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GENOTOXICITY EVALUATION OF PEO-PPO BASED STAR BLOCK COPOLYMER T1107 AND MICELLE ENCAPSULATED ANTIOXIDANTS BY CYTOKINESIS-BLOCK MICRONUCLEUS (CBMN) ASSAY

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ABSTRACT

In the present study, the *in vitro* genotoxicity of star shaped polyethylene oxide-polypropylene oxide (EO-PO) branched octablock copolymer Tetronic®1107 (T1107; mol.wt.: 15000 g.mol-1) was assessed in human lymphocyte culture using CBMN assay. The lymphocytes were exposed to free and micelle encapsulated antioxidants viz. curcumin (25 µg/ml) and quercetin (15 µg/ml) for 48 h and analysed by the CBMN assay. The micellar drug formulations were prepared in 15% aqueous solution of T1107. There were no statistically significant differences in the frequencies of MNBNCs between untreated and treated cells in either of the treatment used. This suggests that the block copolymer did not have potential genotoxicity.

KEYWORDS: Genotoxicity, CBMN, Tetronics®1107, Curcumin, Quercetin.

INTRODUCTION

Two hydrophobic plant derived natural polyphenolic antioxidants viz. curcumin and quercetin are rapidly gaining popularity due to their perceptible therapeutic activities in numerous diseases including cardiovascular disease, cancer, asthma, diabetes, neurodegeneration, aging and stress.[1-8] Curcumin is derived from the rhizome of turmeric plant (*Curcuma longa*), has been consumed for medicinal purposes for thousands of years[9], and is reported to have antitumor and anticancer activity. Curcumin has persuasive anticancer properties as demonstrated in a plethora of human cancer cell lines/animal carcinogenesis

model. It acts as a free radical scavenger and antioxidant[10] inhibiting lipid peroxidation and oxidative DNA damage.[11] It also lowers blood and liver cholesterol levels, boosts immune system, prevents damage to biological membranes against oxidation, reduces rheumatoid arthritis and is also anti-inflammatory.[8] Curcumin exerts dual actions, both as an antioxidant and cytotoxicant.[12] In addition to its cytotoxic anticancer effects, curcumin reduces oxygen free radicals, prevents lipid peroxidation, and attenuates DNA damage.[13] Quercetin is one of the most abundant dietary flavonoids present in fruits and vegetables.[14] Many biological and pharmacological activities that may be beneficial to human health have been attributed to quercetin, including antioxidant, anticarcinogenic, anti-inflammatory, and cardioprotective activities.[15,16]

Despite the therapeutic potential of these compounds, both curcumin and quercetin have low aqueous solubility that results into low bioavailability, poor permeability and extensive pre-systemic metabolism and thus hampers their use as therapeutic agents.[7,17] A waste numbers of nanocarriers are presently being employed for the delivery of drugs and nucleic acids. The advantages of nanocarriers as drug vehicles include improved solubility and stability of the drugs as well as enhanced uptake by targeted cells. Other unique properties of nanoparticles which allow them to be used as nanomedicine in the field of drug delivery, medical diagnostics, therapeutics and molecular targeting are small size, relatively large surface area, high reactivity and ability to cross blood-brain barrier.[18,19] Polymeric micelles are one of the most versatile nanocarriers to enhance the water solubility, the physicochemical stability and the bioavailability of poorly water soluble and instable drugs[20-22] and with the ability to be administered through variety of routes, such as oral, parenteral, ocular and intranasal, have been explored.[23-27] Thus formulation of curcumin and quercetin in micellar media is an attractive approach to overcome low solubility and limited oral bioavailability.[28]

There is a thriving development of nanoparticles materials for pharmaceutical applications, however studies on their genotoxicity are few.[29] Therapeutic nanoparticles are designed for human use and consequently have to withstand critical toxicological analysis, which plays a pivotal role in the decision on the future practical realization of the respective drug-delivery concepts. Nanotoxicology is still a maturing discipline that often lacks profound analysis of non-acute, sub-lethal effects.

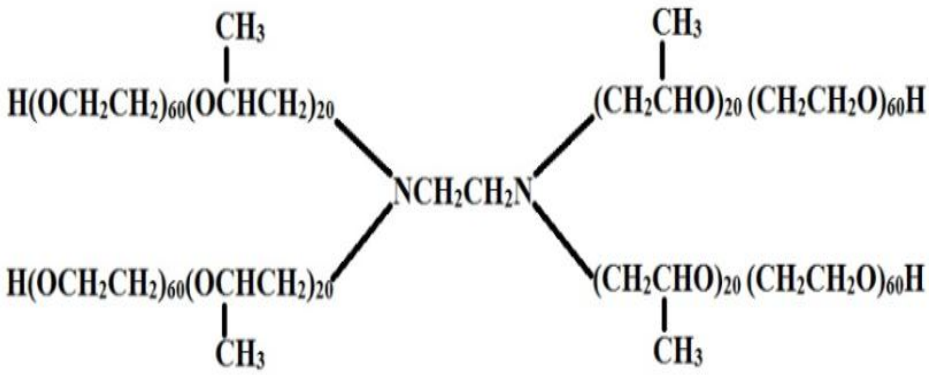
The measurement of MNi has been adopted worldwide as a biomarker of chromosomal damage for genotoxicity testing. The MNi may originate from acentric fragments

(chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division.[30,31] The CBMN assay for measurement of MNi is a sensitive and simple indicator of chromosome damage, both chromosome loss and chromosome breakage, which also provides information on cell cycle regression and cytotoxicity. Currently, this assay is mostly performed with Cyt-B (an inhibitor of actins), which allows the distinction between mono-nucleated cells, which did not divide, and bi-nucleated cells, which completed one nuclear division during *in vitro* culture. The number of nuclei per cell indicates the number of nuclear divisions that have occurred since the addition of Cyt-B. It is recommended that MNi are only counted in bi-nucleated lymphocytes, because these cells have completed one nuclear division.[32,33]

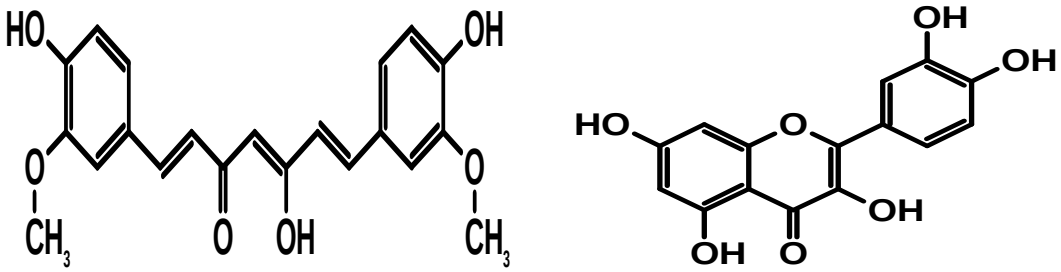
Here, polyethylene oxide-polypropylene oxide (EO-PO) based star shaped, octa block copolymeric micelle Tetronic®1107 (T11107, scheme 1) has been used to solubilize curcumin and quercetin. The present work focuses on evaluating the genetic damage by T1107 on human peripheral lymphocytes alone and with encapsulated antioxidants viz., curcumin and quercetin by means of CBMN assay.

MATERIALS AND METHOD

Materials: T1107 was received as a gift sample from BASF Corp. Parsippany, NJ, USA, and used without any further purification. Curcumin and quercetin were from Sigma Aldrich.



T1107 [Total mol. Wt = 15000 g.mol^{-1}, %PEO = 70, CP > 100 ºC, HLB 18-23, P_{ka} = 5.6].



Curcumin [$C_{21}H_{20}O_6$, mol. wt. 368.38]. Quercetin [$C_{15}H_{14}O_9$, mol. Wt. 338.27].

Scheme 1: Structures of T1107, curcumin and quercetin.[34]

Donors and blood collection

To assess the genotoxic effects, the study was performed on human peripheral lymphocytes of three healthy, non-smoking and randomly selected individuals of either sex aged between 20-30 years. Also care was taken that the participants were without pharmacological treatments for at least three weeks before donation and without any radiological examination performed within the previous three months. The study was approved by the Ethical committee of UTU. Participants were informed of the study aims. Approximately 4–6 ml of blood was drawn by venipuncture in heparinized vials.

CBMN Method

Human peripheral blood lymphocyte cultures were set up according to a standard protocol.[35] The CBMN assay was proceed with a modification of the Fenech method.[36]

Briefly, heparinised whole blood (0.6 mL) obtained by venipuncture from a healthy individual, were added to vials with 5.0 ml of RPMI 1640 (Hikaryo, ready mix). Cultures from each donor were incubated for 72 h at 37 °C. Exactly at 24 h after culture setup different treatments of DMSO, T1107, Curcumin, Curcumin encapsulated T1107 micelles, quercetin and quercetin encapsulated T1107 micelles were given. One tube without any treatment was kept as control. After 44 h of culture, Cyt-B was added at a final concentration of 6 µg/ml. After 72 h, the cultures were terminated by centrifugation at 1500 rpm for 10 min. supernatant was discarded and pellet were exposed to 5 ml of chilled hypotonic potassium chloride (0.075M KCl) for 5-9 min at room temperature followed by chilled fixative (3:1 v/v, methanol: acetic acid) washing till clear white pellet was observed. Obtained clear white pellet were suspended in about half ml of fixative for final slide preparation. For slide preparation, chilled slides were dropped with cell suspension from appropriate height with pasture pipette and air dried followed by immediate coding. Air-dried slides were stained in

Giemsa (2%) for 5-7 minutes. At least 1000 binucleated cells per donor were analysed for the frequency of MN at 100X magnification, according to the criteria described by Fenech.[36] The CBPI was calculated according to Suralles.[37]

Sample preparation

For the treatments of free curcumin and quercetin, solutions were prepared in DMSO. T1107 micellar solutions of both the drugs were prepared in 15% T1107 using sonication method, where both the drugs in respective tubes of 15%T1107 are added in the saturated amounts followed by sonication for 1 h and incubation for 24 h at 37°C. The solutions were then centrifuged and filtered through 22 µ filter. Amount of drugs micellized were analysed using UV-Spectrophotometer and required amount for the treatment is diluted further with blank solution of 15% T1107.

Treatment protocols

Venous blood samples were collected in coded heparinized tubes. Seven separate culture vials were set up from each of the three blood samples collected. First culture vial was kept untreated so as to act as control. After 24 h of initiation, rest of the culture vials were separately treated with DMSO (25 µl, vehicle control for free drugs), T1107 (15%, 25 µl, polymeric micelle), curcumin (25 µg/ml), quercetin (15 µg/ml), micellar curcumin (25 µg/ml) and micellar quercetin (15 µg/ml). At 44th hr, 6 µg/ml of Cyt-B was added to all four vials. The presence of MNi was recorded as per the standard guidelines.[33] Dose selection was done on the basis of recommended dietary allowance (RDA) for oral delivery of both curcumin and quercetin to healthy human subject that is 1-2 gm and 1 gm respectively and average weight of human taken into consideration was 70 kg.

Statistical Analysis: Data were analyzed by t-test, p value 0.05 was considered statistically significant.

RESULTS

In vitro genotoxicity of T1107 alone, and free as well as micellar drugs were evaluated by CBMN assay. Curcumin and quercetin dosage used were 25 µg/gm and 15 µg/gm respectively. Free drugs were given using DMSO as a carrier. The criteria by Fenech[38] was followed for the analysis of MNi. During scoring of slides, both cytotoxicity score as well as DNA damage indices were considered (Figure 1).

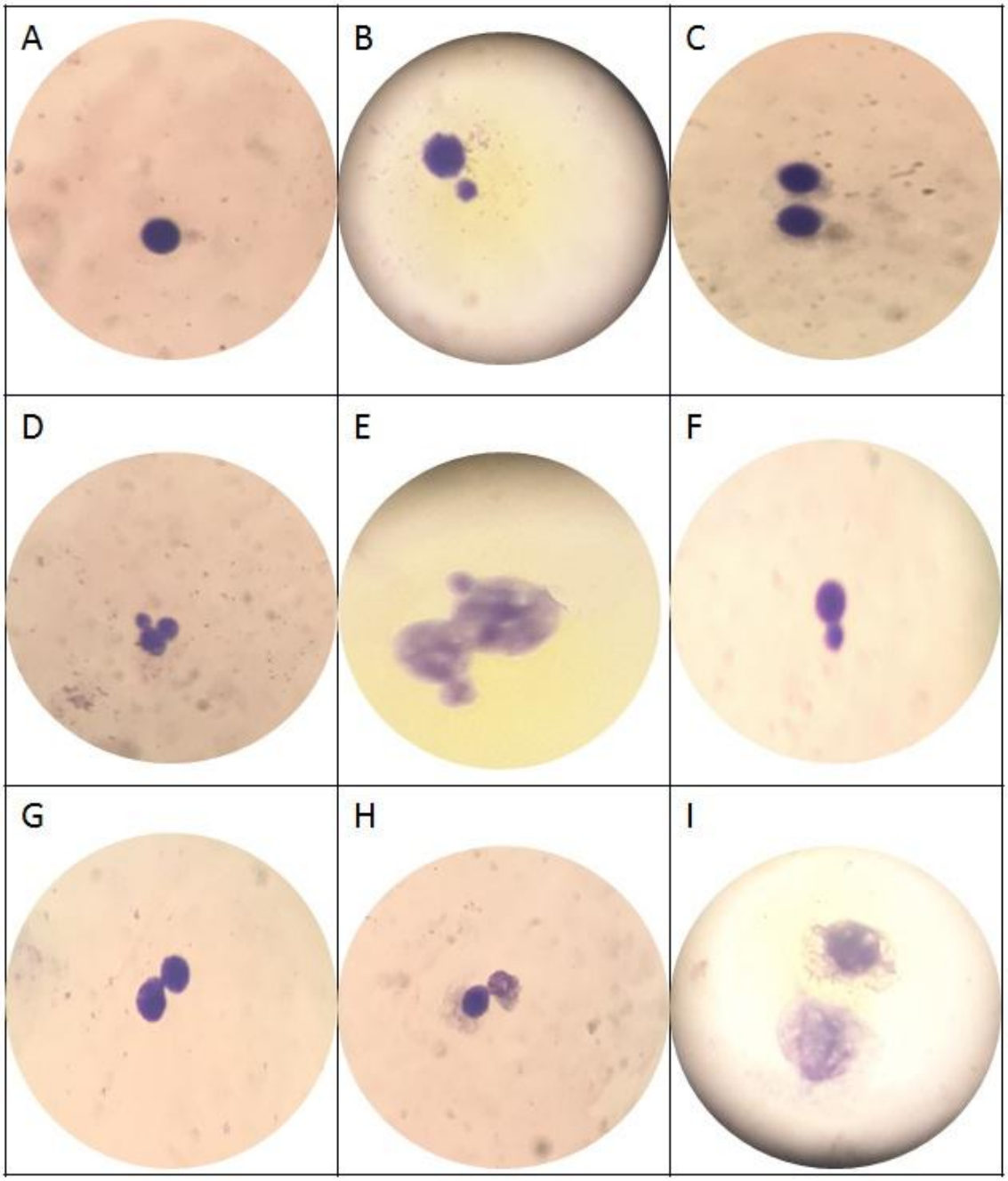


Figure 1: A: Mononucleated cell; B: Mononucleated cell with single micronuclei; C: Binucleated cell; D: Binucleated cell with single micronuclei; E: Binucleated cell with double micronuclei; F: Binucleated cell with nuclear bud; G: Binucleated cell with nucleoplasmic bridge; H: Apoptotic cell; I: Necrotic cell.

Cytotoxicity measurement parameters included number of BN, MLN, apoptotic and necrotic cells, while presence and frequency of MNi in either MN, BN or MLN cells were considered as DNA damage indices. Further number of cells with NPB and Nbud were also recorded**.** Results are presented in Table 1.

Table. 1: Genotoxicity profile of *in vitro* cultured human lymphocytes after drug treatment.

Treatment	Cytostatic/cytotoxicity score					DNA damage indeces in BN cells		
	Total no. of					Total No. of		
	Cells Scored	BN Cells	MLN Cells	Apoptotic Cells	Necrotic Cells	MNi	NPB	NBud
Control	967	930	32	3	2	18	16	11
%	100	96.17	3.31	0.31	0.21	1.86	1.65	1.14
DMSO	1024	984	36	3	1	21	17	15
%	100	96.09	3.52	0.29	0.10	2.05	1.66	1.46
T1107	1020	976	38	2	4	20	19	12
%	100	95.69	3.73	0.20	0.39	1.96	1.86	1.18
Curcumin	1034	960	66	3	5	87*	31	22
%	100	92.84	6.38	0.29	0.48	8.41	3.00	2.13
Micellar curcumin	1012	965	42	3	2	50	20	16
%	100	95.36	4.15	0.30	0.20	4.94	1.98	1.58
Quercetin	1021	940	72	4	5	90*	24	18
%	100	92.07	7.05	0.39	0.49	8.81	2.35	1.76
Micellar quercetin	1001	956	39	4	2	44	17	14
%	100	95.50	3.90	0.40	0.20	4.40	1.70	1.40

BN= Binucleated; MNi= Micronuclei; MLN= Multinucleated; NBud= Nuclear buds; NPB= Nucleoplasmic bridge.

At, $^{*}P<0.05$, statistically significant difference in comparison with control.

At the tested concentration, alone treatment of DMSO (vehicle) did not significantly induce any increase in micronuclei frequency as compared to control. Similarly individual treatment of T1107 did not alter the MNi frequency as compared to control. Separate treatments of both drugs significantly elevated the MNi frequency as compared to control, indicating genotoxic potential at the tested concentration. However, when cultures were treated with micellar drugs at the same concentrations, total MNi frequency remained statistically unaltered as compared to control. This indicates reduction in genotoxicity by encapsulated formulations of both tested drugs.

DISCUSSION

Assessment of DNA and chromosome damage is important because the damage influences cell functions, resulting in various diseases and aging. Among all, MNi assay has been widely used for revealing of aneugenic and clastogenic effects of harmful chemicals. MNi are formed at the cell division in anaphase stage when whole chromosome or fragment of chromosome breaks due to the lack of centromere.[39] According to Zeiger[40], before a

substance is claimed to have antigenotoxic/antimutagenic effects, it should also be evaluated for genotoxicity and rigorously tested with appropriate protocols.

A quick expansion of nanotechnology elevates several environmental, health, and safety concerns which should be understood, examined, and controlled.[18,19] The avoidance of cyto- and genotoxicity of nanocarriers is the main task in nanomedicine. The study of possible dangers of nanocarriers led to the materialization of two novel branches of bionanoscience: nanotoxicology and in recent times nanogenotoxicology.[41-44] Nanotoxicology involves the study of mechanisms of cytotoxicity of nanomaterials and nanogenotoxicology focuses on evaluating the potential of nanomaterials on DNA damage. Cytotoxicity of nanocarriers is relatively extensively investigated for different nanomaterials whereas genotoxicity of nanocarriers were analysed scantily.[41-53]

Genotoxicity of drug carriers can be circumvented if carriers are to be used for prolonged chronic treatment e.g. for the delivery of cytotoxic drugs (*e.g.* for chemotherapy of cancer) and are to be delivered specifically to the targeted site of action (*e.g.* tumor), then their own cyto- and genotoxicity is of fewer importance. However, when the carriers are employed for the delivery of non-cytotoxic drugs and may be gathered in healthy tissues, then it is decisively vital to prevent cytotoxicity of nanocarriers and their undesirable genetic effects. Very few reports are available for nanoparticle genotoxicity. Dendrimers have been accounted to induce not only cell death but also moderate genotoxic effects.[45] Genotoxicity of iron oxide nanoparticles has also been reported in recent times.[51]

Various PEO-PPO based amphiphilic block copolymers are biocompatible, biodegradable materials and being researched to be used in drug delivery and have been approved by FDA. However, there is only scarce knowledge about their behavior in cells and tissues and probable mechanisms underlying their toxicity.[54] Additionally potential genotoxicity of non-toxic nanoparticles has not been systematically assessed while evaluating the bioactivity and biocompatibility of materials. No reports have been found showing genotoxicity of PEO-PPO based block copolymers till now. In our previous study also they are not found to show any cytotoxicity against Caco-2[55] and CHO-K1[56] cell line. Over here the idea was to check the genotoxic effect of T1107 and whether they suppress or induce genotoxic behavior of the antioxidants curcumin and quercetin which are being used in higher concentrations to treat lymphocyte cells. But no significance difference has been found in MNi frequency between T1107 treated lymphocyte cultures as compared to control. Similar CBMN assay was being

carried out by Lili He et al.[29] on CHO cells using PLGA nanoparticles but no increase in frequencies of MN was found.

In vivo exposure studies of curcumin does not found to show any genotoxicity or clastogenicity, believed to be due to its antioxidant properties, which has been evaluated by micronuclei[57], chromosome aberrations[58,59], and recombination assays.[60] Other *in vivo* studies also do not show any genetic damage induced by curcumin.[57-61] Some of the studies even reported on genoprotection by curcumin.[61,62] Corona-Rivera *et al*[63] found protective effect of curcumin against copper induced genotoxicity in terms of micronuclei assay and comet in Balb-C mice. Polasa *et al.*[61], reported inhibition of benzo(a)pyrene-induced damage in the presence of curcumin by the comet assay. Shukla *et al.*[62] revealed the antimutagenic potential of curcumin towards benzo(a)pyrene- and cyclophosphamide-induced genotoxicity in microbial and mammalian test systems (chromosomal aberrations, micronuclei, and sister chromatid exchanges). In cultured human lymphocyte cells Shafaghati *et al.*[64] reported inhibitory effect of curcumin against 131-iodine induced micronuclei induction. Alaikov *et al.*[65] showed that curcumin has protective and anticlastogenic activity by enhancing the scavenging of free radicals against cisplatin. At the concentrations of 100 and 200 mg/kg body weight, curcumin has been shown to reduce the number of aberrant cells in cyclophosphamide- induced chromosomal aberration in Wistar rats.[62]

Curcumin was found to enhance *g*-radiation-induced chromosome aberration in Chinese hamster ovary. Curcumin has also been shown to be non-protective against hexavalent chromium-induced DNA strand break. Rather, the effects of chromium and curcumin correspondingly adds to DNA breaks in human lymphocytes and gastric mucosal cells.[62] Curcumin was also found to be weakly clastogenic in bone marrow cells of acutely treated mice.[58]

It has been found that at low concentrations of 1.0, 2.5 and 5µg/mL, curcumin is not genotoxic; however, at high concentrations of 10 µg/mL, curcumin presents genotoxic potential, as observed by the significant induction of MN in PC12 cells.[66] The genotoxic as well as antigenotoxic properties of curcumin on HepG2 cell line (a relevant *in vitro* model to detect the cytoprotective, antigenotoxic, and cogenotoxic agents) was detected at higher (8 and 16 µg/ml) and lower (2 µg/ml) levels. Results of MNi assay revealed that at the high tested concentrations, curcumin displayed a small but significant increase in the frequency of MNi; while at lower tested concentration, it significantly reduced the MNi formation induced

by the chemotherapeutic agent cyclophosphamide. This suggested that low level of curcumin is not clastogenic, whereas higher concentration of curcumin is genotoxic. Thus it can be predicted that curcumin shows both genotoxicity and antigenotoxicity depending on its concentration.[67] It has been found that low levels of curcumin did not increase the reactive oxygen species (ROS) formation and caused no damage to DNA in human HepG2 cells, but at high doses, curcumin imposed oxidative stress and damaged DNA in a dose-dependent manner.[67,68] Thus, it can be concluded that the anti-oxidant and pro-oxidant properties according to its concentration may be the main reason responsible for its biphasic effects i.e. genotoxicity and antigenotoxicity.[67,68]

In our study, curcumin was used at a very higher concentrations of 25 µg/ml, dose selected on the basis of recommended dietary allowance (RDA) considering the weight of healthy human weight of 70 kg. We found that free curcumin significantly increases the frequency of MNi, which is in agreement of published data. On the other hand, it has been found that T1107 micelle encapsulated curcumin was not showing any increase in frequency of MNi as compared to control culture.

Flavonoids are found to be potentially harmful compounds, as some have been shown to be mutagenic in bacteria and mammalian test systems.[69-71] Though molecular mechanisms of the mutagenicity are not known, different studies[69,70,72] demonstrated that they can act as pro-oxidants. This pro-oxidant activity is believed to deplete the nuclear antioxidant defense and lead to oxidative DNA damage, which may be responsible for their mutagenicity. Nevertheless, the records on genotoxicity of flavonoids are partial, and ambiguous results have been obtained with respect to mutagenicity observed with mammalian cell tests.[71,73]

Quercetin has been shown to be a steady *in vitro* mutagen and genotoxicant. In order to evaluate its mutagenic and genotoxic prospectives, quercetin has been widely tested under *in vitro* and *in vivo* experimental conditions. Quercetin has been shown to be cytotoxic and have DNA damaging ability in vitro.[74-78] Furthermore, quercetin has been reported to be carcinogenic.[79-81] Ambrose and co-workers[73] in 1952 reported toxicity of quercetin for the first time. On several studies, it has been known that relatively high concentrations of quercetin itself induce chromosomal damage or cytotoxicity, in contrast, low concentrations of quercetin prevent chromosomal damage induced by H_2O_2.[82]

Few of the studies on flavonoids reported the mutagenicity of quercetin in Ames test[69], which was believed to be due to its capacity to cause base-pair substitutions and frame-shift mutations, found to induce chromosomal aberrations and sister chromatid exchanges in CHO cells.[71] But on the other hand contradictory results were obtained in the micronucleus test in vivo.[69,70,73] DNA damage by quercetin may occur due to more than one mechanism.[83] Sahu and Gray[70] explained the reason for quercetin genotoxicity that is the production of reactive oxygen species by redox cycling. Quercetin and other phenolic compounds are found to give rise to the superoxide anion by auto-oxidation, which in turn may lead to the formation of H_2O_2[76] and consequently damage DNA.[84] Several other studies have also confirmed quercetin genotoxicity in various short-term assays[73,83], but no consistent results had been shown in relation to carcinogenicity.[85]

Under *in vitro* conditions, quercetin was found to consistently display positive mutagenic activity on the most standard strains of *Salmonella typhimurium.*[74,83,85-100] In *Escherichia coli*, quercetin has been found to induce SOS activity, reverse mutations, and DNA single strand breaks with or without metabolic activation.[87,94,99,101-105] These positive mutagenic and genotoxic responses in bacterial test systems have been confirmed in eukaryotic cells, including yeast[87] and mammalian cells (i.e., mouse, hamster, rat and human)[72,75,76,94,98,106-109], in which sister chromatid exchanges, chromosomal aberrations, unscheduled DNA synthesis (UDS), and micronucleus formation were among some of the endpoints evaluated.

Here, in this study also quercetin at the used concentration of 15µg/ml is found to increase MNi frequency as compared to control, which is again found to be in agreement with literature. However, while encapsulating quercetin in T1107 micelles, the treated lymphocyte culture did not show any significant increase in MNi frequency.

CONCLUSION

The results of present study indicate that above treatment of separate drugs at tested concentrations significantly increase the MNi frequency as compared to control. This suggests the genotoxic potential of both antioxidants curcumin and quercetin at higher concentrations. However, when the cultures were treated with same compounds at same concentrations in micellar form, there was no increase in MNi frequency. This makes T1107 micelle encapsulated drugs viable alternatives for using both the drugs in higher concentrations without any genotoxicity and hence suggests T1107 as a promising delivery system for curcumin and quercetin for clinical applications.

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