In vitro cytotoxicity of "mswaki" fibre on human gingival fibroblasts

Carneiro LC[1] & Botha SJ[2]

[1]Department of Restorative Dentistry, School of Dentistry, Muhimbili University College of Health Sciences, Dar es salaam. Tanzania . [2] Centre for Stomatological Research, School of Dentistry, University of Pretoria, Pretoria, South Africa.

Carneiro LC & Botha SJ *In vitro* cytotoxicity of "mswaki" fibre on human gingival fibroblasts Tanz Dent J 2007; 14 (2): 61-7

Abstract

Aim: This study determined the *in vitro* cytotoxicity of mswaki fibres on human gingival fibroblasts (HGF). **Methods:** Two types of "mswaki" twigs (*Salvadora persica* and *Euclea natalensis*) were used. Each twig was swabbed with 70% ethanol, the bark was then removed and approximately 1cm pieces of fibre were cut and stored separately. Two HGF cell lines (GW3 and GW6) were cultured, harvested and seeded into 12mm tissue culture wells at a concentration of 3-5 X 10^5cells.ml^{-1} and allowed to form a monolayer by incubating them for 24h at 37°C in a humidified atmosphere of 5% CO_2 and of 95% air. To determine cytotoxicity, cut pieces of both twigs were placed into the tissue culture wells with established HGF and excess media was removed for closer proximity. Using an inverted microscope, cell reactions were observed at 10 and 30min and 1, 2, 4, 24, and 48h and cell lysis determined when a change in cell morphology was observed. **Results:** None of the employed HGF cell lines showed any change in cell morphology for either of the inoculated mswaki fibres during the study period. Cells appeared healthy and no changes in morphology were observed indicative of cell lysis. Cell proliferation for both cell lines at 24 and 48h showed no significant difference from controls regardless of the presence of mswaki fibre. **Conclusion:** Both miswaki fibres were observed not to have exhibited any cytotoxic effect hence proof of their suitability as a traditional toothbrush in the maintenance of oral hygiene.

Key Words: *In vitro*, cytotoxicity, mswaki, human gingival fibroblasts

Correspondence: Dr Lorna C Carneiro, P. O. Box 65451, Dar es salaam, Tanzania.
Email: lcarneiro@muchs.ac.tz

Introduction

"Miswaki", a traditional toothbrush used for the maintenance of oral hygiene, are made from straight pieces of twigs of specific tenderness and fibrous quality. They have been used for centuries as a tooth-cleaning device (1) in many Sub-Saharan countries (2), the Middle East (3,4,5) and Indian Subcontinent (2,6).

The widespread use of miswaki can be attributed to their inexpensiveness (7), customary use (8) as well as religious reasons (9). Their use is more prevalent in rural populations (6,10) and more amongst male than females (11,12) and among the older generation (12). In Tanzania, the mswaki is also regarded to be the most commonly used traditional tooth cleaning device and mostly amongst adults in rural Tanzania. Its use is significantly determined by education and occupational status as well as tribal origin (13).
Useful properties of the mswaki fibre which have been demonstrated include antiplaque, antiperiopathic, anticaries and antibacterial effect (3). Apart from being reported to have an agreeable taste, it is also known to stimulate the flow of saliva (14). These properties have led to the mswaki being recommended and encouraged as an effective tool for oral hygiene (15).

Most studies in regard to the "mswaki" have been based on research that deals with the extracts from the "mswaki" (2,16,17) and only one study to date has reported the effects of "freshly cut mswaki" (18). Since it is the mswaki fibres of the formed tuft that come into close contact with the gingival tissues the aim of this study was to investigate the *in vitro* cytotoxicity of the fibres of two different types of "Mswaki" on human gingival fibroblasts (HGF).

Materials and Methods

Preparation of Mswaki samples

Two different types of "mswaki", *Euclea natalensis* (EN) and *Salvadora persica* (SP) (Fig I) were purchased from a local market in Tanzania, stored separately in sealed sterile plastic bags and preserved in a refrigerator. The local names of the twigs *mdaha* and *mkulubuku*,

were verified by the Traditional Medicine Research Unit, Muhimbili University College of Health Sciences. Preparation of the mswaki fibres entailed swabbing of the twigs with 70% ethanol, cutting and discarding a small length of one end and removing the bark of the remaining piece. The exposed inner fibres of the latter piece were then frayed and cut into lengths of approximately 1cm long and stored in labeled sterile containers for later use (Fig II).

Preparation of Cell culture

Healthy gingival tissue acquired from two patients undergoing periodontal surgery (crown lengthening) was used as the primary source of human gingival fibroblast cell lines (GW3 and GW6) (23). The medium used for culturing both cell lines was Eagles Minimum Essential Medium (EMEM) with Non Essential Amino Acids (NEAA) and 10% fetal calf serum and 100U/ml Penicillin G and 100MCG/ml streptomycin sulphate. Confluent cultures were washed with phosphate buffered saline and cells were detached by trypsinization with 3mls of 0.5% trypsin and 0.2% Ethylene Tetra Acetic Acid (EDTA). The cells obtained after centrifugation were seeded into measured tissue culture media (19) so as to achieve a known concentration for seeding. Each of the cell lines was seeded in duplicate at a concentration of 3-5 X 10^5cells.ml^{-1} into 12mm tissue culture wells. On each plate containing 24 wells, GW3 cell line was placed into the first 3 columns (12 wells) and GW6 into the remaining three columns (Fig III). The seeded cells in the wells were allowed to form a monolayer by incubating the tissue culture wells for 24 hour at 37°C in a humidified atmosphere of 5% CO_2 and of 95% air.

Determining cytotoxicity of the mswaki fibres

Using sterile tweezers, mswaki fibres of EN were then added into wells of the seeded plates of the second and fifth columns and those of SP into the third and sixth columns. The first and fourth columns served as control (Fig III). To ensure close contact of the fibres with the cells, excess culture media was removed from all wells using a sterile pipette. Using an inverted microscope cells were observed after 10 and 30min, 1, 2, 4, 24, and 48h of contact with the mswaki fibre and photographs were taken at X20 and X40 magnification. Normal fibroblast cells may appear rounded, stellate or elongated depending on the stage of attachment while Cytotoxicity was determined if there was an observed change in cell morphology or evidence of cell lysis (disruption of cell membranes). No statistical analysis were performed as data obtained were non parametric.

Results

Overall, there was no difference in cell structure between the two HGF cell lines (GW3 and GW6) prior or after the addition of the mswaki fibres. Cells appeared healthy and no visual changes in morphology were observed that suggested lysis.

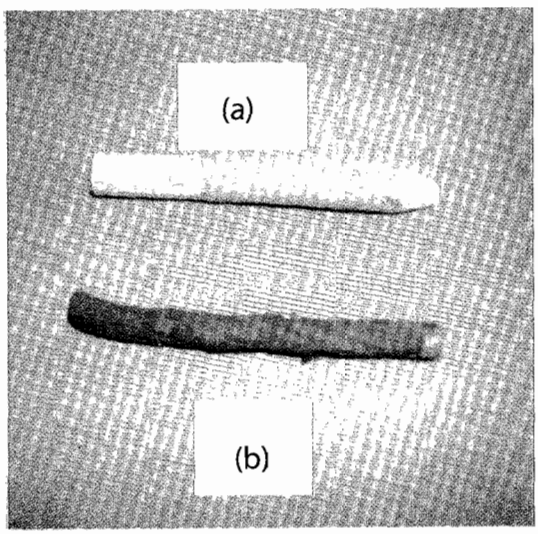

Figure I. Showing Miswaki sticks that are used for oral hygiene. a) Salvadora persica and b) Euclea natalensis

As shown in Fig IV, the monolayer of HGF cells (GW3) with the SP mswaki fibre appeared healthy and displayed no change in cell morphology after 4h of contact.

A paratrooper was home on leave. "How many jumps have you madw?" one of his friends asked.

"Only one," admitted the paratrooper. "My service record shows 20 but on the other 19, I was pushed."

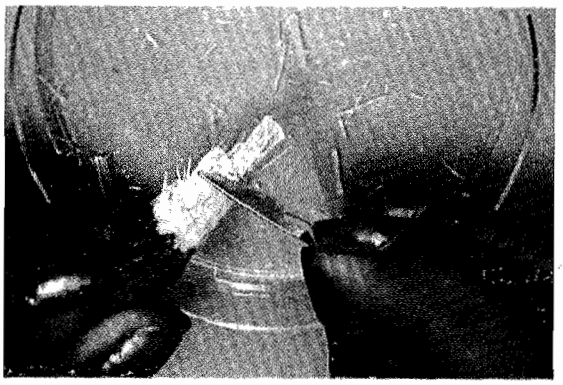

Figure II. Fibres of the mswaki (Salvadora persica) being cut into lengths of approximately 1cm after cleaning and removal of the outer bark.

At 24h the GW6 cells with the mswaki fibre (EN) appeared more compact and maintained normal morphology, including cells that were adjacent and in contact with the fibre. Dividing cells were observed on the surface layer (Fig V).

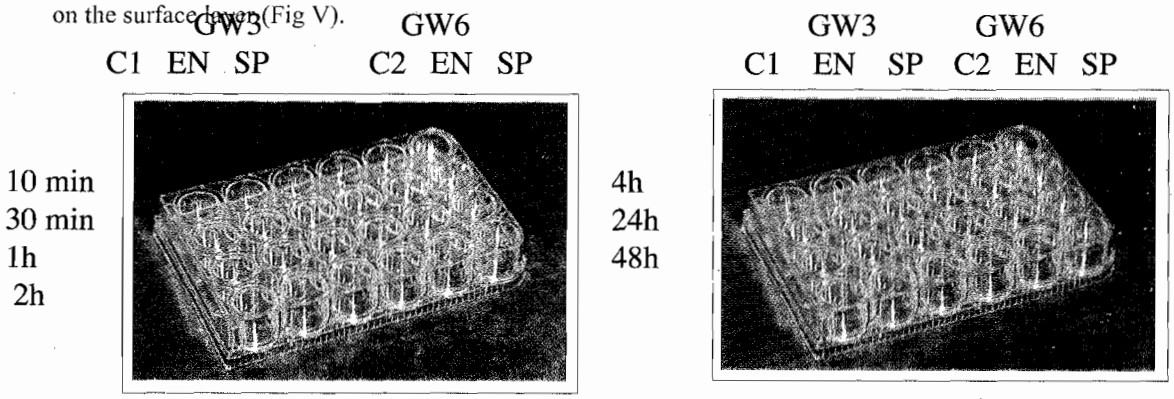

Figure III: Tissue Culture plate showing the 24 wells of 12mm diameter with HGF and mswaki fibres. (GW3 – Human gingival fibroblast cell line - patient 1; GW6 – Human gingival fibroblast cell line – patient 2; C_1 – Control cell line GW3; C_2 – Control cell line GW6; EN – Euclea natalensis mswaki fibre; SP - Salvadora persica mswaki fibre.

In Fig VI a denser layer of GW3 cells with mswaki fibre (EN) is observed after 48h, indicating that further cell division had occurred. The cell morphology remained the same exhibiting no evidence of lysis.

Discussion

In many countries *Salvadora persica* is the most common source for mswaki (20) and Tanzania is no exception. Besides *Salvadora persica*, *Euclea natalensis* is also another commonly used "mswaki" in Tanzania and hence both species were investigated in this study. *Salvadora persica* is an evergreen perennial halophyte capable of growing under extreme conditions, from very dry environments to highly saline soils (21) while *Euclea natalensis* is related to the African Zanzibari community as it is usually found in coastal forest, woodland and on hills, where it grows as a shrub or tree (22).

The mswaki stick is about 15 to 20cm in length and prepared by chewing one end of the stick until a fibrous tuft is formed (23). It is this tuft that is then used in the maintenance of oral hygiene but after it has been used a few times the fibres get soft and the used bristles are cut away and a new part for brushing is prepared (14).

The mswaki is also known to have an agreeable taste and stimulates the flow of saliva, but after it has been used a few times coupled with drying up, the slightly bitter taste is lost.

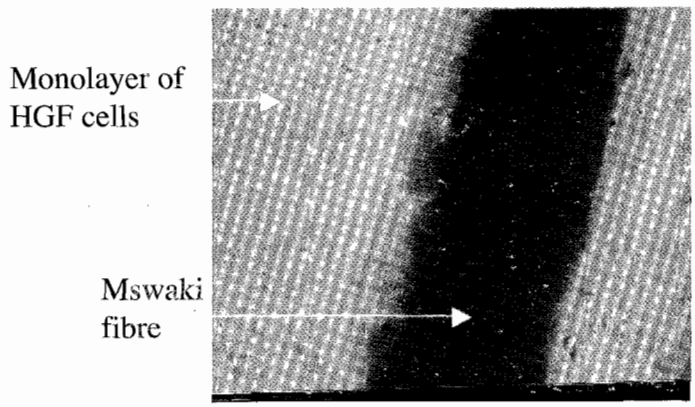

Fig IV. Slide showing a monolayer of HGF cells (GW3) at X40 magnification with the SP mswaki fibre at 4h. Cell morphology appears normal and intertwining of cells is noted. Indication of cell multiplication is apparent.

Comparisons with different studies could not be done due to differences in employed methods, type and form of test material used. Most studies have reported the effect of the extract from the mswaki fibre on cultured cells and it is possible that the observed results may have been influenced by the solvent used in obtaining the extract. The choice of solvent will determine which constituents will be extracted and the solvent may itself cause cell lysis. To overcome the effect of a solvent and to ensure close proximity with cultured cells, the contact method was justified.

Prior to handling of the twig, the risk of contamination was minimized by hand washing, swabbing of the twig with ethanol, cutting and discarding of a small length of one end and removal of the outer bark. The exposed fibres were then cut into lengths of about 1cm to ensure adequate fit into culture wells (12mm).

Storage of the *Salvadora persica* mswaki twig in a refrigerator maintained its freshness which was verified by its whitish brown colour as in accordance with Greenway (24) who stated that a dark brown color indicated that the mswaki was no longer fresh. Although Etebu, Tasie and Daniel-Kalio (25) reported that the percentage of fungal colonization increased with increase in storage period, Almas, Al-Bagieh and Akapata (26) found no difference in antimicrobial effect between fresh and one month old miswak. The *Euclea natalensis* stick had a dark coloured outer bark with an orange tinge on its inside (Fig 1), and its use in this study was mainly as a control to findings portrayed by the *Salvadora persica* stick.

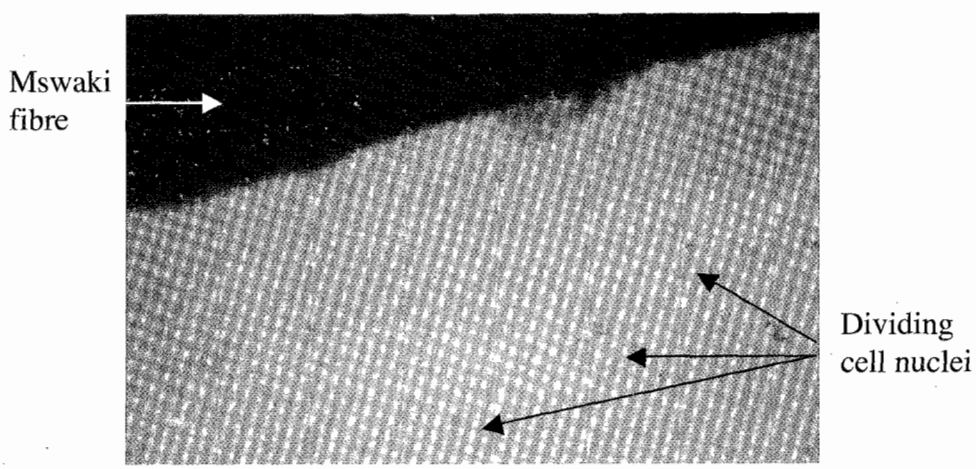

Fig. V. Slide showing a monolayer of HGF cells (GW6) with the mswaki fibre (EN) at 24h (X20 magnification). Dividing cell nuclei are seen at the surface, cells appear to have normal morphology and no evidence of cell lysis.

There are various methods used in the determination of cytotoxicity, each with its strengths and weaknesses. The visual method was chosen as it gives accurate results with a minimum amount of equipment. The obtained results are non-parametric and regarded as reliable based on the researcher's experience in cell culturing. In this study two different sources of cultured fibroblast cells were used to assess cytotoxicity so as to control for the effect of genetic makeup. In assessing the *in vitro* cytotoxicity the direct contact method was preferred to the indirect method as the contact method allows lipid soluble molecules to be absorbed during contact of the fibres with the gingival cells. The wetting of the mswaki fibre by culture media acted as a solvent and the removal of the excess media brought into close proximity the gingival cells and the mswaki fibres. The contact method was also preferred as not all substances within the mswaki fibre can be extracted by solvents and the use of a suitable solvent could be toxic to cells.

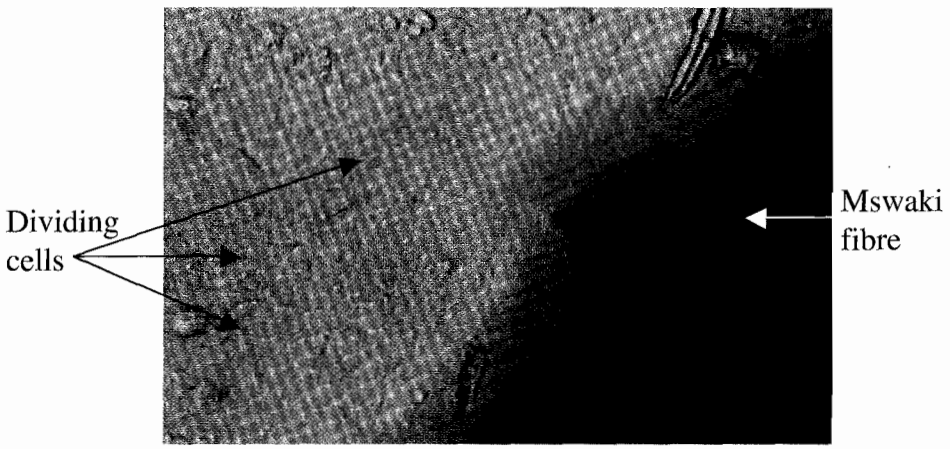

Fig. VI. Slide at X40 magnification showing a denser layer of HGF cells (GW3) with the Mswaki fibre (EN) at 48h. Dividing cells are observed on the surface.

Analysis of cell behaviour carried out under well-controlled conditions allows conclusions about the bio-acceptability of a material and employs cellular morphology as an accepted parameter of bio-acceptability tests (27). When adherent cells (HGF) are cultured in vitro, they maintain a characteristic shape by adhesion both to neighbouring cells and to the extracellular matrix (28). Regardless of the added mswaki fibres, the cell density increased over time as evident from observed cell multiplication at surface, cell morphology was maintained, and no cell lysis was observed in any of the samples. Proliferation of cells represents the final stage of the cellular processes involved in the viability and survival properties of cells and can be summarized in sequential stages of contact, attachment, spreading and proliferation (29).

The mswaki fibre was also used by Mohammad and Turner in their study (18). They did not employ the direct contact method but used the tissue culture-agar overlay method (30) and reported cytotoxicity after 24 hours which was not observed in this study. The direct contact method allows for lipid soluble molecules to be absorbed during contact of the mswaki fibre with the gingival cells. The absence of cytotoxicity reported in this study could also be related to minimal constituents released from the single mswaki fibre within the culture well.

Conclusions

From this study the mswaki fibres were observed to have exhibited no cytotoxic effect on HGF cells, thus their use as a tool in the maintenance of oral hygiene can be recommended. Other studies should be directed into analyzing the cytotoxic effect of many more mswaki fibres within a culture well.

Acknowledgement

The authors would like to acknowledge the staff from the Centre for Stomatological Research, School of Dentistry, University of Pretoria for rendering their assistance in one way or another.

References

1. Eid MA & Selim HA. A retrospective study on the relationship between miswak chewing stick and periodontal health. *Egypt Dent J* 1994;40:589-92
2. Almas K. The antimicrobial effects of extracts of *Azadirachta indica* (Neem) and *Salvadora persica* (Arak) chewing sticks. *Indian Journal of Dental Research* 1999;10:23-26
3. al-Bagieh NH, Idowu A & Salako NO. Effect of aqueous extract of miswak on the *in vitro* growth of *Candida albicans*. *Microbios* 1994;80:107-113

4. Hardie J & Ahmed K. The miswak as an aid in oral hygiene. *Journal of the Philippines Dental Association* 1995;47:33-38
5. Darout IA, Albandar JM & Skaug N. Periodontal status of adult Sudanese habitual users of miswaki chewing sticks or toothbrushes. *Acta Odontol Scand* 2000;58:25-30.
6. Asadi SG & Asadi ZG. Chewing sticks and the oral hygiene habits of the adult Pakistani population. *International Dental Journal* 1997;47:275-8
7. Hattab FN. Meswak: The natural toothbrush. *J Clin Dent* 1997;8:125-9.
8. Almas K, al-Amri M, al-Eid A, al-Shahrani S.Oral hygiene, dietary pattern and smoking habits of Bedouin (nomadic Arabs) population in Saudi Arabia. *Odontostomatol Trop* 2003;26:19-23.
9. Eid MA, Selim HA, al-Shammery AR.Relationship between chewing sticks (Miswak) and periodontal health. Part 1. Review of the literature and profile of the subjects. *Quintessence Int* 1990 Nov;21:913-7.
10. Cai L, Wei GX, van der Bijl P, Wu CD. Namibian chewing stick, *Diospyros lycioides*, contains antibacterial compounds against oral pathogens. *J Agric Food Chem* 2000;48:909-14.
11. Almas K, Al-Malik TM, Al-Shehri MA, Skaug N.The knowledge and practices of oral hygiene methods and attendance pattern among school teachers in Riyadh, Saudi Arabia. *Saudi Med J* 2003;24:1087-91.
12. Al-Otaibi M, Zimmerman M, Angmar-Mansson B.Prevailing oral hygiene practices among urban Saudi Arabians in relation to age, gender and socio-economic background. *Acta Odontol Scand* 2003;61:212-6.
13. Sarita PT & Tuominen R. Tooth cleaning methods and their effectiveness among adults in rural Tanzania. *Proc Finn Dent Soc* 1992;88:139-145.
14. Attar SA. The miswak-nature's toothbrush. *Bull His Dent* 1979;27:1.
15. Wu CD, Darout IA, Skaug N. Chewing sticks: timeless natural toothbrushes for oral cleansing. *J Periodontal Res* 2001;36:275-84.
16. Kemoli AM, van Amerongen WE, de Soet JJ. Antimicrobial and buffer capacity of crude extracts of chewing sticks (Miswaki) from Kenya. *ASDC J Dent Child* 2001;68:183-8, 152.
17. Almas K. The effects of extracts of chewing sticks (*Salvadora persica*) on healthy and periodontally involved human dentine: a SEM study. *Indian Journal of Dental Research* 2001;12:127-32.
18. Mohammad AR & Turner JE. *In vitro* evaluation of Saudi Arabian toothbrush tree (*Salvadora persica*). *Odontostomatol. Trop* 1983;VI(3):145-148.
19. Botha SJ. The definition and the implementation of an *in vitro* fibroblast model to evaluate the bio-acceptability of titanium surfaces in dentistry. PhD thesis, *Faculty of Dentistry, University of Pretoria*, (1995) p 44-46, 115-136.
20. Hattab FN. Meswak: the natural toothbrush. *J Clin Dent* 1997;8:125-9.
21. Maggio A, Reddy MP, Joly RJ. Leaf gas exchange and solute accumulation in the halophyte *Salvadora persica* grown at moderate salinity. *Environ Exp Bot* 2000;44:31-38.
22. Stander I, Van Wyk CW.Toothbrushing with the root of *Euclea natalensis*. *J Biol Buccale* 1991;19:167-72.
23. Mumghamba EGS, Markkanen HA & Honkala E. Risk factors for periodontal diseases in Tanzania. *Journal of Clinical Periodontology* 1995;22:347-54
24. Greenway PL. Vegetable fibres and flosses in East Africa. *E.Afr. Agric. J* 1950;15:146
25. E Etebu, AA Tasie, LA Daniel-Kalio. Oral Microbiology Post-harvest fungal quality of selected chewing sticks. *Oral Diseases*. 2003;9:95.
26. Almas K, Al-Bagieh N, Akpata ES. In vitro antibacterial effect of freshly cut and 1-month-old Miswak extracts. *Biomed Letters*. 1997;56:145-149
27. Schmidt C, Kaspar D, Sarkar MR, Claes LE & Ignatius AA. A scanning electron microscopy study of human osteoblasts morphology on five orthopedic metals. *Journal of Biomedical Materials Research* 2002;63:252-261.
28. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell*. 1996;84:345-57.
29. Kamen PR. Attachment of oral fibroblasts to HTR polymer. *Compendium Continuing Education Dentistry*, 1988;Suppl No **10**:S350-352.
30. Guess WL, Rosenbluth SA, Schmidt B and Autian J. Agar diffusion method for toxicity screening of plastics on cultured cell monolayers. *J Phamac* 1965;54:1545.