Original Research Article

Cytotoxic Analysis of Various Root Canal Irrigants at Cellular Level.

ABSTRACT

Aim:- To evaluate and compare the cytotoxic effects of commercially available root canal irrigants sodium hypochlorite, chlorhexidine and a herbal extract, *Morinda tinctoria.*

Study design:- Concerned to the biological perspective, root canal irrigants must aid in the complete disinfection of the root canal and be biocompatible when come in contact with the vital periapical tissues. Hence the study was done to analyse the cytoxicity of different root canal irrigants at cellular level.

Place and Duration of Study: Department of Pedodontics and Preventive dentistry, GITAM dental college and hospital in collaboration with Chaitanya Medical centre, Visakhapatnam and Department of Oral Pathology,GITAM Dental college.

Methodology: Forty nine samples with 2ml of RBC suspension were randomly assigned to seven groups. 100µl each of 3% NaOCl, 2%CHX and 60mg/ml concentration of *Morinda tinctoria* and their 1:1dilutions were tested on RBC suspension. Normal saline is selected as control. Peripheral smear was made to assess the morphological abnormalities of viable cells. After centrifugation of each test tube, the supernated volume is estimated for haemoglobin concentration representing cytotoxicity. The results obtained were subjected to statistical analysis.

Results: Cytotoxicity varies in the following order: 2% Chlorhexidine> 1:1dil CHX > 3% NaOCl > 1:1 dil. 3% NaOCl= 60mg/ml *M.tinctoria* > 1:1 dil of 60mg/ml *M.tinctoria*. Results showed that statistically significant difference exists between cytotoxicity of tested irrigating solutions.

Conclusion: Considering the undesirable effects of the conventional root canal irrigants and the global scenario with changing trends in search of non-toxic plant extracts, Morinda tinctoria could be an alternative root canal irrigant with least toxicity.

Keywords:- *Sodium hypochlorite, Chlorhexidine, Morinda tinctoria, cytotoxicity, Red blood cells.*

1. INTRODUCTION

The endodontic therapy focuses towards complete debridement of the root canal system preventing further re-emergence of the bacterial infection. This is required to establish a condition conducive to healing, thus maintaining the periodontium healthy. This consecutively relieves the pain and discomfort caused by pulp inflammation or infection. Multifarious root canal irrigants have been encouraged for disinfecting the complex root canal system. Instead of alleviation, endodontic implications such as inadvertent extrusion of these chemical disinfectants beyond the periapical region either due to aggressive instrumentation or in case of wide open apices through external resorption or unnoticed perforation will generate unusual iatrogenic complications[1].

Sodium hypochlorite was the most prevailing root canal irrigant over four decades, readily available as 3% NaOCl. It is illustrious as an effective antimicrobial agent [2] with distinctive tissue dissolving capacity aiding in biomechanical preparation of complex root canal system[3]. Periapical extrusion of sodium hypochlorite in wide open apices leads to undesirable destructive tissue reactions[4]. The sequel of reactions that can be expected include excruciating pain with constant discomfort, diffused swelling, profuse bleeding episode and ecchymosis[5].

Chlorhexidine gluoconate was considered as an effectual antimicrobial agent in endodontics for the past 2 decades. It is commercially available as a root canal irrigant in the concentration of 2%. It is renowned due to its broad spectrum antimicrobial efficacy with a unique feature of being adsorbed onto the dentin and provides antimicrobial substantivity[6]. It is considered as a potential endodontic irrigant alternative to sodium hypochlorite in teeth with very patent apices[7]. The

lack of tissue dissolving capacity limits its usage in complex root canal system[6]. Contact dermatitis is common side effect of Chlorhexidine and even immediate hypersensitivity has been reported with it [8].

As a shift towards nature, secondary metabolite constituents of herbals have a substantial history of use in modern 'western' medicine. World Health Organisation has sorted 2100 plants with therapeutic importance, in which 2,500 species belong to India. In order to capture the wisdom of resurging traditional herbal medicines, there is wide range of investigations with herbal alternatives were put forward in dentistry[9]. One such appraisal recently reported was with *Morinda tinctoria.*

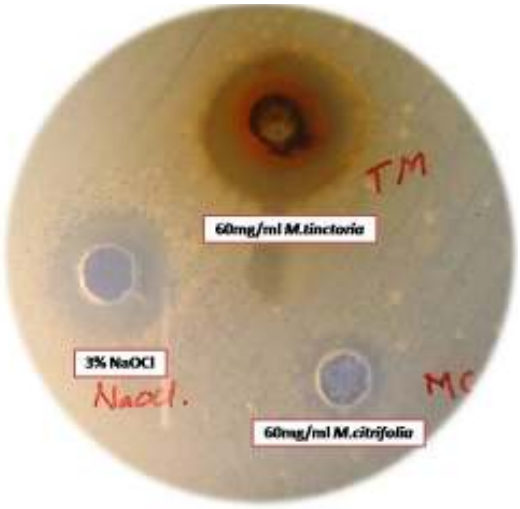
Morinda tinctoria commonly known as Indian mulberry belongs to family *Rubiaceae*. It is ever green flowering shrub, locally known as Togaru. *Morinda tinctoria* has wide range of medicinal properties that includes antimicrobial, anti-inflammmatory, anti-convulsant, cytoprotective and anti-oxidant properties[10]. Phytochemical constituents includes carbohydrates, alkaloids, flavonoids, steroids, tannins and phenols, saponnins, fixed oils and fats, proteins, volatile oils etc responsible for its medicinal acitivity[11] (Table 1).

Table1:- The therapeutic applications of various parts of Morinda tinctoria

Parts	**Therapeutic applications [12]**
Leaves	Dyspepsia, diarrhoea, ulceration, stomatitis, digestion, wounds, fever etc
Roots	Boils
Unripe fruits	Rheumatism, dysentery, vomiting, diarrhea, choleraetc

It was stated that the most desirable root canal irrigant would be the one that combines maximal antimicrobial effect with minimal toxicity [5]. An invitro study has been reported that M. tinctoria at 60mg/ml concentration has better antimicrobial efficacy compared to 3% NaOCl and 60 mg/ml concentration of M. citrifolia against most resistant endodontic pathogen, *E.faecalis* (figure 1)[10].

Figure 1:- Antimicrobial efficacy of 60mg/ml concentrations of M.tinctoria, M.citrifolia compared with 3% NaOCl



Morinda tinctoria at a concentration of 60mg/ml has antimicrobial substantivity lasted for about 12 days against *Enterococcus faecalis* which can be comparable to chlorhexidine efficiency indicating its ability to inhibit the growth of *E.faecalis* for longer period (figure 2).

Figure 2 :- Zones of inhibition with 60mg/ml concentration of *Morinda tinctoria* against E.faecalis after (a) 24hours and (b) 12 days period

(a)

(b)

Shweta Verma, Munish Goel, Shikha Bala, Mahender Singh (2012) has reviewed many invitro and invivo studies evaluating the cytotoxicity and mutagenicity of different root canal irrigants through various techniques in a structured approach and concluded that in vitro methods adequately measure cytotoxicity and therefore could reasonably be used as a screening tool to evaluate biocompatibility of newer test materials [13]. Hence the present study was conducted invitro as a preliminary based analysis in search of least cytotoxic root canal irrigant which is effective antimicrobially in the root canal at cellular level.

OBJECTIVES OF THE STUDY:-

1) To estimate the cytotoxic effects of conventional root canal irrigants 3% NaOCl and 2% CHX and 60mg/ml *M.tinctoria* for endodontic irrigation.

2) To compare the cytotoxic effects of the commercially available root canal irrigants with potential herbal alternative.

2. METHODOLOGY

Commercially available 3% sodium hypochlorite (Prime Dental Products Pvt Ltd) and 2% Chlorhexidine gluconate (Asep RC) obtained from Stedman Pharmaceuticals Pvt. Ltd. The aerial parts (leaves and twigs) of *Morinda tinctoria* were collected from the plants available in GITAM University campus, Visakhapatnam.

Preparation of plant extract:-

The aerial parts obtained were shade dried and coarsely grounded. 10gms of powder was suspended in ethanol solvent and subjected to Soxhlet hot continuous extraction. The solvent was maintained at a temperature of 60^0C and about 7-8 cycles were carried out for complete extraction of the phytochemical compounds. The solvent containing the plant extract was evaporated to dryness in a rotaevapoarator[12]. The known quantity of extract was dissolved in saline (0.9% NaCl) for preparing the required concentration i.e., 60mg/ml[14].

Preparation of RBC suspension:-

Red blood cells were selected to evaluate the cytotoxicity. Fresh blood from human volunteer was drawn into heparinised containers, spun at 1000 rpm for 10minutes, the plasma was discarded and the packed cell volume obtained was washed twice in Dulbecco's phosphate buffered saline by centrifugation. The final hematocrit of the RBC suspension was adjusted to 45%[15].

Cytotoxic analysis:-

3% sodium hypochlorite(NaOCl) and 2% chlorhexidine gluconate(CHX) and herbal extract at higher concentration were tested against viable cells for cytotoxic evaluation. The concentrated solutions and their dilutions were grouped as follows: Group 1 - 3% NaOCl; Group 2- 1:1 dilution of 3% NaOCl (1.5% NaOCl)); Group 3 - 2% CHX; Group 4 - 1:1 dilution of 2% CHX (1%CHX); Group 5 - 60mg/ml Morinda tinctoria; Group 6 - 30mg/ml Morinda tinctoria (1:1 dilution); Group 7- saline (control). 100µl of each root canal irrigants was added to 2ml of the diluted RBC suspension in individual test tubes separately. All the test tubes were incubated for 3 minutes[16]. Morphological alterations in the RBC were evaluated after staining the peripheral smear with Lesihman's stain[14]. Tubes were then centrifuged at 1000rpm for 10min and the supernated volume obtained was subjected to haemoglobin estimation measured by hematology analyzer (Sysmex

automated hematology analyzer KX-21N) which uses non-cyanide hemoglobin analysis method[17]. The readings obtained were tabulated. The data obtained was subjected to statistical analysis using anova and tukey's post-hoc analysis for pair wise comparison.

3. RESULTS

Because of hemolysis, the mean increase in the hemoglobin concentration was 1.3143gm% for CHX, 0.4571 gm% for 3%NaOCl and 0.0857 gm% for 60mg/ml M.tinctoria. Whereas 0.5286 gm% for diluted 2%CHX, 0.0857 gm% for diluted 3% NaOCl and 0.0143 gm% for 1:1 diluted 60mg/ml M.tinctoria. Almost there is no hemolysis with saline. The results obtained were statistically highly significant with a p value of 0.000 and F ratio of 24.801. It is observed that there is decrease in the hemolysis on diluting the concentrated solutions. The rise in the haemoglobin concentration due to hemolysis with 60 mg/ml concentrated M.tinctoria is similar to that of hemolysis caused by 1:1 diluted 3% NaOCl. The hemolysis occurred with 1:1 dilution of 3% NaOCl, 60mg/ml of Morinda tinctoria or 30mg/ml of Morinda tinctoria were similar when compared to that of saline which was not statistically significant. 3% sodium hypochlorite, 2% Chlorhexidine and 1:1 dilution of 2% Chlorhexidine showed significant difference in the cytotoxic effects when compared with saline.(Table 2 and 3,figure 3).

Table 2:- Statistical analysis showing the comparison of hemoglobin percentage between 3% NaOCl , 2% CHX and 60mg/ml Morinda tinctoria and their dilutions

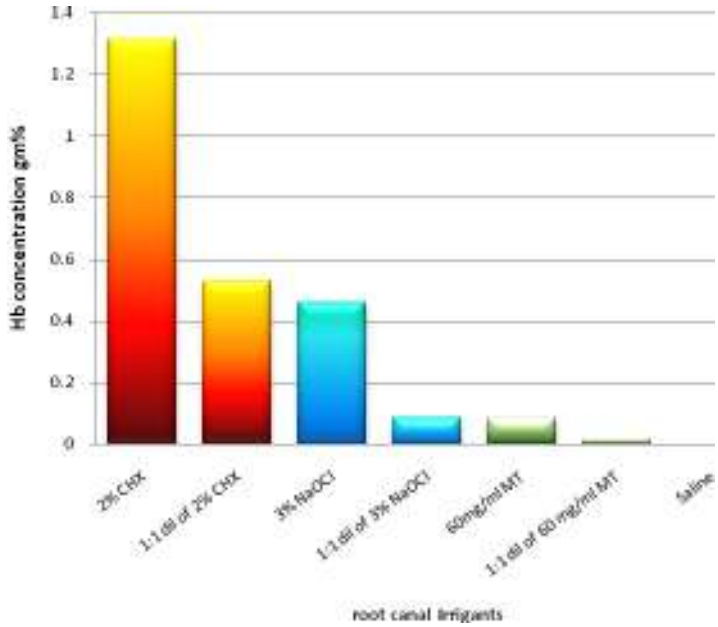
Sl.no	Group	N	Min.	Max.	Mean*± Std. Deviation
1	3% NaOCl	7	0.30	0.60	0.4571 ± 0.11339
2	1:1 dil of 3% NaOCl	7	0.00	0.10	0.0857 ± 0.03780
3	2% CHX	7	1.00	1.70	1.3143 ± 0.26095
4	1:1 dil of 2%CHX	7	0.20	0.90	0.5286 ± 0.25635
5	60mg/ml Morinda tinctoria(MT)	7	0.00	0.20	0.0857 ± 0.10690
6	1:1 dil of 60mg/ml of MT	7	0.00	0.10	0.0143 ± 0.03780
7	Saline	7	0.00	0.00	0.0000 ± 0.00000

Table 3:- showing the post- Hoc statistical analysis for pair- wise comparison between saline and the test groups.

Group (a)	Group (b)	Mean Difference (a-b)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Saline (control)	3% NaOCl	-0.45714*	0.08105	0.000	-0.7080	-0.2062
	1:1 dil of 3% NaOCl	-0.08571*	0.08105	0.937	-0.3366	0.1652
	2% CHX	-1.31429*	0.08105	0.000	-1.5652	-1.0634
	1:1 dil of 2%CHX	-0.52857*	0.08105	0.000	-0.7795	-0.2777
	60mg/ml Morinda tinctoria(MT)	-0.08571*	0.08105	0.937	-0.3366	0.1652
	1:1 dil of 60mg/ml of M.tinctoria	-0.01429*	0.08105	1.000	-0.2652	0.2366

* The mean difference is significant at the 0.05 level

Figure 3:- Graph showing the intergroup and intragroup comparison of hemoglobin percentage between 3% NaOCl , 2% CHX and 60mg/ml Morinda tinctoria and their dilutions



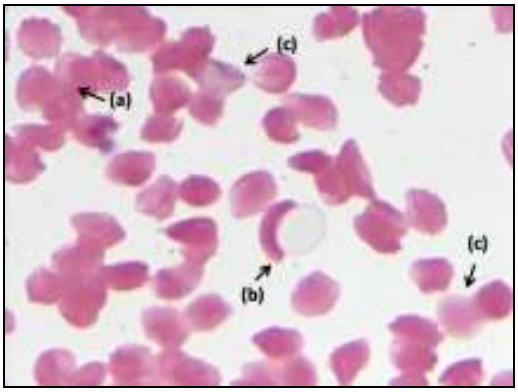
Microscopic features(figure 4):-

The peripheral smears of the RBC suspension were evaluated under oil immersion microscopy at 100X magnification. The smear with RBC treated with 3% sodium hypochlorite stained after 3 minutes incubation revealed poikilocytosis and anisocytosis. Irregular clumping, agglutination and sickling of few RBC's were also seen. Toxic changes in the white blood cells encompass cytoplasmic vacuolization and often caused cell lysis. Ghost cells resembling fat or oil droplets with colourless spherical membrane are present. These cell occurrence might be due to the loss of haemoglobin pigment invariably with altered cell permeability[18].

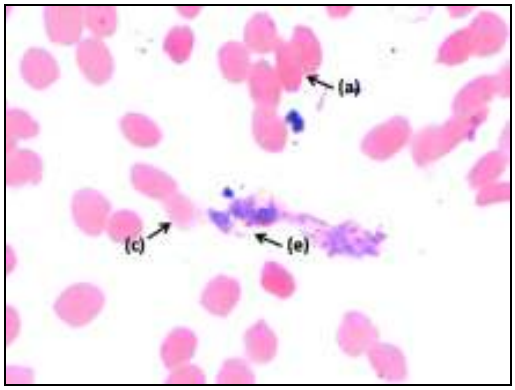
When 3% Naocl was evaluated at 1:1 dilution treated RBC in peripheral smear evaluation revealed poikilocytosis. Both irregular clumping and regularly spaced RBC adhering side to side (rouleux formation) is seen. Ghost cells are relatively scarce compared to 3% NaOCl. WBC cytoplasmic vucuolation and cell lysis are seen.

In case of 2% Chlorhexidine, peripheral smear examination revealed poikilocytosis with cytoplasmic vauolation in the RBC and increased number of ghost cells. Irregular clumping of RBC is also seen. White blood cells with cytoplasmic vacuolation and cell lysis are seen. RBC treated with 2% CHX at 1:1 dilution exhibited poikilocytosis. Ghost cells were reduced on dilution but comparatively more than the other test solutions. There is an intact rouleux formation without any irregular clumping of RBC.

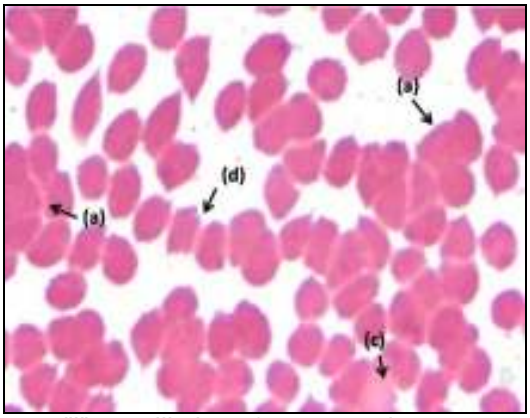
Figure 4:- peripheral smear i) and ii) showing the morphological alteration of RBC's with 3% NaOCl iii) with 1:1 dilution of 3% NaOCl iv) with 2% CHX v) with 1:1 dilution of 2% CHX vi) with 60mg/ml M.tinctoria vii) with 1:1dilution of 60mg/ml M.tinctoria viii) with saline.



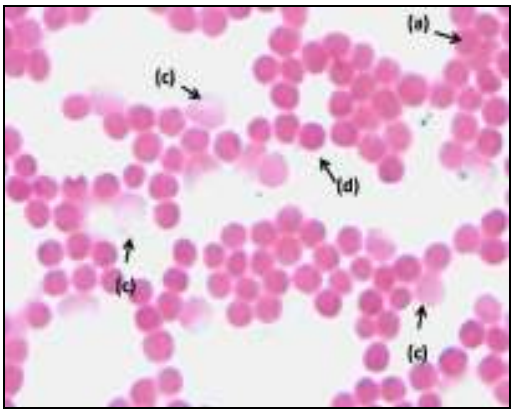
(i) 3% NaOCl



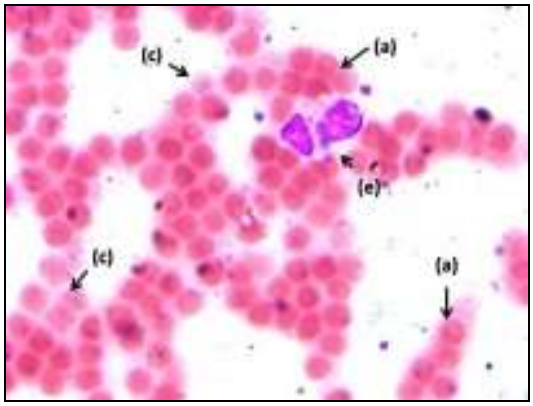
(ii) 3% NaOCl



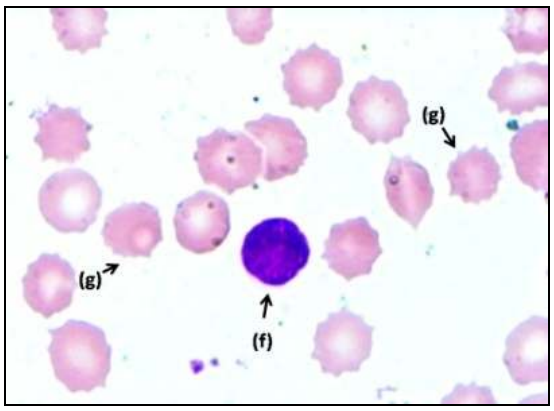
(iii) 1:1 dilution of 3% NaOCl



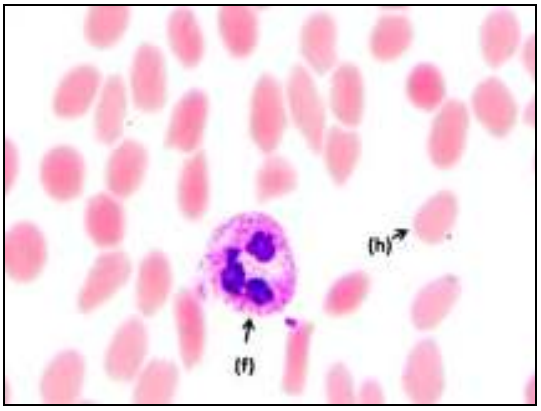
(iv) 2% CHX



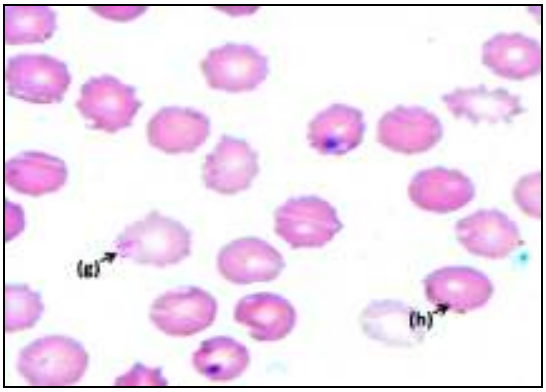
(v) 1:1 dilution of 2% CHX



(vi) 60mg/ml M.tinctoria



(vii) 1:1dilution of 60mg/ml M.tinctoria



(viii) Saline

Labelling:- a) agglutination b) sickled RBC c) ghost cells d) rouleux e) lysed WBC f) intact WBC g) echinocytes h) Discocytes

Smear with Morinda tinctoria herbal extract at the concentration of 60mg/ml revealed poikilocytosis that includes echinocytes. WBC and RBC surface integrity was intact without any lysis and devoid of cytoplasmic vacuolation. Smear with 1:1 diluted 60mg/ml Morinda tinctoria has shown reduced number of echinocytes with intact cell surface integrity. There is no cell lysis or cytoplasmic vacuolation of WBC.

Smear with RBC suspension when treated with isotonic saline revealed intact red blood cell surface. Echinocytes are seen in the peripheral smear. WBC cells were intact without any cytoplasmic vacuolation or disruption.

4. DISCUSSION

The foremost function of a root canal irrigant is to completely disinfect the root canal system without any caustic effects to the periapical tissues. Earlier physiological saline is been used as root canal irrigant but it leaves the bacteria in the root canal alive[19,20], the residual bacteria continue to grow and thrive if not destroyed within the root canal. Hence it does not produce chemical destruction of the microbial matter and dissolution of mechanically inaccessible tissues. But its advantage is that even if it is inadvertently extruded out of the canal during irrigation, it is less likely to produce tissue damage and less chances of acute inflammatory responses because the osmolality of the isotonic saline is equal to that of blood. Hence physiological saline is chosen as a control.

Sodium hypochlorite, though widely used and popular for its tissue dissolving capacity[3] and antimicrobial efficacy[2], it has been cited that even 0.25% was tissue toxic and irritating to the periapical tissues[5]. Other disadvantages reported were cytotoxicity, adverse reactions like contact dermatitis, contact urticaria, photosensitivity, desquamative gingivitis, discoloration of teeth, dysguesia, and ototoxicity [6,21].

Similarly Chlorhexidine, the other commercially available root canal irrigant is well known for its high antimicrobial efficacy but literature has revealed that the bactericidal concentrations of Chlorhexidine were lethal to canine embryonic fibroblasts whilst non- cytotoxic concentrations lack the ability to inhibit the growth of bacteria[6]. According to Boyce (1995), chlorhexidine at low concentration of 0.05% is uniformly toxic to both cultured human cells and microorganisms [22].

Recently a herbal alternative, *Morinda tinctoria* at a concentration of 60mg/ml showed greater antimicrobial efficacy compared to sodium hypochlorite [10] and has long term Antimicrobial substantivity for about 12-15 days against resistant facultative anerobe, *Enterococcus faecalis.*

The present study was undertaken in search of potential antimicrobial root canal irrigant with minimal cytotoxicity at cellular level. 3% NaOCl, 2%CHX, 60mg/ml Morinda tinctoria and their respective 1:1 dilutions were subjected to cytotoxic analysis at cellular level.

Red blood cells were chosen as biological model to evaluate cytotoxic effects as these cells can be easily isolated using least invasive procedure. The red blood cell membranes are semi-permeable barriers and the osmotic gradient established on either side of membrane causes the fluid to flow into and out of the cells. The amount of osmotic pressure depends up on the difference between the concentrations of non-diffusable ions on each side of the membrane [23].

When the cells are subjected to hypertonic solution, they undergo rapid osmotic efflux of water leading to crenation and finally collapse. On the other hand, in hypotonic solution, the cells swell and lyse liberating the cell constituents into the suspension media which results in morphological alteration. Hence an altered morphological characteristic is considered to be one of the parameter to evaluate the cytotoxic effects.

Pashley (1985) considered the protein estimation with lowry method to evaluate the cytotoxic effects. But Chlorhexidine precipitated when mixed with the reagents used in the lowry's method that interfere with spectrophotometric analysis. Lowry's method cannot be used to evaluate the cytotoxicity of herbal extracts also as they themselves constitute proteins[15].

The haemoglobin which is the major intracellular constituent of the RBC can be easily quantified spectrophotometrically once liberated out of the cell. In case of osmotic gradient, the cell becomes a ghost cell, having lost all or most of its haemoglobin content. Hence haemoglobin estimation is considered as other parameter for cytotoxic analysis at cellular level. Pashley (1985) reported that sodium hypochlorite has bleaching effect on the haemoglobin liberated during hemolysis on longer incubation [15]. In order to avoid bleaching effect of the NaOCl, the incubation period was restricted to 3 minutes and this was according to Shibani Shetty and K. Nitesh Rai (2012) [16].

In the present study, the results obtained revealed that there in increased hemolysis with 2% CHX compared to other tested solution. This might be due to the increased osmotic permeability and altered osmotic pressure. This might cause the influx of the fluids and liberation of the haemoglobin out of the cell leading to the formation of ghost cells. On diluting the concentrated 2% CHX, the osmotic pressure is decreased due to the isotonicity of the saline. Hence there is decreased hemolysis on dilution. But even on dilution, results has shown that the cytotoxic effects are greater compared to other tested irrigants.

Haemoglobin released due to hemolysis with 3% NaOCl is comparatively less than 2% CHX but high when compared to 60mg/ml of M.tinctoria and physiological saline. According to Pashley (1985), sodium hypochlorite does not alter the osmotic pressure gradient because of its isotonicity[15], Hence the hemolysis and the morphological alteration that occurred might be due to the strong oxidizing effect of NaOCl on the cell membrane rather than osmolysis. On dilution of 3%NaOCl, the decreased oxidizing effect might have resulted in decreased hemolysis in the present study.

Cemil Yesilsoy (1995) investigated antimicrobial and toxic effects of 3 dilutions of sodium hypochlorite (0.5%, 2.5% and 5.25%) and concluded that when the sodium hypochlorite was diluted to clinically relavant level(2.5% and 0.5%), it was antimicrobially much less effective[5]. They also found that full strength sodium hypochlorite (5.25% and 2.5%) and the Chlorhexidine group (0.12%) showed chronic foreign body reaction at 2 week time period [7].

Oliveira(2007) compared the antimicrobial efficacy of two different concentrations of NaOCl (5.25% and 1.5%) with 2% CHX gel against E. faecalis [24] and has shown that 5.25% NaOCl and 2% CHX gel had antimicrobial action against E.faecalis immediately and 7 days after instrumentation, whereas 1.5% NaOCl reduced the E. faecalis CFU only after instrumentation, thus concluded that the higher the concentration of sodium hypochlorite the better its antimicrobial action. Gomes and co-workers(2001) assessed the antimicrobial effectiveness of different concentrations of NaOCl (0.5%, 1%, 2.5%, 4% and 5.25%) and two forms of chlorhexidine gluconate (gel and liquid) in three concentrations (0.2%, 1% and 2%) in the elimination of E. faecalis and concluded that lower concentration requires greater contact time to inhibit the bacterial growth compared to higher concentration[25] similarly equimolar to that of saline[15]. There is almost no hemolysis when treated with saline as it is isotonic and has no other chemical effects.

Echinocytic transformation is noticed as a histopathological finding with saline and the herbal extract. According to Gerald Lim H.W (2002), factors such as anionic amphipaths, high salt, high pH, ATP depletion, proximity to the glass surface etc causes small changes in the relaxed area between the two leaflets of plasma membrane and also affect the membrane skeleton inducing a series of crenated shapes called Echinocytes, characterized by convex rounded protrusions or spicules[26]. Saline removes the protective coating of the plasma and can crenate the discocyte systematically and reversibly at constant area and volume. G. Brecher and M. Bessis(1972) suggested that addition of 20% fresh plasma to saline subsequently reverse the echinocyte to discocyte[27].

Simpson L. 0. and Shand B. I. (1983) stated that the presence of echinocytes is the most conspicuous feature of blood with hyperproteinaemic conditions[28]. Cholesterol enrichment (Gerald Lim H.W 2002), fattyacid accumulation (G. Brecher and M. Bessis 1972) also causes crenation of RBC [26,27]. Crenation of red blood cells invivo is less because the fattyacids clear rapidly. The lipoproteinaceous phytochemical constituents of the herbal extract, Morinda tinctoria might lead to the echinocytic transformation of the red blood cells.

Unknown suboptimal hemolysis due to interaction with the phytochemical constituents of M.tinctoria with RBC is responsible for the haemoglobin liberation. The amount of haemoglobin released during hemolysis with 60mg/ml M.tinctoria was less compared to the concentrated conventional root canal irrigants tested i.e., 3%NaOCl and 2% CHX. But the haemoglobin released during hemolysis with 60mg/ml M.tinctoria is similar to that of 1:1 diluted 3% NaOCl.

From the present study, Chlorhexidine is most cytotoxic at cellular level even on dilution compared to other irrigants. 3% Sodium hypochlorite is cytotoxic but its cytotoxicity decreases on dilution. Ronald E. Hand (1978) analysed the effect of dilution on the necrotic tissue dissolving property of sodium hypochlorite and concluded that dilution of sodium hypochlorite greatly decreases the necrotic tissue dissolving capacity [29]. Thus the available scientific evidence[5,7,15,24,25] shows that the dilution of sodium hypochlorite adversely affects its necrotic tissue dissolving capacity, its antimicrobial property and its ability to aid in the mechanical debridement.

Hence the outcome of the present study reveals that 60mg/ml concentration of Morinda tinctoria is considered to be having high antimicrobial efficacy [12] and least cytotoxic effects compared to 3% NaOCl and its cytotoxic effects.

5. CONCLUSION

Considering factors such as high antimicrobial efficacy with long term substantivity, least cytotoxicity even on fragile RBC, Morinda tinctoria at higher concentration could be potential alternative to conventional root canal irrigants and might be an adjunctive to the mechanical debridement in endodontic procedures. As the present study was conducted on RBC

as preliminary trial to evaluate the cytotoxicity, further investigation should be carried out to assess potential of Morinda tintoria to be biocompatible and effectively disinfect the root canal system.

REFERENCES

1. Matthias Zehnder: Root Canal Irrigants: Journal of Endodontics, 2006 may; 32(5):389-398.

2. Rupali Karale, Ajay Thakore, VK Shetty. An evaluation of antibacterial efficacy of 3% sodium hypochlorite, high-frequency alternating current and 2% chlorhexidine on Enterococcus faecalis: An in vitro study. Journal of Conservative Dentistry 2011 Jan-Mar; 14(1): 2-5.

3. Terence M. Gordon, Denise Damato, AS and Paul Christner. Solvent effect of various dilutions of sodium hypochlorite on vital and necrotic tissue. Journal of Endodontics 1981 Oct; 7(10): 466-469.

4. Ulrich Klein, Donald J. Kleier. Sodium Hypochlorite Accident in a Pédiatrie Patient. Pediatric Dentistry 2013 Nov/Dec; 35(7): 534-538.

5. Cemil Yesilsoy, Eugene Whitaker, Deborah Cleveland, Eric Phillips and Martin Trope. Antimicrobial and toxic effects of established and potential root canal irrigants. Journal of Endodontics 1995; 21(10): 513-515.

6. Zahed Mohammadi. Chlorhexidine gluconate, its properties and applications in endodontics. Iranian Endodontic Journal 2008;1:113-115.

7. Michael J.Jeansonne and Robert R. White. A comparison of 2% Chlorhexidine Gluconate and 5.25% Sodium hypochlorite as antimicrobial endodontic irrigants. Journal of Endodontics 1994; 20(6) : 276-278.

8. A. Nakonechnaa, P. Dorea, T. Dixonb, S. Khanc, S. Deacockc, S. Holdinga, M. Abuzakouka. Immediate hypersensitivity to chlorhexidine is increasingly recognised in the United Kingdom. Allergol Immunopathol (Madr). 2012. http://dx.doi.org/10.1016/j.aller.2012.08.001

9. Maurya Umashanker and Srivastava Shruti. Traditional indian herbal medicine used a antipyretic, antiulcer, anti-diabetic and anticancer: a review. International Journal of Research in Pharmacy and Chemistry 2011; 1(4):1152-1159.

10. Jayalakshmi Pandranki, Narsimha Rao V.V, Gutta Vijayalakshmi, K. Padma Dorothy. Ethnobotanical Approach against Resistant Endodontic Pathogens using Morinda Species – An Antimicrobial Study. Int J Biol Med Res. 2013; 4(4) :3661-3666.

11. Janakiraman Kavithasrilakshmi, Venkatramani Meenaa, Sridharan Sriram and Chinnagounder Sasikumar Phytochemical screening and antimicrobial evaluation of Morinda tinctoria Roxb. against selected microbes. International journal of pharmaceutical innovation 2012 Mar; 2(2) : 1-7.

12. Deepthi K, Umadevi P, Vijayalakshmi G, Vinod Polarao B. Antimicrobial activity and phytochemical analysis of Morinda tinctoria Roxb. Leaf extracts. Asian Pacific Journal of Tropical Biomedicine 2012; 2(3): S1440-S1442.

13. Shweta Verma, Munish Goel, Shikha Bala, Mahender Singh . Issues Of Biocompatibility Associated With Commonly Used Endodontic Irrigants: A Review. Indian Journal of Dental Sciences. 2012 Oct;4(4):109-113.

14. Kohle A.S, Nanda Patil, Shodh, Samiksha and Mulyunkan. Cytotoxicity of aqueous extract of Arecanut and tobacco on Human Erythrocytes (red Blood corpuscles) International research Journal, 2008 Nov; 2(5): 539-540.

15. Pashley E.L, Birdsong N.L, Bowman K and D.H.Pashley. Cytotoxic effects of NaOCl on vital tissue. Journal of Endodontics 1985, Dec; 11(12): 525-528.

16. Shibani Shetty K Nitesh Rai. Comparison of cytotoxicity of endodontic irrigants at various concentrations - An invitro study. Endodontology 2012 Dec ; 24(2): 13-20.

17. Yesar M.H.Al-Shamma, Nagam Yehya Gafel, Fouad Shareef Dleich, Sami R. Al-Katib. Evaluation of Sysmex Automated Hematological Analyzer (KX-21N) for the measurement of Blood Hemoglobin Level. Kufa Med.Journal 2012;15(1):1-5.

18. Joseph F. Hoffman. On Red Blood Cells, hemolysis and resealed Ghosts. Advances in experimental medicine and biology 1992; 326: 1-15.

19. Poonam Shingare, Vishwas Chaugule. Comparative evaluation of antimicrobial activity of miswak, Propolis, sodium hypochlorite and saline as root canal irrigants by microbial culturing and quantification in chronically exposed primary teeth. GERMS 2011 Dec; 1(1): 12-21.

20. Ingle J.I, Bakland L.K. Endodontics:6th edition. Elsevier Canada; 2008: pg -994.

21. Clyde L, Sabala BS and Steven E. Powell. Sodium hypochlorite injection into periapical tissues. Journal of Endodontics 1989; 15(10): 490-492.

22. Boyce ST, Warden GD, Holder IA. Cytotoxicity testing of topical antimicrobial agents on human keratinocytes and fibroblasts for cultured skin grafts. Burn Care and Rehabil 1995; 16:97–103.

23. Jay A.W.L and S. Rowlands. The stages of osmotic hemolysis. J. Physiology 1975; 252 : 817-882.

24. Oliveira DP, Barzibam JV, Trope M et al. In vitro antibacterial efficacy of endodontic irrigants against Enterococcus faecalis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007 103: 702-706.

25. Gomes BP, Ferraz CC, Vianna ME, Berber VB, Teixeira FB, Souza-Filho FJ. In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of Enterococcus faecalis. Int Endod J. 2001;34:424–8.

26. Gerald Lim H. W., Michael Wortis and Ranjan Mukhopadhyay. Stomatocyte–discocyte–echinocyte sequence of the Human red blood cell: Evidence for the bilayer– couple hypothesis from membrane mechanics. Proc. Natl. Acad. Sci 2002;99(26):16766–16769.

27. G. Brecher and M. Bessis. Present status of Spiculed Red Cells and their relationship to the Discocyte-Echinocyte transformation: A Critical Review. 1972; 40: 333-344.

28. Simpson L. 0 and B. I. Shand. Echinocytes in the blood of hyperproteinaemic mice with proteinuria. Is the effect of such cells on blood viscosity the cause of the proteinuria? Br. J. exp. Path. 1983; 64: 594-598.

29. Ronald E. Hand, Michael L. Smith, and John W. Harrison, Tacoma, Wash. Analysis of the effect of dilution on the necrotic tissue dissolution property of sodium hypochlorite. Journal of Endodontics 1978; 4(2): 61-64.