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Research Article

LOW LEVEL OF CYCLOOXYGENASE-2 TRANSCRIPT IN THE SPLEEN OF LYMPHOMA RATS SUPPLEMENTED WITH GARLIC POWDER

NURUL SAS[1], HAZILAWATI H[1]*, SAJJARATTUL NNA[1], HUTHEYFA AH[1], ROSLY SM[2], WAN SWZ[2], NORHAIZAN ME[3], SHANMUGAVELU S[2], NOORDIN MM[1], JASNI S[4]

[1]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor, Malaysia. [2]BEEF Programme, Animal Science Research Centre, Malaysian Agricultural Research and Development Institute, Serdang, Selangor, Malaysia. [3]Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. [4]Department of Paraclinical Studies, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan Kota Bharu, Kelantan, Malaysia. Email: hazilawati@upm.edu.my

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ABSTRACT

Cyclooxygenases (COXs) regulate tumor cell proliferation and metastasis in many types of cancers including hematological cancers. Organosulfurs derived from garlic have a potential to inhibit the expression of COX-2 in cancer patients.

Objective: In this study, we evaluate the transcription levels of COX-2 in the spleen of lymphoma rats supplemented with garlic powder.

Methods: Three groups of rats were equally divided into control (n=3), lymphoma (n=3), and lymphoma supplemented with garlic powder (n=3) groups. Lymphoma was induced via administration of *N*-methyl-*N*-nitrosourea (MNU) intraperitoneally 4 times in 2 week periods. Garlic powder mixed with ground commercial rat diet was given daily at 5% of feed intake, starting at day 1 of MNU exposure. All rats were kept for 24 weeks before spleen samples were collected and extracted for total RNA. The transcription levels of COX-2 transcript in the total RNA were determined using quantitative real-time reverse transcriptase polymerase chain reaction assay. The total RNA was converted into cDNA followed by amplification of COX-2 and beta-actin genes.

Results: Results of the amplification of COX-2 transcripts were normalized with the housekeeping gene, beta-actin. The relative transcription level of COX-2 transcript in the spleen of lymphoma rats was 1.941±0.131 fold higher ($p<0.05$) than control rats (1.00±0.001 fold), while the transcription levels in the spleen of lymphoma rats supplemented with garlic was significantly lower (0.423±0.239 SE fold) than the lymphoma rats that received no supplementation of garlic powder.

Conclusion: The findings suggest that garlic powder reduces the transcription of COX-2 transcript in the spleen of lymphoma rats

Keywords: Garlic, Cyclooxygenase-2, *N*-methyl-*N*-nitrosourea, Splenic lymphoma, Real-time reverse transcriptase polymerase chain reaction assay.

INTRODUCTION

The use of herbal medicinal products and supplements has increased tremendously with not less than 80% or a total 4 billion population worldwide relying as a part of primary healthcare [1]. Cancers were the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer-related deaths in 2012 [2]. The number of new cases is expected to rise by about 70% over the next 2 decades. Nowadays, most of the research works on anti-cancer drugs are targeted on plants and plant-derived natural products. Many natural products and their bioactive compounds have been identified as potent anti-cancer agents, and the anti-cancer properties of various plants are being continuously identified [3].

Garlic (*Allium sativum L.*), traditionally known for its antibiotic and fungicidal activities, has been proposed to have additional medicinal properties including an ability to reduce platelet aggregation, decrease blood lipid concentrations, and reduce cancer risk [4]. Among *Allium* family (garlic, leek, and onion), extract of garlic exhibited the strongest inhibition of tumor cell proliferation with complete growth inhibition of renal, mammary, and prostate cancer cell lines [5,6]. The potent bioactive compounds of garlic that possess anticancer activities are derived from allyl sulfide derivatives. Different allyl sulfide derivatives (allicin, methyl allyl trisulfide, and diallyl trisulfide [DATS]) have been suggested to modulate an increasing number of molecular mechanisms in carcinogenesis [7]. Allicin, for example, exhibited its chemopreventive effects by inhibiting proliferation and inducing apoptosis of gastric cancer cell lines via arresting cell cycle at G2/M phases [8].

Cyclooxygenases (COXs) are rate-limiting enzymes, which catalyze the conversion of arachidonic acid into prostaglandins (PG) and contribute to the inflammatory process. Overexpression of COX-2, which is always associated with a poor prognosis, has been observed in a wide range of preneoplastic and malignant conditions; for example, colorectal, breast, pancreatic, and lung cancers [9]. Based on findings reported by Barisik *et al*. [10], COX-2 plays a role in carcinogenesis through one of its major products known as prostaglandin E2 (PGE-2). The COX-2/PGE-2 signaling modulates the regulation of tumor microenvironment that influences tumor cell proliferation and metastasis [11]. High level of PGE-2 is described in hematopoietic malignancies such as leukemia and lymphoma (12).

Hematological malignancies such as chronic lymphocytic leukemia, chronic myeloid leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, and multiple myeloma expressed high levels of COX-2 protein/transcripts, which correlate with poor patient prognosis [13-15]. Expression of COX-2 enhances survival and proliferation of cells [16], while negatively influencing anti-tumor immunity [17]. It modulates cellular immune responses by suppressing the roles of natural killer cells, cytotoxic T-lymphocytes, and T regulatory cells in cancer metastasis [18]. It indeed aids cancer cells to avoid immune responses by producing factors, for example, vascular endothelial growth factor (VEGF) that enhances angiogenesis and

metastasis [19]. The objectives of this study are (1) To evaluate the transcription levels of COX-2 in the spleen of lymphoma rats and (2) to evaluate the effect of garlic powder on the transcription level of COX-2 transcript in the spleen of lymphoma rats.

METHODS

Experimental design

Research conducted using animals was approved by the Animal Ethic Committee, Malaysian Agricultural Research and Development Institute (MARDI). A total of nine 6-week-old female Sprague–Dawley rats were used in this study. The rats were housed in polypropylene plastic cages in a controlled temperature colony animal room set at 22-27°C, relative humidity controlled at a range of 40-70%, and a 12-hr light/dark cycle. The animal study was conducted at the Animal Metabolism and Toxicology Research Centre, MARDI, Serdang, Selangor. The rats were maintained on a commercial rat diet and allowed access to drinking water throughout the period of study. The rats were acclimatized for two weeks before they were equally divided into three groups which were control (rats were injected with normal saline), lymphoma (n=3), and lymphoma plus garlic powder groups.

Carcinogen, its preparation and administration

N-Methyl-*N*-nitrosourea (MNU) (Sigma Aldrich, USA) with a molecular mass of 103.08 g/mol was used for induction of lymphoma. It was freshly prepared by dissolved in normal saline and given to the rats via intraperitoneal route. The dosage of MNU was equal to 60 mg/kg of each rat's body weight. The treatment was given twice a week in two consecutive weeks with a total dose of 240 mg/kg. The rats were monitored twice a day after administration of the carcinogen for the continuous 24 weeks.

Garlic powder, its preparation and administration

Garlic cloves obtained from a local market were finely chopped and dried in an oven at 55-60°C. The dried garlic cloves were ground into powder and mixed with ground commercial rat diet and supplemented to the rats daily for 24 weeks at 5% feed intake [5,20].

Spleen sample

At 24th week of the experimental period, all rats were humanely sacrificed by complete exsanguination under anesthesia with combination of ketamine at 75 mg/kg of rat's body weight and xylazine at 10 mg/kg of rat's body weight. Spleen samples were collected from all rats and kept in RNAlater solution (Ambion, Inc., Austin) and kept at −80°C before total RNA extraction was performed.

RNA extraction

Total cellular RNA was isolated from each spleen using the RNAeasy Mini Kit (Qiagen, USA) according to the manufacturer's instruction. The extracted RNA was eluted in 50 uL of RNAase-free water to obtain high volume of total RNA. The total RNA of each sample was quantified using a spectrophotometer (Eppendorf, German) via ultraviolet light absorbance at A260/A280 nm. The RNA concentration was kept in −80°C until further converted into cDNA.

Amplification of COX-2 transcripts via quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay

Two-step qRT-PCR was used to amplify the gene of interest and housekeeping gene. First, total RNA was converted into cDNA using the GeneAmp RNA Core Kit (Applied Biosystem USA) as per manufacturer's instruction. Amplification of COX-2 transcript was normalized with a housekeeping gene, beta-actin. The sense and antisense primer sequences of COX-2 were 5'-GCCACCTCTGCG ATGCTCTT-3' and GTGTTTGGGGTGGGC TTCAG-3', respectively [21]. The forward and reverse primer sequences for beta-actin were 5'-ATC GCT GAC AGG ATG CAG AAG-3' and 5'-AGA GCC ACC AAT CCA CAC AGA-3', respectively [22].

Master mix of the qRT-PCR was carried out in a 10 uL mixture containing 5 uL of SsoFast EvaGreen Supermix (Bio-Rad, USA), 1 uL of forward and reverse primers (0.2 uM), 1 uL of 3-4 ng cDNA, and 2 uL of RNase/Dnase-free water. The amplification conditions for COX-2 gene were started with 1 cycle of 15 minutes predenaturation at 95°C followed by 34 cycles of 10 seconds denaturation at 94°C, 30 seconds of annealing at 50°C, and 45 seconds of extension at 72°C. The amplification conditions of beta-actin gene was carried out at 1 cycle of 95°C for 15 minutes (predenaturation), followed by 34 cycles of 94°C for 10 seconds (denaturation), 51.8°C for 30 seconds (annealing), and 72°C for 45 seconds (extension). The amplification was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). Data were collected during annealing steps. The normalization factors were calculated automatically by the CFX96 Real-Time PCR Manager Software (Bio-Rad, USA). The formula is as follows:

$$\text{Normalization factor}_{\text{sample (GOI)}} = (RQ_{\text{sample (ref 1)}} \times RQ_{\text{sample (ref 2)}} \times \ldots RQ_{\text{sample (ref n)}})^{1/n}$$

Note: RQ=Relative quantity; n=Number of reference targets; GOI=Gene of interest (COX-2).

The transcription levels of gene of interest, COX-2, were normalized to the transcription levels of the housekeeping gene. The normalization was calculated by the CFX96 Real-Time PCR Detection System (Bio-Rad, USA) and the formula is as follows:

$$\text{Normalisation expression}_{\text{sample (GOI)}} = \frac{RQ_{\text{sample (GOI)}}}{\left(RQ_{\text{sample (ref 1)}} \times RQ_{\text{sample (ref 2)}} \times \ldots RQ_{\text{sample (ref n)}}\right)^{1/n}}$$

Note: RQ=Relative quantity of sample; Ref=Reference target in the experiment (Beta actin); GOI=Gene of interest (COX-2).

Standard curves for beta-actin and COX-2 genes were developed in our previous studies [22].

Statistical analyses

Means and standard errors of the relative transcription fold of COX-2 transcripts of each group were analyzed using one-way ANOVA (SPSS Statistic 21.0) and presented as mean±standard error mean. The statistical differences between groups were further analyzed using Tukey's test with $P<0.05$ was considered significant.

RESULTS

Results of the amplification of COX-2 and beta-actin transcripts in the spleen of rats in control, lymphoma, and lymphoma supplemented with garlic powder groups are shown in Figs. 1 and 2. The relative quantification of the transcription of COX-2 transcript is shown in Fig. 3. It showed that lymphoma rats had a significant higher transcription of COX-2 transcript (1.941±0.131 fold) as compared to control rats (1.00±0.001 fold). Interestingly, the transcription level of COX-2 in the spleen of lymphoma rats supplemented with garlic powder was comparable to control, and significantly lower (0.423±0.239 fold) than lymphoma rats. The level of COX-2 in lymphoma rats supplemented with garlic was indeed lower than control, albeit it was not significantly different ($P<0.05$).

DISCUSSION

Lymphoma is a malignant disease of the lymphoid system. The malignant lymphocytes in the lymph nodes replace the normal lymphocytes, multiply, and spread to the other organs such as liver, heart, lungs, and kidneys. We have shown the development of peripheral T-cell lymphoma in rats treated with MNU [23]. This carcinogen is one of the *N*-nitroso compounds which are known for almost 40 years present in foods treated with sodium nitrate. The carcinogenicity of sodium nitrate has been investigated in a 2-year study by Maekwa *et al.* [24]. They revealed that MNU causes mutation of the DNA through direct alkylation damage in DNA. When the excessive DNA damage is not

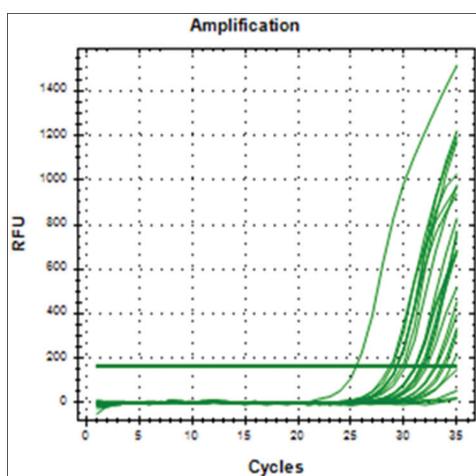

Fig. 1: Amplification curve of cyclooxygenase-2 transcripts from rats' spleens of control, lymphoma, and lymphoma plus garlic groups. RFU denotes relative fluorescence

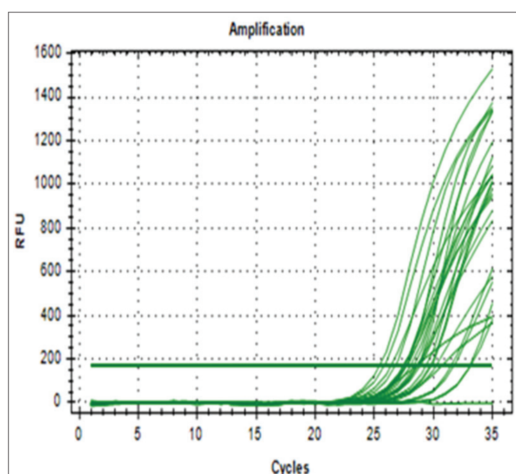

Fig. 2: Amplification curve of beta-actin transcripts from rats' spleens of control, lymphoma, and lymphoma plus garlic groups. RFU denotes relative fluorescence units

repaired, it leads to accumulation of mutations and may enhance cancer risk in target organs. The carcinogen targets various organs in a variety of animal species. In rats, MNU induced lymphoma and leukemia [25] and also induced mammary gland tumors that mimic human breast cancer [26]. Swann *et al.* [27] proved a single dose of MNU, which is completely metabolized in the body within 5 to 6 hours, which is sufficient to induce renal tumor in rats. This carcinogen has also been reported to induce peripheral T-cell lymphoma [23], early phase of chronic leukemia [22], acute promyelocytic leukemia [28], and splenic lymphoma [23,29] in rats. In this study, induction of splenic lymphoma in Sprague–Dawley rats was performed by administration of MNU via intraperitoneal route using similar method as described by Franchi *et al.* [25], Hutheyfa *et al.* [23], Nursyuhada *et al.* [22], Gal *et al.* [30], and Sajjaratul *et al.* [29].

Garlic has been used for decades in traditional medicine [31]. It can prevent the development of cancer and cardiovascular diseases by modifying risk factors such as hypertension, high blood cholesterol, and thrombosis [32-34]. The pharmacological effects of garlic are attributed to the presence of pharmacologically active organosulfur compounds including diallyl sulfide, diallyl disulfide, DATS, dipropyl sulfide, allicin, and ajoene. The evidence of garlic acts as an anti-carcinogenic that comes from both clinical and epidemiological significance. Numerous cohort and case–control studies have been

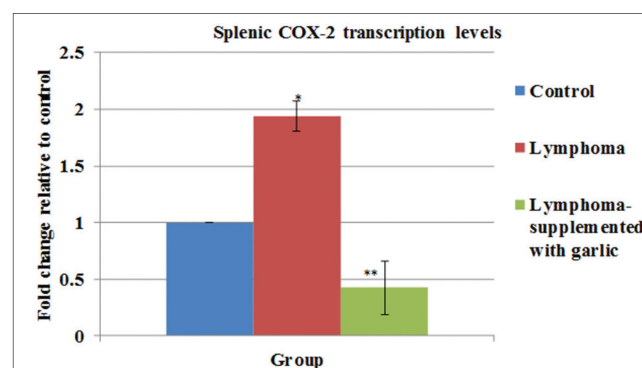

Fig. 3: The transcription levels of cyclooxygenase-2 in the spleen of Sprague–Dawley rats at 24th week post N-methyl-N-nitrosourea exposure. *Denotes significantly difference from control group (p<0.05). **Denotes significantly difference from lymphoma group (p<0.05). Each value is expressed as mean±standard error mean: n=3

conducted over the last 30 years, linking the etiology of cancer to the consumption of *Allium* vegetables, particularly garlic. A meta-analysis of 21 cohort and case–control studies published for the period of 1966-2010 confirmed the association between high consumption of *Allium* vegetables and reduction in the risk of gastric cancer [35]. Similar study conducted by Ngo *et al.* [36] on 7 colorectal studies confirmed an inverse association between cancer risk and high consumption of *Allium* vegetables including garlic. As similar to other anti-cancer agents, the mechanisms of garlic in fighting against cancers are mainly involved in modulating enzymatic activity, restraining of abnormal DNA formation, and scavenging of free radicals [37]. Overexpression of reactive oxygen species (ROS), reactive nitrogen species (RNS), and inducible form of nitric oxide synthase (iNOS), observed during chronic inflammatory conditions, plays a fundamental role in the initiation, growth promotion, and progression of cancer [38,39]. Garlic neutralizes the ROS, RNS and iNOS through its antioxidant activities, which are mainly possess by its organosulfur coumpounds. DATS, for example, inhibits cell growth of human skin cancer cell lines (melanoma and basal cell carcinoma) by decreasing the levels of intracellular ROS and DNA damage, and inducing G2/M arrest, endoplasmic reticulum (RE) stress, and mitochondria-mediated apoptosis, including the caspase-dependent and -independent pathways [40].

Apart from its anti-oxidant activities, Schafer *et al.* [41] proposed that garlic exhibits anti-carcinogenesis through its immunomodulatory effects. It initiates a shift in balance between the proinflammatory and immunosuppressive environments that enhances anti-tumor response leading to suppression of an emerging tumor. The proinflammatory cytokines (e.g. interleukin-6 [IL6], IL1-beta, tumor necrosis factor-alpha, and IL17) promote tumor development and the pro-apoptotic/anti-inflammatory cytokines (e.g. TRAIL, IL10, and transforming growth factor-beta) inhibit carcinogenesis [42]. It has been shown that the transcription of iNOS is upregulated by the proinflammatory cytokines and downregulated by the anti-inflammatory cytokines [43]. Possessing similar pathophysiological conditions as iNOS [44], COX-2 is highly expressed in cancer patients [45,46]. In lymphoma patients, Wun *et al.* [45] and Hazar *et al.* [46] reported that there is a clinical correlation between COX-2 expression and prognostic factors. Wang *et al.* [47] demonstrated that garlic essentially contains a certain amount of COX inhibitors, and the expression of COX-2 protein was found to be very little in colon cancer rats treated with garlic [48]. The finding is in accordance to the findings reported in this study. We detected very low level of COX-2 transcript in lymphoma rats that were supplemented daily with garlic powder. The level was rather lower than the level of COX-2 in the spleen of control rats. Other than garlic, there are numerous other herbs that were reported by Hong

et al. [49] to act as COX-2 inhibitor, for example, turmeric (curcumin) and cinnamon (polyphenols) [49]. The herbs inhibit cell growth and modulate angiogenic factors by downregulating the expression of COX-2 in pancreatic, lung adenocarcinoma [50], and melanoma cell lines [51].

COX-2 initiates the synthesis of numerous products, including PGE-2, which is reported to play a role in contributing to the development of cancer [52,53]. Any forms of alterations that induce the expression of COX-2 tends to cause overproduction of PGE-2, which facilitates the growth of cancer cells [52,53]. In carcinoma, Li *et al*. [54] demonstrated the role of COX-2/PGE-2 signaling in mediating tumorigenesis of a carcinoma stem cell. Many cancers have been reported to be associated with the high expression of COX-2 including colon cancer [55], gastric carcinoma [56], mucosa-associated lymphoid tissue (MALT)-lymphoma [57], and malignant T-cell lymphoma [58], which support the involvement of COX-2 in carcinogenesis. As far as garlic is concerned, its organosulfur compounds have been shown to inhibit the production of COX-2 in colon cancer rats [48]. It also inhibits the expression of iNOS in mouse macrophage cell line (RAW264.7) [59], PGE-2 in the stimulated macrophage cell line [60], and proinflammatory cytokines in the intestinal epithelial cell line [61]. The compounds have also been known to increase the activity of enzymes involved in the metabolism of carcinogens [62], and exhibited anti-oxidative activities [63] as well as anti-inflammatory effects in both *in vitro* and *in vivo* studies [61,63-65]. Apart from its role in cancer initiation, COX-2 is also known to have a role in promoting tumor cell invasion and angiogenesis [66]. VEGF, one of the growth factors that involves in angiogenesis, is produced from the downstream proangiogenic actions of COX-2, which are mediated by PGE-2, prostaglandin I2, and thromboxaneA2 (TXA-2) [19]. Overexpression of COX-2 protein [66] and COX-2 mRNA [67] was found highly correlated with high density of microvessel immunostained with CD-34 in samples collected from patients with gastric cancer [66]. Similarly, in 2013, Koh *et al*. detected overexpression of COX-2 and VEGF that were positively correlated with angiogenesis and tumor progression in the lymph nodes of classical Hodgkin lymphoma patients [68]. Both findings are further supported by our previous finding and current finding on the high transcription level of VEGF [29] and COX-2 RNA transcripts, respectively, in the spleen of lymphoma rats.

In this study, qRT-PCR was used to quantify the transcription levels of mRNA transcript. It is a technique for mRNA quantification that requires appropriate and extensive optimization and development of standard curves for both genes of interest and housekeeping to obtain reliable data [69]. This technique is the most sensitive method for RNA quantification as it allows the detection of small variation of gene expression and rare transcripts [70]. An example of chemistry available for quantitative PCR (qPCR) is an EvaGreen dye. This dye exhibits very low PCR inhibition. Higher concentration of this dye in the SsoFast EvaGreen Supermix kit (Bio-Rad, USA) generated greater fluorescent signal and increased sensitivity even for detection of a single target molecule. Detection and quantification of mRNAs were performed by real-time qPCR using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). qRT-PCR, an important tool to detect desirable and specific gene, provides rapid and accurate quantification of PCR products [71]. In this study, the SsoFast EvaGreen kit (Bio-Rad, USA) was used to amplify COX-2 transcript. The optical data were analyzed using variable parameters from the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The use of qRT-PCR technology to assess gene transcription levels required an accurate normalization of data to avoid misinterpretation of results and erroneous analyses of the results [72]. The accuracy of results obtained from qRT-PCR is dependent on the accurate normalization of the transcript or gene of interest using stably expressed genes known as reference or housekeeping genes [73]. Results of our studies show a very minimal transcription of COX-2 in the spleen of lymphoma rats supplemented with garlic powder. The level was indeed lower than the control rats, although it was not significant, indicating qRT-PCR is a very sensitive method to detect low level of gene expression.

CONCLUSION

COX-2 transcript was detected to be significantly higher in the spleen of lymphoma rats compared to control rats. Importantly, the transcription level was significantly lower in lymphoma rats that were supplemented with garlic powder at 5% of feed intake compared to lymphoma rats that received no supplementation of garlic powder. Results suggest that garlic powder reduces the transcription of COX-2 in the spleen of lymphoma rats.

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REFERENCES

1. World Health Organization (WHO). Report of the National Policy on Traditional Medicine and Regulation of Herbal Medicine. Geneva: World Health Organization; 2005.
2. Bernard S, Christopher PW. World Cancer Report 2014. Lyon: International Agency for Cancer Research; 2014.
3. Kathiresan K, Boopathy NS, Kavitha S. Costal vegetation - An underexplored source of anticancer drugs. Natl Prod Radiance 2006;5(2):115-9.
4. Ariga T Oshiba S. Platelet aggregation inhibitor in garlic. Lancet 1981;317:150-1.
5. Singh V, Belloir C, Siess MH, Le Bon AM. Inhibition of carcinogen-induced DNA damage in rat liver and colon by garlic powders with varying alliin content. Nutr Cancer 2006;55(2):178-84.
6. Boivin D, Simon LD, Jessica J, Edith B, Martine C, Albert M, *et al*. Anti-proliferative and antioxidant activities of common vegetables: A comparative study. Food Chem 2009;112(2):374-80.
7. Capasso A. Antioxidant action and therapeutic efficacy of *Allium sativum* L. Molecules 2013;18(1):690-700.
8. Tao M, Gao L, Pan J, Wang X. Study on the inhibitory effect of allicin on human gastric cancer cell line SGC-7901 and its mechanism. Afr J Tradit Complement Altern Med 2013;11(1):176-9.
9. Secchiero P, Barbarotto E, Gonelli A, Tiribelli M, Zerbinati C, Celeghini C, *et al*. Potential pathogenetic implications of cyclooxygenase-2 overexpression in B chronic lymphoid leukemia cells. Am J Pathol 2005;167(6):1599-607.
10. Barisik NO, Bozkurt S, Gumus M, Kaygusuz I, Karadayi N, Bas E, *et al*. Expression and prognostic significance of Cox-2 and p-53 in Hodgkin lymphomas: A retrospective study. Diagn Pathol 2010;5:19.
11. Katoh H, Hosono K, Ito Y, Suzuki T, Ogawa Y, Kubo H, *et al*. COX-2 and prostaglandin EP3/EP4 signaling regulate the tumor stromal proangiogenic microenvironment via CXCL12-CXCR4 chemokine systems. Am J Pathol 2010;176(3):1469-83.
12. Breyer RM, Bagdassarian CK, Myers SA, Breyer MD. Prostanoid receptors: Subtypes and signalling. Ann Rev Pharmacol Toxicol 2001;41:661-90.
13. Stratton MS, Alberts DS. Current application of selective COX-2 inhibitors in cancer prevention and treatment. Oncology (Williston Park) 2002;16 5 Suppl 4:37-51.
14. Bernard MP, Bancos S, Sime PJ, Phipps RP. Targeting cyclooxygenase-2 in hematological malignancies: Rationale and promise. Curr Pharm Des 2008;14(21):2051-60.
15. Asproni P, Vignoli M, Cancedda S, Millanta F, Terragni R, Poli A. Immunohistochemical expression of cyclooxygenase-2 in normal, hyperplastic and neoplastic canine lymphoid tissues. J Comp Pathol 2014;151(1):35-41.
16. Cao Y, Prescott SM. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. J Cell Physiol 2002;190(3):279-86.
17. Chan MW, Wong CY, Cheng AS, Chan YW, Chan KK, To FK, *et al*. Targeted inhibition of COX-2 expression by RNA interference suppresses tumour growth and potentiates chemosensitivity to cisplatin in human gastric cancer cells. Oncol Rep 2001;18:1557-62.
18. Liu B, Qu L, Yan S. Cyclooxygenase-2 promotes tumor growth and suppresses tumor immunity. Cancer Cell Int 2015;15:106.
19. Gately S, Li WW. Multiple roles of COX-2 in tumor angiogenesis: A target for antiangiogenic therapy. Semin Oncol 2004;31 2 Suppl 7:2-11.

20. Le Bon AM, Vernevaut MF, Guenot L, Kahane R, Auger J, Arnault I, *et al*. Effects of garlic powders with varying alliin contents on hepatic drug metabolizing enzymes in rats. J Agric Food Chem 2003;51(26):7617-23.
21. Nie SN, Sun HC, Wu XH, Qian XM. Cyclooxygenase 2, pS2, inducible nitric oxide synthase and transforming growth factor alpha in gastric adaptation to stress. World J Gastroenterol 2004;10(23):3537-41.
22. Nursyuhada H, Hazilawati H, Hutheyfa AH, Rosly SM, Shanmugavellu S, Noordin MM, *et al*. Detection of Bcl-2 in leukemic rats using an Eva Green real-time RT-PCR assay. Pertanika J Trop Agric Sci 2011;34:372-80.
23. Hutheyfa AH, Hazilawati H, Rosly SM, Jasni S, Noordin MM, Shanmugavelu S. Histopathological features of peripheral T-cell lymphoma in Sprague-Dawley rats induced with N-methyl-N-nitrosourea. Pertanika J Trop Agric Sci 2011;34:357-68.
24. Maekawa A, Ogiu T, Onodera H, Furuta K, Matsuoka C, Ohno Y, *et al*. Carcinogenicity studies of sodium nitrite and sodium nitrate in F-344 rats. Food Chem Toxicol 1982;20(1):25-33.
25. da Silva Franchi CA, Bacchi MM, Padovani CR, de Camargo JL. Thymic lymphomas in Wistar rats exposed to N-methyl-N-nitrosourea (MNU). Cancer Sci 2003;94(3):240-3.
26. Tsubura A, Lai LC, Miki Y, Tomo S, Norihisa U, Yuri T, *et al*. Animal models of N-Methyl-N-nitrosourea-induced mammary cancer and retinal degeneration with special emphasis on therapeutic trials. Int J Exp Clin Pathophysiol Drug Res 2011;25(1):11-22.
27. Swann FP, Magee PN. Nitrosamine induced carcinogen. The alkylation of nucleic acids of the rat by N-methyl-N-nitrosourea, dimethylnitrosamine, dimethyl sulphate and methyl methanesulphate. Biochem J 1968;110:39-47.
28. Chang YC, Hsu JD, Lin WL, Lee YJ, Wang CJ. High incidence of acute promyelocytic leukemia specifically induced by N-nitroso-N-methylurea (NMU) in Sprague-Dawley rats. Arch Toxicol 2012;86(2):315-27.
29. Sajjaratul NN, Hazilawati H, Rosly MS, Hakimi MK, Nursyuhada H, Yusnaini MY, *et al*. Effect of different routes of *N*-methyl-*N*-nitrosourea (MNU) exposure on the levels of vascular endothelial growth factor (VEGF) transcript in spleen of rats. Malays J Vet Res 2014;5:184-5.
30. Gal AF, Miclăuș V, Taulescu M, Bolfă P, Tăbăran F, Bouari C, *et al*. *Concurrent mammary adenocarcinoma and lymphoma in MNU-induced tumo*urigenesis in a Sprague-Dawley female rat. Bull UASMV Vet Med 2012;69:1-2.
31. Zeng T, Zhang CL, Zhao XL, Xie KQ. The roles of garlic on the lipid parameters: A systematic review of the literature. Crit Rev Food Sci Nutr 2013;53(3):215-30.
32. Neil A, Silagy C. Garlic: Its cardio-protective properties. Curr Opin Lipidol 1994;5(1):6-10.
33. Rahman K. Historical perspective on garlic and cardiovascular disease. J Nutr 2001;131(3s):977S-9.
34. Tanaka S, Haruma K, Yoshihara M, Kajiyama G, Kira K, Amagase H, *et al*. Aged garlic extract has potential suppressive effect on colorectal adenomas in humans. J Nutr 2006;136 3 Suppl:821S-6.
35. Zhou Y, Zhuang W, Hu W, Liu GJ, Wu TX, Wu XT. Consumption of large amounts of Allium vegetables reduces risk for gastric cancer in a meta-analysis. Gastroenterology 2011;141(1):80-9.
36. Ngo SN, Williams DB, Cobiac L, Head RJ. Does garlic reduce risk of colorectal cancer? A systematic review. J Nutr 2007;137(10):2264-9.
37. Thomson M, Ali M. Garlic [*Allium sativum*]: A review of its potential use as an anti-cancer agent. Curr Cancer Drug Targets 2003;3(1):67-81.
38. Mocellin S, Bronte V, Nitti D. Nitric oxide a double edged sword in cancer biology: Searching for therapeutic opportunities. Med Res Rev 2007;27(3):317-52.
39. Wink DA, Hines HB, Cheng RY, Switzer CH, Flores-Santana W, Vitek MP, *et al*. Nitric oxide and redox mechanisms in the immune response. J Leukoc Biol 2011;89(6):873-91.
40. Wang HC, Yang JH, Hsieh SC, Sheen LY. Allyl sulfides inhibit cell growth of skin cancer cells through induction of DNA damage mediated G2/M arrest and apoptosis. J Agric Food Chem 2010;58(11):7096-103.
41. Schäfer G, Kaschula CH. The immunomodulation and anti-inflammatory effects of garlic organosulfur compounds in cancer chemoprevention. Anticancer Agents Med Chem 2014;14(2):233-40.
42. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest 2007;117(5):1175-83.
43. Kleinert H, Schwarz PM, Förstermann U. Regulation of the expression of inducible nitric oxide synthase. J Biol Chem 2003;384:1343-64.
44. Massi D, Franchi A, Sardi I, Magnelli L, Paglierani M, Borgognoni L, *et al*. Inducible nitric oxide synthase expression in benign and malignant cutaneous melanocytic lesions. J Pathol 2001;194(2):194-200.
45. Wun T, McKnight H, Tuscano JM. Increased cyclooxygenase-2 (COX-2): A potential role in the pathogenesis of lymphoma. Leuk Res 2004;28(2):179-90.
46. Hazar B, Ergin M, Seyrek E, Erdogan S, Tuncer I, Hakverdi S. Cyclooxygenase-2 (Cox-2) expression in lymphomas. Leuk Lymphoma 2004;45(7):1395-9.
47. Wang XH, Shao DH, Liang GW, Zhang R, Xin Q, Zhang T, *et al*. Cyclooxygenase inhibitors in some dietary vegetables inhibit platelet aggregation function induced by arachidonic acid. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2011;19(5):1260-3.
48. Archana S, Samit G, Shamee B, Sukta D. Experimental evaluation of anticarcinogenic effect of garlic in rats colon cancer. Asian Pac J Cancer 2004;5:126-32.
49. Hong CH, Hur SK, Oh OJ, Kim SS, Nam KA, Lee SK. Evaluation of natural products on inhibition of inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) in cultured mouse macrophage cells. J Ethnopharmacol 2002;83(1-2):153-9.
50. Lev-Ari S, Starr A, Vexler A, Karaush V, Loew V, Greif J, *et al*. Inhibition of pancreatic and lung adenocarcinoma cell survival by curcumin is associated with increased apoptosis, down-regulation of COX-2 and EGFR and inhibition of Erk1/2 activity. Anticancer Res 2006;26(6B):4423-30.
51. Kwon HK, Jeon WK, Hwang JS, Lee CG, So JS, Park JA, *et al*. Cinnamon extract suppresses tumour progression by modulating angiogenesis and the effector function of CD8+ T cells. Cancer Lett 2009;278(2):174-82.
52. Brock TG, McNish RW, Peters-Golden M. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E2. J Biol Chem 1999;274(17):11660-6.
53. Harris RE. Cyclooxygenase-2 (COX-2) and the inflammogenesis of cancer. Subcell Biochem 2007;42:93-126.
54. Li HJ, Reinhardt F, Herschman HR, Weinberg RA. Cancer-stimulated mesenchymal stem cells create a carcinoma stem cell niche via prostaglandin E2 signalling. Cancer Discov 2012;2(9):840-55.
55. Banning A, Florian S, Deubel S, Thalmann S, Müller-Schmehl K, Jacobasch G, *et al*. GPx2 counteracts PGE2 production by dampening COX-2 and mPGES-1 expression in human colon cancer cells. Antioxid Redox Signal 2008;10(9):1491-500.
56. Soni K, Shashi PM, Shyam BP, Suresh SY, Mitali D, Mohan K, *et al*. Differential expression of COX2: A potential marker for clinical phenotypes of gastric cancer. Int J Adv Res 2014;2(2):278-88.
57. Konturek PC, Konturek SJ, Pierzchalski P, Starzyńska T, Marlicz K, Hartwich A, *et al*. Gastric MALT-lymphoma, gastrin and cyclooxygenases. Acta Gastro Enterol Belg 2002;65(1):17-23.
58. Kopp KL, Kauczok CS, Lauenborg B, Krejsgaard T, Eriksen KW, Zhang Q, *et al*. COX-2-dependent PGE(2) acts as a growth factor in mycosis fungoides (MF). Leukemia 2010;24(6):1179-85.
59. Massi DV, Alexandra KK, Hildebert W, Angelika MV. Effect of allicin and ajoene, two compounds of garlic, on inducible nitric oxide synthase. Atherosclerosis 1998;139(2):333-9.
60. Chang HP, Chen YH. Differential effects of organosulfur compounds from garlic oil on nitric oxide and prostaglandin E2 in stimulated macrophages. Nutrition 2005;21(4):530-6.
61. Lang A, Lahav M, Emad S, Iris B, Herma HF, Benjamin A, *et al*. Allicin inhibits spontaneous and TNF-α induced secretion of proinflammatory cytokines and chemokines from intestinal epithelial cells. Clin Nutr 2004;23(5):1199-208.
62. Oommen S, Anto RJ, Srinivas G, Karunagaran D. Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells. Eur J Pharmacol 2004;485(1-3):97-103.
63. Pari L, Murugavel P, Sitasawad SL, Kumar KS. Cytoprotective and antioxidant role of diallyl tetrasulfide on cadmium induced renal injury: An *in vivo* and *in vitro* study. Life Sci 2007;80(7):650-8.
64. Son EW, Mo SJ, Rhee DK, Pyo S. Inhibition of ICAM-1 expression by garlic component, allicin, in gamma-irradiated human vascular endothelial cells via downregulation of the JNK signalling pathway. Int Immunopharmacol 2006;6(12):1788-95.
65. Murugavel P, Pari L. Effects of diallyl tetrasulfide on cadmium-induced oxidative damage in the liver of rats. Hum Exp Toxicol 2007;26(6):527-34.
66. Uefuji K, Ichikura T, Mochizuki H. Cyclooxygenase-2 expression is related to prostaglandin biosynthesis and angiogenesis in human gastric cancer. Clin Cancer Res 2000;6(1):135-8.
67. Uefuji K, Ichikura T, Mochizuki H. Expression of cyclooxygenase-2 in human gastric adenomas and adenocarcinomas. J Surg Oncol 2001;76(1):26-30.
68. Koh YW, Park C, Yoon DH, Suh C, Huh J. Prognostic significance of

COX-2 expression and correlation with Bcl-2 and VEGF expression, microvessel density, and clinical variables in classical Hodgkin lymphoma. Am J Surg Pathol 2013;37(8):1242-51.
69. Tong Z, Gao Z, Wang F, Zhou J, Zhang Z. Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 2009;10:71.
70. Alexandre HP, Caroline BM, Eric A, Florence BM, Frederic B, Cellier Dominic C, *et al*. Reverse transcriptase quantitative polymerase chain reaction: Description of a RIN-based algorithm for accurate data normalization. BMC Mol Biol 2009;10:31.
71. Jebbink J, Bai X, Rogers BB, Dawson DB, Scheuermann RH, Domiati-Saad R. Development of real-time PCR assays for the quantitative detection of Epstein-Barr virus and cytomegalovirus, comparison of TaqMan probes, and molecular beacons. J Mol Diagn 2003;5(1):15-20.
72. Marum L, Miguel A, Ricardo CP, Miguel C. Reference gene selection for quantitative real-time PCR normalization. Publ Lib Sci 2012;7:1371.
73. Guénin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L. Normalization of qRT-PCR data: The necessity of adopting a systematic, experimental conditions-specific, validation of references. J Exp Bot 2009;60(2):487-93.