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Antioxidative and Antiplatelet Effects of Aqueous Inflorescence *Piper betle* Extract

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Piper betle, belonging to the *Piperaceae* family, is a tropical plant, and its leaf and inflorescence are popularly consumed by betel quid (BQ) chewers in Taiwan and many other South and Southeast Asian countries. However, little is known about the biochemical properties of inflorescence *Piper betle* (IPB) toward reactive oxygen species (ROS) and platelet functions. In the present work, aqueous IPB extract was shown to be a scavenger of H_2O_2, superoxide radical, and hydroxyl radical with a 50% inhibitory concentration (IC_{50}) of about 80, 28, and 73 μg/mL, respectively. IPB extract also prevented the hydroxyl radical induced PUC18 plasmid DNA breaks at concentrations higher than 40 μg/mL. Since ROS are crucial for platelet aggregation, we further found that IPB extract also inhibited the arachidonic acid (AA) induced and collagen-induced platelet aggregation, with an IC_{50} of 207 and 335 μg/mL, respectively. IPB extract also inhibited the AA-, collagen- (>100 μg/mL of IPB), and thrombin (>250 μg/mL of IPB)-induced thromboxane B_2 (TXB_2) production by more than 90%. However, IPB extract showed little effect on thrombin-induced aggregation. These results indicated that aqueous components of IPB are potential ROS scavengers and may prevent the platelet aggregation possibly via scavenging ROS or inhibition of TXB_2 production.

KEYWORDS: Antioxidant; betel chewing; health; inflorescence *Piper betle*; platelet aggregation; reactive oxygen species

INTRODUCTION

Piper betle is a tropical plant that grows in Malaysia, Taiwan, and other Southeast Asian countries. Inflorescence *Piper betle* (IPB) and *Piper betle* leaf (PBL), which contains eugenol and hydroxychavicol, were often added into the betel quid (BQ) to improve aromatic flavor. This BQ chewing habit is popular in India, Pakistan, South Africa, and Southern Asian areas and in India immigrants of England, New Zealand, and Australia. There are about 200−600 million BQ chewers in the world, whereas the presence of about 2 million BQ chewers in Taiwan is reported (*1*−*3*). An average of 5−20 quids are chewed each day (*1*) with an average chewing time of 15−25 min of each quid. This oral habit is more popular in aborigines, laborers, and taxi and truck drivers because of stimulation of the central and autonomic nervous system, which produces a sense of well-being, euphoria, alertness, and sweating and increases the ability to work (*1*, *2*). Betel quid usually contains areaca nut (AN), lime, PBL, tobacco, and other sweetening agents (*1*, *4*). In Taiwan and Papua New Guinea, BQ is generally composed of a fresh green (raw) AN, lime, and a piece of IPB (*2*, *4*, *5*). The lime, which is manipulated in the form of a paste, contains mainly calcium hydroxide, catechu, or other additives (*1*). These additives may neutralize the acidity of AN, reduce its astringency, and improve flavor. No tobacco is added into the chewing mixture in Taiwan.

BQ chewing is well recognized as the major causation factor of oral leukoplakia, oral submucous fibrosis (OSF), and oral cancer in Taiwan, South Africa, India, and many Southeast

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Asian countries (*1*, *4*). Most of the previous studies have investigated mainly the toxicity of AN and its derivatives such as AN-specific nitrosamines and reactive oxygen species (ROS) production (*1*, *6*). Recently some BQ planters and sellers want to make BQ substitutes by excluding the lime component but including new components to decrease the toxicity of BQ. However, little is known about the biochemical properties of IPB toward ROS and platelet functions and whether BQ should exclude or include IPB. IPB has been shown to exert cytotoxicity and genotoxicity to oral mucosal cells at relatively high concentrations (>800 μg/mL) (*7*). Chewing BQ containing IPB has been shown to markedly elevate the oral cancer incidence in Taiwan possibly because of its content of potential human carcinogen safrole in its organic fraction (*2*). However, aqueous IPB extract is not able to induce unscheduled DNA synthesis of cultured oral keratinocytes, indicating lack of DNA breaking capacity (*8*). PBL and IPB components reduce the methyl mercaptan induced halitosis (*9*) and inhibit the nitrosation of areca alkaloids, a process that produces AN-specific carcinogenic nitrosamines (*10*). PBL and IPB components also suppress the capacity of catechol to induce adrenal chromaffin cells secreting catecholamine, which exhibits profound effects on human endocrine, metabolic, cardiovascular, and immune functions (*11*). IPB extract further induces hypotensive and bradycardiac or tachycardiac effect in rats (*12*). Leaf and stalk extract of *Piper betle* suppresses the spermatozoa maturation and induces infertility of Swiss male albino mice (*13*, *14*). These results indicate that consumption of IPB may potentially affect the systemic health of BQ chewers. Recently, the mechanisms of cancer and arteriosclerosis have been shown to have similar characteristics (*15*). However, whether BQ chewing will have an impact on the health of the local and systemic cardiovascular systems is still not elucidated.

Local hypercoagulation and coagulopathy are critical factors in the pathogenesis of OSF (*16*−*20*). Intriguingly, OSF patients show higher serum levels of factor VIII and circulating platelet aggregates than that of healthy subjects (*20*). Production of ROS in the saliva while chewing BQ has been reported (*21*, *22*). Moreover, ROS are shown to be critical in the pathogenesis of oral cancer (*22*, *23*) and blood coagulation (*24*−*26*). We recently find that AN extract induces platelet aggregation and thromboxane B_2 (TXB_2) production, whereas PBL extract inhibits platelet activation (*27*). To better understand whether IPB may positively or negatively affect the BQ chewing related oral mucosal diseases, the biochemical properties of aqueous IPB extract toward free radical production, ROS-induced DNA strand breaks, and platelet aggregation were investigated. Results of the present study facilitate our understanding the effects of BQ chewing on the health of cardiovascular systems and oral cavity.

MATERIALS AND METHODS

Materials. Fresh IPB were obtained from local market in Taiwan. IPB extract was prepared as described with slight modification (*7*, *8*). Briefly, 50 g of IPB (wet weight) was grinded in 1 L of ice-cold doubly distilled water by an engine-driven blender for 10 min and then extracted at 4 °C for 4 h. After centrifugation, the supernatant was filtered two times with Advantec filter paper (Toyo Roshi Kaisha LTD, Japan). IPB extract was then lyophilized, weighed, and redissolved in doubly distilled water prior to use. Ferrous chloride, arachidonic acid (AA), collagen, thrombin, luminol, and lucigenin were products of Sigma (Sigma Chemical Company, St. Louis, MO). PUC18 DNA was purchased from Bayou Biolab (LA). Agarose, TBE buffer, and ethidium bromide were from HT Inc., (U.K.). TXB_2 enzyme linked immunosorbent assay (ELISA) kits were purchased from Cayman Chemical Company (Ann Arbor, MI).

Scavenger of H_2O_2. The reaction was carried out in a mixture of 200 or 250 μL containing 133 μL of 50 mM Tris-HCl buffer (pH 7.4), 100 μL of 2 mM lucigenin, and various concentrations of IPB. Subsequently, reaction was started by the injection of 17 μL of 30% H_2O_2 (final 2% H_2O_2). A luminometer (Orion microplate luminometer, Berthold DS, Tforzheim, Germany) was used to measure the H_2O_2-induced lucigenin chemiluminescence.

Scavenger of Superoxide Radical. The reaction was carried out in a mixture of 250 μL containing 150 μL of 50 mM Tris-HCl buffer (pH 7.4), 60 μL of 2 mM lucigenin, and various concentrations of IPB (20−600 μg/mL). Subsequently, 10 μL of xanthine oxidase (XO, 0.02 U/mL) was added. Reaction was started by the addition of 30 μL of xanthine (0.33 M). The superoxide-induced lucigenin chemiluminescence was measured by a microplate luminometer (*27*−*29*).

Scavenger of Hydroxyl Radical. The reaction was carried out in a reaction mixture (250 μL) containing 10 mM luminol (pH 7.4), various concentrations of IPB extract (final 20−600 μg/mL), and $FeCl_2$ (100 μM). H_2O_2 was subsequently added into the assay well, and chemiluminescence was measured with a microplate luminometer.

DNA Strand Breaking Activity. Reaction was conducted in a reaction mixture containing 5 μL of 50 mM Tris-HCl buffer (pH 7.4), 5 μL of PUC18 plasmid DNA (1 μg), 5 μL of of IPB extract (final concentration of 40−400 μg/mL), 5 μL of 3% H_2O_2, and 5 μL of 100 μM $FeCl_2$ at 37 °C for 30 min. After 30 min, the mixture was subjected to gel electrophoresis in 0.8% agarose/Tris-borate−EDTA buffer and run at 100 V for 20 min, using Mupid-2 electrophoretic equipment. DNA was visualized and photographed by a UV apparatus (Alpha Innotech Corporation, CA) (*27*−*29*).

Effect of IPB Extract on Platelet Aggregation. Washed rabbit platelets were prepared as described previously (*27*, *30*, *31*) and suspended in Tyrode's solution containing 1 mM of calcium and 0.35% BSA (3×10^8 platelets/mL). Platelet aggregation was measured by the turbidimetric method of Born and Cross (*32*), and the percent aggregation was calculated as described by Teng and Ko (*33*). Briefly, platelets were preincubated with IPB extract for 3 min followed by the addition of thrombin (0.1 U/mL), AA (100 μM), and collagen (10 μg/mL). The aggregation was recorded by a Lumi aggregometer (model 1020, Payton, Ontario, Canada).

Effect of IPB Extract on Thromboxane B_2 (TXB_2) Production of Platelets. After 3 min of aggregation experiments, EDTA (2 mM) and indomethacin (50 μM) were added to the platelet suspension. After centrifugation (14 000 rpm, 2 min) with an Eppendorf centrifuge (model 5414, Merck), TXB_2 concentrations of the aliquots were measured with ELISA kits (Cayman) according to the manufacture's instruction (*27*, *30*, *31*).

Statistical Analysis. Results of the IPB extract as ROS scavenger and on platelet aggregation were expressed as percentage of inhibition (mean ± SE). The IC_{50} was calculated by regression analysis. Four or more separate experiments were performed, and results were expressed as the mean ± SE. Statistical analysis was conducted using a paired Student's *t*-test. A *P* value less than 0.05 was considered to constitute differences between control and experimental groups.

RESULTS

Antioxidative Capacities of IPB Components. IPB extract can be a potent H_2O_2 scavenger. As shown in **Figure 1a**, IPB extract suppressed the lucigenin-enhanced H_2O_2 chemiluminescence with an IC_{50} of about 80 μg/mL. At concentrations of 40 and 200 μg/mL, IPB extract inhibited the H_2O_2-induced chemiluminescence by 49% and 53%, respectively.

IPB extract was also a scavenger of the superoxide radical. Reaction by xanthine and XO produced superoxide radicals as revealed by the increase of lucigenin-enhanced chemiluminescence (data not shown). The presence of IPB extract markedly inhibited the X/XO produced superoxide radicals in a dose-dependent fashion (**Figure 1b**). The IC_{50} of the IPB extract against superoxide radicals was about 28 μg/mL.

IPB extract was further a scavenger of hydroxyl radicals produced by H_2O_2 and $FeCl_2$. The hydroxyl radical induced

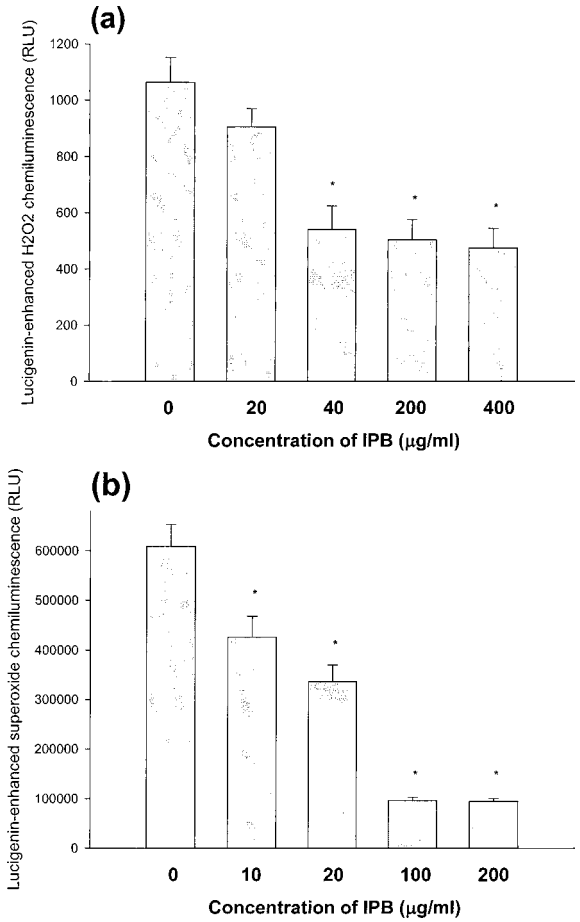

Figure 1. IPB extract as H_2O_2 and superoxide radical scavenger. (a) Externally added H_2O_2 was used to produce the lucigenin chemiluminescence and its prevention by IPB components. The peak value of chemiluminescence (RLU) in each test was measured ($n = 4$). (b) Xanthine and xanthine oxidase were used to generate superoxide radicals and their inhibition by IPB extract, as recorded by a luminometer. The lucigenin-enhanced superoxide chemiluminescence during the first 10 s was averaged for data presentation. The results were expressed as relative light units (RLU) (mean ± SEM) ($n = 9$). The asterisk (∗) denotes a marked difference between the control and the experimental groups.

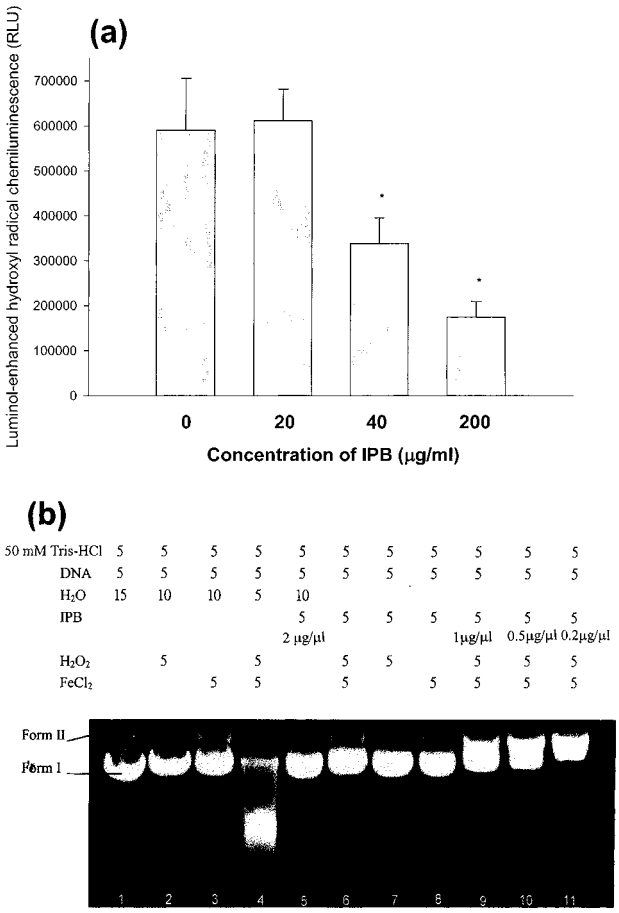

Figure 2. IPB extract was a scavenger of hydroxyl radicals. Reaction by H_2O_2 and $FeCl_2$ was used to generate hydroxyl radicals. (a) The peaks of hydroxyl radical induced luminol chemiluminescence were measured. Results were expressed as relative light units (RLU) (mean ± SEM) ($n = 4$). The asterisk (∗) denotes a marked difference between the control and the experimental groups. (b) Hydroxyl radical induced breaks of PUC18 plasmid DNA and its prevention by IPB extract. H_2O_2 and $FeCl_2$ (Fenton reaction) were used to generate hydroxyl radicals, leading to DNA breaks on PUC18 DNA. Form I (supercoil), form II (open circular) DNA bands, and DNA smear were visualized by UV box and photographed. The result was one representative experiment: lane 1, untreated PUC18 DNA; lane 2, DNA with 6% H_2O_2; lane 3, DNA with 100 μM $FeCl_2$; lane 4, DNA breaks induced by H_2O_2 with $FeCl_2$; lane 5, DNA incubated with IPB extract alone; lanes 6, 9, 10, 11, DNA breaks in the presence of 400, 200, 100, and 40 μg/mL (final concentration) of IPB, respectively.

luminol chemiluminescence was obviously inhibited by IPB extract (>40 μg/mL) (**Figure 2a**). The IC_{50} of IPB extract was about 73 μg/mL. Consistently, IPB extract also prevented the hydroxyl radical induced DNA breaks on PUC18 plasmid DNA. As shown in **Figure 2b**, most of the purchased PUC18 plasmid DNA was in supercoil form (form I) and only a small portion of plasmid DNA was in open circular form (form II) (**Figure 2b**, lane 1). IPB extract, H_2O_2, and $FeCl_2$ alone showed little DNA breaking capacity (**Figure 2b**, lanes 2, 3, and 5). When incubated with H_2O_2 and $FeCl_2$, evident DNA breaks were noted. Most of the plasmid DNA has been broken into small pieces as revealed by the presence of DNA smear (**Figure 2b**, lane 4). In the presence of IPB extract (>40 μg/mL), no DNA smear was noted, indicating the preventive effect of IPB components (**Figure 2b**, lanes 6, 9, 10, and 11). At final concentrations higher than 400 μg/mL, the hydroxyl radical induced DNA breaks were completely protected.

Antiplatelet Effect of IPB Components. Since ROS have been shown to be crucial for platelet aggregation (*24, 25*), we therefore investigated whether IPB components may modulate platelet aggregation. As shown in **Figure 3a**, IPB extract inhibited the AA-induced platelet aggregation by 47% and 82%, respectively, at concentrations of 200 and 250 μg/mL. The IC_{50} of IPB against platelet aggregation was about 207 μg/mL. IPB extract also inhibited the collagen-induced platelet aggregation in a dose-dependent fashion, with an IC_{50} concentration of 335 μg/mL (**Figure 3b**). In contrast, IPB extract showed little effect on thrombin-induced platelet aggregation (data not shown). IPB extract may inhibit the AA-induced platelet aggregation in a dose-dependent manner.

Effect of IPB Extract on TXB2 Production of Platelets. TXB_2 production is crucial for regulation of platelet aggregation. We further elucidated whether IPB extract modulates the AA-, collagen-, and thrombin-induced TXB_2 production by platelets. As shown in **Figure 4a**, IPB extract markedly suppressed the AA-induced TXB_2 production at concentrations higher than 100 μg/mL. Collagen-induced TXB_2 production was also inhibited by IPB extract at concentrations higher than 100 μg/mL (**Figure 4b**). Interestingly, IPB extract was a potent inhibitor of

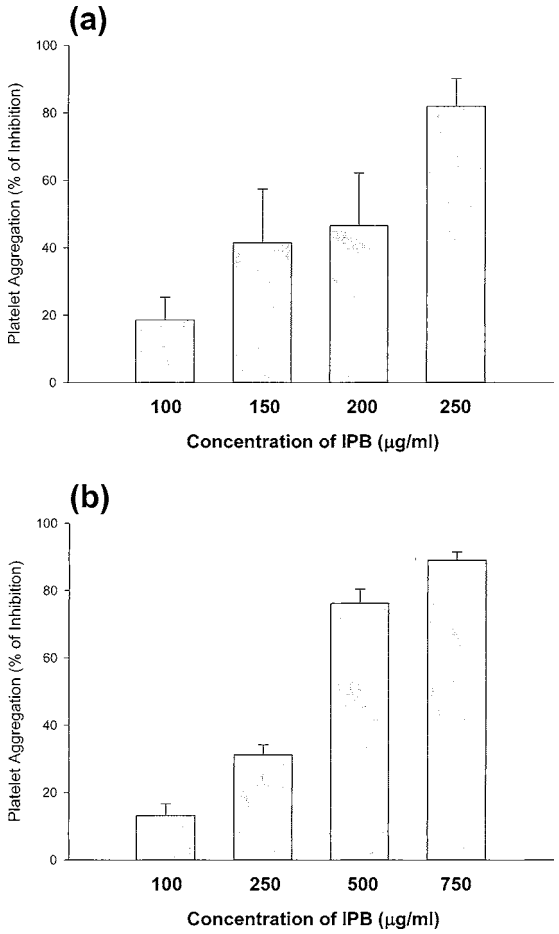

Figure 3. Effect of IPB extract on platelet aggregation induced by AA and collagen: (a) inhibition of AA-induced platelet aggregation by IPB extract; (b) inhibition of collagen-induced platelet aggregation by IPB extract. Results were expressed as percentage of inhibition (mean ± SE) ($n = 4$).

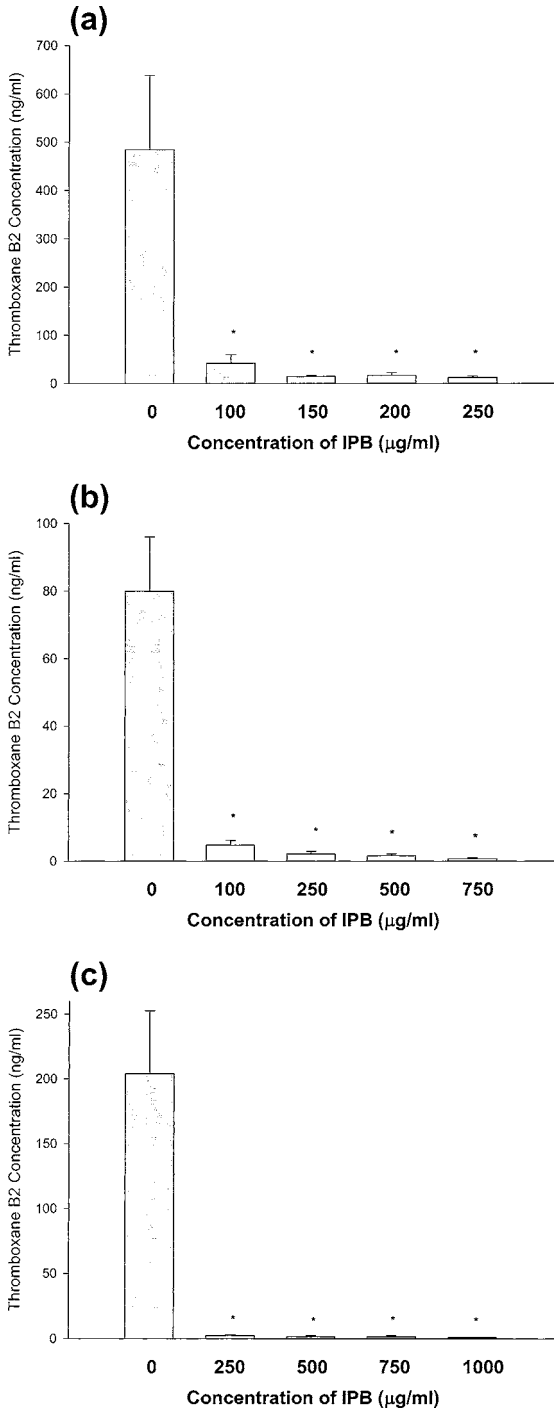

Figure 4. Effect of IPB extract on (a) AA-, (b) collagen-, and (c) thrombin-induced thromboxane B_2 production of platelets. Results were expressed as mean ± SE (ng/mL) ($n = 4$). The asterisk (∗) denotes a marked difference between the control and the experimental groups.

thrombin-induced TXB_2 production. Even at a concentration of 250 μg/mL, thrombin-induced TXB_2 production was nearly completely inhibited (**Figure 4c**), whereas IPB extract showed little effect on platelet aggregation even at a concentration of 1000 μg/mL (data not shown).

DISCUSSION

Inflorescence *Piper betle* (IPB) was a major BQ component in Taiwan and Papua New Guinea (*2*, *5*). Chewing BQ containing IPB has been shown to elevate the incidence of oral cancer (*2*) perhaps because of its content of safrole, a potential hepatocarcinogen. Higher concentrations (>1 mg/mL) of aqueous IPB extract may induce DNA breaks of OMF (*7*) and lead to cytotoxicity on oral keratinocytes (*8*). However, IPB extract (<800 μg/mL) induces no evident unscheduled DNA synthesis on cultured human oral keratinocytes (*8*). In the present study, aqueous IPB extract exerts a scavenging effect on H_2O_2, superoxide radicals, and hydroxyl radicals, with similar potency. But it seems that IPB protects DNA from breaking but not from opening because form II plasmid DNA seems abundant when 40−200 μg/mL of IPB was used (**Figure 2b**). This may be due to Tris-borate−EDTA buffer used for agarose gel electrophoresis, which shows less resolving capacity to discriminate form II and form III DNA. IPB scavenges the ROS at concentrations ranging from 20 to 100 μg/mL, a concentration showing little cytotoxicity on oral mucosal cells (*7*, *8*). This indicated that aqueous IPB components are potential antioxidants in the BQ. Although inclusion of IPB in the BQ may promote oral cancer risk (*2*), this may be due to exposure of higher IPB concentrations or attributed to its content of safrole. Since ROS production is related to the pathogenesis in a number of diseases, including cancer, arteriosclerosis, ischemia reperfusion injury, tissue fibrosis, etc. (*34*, *35*), the aqueous fraction of IPB may have a potential preventive effect toward these disorders.

Hypercoagulation has been suggested to be a major contributing factor of OSF (*16*−*19*). Platelet aggregation is associated with the production of TXB_2 and ROS, such as H_2O_2 and

superoxide radicals (*24*, *25*). H_2O_2 produced by platelets can be converted to highly reactive hydroxyl radicals via the Fenton reaction (*24*, *25*). Inhibition of this response by catalase, which removes H_2O_2, or by desferrioxamine, which binds catalytic metals and renders them inactive, or by hydroxyl radical scavengers deoxyribose and mannitol prevented the collagen-induced platelet aggregation (*24*, *25*). Recently, we have found that AN components induce platelet aggregation and thromboxane B2 production (*27*), a potential factor for inducing hypercoagulation in OSF patients. We therefore further tested whether the aqueous IPB fraction was the responsible factor for hypercoagulation in the BQ. Interestingly, the IPB extract inhibited the AA- and collagen-induced platelet aggregation. This is generally in accordance with the statement that ROS may stimulate platelet aggregation, whereas the IPB extract as a potent ROS scavenger may inhibit platelet aggregation. The antiplatelet effect of IPB occurred at concentrations of 207−335 μg/mL, markedly higher than its antioxidant property, indicating the presence of additional mechanisms responsible for the antiplatelet effect of IPB. In contrast, the IPB extract showed little effect on thrombin-induced platelet aggregation possibly because of the presence of multiple pathways for the thrombin-induced platelet activation. These results indicate that consumption of IPB by BQ chewers may potentially inhibit platelet aggregation and prevent thrombus formation. Further epidemiological studies or animal experiments are needed to clarify the antiplatelet effects of IPB and its clinical relevance.

TXB_2, a cyclooxygenase metabolite, is also a crucial mediator for AA-, collagen-, and thrombin-induced platelet aggregation (*24*, *25*). We therefore further delineate whether inhibition of AA- and collagen-induced platelet aggregation by IPB extract is associated with TXB_2 production. Accordingly, IPB extract evidently inhibited the AA- and collagen-induced TXB_2 production to near 100% at concentrations higher than 100 μg/mL. Consistently, collagen- and AA-induced platelet aggregation has been found to be mediated by ROS production and TXB_2 production (*24*, *25*). Interestingly, IPB (>250 μg/mL) completely inhibited the thrombin-induced TXB_2 production, whereas IPB exerted no marked suppression on platelet aggregation. This reveals that thrombin may induce platelet aggregation via cyclooxygenase-dependent and -independent pathways.

Taken together, IPB may inhibit TXB_2 production and platelet aggregation. This can be partly due to scavenging by IPB of ROS, which is a mediator of platelet aggregation (*24*−*26*). Whether inhibition of platelet aggregation by IPB components was mediated by direct inhibition of cyclooxygenase activity or via inducing cAMP and cGMP levels should be further addressed. There is much evidence indicating the usage of antioxidants to retard the progression of arteriosclerosis and to ameliorate systemic disorders (*34*, *35*). However, IPB is compartmentalized in the oral cavity. Whether consumption of aqueous IPB components during BQ chewing may prevent ROS-related oral mucosal damage and potentially prevent thrombosis in vivo needs to be further supported by additional data showing the partition of the IPB components to the blood or the effect of IPB on the rate of healing of mouth sores. More epidemiological studies will be helpful to assess the properties of IPB components and their clinical relevance to the health status of BQ chewers.

ABBREVIATIONS USED

AA, arachidonic acid; AN, areca nut; BQ, betel quid; BSA, bovine serum albumin; IPB, inflorescence *Piper betle*; IC_{50}, 50% inhibitory concentration; OSF, oral submucous fibrosis; ROS, reactive oxygen species; TXB2, thromboxane B2; XO, xanthine oxidase.

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