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REVIEW ARTICLE

Plant extracts inhibit ADP-induced platelet activation in humans: their potential therapeutic role as ADP antagonists

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Abstract Adenosine diphosphate (ADP) plays a pivotal role in platelet activation. Platelet hyperactivity is associated with vascular disease and also has a key role in haemostasis and thrombosis. ADP activates platelets through three purinoceptor subtypes, the G_q-coupled $P2Y_1$ receptor, G_i-coupled $P2Y_{12}$ receptor and $P2X_1$ ligand-gated cation channel. Platelet ADP purinergic receptors are therefore suitable targets for antiplatelet drugs. Thienopyridines such as clopidogrel and ticlopidine, as well as other ADP receptor antagonists like prasugrel, ticagrelor, cangrelor and elinogrel have demonstrated clinical benefits via the inhibition of the selective purinergic ADP receptor, $P2Y_{12}$. However, they still have limitations in their mode of action and efficacy, like increased risk of bleeding. Thus, the ongoing pursuit to develop newer and more effective antiplatelet agents continues. There is a growing interest in the purinergic antiplatelet properties exhibited by plant extracts. This article considers the following: pomolic acid isolated from *Licania pittieri*, brazilin isolated from the heartwood of *Caesalpinia sappan* L, phylligenin isolated from the twigs of *Muraltia vulpina*, bark oil of *Gonystylus velutinus*, seed and bark extracts from *Aesculus hippocastanum L*. and red wine phenolics and catechins isolated from green tea. Moreover, the method used to investigate platelet purinergic receptors should be considered, since using a more sensitive, high-resolution platelet sizer can sometimes detect platelet variations when the light transmission method was not able to do so. The exact mechanisms by which these plant extracts work need further investigation. They all however inhibit ADP-induced activation in human platelets. This could explain, at least in part, the protective effect of plant extracts as antiplatelet agents.

Keywords Adenosine diphosphate (ADP) · Platelet · Plant extract · Antiplatelet drugs · Vascular disease

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Adenosine-5′-diphosphate (ADP) plays a crucial role in platelet activation [1, 2]. In addition, platelet hyperactivity is known to be associated with vascular disease and has a key role in haemostasis and thrombosis [3, 4]. ADP is considered a weak platelet agonist, since it induces platelet shape change (PSC, an early phase of platelet activation) and reversible aggregation. Nevertheless, when ADP is secreted from its storage pools in platelet dense granules, it amplifies platelet responses induced by other platelet agonists [5, 6]. It is well established that ADP activates platelets through three purinoceptor subtypes, namely $P2Y_1$, $P2Y_{12}$ and $P2X_1$ [1, 7, 8]. The $P2Y_1$ purinergic receptor is coupled to G_q, which is involved in PSC and reversible aggregation, due to a transient increase in cytoplasmic Ca^{2+}. The $P2Y_{12}$ purinergic receptor is coupled to G_i which mediates the inhibition of adenylyl cyclase and amplifies the platelet aggregation response [6]. The $P2X_1$ ligand-gated cation channel is activated by adenosine triphosphate (ATP) and is also involved in PSC [7, 9]. The simultaneous activation of both G_q and G_i pathways by ADP is necessary to elicit normal platelet aggregation [6]. Moreover, the limited distribution of the $P2Y_{12}$ receptor, primarily expressed on platelets, makes it an especially attractive pharmacologic target for antithrombotic drugs [10–12].

Over the last several decades, the thienopyridine family of $P2Y_{12}$ receptor antagonists has provided the scientific data confirming the clinical benefit of selective purinergic ADP receptor, $P2Y_{12}$, inhibition [1, 13]. In fact, the $P2Y_{12}$ receptor is the target for thienopyridine compounds like ticlopidine and clopidogrel which have been used in the treatment of patients at risk for major adverse cardiovascular event (MACE) [14–16]. Ticlopidine was first synthesized in 1974 [17], and clopidogrel was its successor [18]. Although thienopyridines have demonstrable antithrombotic efficacy, they still have limitations in their mode of action and efficacy. For example, both ticlopidine and clopidogrel are pro-drugs that need to be metabolised to its active metabolite. Also, antiplatelet agents like aspirin may cause gastric erosions and gastric ulcers, and patients may present with anaemia and gastric haemorrhage [19, 20]. These limitations have led to the search for other therapeutic $P2Y_{12}$ antagonists.

Prasugrel, is a new FDA-approved ADP receptor antagonist. The metabolism of prasugrel to its active metabolite is much more efficient and consistent than that of clopidogrel; hence, it has a faster onset of action [21]. Prasugrel was also shown to be superior to clopidogrel in inhibiting $P2Y_{12}$-dependent platelet function [21]. This accounts for the lower incidence of MACE, but the disadvantage is that it also increased bleeding among acute coronary syndrome (ACS) patients who required percutaneous coronary intervention (PCI) [21]. Furthermore, the results of the TRITON TIMI-38 trial showed that prasugrel was a more potent antiplatelet agent than clopidogrel when used only in high-risk patients or for a short period, while clopidogrel treatment is preferred in the remaining patient groups [22]. It is now known that clopidogrel treatment is ineffective in about 30 % of patients, thus other antithrombotic treatments have also been FDA-approved. One such drug is ticagrelor (previously known as AZD6140), a direct $P2Y_{12}$ antagonist, which was shown to induce an appreciable inhibition of platelet function when given to patients who were poor responders to clopidogrel [12, 13, 23]. Ticagrelor has a rapid onset of actions and achieves a maximal inhibition of platelet function in about 2 h with much less inter-individual variability when compared to clopidogrel [23, 24]. Moreover, the DISPERSE study showed that overall, ticagrelor was superior to clopidogrel with regard to antiplatelet efficacy [25]. However, although ticagrelor shows some advantages over clopidogrel, the DISPERSE study also demonstrates that the incidence of bleeding (from 29 to 52 %) events was dose-related in ticagrelor-treated patients (50 mg b.i.d., 100 mg b.i.d., 200 mg b.i.d., or 400 mg q.d.), as compared to 32 % in clopidogrel-treated patients (75 mg once daily) [25].

Cangrelor (previously known as ARC69931[MX] is another direct and reversible $P2Y_{12}$ antagonist, which can be given intravenously. It is immediately active since it does not have to be converted to an active metabolite [12, 13, 19]. Pharmacological studies showed that cangrelor inhibited ADP-induced aggregation in washed human platelets (IC_{50}= 9.4 with ADP 30 μM) [13, 26]. Moreover, Jagroop et al. [8] demonstrated that ARC69931[MX] inhibited ADP-induced PSC in a time-dependent manner in human platelets. This was the first group to actually quantify PSC inhibition by ARC69931[MX] or any other $P2Y_{12}$ blocker. It is possible that ARC69931[MX] affects PSC due to its immediate effect and its high affinity for the $P2Y_{12}$ receptors. Moreover, the findings of Jagroop et al. [8] suggest that the clinical effectiveness of $P2Y_{12}$ antagonists may be linked to the fact that they inhibit both the early and late phases of platelet activation.

In addition to prasugrel, ticagrelor and cangrelor, the fourth $P2Y_{12}$ inhibitor which has been investigated is elinogrel, which has direct and reversible actions. Elinogrel is the first $P2Y_{12}$ antagonist to be available in both intravenous and oral formulations to enable a smooth transition from short- to long-term treatment [27, 28]. These four newly developed $P2Y_{12}$ inhibitors offer a more potent, more predictable and faster onset of action than clopidogrel [12–14]. However, their risk of major and even fatal bleeding needs to be considered, especially in elderly patients with comorbidities [29]. It should also be considered that in clinical trials, to assess the effectiveness of $P2Y_{12}$ inhibitors, patients perceived to be at a higher risk of bleeding are usually excluded [29]. Thus, the ongoing pursuit to develop newer and more effective antiplatelet agents continues. As a result, there is a growing interest in the properties of plant extracts, and their antiplatelet effect on purinergic receptors. There is also a focus on the development of safe and effective antithrombotic drugs from plant-derived extracts. Furthermore, the importance of ADP in haemostasis and thrombosis greatly underscores the significance of understanding the function of platelet purinergic receptors with regard to drug development from plants.

The leaves of *Licania pittieri* plants (belonging to the Chrysobalanaceae family) are considered medicinal in South America and have historically been used to treat diseases such as diabetes and hypertension [30]. In an interesting study, Alvarado-Castillo et al. [31] evaluated pomolic acid (PA), a triterpenoid isolated from *L. pittieri*, as a competitive antagonist of ADP-induced aggregation of human platelets. The authors used light transmission aggregometry to assess the ability of PA (150 nM) to inhibit PSC induced by a selective $P2Y_1$ receptor agonist, MRS 2365 (25 μM). They found that PA was not an antagonist of the $P2Y_1$ receptor [31]. It would however be interesting to use another technique like a more sensitive, high-resolution platelet sizer to assess if there is indeed any $P2Y_1$ purinergic effect associated with the plant isolate PA on PSC in human platelets [8]. There was also evidence in this study showing that PA (25–100 nM) acts as a

competitive antagonist of ADP-induced (10^{-6}–10^{-3} M) platelet aggregation in platelet-rich plasma (PRP). Thus, the authors concluded that PA may be a potent competitive antagonist of the $P2Y_{12}$ receptor, a pharmacological characteristic shared by the new generation of $P2Y_{12}$ receptor antagonists that are currently being investigated in clinical trials for their antiplatelet activity [31]. These results also provide evidence to suggest that purinergic receptor blockers isolated from the plants *L. pittieri* may be beneficial for their use as antiplatelet agents in humans [31]. However, further investigations are warranted to provide more data in support of this evidence.

With regard to using a more sensitive platelet sizer with a high-resolution channelyzer, I have shown that a $P2Y_1$ receptor antagonist MRS 2179 (1.06–10.25 μmol/l) blocked ADP-induced (0.2–0.4 μM) PSC in human platelets by up to 100 % (median IC_{50} 3.16 μmol/l) [8]. To the author's knowledge, this was the first study to demonstrate an IC_{50} for a $P2Y_1$ receptor blocker using the human PSC phenomenon. These results are in line with the findings by Alvarado-Castillo et al. [31] who showed that MRS 2365-induced (25 μM, a $P2Y_1$ agonist) PSC was completely blocked by MRS 2500 (100 nM, a $P2Y_1$ antagonist) using human platelets in PRP. It was also demonstrated that the $P2Y_{12}$ receptor antagonist AR-C69931MX (10 μM) significantly inhibited (p =0.01 to 0.001) ADP-induced (0.2–0.4 μM) PSC [8]. The inhibition was of the order of 2.9 %, which provides support that this 'relatively small' change was detectable because of the sensitivity of the high-resolution (0.07 fl) channelyzer used. This allows lower concentrations of agonists and antagonists, and also has greater sensitivity to detect variations in PSC [32–36]. Overall, the study by Jagroop et al. [8] suggested that both the $P2Y_1$ and $P2Y_{12}$ ADP receptors play a role in controlling PSC in humans [8]. It is possible that Alvarado-Castillo et al. [31] using the less sensitive optical technique to assess PSC could not detect such a relatively small $P2Y_1$-dependent PSC inhibition.

In a noteworthy study, it is reported that PA proved to be a potent inhibitor of epinephrine-induced human platelet aggregation (IC_{50} 20 nM) [30]. These were the first results to show the anti-aggregating effects of PA, suggesting its potential role in cardiovascular therapy. It is also possible that non-ADP mechanisms are involved. On the other hand, it can be suggested that PA could be acting on any ADP released by epinephrine. The mechanisms involved are perhaps complex because PA also acts as a vasorelaxant on norepinephrine-induced contraction in rat aortic rings. This effect was dependent on intact endothelium [37]. Furthermore, Barradas et al. [33] could not demonstrate an increase in median platelet volume in human platelets after adding epinephrine in vitro. Nevertheless, epinephrine enhanced serotonin-induced PSC [38]. Therefore, it may be possible to elucidate the effect of PA on epinephrine-induced platelet activation, assuming that PA does not influence serotonin-induced PSC. Estrada et al. [30] also reported that PA does not affect aggregation induced by several agonists (e.g. platelet-activating factor, collagen, U46619 (a thromboxane A_2 analogue) and arachidonic acid). Furthermore, Barradas et al. [33] showed that all these agonists can induce PSC. It is very likely that PSC induced by the latter agonists is enhanced by epinephrine. This would help define the specific mode of action of PA on PSC. Overall, the findings of Jagroop et al. [8] suggest that both the $P2Y_1$ and $P2Y_{12}$ ADP receptors play a role in controlling PSC in humans. It would be interesting to assess the effect of PA on PSC using a high-resolution channelyzer. Further investigations are needed to understand the molecular mechanisms by which PA affects PSC as well as platelet aggregation.

Brazilin is isolated from the heartwood of *Caesalpinia sappan* L. (sappan wood, Leguminosae) [39] and for many years has been used as a traditional oriental or folk medicine. Brazilin has been involved in the treatment for sprains, convulsions and diabetic complications, and was used to improve blood circulation. In addition, brazilin was supposed to have antithrombotic activities, and it is interesting to note that sappan wood has been used as a remedy for thrombosis in the oriental traditional medicine [40]. Chang et al. [39] are the first to show the effect of brazilin in human platelet activation. They demonstrated that brazilin (1 to 50 μM) potentiated collagen-induced (0.1 μg/ml) platelet aggregation in washed human platelets, using the turbidimetric method for assessing aggregation [39]. Moreover, higher concentrations of brazilin (20 to 50 μM) directly triggered platelet aggregation in a concentration-dependant manner. They also demonstrated that ATP (50 μM, an ADP antagonist) inhibited platelet aggregation stimulated by ADP (20 μM) more effectively than that stimulated by brazilin (50 μM). These results indicated that brazilin-induced platelet aggregation was not mediated even partially by ADP receptors, but that brazilin acts as a collagen receptor agonist [39]. In contrast, another study reported that brazilin (10^{-5}–10^{-4} M) inhibited thrombin- (0.5 U/ml), collagen- (1 mg/ml), and ADP-induced (2 μM) platelet aggregation in washed rat platelets [40]. The authors suggested that this discrepancy might be due to species-specific characteristics of platelets. If this is the case, then further investigations are needed. Chang et al. [39] stated that the plant-based natural product brazilin at relatively low concentrations (1–10 μM) has the potential to act as therapeutic antithrombotic agent for targeting collagen (0.1 μM) receptors. They also suggested that brazilin may also be a useful tool for the study of detailed mechanisms in collagen receptor-mediated platelet activation. Thus, the literature shows that brazilin which is of natural origin has the capacity to act on various platelet receptors. Undoubtedly, it is also of interest to determine if brazilin does indeed affect the P_2 purinergic receptors in human platelets, as it can

prospectively lead to the development of new 'natural' ADP receptor antagonists.

Mitrephora plants (of the Annonaceae family), located in Borneo, Philippines, north China, the west of India, and the south-east of Australia, have been investigated for their chemical components [41, 42]. In an exciting study, Moharam et al. [41] examined the biological activity of the methanol extract of the twigs of *Muraltia vulpina*. Phylligenin is one of the five compounds isolated from the twigs of *M. vulpina* and was shown to inhibit arachidonic acid- (AA, 0.5 mM) and ADP-induced (10 μM) aggregation in human platelets (in vitro) in a dose-dependent manner. Phylligenin demonstrated >60 % inhibition of ADP-induced aggregation, with an IC_{50} of 121.8 μM [41]. These results are of importance since the actions of endogenous agonists like AA and ADP can induce platelet aggregation in several cardiovascular disease states. Moreover, phylligenin has the potential to act as an antiplatelet agent possibly through purinergic receptors to inhibit platelet activation. The exact mechanism of the action of phylligenin is still unclear, although it has similar effects to aspirin, i.e. it inhibits thromboxane A_2 formation [41, 42]. Furthermore, aspirin, a potent cyclooxygenase inhibitor, was used as a positive control for phylligenin and demonstrated a lower IC_{50} (24.8 μM) [41]. With the common knowledge that aspirin may cause gastric erosions and gastric ulcers, and that patients may suffer with anaemia and gastric haemorrhage, the search for more potent yet safe antiplatelet agents continues. In addition, since phylligenin has demonstrated ADP-induced platelet inhibition, further investigations should be carried out into the effects these oils (isolated from the twigs of *M. vulpina*) may have on ADP purinergic receptors.

Additional evidence of antiplatelet activity via the ADP purinergic receptors with 'plant-derived' extracts can be seen with essential oils of five *Goniothalamus* species found in tropical Southeast Asia, throughout Indochina and Malaysia [42]. Different parts of these plants are used in traditional medicine to treat conditions such as asthma, rheumatism, fever, malaria, cholera and stomach aches. The in vitro inhibitory effect of various oils from *Goniothalamus* species was observed for the first time, by Moharam et al. [42]. The bark oil, identified as *Gonystylus velutinus* (100 μg/ml, distilled from *Goniothalamus*) showed strong inhibition (51 %) of human platelet aggregation in whole blood, induced by ADP (10 μM, with an IC_{50} of 87.7 μg/ml). There was also inhibition (50 %) of AA-induced platelet aggregation (with an IC_{50} of 93.6 μg/ml). Aspirin, which was used as the positive control in the study, was found to be a more potent cyclooxygenase inhibitor, since its IC_{50} was much lower (4.5 μg/ml or 24.8 μM) than those of the oils investigated [42]. Furthermore, it is suggested that the antiplatelet activity of the *Goniothalamus* oils may not be due solely to any individual component but could be due to the synergistic effect of a combination of its compounds. This theory is supported by another experimental model (in mice) that shows for the first time that *Lavandula hybrida* oil (also known as lavender oil) has antiplatelet/antithrombotic properties which could also be due to a synergistic effect of its components [43]. Further studies are required to investigate the overall antiplatelet, synergistic effects caused by the combination of components making up the essential oils of the *Goniothalamus* species. It should however be noted that the collection of plant material from the *Goniothalamus* species is fresh from the forests and that its preparation and isolation into essential oils are time consuming and complicated, involving various stages of gas chromatography (GC) and gas chromatography–mass spectrometry (MS) [42]. Thus, it is worth considering that the quality and effectiveness of various batches of essential oils may not be uniform between different research groups and may thus influence results. There is however the 'Wiley GC–MS Library' database of some existing plant components which can be accessed by researchers to identify and compare relative retention indices of some isolated oils [42]. The information provided in the literature shows that the *Goniothalamus* species of trees and shrubs may prove to be a beneficial source for the therapeutic development of ADP receptor antagonists or other antiplatelet agents.

The extract from seeds and bark of the horse chestnut tree, *Aesculus hippocastanum L*. Hippocastanaceae, has been used as herbal medicine in Europe against chronic venous insufficiency, i.e. varicose veins accompanied with pain, oedema pruritus and a sense of heaviness [44]. Clinical studies have shown that oral horse chestnut extract was as effective as compression stockings in chronic venous insufficiency [45, 46]. The antithrombotic effect of the extract from the horse chestnut tree was first observed more than 40 years ago, but no thorough investigations have since been carried out [47]. Nonetheless, in 2010, Felixsson et al. investigated the pharmacological effects of horse chestnut extract [44]. It was shown that the mechanism of action was via the ADP receptors. That is to say that the extract from the horse chestnut tree (5 μl) inhibited ADP-induced (10^{-6} or 10^{-5} M) platelet aggregation in human platelet-rich plasma [44]. Moreover, when ketanserin (a $5HT_{2A}$ receptor antagonist) was added to the horse chestnut extract-treated platelets, aggregation was further reduced. The authors suggest that platelet inhibition by horse chestnut extract appears to be the sum of both inhibitory (due to flavonoids) and stimulatory (due to $5HT_{2A}$ receptor-mediated) effects [44]. It was clear from this study that horse chestnut extract had inhibitory action on ADP-induced human platelet aggregation. But, this antithrombotic effect also needs to be studied together with possible drug interactions like other antiplatelet drugs, commonly used in vascular disease. Thus, the clinical use of horse chestnut extract as an antiplatelet drug or ADP receptor antagonist remains to be further investigated.

Moderate and prolonged consumption of red wine is associated with a decrease in cardiovascular morbidity and mortality [48]. This may be linked to a lowering of the risk for stroke and sudden death, which have collectively come to be known as 'The French Paradox', highlighting the low incidence of coronary heart disease in a country known for its high-fat intake, little exercise and widespread cigarette smoking [49]. This phenomenon could be due to the presence of polyphenolic compounds in red wine. In an interesting study by de Lange et al. [49], the ingredients found in red wine were shown to have an inhibitory effect on ADP-induced (5 μM) platelet aggregation. This finding is consistent with other studies that suggest that wine and its polyphenolics, in particular, could significantly inhibit platelet aggregation and that this could explain, at least in part, the protective effect of red wine against atherosclerosis and coronary heart disease [50, 51]. Red wine contains polyphenols that belong to a complex group of compounds that determine its characteristics and qualities. Flavonoids and non-flavonoids are the two main groups of phenols. In grapes, the polyphenols' function is to scavenge free radicals and thus prevent the fruit from decay [52]. The inhibition of platelet function may provide some explanation for the cardio protective effects associated with red wine. Supporting studies have also shown that the consumption of polyphenolic-rich beverages and the addition of polyphenols to platelets in vitro are associated with the inhibition of platelet aggregation [53, 54]. Moreover, substantial progress in understanding the inhibition of ADP-induced platelet activation, as demonstrated by polyphenols, can play a key role in the development of novel antiplatelet drugs that target P2 receptors [55]. There has been great interest in exploiting cardiovascular purine P2 receptors as therapeutic targets [56–58].

There is strong support that cigarette smoking is associated with cardiovascular events like myocardial infarction, peripheral vascular disease, stroke and even sudden death [59]. Hence, following on from The French Paradox, attention is now drawn to the 'The Asian Paradox', where, despite the high consumption of tobacco, a low incidence of atherosclerosis and lung cancer per capita is observed, particularly in Asia and Japan [60]. It has been suggested that this may be a result of the high consumption of green tea in Asia. Furthermore, most benefits occurred in regions where approximately 1.2 l of green tea is consumed every day [61]. Green tea is derived from the leaves of the plants *Camellia sinensis* and contains nearly 4,000 bioactive chemical compounds, one-third of which are polyphenols. The major catechin in green tea is (−)-epigallocatechin gallate (EGCG) and accounts for 10 % of the total weight. It has been shown that green tea catechins (GTC, 0.01–1 mg/ml) inhibited ADP- (20 μM) as well as collagen- (50 μg/ml), thrombin- (0.5 U/ml) and calcium ionophore A23187-induced (1 μM), aggregation of human platelets in vitro [62]. These results suggest that GTC and EGCG have antithrombotic activities and the modes of action may be due to their antiplatelet activities, which may account for the beneficial effect of green tea in cardiovascular disease [60]. Clearly, the role of platelet ADP purinergic receptor inhibition needs to be further examined. Therefore, a rigorous assessment of the effects of GTC on purinergic receptors in well-controlled human trials will be required for a better understanding of the effects of green tea in cardiovascular health [63].

In summary, this article gives a brief overview of some current thienopyridines and other $P2Y_{12}$ receptor antagonists, highlighting their main disadvantages. Their delayed onset of actions and the increased risk for bleeding among this class of drugs are still of major concern [64]. Hence, these limitations have led to the search for new and improved ADP receptor antagonists. This review outlines a range of plant extracts and their inhibitory effect on ADP-induced platelet activation. The data from these papers indicate that plant extracts may potentially lead to the therapeutic development of 'new' ADP receptor antagonists. But, in some cases, the exact mechanisms of action are still unclear. These new plant extracts as drug candidates will have to prove beneficial in comparison to the currently FDA-approved ADP antagonists. Basically, they will have to display faster onset and offset of action, better efficacy and less prolongation of bleeding time. Thus, further investigations and toxicity studies in well controlled human trials are needed.

Conflict of interest The author reports no conflict of interest.

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