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Original article

Crocetin treatment inhibits proliferation of colon cancer cells through down-regulation of genes involved in the inflammation

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ABSTRACT

Background: The current study was designed to investigate the effect of crocetin on the proliferation inhibition of colon cancer cells and the underlying mechanism.
Methods: MTT assay showed inhibition of proliferation of colon cancer cells in a dose based manner by crocetin treatment. At 30 μM concentration of crocetin proliferation rate of colon cancer cells was reduced to 14% after 24 h. Flow cytometry and fluorescence microscopy revealed induction of apoptosis in colon cancer cells on treatment with crocetin. The tube formation was suppressed significantly in the cultures of HUVEC treated with 30 μM concentration of crocetin compared to the control cultures.
Results: The results from transwell assay revealed a significant reduction in the population of DU-145 cells passing through filters of transwell on treatment with crocetin compared to the control cells. Treatment of the DU-145 cells with crocetin caused a significant reduction in the expression levels of NF-κB, VEGF and MMP-9. The results from RT-PCR analysis revealed a significant reduction in the expression of genes involved in inflammation including, HMGB1, IL-6 and IL-8 on treatment of DU-145 cells with crocetin. However, the expression of NAG-1 gene was increased by crocetin treatment in DU-145 cells significantly compared to the control cells.
Conclusion: Crocetin inhibits growth of colon cancer cells and prevents tube formation through induction of apoptosis. Therefore, crocetin can be used efficiently for the treatment of colon cancer.

1. Introduction

Colon cancer is the malignant carcinoma present in the lining of the colon and is the third most commonly detected cancer (Tenesa and Dunlop, 2009). In United States colon cancer is the one of the leading causes of deaths arising due to cancer. Among the colon cancer patients around 15% have been found to possess liver metastases even at the time of diagnosis (Manfredi et al., 2006). The early stage of colon cancer can be treated by using radiotherapy, surgical extraction and androgen ablation. Thus the treatment of colon cancer demands for the identification of novel molecules which can efficiently inhibit the proliferation, invasion and metastasis of the disease.

Plants and herbs have long traditional medicinal importance for the treatment of various types of cancers because of least or no harmful side effects (Rasul et al., 2012; Wang et al., 2012; Lin et al., 2013; Antonisamy et al., 2015; Balamurugan, 2015; Rathi et al., 2015; Serasanambati and Chilakapati, 2016; Puthur, 2016). Crocetin, a low molecular weight compound is the major component present in saffron and belongs to the family of carotenoids (Bolhassani et al., 2014). Biological evaluation of crocetin revealed its role as a promising antioxidant (Ordoudi et al., 2009), anti-inflammation (Nam et al., 2010), heart disease preventer (Cai et al., 2009), hepatoprotective (Yang et al., 2011) and neuroprotective (Khan et al., 2012) agent. Studies have demonstrated that crocetin inhibits proliferation of various types of carcinoma cell lines including, breast, gastric and colon cancer cells (Bathaie et al., 2013). Crocetin treatment of the lung cancer animal model led to the prevention of development of pathological features (Magesh et al., 2009). In animal model of gastric cancer, treatment with crocetin resulted in the inhibition of tumor growth efficiently (Bathaie

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et al., 2013). The current study was aimed to investigate the effect of crocetin on the proliferation, apoptosis induction and expression of inflammatory genes in the colon cancer cells. Crocetin treatment of the colon cancer cells caused inhibition of proliferation, induction of apoptosis, suppressed migration and invasion potential and inhibited the expression of inflammatory genes.

2. Materials and methods

2.1. Drug and chemicals

Crocetin was supplied by Sigma-Aldrich (St. Louis, MO) and its stock solution was prepared in dimethyl sulfoxide. The solution was stored at −10 °C before use in the experiment.

2.2. Cell lines and culture

The human colon cancer cells HCT116 and were purchased from American Type Cell Culture (ATCC, Manasas, VA). The cells were cultured in RPMI-1640 (HyClone, Logan, UT) containing 10% fetal bovine serum (FBS, HyClone). The medium was supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone). The plates were cultured in a humidified atmosphere with 5% CO_2 and 95% air at 37 °C.

2.3. MTT assay for cell proliferation

The colon cancer cells, HCT116 and were distributed in 24-well culture plates at a density of 2×10^6 cells per well. The cells were cultured for 24 h in RPMI-1640 medium. The medium was then removed and replaced with the new medium containing various concentrations of crocetin. The control cells were grown in presence of DMSO alone. MTT [3-(4,5-dimethylthiazol2-yl)-2,5-diphe nyltetrazolium bromide] (Sigma-Aldrich) at a concentration of 0.5 mg/ml was added to the wells and the cells were incubated for 2 h at 37 °C. DMSO was added to the wells for dissolution of formazan granules produced by the live cells and the absorbance measurements were performed at 540 nm using a multi-well reader (Bio-Rad, Richmond, CA).

2.4. Detection of apoptotic by flow cytometry

Apoptosis induction in colon cancer cells was analyzed by Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit I (BD Pharmingen). The cells were incubated with various concentrations of crocetin for 24 h. Then cells were subjected to washing two times inice-cold PBS followed by treatment with 500 μl binding buffer. To each of the plate, annexin V-FITC solution (5 μl) and PI (5 μl) was added at 37 °C and incubated for1 h. Flow cytometry was performed for the analysis of induction of in the cell cultures.

2.5. Analysis of tube formation

The medium was prepared with colon cancer cells incubated in presence or absence of crocetin. The HUVEC cells were then incubated with the colon cancer cells in 96-well tissue culture plates subjected to pre-coating using matrigel (BD Biosciences, San Jose, CA). The plates were incubated for 24 h in humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.6. Analysis of cell invasion

For the analysis of effect of crocetin on the invasion potential of colon cancer cells transwell inserts coated with the matrigel (BD Biosciences) were used. The cells were distributed on the transwell inserts at a density of 2×10^7 and incubated with crocetin or with DMSO (control) for 24 h duration. Cell invasion was determined by using HEMA stain. The HEMA stained cells were counted and the images were captured using light microscope.

2.7. Western blot analysis

Colon cancer cells were treated with various concentrations of crocetin for 24 h, the cells were harvested and rinsed in cold PBS. The cells were then treated with lysis buffer [40 mMTris (pH 8.0), 120 mMNaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF)]. After incubation, the supernatant was collected and the concentration of proteins was measured (Pierce, Rockford, IL). The proteins in equal amounts were subjected to 6–15% SDS–PAGE and subsequently transferred to PVDF membrane. Non-specific sites in the membranes were blocked by incubation with 5% non-fat dry milk, Tris-buffered saline and Tween-20 buffer (TBS-T) for a duration of 1 h. Incubation of the membranes with primary antibodies was performed overnight at 4 °C. Then membranes were subjected to washing using 4X TBS-T buffer and subsequently incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) secondary antibody for 1 h. Again, the membrane washing was performed using 1X TBS-T buffer. The complexes formed by interaction between antigen-antibody were analyzed using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Biosciences, Pittsburgh, PA).

2.8. Statistical analysis

The results obtained are presented as the mean ± SD of three experiments performed independently. The data obtained were analyzed by Student's *t*-test. The values are considered significantly different at $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Inhibition of colon cancer cell proliferation by crocetin

Treatment of the colon cancer cell lines, LNCaP and DU-145 with crocetinfor 24 h exhibited inhibitory effect on the rate of proliferation. The cells were treated with 5, 10, 15, 25 and 30 μM concentrations of crocetin and then analyzed for proliferation. The rate of proliferation was inhibited significantly ($p < 0.05$) from 10 μM concentration of crocetin in both the cell lines (Fig. 1).

3.2. Induction of apoptosis in colon cancer cells by crocetin

DU-145 cells were treated with 5, 10, 15, 25 and 30 μM concentrations of crocetin for 48 h and then stained using Annexin V-FITC and propidium iodide. An increase in the proportion of apoptotic cells was observed with the increase in concentration of crocetin from 5 to 30 μM using fluorescence microscopy (Fig. 2A). Dose dependent increase in the population of apoptotic cells by crocetin treatment was further confirmed using flow cytometric examination (Fig. 2B).

3.3. Inhibition of tube formation of HUVEC and invasion potential of DU-145 cells by crocetin

HUVEC were incubated with DU-145 cells in a medium containing 30 μM concentration of crocetin. The tube formation was suppressed significantly in the cultures of HUVEC treated with 30 μM

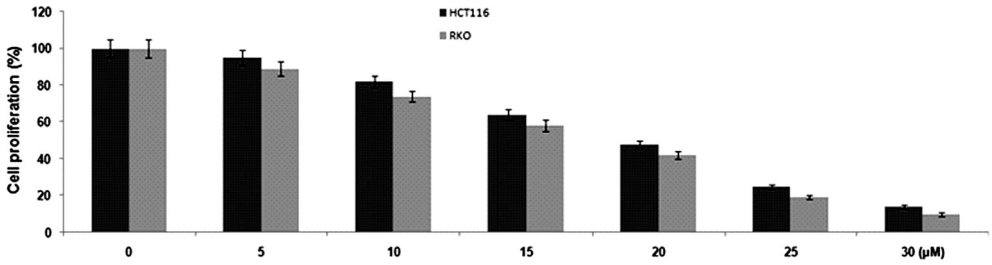

Fig. 1. Crocetin exhibits inhibitory effect on the proliferation of colon cancer cells. The cells were cultured for 24 h in 96-well plates and then incubated for 24 h with crocetin. Proliferation of cells was measured using MTT assay. The experiments were performed in triplicates. ($p < 0.05$) compared with the control cells.

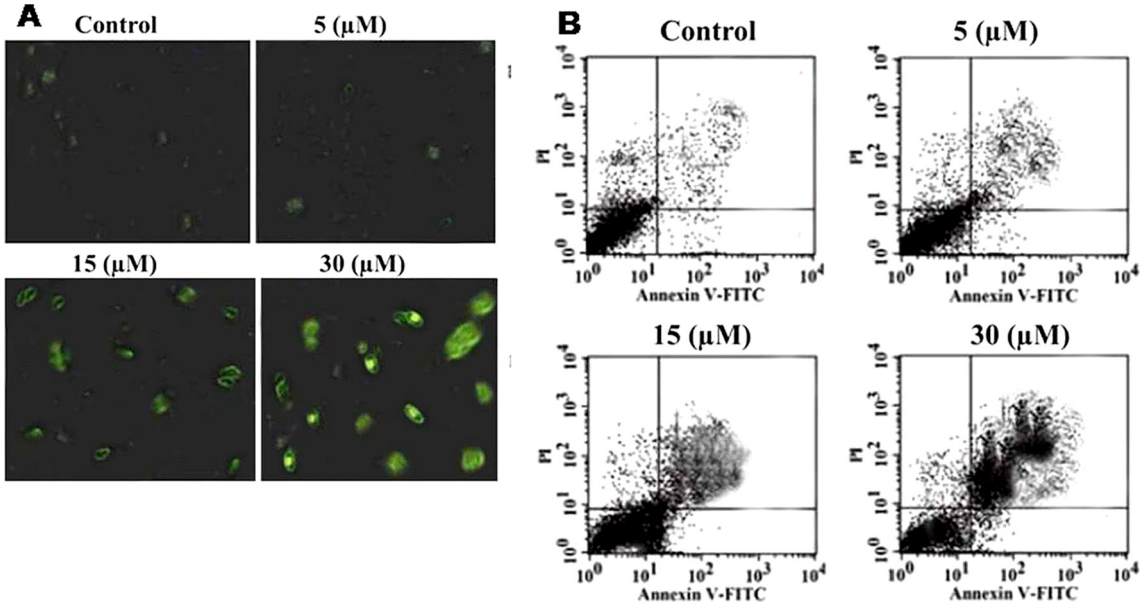

Fig. 2. Crocetin treatment induces apoptosis in colon cancer cells. The cells were treated with various concentrations of crocetin for 24 h and then subjected to annexin V-FITC and propidium iodide staining for detection of apoptosis. Following treatment the cells were visualized by fluorescence microscopy (A) and (flow cytometry) for apoptosis induction.

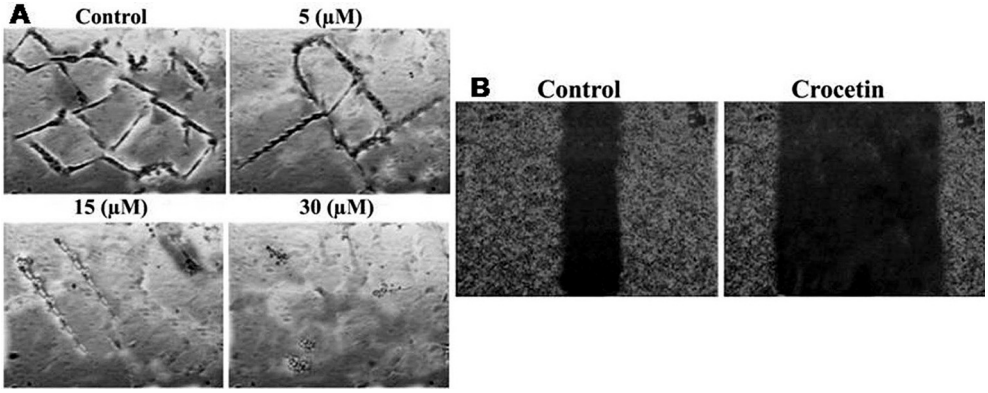

Fig. 3. Crocetin treatment inhibits tube formation potential of HUVEC and invasion tendency of colon cancer cells. (A) The cells were incubated for 24 h with crocetin, rinsed in PBS and then cultured with HUVEC using matrigel pre-coated plates. Crocetin caused a significant reduction in the tube formation potential of HUVEC cells. (B) The colon cancer cells were put onto the matrigel coated trans wells with or without crocetin for 24 h. Invasion of the cells was analyzed using HEMA stain.

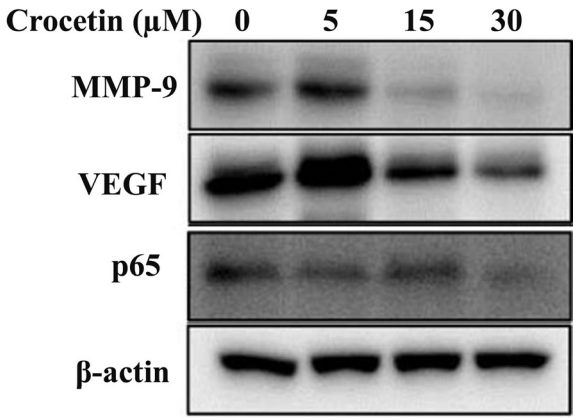

Fig. 4. Crocetin treatment inhibits level of p65, VEGF and MMP-9 in colon cancer cells. The cells were incubated for 24 h with crocetin and then analyzed by western blot assay.

concentration of crocetin compared to the control cultures (Fig. 3A). The results from transwell assay revealed a significant reduction in the population of DU-145 cells passing through filters of transwell on treatment with crocetin compared to the control cells (Fig. 3B).

3.4. Crocetin treatment suppresses the expression levels of NF-κB, VEGF and MMP-9 in DU-145 cells

Treatment of the DU-145 cells with crocetin caused a significant reduction in the expression levels of NF-κB, VEGF and MMP-9. The reduction in the expression of NF-κB, VEGF and MMP-9 was found to be dependent on the concentration of crocetin (Fig. 4).

3.5. Crocetin treatment alters the expression genes involved in inflammation

The results from RT-PCR analysis revealed a significant reduction in the expression of genes involved in inflammation including, HMGB1, IL-6 and IL-8 on treatment of DU-145 cells with crocetin. However, the expression of NAG-1 gene was increased by crocetin treatment in DU-145 cells significantly compared to the control cells (Fig. 5).

4. Discussion

Colon cancer, malignant tumor in the lining of colon is the leading cause of deaths arising due to cancer and a challenge to clinicians throughout the world. Among the colon cancer patients around 15% have been found to possess liver metastases even at the time of diagnosis. Thus the development of novel treatment strategies for colon cancer demands for the identification of new molecules. In the current study effect of crocetin on proliferation, invasion and metastasis potential of colon cancer cells was investigated. The results demonstrated that crocetin treatment inhibits colon cancer cell proliferation through down regulation of inflammatory gene expression. There are reports that crocetin treatment causes inhibition of cell proliferation in various types of carcinoma cells including, breast, gastric and colon cancer cells (Magesh et al., 2009; Bathaie et al., 2013). In the current study, crocetin treatment of colon cancer cells inhibited proliferation significantly ($p < 0.05$) at 10 μM concentration. The processes like carcinoma cell angiogenesis and invasion are major factors contributing to the metastasis of cancer cells to the distant organs (Weidner et al., 1993; Wakui et al., 1992). The current study demonstrated that tube formation was suppressed significantly in the cultures of HUVEC treated with 30 μM concentration of crocetin compared to the control cultures. The results from trans well assay revealed a significant reduction in the population of DU-145 cells passing through filters of trans well on treatment with crocetin compared to the control cells. Hereditary as well as environmental factors lead to the chronic inflammation and contribute towards the carcinoma progression (De Marzo et al., 2007). Crocetin is a potent antioxidant and anti-inflammatory agent (Nam et al., 2010). Our results revealed a significant reduction in the expression of genes associated with inflammation like HMGB1, interleukin-6 and interleukin-8 on treatment of colon cancer cells with crocetin. The expression of NAG-1 gene was increased by crocetin treatment in DU-145 cells significantly compared to the control cells.

For the establishment of cancer environment inflammatory cytokines and chemokines play an important role. The expression of interleukin-6 and interleukin-8 is significantly higher in the cancer cells (Veltri et al., 1999; Pfitzenmaier et al., 2003; Uehara et al., 2005). The current study revealed a significant reduction in the expression of inflammatory cytokes, interleukin-6 and interleukin-8 in colon cancer cells on treatment with crocetin. HMGB1 plays a vital role in the attraction of cells involved in inflammation such as monocytes and macrophages as well as induces expression of cytokines (Erlandsson Harris and Andersson, 2004; Andersson et al., 2000). It has been observed that inhibition of HMGB1 gene caused induction of apoptosis in cancer cells (Gnanasekar et al., 2009). In the current study crocetin treatment caused a significant reduction in the expression of HMGB1 gene in the colon cancer cells.

In conclusion, crocetin treatment inhibits proliferation and induces apoptosis in colon cancer through inhibition of inflammatory gene expression. Thus crocetin can be used for the treatment of colon cancer.

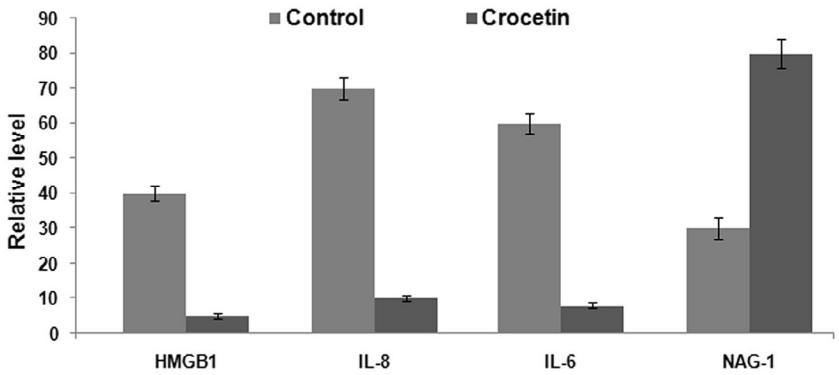

Fig. 5. Crocetin treatment inhibits inflammatory gene expression in colon cancer cells. The cells at a density of 2×10^7 were treated with various concentrations of crocetin for 24 h and then subjected to RT-PCR analysis.

Conflict of interest

The authors declare no conflict of interest.

References

Andersson, U., Wang, H., Palmblad, K., Aveberger, A.C., Bloom, O., Erlandsson-Harris, H., Janson, A., Kokkola, R., Zhang, M., Yang, H., Tracey, K.J., 2000. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J. Exp. Med. 192, 565–570.

Antonisamy, P., Duraipandiyan, V., Ignacimuthu, S., Kim, J.-H., 2015. Anti-diarrhoeal activity of friedelin isolated from *Azima tetracantha* Lam. in Wistar rats. South Ind. J. Biol. Sci. 1, 34–37.

Balamurugan, R., 2015. Smilax chinensis Linn. (Liliaceae) root attenuates insulin resistance and ameliorate obesity in high diet induced obese rat. South Ind. J. Biol. Sci. 1, 47–51.

Bathaie, S.Z., Hoshyar, R., Miri, H., Sadeghizadeh, M., 2013. Anticancer effects of crocetin in both human adenocarcinoma gastric cancer cells and rat model of gastric cancer. Biochem. Cell Biol. 91, 397–403.

Bolhassani, A., Khavari, A., Bathaie, S.Z., 2014. Saffron and natural carotenoids: biochemical activities and anti-tumor effects. Biochem. Biophys. Acta. 1845, 20–30.

Cai, J., Yi, F.F., Bian, Z.Y., Shen, D.F., Yang, L., Yan, L., Tang, Q.Z., Yang, X.C., Li, H., 2009. Crocetin protects against cardiac hypertrophy by blocking MEK-ERK1/2 signalling pathway. J. Cell Mol. Med. 13, 909–925.

De Marzo, A.M., Platz, E.A., Sutcliffe, S., Xu, J., Grönberg, H., Drake, C.G., Nakai, Y., Isaacs, W.B., Nelson, W.G., 2007. Inflammation in prostate carcinogenesis. Nat. Rev. Cancer 7, 256–269.

Erlandsson Harris, H., Andersson, U., 2004. Mini-review: the nuclear protein HMGB1 as a proinflammatory mediator. Eur. J. Immunol. 34, 1503–1512.

Gnanasekar, M., Thirugnanam, S., Ramaswamy, K., 2009. Short hairpin RNA (shRNA) constructs targeting high mobility group box-1 (HMGB1) expression leads to inhibition of prostate cancer cell survival and apoptosis. Int. J. Oncol. 34, 425–431.

Khan, M.B., Hoda, M.N., Ishrat, T., Ahmad, S., Moshahid Khan, M., Ahmad, A., Yusuf, S., Islam, F., 2012. Neuroprotective efficacy of Nardostachysjatamansi and crocetin in conjunction with selenium in cognitive impairment. Neurol. Sci. 33, 1011–1020.

Lin, Y., Peng, N., Li, J., Zhuang, H., Hua, Z.C., 2013. Herbal compound triptolide synergistically enhanced antitumor activity of amino-terminal fragment of urokinase. Mol. Cancer 12, 54.

Magesh, V., DurgaBhavani, K., Senthilnathan, P., Rajendran, P., Sakthisekaran, D., 2009. In vivo protective effect of crocetin on benzo(a)pyrene-induced lung cancer in Swiss albino mice. Phytother. Res. 23, 533–539.

Manfredi, S., Lepage, C., Hatem, C., Coatmeur, O., Faivre, J., Bouvier, A.M., 2006. Epidemiology and management of liver metastases from colorectal cancer. Ann. Surg. 244, 254–259.

Nam, K.N., Park, Y.M., Jung, H.J., Lee, J.Y., Min, B.D., Park, S.U., Jung, W.S., Cho, K.H., Park, J.H., Kang, I., Hong, J.W., Lee, E.H., 2010. Anti-inflammatory effects of crocin and crocetin in rat brain microglial cells. Eur. J. Pharmacol. 648, 110–116.

Ordoudi, S.A., Befani, C.D., Nenadis, N., Koliakos, G.G., Tsimidou, M.Z., 2009. Further examination of antiradical properties of Crocus sativus stigmas extract rich in crocins. J. Agric. Food Chem. 57, 3080–3086.

Pfitzenmaier, J., Vessella, R., Higano, C.S., Noteboom, J.L., Wallace Jr., D., Corey, E., 2003. Elevation of cytokine levels in cachectic patients with prostate carcinoma. Cancer 97, 1211–1216.

Puthur, J.T., 2016. Antioxidants and cellular antioxidation mechanism in plants. South Ind. J. Biol. Sci. 2, 14–17.

Rasul, A., Yu, B., Khan, M., Zhang, K., Iqbal, F., Ma, T., Yang, H., 2012. Magnolol, a natural compound, induces apoptosis of SGC-7901 human gastric adenocarcinoma cells via the mitochondrial and PI3K/Akt signaling pathways. Int. J. Oncol. 40, 1153–1161.

Rathi, M.A., Meenakshi, P., Gopalakrishnan, V.K., 2015. Hepatoprotective activity of ethanolic extract of alysicarpus vaginalis against nitrobenzene-induced hepatic damage in rats. South Ind. J. Biol. Sci. 1, 60–65.

Serasanambati, M., Chilakapati, S.R., 2016. Function of nuclear factor Kappa B (NF-kB) in human diseases – a review. South Ind. J. Biol. Sci. 2, 368–387.

Tenesa, A., Dunlop, M.G., 2009. New insights into the aetiology of colorectal cancer from genome-wide association studies. Nat. Rev. Genet. 10, 353–358.

Uehara, H., Troncoso, P., Johnston, D., Bucana, C.D., Dinney, C., Dong, Z., Fidler, I.J., Pettaway, C.A., 2005. Expression of interleukin-8 gene in radical prostatectomy specimens is associated with advanced pathologic stage. Prostate 64, 40–49.

Veltri, R.W., Miller, M.C., Zhao, G., Ng, A., Marley, G.M., Wright Jr., G.L., Vessella, R.L., Ralph, D., 1999. Interleukin-8 serum levels in patients with benign prostatic hyperplasia and prostate cancer. Urology 53, 139–147.

Wakui, S., Furusato, M., Itoh, T., Sasaki, H., Akiyama, A., Kinoshita, I., Asano, K., Tokuda, T., Aizawa, S., Ushigome, S., 1992. Tumour angiogenesis in prostatic carcinoma with and without bone marrow metastasis: a morphometric study. J. Pathol. 168, 257–262.

Wang, L., Lu, A., Meng, F., Cao, Q., Shan, B., 2012. Inhibitory effects of lupeal acetate of Cortex periplocae on N-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis. Oncol. Lett. 4, 231–236.

Weidner, N., Carroll, P.R., Flax, J., Blumenfeld, W., Folkman, J., 1993. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am. J. Pathol. 143, 401–409.

Yang, R., Vernon, K., Thomas, A., Morrison, D., Qureshi, N., Van Way, C.W., 2011. Crocetin reduces activation of hepatic apoptotic pathways and improves survival in experimental hemorrhagic shock. JPEN J. Parenter. Enteral Nutr. 35, 107–113.