta 200C produce genotoxic effect and apoptosis in tumor cells by generation of reactive oxygen species.

Acknowledgements

Authors are thankful to Mr. M. Muthuvel, Radiation safety Officer, Amala Cancer Hospital & Research Centre, Thrissur, India for providing necessary irradiation facilities. This work was funded by a grant from Samueli Foundation, USA.

References

Alexandre J, Hu Y, Lu W, Pelicano H, et al (2007). Novel action of paclitaxel against cancer cells: bystander effect mediated by reactive oxygen species. *Cancer Res*, **67**, 3512-7.

Banerji P, Banerji P (2001). Intracranial cysticercosis: an effective treatment with alternative medicines. *In Vivo*, **15**, 181-4.

Bohuslavizki KH, Hansel W, Kneip A, et al (1992). Potassium channel blockers from Ruta- a new approach for the treatment of multiple sclerosis. *Gen Physiol Biophysiol*, **11**, 507-12.

Chang YF, Chi CW, Wang JJ (2006). Reactive oxygen species production is involved in quercetin induced apoptosis in human hepatoma cells. *Nutr Cancer*, **55**, 201-9.

Chiu KW, Fung AY (1997). The cardiovascular effects of green beans (*Phaseolus aureus*), common rue (*Ruta graveolens*) and kelp (*Laminaria japonica*) in rats. *Gen Pharmacol*, **29**,

859-62.
Colic M, Pavelic K (2000). Molecular mechanisms of anticancer activity of natural dietetic products. *J Mol Med*, **78**, 333-6.
Da Silva J, Herrmann SM, Heuser V, Peres W, Marroni NP (2002). Evaluation of the genotoxic effect of rutin and quercetin by comet assay and micronucleus test. *Food Chem Toxicol*, **40**, 941-7.
El Agraa SE, El Balwi SM, Adam SE (2002). Preliminary observations on experimental *Ruta gravoelens* toxicosis in Nubian goats. *Trop Anim Health Pro*, **34**, 271-81.
Furniss D, Adams T (2007). Herb of grace: An unusual cause of phytophotodermatitis mimicking burn injury. *J Burn Care Res*, **28**, 1.
Harikumar KB, Sunila ES, Kuttan G, et al (2007).Inhibition of chemically induced carcinogenesis by drugs used in homeopathic medicine. *Asian Pac J Cancer Prev*, **8**, 98-102.
Ivanova A, Mikhova B, Majdenski H, et al (2005). Antimicrobial and cytotoxic activity of *Ruta graveolens*. *Fitoterapia*, **76**, 344-7.
Josely G, Kuttan R (1996). Lack of induction of chromosome aberrations and micronuclei by N (1,3-dimethylbutyl)-N-henylparaphenlenediamine in mouse and bone marrow cells. *Mutat Res*, **370**, 191-4.
Konca K, Lankoff A, Banasik A, et al (2003). Across platform public domain pc image analysis program for the comet assay. *Mutat Res*, **534**, 15-20.
Maurya DK, Salvi VP, Nair CKK (2005). Radiation protection of DNA by ferrulic acid under *in vitro* and *in vivo* conditions. *Mol Cell Biochem*, **280**, 209-17.
Mizutani H, Oikawa TS, Hiraku Y,et al (2005). Mechanism of apoptosis induced by doxorubicin through the generation of hydrogen peroxide. *Life Sci*, **76**, 1439-53.
Ojala T, Remes S, Haansuu P, et al (2000). Antimicrobial activity of some coumarin containing herbal plants growing in Finland. *J Ethnopharmacol*, **73**, 299-305.
Oliva A, Meepagala KM, Wedge DE, et al (2003). Natural fungicides from *Ruta graveolens* L. leaves including a new quinolone alkaloid. *J Agri Food Chem*, **51**, 890-6.
Pathak S, Multani AS, Banerji P, et al (2003). Ruta 6 selectively induces cell death in brain cancer cells but proliferation in normal peripheral blood lymphocytes: A novel treatment for human brain cancer. *Int J Oncol*, **23**, 975-82.
Paulini H, Eilert U, Schimmer O (1987). Mutagenic compounds in an extract from Rutae herba (*Ruta graveolens* L). I. Mutagenicity is partially caused by furoquinoline alkaloids. *Mutagenesis*, **2**, 271-3.
Preethi KC, Kuttan G, Kuttan R (2006). Antitumor activity of *Ruta graveolens* extract. *Asian Pac J Cancer Prev*, **7**, 439-43.
Raghav SK, Gupta B, Agarwal K, et al (2005). Anti-inflammatory effect of *Ruta graveolens* L. in murine macrophage cells. *J Ethnopharmacol*, **104**, 234-9.
Sahu SC, Gray GC (1996). Prooxidant activity of flavonoids: effects on glutathione and glutathione-S-transferase in isolated rat liver nuclei. *Cancer Lett*, **104**, 193-6.
Schmid W (1975). The micronucleus test. *Mutat Res*, **31**, 9-15.
Singh NP (2000). Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. *Mutat Res*, **455**, 111-27.
Sunila ES, Kuttan G, Preethi KC, et al (2007). Effect of homeopathic medicines on transplanted tumors in mice. *Asian Pac J Cancer Prev*, **8**, 24-6.
Sunila ES, Kuttan R, Preethi KC, et al (2007). Action of dynamized medicines in cell culture. *Evid Based Comple Alter Med*, doi: 10.1093/ecam/nem082.
Thresiamma KC, Josely G, Kuttan R (1998). Protective effect of curcumin, ellagic acid and bixin on radiation induced genotoxicity. *J Exp Clin Cancer Res*, **17**, 431-4.
Webster RP, Gawde MD, Bhattacharya RK (1996). Protective effect of rutin, a flavonol glycoside, on the carcinogen induced DNA damage and repair enzymes in rats. *Cancer Lett*, **109**, 185-91.
Wessener D, Hofmann H, Ring J (1999). Phytophotodermatitis due to *Ruta graveolens* applied as protection against evil spells. *Con Dermat*, **41**, 232.