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Abstract

Background: The present study has evaluated the Emodin efficacy on the Akt, MAPK, ERK and DNMT expression pattern during 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinoma in golden Syrian hamsters, in order to explore its antitumor potential.

Materials and methods: Oral tumors were developed in the buccal pouches of golden Syrian hamsters using the carcinogen, DMBA.

Results: While the incidence of tumor formation was 100% in hamsters treated with DMBA alone, the tumor formation was not noticed in DMBA+ Emodin treated hamsters. Also, Emodin reduced the severity of precancerous pathological lesions such as dysplasia, in the hamsters treated with DMBA. Emodin administration corrected the abnormalities in the expression pattern of Akt, MAPK, ERK and DNMT in the buccal mucosa of hamsters treated with DMBA.

Conclusions: The present study thus suggests that the tumor preventive potential of Emodin is partly related to its modulating effect on the Akt, MAPK, ERK and DNMT expression pattern, as these molecular markers have a pivotal role in the process of cell proliferation, inflammation, invasion, and apoptosis.

Keywords: Oral carcinoma, Akt, MAPK, DNMT.

Introduction

Oral squamous cell carcinoma, a cancer of the oral cavity, is one of the ten most common malignant neoplasms and is the 5th most frequent cancer worldwide (Markopoulos, 2012). The development of oral cancer is usually preceded by distinct precancerous lesions such as leukoplakia, erythroplakia and oral submucous fibrosis. However, the potential or degree of malignant transformation varies from one another (Carnelio et al., 2011). Oral carcinoma has a spectrum of risk factors, which include tobacco, alcohol, diet and nutrition, betel quid, viruses, immune deficiency and poor oral hygiene (Khyani et al., 2015). Tobacco chewing together with tobacco smoking, betel quid chewing and alcohol abuse is recognized as the strongest risk factor of oral cancer (Sridharan, 2014). Several epidemiological and aetiological studies pointed out the synergistic efficacy of tobacco and alcohol in the development of oral carcinoma (Patil et al., 2013). Although oral cancer is common in several countries, the highest incidence is reported every year from India, Pakistan, Sri Lanka, France and Hungary (Vigneswaran and Williams, 2014). The behavioral risk factors, tobacco and alcohol, accounts for 75-95% of all oral cancers in India (Gupta et al., 2013). Creating awareness on the risk factors of oral cancer to the public could help to reduce the annual incidence, since the risk factors are avoidable one (El Rhazi et al., 2014). It has also been pointed out that early diagnosis will improve the survival outcome of the patients (80%) and quality of life than diagnosis at late stage [30%] (Baykul et al., 2010).

Golden Syrian hamsters serve as an ideal model to study the tumor inhibitory potential of natural products, especially in oral carcinoma (Steele and Lubet, 2010). Hamsters possess buccal pouches (a pocket like anatomy) on their oral cavity, which can retain the carcinogen for the longest time when applied topically and thus tumors develop within a shorter duration (Chen et al., 2012). DMBA induces buccal pouch carcinogenesis in the hamsters through inducing chronic inflammation, causing extensive oxidative DNA damage and by inducing DNA mutations (Ismail et al., 2016). Oral cancer chemoprevention researchers prefer DMBA induced hamster buccal pouch carcinogenesis model, as this model exhibits close resemblance and similarities with human oral tumor in many aspects, including at histopathological and molecular levels (Manoharan et al., 2016; Casto et al., 2013).

P13K/Akt and ERK/MAPK pathways perform a pivotal role in the regulation of tumor growth, progression, invasion and metastasis (Gan et al., 2010). As P13K/Akt pathways play crucial and critical role in both cell survival and apoptotic pathway, their abnormal regulation or expression could mediate the neoplastic transformation in various cancers including oral carcinoma (Chang et al., 2013). The status of p-Akt has been focused as an independent prognostic marker in various cancers. Inhibition of P13K/Akt pathway was resulted in the induction of apoptotic cascade in various cancers (Matsuoka and Yashiro, 2014). P13K/Akt pathway was significantly activated during oral carcinogenesis. Tumor growth inhibition could thus be achieved by blocking the activation of Akt or ERK signaling pathways (Chappell et al., 2011).

MAPK family plays an important role in cell differentiation, proliferation, inflammation and apoptosis. This family also consists of ERK and P³⁸. Profound studies pointed out that P³⁸ MAPK are activated by a bundle of stimuli such as growth factors, cytokines and chemical stresses, resulting in cell proliferation, migration and apoptosis (Gerthoffer et al., 2012). MAPK has also a pivotal role in the regulation of angiogenesis. ERK, a subfamily of the MAPK family, plays an important role in the regulation of cell differentiation and proliferation (Munshi and Ramesh, 2013). ERK has been identified as a positive regulator of cell cycle and it stimulates cyclic dependent kinases - cyclin D complex, which is essential for the progression of the cell cycle (Villanueva et al., 2007). Several studies have pointed out that the accumulation of activated ERK (pERK) in the nucleus could result in genomic

instability as well as promotes tumorigenesis. ERK also has a role in the stimulation of tumor suppressor pathways (Bobrovnikova et al., 2010). DNA methylation, a major factor in epigenetic phenomenon, plays prominent role in the process of carcinogenesis. The enzyme DNA methyl transferase (DNMT) regulates the process of DNA methylation (Jin et al., 2011). Profound studies on various cancers documented the over expression of DNMT (Estève et al., 2016). Thus, DNMT has been utilised as a major target for cancer treatment.

Emodin, an anthraquinone derivative, is present in the root and rhizome of several Chinese medicinal plants and used in traditional Chinese medicine to treat numerous illnesses (Yang et al., 2014). Profound studies documented the pharmacological, biochemical and therapeutic effects of Emodin using experimental animal models or via in vitro approach. Emodin explored its cytotoxic potential against various tumor cell lines including human lung squamous carcinoma cells, human cervical cancer cells, human hepatoma cells, human breast cancer cells and human cervix epithelioid carcinoma cells (Zu et al., 2015; Yaoxian et al., 2013; Huang et al., 2013; Huang et al., 2013). Emodin exerted apoptotic potential in various cancer cell lines. In vivo studies demonstrated its antidiabetic, hepatoprotective and anticancer properties (Zhao et al., 2009; Lin et al., 2012). The present study explores the Emodin efficacy on the Akt, MAPK, ERK and DNMT expression pattern during 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinoma.

Materials and methods

Animals

We procured forty golden Syrian hamsters, (male; 80-120g) from National Institute of Nutrition, Hyderabad, India and were maintained according to the Institutional ethical committee guidelines in the Central Animal House, Annamalai University (Registration Number 160/1999/ CPCSEA).

Tumor induction

The present study utilized DMBA, the site specific and organ specific carcinogen to produce tumors in the buccal pouches of golden Syrian hamsters. Topical application of this carcinogen (0.5% in liquid paraffin) three times a week for 14 weeks developed well differentiated squamous cell carcinoma (confirmed by oral pathologist, Annamalai University)

Experimental design

The experimental hamsters were divided into four groups of ten in each and housed in the polypropylene animal cages (5 animals/cage). The experimental hamsters were provided with pellet diet and water *ad libitum*. The experimental protocol followed in the present study is given in the figure 1.

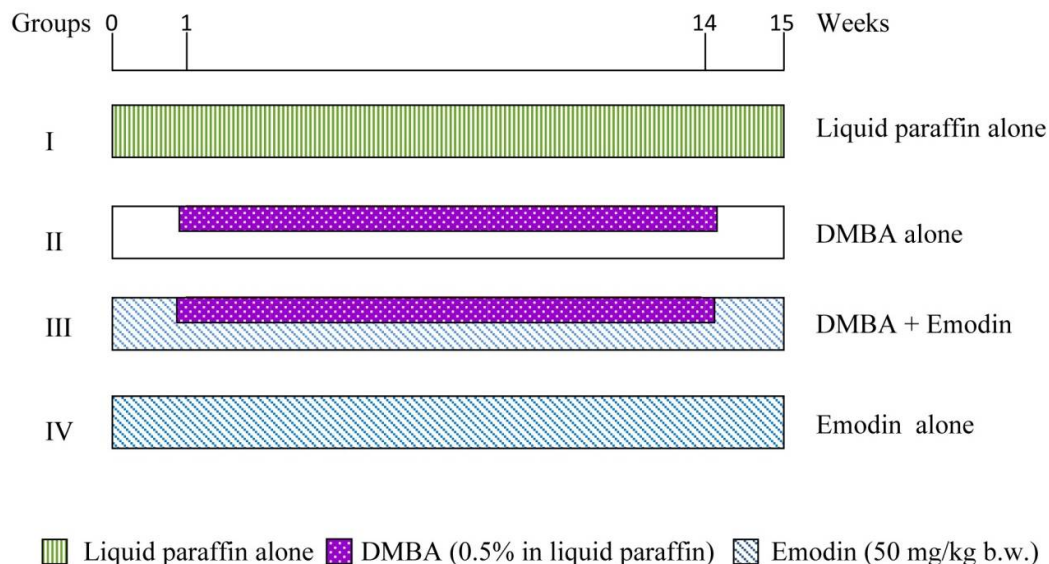


Figure 1: Experimental protocol

Group I hamsters received liquid paraffin alone on their buccal pouches (topical application, three times a week for 14 weeks) and served as a vehicle treated control. The buccal pouches of group II hamsters were exposed to 0.5% DMBA in liquid paraffin alone (Topical application; three times a week for 14 weeks) and served as carcinogen treated control. Group III hamsters were exposed to DMBA, as in group II, and were orally administered with Emodin (50mg/kg b.w), three times a week for 14 weeks;

on alternate days of DMBA painting. Group IV hamsters received Emodin alone orally (three times a week for 14 weeks). After the experimental period, the buccal mucosa was excised from all the experimental hamsters and subjected to Western blotting to assess the expression pattern of p-Akt, p-ERK, p-P³⁸ MAPK, DNMT1, DNMT3a and DNMT3b

Western blotting

The proteins were extracted from the buccal mucosa tissues and quantified (Bradford, 1976). The tissue extract was then subjected to polyacrylamide gel electrophoresis (PAGE) to separate the various proteins. Electroblothing was employed to transfer the separated protein bands onto PVDF membrane. The membrane containing protein blots were then incubated with the corresponding primary antibodies (p-Akt, p-ERK, p-P³⁸ MAPK, DNMT1, DNMT3a and DNMT3b, Cell Signaling Technology, Danvers, MA, USA). After the incubation period the blot was treated with the secondary antibodies labelled with horseradish peroxidase (Santa Cruz Biotechnology, USA). The immune complex formed was then treated with diaminobenzidine, and the bands were scanned and analysed densitometrically (Bio-Rad Image Lab™ software version 4.1 software).

Statistical analysis

The statistical significance between the groups was assessed using One way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The results obtained were considered statistically significant if the p value was less than 0.05 between the two groups.

Results

Buccal mucosa p-Akt, p-ERK, p-P³⁸ MAPK, DNMT1, DNMT3a and DNMT3b expression pattern obtained using Western blotting and their densitometry analysis in the control and experimental hamsters are given in the figures 2-5. Western blot analysis revealed over-expression of the above said molecular markers in the hamsters treated with DMBA alone when compared to control hamsters. Emodin administration orally at the dose of 50mg/kg b.w to the hamsters treated with DMBA brought back the expression pattern of all the markers to near normal range.

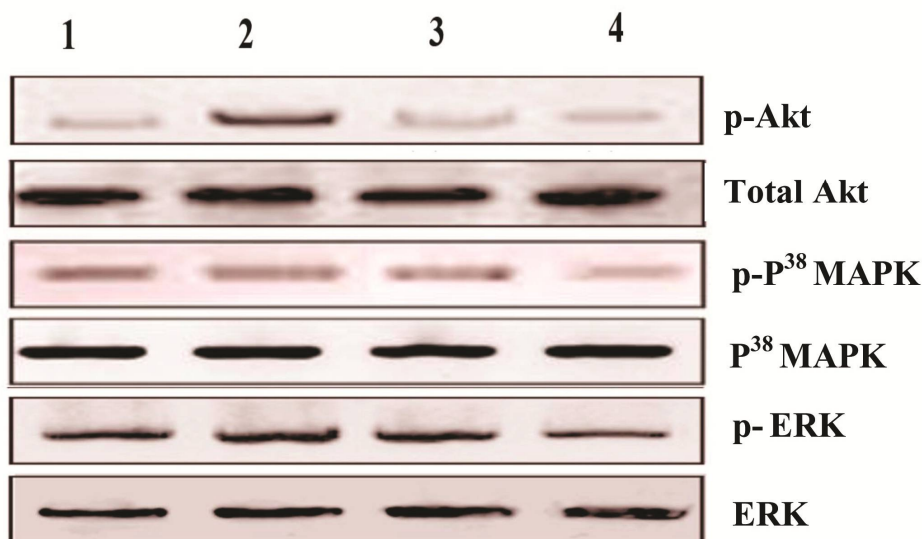


Figure 2: Expression pattern of p-Akt, p-P³⁸ MAPK and p-ERK in the buccal pouch tissues of control and experimental animals in each group. Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA + Emodin, Lane 4: Emodin alone.

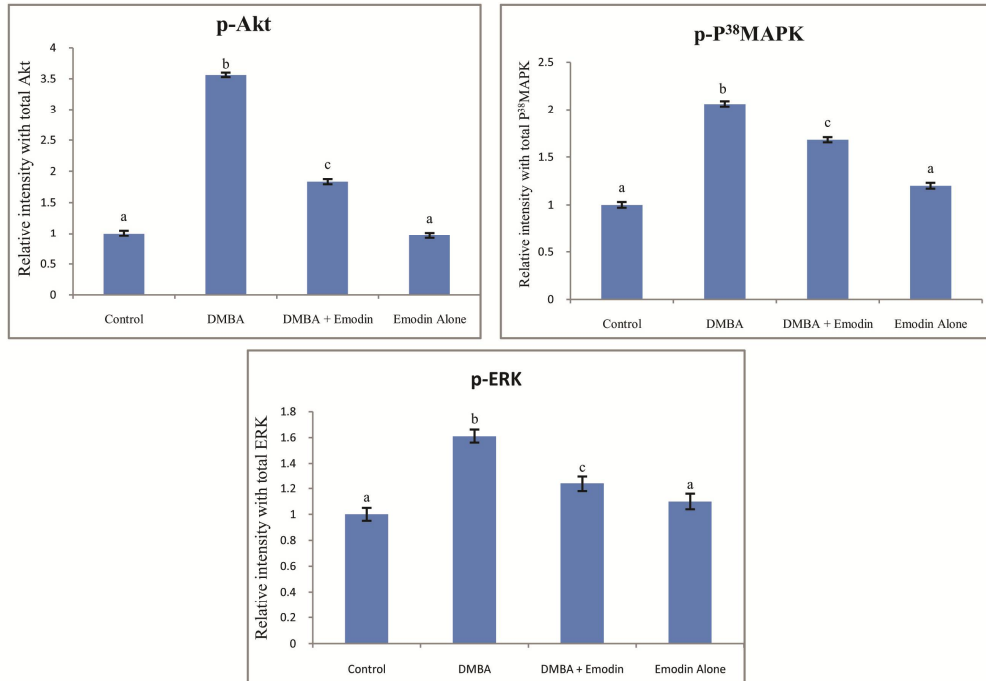


Figure 3: Densitometric analysis for p-Akt, p-P³⁸ MAPK and p-ERK expression pattern in control and experimental animals in each group. Data presented are the mean \pm SD (n=10). Common superscripts between two groups - not significant. Different superscripts between two groups - significant p<0.05.

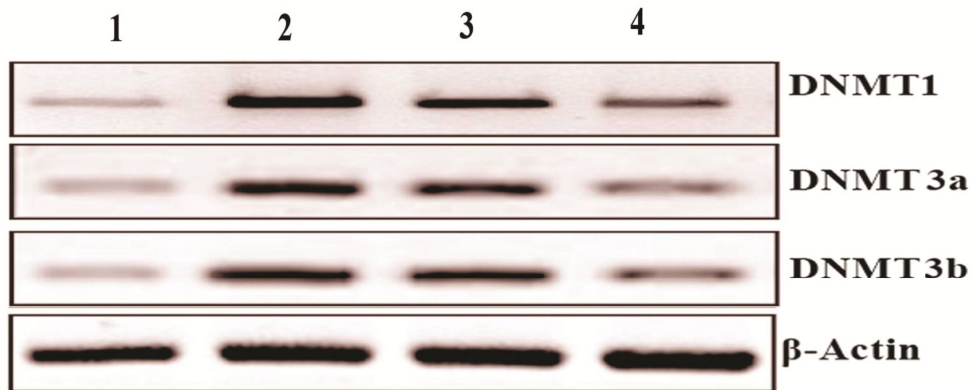


Figure 4: Expression pattern of DNMT1, DNMT3a and DNMT3b in the buccal pouch tissues of control and experimental animals. Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA + Emodin, Lane 4: Emodin alone.

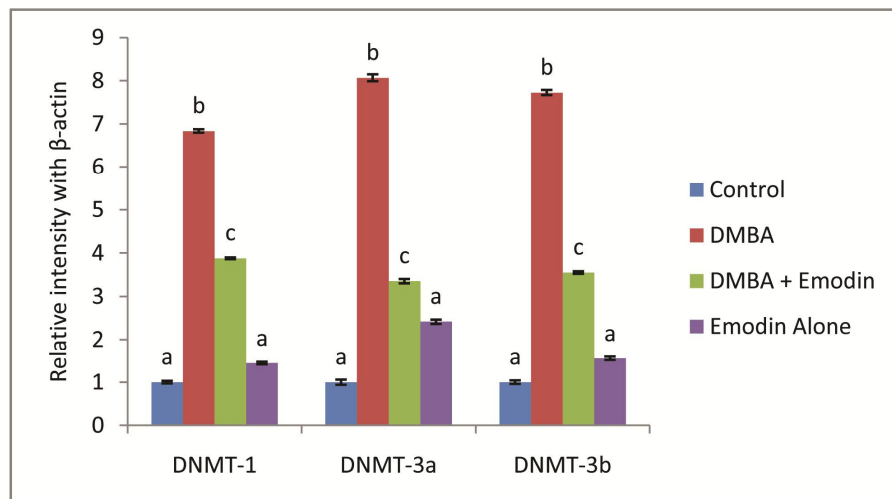


Figure 5: Densitometric analysis for DNMT1, DNMT3a and DNMT3b expression pattern in control and experimental animals in each group. Data presented are the mean \pm SD (n=10). Common superscripts between two groups - not significant. Different superscripts between two groups - significant $p < 0.05$.

Discussion

Oral cancer arises due to the abnormalities in multiple molecular pathways, which include pathways of cell proliferation, apoptosis, inflammation and angiogenesis (Manoharan et al., 2015). Extensive studies utilized these molecular pathways to evaluate the antitumor potential of the medicinal plants, their active constituents and synthetic agents (Schetter et al., 2010). The present study has utilized the Akt, MAPK, ERK and DNMT pathways to explore the Emodin efficacy in the inhibition of tumor formation during 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinoma.

Akt and MAPK pathways are involved in the regulation of genes that play pivotal role in cell proliferation, inflammation, invasion, autophagy and apoptosis. Apoptosis and arrest of cell growth can be achieved by blocking Akt activation in tumor cells (Jiang and Liu, 2009). The Akt pathway plays an important role in the regulation of cell-cycle progression and tumor cell growth (Antico Arciuch et al., 2012). A large number of studies showed increased activity of Akt, a well known serine/ threonine kinase, in various types of malignancies including oral cancer (Lee, 2014). Upregulation of Akt/P³⁸ MAPK signaling pathway has been reported in oral carcinoma HSC-3 cells. Akt mediates its self proliferative role via inhibiting pro-apoptotic proteins or promoting anti-apoptotic genes (Kalimuthu and Se-Kwon, 2013). Akt pathway was up-regulated in 30 to 60% of prostate carcinogenesis (Shtivelman et al., 2014).

A pro-apoptotic as well as anti-apoptotic role for P³⁸ MAPK has been reported in tumor cells. Multiple evidences explored deregulation in P³⁸MAPK signaling pathway, which has been associated with malignant transformation (Wagner and Nebreda, 2009). Deregulation of MAPK pathway has been reported in several cancers, particularly in more than 50% of hepatocellular carcinomas. It has been reported that the mammary cancer metastasis can be inhibited by inhibiting P³⁸ expression (Dhillon et al., 2007).

ERKs occupy the central position of diverse cellular signaling pathways that are involved in cell growth, proliferation and survival (Hong et al., 2015). Deregulation in the ERK / MAPK pathway is associated with the poor prognosis of oral cancer. ERK1/2 and P³⁸, the subfamilies of MAPK, were deregulated in various carcinogenesis (Mebratu and Tesfaigzi, 2009). Aberrant ERK signaling could therefore lead to malignant transformation. ERK activation resulted in the activation of the spectrum of genes including PCNA, which in turn favors malignant transformation (Jin and Robertson, 2013).

There are three classes of DNMTs in the mammalian cells, DNMT1, DNMT3a and DNMT3b. DNMT1 is a maintenance methyltransferase and DNMT3a and DNMT3b are de novo methyltransferases (Liang et al., 2002). All three types of DNMT were abnormally expressed in cancerous conditions (Skowronski et al., 2010). DNMT1 plays prominent and pertinent role for DNA methylation during DNA replication. DNMT1 over expression was reported in lung, colorectal, prostate, breast, gastric and oral cavity cancers (Delpu et al., 2013). It has been pointed out that DNMT inhibition might lead to reactivation of the silenced genes. Down-regulation of p53 has been associated with up-regulation of DNMT1 (Au Yeung et al., 2010).

Extensive studies reported the effect of Emodin on the Akt, MAPK, ERK and DNMT expression pattern in various cancer cell lines. Lin et al., (2016) pointed out that Emodin induced apoptotic process in the hepatic cancer cell lines via P13K/Akt and MAPK signaling pathways. Liu et al., (2015) explored the inhibitory effect of Emodin on the expression of P13K/Akt in the mechanical stress induced hypertrophic scarring. Emodin prevented the mammary cancer cell proliferation via down-regulating P13K/Akt protein expression (Sui et al., 2014). It has been reported that Akt down-regulation by Emodin is due to the inhibition of the components of the P13K pathway (Olsen et al., 2007). Zheng et al., (2015) observed that Emodin downregulated Akt expression in K562/Adr cells. Way et al., (2014) demonstrated that Emodin effectively inhibited the epithelial-mesenchymal transition in head and neck cancer cells via inhibiting Akt pathways. Inhibition of Akt activation has been suggested as a major mechanism of antitumor effect of Emodin against pancreatic cancer in mice (Wei et al., 2011). It has been pointed out that Emodin attenuated the

phosphorylation of PI3K/Akt and ERK in HPG2 cells (Cui et al., 2016). Emodin exerted its antiinvasive property via down-regulating ERK1/2 and Akt/PKB activation in glioma cells (Kim et al., 2005). Inactivation of ERK and Akt has been demonstrated in human lung adenocarcinoma cells (Su et al., 2005). Emodin downregulated the expression of DNMT1 and DNMT3 in pancreatic cancer cells. (Pan et al., 2016; Zhang et al., 2015). We, for the first time, demonstrated the modulating effect of emodin on the Akt, MAPK, ERK and DNMT expression pattern in experimental oral carcinogenesis.

In the present study, we have explored the activation of Akt, ERK, P³⁸ MAPK and DNMT, which have a crucial role in the process of cell proliferation, apoptosis and cell differentiation in the tumor bearing hamsters (DMBA alone treated hamsters). Emodin administration at a dose of 50mg/kg b.w to hamsters treated with DMBA inhibited the activation of all these molecular markers, as evidenced by the down regulation of these markers (Western blotting). The present study thus suggests that Emodin might have modulated the Akt, ERK, P³⁸ MAPK and DNMT pathways towards suppression of tumor formation during DMBA induced oral carcinogenesis.

Conclusion

The present study concludes that the antitumor potential of Emodin might have partly attributed to its inhibitory effect on the activation of Akt, ERK, P³⁸ MAPK and DNMT pathways during DMBA induced hamster buccal pouch carcinogenesis.

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References

1. Antico Arciuch, V.G., Elguero, M.E., Poderoso, J.J. and Carreras, M.C. (2012). Mitochondrial regulation of cell cycle and proliferation. *Antioxid Redox Signal.* **16**(10):1150–1180.
2. Au Yeung, C.L., Tsang, W.P., Tsang, T.Y., Co, N.N., Yau, P.L. and Kwok, T.T. (2010). HPV-16 E6 upregulation of DNMT1 through repression of tumor suppressor p53. *Oncol Rep.* **24**:1599-1604.
3. Baykul, T., Yilmaz, H.H., Aydin, U., Aydin, M.A., Aksoy, M. and Yildirim, D. (2010). Early diagnosis of oral cancer. *J Int Med Res.* **38**(3):737-49.
4. Bobrovnikova, M.E., Grigoriadou, C., Pytel, D., Zhang, F., Ye, J., Koumenis, C. and Diehl, J.A. (2010). PERK promotes cancer cell proliferation and tumor growth by limiting oxidative DNA damage. *Oncogene.* **29**(27):3881–3895.
5. Bradford, M.M. (1976). A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**(1):248-254.
6. Carnelio, S., Rodrigues, G.S., Shenoy, R. and Fernandes, D. (2011). A brief review of common oral premalignant lesions with emphasis on their management and cancer prevention. *Indian J Surg.* **73**(4):256–261.
7. Casto, B.C., Knobloch, T.J., Galioto, R.L., Yu, Z., Accurso, B.T. and Warner, B.M. (2013). Chemoprevention of oral cancer by lyophilized strawberries. *Anticancer Res.* **33**(11):4757–4766.
8. Chang, K.Y., Tsai, S.Y., Chen, S.H., Tsou, H.H., Yen, C.J., Liu, K.J. and Chang, J.Y. (2013). Dissecting the EGFR-PI3K-AKT pathway in oral cancer highlights the role of the EGFR variant III and its clinical relevance. *J Biomed Science.* **20**(1): 43.
9. Chappell, W.H., Steelman, L.S., Long, J.M., Kempf, R.C., Abrams, S.L., Franklin, R.A. and McCubrey, J.A. (2011). Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. *Oncotarget.* **2**(3):135–164.
10. Chen, X., Zhang, X., Lu, Y., Shim, J.Y., Sang, S., Sun, Z. and Chen, X. (2012). Chemoprevention of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced Hamster Cheek Pouch Carcinogenesis by a 5-Lipoxygenase Inhibitor. *Garcinol. Nutr Cancer.* **64**(8):1211–1218.
11. Cui, Y., Lu, P., Song, G., Liu, Q., Zhu, D. and Liu, X. (2016). Involvement of PI3K/Akt, ERK and p38 signaling pathways in emodin-mediated extrinsic and intrinsic human hepatoblastoma cell apoptosis. *Food Chem Toxicol.* **92**:26-37.
12. Delpu, Y., Cordelier, P., Cho, W.C. and Torrisani, J. (2013). DNA methylation and cancer diagnosis. *Int J Mol Sci.* **14**(7):15029–15058.
13. Dhillon, A.S., Hagan, S., Rath, O. and Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene.* **26**(22):3279-3290.
14. El Rhazi, K., Bennani, B., El Fakir, S., Boly, A., Bekkali, R., Zidouh, A. and Nejari, C. (2014). Public awareness of cancer risk factors in the Moroccan population: a population-based cross-sectional study. *BMC Cancer.* **14**:695.
15. Estève, P.O., Zhang, G., Ponnaluri, V.K.C., Deepti, K., Chin, H.G., Dai, N. and Pradhan, S. (2016). Binding of 14-3-3 reader proteins to phosphorylated DNMT1 facilitates aberrant DNA methylation and gene expression. *Nuc Acids Res.* **44**(4):1642–1656.
16. Gan, Y., Shi, C., Inge, L., Hibner, M., Balducci, J. and Huang, Y. (2010). Differential roles of ERK and Akt pathways in regulation of EGFR-mediated signaling and motility in prostate cancer cells. *Oncogene.* **29**(35):4947-58.
17. Gerthoffer, W.T., Schaafsma, D., Sharma, P., Ghavami, S. and Halayko, A.J. (2012). yearnotcorrespond Motility, survival and proliferation. *Compr Physiol.* **2**(1):255–281.
18. Gupta, B., Ariyawardana, A. and Johnson, N.W. (2013). Oral cancer in India continues in epidemic proportions: evidence base and policy initiatives. *Int Dent J.* **63**(1):12-25.
19. Hong, B., Li, H., Zhang, M., Xu, J., Lu, Y., Zheng, Y. and Yi, Q. (2015). p38 MAPK inhibits breast cancer metastasis through regulation of stromal expansion. *International Journal of Cancer. J Int Du Cancer.* **136**(1): 34–43.

20. Huang, F.J., Hsuuw, Y.D. and Chan, W.H. (2013). Characterization of Apoptosis Induced by Emodin and Related Regulatory Mechanisms in Human Neuroblastoma Cells. *Int J Mol Sci*, **14**(10), 20139–20156.
21. Huang, P.H., Huang, C.Y., Chen, M.C., Lee, Y.T., Yue, C.H., Wang, H.-Y. and Lin, H. (2013). Emodin and aloe-emodin suppress breast cancer cell proliferation through ER α inhibition. *Evid Based Complement Alternat Med*. **2013**:e376123.
22. Ismail, T., Calcabrini, C., Diaz, A. R., Fimognari, C., Turrini, E., Catanzaro, E. and Sestili, P. (2016). Ellagitannins in cancer chemoprevention and therapy. *Toxins*. **8**(5):e151.
23. Jiang, B.H. and Liu, L.Z. (2009). PI3K/PTEEN signaling in angiogenesis and tumorigenesis. *Adv Cancer Res*. **102**:19–65.
24. Jin, B., and Robertson, K. D. (2013). DNA Methyltransferases (DNMTs), DNA damage repair, and cancer. *Adv Exp Med Biol*. **754**:3–29.
25. Jin, B., Li, Y. and Robertson, K.D. (2011). DNA methylation: superior or subordinate in the epigenetic hierarchy?. *Genes Cancer*. **2**(6):07–617.
26. Kalimuthu, S. and Se-Kwon, K. (2013). Cell survival and apoptosis signaling as therapeutic target for cancer: Marine bioactive compounds. *Int J Mol Sci*. **14**(2):2334–2354.
27. Khyani, I.A.M., Qureshi, M.A., Mirza, T. and Farooq, M.U. (2015). Salivary detection of human Papilloma virus 16 & 18 in pre-malignant and malignant lesions of oral cavity: Is it feasible in Pakistani context of socio-cultural taboos. *PaK J Med Sci*. **31**(5):1104–1109.
28. Kim, M.S., Park, M.J., Kim, S.J., Lee, C.H., Yoo, H., Shin, S.H., Song, E.S. and Lee, S.H. (2005). Emodin suppresses hyaluronic acid-induced MMP-9 secretion and invasion of glioma cells. *Int J Oncol*. **27**(3):839-846.
29. Lee, A.S. (2014). Glucose regulated proteins in cancer: molecular mechanisms and therapeutic potential. *Nature Rev. Cancer*. **14**(4):263–276.
30. Liang, G., Chan, M.F., Tomigahara, Y., Tsai, Y.C., Gonzales, F.A., Li, E and Jones, P.A. (2002). Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Mol Cell Biol*. **22**(2):480–491.
31. Lin, S.Z., Wei, W.T., Chen, H., Chen, K.J., Tong, H.F., Wang, Z.H. and Liu, D.L. (2012). Antitumor activity of emodin against pancreatic cancer depends on its dual role: promotion of apoptosis and suppression of angiogenesis. *PLoS ONE*. **7**(8):e42146.
32. Lin, W., Zhong, M., Yin, H., Chen, Y., Cao, Q., Wang, C. and Ling, C. (2016). Emodin induces hepatocellular carcinoma cell apoptosis through MAPK and PI3K/AKT signaling pathways in vitro and in vivo. *Oncol Rep*. **36**(1):961-967.
33. Liu, C. (2015). Inhibition of mechanical stress-induced hypertrophic scar inflammation by emodin. *Mol Med Rep*. **11**(6):4087–4092.
34. Manoharan, S., Karthikeyan, S., Essa, M.M, Manimaran, A. and Selvasundram, R. (2016). An overview of oral carcinogenesis. *Int J Nutr Pharmacol Neurol Dis*. **6**:51-62.
35. Manoharan, S., Rajasekaran, D., Prabhakar, M. M., Karthikeyan, S. and Manimaran, A. (2015). Modulating Effect of Enicostemma littorale on the Expression Pattern of Apoptotic, Cell Proliferative, Inflammatory and Angiogenic Markers During 7, 12-Dimethylbenz (a) Anthracene Induced Hamster Buccal Pouch Carcinogenesis. *Toxicol Int*. **22**(1):130–140.
36. Markopoulos, A.K. (2012). Current aspects on oral squamous cell carcinoma. *Open Dent J*. **6**:126–130.
37. Matsuoka, T. and Yashiro, M. (2014). The role of PI3K/Akt/mTOR signaling in gastric carcinoma. *Cancers*. **6**(3):1441–1463.
38. Mebratu, Y. and Tesfaiigzi, Y. (2009). How ERK1/2 activation controls cell proliferation and cell death is subcellular localization the answer?. *Cell Cycle*. **8**(8):1168–1175.
39. Munshi, A. and Ramesh, R. (2013). Mitogen-activated protein kinases and their role in radiation response. *Genes Cancer*. **4**(9-10):401–408.
40. Olsen, B.B., Bjørning-Poulsen, M. and Guerra, B. (2007). Emodin negatively affects the phosphoinositide 3-kinase/AKT signalling pathway: a study on its mechanism of action. *Int J Biochem Cell Biol*. **39**(1):227-237.
41. Pan, F.P., Zhou, H.K., Bu, H.Q., Chen, Z.Q., Zhang, H., Xu, L.P. and Chen, L. (2016). Emodin enhances the demethylation by 5-Aza-CdR of pancreatic cancer cell tumor-suppressor genes P16, RASSF1A and ppENK. *Oncol Rep*. **35**(4):1941–1949.
42. Patil, P.B., Bathi, R., and Chaudhari, S. (2013). Prevalence of oral mucosal lesions in dental patients with tobacco smoking, chewing, and mixed habits: A cross-sectional study in South India. *J Family Community Med*. **20**(2):130–135.
43. Schetter, A.J., Heegaard, N. H. H. and Harris, C. C. (2010). Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis*. **31**(1):37–49.
44. Shtivelman, E., Beer, T. M. and Evans, C. P. (2014). Molecular pathways and targets in prostate cancer. *Oncotarget*. **5**(17):7217–7259.
45. Skowronski, K., Dubey, S., Rodenhiser, D. and Coomber, B.L. (2010). Ischemia dysregulates DNA methyltransferases and p16^{INK4a} methylation in human colorectal cancer cells. *Epigenetics*. **5**(6):547–556.
46. Sridharan, G. (2014). Epidemiology, control and prevention of tobacco induced oral mucosal lesions in India. *Indian J Cancer*. **51**(1):80-85.
47. Steele, V.E. and Lubet, R.A. (2010). The use of animal models for cancer chemoprevention drug development. *Semin Oncol*. **37**(4):327–338.
48. Su, Y.T., Chang, H.L., Shyue, S.K. and Hsu, S.L. (2005). Emodin induces apoptosis in human lung adenocarcinoma cells through a reactive oxygen species-dependent mitochondrial signaling pathway. *Biochem Pharmacol*. **70**(2):229-41.
49. Sui, J.Q., Xie, K.P., Zou, W. and Xie, M.J. (2014). Emodin inhibits breast cancer cell proliferation through the ER α -MAPK/Akt-cyclin D1/Bcl-2 signaling pathway. *Asian Pac J Cancer Prev*. **15**(15):6247-6251.
50. Vigneswaran, N. and Williams, M.D. (2014). Epidemiological trends in head and neck cancer and aids in diagnosis. *Oral Maxillofac Surg Clin North Am*. **26**(2):123–141.
51. Villanueva, J., Yung, Y., Walker, J.L. and Assoian, R. K. (2007). ERK activity and g1 phase progression: Identifying dispensable versus essential activities and primary versus secondary targets. *Mol Biol Cell*. **18**(4):1457–1463.
52. Wagner, E.F. and Nebreda, A.R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer*. **9**(8):537-549.
53. Way, T.D., Huang, J.T., Chou, C.H., Huang, C.H., Yang, M.H. and Ho, C.T. (2014). Emodin represses TWIST1-induced epithelial-mesenchymal transitions in head and neck squamous cell carcinoma cells by inhibiting the β -catenin and Akt pathways. *Eur J Cancer*. **50**(2):366-378.

54. Wei, W.T., Chen, H., Ni, Z.L., Liu, H.B., Tong, H.F., Fan, L., Liu, A., Qiu, M.X., Liu, D.L., Guo, H.C., Wang, Z.H. and Lin, S.Z. (2011). Antitumor and apoptosis-promoting properties of emodin, an anthraquinone derivative from *Rheum officinale* Baill, against pancreatic cancer in mice via inhibition of Akt activation. *Int J Oncol.* **39**(6):1381-1390.
55. Yang, F., Yuan, P., Hao, Y.Q. and Lu, Z.M. (2014). Emodin enhances osteogenesis and inhibits adipogenesis. *BMC Complement Altern Med.* **14**:e74.
56. Yaoxian, W., Hui, Y., Yunyan, Z., Yanqin, L., Xin, G. and Xiaoke, W. (2013). Emodin induces apoptosis of human cervical cancer hela cells via intrinsic mitochondrial and extrinsic death receptor pathway. *Cancer Cell Int.* **13**:e71.
57. Zhang, H., Chen, L., Bu, H.Q., Yu, Q.J, Jiang, D.D., Pan, F.P., Wang, Y., Liu, D.L. and Lin, S.Z. (2015). Effects of emodin on the demethylation of tumor-suppressor genes in pancreatic cancer PANC-1 cells. *Oncol Rep.* **33**(6):3015-3023.
58. Zhao, X.Y., Qiao, G.F., Li, B.X., Chai, L.M., Li, Z., Lu, Y.J. and Yang, B.F. (2009). Hypoglycaemic and hypolipidaemic effects of emodin and its effect on L-type calcium channels in dyslipidaemic-diabetic rats. *Clin Exp Pharmacol Physiol.* **36**(1):29-34.
59. Zheng, H.Y., Lin, W.Q., Hu, J.D., Lin, M.H. and Xie, L.J. (2015). Emodin Induces Apoptosis of K562/Adr Cells Probably through Akt-Caspase 3 Signal Pathway. *Zhongguo Shi Yan Xue Ye Xue Za Zhi.* **23**(6):1556-1559.
60. Zu, C., Zhang, M., Xue, H., Cai, X., Zhao, L., He, A. and Zheng, X. (2015). Emodin induces apoptosis of human breast cancer cells by modulating the expression of apoptosis-related genes. *Oncol Lett.* **10**(5):2919–2924.