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5-bromoprotocatechualdehyde suppresses growth of human lung cancer cells through modulation of ROS and the AKT/MAPK signaling pathway

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Abstract Early-stage lung cancer is the deadliest form of the disease. In this study, we investigated the anticancer activity of 5-bromoprotocatechualdehyde (BPCA) extracted from the seaweed *Polysiphonia morrowii* Harvey (*P. morrowii*) in lung cancer H460 cells. We extracted *P. morrowii* powder thrice with 80% aqueous methanol and separated the extract using high-performance liquid chromatography. We then tested BPCA's effects on cell viability, apoptosis, reactive oxygen species (ROS) generation, and protein expression Our results showed that BPCA inhibited tumor cell growth and ROS production and induced apoptosis through mitogen-activated protein kinase (MAPK) and AKT signaling pathways in lung cancer cells. When BPCA was combined with hydrogen peroxide, ROS production and apoptosis increased even further due to the regulation of AKT signaling and JNK-MAPKs pathways. These findings suggest that BPCA induces lung-cancer-cell death through ROS-mediated phosphorylation in AKT/MAPK signaling. This could lead to the development of new and effective treatments for early-stage lung cancer.

Keywords : 5-bromoprotocatechualdehyde, Cancer, ROS, MAPK pathway, *Polysiphonia morrowii* Harvey

Introduction

Normal cells constantly generate reactive oxygen species (ROS) intrinsically and in response to external sources, such as infrared radiation, virus infection, and hormones. ROS reacts with intracellular constituents and may induce cancer through DNA damage leading to mutation and subsequent abnormal cell proliferation. (Jena 2012, Matés, Segura et al. 2012, Choi 2013). Cancer is a significant worldwide health problem, and, globally, lung cancer is the most common cause of cancer death (Bostancıoğlu, Demirel et al. 2013). Smoking is a major cause of lung cancer (Peto, Darby et al. 2000). Most lung cancer patients display non-small cell lung cancer (NSCLC) that accounts for 80% of all lung cancers (Jemal, Siegel et al. 2010).

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NSCLC patients are commonly treated with antineoplastic agents, radiation, targeted therapies, and combinations of these therapies. Advances in these and other approaches are continuously changing to continue to improve the prognosis for patients with advanced lung cancer (Blackstock and Govindan 2007). The development of new anticancer agents from natural materials is currently emphasized as a means to lessen impacts on normal cell survival and patient tolerance that are common side effects of chemotherapy. Discovery of natural agents that inhibit the proliferation of cancer cells and induce apoptosis by controlling the cell cycle is an active area of investigation (von Freeden-Jeffry, Vieira et al. 1995, Kawabe 2004, Ouhtit, Gaur et al. 2013). Apoptosis is characterized by blebbing of cell membranes, nuclear fragmentation, karyorrhexis, chromosome condensation, and internucleosomal cleavage (Green and Reed 1998). Mitochondria play an essential role in cancer cell growth and apoptosis (Wyllie, Kerr et al. 1980, Lu, Dempsey et al. 2001). In particular, mitochondria regulate normal oxidative stress and failure in this regulation may lead to apoptosis induction (Tao, Zhang et al. 2019). BPCA is a bromophenol isolated from the marine red alga Polysiphonia morrowii Harvey. Marine red algae contain large amounts of various bromophenols. Previous studies suggest that bromophenols are effective antioxidant and anti-inflammatory agents (Je, Ahn et al. 2009, Kang, Han et al. 2017, Choi, Ye et al. 2018). However, no reports of anticancer activities of BPCA are available. In this study, we confirm that BPCA could be an effective treatment for lung cancer. The chemical may also be effective for prophylaxis of cancer and as an antioxidant in the future.

Meterials and methods

Materials

BPCA is a bromophenol isolated from the marine red alga *Polysiphonia morrowii* Harvey (Figure 1). The red algae *P. morrowii* Harvery was collected from coastal margins of Jeju Island, South Korea. The sample was washed three times with tap water to remove salt. Washing was followed by rinsing with freshwater and vacuum drying at 25°C – 30°C for 72 h. Vacuum-dried sample was homogenized with a grinder before extraction.

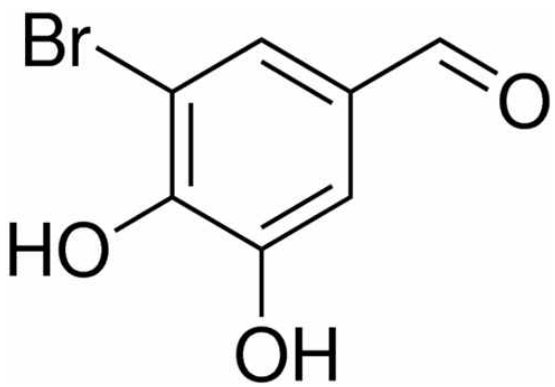

Fig.1 Chemical structure of BPCA.

Extraction and isolation

Dried red algae *P. morrowii* powder was extracted with 80% methanol at room temperature until color

of the powder was lost. The liquid portion of the suspension was obtained via filtration, and the filtrate was concentrated using an evaporator under reduced pressure. The extract was suspended in water, and the aqueous layer was partitioned with chloroform. The chloroform extract was fractionated on a silica column with stepwise elution with a chloroform-methanol mixture (30:1 → 1:1). A combined active fraction was further separated on a Sephadex LH-20 column saturated with 100% methanol. The active fraction was purified with reversed-phase high-performance liquid chromatography (HPLC) using a Waters HPLC system (Alliance 2690; Waters Corp., Milford, MA, USA) equipped with a Waters 996 photodiode array detector and a C18 column (J'sphere ODS-H80, 250 × 4.6 mm, 4 μm; YMC Co., Kyoto, Japan) by stepwise elution with a methanol–water gradient (UV range, 290 nm; flow rate, 1 ml/min). Finally, the purified compound was identified by comparing its ^{1}H and ^{13}CNMR data with ESI-mass spectrum. The compound was dissolved in dimethylsulfoxide (DMSO) and used in experiments in which the final concentration of DMSO in culture medium was adjusted to <0.01%.

Cell cultures

H460 human lung cancer cells were purchased from the Korean Cell Line Bank (Seoul, South Korea). Cells were cultured in RPMI 1640 medium (Welgene) with 10% fetal bovine serum and 1% antibiotics. H460 cells were passaged every two days and incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell viability

Cell viability was measured using the MTT assay. H460 cells were seeded on 24-well plates at a concentration of 1.5 × 10^5 cells/ml. After 24 h, 48 h, and 72 h, cells were treated with different concentrations of BPCA (10, 25, 50, and 100 μM), and incubated for 48 h (5% CO_2 / 37°C). Fifty μl of MTT solution (5 mg/ml) was added per well, and incubation continued for three hours at 37°C. Formazan crystals in each well were dissolved in DMSO. The intensity of purple formazan was determined by measuring absorbance at 540 nm using a microplate reader.

ROS measurement

H460 cells were seeded in 60-mm dishes and co-treated with BPCA (10, 25, and 50 μM) and H_2DCFDA dye at 37°C. After one hour, cells were harvested, and ROS production was detected using a flow cytometer (BD Accuri C6; BD Biosciences, San Diego, CA, USA). Data were analyzed using a flow cytometer with measurement in the FL1 channel. For inhibition of ROS production, cells were pretreated with N-acetyl-L-cysteine (NAC, 2.5 mM,

Sigma-Aldrich, St. Louis, MO, USA) for one hour before BPCA and H_2DCFDA co-treatment.

Apoptosis measurement

H460 cells were seeded in 60-mm dishes and treated with BPCA (10, 25, and 50 μM) for 48 h at 37°C. Cells were then stained with annexin V-FITC (BD Bioscience, San Jose, CA, USA), and placed in the dark at room temperature for 15 min, followed by incubation with 7AAD in the dark at room temperature for 15 min. Annexin V- and 7AAD-positive cells were detected using a flow cytometer (BD Accuri C6; BD Biosciences, San Diego, CA, USA). Inhibition of apoptosis was assessed by pretreating cells with NAC for 30 min before adding BPCA. Data were analyzed using a flow cytometer with measurements in FL1 and FL3 channels.

Western blotting

H460 cells were seeded in 6-well plates at a concentration of 1.5 × 10^5 cells/ml. After 16 h, cells were treated with various concentrations of BPCA (10, 25, and 50 μM), and incubated (37°C / 5% CO_2) for 24 h or 15 min. After incubation, cells were harvested and washed twice with cold PBS. Cells were lysed with RIPA buffer, and equal amounts of protein in total cell lysate were run on 8% – 12% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked and blotted with the relevant primary antibodies. Anti-actin, Bcl2, p-EGFR (Y992), p-EGFR (Y1068), p-EGFR (Y992), p-ERK, ERK, p-JNK and p-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa, CA, USA). Anti-AKT, JNK, p38, p-AKT, PARP, p-STAT3, and STAT3 antibodies were obtained from Cell Signaling (Danvers, MA, USA). The protein bands were visualized using an ECL Western blotting detection kit (BIO-RAD, USA) and Olympus FV10i Self-Contained confocal laser system.

2.8 Statistical analysis

All data were collected in triplicate and reported as means ± SD. Significant differences between the means were determined using the Student's t-test (*$p < 0.1$,** $p < 0.05$,*** $p < 0.01$).

Result

BPCA induced apoptosis

H460 cells were treated with various concentrations (0, 10, 25, 50 and 100 μM) of BPCA for 24 h. BPCA significantly suppressed cell growth at the end of the incubation (Figure 2A). BPCA induced apoptotic cell death was assessed using flow cytometry. BPCA increased numbers of apoptotic cells, as shown by annexin V and 7AAD double-positive cell staining (Figure 2B). Moreover,

we investigated the expression of apoptosis-related proteins using western blot. BPCA decreased the expression of PARP and Bcl2 antibodies (Figure 2C).

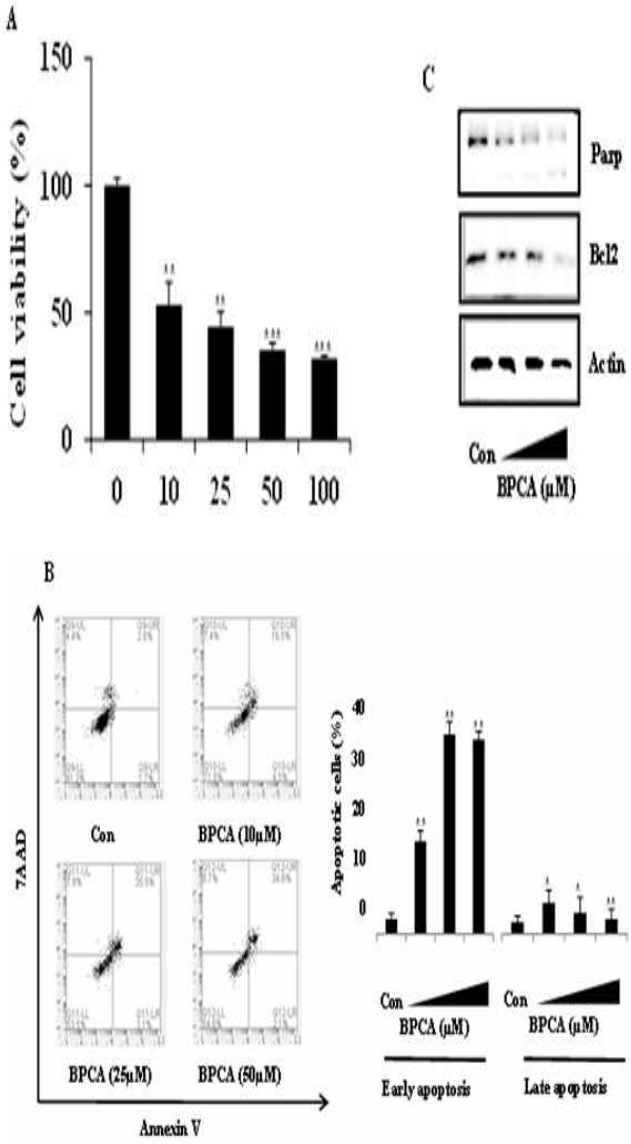

Figure 2. (A) 5-bromoprotocatechualdehyde (BPCA) induces apoptotic cell death: Cells were seeded in 24-well plates and then treated with various concentrations of BPCA (0, 10, 25, 50, and 100 μM) and DMSO. (B) H460 cells were treated with BPCA (0, 10, 25, and 50 μM) for 24 h, and then harvested. Cells were stained with annexin V and 7AAD in a binding buffer at room temperature in the dark. The stained cells were detected using FACSCalibur. The graph shows the examples of annexin V only positive cells (early apoptotic cells) and annexin V and 7AAD double-positive cells (late apoptosis cells) from the total stained cells. (C) The H460 cells were treated with BPCA (0, 10, 25, and 50 μM) for 24 h, and whole lysates were analyzed using Western blot with PARP and Bcl2 antibodies. Actin was used as the internal control.

BPCA induces apoptosis via AKT and MAPK pathways

H460 cells were treated with various concentrations (0, 10, 25, and 50 μM) of BPCA for 15 min and subjected to western blotting. BPCA decreased the phosphorylation of the STAT3, JNK and AKT at the highest concentration (Figure 3).

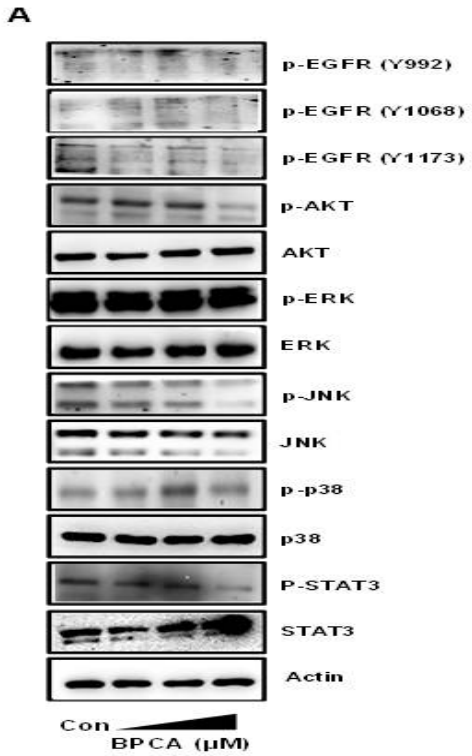

Figure 3. BPCA inhibits the cell signaling pathway: (A) The H460 cells were treated with BPCA (0, 10, 25, and 50 μM)

for 15 min, and the whole lysates were analyzed using western blot with anti p AKT, AKT, p ERK, ERK, p JNK, JNK, p p38, p38, p STAT3 and STAT3 antibodies. Actin was used as the internal control.

BPCA decreases ROS production

H460 cells were treated with various concentrations (0, 10, 25, and 50 μM) of BPCA to confirm the effects of BPCA on ROS production. As shown in Figure 4A, BPCA decreased ROS production by 28.9%, 29.2%, and 30.9% at 10, 25, and 50 μM, respectively, compared with the control. H2O2 is known to induce ROS production. To further confirm the activity of BPCA on ROS production, we used co-treatment with BPCA and H_2O_2. As shown in Figure4B, BPCA significantly decreased ROS production caused by H2O2 treatment.

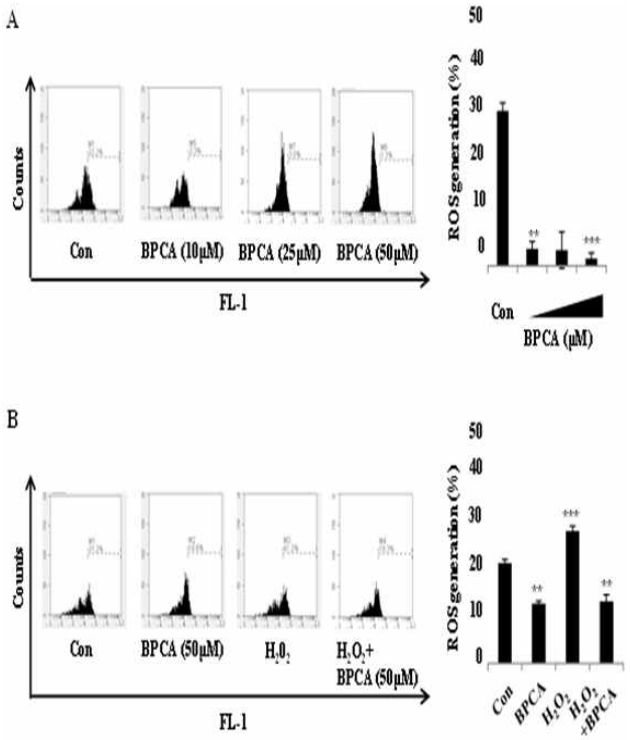

Figure 4. BPCA increases ROS production in cancer cells: (A) The H460 cells were co-treated with (0, 10, 25, and 50 μM) of BPCA and H_2DCFDA dye for 1 h at 37°C. ROS production was detected using FACSCalibur. The graph shows H_2DCFDA-positive cells from the total cells. (B) The cells were pretreated for 1 h with or without H_2O_2, followed by exposure to 50 μM of BPCA and H_2DCFDA dye for 1 h at 37°C.

BPCA inhibits ROS production is the AKT pathway and apoptosis

H_2O_2 induces ROS production through the AKT pathway. We observed that H_2O_2-mediated increases in the phosphorylated AKT reversed after treatment with BPCA (Figure5A). Also, BPCA induced apoptosis at the same level of H_2O_2 (Figure5B) in ROS measurment section. Thus, BPCA inhibits ROS production by decreasing the phosphorylation of AKT and inducing apoptosis.

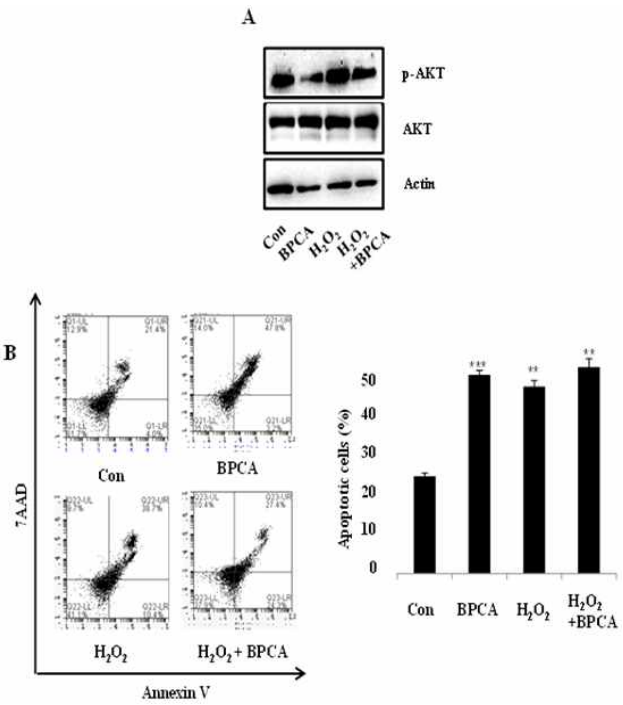

Figure.5 BPCA-induced ROS reduction causes the MAP kinases' inhibition and apoptosis: (A) The H460 cells were pretreated with H_2O_2 for 1 h and then exposed to BPCA (50 μM) for 15min. p AKT and AKT protein expression levels were analyzed using Westernblot. Actin was used as the loading control. (B)The H460 cells were pretreated with H_2O_2 for 1 h ,and then exposed to BPCA (50μM) for 24 h. The cells were stained with annexinV and 7AAD at room temperature in the dark.

Discussion

In this study, we investigated anticancer activity of 5-bromoprotocatechualdehyde (BPCA) from *Polysiphonia morrowii* in H460 lung cancer cells. Mitochondria are associated with apoptosis through redox regulation. ROS are generated during the production of adenosine triphosphate (ATP) by aerobic metabolism in the mitochondria. ROS are potentially toxic products of cellular metabolism and play important roles in cell proliferation, aging, and cancer development (Sankarapandi and Zweier 1999, Zhou, Hileman et al. 2003). An increase in ROS is associated with abnormal cancer cell growth, and ROS may cause tissue injury in a wide range of human diseases, and an excessive amount of ROS can lead to cell death via apoptosis. ROS production damages DNA and leads to gene mutations, and cells that are not removed may progress to malignancy. (Che, Moriya et al. 2013, Poillet-Perez, Despouy et al. 2015). ROS increases to levels incompatible with cell viability, ROS becomes cytotoxic. In cancer cells, this action may lead to death of malignant cells and thus limiting cancer progression (Zhou, Hileman et al. 2003, Yang, Karakhanova et al. 2016). Various studies examined apoptosis in cancer cells due to ROS generation (Hadi, Asad et al. 2000). BPCA showed inhibition of ROS production and simultaneously induced apoptosis in lung cancer cells. This this activity may spare from damage caused by ROS. Cancer cells typically display multiple genetic alterations, and a combination of target-specific agents might be required to effectively eliminate these cells. The phosphorylation of specific proteins (i.e., AKT/MAPK) may lead to cancer cell death (Sarbassov, Ali et al. 2006, Li, Yi et al. 2007). EGFR is a cellular membrane glycoprotein receptor. Upon binding with a signal transducer in the extracellular region, the intracellular tyrosine kinase domain becomes phosphorylated. As a result, STATs are activated, and the activation of these signaling pathways is correlated with the proliferation of cancer cells (J.-P. Spano et al. 2005, Irena, Porebska et al, 2000). The AKT signaling pathway is involved in cell metabolism, growth and survival and proliferation in lung cancers (Li, Wang et al. 2017). Also, aberrant AKT activation has a substantial role in lung cancer metastasis (Saji, Narahara et al. 2011). The mitogen-activated protein kinase (MAPK) system also facilitates cell proliferation and survival (Fang and Richardson 2005, Kinkade, Castillo-Martin et al. 2008). The JNK pathway is a sub-group of MAPK activated by cytokines (Weston and Davis 2007).

JNK promotes apoptosis by inhibiting anti-apoptotic proteins such as Bcl2 (Dhanasekaran and Reddy 2008). BPCA inhibits AKT signaling and, thus, expression of p-JNK and Bcl2. These results may partially account for the inhibition of lung cancer cell proliferation. In conclusion, study results demonstrate that BPCA possesses possible anticancer activities by encouraging cell apoptosis and decreasing ROS production in H460 lung cancer cells.

Acknowledgements

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References

1. Blackstock, A. W. and R. Govindan. 2007. Definitive chemoradiation for the treatment of locally advanced non‐small-cell lung cancer. Journalofclinicaloncology25(26): 4146-4152.
2. Bostancıoğlu, R. B., et al. 2013. Novel ferrocenyl-containing N-acetyl-2-pyrazolines inhibit in vitro angiogenesis and human lung cancer growth by interfering with F-actin stress fiber polimeryzation. Drug and chemical toxicology 36(4): 484-495.
3. Che, X.-F., et al. 2013. 2-Aminophenoxazine-3-one-induced apoptosis via generation of reactive oxygen species followed by c-jun N-terminal kinase activation in the human glioblastoma cell line LN229. Internationaljournalofoncology43(5): 1456-1466.
4. Choi, I. 2013. Reactive Oxygen Species and Cancer. HanyangMedRev33: 118-122.
5. Choi, Y. K., et al. 2018. Bis (3-bromo-4, 5-dihydroxybenzyl) ether, a novel bromophenol from the marine red alga Polysiphonia morrowii that suppresses LPS-induced inflammatory response by inhibiting ROS-mediated ERK signaling pathway in RAW 264.7 macrophages. Biomedicine&pharmacotherapy103: 1170-1177.
6. Dhanasekaran, D. N. and E. P. Reddy 2008. JNK signaling in apoptosis. Oncogene27(48): 6245-6251.
7. Fang, J. Y. and B. C. Richardson 2005. The MAPK signalling pathways and colorectal cancer. Thelancetoncology6(5): 322-327.
8. Green, D. R. and J. C. Reed 1998. Mitochondria and apoptosis. science281(5381): 1309-1312.
9. Hadi, S., et al. 2000. Putative mechanism for anticancer and apoptosis inducing properties of plant derived polyphenolic compounds. IUBMBlife50(3): 167-171.
10. Je, J.-Y., et al. 2009. Antioxidant activity of a red seaweed Polysiphonia morrowii extract. FoodScienceandBiotechnology18(1): 124-129.

11. Jemal, A., et al. 2010. Cancer statistics, 2010. CA:acancerjournalforclinicians60(5): 277-300.

12. Jena, N. 2012. DNA damage by reactive species: Mechanisms, mutation and repair. Journalofbiosciences37(3): 503-517.

13. Kang, N.-J., et al. 2017. Anti-inflammatory effect of 3-bromo-4, 5-dihydroxybenzaldehyde, a component of polysiphonia morrowii, in vivo and in vitro. Toxicologicalresearch33(4): 325-332.

14. Kawabe, T. 2004. G2 checkpoint abrogators as anticancer drugs. Moleculcancertherapeutics3(4): 513-519.

15. Kinkade, C. W., et al. 2008. Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model. TheJournalofclinicalinvestigation118(9): 3051-3064.

16. Li, H.-b., et al. 2007. Magnolol-lnduced H460 cells deathvia autophagy but not apoptosis. Archivesofpharmacalresearch30(12): 1566-1574.

17. Li, L., et al. 2017. Berberine could inhibit thyroid carcinoma cells by inducing mitochondrial apoptosis, G0/G1 cell cycle arrest and suppressing migration via PI3K-AKT and MAPK signaling pathways.Biomedicine&pharmacotherapy95: 1225-1231.

18. Lu, K., et al. 2001. Cryptophycin-induced hyperphosphorylation of Bcl-2, cell cycle arrest and growth inhibition in human H460 NSCLC cells. Cancerchemotherapyandpharmacology47(2): 170-178.

19. Matés, J. M., et al. 2012. Oxidative stress in apoptosis and cancer: an update. Archivesoftoxicology86(11): 1649-1665.

20. Ouhtit, A., et al. 2013. Simultaneous inhibition of cell-cycle, proliferation, survival, metastatic pathways and induction of apoptosis in breast cancer cells by a phytochemical super-cocktail: genes that underpin its mode of action. JournalofCancer4(9): 703.

21. Peto, R., et al. 2000. Smoking, smoking cessation, and lung cancer in the UK since 1950: combination of national statistics with two case-control studies. Bmj321(7257): 323-329.

22. Poillet-Perez, L., et al. 2015. Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy. Redoxbiology4: 184-192.

23. Saji, M., et al. 2011. Akt1 deficiency delays tumor progression, vascular invasion, and distant metastasis in a murine model of thyroid cancer. Oncogene30(42): 4307-4315.

24. Sankarapandi, S. and J. L. Zweier 1999. Evidence against the generation of free hydroxyl radicals from the interaction of copper, zinc-superoxide dismutase and hydrogen peroxide. JournalofBiologicalChemistry274(49): 34576-34583.

25. Sarbassov, D. D., et al. 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Moleculcell22(2): 159-168.

26. Tao, F., et al. 2019. The role of herbal bioactive components in mitochondria function and Cancer therapy. Evidence-BasedComplementaryandAlternativeMedicine2019.

27. von Freeden-Jeffry, U., et al. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as

a nonredundant cytokine. TheJournalofexperimentalmedicine181(4): 1519-1526.

28. Weston, C. R. and R. J. Davis 2007. The JNK signal transduction pathway. Currentopinionincellbiology19(2): 142-149.

29. Wyllie, A., et al. 1980. Cell death: the significance of apoptosis. Internationalreviewofcytology,Elsevier.68: 251-306.

30. Yang, Y., et al. 2016. Mitochondria and mitochondrial ROS in cancer: novel targets for anticancer therapy. Journalofcellularphysiology231(12): 2570-2581.

31. Zhou, Y., et al. 2003. Free radical stress in chronic lymph ocytic leukemia cells and its role in cellular sensitivity to ROS-generating anticancer agents. Blood,TheJournalof theAmericanSocietyof