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ORIGINAL ARTICLE

Anti-inflammatory Properties of *Bixa Orellana* Leaves Extract are Associated with Suppression of Bradykinin-induced Endothelial Hyperpermeability

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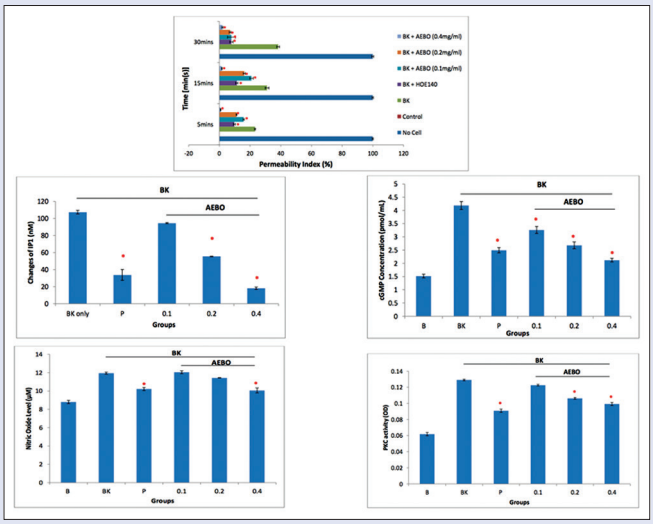
ABSTRACT

Background: Previous study showed that an aqueous extract of *Bixa orellana* L. (Family of *Bixaceae*) leaf (AEBO) is capable of inhibiting bradykinin (BK)-induced inflammation in animal models. **Objective:** This study further investigates the effect of AEBO on BK-induce inflammation *in vitro* model. **Materials and Methods:** The endothelial barrier protective effect of AEBO was examined via an *in vitro* endothelial permeability assay. Human umbilical vein endothelial cell (HUVEC) was first pretreated with AEBO with a concentration range from 0.1, 0.2, and 0.4 mg/mL and then induced with BK. Fluorescein isothiocyanate-conjugated-dextran was used as an indicator of permeability flux. To elucidate its mechanism of action, the phospholipase C (PLC) – nitric oxide (NO) – cyclic guanosine monophosphate (cGMP) signaling pathway and protein kinase C (PKC) activity were evaluated. **Results:** Pretreatment of AEBO significantly ($P < 0.05$) suppressed BK-induced HUVEC hyperpermeability and 0.4 mg/mL possessed the maximal inhibitory effect (87%, 70%, and 57% inhibition rate at 5, 15, and 30 min time point, respectively). Moreover, AEBO has presented remarkable IC_{50} = 0.24 mg/mL for anti-PLC activity, 0.36 mg/mL for anti-NO production, and 0.19 mg/mL for anti-cGMP production. For PKC inhibition, the IC_{50} (0.42 mg/mL) was slightly higher compared to others. **Conclusion:** This study provided supportive evidence for the previous study where AEBO exhibited anti-inflammatory activity against BK *in vivo*. The anti-inflammatory activity of AEBO may partly be associated with the reduction of endothelial hyperpermeability via the suppression of PLC-NO-cGMP signaling and PKC activity.

Key words: Cyclic guanosine monophosphate, endothelial permeability, nitric oxide, phospholipase C

SUMMARY

- Aqueous extract of Bixa orellana' leaves exhibited anti-hyperpermeability effect against bradykinin in human umbilical veins endothelial cells
- The anti-hyperpermeability activity of the extract may partly involve phospholipase C nitric oxide cyclic guanosine monophosphate signaling and protein kinase C activity.



Abbreviations used: AEBO: Aqueous extract of Bixa orellana; BK: Bradykinin; PLC: Phospholipase C; NO: Nitric oxide; cGMP: Cyclic guanosine monophosphate; PKC: Protein kinase C; HUVEC: Human umbilical vein endothelial cell; PBS: Phosphate buffered saline; eNOS: Endothelial nitric oxide synthase.

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INTRODUCTION

Aberrations of endothelial barrier function subsequently increase endothelial permeability, which is one of the most important pathological conditions in the pathogenesis of various inflammatory diseases, including atherosclerosis, cardiac failure, and even cancer formation and metastasis.[1] Number of cytokines and inflammatory mediators, such as vascular endothelial growth factor,[2] reactive oxygen species,[3] histamine,[4] and interferon-γ,[5] are known to increase endothelial permeability. Bradykinin (BK), a vasodilating peptide which is involved in the sensing of pain, also increases vascular permeability.[6]

The basis of pharmacological actions of BK is mediated by at least two subtype receptors, designated as B1 and B2:[7] the B1 receptor is undetectable and is usually expressed following inflammation, whereas B2 is constitutively expressed in various types of tissues and mediates many of the actions in physiological condition.[8] Previous studies postulated that the B2 receptor mainly contributed to endothelial hyperpermeability when exposed to BK. This is because selective B2 receptor antagonist showed markedly attenuated reduction in dye leakage

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from the vasculature.[9,10] Both receptors are G-protein-coupled receptors, and the activation of the B2 receptor could trigger the phospholipase C nitric oxide cyclic guanosine monophosphate (PLC-NO-cGMP) pathway, which is known as a major signaling pathway.[8] Moreover, BK also upregulates protein kinase C (PKC) activity which is an essential step in endothelial hyperpermeability.[11] Due to the fact that treatment for endothelial dysfunction is still lacking, many researchers are trying to discover new therapeutic options for the prevention and treatment of endothelial inflammation.

Our previous studies reported that the extract of *Bixa orellana* L., also known as "annatto," has the potential of inhibiting the vascular hyperpermeability induced by different types of inflammatory mediators both *in vivo* and *in vitro*.[12-15] Despite active research, the mechanism of action of *B. orellana* leaf extract remains largely unknown. Therefore, the aim of this study was to extend the previous *in vivo* study,[12] and further elucidate the signaling pathway involved in the action of *B. orellana* leaf extract in BK-induced endothelial permeability.

MATERIALS AND METHODS

Plant materials and extraction

B. orellana leaves were collected from around Universiti Putra Malaysia, Malaysia in the month of July 2014. The plant was authenticated, and the voucher specimen (NL16) was deposited at the Phytomedicinal Herbarium, Institute of Biosciences, Universiti Putra Malaysia, Selangor, Malaysia. Basically, the aqueous extraction method used in the present study was prepared according to the methods described previously[15] and the aqueous extract of *B. orellana* (AEBO) was from the same batch of the sample from the previous study.[15]

Drugs and chemicals

BK, HOE140, phosphate buffered saline (PBS) and a ×10 trypsin-EDTA solution were purchased from Sigma Chemical Co. Ltd. Malaysia. 1-(6-[[[17 β]-3-Methoxyestra-1,3,5[10]-trien-17-yl] amino] hexyl)-1H-pyrrole-2,5-dione (U-73122), L-NG-nitroarginine methyl ester hydrochloride (L-NAME), 6-(phenylamino)-5,8-quinolinedione (LY 83583) and GF 109203X hydrochloride were purchased from Merck, Malaysia.

Human umbilical veins endothelial cell culture

Human umbilical veins endothelial cell (HUVEC) and its growth media (M 200) supplemented with a low-serum growth supplement were purchased from Cascade Biologic (Portland). The cultures were maintained at 37°C and 5% CO_2. Only cells from passages 1–5 were used in the experiments.

In vitro endothelial permeability assay

In vitro vascular permeability Assay kit (Chemicon® US, in 24-well format) was used as described.[15] A 24-well transwell inserts were coated with collagen, and HUVEC were seeded at 1×10^6 cells/mL and culture until the formation of an integrated cell monolayer. An integrated cell monolayer was verified by microscopic monitoring. Cells were pretreated with AEBO at 0.1, 0.2, and 0.4 mg/mL for 12 h before inducing with 1 μM of BK. Permeability was determined by adding fluorescein isothiocyanateconjugated Dextran (FITC-Dextran) to the upper chamber of each well. Readings were taken from the bottom chamber at 5, 15, and 30 min time points using Tecan Infinite M200 spectrofluorometer (Switzerland) (excitation/emission wavelengths of 485/530 nm). HOE 140 (10 μM), a BK inhibitor was used as a positive control.

The permeability index (%) was calculated as:

$$\frac{(\text{experimental clearance}) - (\text{spontaneous clearance})}{(\text{clearance of the filter alone}) - (\text{spontaneous clearance})} \times 100$$

Indirect determination of phospholipase C activity

PLC activity was determined by the quantification of inositol phosphate using a commercial IP-One ELISA kit (Cisbio, USA) and the assay procedures were followed as described in previous studies.[15] U-73122 (10 μM), a PLC inhibitor was used as positive control. A concentration of inositol phosphate was calculated from the standard curve which runs together with the sample.

Nitrite and nitrate assay

NO accumulation (sum of nitrite and nitrate) was used as an indicator of NO production in the cell culture medium by using a nitrite/nitrate assay kit (Roche, Malaysia). The assay protocol was followed as described in Yong *et al.*[15] L-NAME (10 μM), an endothelial NO synthase (eNOS) inhibitor, was used as a positive control.

Cyclic guanosine monophosphate assay

The total soluble cGMP produced by HUVEC was measured using a cGMP enzyme immunoassay (R and D System, USA) according to the manufacturer's instructions and the procedure put forth by Yong *et al.*[15] LY 83583 (10 μM), an inhibitor of soluble guanylate cyclase (GC) and of cGMP production was used as positive control.

Protein kinase C assay

Effect of AEBO on BK-induced PKC activity in HUVEC was determined by an ELISA kit (Enzo Life Sciences, Malaysia). The experiment's protocol was followed as described in Yong *et al.*[15] GF 109203X hydrochloride (10 μM), a competitive inhibitor of PKC was used as positive control.

Statistical analysis

All experiments were conducted in triplicate, and all values in the figures and test results are expressed as a mean ± standard error of the mean. All data were analyzed using the Statistical Package for Social Sciences (SPSS 22, IBM Corporation, NY, USA). Differences between values were assessed for significance using one-way analysis for variance and further analyzed using Dunnet's test. $P < 0.05$ was considered statistically significant.

RESULTS

Bradykinin-induced increased human umbilical veins endothelial cell permeability was significantly suppressed by pretreatment of aqueous extract of *Bixa orellana*

In vitro HUVEC permeability assay result showed that the HUVEC permeability significantly increased after induced by BK when compared with the basal level [Figure 1]. The pretreatment of HUVEC with AEBO suppressed the BK-induced increased permeability in HUVEC at all-time points. Interestingly, AEBO at a concentration of 0.4 mg/mL showed maximal inhibition among all the concentrations ($P < 0.05$), almost restoring the permeability to the basal level [Figure 1]. HOE 140 also produced significant inhibition ($P < 0.05$) concerning BK alone group. Furthermore, 0.1 and 0.2 mg/mL of AEBO showed a comparable effect ($P > 0.05$) with HOE 140.

Aqueous extract of *Bixa orellana inhibited* phospholipase C *activity*

To study the mechanism of AEBO, the PLC– NO– cGMP signaling pathway was examined at cellular level. The production of inositol phosphate was measured to indicate PLC activity in the current study. BK significantly ($P < 0.05$) increased the production of inositol phosphate, and this indicates that BK elevated PLC activity in endothelial cells [Figure 2]. However, pretreatment of the AEBO inhibited BK-induced PLC activity, especially at 0.2 and 0.4 mg/mL. The highest inhibition was produced by 0.4 mg/mL of AEBO with an 82% inhibition rate ($P < 0.05$). AEBO at 0.1 mg/mL had no effect on BK regulating the endothelial PLC activity, and there was no significant ($P > 0.05$) difference compared with the BK alone group.

Aqueous extract of *Bixa orellana reduced nitric oxide accumulation*

The data revealed that the NO value of L-NAME and 0.4 mg/mL of AEBO significantly decreased ($P < 0.05$) compared with the BK-only group while that of the BK-only group markedly increased in comparison with the basal group [Figure 3]. On the other hand, pretreatment of HUVEC with 0.1 and 0.2 mg/mL of AEBO failed to suppress the production of NO-induced by BK.

Aqueous extract of *Bixa orellana* decreased cyclic guanosine monophosphate production

cGMP levels in HUVEC was increased significantly ($P < 0.05$) by BK from the basal level of 1.42 ± 0.01 pmol/mL to 2.74 ± 0.07 pmol/mL [Figure 4]. This increase by BK was inhibited by AEBO in a

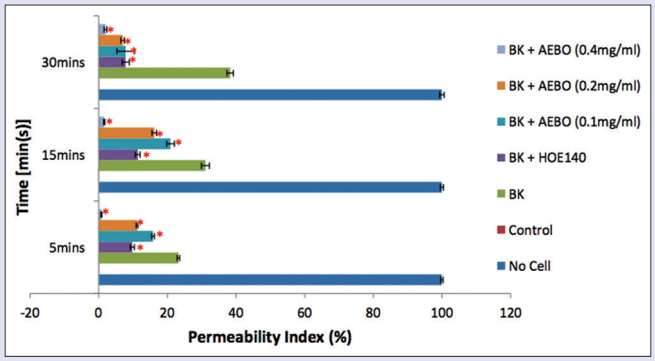

Figure 1: AEBO suppressed BK-induced increased HUVEC permeability in three different time points. Permeability in HUVEC was measured as flux of FITC-Dextran from upper to lower chambers. Three independent experiments were performed in triplicate. Data are expressed in mean ± standard error of the mean. *$P < 0.05$ considered statistically significant versus bradykinin group in each time points. AEBO: Aqueous extract of *Bixa orellana*; BK: Bradykinin; HUVEC: Human umbilical veins endothelial cell; FITC: Fluorescein isothiocyanate-conjugated

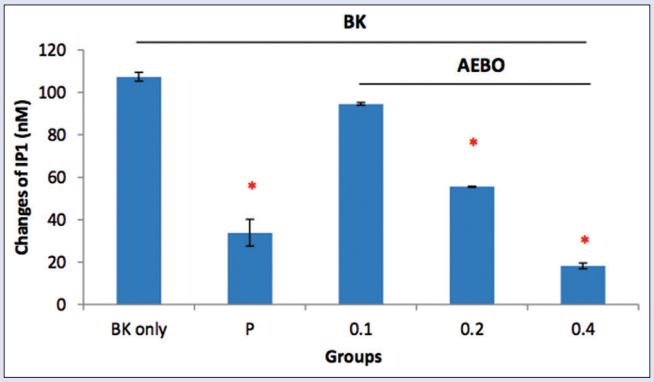

Figure 2: Effect of AEBO on the PLC activity was detected by measuring inositol phosphate production in HUVEC. Three independent experiments were performed in triplicate. Data are expressed in mean ± standard error of the mean. *$P < 0.05$ considered statistically significant versus bradykinin group. AEBO: Aqueous extract of *Bixa orellana*, P: Positive group (U-73122 10μM), BK = Bradykinin; AEBO: Aqueous extract of *Bixa orellana*; HUVEC: Human umbilical veins endothelial cell; PLC: Phospholipase C

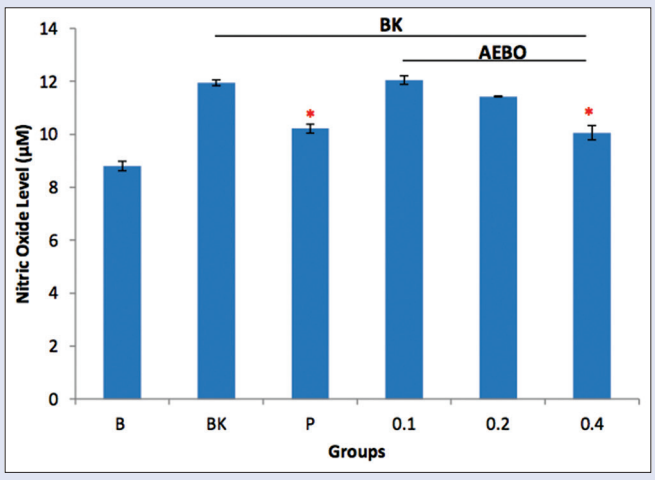

Figure 3: The effect of AEBO on nitric oxide production induced by BK in HUVEC. Three independent experiments were performed in triplicate. Data are expressed in mean ± standard error of the mean. *$P < 0.05$ considered statistically significant versus bradykinin group. B: Basal group, BK: Bradykinin group, P = L-NAME, AEBOL: Aqueous extract of *Bixa orellana*; HUVEC: Human umbilical veins endothelial cell

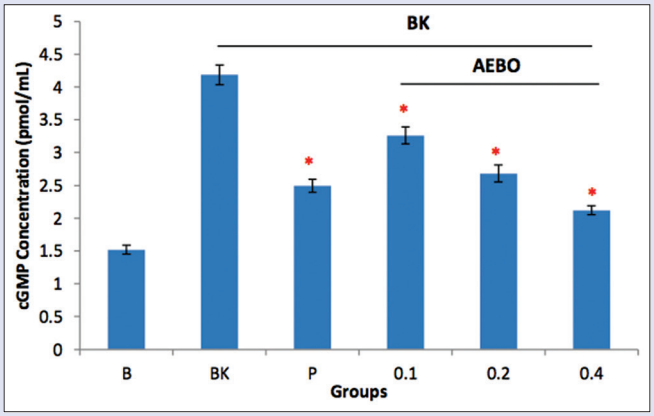

Figure 4: Effect of AEBO on BK-induced cGMP production on HUVEC. Values are expressed in means ± standard error of the mean of triplicate, and were obtained in at least three independent experiments. Data are analyzed using one-way analysis for variance. *$P < 0.05$ (B: Basal level; BK: Bradykinin, P: Positive control, 10 μM of LY 83583, AEBO: Aqueous extract of *Bixa orellana*; cGMP: Cyclic guanosine monophosphate; HUVEC: Human umbilical veins endothelial cell)

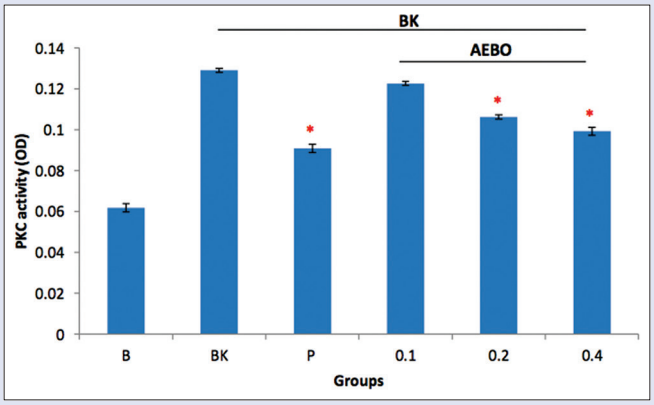

Figure 5: The effect of AEBO on BK-activated PKC activity on HUVEC. Data expressed in means ± standard error of the mean of triplicate, and were obtained in at least three independent experiments. Data are analyzed using one-way analysis for variance. *$P < 0.05$ (B: Basal level; BK: Bradykinin; P: Positive control, 10 μM of GF109203X hydrochloride, AEBO: Aqueous extract of *Bixa orellana*; HUVEC: Human umbilical veins endothelial cell; BK: Bradykinin; PKC: Protein kinase C)

dose-dependent manner. Maximal inhibition was found at 0.4 mg/mL of AEBO with 77% inhibition rate, while 0.1 and 0.2 mg/mL of AEBO showed 35% and 56% inhibition rates, respectively. The positive control, LY83583, an inhibitor of soluble GC and of cGMP production also showed a significant reduction ($P < 0.05$) of cGMP level against BK in HUVEC.

Aqueous extract of *Bixa orellana reduced* protein kinase C *activity*

PKC activity was significantly ($P < 0.05$) elevated when stimulated with BK in HUVEC [Figure 5]. Preincubation with AEBO at 0.2 and 0.4 mg/mL significantly ($P < 0.05$) suppressed PKC activity, where the maximal inhibitory rate produced by 0.4 mg/mL was 44% ($P < 0.05$). However, 0.1 mg/mL of AEBO failed to down-regulate PKC activity given HUVEC exposure to BK. GF 109203X hydrochloride used as positive control showed significant reduction ($P < 0.05$) in PKC activity compared with BK alone group.

DISCUSSION

A healthy endothelium is important in maintaining vascular homeostasis. Aberrations of endothelial barrier function and vascular hyper-permeability are always associated with numerous detrimental diseases, for instance, hypertension, coronary artery disease, heart failure,[16] or even cancer proliferation and metastasis. Due to its predominant role in the pathogenesis of numerous of diseases, endothelia are an attractive therapeutic target, especially for cardiovascular diseases.

There are a number of inflammatory mediators, cytokines, and chemokines that could alter the endothelial barrier function, and thus lead to increase vascular permeability. BK is not only known as a potent vasoactive which elicits pain but it is also potent in increasing vascular permeability.[17] The present data demonstrate that pretreatment of AEBO is capable of suppressing FITC-Dextran across HUVEC when challenged with BK. This result tallies with the previous study where AEBO was shown to reduce the leakage of Evans's Blue dye when the animal was challenged with BK.[12] This again proved that AEBO can suppress the permeability of small blood vessels in an inflamed condition. BK is not only able to increase HUVEC permeability,[18] but it also increases blood-brain barrier permeability,[19] pulmonary vascular permeability,[20] and even increased vascular permeability in ascitic tumors.[21] Previous studies demonstrated that activation of BK subtype 2 receptors (B2) by exogenous BK is contributing to vascular hyperpermeability in animals.[22] However, this pathologic condition was blocked by HOE 140, a highly selective antagonist at the B2 receptor.[23] In our studies, BK significantly increased endothelial permeability; however, pre-treatment of AEBO was shown to markedly attenuate FITC-dextran leakage from the upper to the lower chamber, and the result is comparable with HOE 140. This proves that BK increased endothelial permeability via the B2 receptor because a B2 receptor antagonist inhibited BK-induced hyperpermeability. Thus, AEBO can suppress endothelial hyperpermeability induced by BK, which may be associated with B2 receptor inactivation or inhibition.

To elucidate the mechanism of action of AEBO on how to suppress endothelial hyperpermeability induced by BK, the PLC-NO-cGMP signaling pathway was investigated. A number of phosphoinositide-specific PLC isoforms were found to be expressed in HUVEC.[24] The binding of BK to B2 receptors on the surface of endothelial cells will subsequently lead to the activation of PLC and eNOS.[25] This results in the release of NO and also production of cGMP.[26,27] Interestingly, Aschner and his group[11] reported that endothelial hyperpermeability induced by BK is independent of PLC activation. This contradiction could be explaining by different experimental setting among the researchers. G protein is activated depending on the cell type on which BK exerts its action.[25] In the current study, we measured cellular PLC activity indirectly by determining the level of inositol phosphate produced by endothelial cells when challenged with BK. BK was able to upregulate PLC activity when compared with BK group alone. However, AEBO at 0.2 and 0.4 mg/mL significantly reversed PLC activity by more than 80% (by 0.4 mg/mL). This could suggest AEBO-suppressed endothelial hyperpermeability induced by BK through inhibit PLC activity. NO synthesis by eNOS from the endothelial cells, with a very short half-life free radical participated in the regulating of vascular functions, including vascular tone and blood flow. However, excessive production of NO is always associated with pathologic condition, such as NO produced by macrophages, induced by endotoxins, causing an increase in vascular permeability. A previous study also reported that inhibition of NO production could lead to vascular protein leakage and increased microvascular permeability in animal models.[28] These suggested that NO plays both pro- and anti-inflammatory properties and this depends on the level of NO and "where" it was produced.[29] Extensive studies demonstrated that NO enhanced endothelial permeability in response to BK.[22,30] This is similar to our current result where BK increased NO production in HUVEC, but this condition was suppressed by the pre-treatment of AEBO. Surprisingly, only 0.4 mg/mL of AEBO significantly reduced the NO level by 50% inhibition rate. This might be due the fact that relatively high concentrations are needed to achieve effective NO inhibition, as eNOS activation can be due to other signaling pathways, such as protein kinase A, Akt, and AMPK.[31]

The involvement of AEBO in downstream PLC signaling pathway was further investigated by measuring the level of soluble cGMP. The activation of soluble GC by NO in healthy endothelium leads to the production of intracellular cGMP concentration. This level of cGMP is essential to maintain basal permeability. On the contrary, elevation of cGMP production is associated with increased endothelial permeability, and this had been shown by the current data [Figure 4]. The extensive literature reported that increased endothelial permeability by different inducers occurred through a cGMP-dependent pathway.[32-34] In the experiment done by He, the group even reported that the elevation

of cGMP levels using cGMP analogs will transiently increase basal permeability in both frog and rat mesentery microvessels.[26] At the same time, inhibition of the GC using LY-83583 (inhibitor of soluble GC and of cGMP production) attenuated the BK-induced increase permeability in rats.[26] Our current data again showed that the reduction of cGMP level in the BK-induced group and this indicated an elevation of cGMP level is essential to increase endothelial permeability. However, all concentrations of AEBO were capable of suppressing the production of cGMP when HUVEC was induced by BK, with 0.4 mg/mL of the extract showing the highest inhibition rate compared with the rest. All the data suggested that AEBO participates in the suppression of endothelial permeability induced by BK may via PLC-NO-cGMP signaling pathway.

Aschner *et al.*[11] observed that PKC-dependent pathway is required for increased in endothelial permeability induced by BK. On top of that, a finding from Murray *et al.*[35] also suggested that PKC may mediate increases in vascular permeability in response to BK due to the reason that sphingosine (PKC inhibitor) markedly attenuated responses to BK in an animal setting. In the present study, we observed that BK significantly upregulated PKC activity in vascular endothelial cells, but this was reduced by the pretreatment of AEBO. The reduction is significant; however, inhibition rate is <50% even in the highest concentration (0.4 mg/mL). This may be due to AEBO not being a specific target in PKC activity.

Based on the previous GC-MS analysis,[14] the major compound presented in the AEBO is acetic acid and Ruiz and Gomes[36] had documented that low concentration of acetic acid exhibited anti-histamine activity. This could be explained the anti-hyperpermeability effect of AEBO in the current study. Nonetheless, the possibility of the other compounds or minor compounds which are yet discovered in the *B. orellana* leaves exhibiting anti-inflammatory properties cannot be excluded. Current results are important and contribute toward the validation of the traditional use of this plant in the treatment of inflammatory disorders. In addition, it could be also a potential therapeutic agent to treat vascular hyper-permeability-related diseases.

CONCLUSION

The present data has shown that AEBO plays an inhibitory role in vascular inflammation, especially vascular permeability. It was clarified that AEBO suppresses BK-induced endothelial hyperpermeability via inhibiting the PLC-NO-cGMP pathway. However, the molecular mechanism including receptor identification of AEBO involved remains to be clarified and is a very attractive future target.

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Conflicts of interest

There are no conflicts of interest.

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