Original Article

Genotoxic effects of some l-[(benzofuran-2-yl)-phenylmethyl]-imidazoles on MCF-7 cell line

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Abstract

Increased exposure to estrogen has been associated with the risk of breast cancer. Substituted benzofuran derivatives with inhibitory effects on estrogen synthesis could be considered as a potential approach to reduce the risk of breast cancer. The study of cytotoxic effects of these compounds has suggested involvement of other mechanisms such as DNA damage. In the current study we have investigated genotoxic effects of some benzofuran derivatives on MCF-7 cell line. The MCF-7 cell line was exposed both to benzofuran phenylmethyl imidazole and its 4- fluoro, 4-chloro, 2-methoxy and 2-methyl derivatives for 2 h. The Comet assay was used to examine DNA damage due to this exposure. We also studied the DNA repair capacity after 2 h exposure to genotoxic concentrations of these compounds and their recovery were evaluated after 17 and 24 h, using the comet assay. The results indicated that genotoxic effects of these compounds appeared in concentrations of 10^{-8} to 10^{-6} M. The 4- fluoro and 4-chloro derivatives exhibited the highest genotoxicity and the unsubstituted benzofuran phenylmethyl imidazole had the lowest effect. The 4- fluoro, 4-chloro and 2-methyl derivatives were recovered after 24 h while 2-methoxy and the unsubstituted derivatives were recovered after 17 h. The results showed that these compounds are genotoxic and the concentration of tested benzofuran derivatives with genotoxic effects are not close to their enzyme inhibitory concentration. Moreover, our study shows that the DNA damages are repairable. Therefore, it seems that the investigated compounds have the potentials as therapeutic agents.

Keywords: DNA damage; Benzofuran-phenylmethyl imidazoles; Comet assay; MCF-7; DNA repair

INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer deaths in women (1,2). The involvement of estrogen in initiating and promoting breast cancer has been known for a long time (3,4). Inhibition of the synthesis or blocking the action of estrogens is an attractive strategy for therapeutic intervention. Antiestrogens inhibit the growth of breast tumors by competitive antagonism of estrogen at its receptor site (5). Whereas aromatase inhibitors block the enzyme aromatase that catalyses the conversion of androgens into estrogens, they can suppress plasma estrogen levels in postmenopausal women with estrogen receptor-positive tissues (6-8). Aromatase inhibitors have been used not only for advanced breast cancer, but also as optimal adjuvant hormonal therapy for these patients (7,9,10).

There are several generations of aromatase inhibitors in the market (11). Substituted l-[(benzofuran-2-yl)-phenylmethyl]-imidazoles are a new group of potent aromatase inhibitors which bind to active site of enzyme through nitrogen pair electrons of heterocyclic ring (12). Although these compounds have initially been synthesized as antifungal agents (13), further researches have shown that these compounds are more potent aromatase inhibitors than aminoglutethimide (14). Assessment of the cytotoxic effects of some l-[(benzofuran-2-yl)-phenylmethyl]-imidazoles showed that these compounds act as cytotoxic agents on MCF-7 and HeLa cell lines (13).

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For a new chemical to get into the market as a therapeutic agent, a thorough investigation of their safety profile and efficacy are required. Characterization of the basic toxicological profile of a new entity is one of the basic issues in drug safety evaluation. Genotoxicity assays have become an integral component of drug application process (15). To the best of our knowledge, benzofuran phenylmethyl imidazole derivatives genotoxicities have not yet been investigated .

In this study, genotoxic effects and DNA repair following the administration of some l-[(benzofuran-2-yl)-phenylmethyl]-imidazoles were investigated.

MATERIALS AND METHODS

MCF-7 (the human mammary carcinoma cell line) was purchased from Pasteur Institute of Iran. It was grown in a RPMI 1640 medium, which was enriched with 10% heat-inactivated fetal calf serum (FCS) and was mixed with 250 µl of penicillin/streptomycin to prevent the growth of unwanted bacterial microorganisms. Cells were cultured routinely in 75 cm^3 flasks in a humidified atmosphere of 5% CO_2 and 95 % air at 37°C (16). The stock solutions of 1 mM were prepared by dissolving the required amount of substituted l-[(benzofuran-2-yl)-phenylmethyl]-imidazoles in dimethylsulfoxide (DMSO) (13). Then the prepared medium was added to this solution to reach to a medium containing 10% DMSO. In order to prepare final concentration (100 µM), the stock solution was diluted 1:9 with RPMI 1640 medium. Before subculture, cultured cells were disaggregated using a trypsin/EDTA solution to form a homogeneous suspension. Then the homogeneous cell suspension (the number of cells estimated to be 10^6) and prepared RPMI 1640 containing 10% FCS and penicillin/ streptomycin were added to the wells of a plate with a ratio of 1:2 respectively. After addition of different concentrations of each derivative, plate was incubated at 37°C in 5% CO_2 for 2 h. A well containing medium and cell suspension (2:1 respectively) was used as control. A solvent control composed of cell suspension and medium containing maximum concentration of 1% v/v DMSO with a ratio of 1: 2. After incubation for 2 h period, the media was removed from each well and cells were disaggregated using trypsin/EDTA to form a homogenous cell suspension. Then the genotoxicity of these compounds against the MCF-7 cell line were evaluated using comet assay (17). Comet assay is a sensitive, reliable, and rapid method for DNA damage evaluation. The segmented DNA emigrates from the nucleus towards the anode under an electrical current to produce comets (Fig. 1).

Comet assay

About 400 µl of the cell suspension was mixed with 800 µl 1% low melting point agarose. Then 100 µl of the mixture was layered on slides precoated with a 1% normal melting point agarose and then covered with a cover slip. The slides were then placed in a refrigerator for 5 min. Then cover slips were
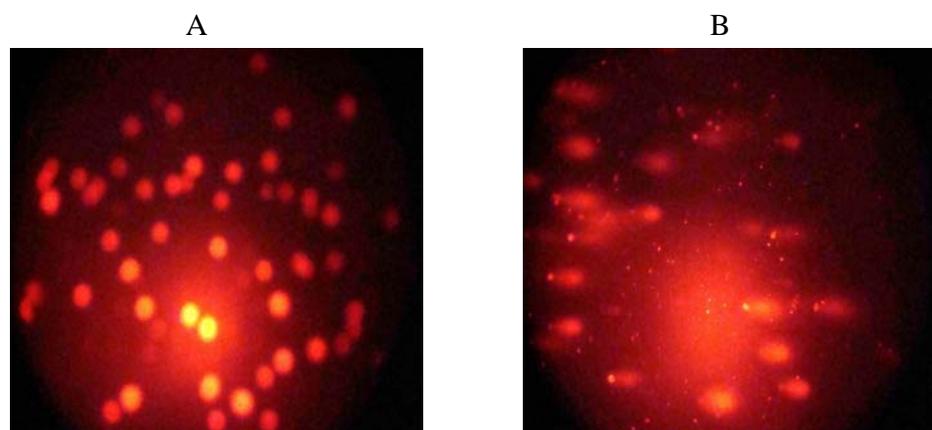

Fig. 1. A) Comet image of untreated MCF-7 cells as negative control. B) Comet image of MCF-7 cells treated with 200 µM H_2O_2 as positive control.

carefully removed and the slides were left in a dark place except for the positive control slides. The positive control slides were treated with 200 µM H_2O_2 solution for 17 min at 4°C, while the negative control slides were remained untreated. Then all the slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 0.2 M NaOH, 10 mM Tris, 1% Triton X-100, pH=10), protected from light and refrigerated at 4°C for 45 min. Slides were then left in freshly prepared electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH> 13,) for 40 min. Electrophoresis was performed using the same solution at 300 mA, 25 V for 45 min. After electrophoresis the slides were neutralized gently with 0.4 M Tris buffer at pH 7.5 for 10 min and stained with 20 µg/ml ethidium bromide for 7 min. Slides were washed and then dried. Two slides were used for each sample and photos were taken from at least 100 cells (50 cells for each slide) using a fluorescent microscope at 400 magnifications. The comet assay parameter and tail moment (TM) were measured and calculated by Comet score software (TriTek Corporation, USA, Version 1.5). Every experiment was repeated thrice (18).

DNA repair capacity

To evaluate DNA repair, after 2 h incubation with the genotoxic concentration of

each samples, the media in each well were replaced with freshly prepared media and was incubated at 37°C and 5% CO_2 for 17 and 24 h. Again the cells were disaggregated to form a homogenous cell suspension and were tested by comet assay as previously described (17).

Statistical analysis

ANOVA followed by Tukey test were used to compare the calculated means of comet tail moments with the respective negative control using SIGMASTAT software. Values were considered to be significantly different if P values were less than 0.05.

RESULTS

After 2 h treatment of MCF-7 cells with different concentrations of 4-fluoro (30, 50, 70, 100 nM), 4-chloro (25, 50, 75, 100 nM), 2-methoxy (100, 150, 200 nM) and 2-methyl (200, 250, 300 nM) benzofuran phenylmethyl imidazole and also its unsubstituted derivative (500, 750, 1000, 1250 nM), the genotoxic effects were evaluated by three parameters of comet assay method including percentage of DNA in the tail, tail length and TM. Since the TM is the product of percentage of DNA confined in the tail multiplied by tail length, this parameter is reported in Figs. 2-7. DNA repair capacity after 2 h exposure to the
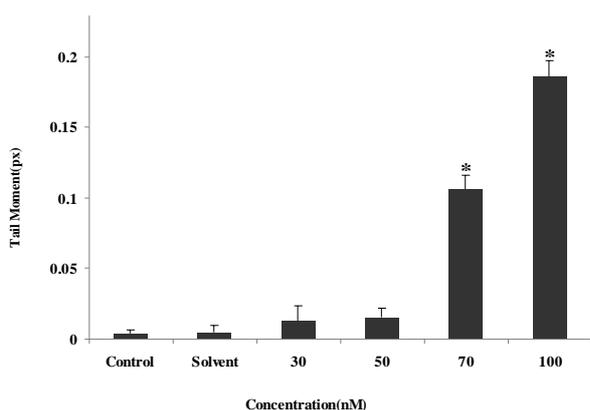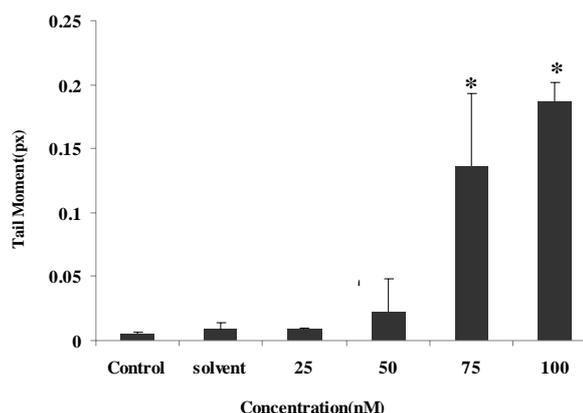

Fig. 2. Comet forming activity of 4- fluoro benzofuran phenylmethyl imidazole in MCF-7 cells. Comet assay, as described in methods, was used to test MCF-7 cells for DNA damage. Concentrations and solvent control were compared with negative control. Every experiment was repeated three times and tail moment was used as a measure of DNA damage. *: P<0.05 compared to the negative control.

Fig. 3. Comet-forming activity of 4-chloro benzofuran phenylmethyl imidazole in MCF-7 cells. Comet assay, as described in methods, was used to test MCF-7 cells for DNA damage. Every experiment was repeated three times and tail moment was used as a measure of DNA damage. *: P<0.05 compared to the negative control.
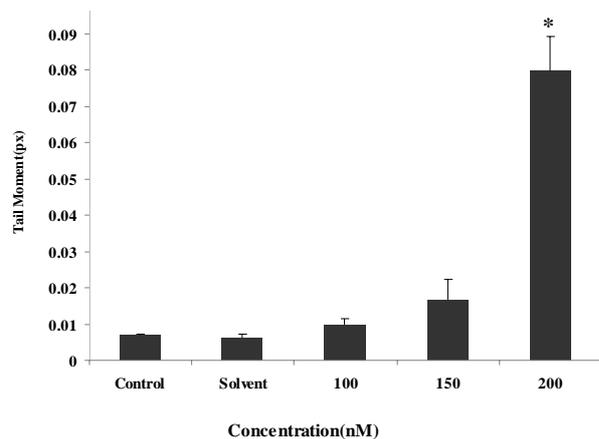
Fig. 4. Comet-forming activity of 2- methoxy benzofuran phenylmethyl imidazole in MCF-7 cells. Comet assay, as described in methods, was used to test MCF-7 cells for DNA damage. Every experiment was repeated three times and tail moment was used as a measure of DNA damage. *: $P<0.05$ compared to the negative control.
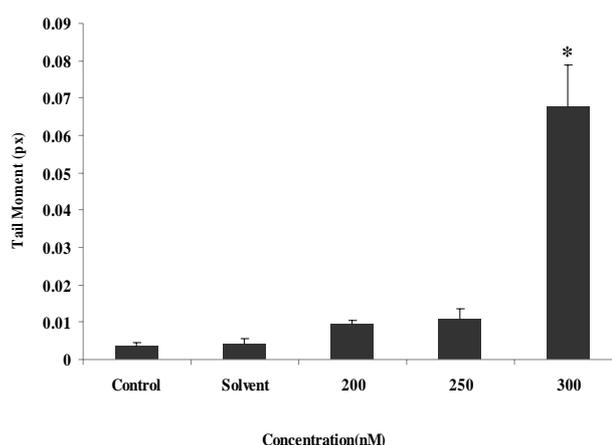
Fig. 5. Comet-forming activity of 2-methyl benzofuran phenylmethyl imidazole in MCF-7 cells. Comet assay, as described in methods, was used to test MCF-7 cells for DNA damage. Every experiment was repeated three times and tail moment was used as a measure of DNA damage. *: $P<0.05$ compared to the negative control.

Fig. 6. Comet-forming activity of unsubstituted benzofuran phenylmethyl imidazole in MCF-7 cells. Comet assay, as described in methods, was used to test MCF-7 cells for DNA damage. Every experiment was repeated three times and tail moment was used as a measure of DNA damage. *: $P<0.05$ compared to the negative control.
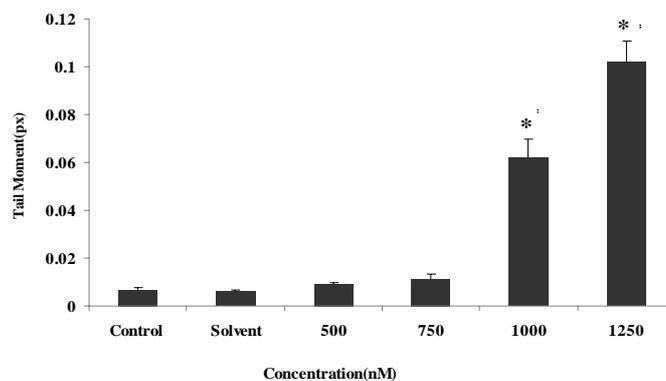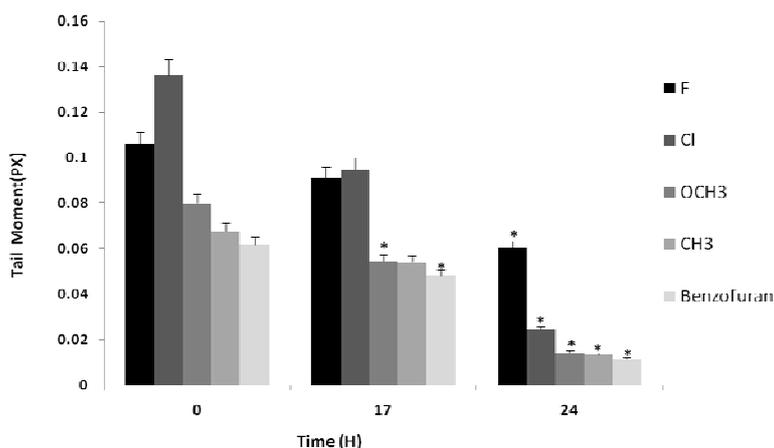

Fig. 7. Repair of DNA damage induced by benzofuran phenylmethyl imidazole (Benzofuran) and its 4- fluoro (F), 4-chloro (Cl), 2-methoxy (OCH3) and 2-methyl (CH3) derivatives in MCF-7 cells. Cells treated with the genotoxic concentration of each compound for 2 h and then with fresh medium for 17 and 24 h. DNA damage was detected by comet assay. Tail moment was used as a measure of DNA damage. *: P< 0.05 as compared with time= 0 h

genotoxic concentration of compounds in 17 and 24 h are shown in Fig. 7. 4-fluoro benzofuran phenylmethyl imidazole at 70 nM concentration had a marked genotoxic effect on the MCF-7 cells compared to the negative control (Fig. 2). The TM which was obtained after treatment with a concentration of 100 nM of this derivative was significantly higher than that of the treatment with 70 nM ($P<0.001$). As shown in Fig. 7, DNA damage induced by genotoxic effect of 4-fluoro derivative (70 nM) was recovered within 24 h ($P<0.05$). Statistical significance between the TM of the 17 h and 24 h was not observed.

2 h treatment of MCF-7 cells with 75 nM 4-chloro benzofuran phenylmethyl imidazole increased the TM significantly ($P<0.05$) as compared to the negative control (Fig. 3). The TM parameter for the 24 h recovery was statistically decreased compared to the 0 h. The decrease in TM for 17 h, however, did not reach to a significant level ($p>0.05$) . The difference between the TMs of 24 h and 17 h was not significant (Fig. 7).

2-Methoxy benzofuran phenylmethyl imidazole at 200 nM concentration showed statistically significant genotoxicity (increase in TM parameter) as compared with the negative control (Fig. 4).

The TM parameters of MCF-7 cells exposed to the 2-Methoxy benzofuran phenylmethyl imidazole at the genotoxic concentration of 200 nM for 17 and 24 h were significantly ($P<0.001$ and $P<0.005$, respectively) different from that of the time zero (Fig 7). The differences in TMs between 24 h and 17 h were also significant ($P<0.001$).

A significant increase in the DNA damage of the MCF-7 cells treated with 300 nM of 2-methyl benzofuran phenylmethyl imidazole was observed when TMs were compared with that of the control cells (Fig. 5). Our results indicated a considerable decrease in TM for 24 h recovery ($P<0.001$). However, this decrease in TM was not significant for 17 h. As shown in Fig. 7, there was a significant difference between the TMs for the recovery time of 17 h and 24 h ($P< 0.05$).

The results also showed that the DNA damage induced by unsubstituted derivative at

concentrations as low as 1000 nM was significant ($P<0.001$) in comparison with negative control (Fig. 6). This damage was even more considerable at concentration of 1250 nM ($P<0.001$).

The TM parameters of MCF-7 cells exposed to the unsubstituted derivative for 17 and 24 h were significantly ($P<0.05$ and $P<0.001$, respectively) different from that of the time zero (Fig 7). The differences in TMs between 24 h and 17 h were also significant ($P<0.001$).

DISCUSSION

Our results indicated that toxic concentrations of substituted l-[(benzofuran-2-yl)-phenylmethyl]-imidazole derivatives were around 10^{-8} -10^{-6}M. The 4-fluoro derivative compound was tested at first. The cytotoxic concentration of this compound has been reported to be 35 µM (13). Accordingly, concentrations of 0.1, 1 and 10 µM of 4-fluoro derivative were selected and comets were observed for all tested concentrations. Therefore, we decided to test much lower concentrations. The genotoxic effects appeared in concentrations of 70 nM, which was close to the enzyme inhibitory concentration of this compound. The tested concentrations for other compounds were also close to their enzyme inhibition concentration.

Cytotoxic effects of these compounds on MCF-7, an aromatase positive cell, and HeLa, an aromatase negative cell, have revealed a mechanism of action other than inhibition of the aromatase enzyme. In agreement with this study, our study shows that DNA damage could potentially be one of the underlying mechanisms.

Whomsley and coworkers demonstrated that human placental aromatase could be inhibited by l-[(benzofuran-2-yl)-phenylmethyl]-imidazole derivatives (19). There exist a remarkable difference between the aromatase inhibitory concentrations of l-[(benzofuran-2-yl)-phenylmethyl] -imidazole derivatives which is also therapeutically effective and the genotoxic concentrations of these compounds observed in the current

study. This implies that these compounds may have good potential to be used as therapeutic agents.

The results of the present study indicated that all tested compounds are able to repair DNA damage. However, some compounds need longer time to perform this function. The IC_{50} value of cytotoxic effects of l-[(benzofuran-2-yl)-phenylmethyl]-imidazole derivatives on MCF-7 cell line have been reported to be around 10^{-5} M (13). This value is about 10-1000 times higher than their genotoxic concentrations found in the present study. It seems that, due to the DNA repair capacity of the cells, there is a remarkable differences between their genotoxic and cytotoxic concentrations. However, there is a possibility that exposure of the cell to the higher concentrations of these derivatives leads to some severe DNA damage which could not be repaired.

In the current work, 4-fluoro and 4-chloro derivatives exhibited the highest genotoxicity while unsubstituted benzofuran phenylmethyl imidazole had the lowest side effects on the MCF-7 cell line. These results are mostly in agreement with the previous report (13) on cytotoxic effects of these compounds on MCF-7 cell line and on the aromatase inhibitory activity of these compounds(19). It has been shown that some benzofuran phenylmethyl imidazole act as a substrate and could bind to active site of aromatase enzyme (14). These results suggest that substituted derivatives of benzofuran phenylmethyl imidazole might fit more properly into the active site of the enzyme which leads to a more effective enzyme inhibition.

CONCLUSION

The distinct differences between the enzyme inhibition and genotoxic concentrations of substituted benzofuran phenylmethyl imidazole derivatives and also their DNA repair capacity make these derivatives suitable for future studies. Therefore, if these compounds could pass other safety and clinical assessments, they would be used as new therapeutic compounds.

ACKNOWLEDGMENT

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893-2917.
2. Pakseresht S, Ingle GK, Bahadur AK, Ramteke VK, Singh MM, Garg S, et al. Risk factors with breast cancer among women in Delhi. Indian J Cancer. 2009;46:132-138.
3. Vankrunkelsven P, Kellen E, Lousbergh D, Cloes E, Beeck L, Faes C, et al. Reduction in hormone replacement therapy use and declining breast cancer incidence in the Belgian province of Limburg. Breast Cancer Res Treat. 2009;118:425-432.
4. Russo J, Russo IH. The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol. 2006;102:89-96.
5. Smith I, Dowsett M . Aromatase Inhibitors in Breast Cancer. N Engl J Med. 2003;348:2431-2442.
6. Hong Y, Chen S. Aromatase, estrone sulfatase, and 17-Hydroxysteroid dehydrogenase: Structure-function studies and inhibitor development. Mol Cell Endocrinol. 2011;340:120-126.
7. Forbes JF, Cuzick J, Buzdar A, Howell A, Tobias JS, Baum M. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. Lancet Oncol. 2008;9:45-53.
8. Criscitiello C, Fumagalli D, Saini K, Loi S. Tamoxifen in early-stage estrogen receptor-positive breast cancer: overview of clinical use and molecular biomarkers for patient selection. Onco Targets Ther. 2010;4:-11.
9. Miller WR. Aromatase inhibitors: mechanism of action and role in the treatment of breast cancer. Semin Oncol. 2003;30:3-11.
10. Howell A. New developments in the treatment of postmenopausal breast cancer. Trends Endocrinol Metab. 2005; 16: 420-428.
11. Dutta U, Pant K. Aromatase inhibitors: past, present and future in breast cancer therapy. Med Oncol. 2008;25:113-124.
12. Vinh TK, Ahmadi M, Lopez Delgado P, Fernandez Perez S, Waiters H, Smith H, et al. 1-[(Benzofuran-2-yl) phenylmethyl] -Triazoles and -Tetrazoles - Potent Competitive Inhibitors of Aromatase. Bioorg Med Chem. 1999;9:2105-2108.
13. Khodarahmi GA, Hassanzadeh F, Jafarian A, Chiniforoosh AH, Hajseyedabutorabi A. Cytotoxic effects of some 1-[(benzofuran-2-yl)-phenylmethyl]

imidazoles on MCF-7 and Hella cell lines. Res Pharm Sci. 2007;2:73-79.

14. khodarahmi GA, Laughton CA, Smith HJ, Nicholis PJ. Enantioselectivity of some 1-[(benzofuran-2-yl)-pHenylmethyl] imidazoles as aromatase (P450 arom) inhibitors. J Enzyme Inhib. 2001;16:401-416.

15. Jena GB, Kaul CL, Ramarao P. Genotoxicity testing, a regulatory requirement for drug discovery and development :impact of ich guidelines. Indian J Pharmacol. 2002;34:86-99.

16. Davis C, Bhana S, Shorrocks A J, Martin FL. Oestrogens induce G1 arrest in benzo[a] pyrene-treated MCF-7 breast cells whilst enhancing genotoxicity and clonogenic survival. Mutage. 2002;17:431-438.

17. Yared E, McMillan TJ, Martin FL. Genotoxic effects of estrogens in breast cells as detected by the micronucleus assay and the Comet assay . Mutagenesis. 2002;17:345-352.

18. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988;175:184-191.

19. Whomsley R, Fernandez E, Nicholls PJ, Smith HJ, Lombardi P, Pestellini V. Substituted 1-[benzofuran-2-yl)-pHenylmethyl]-imidazoles as potent inhibitors of aromatase *in vitro* and in female rats *in vivo*. J Steroid Biochem Mol Biol. 1993;44:675-676.