

# Synergistic Effects of Natural Product Combinations in Protecting the Endothelium Against Cardiovascular Risk Factors

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## Abstract

Endothelial dysfunction is an early hallmark of cardiovascular diseases (CVDs). Monotherapies are limited due to the complex, multifactorial pathways. The multi-component and multi-targeted approach of natural products have the potential to manage CVDs.

This review aims to provide a comprehensive insight into the synergistic mechanism of natural product combinations in protecting the endothelium against various cardiovascular risk factors.

Databases (PubMed, MEDLINE and EMBASE) and Google Scholar were searched, and studies in English published between January 2000 and February 2022 were collated. Clinical and pre-clinical studies of natural product combinations with or without pharmaceutical medicines, compared with monotherapy and/or proposing the underlying mechanism in protecting endothelial function, were included.

Four clinical studies demonstrated that natural product combinations or natural product-pharmaceutical combinations improved endothelial function. This was associated with multi-targeted effects or improved absorption of the active substances in the body. Seventeen preclinical studies showed that natural product combinations produced synergistic (demonstrated by combination index or Bliss independence model) or enhanced effects in protecting the endothelium against hyperlipidemia, hypertension, diabetes mellitus, platelet activation, oxidative stress and hyperhomocysteinemia. The molecular targets included reactive oxygen species, Nrf2-HO-1, p38MAPK, PI3K/Akt and NF-κB.

Thus, the current available evidence of natural product combinations in targeting endothelial dysfunction is predominantly from pre-clinical studies. These have demonstrated synergistic/enhanced pharmacological activities and proposed associated mechanisms. However, evidence from larger, well-designed clinical trials remains weak. More cohesion is required between preclinical and clinical data to support natural product combinations in preventing or slowing the progression of CVDs.

## Keywords

natural products, synergy, endothelial dysfunction, combination index, cardiovascular diseases

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## Background

Cardiovascular diseases (CVDs) are the leading cause of mortality and a major contributor to the disease burden worldwide.<sup>1</sup> According to the latest World Health Organization report, there are over 523 million people with CVDs globally,<sup>1</sup> with an estimated 17.9 million deaths each year. Endothelial dysfunction has been recognized as an early but reversible marker of CVDs. Reducing CVDs risk factors such as hyperlipidemia, hyperglycemia, hypertension, smoking and alcohol consumption

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have been suggested to protect and improve endothelial function and slow the progress of CVDs.<sup>2</sup>

The endothelium is a monolayer of the inner wall of capillaries, arteries and veins that mediates the blood supply<sup>2</sup> through vascular constriction and relaxation.<sup>3</sup> It plays a key role in angiogenesis, inflammation and immune responses under both normal and pathological conditions.<sup>4</sup> Endothelial dysfunction is the earliest pathological event in CVDs, and is characterized by a diminished level of endothelial nitric oxide synthase (eNOS) under the insult of aforementioned CVDs risk factors.<sup>5</sup> Particularly, oxidative stress, which is mediated by excessive reactive oxygen species (ROS), directly quenches eNOS and results in impaired endothelium-dependent vasorelaxation.<sup>5-7</sup> Endothelial function is an important pharmacological target for the early prevention of CVDs and can be used to triage CVD management and to improve outcomes.<sup>8</sup>

Current pharmacological interventions such as nitrites, calcium channel blockers and statins are used to improve endothelial function by directly widening the blood vessels or inhibiting specific CVDs risk factors with a mono-target action. Natural products such as herbal medicines, herbal supplements, nutraceuticals, foods and animal-derived ingredients serve as an abundant source of drug candidates for development. In particular, the multi-component and multi-targeted approach of these phytotherapies provide an attractive alternative or adjunct therapy to single-entity, single-targeted pharmaceuticals for managing complex diseases such as CVDs.<sup>9</sup> The synergistic interactions are perceived to play a pivotal role in their pharmacological actions.<sup>10</sup> Synergy describes a positive interaction among two or more ingredients exerting a higher combinative effect compared to the sum of their individual effects, leading to an enhanced pharmacological outcome and/or reduced toxicity. This concept underpins the philosophy of many traditional medical systems, such as traditional Chinese medicine, whereby multiple herbs are combined in a formula to facilitate the positive interactions of key ingredients which, in turn, enhances the pharmacological effects.<sup>11</sup>

Numerous mathematical models have been developed to determine and quantify synergism in herbal medicines. Among them, combination index (CI) is predominantly used to determine the synergistic effect of two or more agents acting on a specific pharmacological target (ie receptor, gene, protein) compared to the action of each individual agent.<sup>12</sup> In addition, systems biology approaches such as network pharmacology have been used for omics data integration and developing multi-target drugs.<sup>13</sup> Based on the existing literature and databases, network pharmacology aligns bioactive molecules to the targeted genes and signaling pathways which can be verified by molecular docking analysis.<sup>13</sup> The method is often used to screen bioactives in a herbal mixture and provide insight of their multi-targeted actions.<sup>14</sup>

In recent years, a number of clinical and preclinical studies investigated the synergistic effects of natural product combinations for protecting the endothelium *via* reducing CVDs risk factors. Thus, this study aims to review the (synergistic) pharmacological actions of these natural product combinations and elucidate the associated molecular mechanisms.

## Methods

A comprehensive search of peer-reviewed journal articles between January 2000 and February 2022 related to endothelial protective activity of natural product combinations was conducted in Google Scholar, PubMed, MEDLINE and EMBASE.

The search terms for the review needed to address four components. For the search terms related to natural products, these included: “natural medicines”, “natural compounds”, “herb”, “herbal”, “herbal products”, “plant”, “medicinal plant”, “botanical plant”, “phytochemistry”, “phytochemicals”, “nutraceuticals”, “supplements”, “foods”, “functional foods”, “Chinese Herbal Medicine”, “Chinese herbal formula”, “Chinese formula”, “Chinese formulation”, and “Chinese herbal combination”.

The search terms relating to endothelial dysfunction or endothelial protection included “endothelial protective”, “endothelial dysfunction”, “endothelial injury”, “endothelial apoptosis”, “endothelial damage”, “endothelial impairment”, “oxidative stress”, “endothelium”, “vascular protection”, “vascular dysfunction”, “vascular impairment”, “vascular damage”, “atherosclerosis”, “vascular disease”, and “cardiovascular disease”.

The search term, synergy, was enhanced with synonyms and MeSH terms including “synergistic”, “synergism”, “synergize”, “synergise”, “enhance”, “promote”, “compatible”, “augment”, “combine”, “combination”, “improve”, “magnify”, “formulate”, “formulation”, “interact”, “interaction”, and “positive interaction”.

The key words used for pharmaceutical medicines included “Western drugs”, “conventional drugs”, “traditional drugs”, “conventional medicine”, “Western medicine”, “pharmaceutical drugs”, “synthetic drugs” and “drugs”. The search strategy has been included in Supplementary 1.

The identified abstracts from the electronic search were independently reviewed by two authors (MY and XZ) with a third author (VR-N) to facilitate any disagreements.

We included original research articles of natural product combinations: (1) with or without pharmaceutical medicines that used quantitative analyses to determine synergy; (2) studies that implied synergy or enhanced pharmacological activity by comparing combined effects to individual effects as an outcome measurement; and (3) studies that elucidated the interaction by systems biology or bioavailability. Articles were also identified through the reference list of retrieved research articles and reviews. Excluded articles were studies published in languages other than English. Original research articles that investigated combined therapy without any comparison to monotherapies or elucidation of interaction were also excluded.

A standardised data charting form was developed (MY) to extract data from included documents and confirmed independently by a second author (XZ). Data items included author, year of publication, natural product combinations, dose or ratio, type of study, key findings, mechanism of synergy/enhanced activity and method used to determine synergy. This information was summarised for each paper and narratively described.

## Results

### Study Selection

The search strategy resulted in 64 studies, and 17 additional articles were identified through the reference lists of the retrieved research articles and reviews. We excluded five studies as duplicates, and another 55 studies as they lacked

comparison between combined natural product combination and monotherapy or interaction of ingredients/compounds. Finally, 21 studies consisting of four clinical studies and 17 pre-clinical studies were included in this review. The study selection process is illustrated in Figure 1.

### **Investigating Natural Product Combinations in Protecting the Endothelium Against CVDs Risk Factors**

**Hyperlipidemia.** Atherosclerosis is the dominant cause of CVDs, and it is characterized by chronic inflammation and lipid accumulation within the arteries, ultimately restricting blood flow to the artery and leading to heart attack and ischemic stroke.<sup>15</sup> The role of lipids and lipoproteins as causal factors for the progression of atherosclerosis and CVDs is well established.<sup>16</sup> The lipid accumulation in vessel walls is coupled with the apoptosis of endothelial cells, especially in the later stages, contributing to the incidence of plaque rupture and consequent thrombus formation.<sup>17</sup>

Two *in vivo* animal studies and one clinical trial investigated natural product combinations as primary therapy or adjunct therapy on endothelial function against hyperlipidemia. A combined therapy of chitin-glucan (CG) and pomegranate peel extract (PPE) in protecting endothelial function, mediating gut microbiota and eNOS against hyperlipidemia, was investigated in male apolipoprotein E knock-out mice fed with a high fat diet.<sup>18</sup> CG is a prebiotic which has been shown to help regulate the human gut microbiota, and PPE is rich in polyphenols with potent antioxidant activity. The results demonstrated that the CG (5%)-PPE (0.5%) combination for eight weeks markedly increased eNOS in mesenteric arteries and the heme-nitrosylated hemoglobin blood levels, whereas CG monotherapy did not show a significant change. The endothelial improvement of the combined treatment was significantly higher compared to the high-fat diet fed ApoE<sup>-/-</sup> mice group ( $p < 0.05$ ). Although the underlying mechanism was not examined, it was suggested that the modulation of *Lactobacillus* and *Alistipes* in the gut lead to reduced circulatory inflammation. The combined therapy also exhibited a moderate effect in reducing lipids in plasma and liver tissue. Whether the combined therapy was superior to PPE monotherapy was not discussed in the study.

A study by Li et al (2010) investigated the protective effect of a fenofibrate-allicin combination on endothelial function in Wistar rats with high fat diet-induced hyperlipidemia. Fenofibrate is a fibric acid derivative used to treat abnormal blood lipid levels.<sup>19</sup> Allicin is a compound from *Allium sativum* (garlic) and is a popular remedy for hypertension and hyperlipidemia.<sup>20,21</sup> The animals were allocated to receive allicin (60 mg/kg per day), fenofibrate (80 mg/kg per day) and a combination of fenofibrate (30 mg/kg per day) and allicin (20 mg/kg per day) over eight weeks. The results demonstrated that fenofibrate alone and the combined therapy enhanced the high-density lipoprotein levels compared to the high-fat diet control. Although the single and combined treatment of allicin and fenofibrate significantly increased the NO

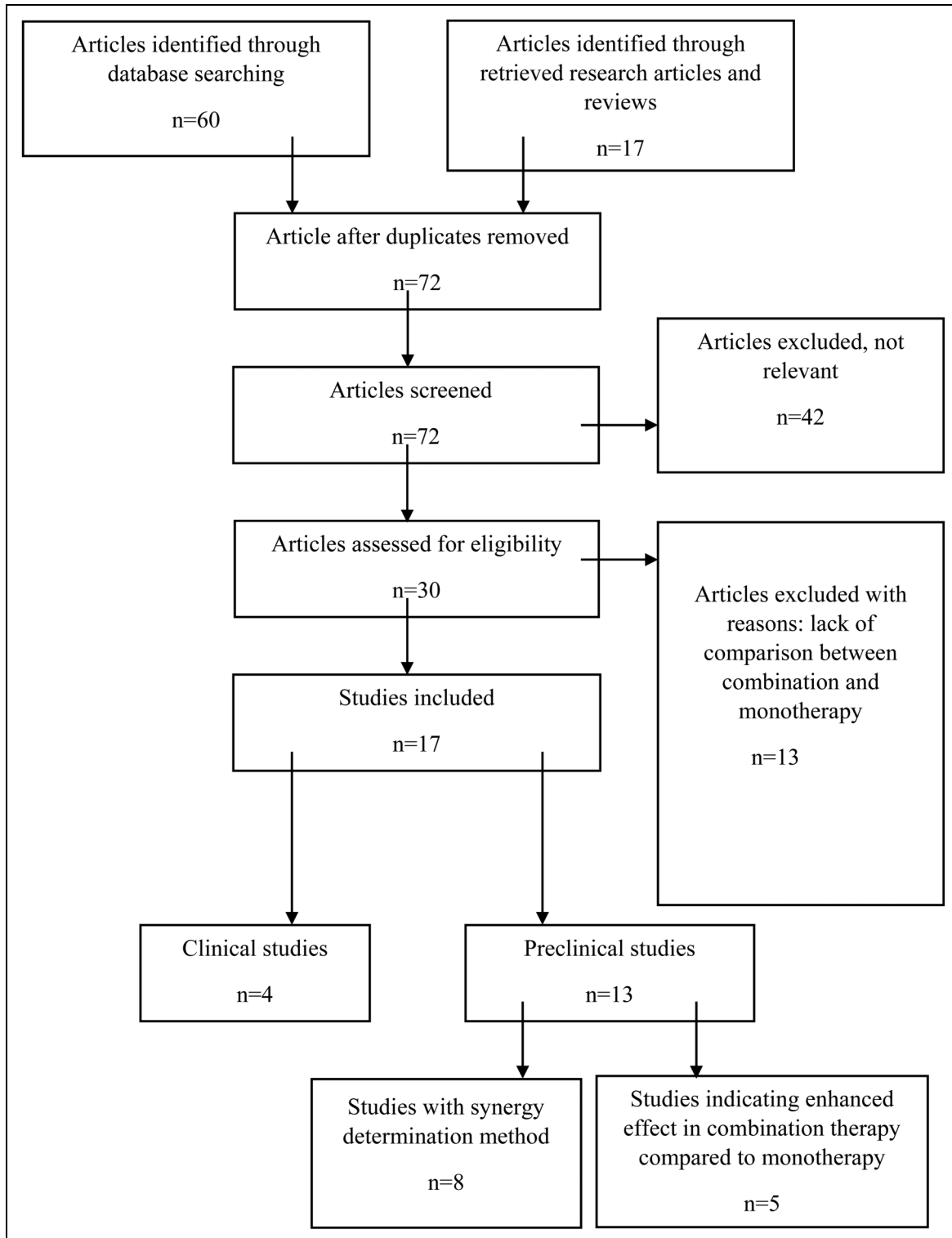
levels, the combined therapy showed a greater effect on endothelium-dependent vascular relaxation responses in comparison to fenofibrate alone ( $p < 0.05$ ). This suggests that the endothelial protective effect of the fenofibrate-allicin combination is superior than each monotherapy in hyperlipidemic rats.<sup>22</sup>

A study by Toyama et al (2014) evaluated an eicosapentaenoic acid (EPA)-statin combination for endothelial dysfunction in patients with coronary artery disease (CAD). EPA, an omega-3 fatty acid, has been shown to improve endothelial function in patients with CAD<sup>23</sup> and lower blood triglyceride concentrations.<sup>24,25</sup> Statins are standard pharmaceutical medicines used to reduce cholesterol levels.<sup>26</sup> Eighty patients were randomly allocated to receive 1800 mg of EPA plus statin daily (dose not specified,  $n = 40$ ) or statin alone (dose not specified,  $n = 40$ ) over 3 months. There was a significant improvement in flow mediated vasodilation (FMD) in the combined therapy group ( $p = 0.02$ ), whereas no significant change was observed in the statin-only treatment group ( $p > 0.05$ ). The results also suggested that the EPA-statin combination produced more profound effects on improving endothelial function and blood lipid level in CAD patients compared to statin monotherapy.<sup>27</sup>

**Hypertension.** Hypertension is a common condition and one of the strongest risk factors for CVDs.<sup>28</sup> In hypertensive subjects, oxidative stress, inflammation and impairment of vascular flow are often involved in the progression of endothelial dysfunction, leading to vascular re-modelling, atherosclerotic plaque development and an increased risk of CVDs.<sup>29</sup>

One *in vivo* study investigated the combined activity of *Uncaria* and *Semen Raphani* on the vascular endothelium in spontaneously hypertensive rats.<sup>30</sup> *Uncaria* (U) is a rubiaceae plant growing in South China and has been used for hypertension, cerebral ischemia and sedation<sup>31,32</sup> with the alkaloid components acting on the cardiovascular and central nervous system.<sup>31</sup> The dried ripe seed of *Raphanus sativus* L. (R) is used in traditional Chinese medicine to treat constipation, chronic tracheitis and hypertension.<sup>33</sup> A study from Li et al (2015) demonstrated that U (38.525 mg/kg/day of *Uncaria* alkaloids) and R (46.230 mg/kg/day) effectively decreased systolic, diastolic and mean arterial pressure in hypertensive rats which were at least comparable to each monotherapy. In addition, the combination improved the endothelial integrity of the thoracic aorta and mesenteric artery as observed by electron microscopy. The combination also normalized the plasma biomarkers of endothelial damage compared to the hypertensive rats group ( $p < 0.05$ ), and the regulatory effects were greater than each monotherapy.<sup>30</sup> It was suggested that the enhanced action of the combination was achieved through the compatibility of the two herbs: U contributes to the antihypertensive activity, whereas R is involved in reducing the inflammation.

**Diabetes mellitus.** In diabetes mellitus, hyperglycemia is the main causal factor of endothelial dysfunction and the consequent progression of vascular issues.<sup>34</sup> In diabetic induced-endothelial dysregulation, the reduced bioavailability of vasodilators, particularly



**Figure 1.** Flow diagram of retrieved journal articles.

eNOS, has been reported. This has been attributed to the excessive production of ROS and oxidative stress produced at sites of injury, and induced inflammation which directly impairs the endothelial function and increases the risk of CVDs.<sup>35</sup> At present, CVDs is the biggest cause of death among people with diabetes mellitus.

One *in vivo* animal study and one clinical study in humans evaluated the effect of combined natural products in improving endothelial function in diabetic subjects. *Angelica sinensis* (Oliv.) Diels and *Astragalus membranaceus* (Fisch.) Bge. are the two principal herbs in Dang-Gui-Bu-Xue-Tang which is a

classic traditional Chinese medicine formula used for diabetes mellitus, inflammatory and ischemic diseases.<sup>36–38</sup> Ferulic acid and astragaloside IV, key bioactive components of *A. sinensis* and *A. membranaceus*, respectively, independently exhibit cardio-protective properties through their immunomodulatory, antioxidant and anti-inflammatory activities.<sup>39</sup> In a diabetic rat model, the ferulic acid-astragaloside IV combination was evaluated for the treatment of endothelial dysfunction. Histological morphology showed that the combined therapy (each 50 mg/kg/day) preserved the endothelial structure and endothelial cell integrity against the streptozotocin-induced hyperglycemia, which was comparable to that of the control and metformin (positive control) groups. The combination showed a remarkable vascular protective effect indicated by higher eNOS and NO levels compared to the diabetic rats group. The combination also showed the greatest effect in reducing lipid levels and inflammatory biomarkers compared to each single compound, although ferulic acid alone was also effective.<sup>39</sup>

Fish oil is a popular supplement for improving wellbeing, partly attributed to the lipid-lowering effect of omega-3 fatty acids.<sup>40</sup> Proprietary chromium complex (PCC) consists of trivalent chromium chloride and an aqueous extract of *Phyllanthus emblica* L. fruit with polyphenolic compounds. PCC is known to protect cardiovascular function through its insulin regulatory, hypolipidemic and antioxidant activities.<sup>41</sup> A randomized, double-blind, placebo-controlled clinical trial was conducted to evaluate the effect of a fish oil-PCC combination in alleviating endothelial dysfunction in participants with type 2 diabetes mellitus. Fifty-three participants were randomly assigned to receive fish oil alone (2.000 mg/day), fish oil (2.000 mg/day) plus PCC (10 mg/day, 200 µg of Cr<sup>3+</sup>), or fish oil (2.000 mg/day) plus PCC (20 mg/day, 400 µg of Cr<sup>3+</sup>) over 12 weeks. The results showed that adding a small dose of PCC (20 mg, 400 µg of Cr<sup>3+</sup>) to fish oil markedly enhanced the antioxidant responses (malondialdehyde, nitric oxide, glutathione), inhibited inflammatory proteins (high sensitivity C-reactive protein, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and endothelin-1) and regulated lipid profile (decreased total cholesterol, low-density lipoprotein, very-low-density lipoprotein, triglyceride, and increased high-density lipoprotein). Thus, the combined therapy resulted in an improved endothelial function. It was concluded that PCC significantly enhanced the efficacy of fish oil in protecting the endothelium in comparison to fish oil alone. However, a PCC monotherapy group was not included in the study and thus, the superiority of the combination was not fully determined.<sup>41</sup>

**Platelet activation.** Activated platelets are often associated with thrombotic occlusion of ruptured or eroded plaques in atherosclerosis<sup>42</sup> and contribute to the early onset of atherosclerotic lesions under inflammatory conditions: Endothelial cells interact with activated platelets which promote the secretion of chemokines, cytokines, metalloproteinases, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) superfamily ligands and other mediators. These substances induce monocyte/leukocyte recruitment and

activation, extracellular matrix proteins degradation and exacerbate endothelium dysfunction, and contribute to the progression of atherosclerosis.<sup>43</sup>

One *in vivo* study determined the anti-platelet activity of a traditional Chinese herbal formula consisting of *Salvia miltiorrhiza* Bge. and *Phellinus baumii*. *S. miltiorrhiza* is a principal herb used in many Chinese medicine formulas for the treatment of cardiovascular complications attributed to its pharmacological benefits on microcirculation, coronary vasodilatation and platelet adhesion and aggregation.<sup>44,45</sup> *P. baumii* is a fungus that has been reported to possess antioxidant property.<sup>46</sup> A study by Lim et al (2016) examined the endothelial protective effect of this combination in a collagen-epinephrine mixture-induced platelet activation rat model. The seven-day pretreatment of *P. baumii* (500 mg/kg) – *S. miltiorrhiza* (100 mg/kg) combination showed an enhanced eNOS signal on the aortic wall compared to each monotherapy. This effect was comparable to that of aspirin (positive control) which is known for its antiplatelet activity. In addition, the combined treatment remarkably increased eNOS phosphorylation and intracellular NO (both  $p < 0.05$ ) in human umbilical vein endothelial cells (HUVECs). The combined effect was likely attributed to the anti-inflammatory activity of tanshinone IIA and increased NO availability action of protocatechuic acid, a key bioactive component of *S. miltiorrhiza* and *P. baumii*, respectively. The outcome of this study supported the two natural product combination in improving platelet activation-induced endothelial impairment.<sup>47</sup>

**Oxidative stress.** Oxidative stress plays a fundamental role in endothelial dysfunction and progressing atherosclerosis.<sup>48</sup> Elevated oxidative stress such as ROS can directly quench the production of eNOS, resulting in the loss of eNOS activity and impaired vascular integrity and homeostasis.<sup>49</sup> Additionally, ROS disturb lipoprotein metabolism and contribute to atherosclerotic vascular lesions.<sup>50</sup> Thus, oxidative stress is highly implicated in many CVD conditions including myocardial infarction, ischemia/reperfusion and heart failure.

Seven preclinical studies were found to support the combined effects of herbal extracts and/or bioactive herbal compounds in protecting endothelial function against oxidative stress. For over ten years in China, the Shuanglong formula, consisting of *S. miltiorrhiza* and *Panax ginseng* C. A. Mey. (7:3 ratio), has been used for treating myocardial infarction (MI) and angina pectoris. A study by Liang et al (2012) showed that the seven-day treatment of Shuanglong markedly reduced the infarct areas in rats with MI. Systems biology analysis showed that total salvianolic acids from *S. miltiorrhiza* and total ginsenosides from *P. ginseng* were the key components contributing to the pharmacological effect of the formula. The data partly confirmed that the combination of salvianolic acids and ginsenosides promoted cell regeneration and myocardial angiogenesis in HUVECs against lipopolysaccharide-induced oxidative damage.<sup>51</sup>

As a popular traditional herbal combination, *S. miltiorrhiza*-*Panax notoginseng* (Burk.) F.H.Chen has shown pharmacological

benefits in the treatment of cardiovascular-related complications.<sup>52,53</sup> In an *in vitro* study by Zhou et al (2019), the synergy of nine combinations (1:9, 2:8, 3:7, ...9:1, w/w) were investigated in protecting endothelial survival against oxidative stress (H<sub>2</sub>O<sub>2</sub>) using human endothelial EA.hy926 cells. The results showed that the 6:4 combination exhibited synergistic effects (CI<1) in restoring cell viability compared to each herb alone. Additionally, the synergistic interaction of these two herbs was likely attributed to the positive interaction of salvianolic acid A from *S. miltiorrhiza* and ginsenoside Rb1 from *P. notoginseng*, whereby the combination exhibited synergistic effect in improving EA.hy926 cell viability against H<sub>2</sub>O<sub>2</sub> injury.<sup>54</sup>

Yang et al (2018) also investigated a salvianolic acid B-ginsenoside Re combination, the two key bioactive compounds from *S. miltiorrhiza* and *P. notoginseng*, respectively. The salvianolic acid B (60 µg/mL)-ginsenoside Re (120 µg/mL) combination exhibited an optimal and synergistic protective effect against oxidised low-density lipoprotein (oxLDL)-induced endothelial apoptosis as evidenced by the CI model. Network pharmacology and *in vitro* experimental validation showed that the observed effects were attributed to the promotion of antioxidant enzymes and NF-κB-mediated anti-inflammatory pathway.<sup>55</sup>

*Vitis vinifera* L. leaves are traditionally used in Ayurvedic medicine to treat diarrhea, vomiting, wounds and bleeding. Modern studies have shown its potent effect against oxidative stress. In Asia, *Centella asiatica* (L.) Urb. is a traditional medicinal plant used for wound-healing and scar management, partially attributed to its anti-inflammatory and antioxidant properties. A study from Jeon et al (2020) showed that a mixture of *V. vinifera* L. leaf and *C. asiatica* ethanolic extract (3:1) reduced ROS production and decreased nitric oxide level in HUVECs. This contributed to the enhanced integrity of the endothelial tight junction against oxidative damage (H<sub>2</sub>O<sub>2</sub>). The combined activities were generally higher than each monotherapy although no statistical analysis was shown.<sup>56</sup>

Nalinratana et al (2014) investigated the synergistic effect of the nine herbal ingredients in a Thai herbal preparation, Phikud Navakot (see Table 1 for the full ingredients) which is indicated for circulatory disorders. Phikud Navakot was extracted in 50% ethanol or water, and the extracts were examined in a H<sub>2</sub>O<sub>2</sub> induced-oxidative stress model using human endothelial ECV304 cells. Synergy analysis using the CI model suggested that the hydroethanolic extract exhibited a stronger efficacy (manifested by a lower IC<sub>50</sub> value) and synergy (0.421<CI<0.831) in reducing the free radicals, including superoxide, hydroxyl, nitric oxide radicals and H<sub>2</sub>O<sub>2</sub>, compared to the aqueous extract (0.752<CI<1.555). The observed synergy is likely to be associated with the interactions of the relatively more lipophilic components in the hydroethanolic extract.<sup>57</sup>

Curcumin is a polyphenol derived from turmeric (*Curcuma longa* L.).<sup>66</sup> Resveratrol is a natural phenol sourced from grapes, peanuts and red wine.<sup>67</sup> Both curcumin and resveratrol are two well-known nutraceuticals with strong antioxidant activity and shown to protect cells from oxidative stress. A recent *in vitro* study by Zhou et al (2021) demonstrated

that a curcumin-resveratrol (8:2 w/w) combination produced a synergistic protective effect (as analysed by CI models) against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in human endothelial EA.hy926 cells, by inhibiting ROS production, restoring cell viability and reducing caspase-3 activity. Moreover, this study related the possible synergistic mechanism to the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) translocation and increased expressions of Nrf2-regulated antioxidant enzymes including heme oxygenase-1 (HO-1), nicotinamide adenine dinucleotide (NAD) and superoxide dismutase (SOD).<sup>60</sup>

*Olea europaea* L. leaf has been clinically shown to exhibit hypotensive activities through vascular smooth muscle modulation.<sup>68</sup> *Hibiscus sabdariffa* L. flower is suggested to attenuate atherosclerosis via the antioxidant and antihyperlipidemic activities of its polyphenols.<sup>69,70</sup> An *in vitro* study revealed that the combination of *O. europaea* L. leaf and *H. sabdariffa* L. flower extract restored cell viability and reduced ROS level in HUVECs. In the subsequent *in vivo* study, the combination of these two herbs (13:2, w/w) showed a greater vascular relaxation in K<sup>+</sup> depolarized aorta smooth muscle of guinea pig (IC<sub>50</sub> = 5.89 mg/mL) than *H. sabdariffa* L. alone (IC<sub>50</sub> = 6.63 mg/mL), but this effect was potentially less potent than that of *O. europaea* L. leaf alone (IC<sub>50</sub> = 5.15 mg/mL).<sup>59</sup>

**Hyperhomocysteinemia.** Homocysteine (Hcy) is an amino acid in the blood formed during the metabolism of the essential amino acid methionine.<sup>50</sup> A high Hcy level, termed hyperhomocysteinemia, is associated with endothelial dysfunction and a higher risk of CVDs.<sup>71</sup> Although the molecular basis of the association remains unclear, the vascular toxicity induced by hyperhomocysteinemia may involve the interfered production of eNOS, deregulation of essential endothelial neurotransmitter, increased expression of ROS,<sup>71</sup> and impaired endothelial cell survival by forming S-adenosylhomocysteine.<sup>72,73</sup>

Two *in vitro* studies discussed the protective effect of a herbal extract combination and a phytochemical combination against Hcy-induced endothelial damage. An *in vitro* study by Zhou et al (2019) investigated the *S. miltiorrhiza* and *P. notoginseng* combination in protecting EA.hy926 cells against Hcy-adenosine-TNF-α-induced apoptosis. The results of the CI analysis demonstrated that the 6:4 combination of the two herbs was optimal in improving cell survival and reducing apoptotic level, with the effect greater than each monotherapy.<sup>54</sup> The combinations 2:8 and 3:7 produced a significant synergistic effect in promoting endothelial cell growth, scratch wound closure and tube formation in EA.hy926 cells against Hcy-adenosine induced impairment.<sup>45</sup> The wound healing ability of the combination was substantially attenuated by phosphatidylinositol-3 kinases (PI3K), mitogen-activated protein kinase (MEK) and extracellular signal-regulated kinases (ERK) inhibitors which inferred the potential mechanistic pathways.

More recently, a study demonstrated that a ginsenoside Rb2-ginsenoside Rg3 combination produced a greater protective effect against Hcy-induced damage compared to monotherapy alone. The gene correlation network analysis showed that

**Table 1.** Preclinical and Clinical Studies of Natural Product Combinations with Determined or Indicated Synergistic Activities in Improving or Protecting Endothelial Function.

Extracts or compounds (Dose or ratio)	Type of study and subjects	Key results	Mechanism of synergy or enhanced activity	Method of synergy determination
Ginsenoside Rb2 (50 $\mu$ M)-ginsenoside Rg3 (5 $\mu$ M) <sup>58</sup>	<i>In vitro</i> : HUVECs	Synergistically promoted HUVECs proliferation and tube formation against Hcy-induced damage	Modulation of CXCR1/2 and CXCL8 (IL8)-mediated PI3K/Akt and MAPK/ERK signaling pathways	Bliss independence method
<i>S. miltiorrhiza</i> - <i>P. notoginseng</i> (2:8 and 3:7, w/w) water extract <sup>45</sup>	<i>In vitro</i> : EA.hy926 cells	Synergistically promoted cell proliferation, wound healing and tube formation in EA.hy926 cells	Modulation of PI3K, MEK and ERK pathways	CI and isobologram models
<i>S. miltiorrhiza</i> - <i>P. notoginseng</i> (6:4, w/w) water extract <sup>54</sup>	<i>In vitro</i> : EA.hy926 cells	Synergistically enhanced cell viability against the impairment from Hcy – adenosine–TNF- $\alpha$ and H <sub>2</sub> O <sub>2</sub> induced oxidative stress	Synergistically reduced caspase-3 and ROS levels	CI
<i>Vitis vinifera</i> L.- <i>Centella asiatica</i> (3:1) ethanolic extract <sup>56</sup>	<i>In vitro</i> : HUVECs	Enhanced integrity of endothelial tight junction against oxidative damage (H <sub>2</sub> O <sub>2</sub> )	Greater suppression of ROS production and reduced nitric oxide level compared to each monotherapy	Not measured
<i>Olea europea</i> L. leaf- <i>Hibiscus sabdariffa</i> L. extracts (13:2 w/w) water and methanolic extract <sup>59</sup>	<i>In vivo</i> : guinea pigs <i>In vitro</i> : HUVECs	Higher vasorelaxant activity in atrial of guinea pigs	Higher antioxidant and cytoprotective activities via reducing ROS and improving cell viability compared to single extract	Not measured
Phikud Navakot, a mixture* of nine herbal plants water and ethanolic extract <sup>57</sup>	<i>In vitro</i> : human endothelial ECV304 cells	Synergistic endothelial protective effect in reducing free radicals	Hydroethanolic exhibited stronger effect than aqueous extract, suggesting chemical composition influences synergy	CI
Curcumin-resveratrol (8:2, v/v) <sup>60</sup>	<i>In vitro</i> : EA.hy926 cell line	Synergistic protective effect against H <sub>2</sub> O <sub>2</sub> -induced oxidative stress	Reduced caspase-3 activity and ROS production, increased Nrf2 translocation, expressions of HO-1 and Nrf2 proteins, NAD production and SOD activity, Nrf2-HO-1 activation and elevation of antioxidant enzymes	CI
Ginsenoside Re (120 $\mu$ g/mL)-salvianolic acid B (60 $\mu$ g/mL) <sup>55</sup>	<i>In vitro</i> : HUVECs	Synergistic protective effect against Ox-LDL-induced endothelial apoptosis	Further promotion of antioxidant enzymes and inhibition of NF- $\kappa$ B mediated inflammatory pathways	Central composite design with response surface methodology; CI
Chinese herbal formula: Danggui Buxue Tang (DBT), Astragali Radix and Angelicae Sinensis Radix at a ratio of 5:1 boiled with 8 volumes of water <sup>61</sup>	<i>In vitro</i> : HUVECs	DBT induced NO production and eNOS phosphorylation in HUVECs, whereas such effects were much less in the herb extract (without boiling) or single herb	Akt activation	Not measured

(continued)

Table 1. (continued)

Extracts or compounds (Dose or ratio)	Type of study and subjects	Key results	Mechanism of synergy or enhanced activity	Method of synergy determination
<i>S. miltiorrhiza</i> and ligustrazine <sup>62</sup>	<i>In vitro</i> : HUVECs	Combination mitigated inflammation in HUVECs, which was likely to be attributed by ligustrazine, whereas <i>Salvia miltiorrhiza</i> only showed a slight effect.	Regulating IL-6 gene and CXCL10	Bioinformatic analysis
Ferulic acid (50 mg/kg)-astragaloside IV (50 mg/kg) <sup>39</sup>	<i>In vivo</i> : male Wistar rats (n = 60)	Higher endothelial protective effects against hyperlipidemia and hyperglycemia	Suppressed NF- $\kappa$ B pathway with reduced TNF- $\alpha$ , MCP-1, p-NF- $\kappa$ B p65 and immunoreactive score	Not measured
CG (5%)-PPE (0.5%) <sup>18</sup>	<i>In vivo</i> : male Apolipoprotein E knock-out mice (n = 17)	Combined treatment was stronger than the monotherapy of CG on the endothelium	Suppressed anti-inflammatory effects, improved endothelial function in mesenteric arteries through availability and higher production of NO, and modulation of gut microbiota	Not measured
<i>S. miltiorrhiza</i> - <i>P. ginseng</i> (3:7) <sup>51</sup>	<i>In vivo</i> : MI rat model	Enhanced pharmacological effects of total ginsenosides and salvanolic acids predicted by systems biology and validated by experimental data	Predicted key bioactive components of salvanolic acids and ginsenosides to achieve an optimal minimal phytochemical composition	System biology
<i>Phellinus baumii</i> (500 mg/kg)- <i>S. miltiorrhiza</i> (100 mg/kg) ethanolic extraction <sup>47</sup>	<i>In vivo</i> : male Sprague-Dawley rats (n = 40)	Strongest improvement of eNOS in the combination group compared to the monotherapies	Further enhanced eNOS, inhibited pro-inflammatory cytokines and adhesion molecules	Not measured
Total <i>Uncaria alkaloids</i> and soluble <i>Semen Raphani alkaloid</i> <sup>30</sup>	Spontaneously hypertensive rats	Combination lowered activated circulating endothelial cells, improved endothelial integrity of thoracic aorta and mesenteric artery, and normalized plasma biomarkers of endothelial damage	Decreased mRNA levels of VCAM-1, SelL, TFPI, and Sel-P, elevated mRNA expressions of FGF-1 and THBD of the thoracic aorta	Not measured
Fenofibrate (30 mg/kg per day)-allicin (20 mg/kg per day) <sup>22</sup>	<i>In vivo</i> : male Wister rats with high fat diet-induced hyperlipidemia (n = 60)	Combined treatment enhanced protective effects on endothelial function	Significantly increased NO levels	Not measured
Luteolin (500 mg/kg)-curcumin (500 mg/kg) <sup>63</sup>	<i>In vivo</i> and <i>in vitro</i> : aortic endothelium isolated from mice (n = 40); Human EA.hy926 cells	Synergistically protected endothelial cells and mice against vascular inflammation	Synergistically inhibited TNF- $\alpha$ which was related to further inhibited NF- $\kappa$ B translocation	CI
<i>Phellinus baumii</i> - <i>S. miltiorrhiza</i> (900 mg/day) water and ethanolic extract <sup>64</sup>	Clinical: randomized, double-blind, controlled trial on healthy chronic smokers (n = 72)	Combined ingestion exhibited enhanced improvement of blood pressure and vascular stiffness in chronic smokers compared to monotherapy	Crosstalk of six bioactives which interacted with 15 key targets of CVDs	Not measured
Cocoa flavanols (820	Clinical: randomized,	Combined treatment	Methylxanthine improved	Not measured

(continued)



**Table 1.** (continued)

Extracts or compounds (Dose or ratio)	Type of study and subjects	Key results	Mechanism of synergy or enhanced activity	Method of synergy determination
mg)-methylxanthine (220 mg) water extract <sup>65</sup>	double-masked controlled trail on healthy individuals (n = 47)	improved flow mediated dilatation more significantly than intake of cocoa flavanols alone	absorption of cocoa flavanols in the body when administered together	
Fish oil (2000 mg)-PCC (20 mg) <sup>41</sup>	Clinical: randomized, double-blinded, placebo-controlled trial on type 2 diabetes mellitus individuals (n = 59)	Improved cardiovascular parameters compared to fish oil alone; attributed to endothelial antioxidant, anti-inflammatory and lowered lipids level	Not determined	Not measured
EPA (800 mg)-statin <sup>27</sup>	Clinical: CAD patients (n = 80)	Improved FMD; combined treatment improved endothelial function in CAD patients	Not determined	Not measured

Abbreviations: Akt: protein kinase B; CAD: coronary artery disease; CVDs: cardiovascular diseases; CG: chitin-glucan; CI: combination index; CXCR1: C-X-C motif chemokine receptor 1; CXCL8: C-X-C motif chemokine ligand 8; CXCL10: C-X-C motif chemokine ligand 8. ERK: extracellular signal-regulated kinases; eNOS: endothelial NO synthases; FMD: flow mediated vasodilation; Hcy: homocysteine; HUVECs: human umbilical vein endothelial cells; H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide; MAPK: mitogen-activated protein kinase; MCP-1: monocyte chemoattractant protein 1; MEK: mitogen-activated protein kinase kinase; MI: myocardial infarction; NAD: nicotinamide adenine dinucleotide; NF- $\kappa$ B: nuclear factor kappa-light-chain-enhancer of activated B cells; NO: nitric oxide; Nrf2: nuclear factor erythroid 2-related factor 2; ox-LDL: oxidized low-lipid protein; PI3K: phosphatidylinositol-3-Kinase; p38MAPK: protein kinases and the 38-kDa mitogen-activated protein kinase; PPE: pomegranate peel extract; ROS: reactive oxygen species; SOD: superoxide dismutase; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; VCAM-1: vascular cell adhesion protein 1. \*Phikud Navakot is a mixture of nine herbal plants (roots of *Angelica dahurica*, *Angelica sinensis* and *Saussurea costus*; the rhizomes of *Atractylodes lancea*, *Ligusticum chuanxiong*, and *Picrorhiza kurroa*; the roots and rhizomes of *Nardostachys jatamansi*; the aerial parts of *Artemisia pallens*; and the galls of *Terminalia chebula*).

the synergistic interaction of Rb2/Rg3 to promote endothelial cell proliferation was mediated through the chemokine receptor type 1/2 and chemokine (C-X-C motif) ligand-8-mediated PI3K/Akt and MAPK/ERK signaling pathways.<sup>58</sup>

**Inflammation.** Inflammation is a major initiator of endothelial dysfunction and progression of CVDs.<sup>74</sup> Inflammation in the endothelium is triggered by toxins, injury or inflammatory mediators in the blood, and terminated when the injurious stimulus is removed and the mediators are dissipated or inhibited.<sup>75</sup> However, atherosclerosis is a chronic inflammatory process that involves multiple factors including circulating inflammatory cytokines, TNF- $\alpha$ , ROS and oxidized-LDL. These mediators activate endothelial cells and lead to endothelial dysfunction and a pro-thrombotic state. Thus, targeting TNF- $\alpha$ -mediated endothelial inflammation may ameliorate endothelial dysfunction and reduce the risk of CVDs.<sup>76</sup>

Luteolin is a polyphenolic flavone isolated from many herbs and foods, and it has shown potent anti-inflammatory activity.<sup>77</sup> Curcumin has been extensively studied for its anti-inflammatory property and is deemed as one of the most promising cytokine-suppressive agents.<sup>78</sup> A recent *in vitro* study showed that a luteolin (1  $\mu$ M) - curcumin (0.5  $\mu$ M) combination synergistically inhibited TNF- $\alpha$ -induced monocyte adhesion in EA. hy926 cells (CI<1) and suppressed TNF- $\alpha$ -induced NF- $\kappa$ B p65 nuclear translocation. This inhibitory effect was not observed when luteolin and curcumin was used alone. In addition, the *in vivo* results showed that a two-week treatment of combined luteolin

(500 mg/kg)-curcumin (500 mg/kg) in C57BL/6 mice synergistically prevented TNF- $\alpha$ -stimulated adhesion of mouse monocytes to aortic endothelium, with the mechanism potentially related to the reduced aortic protein expressions of vascular cell adhesion protein 1 and monocyte chemoattractant protein-1. Thus, it is speculated that atherosclerosis was ameliorated by the synergistic endothelial protection against inflammation by the luteolin-curcumin combination.<sup>63</sup>

In another *in vitro* study, *S. miltiorrhiza* combined with ligustrazine, (a bioactive compound from *Ligusticum chuanxiong* Hort) demonstrated a pronounced anti-inflammatory effect on HUVECs. The combined effect was stronger than each monotherapy, with *S. miltiorrhiza* showing minor action, and the combined effect mainly attributed to ligustrazine. Bioinformatic analysis revealed that IL-6 gene and C-X-C modify chemokine ligand 10 (CXCL10) appeared to be involved in the mechanism.<sup>62</sup>

**Prevention of endothelial dysfunction against cigarette smoking in healthy subjects.** Smoking is strongly linked with mortality in atherosclerotic CVDs.<sup>79</sup> It has long been recognized that cigarette smoking adversely affects endothelial function which is mainly attributed to mitochondrial oxidative stress and inflammation [activation of cyclooxygenase (COX)-1 and COX-2],<sup>80-82</sup> leading to arterial stiffness.<sup>83</sup>

One randomized clinical trial investigated the synergistic activity of *Phellinus baumii*-*S. miltiorrhiza* combination in regulating endothelial-mediated dilation in 72 healthy smokers.<sup>64</sup> The results showed that a four-week treatment of the herbal

combination at 900 mg/day significantly improved pulse wave velocity, reduced systolic blood pressure and increased endothelial eNOS activation ( $p < 0.01$ ). The network pharmacology analysis illustrated that the bioactive components in *Phellinus baumii* (caffeic acid, ellagic acid and protocatechuic acid) and *S. miltiorrhiza* (tanshinone IIA, tanshinone I and cryptotanshinone) had crosstalk on 15 key biological targets, including Endothelin 1, Arginase 2 and NOS3, which improved vasodilation relevant to major metabolomic pathways.<sup>64</sup> However, the described actions of the bioactives related to the key gene targets were not confirmed.

**Prevention of endothelial dysfunction in healthy subjects.** Cocoa flavanols are bioactive compounds found in the cacao plant and have shown beneficial effects on cardiovascular health.<sup>65,84,85</sup> Sansone R et al (2017) investigated the clinical efficacy of combining methylxanthine (a bronchodilator drug) and cocoa flavanol in enhancing cardiovascular function through four clinical studies (3 randomized, double-masked cross design and 1 parallel crossover studies) in healthy volunteers.<sup>65</sup> The collective results demonstrated that the combined methylxanthines (220 mg) and cocoa flavanols (820 mg) treatment improved FMD more significantly than cocoa flavanols alone. Additionally, the combination decreased blood pressure and brachial pulse wave velocity and enhanced circulating angiogenic cells compared to baseline and cocoa flavanol alone. It was noteworthy that the co-administration enhanced the absorption and plasma concentration of epicatechin metabolites of cocoa flavanol in comparison to cocoa flavanol treatment alone. Similar results were obtained when pure caffeine, methylxanthine and epicatechin were consumed together. Thus, it was suggested that methylxanthine facilitated the increased plasma concentration of epicatechin as an active metabolite, which, in turn, enhanced the vascular effects of the cocoa flavanols.<sup>65</sup> Importantly, no adverse effects were recorded in any of the studies. Although these results are in healthy subjects, the combination may be pharmacologically beneficial in preventing vascular dysfunction.

### Potential Molecular Targets of Herbal Medicines Combinations in Protecting Endothelial Function

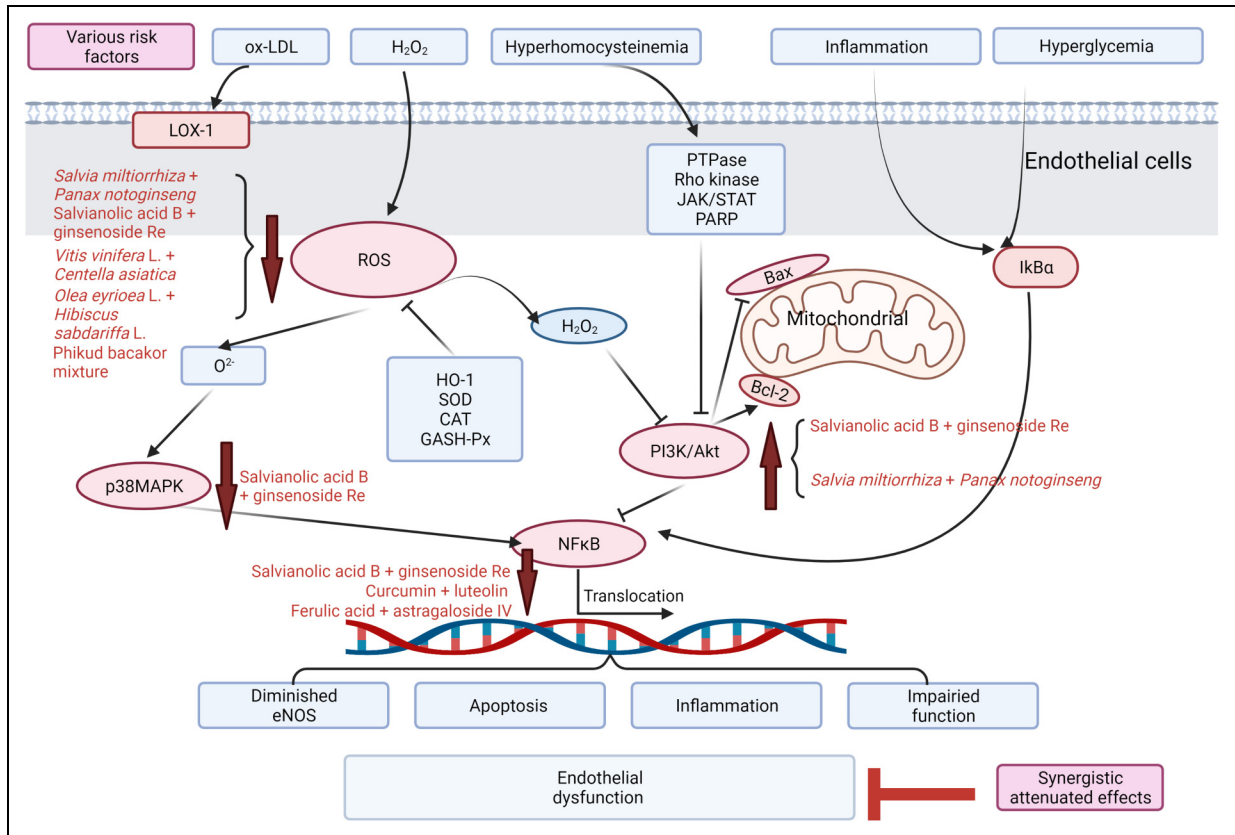
Endothelial dysfunction induced by various CVDs risk factors involve a myriad of deleterious processes and pathways. It is known that, upon the various stimulants such as lipids, inflammation and homocysteine, intracellular NADPH oxidase is activated to produce numerous types of ROS that can directly diminish eNOS and provoke the caspase cascade. ROS also activates several downstream pathways leading to the upregulated p38MAPK, inhibited PI3K/Akt pathways and the subsequent NF $\kappa$ B-mediated inflammatory response which further induces cell apoptosis and apoptotic-related protein expressions.<sup>54</sup> The retrieved studies revealed that the common molecular targets for natural product combinations (with potential synergy) included ROS, NF $\kappa$ B, p38MAPK and PI3K/Akt.

Figure 2 illustrates the molecular mechanisms underlying the synergistic effect of selected natural products. The following sections explain how the natural product bioactives interact at the molecular targets.

**Decreased ROS and activated Nrf2 against oxidative stress.** Five *in vitro* studies have demonstrated that ROS is the common target for natural product combinations against oxidative stress-induced endothelial dysfunction. Zhou et al (2019) showed that the *S. miltiorrhiza* - *P. notoginseng* combination, in the ratio of 6:4, significantly reduced ROS production in EA.hy926 cells against H<sub>2</sub>O<sub>2</sub> when the concentration was lower than 80.51  $\mu$ g/mL. The inhibitory effect on ROS was synergistically (CI<1) stronger than each individual herb.<sup>54</sup> Another study revealed that the bioactive compounds from these two herbs, salvianolic acid B and ginsenoside Re, respectively, further enhanced ROS inhibitory activity in HUVEC cells. Salvianolic acid B was likely to play a leading role as the individual activity was stronger than that of ginsenoside Re. Additionally, three herbal combinations, including *Vitis vinifera* L. - *Centella asiatica*, *Olea europaea* L. - *Hibiscus sabdariffa* L. and *Phikud Navakot* mixture, demonstrated a greater ROS inhibitory effect against H<sub>2</sub>O<sub>2</sub> compared to their single components.<sup>56,57,59</sup>

Oxidative stress induces ROS expression, leading to oxidative modification or crosslinking of cysteine residues within kelch-like ECH-associated protein 1 (Keap1), which results in the dissociation of Keap 1 from Nrf2 and permits the translocation of Nrf2 from cytoplasm into the nucleus.<sup>86</sup> Nrf2 in the nucleus binds to antioxidant response elements (ARE), thereby activating the transcription of many genes encoding antioxidant proteins including HO-1, NAD(P)H dehydrogenase [quinone] 1 and SOD. These proteins scavenge free radicals and protect cells against oxidative stress through various molecular pathways.<sup>87</sup> Thus, Nrf2 activation is a key target for endothelial protective activity against oxidative stress. Zhou et al (2021) showed that CR synergistically induced Nrf2 translocation,<sup>60</sup> which then exhibited increased expressions of HO-1, SOD and NAD, leading to reduced ROS and apoptosis in EA.hy926 cells. These findings highlighted Nrf2 as a novel molecular target for synergistic, cytoprotective action in the endothelium. In addition, a salvianolic acid B and ginsenoside Re combination exhibited enhanced endothelial protective activity *via* the Nrf2-regulated antioxidant pathway. Consequently, increased antioxidant enzymes activities, including superoxide dismutase, plasma glutathione peroxidase and catalase, were observed in Ox-LDL-stimulated HUVEC cells.<sup>55</sup>

**Regulated PI3K/Akt and MAPK pathways against hyperhomocysteinemia and hyperlipidemia.** Three studies have reported that PI3K/Akt and MAPK were targeted by natural product combinations to attenuate endothelial injury and restore angiogenesis function against hyperhomocysteinemia or hyperlipidemia. Zhou et al (2017) revealed that homocysteine and adenosine downregulated the PI3K pathway which was reversed by a *S. miltiorrhiza*-*P. notoginseng* (2:8, w/w) combination in EA.hy926 cells. A study by Yang 2018 revealed that Akt was the common target for salvianolic



**Figure 2.** Schematic diagram illustrating key molecular targets for herbal medicines combinations in endothelial cells against various risk factors. T-bars represent inhibition and arrows represent activation.

acid B and ginsenoside Re against Ox-LDL-induced endothelial injury. Although both single compounds showed significant effects in restoring Akt phosphorylation, the effect of their combination was found to be the strongest. Similarly, a salvianolic acid B-ginsenoside Re combination produced a stronger downregulation of p38MAPK when compared to the single compounds.<sup>55</sup>

Danggui Buxue Tang, an ancient Chinese herbal decoction, is prepared by mixing Astragali Radix (AR) and Angelicae Sinensis Radix (ASR) at a weight ratio of 5:1 and boiled with 8 volumes of water. The decoction is traditionally used to promote blood circulation and relaxation of blood vessels. The study from Gong et al (2015) showed that the decoction induced nitric oxide production and phosphorylation in HUVECs, whereas such effects were insignificant for each individual herb and when mixing individually extracted AR and ASR. This finding suggested that the synergy may be associated with the preparation of these two herbs in the specific ratio during boiling which cannot be represented by simply mixing the two key herbs. The formula's effect on NO induction was fully blocked by a PI3K/Akt inhibitor which suggested the underlying mechanism is, at least partially, relevant to the activation of the Akt pathway.<sup>61</sup>

Reduced expressions of cell adhesion molecules *via* inhibited NF-κB translocation. NF-κB is a key downstream effector of

p38MAPK and plays a central role in inflammatory responses. It contributes to the damaged endothelial cells, recruitment of cell adhesion molecules and the development of atherosclerosis. An *in vitro* study by Yang et al (2018) reported that Ox-LDL markedly activated NF-κB translocation leading to increased endothelial apoptosis.<sup>55</sup> Pretreatment with the salvianolic acid B-ginsenoside Re combination significantly inhibited the NF-κB translocation which was stronger than the individual compounds.<sup>55</sup>

Both curcumin and luteolin have shown potent anti-inflammatory activity associated with the NF-κB pathway.<sup>88</sup> Zhang et al (2019) revealed that the combination of curcumin and luteolin significantly reduced the nuclear expression of NF-κB without affecting total cellular protein.<sup>63</sup> The combined effect was significantly stronger than each compound alone. Similarly, a ferulic acid-astragaloside IV combination markedly reversed the increased phosphorylated NF-κB protein expressions in streptozotocin-induced diabetic mice and the effect was more profound than the individual compounds.<sup>39</sup>

## Discussion and Conclusion

Endothelial dysfunction is a complex pathophysiological process and involves numerous mechanistic pathways. While single pharmaceutical drugs can address individual targets,

combination therapies that can address the multimodal pathways may offer a more practical pharmacological strategy for endothelial dysfunction. The present study reviewed the scientific evidence of natural product combinations and generally showed a multi-component-multi-targeted approach with improved pharmacological activity in protecting the endothelial function against various cardiovascular risk factors.

Many herbs with a long history, such as *S. miltiorrhiza*, *P. baumii*, *P. ginseng* and *P. notoginseng*, have been traditionally used as the principal ingredients in herbal formulations for the prevention and treatment of CVDs. The Pharmacopoeia of the People's Republic of China outlines the preparation of the herbs/extracts, their combinations and formulation. Nutraceuticals including cocoa flavanols,<sup>89</sup> fish oil,<sup>90,91</sup> curcumin<sup>92,93</sup> and luteolin<sup>94</sup> have also shown pharmacological benefits for CVDs. However, many of these studies did not provide reasoning for the basis of the combination.

It has been a challenge to evaluate the effectiveness of these natural products in combinations. Animal studies and clinical trials to assess synergy in natural product combinations were scarce. Seven animal studies and four clinical trials included in this review provided some preliminary evidence to support synergy in herbal combinations for improving endothelial dysfunction. Potential synergy was largely based on a statistical significance of *p* value when comparing means among groups. However, a validated mathematical method (ie CI and isobologram models) to assess the interactions in the combination would substantiate their claims for synergy. In addition, there needs to be a connection between preclinical and clinical data to offer definitive evidence for the effectiveness of natural product combinations.

Integrating natural product interventions with main pharmaceutical medicine is gaining popularity in clinical practice. Three studies included in this review investigated the interactions of natural products and mainstream pharmaceutical medicines. The results demonstrated enhanced bioactivity in improving endothelial function compared to pharmaceutical medicines used alone.<sup>22,27,65</sup> The outcomes of these studies highlight the potential of natural product-pharmaceutical medicine combinations in treating CVDs. However, the safety aspects of these combinations should not be neglected, and thus more research is warranted to determine the possible harmful interactions to guide clinical practice.

Two methods used to quantify synergistic interactions of natural product combinations retrieved from the studies included CI and network pharmacology, with CI being the most applied method. It has the advantages of simple conduction, robust analysis and economic viability in experimental practice.<sup>95</sup> However, this model requires a complete dose-response curve to provide accurate results and thus, has not been extensively used in *in vivo* and clinical studies due to time and research costs. Network pharmacology methods may address the challenges of searching for the bioactive molecules in a natural product mixture and relating this to the mechanisms of the multi-targeted approach of a mixture. However, the mechanisms of action illustrated by network pharmacology are largely based on the existing

literature and databases, and the conclusion should be confirmed by experimental verification. The approach was illustrated in a study by Liang et al (2012) where the network pharmacology identified the main bioactive components in a *Salvia miltiorrhiza*-*Panax ginseng* combination (3:7) contributing to the pharmacological effects in MI. Experimental validation in an *in vivo* study subsequently confirmed the main bioactives predicted from the network pharmacology, and their effects on the most relevant targets.<sup>51</sup>

The elucidation of molecular mechanism(s) and common pharmacological targets helps to understand synergistic interactions in natural products and other drug agents. Several *in vitro* and *in vivo* studies included in this review demonstrated synergy or enhanced activities in various natural product combinations. In these studies, several molecular targets and signaling pathways such as ROS, PI3K/Akt, NF- $\kappa$ B and p38/MAPK illustrated how synergy occurs at the molecular level to attenuate the endothelial injury. Despite this, the synergistic studies of complex combinations are still at an early stage; many other key targets such as plasminogen activator inhibitor, cytokines, growth factors and proteolysis that regulate the endothelial function have not been examined. Furthermore, the modulation of the relevant gene and mRNA by these combinations is largely unknown.

Most of the studies included in this review only examined pharmacodynamic interactions using mathematical or systems biology models. The pharmacokinetic research of synergy is currently scarce and limited to bioavailability, which may occur at the absorption (uptake and efflux system) and metabolism (cytochrome P450 enzymes) levels.<sup>96</sup> The increased bioavailability may lead to increased effectiveness and/or increased toxicity. Thus, the pharmacokinetic investigation is of great clinical significance. Although one clinical study demonstrated a positive interaction of cocoa flavanols-methylxanthine at the absorption level,<sup>65</sup> distribution, metabolism and elimination were not investigated. In addition, the lack of rigorous statistical analysis tool(s) to accurately determine the synergistic interactions of a combination remains a challenge in a pharmacokinetic study. This is especially important when certain compounds are increasing or decreasing the amount of a pharmaceutical drug in the body, making it more toxic or less effective, respectively.

Another interesting point to note is the extraction method of the herbal preparations. The water extraction is the most used in traditional medicine practice and would yield predominantly polar molecules. However, accumulated evidence has shown extraction using organic solvents could lead to improved bioactivity of the combination as a greater range of compounds would be available. One *in vitro* study included in this review illustrated that extraction solvents determined the chemical composition of bioactive components in the formulation which impacted the pharmacological outcome significantly. It is worth noting that most included studies did not define and/or standardise the chemical compositions in the combination, and this would make it difficult to reproduce the results and yield consistent pharmacological outcome.

In conclusion, this review summarises the preclinical and clinical evidence of several natural product combinations for the prevention and treatment of endothelial dysfunction induced by several CVDs risk factors including hyperlipidemia, diabetes mellitus, platelet activation, oxidative stress and hyperhomocysteinemia. The demonstrated synergy was solely investigated in preclinical studies using CI and system biological methods. The associated molecular mechanisms were mainly related to Nrf2, PI3K/Akt and NF- $\kappa$ B pathways. Although the results are supportive for using natural product combinations to address endothelial dysfunction, conclusive evidence for synergism in each natural product combination remains low. Future research focusing on systematically evaluating mechanism-based synergy using combined *in vitro/in vivo* models and validating the synergistic interactions in clinical studies is warranted. However, the findings of this review may shed light on the development of novel and more advantageous pharmacological combinations against endothelial dysfunction to prevent or slow the progress of CVDs.

#### List of abbreviations

ARE	antioxidant response elements
AR	Astragali Radix
ASR	Angelicae Sinensis Radix
CAD	coronary artery disease
CG	Chitin-glucan
CI	Combination index
CVDs	Cardiovascular diseases
DBT	Danggui Buxue Tang
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
ERK	extracellular signal-regulated kinases
FMD	Flow mediated vasodilation
Hcy	Homocysteine
HO-1	Heme oxygenase-1
HUVECs	Human umbilical vein endothelial cells
ICON	Improving Cardiovascular Outcome Network
Keap1	Kelch-like ECH-associated protein 1
MEK	mitogen-activated protein kinase
MI	Myocardial infarction
NAD	nicotinamide adenine dinucleotide
Nrf2	Nuclear factor erythroid 2-related factor 2
OxLDL	oxidized low-density lipoproteins
PCC	Proprietary chromium complex
PI3K	phosphatidylinositol-3 kinases
PPE	Pomegranate peel extract
R	<i>Raphanus sativus</i> L.
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TNF- $\alpha$	tumor necrosis factor- $\alpha$

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#### Author Contributions

X.Z designed, organized, and supervised the study. M.Y and X.Z searched all the relative article and drafted the manuscript. X.Z, V.R-N, D.C and M.Z analyzed and revised the manuscript. All authors read and approved the final manuscript.

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#### Ethical Approval

Not applicable

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#### Supplemental Material

Supplemental material for this article is available online.

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