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Submission date: 30-Jun-2021 11:45AM (UTC+0800)
Submission ID: 1614005583
File name: The_Number_of_Macrophages.pdf (558.8K)
Word count: 2676
Character count: 15054

Indonesian Journal of Cancer Chemoprevention, June 2012
ISSN: 2088–0197
e-ISSN: 2355-8989

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The Number of Macrophages and Heterophils on Chick Embryo Chorioallantoic Membrane After *Gynura procumbens* (Lour) Merr Extract Treatment and bFgF Induction

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Abstract

Antiangiogenesis (inhibition of new blood vessels formation) has become a strategy to inhibit cancer development. The aim of this experiment was to investigate antiangiogenic effect of *Gynura procumbens* (Lour) Merr focusing on the decreasing of the number of macrophages and heterophils on chick embryo chorioallantoic membrane. Nine-days-aged-eggs were divided into six groups (eight eggs each group). Group I (positive control) eggs were induced with bFGF+Tris HCl. Group II (negative control) eggs were treated with DMSO+Tris HCl. Group III (treatment I) eggs were induced with 60 ng bFGF and treated with ethanolic extract of *G. procumbens* leaves with the dose of 60 µg. The following treatment groups, i.e. group IV (treatment II), group V (treatment III), and group VI (treatment IV) were treated with increasing dose of extract, starting from 75 µg, 90 µg, and the last was 110 µg. Eggs were incubated until they reach the age of twelve days to observe macrophages, while to observe heterophils, eggs were incubated until the age of seventeen days. Based on haematoxylin-eosin staining, macrophages in the treatment groups were less than the control positive group (bFGF+Tris HCl), but based on giemsa staining, the effect of *Gynura procumbens* in decreasing the number of heterophils could not be observed because some blood smears. These analysis suggest that the ethanolic extract of *Gynura procumbens* leaves can perform as antiangiogenic agent decreasing the number of macrophages.

Keywords: *antiangiogenic, macrophages, heterophils, Gynura procumbens*

INTRODUCTION

Angiogenesis is new blood formation as part of normal process in the body, playing and important role in growth and development. Angiogenesis occurs during recovery, such as during the formation of new tissue after damage. Unfortunately, angiogenesis is also involved in carcinogenesis, that turns cancer into its uncontrolled malignant state (Folkman, 1998).

Angiogenesis involves cells engaged with inflammation, including macrophages and heterophils, facilitated by matrix metalloproteinase (MMP) (Zijlstra *et al*., 2004; Zijlstra *et al*., 2005). According to the study conducted by Deryugina and Quigley (2006) discussed in Ardi *et al*. (2007), the role of MMP in the development of tumour and metastasis is complex. MMP acts as the main mediator in growth factor activation, e.g. bFGF, receptor bioavailability and signaling, cell adhesion and motility, apoptosis and survival mechanism, angiogenesis and immune response, and also immune surveillance. To date, more than 20 proteases that belongs to MMP family have been discovered (Rundhaug, 2005). MMP-9 secreted by heterophils and MMP-13 secreted by macrophages as part of MMP family involved in angiogenesis, as proangiogenic proteinase (Zijlstra *et al*., 2004; Zijlstra *et al*., 2005).

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Plenty strategies have been applied in cancer treatment. However, existing methods faced various clinical problems. Hence, novel strategies are being developed continuously. One strategy of suppressing carcinogenesis is by inhibiting angiogenesis. Jenie *et al*. (2006) reported that *G.procumbens* leaves possess antiangiogenic activity. *G. procumbens* also showed pharmacological effect to cancer, since its extract contains flavonoid, quercetin, polyphenol, alkaloid, and also other substances that are able to inhibit carcinogenesis (Hamid *et al*., 2009).

This study was conducted to observe *G. procumbens* leave ethanolic extract's ability to suppress cancer growth by inhibiting angiogenesis. Antiangiogenic effect will be observed by evaluating its ability to decrease the number of macrophages and heterophils.

MATERIALS AND METHODS

Materials

Another chemicals used were ethanol 70%, sterile aquabidest, formaline buffer 10% for TAB chorioallantoic membrane preservation, haematoxyllin-eosin for macrophage-histology and counting staining, and methanol-giemsa for heterophils counting staining (Thompson and Samuel, 1966).

Antiangiogenic Activity Observation

Eggshell were marked for air space boundary, embryo location, and the area above the embryo that later would be perforated with a 1x1 cm square window. The embryo location was detected by candling on the eggs. The pole of the eggshells containing air space and the eggshell part above the embryo was washed with ethanol 70%. Then, those two areas were perforated with a minidrill.

The air inside the air space was aspirated by using a rubber ball until the chorioallantoic that adhered to the egg membrane detached. Egg position was mantained horizontal during the process conducted in a dark room by candling, so that the chorioallantoic membrane and the artificial air space formed above the embryo could be seen. Next, eggs were washed with ethanol 70% again and kept horizontally in laminar air flow hood. Artificial air space were positioned at the upper part.

Eggshell above the embryo were perforated with a minidrill to make a 1 cm^2 hole. Test solution was implanted into formed chorioallantoic membrane through this window. There were 6 groups, each containing 8 eggs, half were for machrophages observation, while another four were for heterophils observation. Group 1 was implanted with paper disc containing 60 ng bFGF and tris-HCl as positive control. The 2nd group was implanted with paper disc containing tris-HCl and DMSO 2% as negative control. Group 3, as the first treatment group, was implanted with paper disc containing 60 ng bFGF and 60 μg *Gynura procumbens* leave ethanolic extract (GLE). Another treatment groups were group 4, 5, and 6, implanted with paper disc containing 60 ng bFGF and 75 μg, 90 μg, and 110 μg GLE, respectively.

After treatment, eggs were incubated at 39ºC and 60% RH for 72 hours (Ribatti *et al*., 1997). On the 13th day, four embryonic eggs from each group were kept in the freezer for 24 hours to kill the embryo. Following it, TAB was opened by cutting the shell into two, starting from the area that was close to the air space. Egg content was slowly extracted to prevent chorioallantoic membrane detachment. Chorioallantoic membrane around the paperdisc were then cut and kept in formaline buffer 10% for preservation. The membrane then were used to made the histopathology preparation stained with haematoxyllin-eosin for macrophages number observation. Preparations were conducted in Laboratorium Patologi Fakultas Kedokteran Universitas Gadjah Mada.

The other four eggs remaining from each group were used for heterophils observation. Heterophils could be isolated from 17 days-aged TAB (Zijlstra *et al*., 2005). Embryonic chicken eggs were opened by cutting the shell. Blood was extracted from the blood vessel of the egg to be made into blood preparations with Romanowsky method. The preparations were then used for heterophils counting.

Observation was conducted by using Olympus ® CX-21 microscope with 1000x magnification on six different window of view. Data acquired was analyzed statistically by using One-way Analysis of Variance (ANOVA), continued with Duncan post-hoc test ($p<0.05$).

RESULTS AND DISCUSSION

Effect of GLE on Number of Macrophages

The result of hematoxylin-eosin-stained embryonic chicken egg chorioallantoic membrane macrophage counting is shown on Table I. Histology observation is shown in Fig. 1.

Table 1. Effect of GLE on mean of macrophages number on each group. Differently superscritpted mean shows significant different compared to each other ($p < 0.05$)

Group	Number of Macrophages ($\bar{x} \pm$ SD)
1	$104.50^{a} \pm 16.46$
2	$21.50^{d} \pm 2.65$
3	$76.50^{b} \pm 6.56$
4	$50.25^{c} \pm 7.57$
5	$40.75^{c} \pm 2.87$
6	$24.00^{d} \pm 6.83$

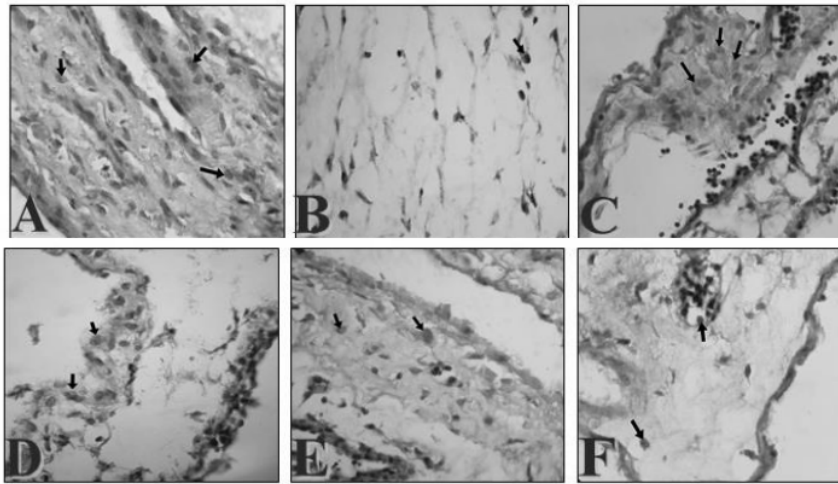

Figure 1. Histology observation on (A) Group 1 as positive control, (B) Group 2 as negative control, (C) Group 3, (D) Group 4, (E) Group 5, and (F) Group 6 as treatment groups. Preparations were stained with haematoxylin-eosin, 1000x magnification. Black arrows shown macrophages.

The mean of the number of macrophages of all GLE-treated groups (Group 3 to 6) with different doses, that are 60 µg, 75 µg, 90 µg, and 110 µg, respectively, shown a significant decrease compared to positive control group (Group 1) ($p < 0.05$). Moreover, Group 6, 110 µg GLE-treated group, had shown a decrease of the number of macrophage that is similar to its normal state, shown by Group 2 as the negative control group.

The number of macrophage in each GLE-treated group showed significant difference among each other ($p < 0.05$). These data showed that GLE could decrease the number of macrophages. It is possible that GLE contains certain substance(s) that possess angiogenesis inhibitory activity by decreasing macrophages infiltration.

A component in *G. procumbens* having antiangiogenic activity is flavonoid. The study support the result reported by Jenie *et al.* (2006), proving that flavonoid contained in *G. procumbens* could inhibit angiogenesis of TAB chorioallantoic membrane. According to the study on *Citrus reticulata* conducted by Chrisnanto *et al.* (2008), treatment of *Citrus reticulata* peels extract containing a huge amount of tangeretin (a polymethoxy flavonoid) and nobiletin yielded less number of macrophages compared to bFGF-only-treated group in microscopic observation of hematoxylin-eosin-stained TAB chorioallantoic membrane histopathological preparations . Macrophages are known to secrete angiogenic factors, such as VEGF, bFGF, and interleukin-8 (IL-8) that are able to induce angiogenesis and Tumor Necrosis Factor-α (TNF-α) that later would increase the number of VEGF, bFGF, and IL-8 receptors (Lee *et al*., 2006). The decrease of the number of macrophages could possibly occured by cell cycle arrest induced by tangeretin (Chrisnanto *et al*., 2008).

It has been reported that several flavonoids, such as apigenin, genistein, and quercetin were able to induce premitotic Growth phase-2/Mitotic phase (G2/M) arrest in several cell lines, while some others were also able to induce Presynthetic Growth phase-1 (G1) arrest (Pan *et al*., 2002). Pan *et al*. (2002) also reported that tangeretin found in *Citrus reticulata* peels could induce G1 arrest by increasing Cyclin-dependent Kinase Inhibitors (CKIs), such as p27 and p21 in colon cancer cell lines. The possible mechanism of cell cycle arrest by the extract might be the same as the mechanism of cell cycle arrest by flavonoids contained in GLE.

GLE could possibly increase CKIs, such as p21, p27, and/or p57, inhibiting Cyclin-dependent Kinases (CDKs), that later would suppress cyclins expression or increase p53 level, resulting in cell cycle arrest (Meiyanto *et al*., 2007) and the decrease of the number of macrophages. p53 a regulatory protein that could induce p21 expression, hence causing G1 arrest (Meiyanto *et al*., 2007). G1 phase is the phase when cells are getting prepared for DNA synthesis together with RNA and protein biosynthesis (Hartono, 2009). Flavonoid contained in *G. procumbens* extract could also suppress topoisomarase I and II expression that play a role in DNA supercoil conversion. Topoisomerase inhibitor may stabilize topoisomerase complex, causing the formation of DNA nick hence undergoes a damage. DNA damage will further increse the expression of proapoptotic proteins, such as Bax and Bak, and decrease antiapoptotic proteins Bcl-2 and Bcl-X_L (Ren *et al*., 2003). Here, we may see that flavonoids play a role in inducing apoptosis.

According to the discussion above, it is very likely that flavonoids in GLE could decrese the number of macrophages by similar mechanism.

Effect of GLE on the Number of Heterophils

The effect of GLE on heterophils could not be observed yet, since the preparations did not allow a valid observation, resulting in insufficient replication. The problem was caused by the lysed nucleus, causing it impossible to differ heterophils and the other leukocytes, worsen by the precitipated staining agent interfering cell observation.

Nucleus lysis may occur because of insufficient duration of fixation. The minimum duration of fixation is 5 minutes. It could be modified, depending on the quality of staining agent used (Bijanti *et al*., 2010). The precipitation of staining agent was caused by the solution that was kept uncovered, the solution that was not filtered properly prior to usage, and dirty object glass (WHO, 2006). The preparations also shown too intense blue color of the cells and blue background. Too intense blue color of the cells may caused by the blood smear that is too thick, insufficient time for the buffer to dissolve, too high buffer pH, too long

staining duration, too high staining agent concentration and viscosity, impurities in staining agent stock, and light exposure on staining agent stock solution.The blue background yielded may be caused by the too quick fixation process and too long duration time prior to fixation (WHO, 2006).

This study concluded that GLE treatment could decrease the number of macrophages of bFGF-induced TAB chorioallantoic membrane histopahological preparations. Unfortunately, the effect of GLE on the number of heterophils could not be observed because of the improper preparation of the blood smears that did not allow a valid observation, resulting in in-sufficient replication.

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