Original Article

CYTOTOXIC EFFECTS OF BIXA ORELLANA BARK EXTRACTS ON HUMAN CELL LINE (HEPG2 CELLS)

Vidusha A[1], Gayatri Devi.R[2], J.Selvaraj3[3]

[1]Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Science (SIMATS), Saveetha University, Chennai, India

[2]Associate Professor, Department of Physiology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Science (SIMATS), Saveetha University, Chennai - 600077, India

[3]Associate Professor, CoMManD, Department of Biochemistry, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Science (SIMATS), Saveetha University, Chennai - 600077, India

DOI: 10.47750/pnr.2022.13.S06.238

Abstract

Introduction: Bixaorellanais a species of tree in the family Bixaceae. The common usage of Bixaorellana include antipyretic, aphrodisiac, anti diarrhoeal, antidiabetic and insect repellant. HepG2 is an immortal cell line used as a model system for studies of liver metabolism and toxicity of xenobiotics, detection of environmental and dietary cytotoxic and genotoxic agents, understanding hepatocarcinogenesis and for drug targeting studies. The main aim of this study is to prepare Bixaorellana bark extract and to determine the cytotoxic effects of the extract on HepG2 cell line.

Materials and Methods: The Bixaorellana bark extract was made using Soxhlet extraction and concentrated using Rotary evaporator. The cell viability of HepG2 cells was measured using MTT Assay. Then the gene expression analysis of the HepG2 cells was done by RNA isolation, complementary DNA synthesis followed by Real time PCR to analyze the anticancer activity.

Results: As the concentration level increases, the cell viability dose dependently decreases. By modulating the expression of apoptotic signaling molecules, the drug was able to inhibit the proliferation of the cancer cells.

Conclusion: This study demonstrated that bark extracts of Bixaorellanawere cytotoxic against BCl 2 mRNA and BCl-xL mRNA induced apoptosis on the human cells. Active components were probably responsible for anticancer activity. MTT assay showed that as the concentration of treated Bixaorellana bark extract increases, the cancer cell viability decreases.

Keywords: BCl2 mRNA and BCl-xL mRNA, Bixaorellana bark extract, HepG2 cancer cell line, Cytotoxicity, MTT Assay.

INTRODUCTION

Cancer is a dreadful disease caused by abnormal and uncontrolled cell division. The cancer cells have the ability to potentially invade other parts of the body. About 6 million new incidences of cancer are reported yearly worldwide and more than 70% of all cancer deaths occurred in low and middle income countries. Deaths from cancer are projected to continue rising worldwide with an estimated 12 million deaths by 2030 [1]. HepG2 cells are Human liver cancer cell lines. Human Hepatocellular Carcinoma (HCC) constitutes about 85% of primary liver cancer recorded in cancer data banks. The synchronous occurrence of HCC may be due to different risk factors, such as chronic viral hepatitis B or hepatitis C infection, aflatoxin exposure, alcohol consumption and iron overload [2]. Even though curative therapy can be achieved by surgical treatments such as resection or liver transplantation and other therapies such as chemotherapy, radiotherapy, thermal ablations and using targeted medicines. However, the treatment of HCC is very disappointing because many tumors stay asymptomatic for a long time and in the absence of effective screening programs. Natural products have a long history of being used as an anti-cancer agent. Many plant compounds isolated from plants are employed in chemotherapy. More than 50%of the drugs used in clinical trials are from natural sources. Many plant metabolites induce apoptosis in cancer cells but not in normal cells, thus making them potential drug leads [3]. Interestingly, a number of non-nutrient chemicals from plants and fruits have also been reported to possess anticancer activity, certain products from plants are known to induce apoptosis in neoplasticism cells but not in normal cells [4]. The development of chemotherapeutic or chemo preventive agents for hepatocellular carcinoma is important in order to help reduce the mortality caused by this disease. Plants have many photo chemicals with various bioactivities, including

antioxidant, anti-inflammatory and anti-cancer functions. Some studies have reported that extracts from natural products such as medicinal herbs, fruits and vegetables have positive effects against cancer compared with other treatment options such as hormonal treatment or chemotherapy and radiotherapy, etc. [5]

Bixaorellana (also known as achiote) is a species of tree in the family Bixaceae. They have simple, broad leaves and are native to Central America. But it is grown in many countries worldwide. Bixaorellanais used in traditional medicine. The tree has been used in Ayurveda, the folk medicine practices of India, where different parts of the plant are thought to be useful as therapy. The popular uses of Bixaorellana are antipyretic, aphrodisiac, anti-diarrhoeal, anti-diabetic and insect repellant [6]. A number of ailments, including tonsillitis, asthma, pneumonia, colonic problems, migraine, jaundice, sunstroke, and burns, have been treated with formulations made from the seeds and leaves of the B.orellana tree [1]. Our team has extensive knowledge and research experience that has translate into high quality of publications [7–20]

Numerous studies have demonstrated the enzymatic antioxidant properties of plant extracts and their ability to reduce lipid peroxidation. Therefore, an initiative has been taken to study the effects of B.orellana bark extract against the extract's cytotoxic effects on the HepG2 cell line.

MATERIALS AND METHODS:

Chemicals:

Trypsin EDTA, fetal bovine serum (FBS), antibiotics- antimycotics, Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) were purchased from Gibco, Canada. JC-1 (5,5,6,6 - tetrachloro - 1,1,3,3 - tetraethyl benzimidazolecarbocyanine iodide) and real time PCR kit (MESA Green) were purchased from Invitrogen, USA. All the chemicals used were extra pure of analytical grade.

Extract preparation:

The Bixaorellanabark powder was Soxhlet extracted with 70% ethanol. The extract was then filtered with Whatman no.1 filter paper and the solvent evaporated at reduced pressure by using a Rotary evaporator apparatus to get a viscous mass, which was then stored at 4˚ until used.

Cell viability assay:

HepG2 cells were seeded at a density of 5x105 cells/wells in 96 well plates and allowed to attach to the well overnight. After incubation, cultured cells were stimulated with various concentrations of Bixaorellana bark extracts in triplicate and incubated at 370C in a 5% humidified CO2 incubator for 24 hours. Subsequently, 3-(4,5- dimethylthiazole 2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubation was continued for a further 4h at 37˚Celsius. To dissolve the formazan formed from MTT, the cells were resuspended in 200 µl dimethyl sulfoxide (DMSO), and the optical density (OD) of the solution was determined using a spectrometer at a wavelength of 570 nm. The experiments were repeated 3 times, independently. The mean optical density (OD) ± SD for each group of replicates was calculated. The entire procedure was repeated 3 times. The inhibitory rate of cell growth was calculated using the equation:

% Growth inhibition = (1 - ODextract treated)/OD negative control x 100.

Gene expression analysis by Real Time PCR:

mRNA expression levels were examined using Real Time PCR. The total RNA was isolated by using Tri Reagent (Sigma). Total RNA (2 µg) from each sample was reverse transcribed using a commercial SuperscriptIII first strand cDNAsynthesis kit (Invitrogen, USA) according to the manufacturer's protocol. Real time-PCR was carried out in a MX3000p PCR system (Stratagene, Europe). Reaction was performed using MESA Green PCR master mix (It contains all the PCR components along with SYBR green dye.) Eurogentec, USA. The specificity of the amplification product was determined by melting curve analysis for each primer pair. The data were analyzed by comparative CT method and the fold change is calculated by 2−CT method described by Schmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

Statistical analysis:

Data were expressed as the means ± SD of 3 individual experiments performed in triplicate. Statistical analysis was performed using the one-way ANOVA and $p<0.05$ was considered to indicate a statistically significant result.

RESULT AND DISCUSSION:

Many contemporary medications are developed using various pharmacologically active components of herbal medicines. Understanding plant toxicity and protecting living beings from natural toxins are both made easier by studying medicinal plants [21]. However, it has been noted that the complete contents of a whole herb show a substantially higher synergistic impact than a single specific active component. Several anticancer researches have focused on the potential of pure substances [22]. The antiproliferative effect of Bixaorellana was estimated by MTT Assay. The color intensity is directly proportional to the amount of line cells. The amount of cell viability is calculated in terms of percentage corresponding to the different concentration of bark extract. At control, the cell viability is 100%. When 50µg/ml of bark extract is used, the cell viability is 80%, 100 µg/ml the viability is 60% and for 200 µg/ml the viability is 50% (Figure 1). This shows that as the concentration of the extract increases, there is a significant decrease in the cell viability, which further implies that the higher concentration level (i.e) 100 microgram and 200 microgram has significantly inhibited at least 50% of the HepG2 cancer cell proliferation compared to the lower concentration (i.e) 50 µg/ml. The optimal dose is found in terms of inhibitory concentration. Therefore, the higher concentration can be considered as the optimal dose.

The pro- and anti-apoptotic byproducts of apoptotic genes control apoptosis. The Fas, Caspase, and Bcl-x family of proteins are among the most significant proteins. The Bax/Bcl-2 pathways are used to achieve many pharmacological components for anti-tumor growth and efficacy in vitro [23,24]. Apoptotic gene expression is very variable in vivo and is linked to the clinical and pathologic uniqueness of tumor growth and metastasis. Bcl-2 and Bax protein expression and clinical prognostic variables, however, have varying relationships [252627].

This study elucidates the underlying mechanisms after finding that B. orellana extract, particularly at concentrations of 200µg/ml, triggers HepG2 cell death. The Bax family is known to play a significant part in cell apoptosis. After being treated to B. orellana extract at escalating doses (0, 50, 100, and 200µg/ml,) for 24 hours, HepG2 cells had their levels of Bax and Bcl-2 mRNA expression evaluated using real-time PCR. In HepG2 cells, B. orellana extract, especially at the 200 µg/ml, treatment dose, reduced the mRNA expression of the pro-apoptotic gene Bax and decreased the expression of the anti-apoptotic gene Bcl-2 in comparison to the negative control. The BCl 2 mRNA gene showed no significant change and BCL-xL mRNA showed a significant change in the concentration level of 100 and 200µg compared to control and low dose (Figure 2).

The bark extract of Bixaorellanapossesses potent anticancer and apoptotic inductive potential against HepG2 cells. There is a significant decrease in the cascade activity after incubation with the bark extract in the human cell line. BCl2 mRNA is a protein that helps control whether a cell lives or dies by blocking a type of cell death called apoptosis. This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptosis death of some cells such as lymphocytes [28]. BCl-xL mRNA gene is found mainly in the cytoplasm of liver cancer or dies by blocking a type of cell death called apoptosis. This gene encodes an integral outer mitochondrial membrane protein that blocks apoptotic death [29]. Trans-Geranylgeraniol, squalene, and beta-sitosterol are the active components in B.orellanathat appear to be the main causes of the cytotoxicity and apoptotic-like properties. B.orellanabark extractcan inhibit proliferation of HepG2 cells and promote apoptosis through Bax/Bcl-2 pathways. There is proof that overexpressing either Bcl-2 or Bcl-Xl would suppress the other, demonstrating that these two proteins are regulated in a mutually exclusive manner.

Extensive research will need to confirm whether B.orellana has the potential to enhance the antitumor effects of anti-melanoma medications in in vivo models. This may give a better idea for the pharmacological uses in the industries.

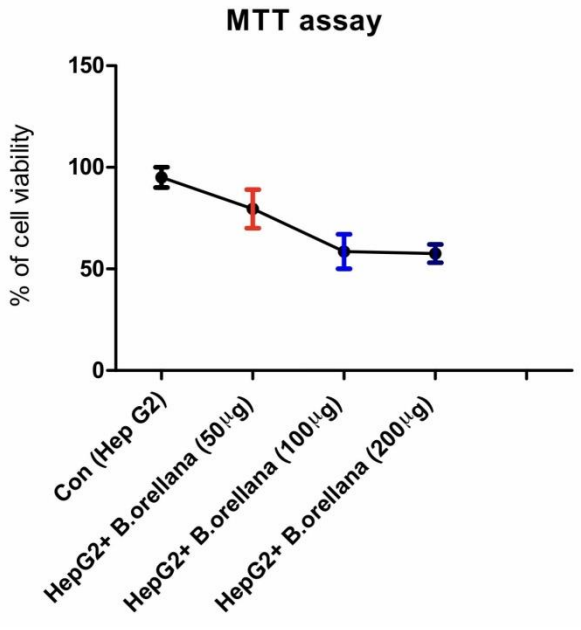

Figure 1: MTT assay

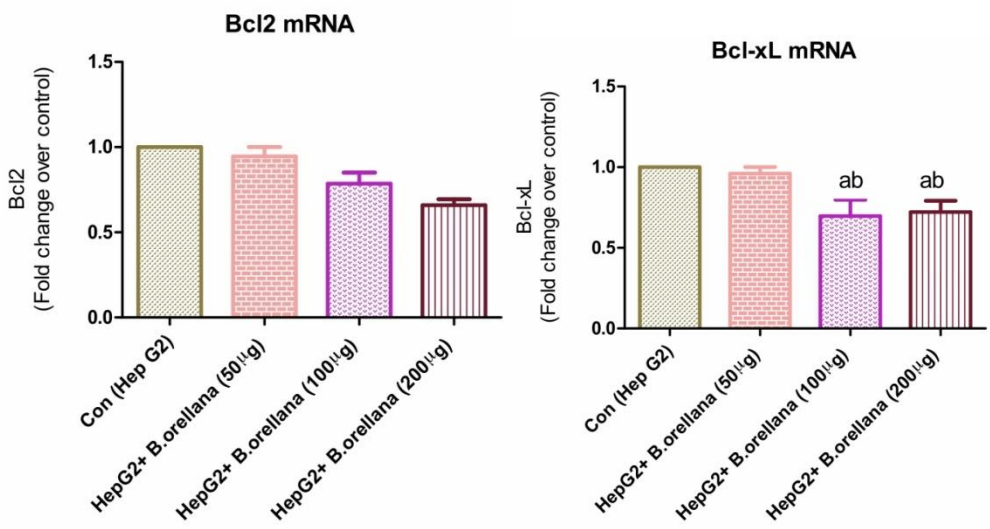

Figure 2: Bcl 2 and Bcl-xl mRNA expression

CONCLUSION:

The current study demonstrated that bark extracts of Bixaorellanawere cytotoxic against BCl2 and BCl-xL mRNA induced apoptosis on human cells. By modulating the expression of apoptotic signaling molecules, the drug was able to inhibit the proliferation of HepG2 cancer cells. Active components are probably responsible for anticancer activity.

Acknowledgement: The authors would like to thank Saveetha Dental college and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha university for providing research laboratory facilities to carry out the study.

Source of funding:

The present project was funded by

- Saveetha Institute of Medical and Technical Sciences
- Saveetha Dental College and Hospital
- Saveetha University
- AishwaryamSpeciality Hospital

Conflict of interest: All the authors declare that there was no conflict of interest in the present study

Author Contributions:

Vidusha A: Literature search, Data collection analysis, Manuscript drafting

Dr.R. GayatriDevi :Data Verification, Manuscript draft

Dr.J.Selvaraj: Data collection analysis, Data Verification, Manuscript draft

REFERENCES

1. Bioassay-guided in vitro study of the antileishmanial and cytotoxic properties of Bixa orellana seed extract [Internet]. Journal of Coastal Life Medicine. 2014. Available from: http://dx.doi.org/10.12980/jclm.2.2014apjtb-2014-0074
2. Kar P. Risk factors for hepatocellular carcinoma in India. J ClinExpHepatol. 2014 Aug;4(Suppl 3):S34–42.
3. Pathania AS, Wani ZA, Guru SK, Kumar S, Bhushan S, Korkaya H, et al. The anti-angiogenic and cytotoxic effects of the boswellic acid analog BA145 are potentiated by autophagy inhibitors [Internet]. Vol. 14, Molecular Cancer. 2015. Available from: http://dx.doi.org/10.1186/1476-4598-14-6
4. Sivalokanathan S. Effects of Terminalia arjuna bark extract on apoptosis of human hepatoma cell line HepG2 [Internet]. Vol. 12, World Journal of Gastroenterology. 2006. p. 1018. Available from: http://dx.doi.org/10.3748/wjg.v12.i7.1018
5. Koschutnig K, Heikkinen S, Kemmo S, Lampi AM, Piironen V, Wagner KH. Cytotoxic and apoptotic effects of single and mixed oxides of β-sitosterol on HepG2-cells [Internet]. Vol. 23, Toxicology in Vitro. 2009. p. 755–62. Available from: http://dx.doi.org/10.1016/j.tiv.2009.03.007
6. Hindawi. Traditional Uses, Chemical Constituents, and Biological Activities of Bixa orellana L.: A Review [Internet]. [cited 2022 Sep 24]. Available from: https://www.hindawi.com/journals/tswj/2014/857292/
7. Felicita AS. Quantification of intrusive/retraction force and moment generated during en-masse retraction of maxillary anterior teeth using mini-implants: A conceptual approach. Dental Press J Orthod. 2017 Sep;22(5):47–55.
8. Jain AR. Prevalence of Partial Edentulousness and treatment needs in Rural Population of South India. World J Dent. 2017 Jun;8(3):213–7.
9. Kumar S. The emerging role of botulinum toxin in the treatment of orofacial disorders: Literature update. Asian J Pharm Clin Res. 2017 Sep 1;10(9):21.
10. Sivamurthy G, Sundari S. Stress distribution patterns at mini-implant site during retraction and intrusion--a three-dimensional finite element study. ProgOrthod. 2016 Jan 18;17:4.
11. Kumar MS, Vamsi G, Sripriya R, Sehgal PK. Expression of matrix metalloproteinases (MMP-8 and -9) in chronic periodontitis patients with and without diabetes mellitus. J Periodontol. 2006 Nov;77(11):1803–8.
12. Azeem RA, Sureshbabu NM. Clinical performance of direct versus indirect composite restorations in posterior teeth: A systematic review. J Conserv Dent. 2018 Jan;21(1):2–9.
13. Krishnan V, Lakshmi T. Bioglass: A novel biocompatible innovation. J Adv Pharm Technol Res. 2013 Apr;4(2):78–83.
14. Sekar D, Lakshmanan G, Mani P, Biruntha M. Methylation-dependent circulating microRNA 510 in preeclampsia patients. HypertensRes. 2019 Oct;42(10):1647–8.
15. Felicita AS, Chandrasekar S, Shanthasundari KK. Determination of craniofacial relation among the subethnic Indian population: a modified approach - (Sagittal relation). Indian J Dent Res. 2012 May;23(3):305–12.
16. Neelakantan P, Subbarao C, Subbarao CV, De-Deus G, Zehnder M. The impact of root dentine conditioning on sealing ability and push-out bond strength of an epoxy resin root canal sealer.IntEndod J. 2011 Jun;44(6):491–8.
17. Jain RK, Kumar SP, Manjula WS. Comparison of intrusion effects on maxillary incisors among mini implant anchorage, j-hook headgear and utility arch. J ClinDiagn Res. 2014 Jul;8(7):ZC21–4.
18. Johnson J, Lakshmanan G, M B, R M V, Kalimuthu K, Sekar D. Computational identification of MiRNA-7110 from pulmonary arterial hypertension (PAH) ESTs: a new microRNA that links diabetes and PAH. Hypertens Res. 2020 Apr;43(4):360–2.
19. Keerthana B, Thenmozhi MS. Occurrence of foramen of huschke and its clinical significance. J Adv Pharm Technol Res. 2016;9(11):1835.
20. Lakshmi T, Krishnan V, Rajendran R, Madhusudhanan N. Azadirachtaindica: A herbal panacea in dentistry - An update. Pharmacogn Rev. 2015 Jan;9(17):41–4.
21. Singh B, Bani S, Gupta DK, Chandan BK, Kaul A. Anti-inflammatory activity of "TAF" an active fraction from the plant Barleria prionitis Linn [Internet]. Vol. 85, Journal of Ethnopharmacology. 2003. p. 187–93. Available from: http://dx.doi.org/10.1016/s0378-8741(02)00358-6
22. Ma XH, Zheng CJ, Han LY, Xie B, Jia J, Cao ZW, et al. Synergistic therapeutic actions of herbal ingredients and their mechanisms from molecular interaction and network perspectives. Drug Discov Today. 2009 Jun;14(11-12):579–88.
23. Cai Y, Sheng ZY, Chen Y, Bai C. Effect of Withaferin A on A549 cellular proliferation and apoptosis in non-small cell lung cancer. Asian Pac J Cancer Prev. 2014;15(4):1711–4.
24. Tang Y, Zhang X, Qi F, Chen M, Li Y, Liu L, et al. Afatinib inhibits proliferation and invasion and promotes apoptosis of the T24 bladder cancer cell

line [Internet]. Vol. 9, Experimental and Therapeutic Medicine. 2015. p. 1851–6. Available from: http://dx.doi.org/10.3892/etm.2015.2314
25. Stark AM, Hugo HH, Tscheslog H, Maximilian Mehdorn H. p53, BCL-2 and BAX in non-small cell lung cancer brain metastases: a comparison of real-time RT-PCR, ELISA and immunohistochemical techniques [Internet]. Vol. 29, Neurological Research. 2007. p. 435–40. Available from: http://dx.doi.org/10.1179/016164107x165282
26. Zhu Y, Lv H, Xie Y, Sheng W, Xiang J, Yang J. Enhanced tumor suppression by an ING4/IL-24 bicistronic adenovirus-mediated gene cotransfer in human non-small cell lung cancer cells [Internet]. Vol. 18, Cancer Gene Therapy. 2011. p. 627–36. Available from: http://dx.doi.org/10.1038/cgt.2011.31
27. Toffart AC, Timsit JF, Couraud S, Merle P, Moro-Sibilot D, Perol M, et al. Immunohistochemistry evaluation of biomarker expression in non-small cell lung cancer (Pharmacogenoscan study) [Internet]. Vol. 83, Lung Cancer. 2014. p. 182–8. Available from: http://dx.doi.org/10.1016/j.lungcan.2013.12.003
28. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch [Internet]. Vol. 2, Nature Reviews Cancer. 2002. p. 647–56. Available from: http://dx.doi.org/10.1038/nrc883
29. Petros AM, Olejniczak ET, Fesik SW. Structural biology of the Bcl-2 family of proteins [Internet]. Vol. 1644, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2004. p. 83–94. Available from: http://dx.doi.org/10.1016/j.bbamcr.2003.08.012
30. Borkataky, Munmi, and Sood KAUSHAL. "Antibacterial, antioxidant and cytotoxic activities of Cinnamomum tamala Nees. Leaves." TJPRC: International Journal of Medicine and Pharmaceutical Sciences 4.6 (2014): 55-62.
31. Raikar, Shanthanu M., Vishal U. Kalebar, and Shakeel Ahmed Adhoni. "Screening of pharmacological and cytotoxic activities of fresh water lake isolated microalgae Chlorella vulgaris as-13 and Chlorella pyrenoidosa AS-6." TJPRC: Int. J. Bio-Technology Res 8.4 (2018): 1-8.
32. Moffatt, Stanley, and Mutune Wangari. "Enhanced Silencing of Bmi-1 and Htert Gene Expression with Ngr-Pei-Coupled Sirna Lipoprotein Nano-Complexes." TJPRC: Journal of Medicine and Pharmaceutical Science (TJPRC: JMPS) 2 (2016): 9-18.
33. Hanif, Farina, Et Al. "In Vitro Growth Inhibition of U87 Glioblastoma Cells by N-(2-Hydroxy Phenyl) Acetamide." TJPRC: International Journal of Medicine and Pharmaceutical Sciences (TJPRC: IJMPS)Vol. 3, Issue 4, Oct 2013, 69-78.
34. Salma, Ahmed Abdulwahid, and Mohammed Younus Naji Al Atbee. "Prevalence of Hepatitis C Virus Genotypes and Viral Load Isolated from Iraqi Patients by Reverse Transcription-Pcr." TJPRC: International Journal of Medicine and Pharmaceutical Sciences (IJMPS) ISSN (P): 2250-0049; ISSN (E): 2321-0095 Vol. 6, Issue 2, Apr 2016, 1-6.
35. Aljammali, Zainab Mahmood. "Effect Of Drugs On Teeth And Gums-A Review." TJPRC: International Journal of Pedodontics and Preventive Dentistry (TJPRC:IJPPD) Vol. 1, Issue 1, Jun 2015, 1-8