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

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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 1.91±0.13 fold higher (p<0.05) than control rats (1.00±0.00 fold), while the transcription levels in the spleen of lymphoma rats supplemented with garlic was significantly lower (0.43±0.23 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 properties, 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 antitumor 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 processes. 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 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=5), 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 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 10 mg/kg of rat’s body weight. Spleen samples were collected from each rat 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.

RESULTS
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'-GCCACCTCTGCGATGCTCTT-3' and GTGTTTGGGGTGGCTTCACTC-3', respectively [21]. The forward and reverse primer sequences for beta-actin were 5'-ATGGCAGAGATCTAGAGAAC-3' and 5'-AGAAGCCACCACATCCAAG-3', respectively [22].

Master mix of the qRT-PCR was carried out in a 10 μL mixture containing 5 μL of SsoFast EvaGreen Supermix (Bio-Rad, USA), 1 μL of forward and reverse primers (0.2 μM), 1 μL of 3-4 ng cDNA, and 2 μL 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 (pre-denaturation), 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:

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\text{Normalization factor} = \left(\frac{RQ_{\text{sample}}}{RQ_{\text{ref 1}}} \times \frac{RQ_{\text{sample}}}{RQ_{\text{ref 2}}} \times \cdots \frac{RQ_{\text{sample}}}{RQ_{\text{ref n}}}\right)^{1/n}
\]

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

Means and standard errors of the relative transcription fold of COX-2 genes 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 were started with 1 cycle of 95°C for 15 minutes (pre-denaturation), 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) and the formula is as follows:

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\text{Normalisation expression}_{\text{sample} (GOI)} = \left(\frac{RQ_{\text{sample}}}{RQ_{\text{ref 1}}} \times \frac{RQ_{\text{sample}}}{RQ_{\text{ref 2}}} \times \cdots \frac{RQ_{\text{sample}}}{RQ_{\text{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).

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 Maekawa 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
repairs, 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, allin, 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 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 inductible 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 compounds. 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-inflammatory 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 12, and thrombomodulin2 (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 kit (Bio-Rad, USA) generated more 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 dependant 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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AQ1: Kindly provide structured abstract as per journal style (Objective, Methods, Results and Conclusion)