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Synergistic therapeutic actions of herbal ingredients and their mechanisms from molecular interaction and network perspectives

X.H. Ma[1], C.J. Zheng[1], L.Y. Han[1], B. Xie[1], J. Jia[1], Z.W. Cao[2], Y.X. Li[2] and Y.Z. Chen[1,2]

[1] Bioinformatics and Drug Design Group, Department of Pharmacy, National University of Singapore, Blk SOC1, Level 7, 3 Science Drive 2, Singapore 117543, Singapore
[2] Shanghai Center for Bioinformatics Technology, 100, Qinzhou Road, Shanghai 200235, PR China

Opinions about the therapeutic efficacy of medicinal herbs differ significantly. Some reported herbal efficacies at low doses of active ingredients suggest a need for investigating whether these are because of placebo or multi-ingredient synergistic effects. This review discusses the opinions, methods and outcomes of herbal synergism investigations and analyzes indications from 48 *in vivo* tests and 106 rigorous clinical trials. Analyses of ingredient-mediated interactions at molecular and pathway levels indicate multi-ingredient synergism in 27 of the 39 reported cases of herbal synergism with available ingredient information. Synergistic actions may be responsible for the therapeutic efficacy of a substantial number of herbal products and their mechanisms may be studied by analyzing ingredient-mediated molecular interactions and network regulation.

There have been conflicting views with respect to the mechanisms of reported therapeutic efficacy of herbal products. One view attributes these efficacies to placebo effects [1–4] and the other to synergism of herbal active ingredients [5–8]. While the exact mechanism of most of them is still unclear, herbal products have been widely explored as supplements, in folk and traditional medicines and as alternatives to conventional medicine [9,10]. Approximately 20% of the US population [11] and 22–23% of preoperative and ambulatory patients [12,13] have used herbal products. Positive outcomes have been observed in *in vivo* studies [14] and clinical trials of herbal extracts (e.g. Ginkgo (*ginkgo biloba*) EGb761 [15], St. John's wort (*Hypericum perforatum*) [16]), whole herbs [17] and multiherb recipes (e.g. PC-SPES [18]). Because of their extensive use, there is an increasing interest and need to evaluate herbal products and their mechanisms of action rigorously.

Significant progress has been made in extracting and analyzing active herbal ingredients [19,20]. Approximately one-third of the top-selling drugs have been derived from plant ingredients [21]. Natural products continue to be valuable sources for drug development [22]. Despite the accumulated knowledge of their ingredients, the mechanism and clinical pharmacology is only known for a small percentage of herbal products [19], which hinders efforts for standardization, evaluation and further exploration of herbal therapeutics. Much more research is needed to understand fully the individual and collective actions of herbal ingredients and therapeutic mechanisms of herbal products.

The concentration of active ingredients in some herbs is lower than therapeutic dosages [6,23], which has led to skepticism and suggestion that herbal therapeutic efficacies are because of placebo effects [1–4]. By contrast, there have been reports of the total contents of a herbal product showing a significantly better effect than an equivalent dose of a single isolated active ingredient [6,24]. Some herbal combinations are more effective than the constituent herb used alone [25]. Positive beliefs about alternative medicines are not necessarily associated with their positive or negative effects [3]. These findings suggest that, while placebo effects may be responsible for the therapeutic efficacies of some herbal products, those of other herbal products may arise from synergistic actions of herbal ingredients [5–8].

Synergy occurs if two or more herbal ingredients mutually enhance each other's effect more significantly than the simple sum of these ingredients [6,7]. Synergism may also arise from the potentiation of pharmacokinetics, such that one ingredient

Corresponding author: Chen, Y.Z. (phacyz@nus.edu.sg)

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enhances the therapeutic effect of another active ingredient by modulating its ADME [5,8], and via coalistic combinations, such that all ingredients involved are inactive individually but become active in combination [26]. Modes of action of synergistic drug combinations have been investigated from drug-mediated molecular interaction (MI) profiles and network regulatory actions [27]. With the accumulated knowledge of herbal ingredients and ingredient-mediated MI profiles [19,20], the same approach may be used for investigating possible occurrences and modes of synergistic actions of herbal ingredients.

It is cautioned that, although connections can be made from the MI profiles of herbal ingredients to possible synergistic actions, many of these interconnections are much more complicated and dynamic than those summarized in this review [28]. The extent of activation of these connections may be influenced by genetic variations, environmental factors, host's behavior and therapeutic scheduling [27]. Therefore, the use of these connections should be more appropriately viewed as a start to a more comprehensive analysis. Moreover, as in the investigations of synergistic drug combinations [27], herbal synergism needs to be evaluated by rigorous synergism analysis methods and validated in clinical trials.

Investigations and opinions about possible mechanism of herbal synergism

Herbal synergism has been frequently reported [5–8], but few reports have offered clear underlying mechanisms [6]. Similar to those of drug combinations [27], both pharmacodynamic or pharmacokinetic herb synergism has been defined in the literature [8]. The majority (74%) of the reported herbal synergism has been derived from direct comparison of effective concentrations [29] or therapeutic effects [30]. In these studies, synergism is assumed to occur if the effective concentration of ingredients in combination is significantly reduced [29] or the effects of ingredients in combination are significantly increased [30] with respect to that of each individual ingredient, which cannot easily distinguish synergistic from additive effects and usually rely on high margins of variation [31]. An increasing number of studies have employed rigorous drug combination analysis methods, such as isobolographic analysis, interaction index and ANOVA that more accurately measure synergistic effects [31].

Attempts have been made to use the MI profiles of herbal ingredients to explain the synergistic effects of some herbal ingredients [5,6,8]. For instance, some active ingredients in St. John's wort (*H. perforatum*) separately inhibit catechol *O*-methyl transferase (COMT), monoamine oxidase (MAO) and monoamine reuptake, but their individual systemic concentration levels are lower than those that would be expected to be effective for *in vitro* activity [8]. Nonetheless, these ingredients combine to produce pharmacodynamic synergy, leading to observable antidepressant effects [8]. Monoamines in CNS are reduced via monoamine reuptake [32] and COMT- and MAO-mediated catabolism [33]. Inhibition of one mechanism may elevate the compensatory activity of another. For instance, COMT inhibition shifts levodopa metabolism toward the MAO-B dependent oxidative pathway [34]. Therefore, the inhibition of one monoamine reduction route is complemented by inhibiting the other two to reduce their compensating activities, leading to synergy. Moreover, the effects of COMT on the release of adrenocorticotrophic hormone (ACTH) in depression depend on the presence of low-expression MAOA variants [35], which are reduced by the inhibition of MAOA to complement further the inhibition of COMT and enhance synergy.

A comprehensive literature search uncovered MI profiles for more than 1876 active ingredients from more than 1239 herbs. These active ingredients interact with at least 970 distinct proteins, many of which are therapeutic targets and ADME-Tox associated proteins. Therefore, as in the case of drug combination studies [27], the amount of currently available MI profile information appears to be sufficient to partly facilitate the detection and study of possible synergistic actions of herbal ingredients.

Indications from clinical trials

Rigorous clinical trials have been conducted for an increasing number of herbal products [15–18]. Our literature search found 104 publications in nonherbal research journals between 2005 and 2008 that report randomized, double-blind, placebo-controlled trials of herbal products (excluding trials of purified individual ingredients that are obviously unrelated to multi-ingredient effects). Although clinical trials have also been reported in herbal research journals, only those in nonherbal research journals were selected to reflect maximally the opinions of the nonherbal research community. These trials involve anywhere from 15 to 1200 patients (with the majority being >50 patients), which are comparable to the numbers of patients in typical phase I (20–80) and phase II (20–300) clinical trials. Overall, 70 (66%), 4 (6%) and 32 (30%) of the trials have shown significantly better, moderately more effective and no better efficacies than placebo, respectively. Information from these trials are summarized in Supplementary Tables S1, S2 and S3, respectively. The outcomes of these trials clearly indicate that placebo effects are improbably the sole factor for the observed therapeutic efficacies of a substantial percentage of herbal products.

Analysis of 15 clinical trials of the nootropic effects of G. biloba extracts in healthy people has shown that some studies use multiple outcomes and report positive effects on one or more of these at particular time points with particular doses, but these findings are either not replicated or have been contradicted by other studies [36]. Similar types of multiple outcome measurements were found in other reported clinical trials of herbal products. Although more standardized outcome measurements are generally desired [36], it is noted that, other than placebo effects, the therapeutic efficacies of herbal products at low levels of active ingredients probably arise from multi-ingredient synergistic effects that are highly sensitive to genetic variations, environmental factors, host's behavior and therapeutic scheduling [27]. Therefore, synergistic effects of some herbal products may be more sensitively and appropriately detected by multiple, rather than single, outcome measurements.

Indications from *in vivo* tests

Herbal products have also been investigated by *in vivo* studies [14]. Our literature search found 53 publications in nonherbal research journals that report *in vivo* studies of herbal products (excluding studies of purified individual ingredients). The information of the *in vivo* studies that show significant and insignificant effects are summarized in Supplementary Tables S4 and S5, respectively.

Overall, 48 (90%) of these *in vivo* studies have shown significant therapeutic effects. While negative *in vivo* results tend to be unreported, a substantial number of published positive *in vivo* results nonetheless show that a substantial percentage of herbal products are therapeutically active at comparable levels of drug leads in similar *in vivo* models.

Some *in vivo* studies have indicated that therapeutic effect of some herbal products may arise from multi-ingredient synergism rather than independent actions of individual ingredients. For instance, in evaluating the effects of the active ingredients tetrasulfide, indirubin and tanshinone IIA of the traditional Chinese medicine (TCM) Realgar-Indigo naturalis anticancer formula on acute promyelocytic leukemia FVB/NJ mice, a statistically significant prolongation of median overall survival was observed in the mice treated with the three-ingredient combination compared with those treated with vehicle control or mono- or bi-therapy of these ingredients [14].

Literature-reported herbal synergism

Our comprehensive literature search identified 39 reported cases of herbal synergism with available active ingredient information. Nineteen cases involved single-herb or extracts of single-herb and 20 cases involved twin-herbs and extracts from twin-herbs, 10 and 9 of these single-herb and twin-herb products have been tested and showed significant effects in at least 1 clinical trial or *in vivo* study. The information about these single-herb and twin-herb products, ingredients, reported synergistic effects, reported outcome of clinical trial or *in vivo* studies, known commercial exploration and possible synergism mechanism derived from ingredient-mediated MI profiles are summarized in Supplementary Tables S6 and S7 respectively. Examples of these products are given in Tables 1 and 2, respectively. Synergism in these products has been identified primarily based on enhanced effects or reduced effective concentrations, many of which exhibit large margins to allow reasonable conclusions to be drawn, but more careful analysis is needed for confidently distinguishing pharmacodynamic synergism and additive effects [31].

In many cases, literature-reported synergism is not sufficiently detailed to provide clues to the potential mechanism; but some reports offer useful clues. For instance, it has been reported that reveratrol and catechin synergistically protect PC12 cells from beta-amyloid protein (AP) toxicity of hydrogen peroxide [29]. beta-AP induces toxicity by activating a tyrosine kinase-based signaling response to produce neurotoxic secretory products, proinflammatory cytokines and reactive oxygen species (ROS) [37]. Therefore, synergy may arise from the collective modulation of tyrosine kinase signaling, which is supported and further revealed by indications from ingredient-mediated MI profiles described in Table 2.

Literature-reported molecular interaction profiles of herbal ingredients

As part of the effort for lead discovery from plants and for studying the molecular mechanism of herbal products, a large number of active ingredients have been extracted from herbs and many of these have been studied for their potential therapeutic effects [19,20]. Our literature search suggests that at least one MI profile has been reported for a substantial percentage (>50%) of herbal ingredients, many of which (40%) describe molecular target and regulatory actions in terms of inhibition, activation and expression level variations. Although the molecular target is not specified, some of the literature-described MI profiles identify specific pathway or process as target of a herbal ingredient and reveal the pharmacodynamic or pharmacokinetic consequence of the interaction. For instance, (−)-epigallocatechin-3-gallate has been described to potently inhibit the tumor cellular proteasome activity, which may contribute to the cancer-preventative effect of green tea [38].

Mechanism of synergistic actions of herbal ingredients suggested by the analysis of their molecular interaction profiles

Analysis of the MI profiles of synergistic drug combinations has shown that pharmacodynamic synergism arises from anticounteractive, complementary and facilitating actions of the drugs involved, and pharmacokinetic potentiation involves positive modulation of drug transport or permeation, distribution or localization and metabolism [27]. Some of the synergic actions of herbal ingredients are expected to be similar to these actions. Anticounteractive actions reduce a network's counteractive activities against a drug's therapeutic effect via interactions against an antitarget or counter-target and negative modulations of a network's robustness, crosstalk and compensatory and neutralizing actions. Complementary actions positively regulate a target/process or negatively regulate a competing mechanism by interacting with multiple points of a pathway and its crosstalk pathways, interacting with multiple sites, states, conformations and mutant forms of the target, collectively modulating target activity and expression, and simultaneously enhancing the positive and reducing the negative effects of the target. Facilitating actions are secondary actions of one drug in enhancing the activity or level of another drug. Potentiative modulation of drug transport or permeation enhances drug absorption via disruption of transport barrier, delay of barrier recovery or reduction of first-pass excretion by inhibiting drug efflux. Potentiative modulation of drug distribution or localization increases drug concentration by blocking drug uptake and inhibiting metabolic processes that convert drugs into excretable forms. Potentiative metabolism modulation stimulates the metabolism of drugs into active forms, or inhibits the metabolism of drugs into inactive forms.

Literature-reported MI profiles appear to offer clues to the possible mechanisms of synergistic actions of herbal ingredients for 12 and 15 of the 19 and 20 reported cases of single-herb and twin-herb synergism. Of these products, seven and five, respectively, have been tested in one or more clinical trials or *in vivo* study, with at least one study on each product showing significant effects. The information for the 12 single-herb and 15 twin-herb products are summarized in Supplementary Tables S6 and S7, and examples of these products are given in Tables 1 and 2, respectively. Synergism of three single-herb and two twin-herb products may be attributed to pharmacokinetic potentiation via enhanced bioavailability. Synergism of 9 single-herb and 13 twin-herb products may be attributable to pharmacodynamic synergy resulting from complementary (12 products), anticounteractive (7 products), facilitating (1 product) and antinegative-effect (2 products) actions.

TABLE 1

Examples of medicinal herbs or herbal extracts whose active ingredients have been reported to produce synergistic effect

Herb or herbal extract (principal ingredients) [PMID]	Reported synergistic effect [PMID]	Pharmacodynamic synergism determination method and reliability of synergism detection result	Literature-reported clinical trial or *in vivo* test results of herb or herbal extract [PMID]	Possible molecular mechanism of synergism [PMID]	Type of synergism
Green tea (*Camellia sinensis*) (polyphenols: (−)-epigallocatechin-3-gallate, catechin, caffeine) [12906756]	Synergy in augmenting and prolonging sympathetic stimulation of thermogenesis in Sprague–Dawley rats [10702779]	Comparison of effects, effect 2.4-fold stronger in combination. Synergism detection result reliable	Randomized trials of ten healthy men in three groups each taking green tea extract, caffeine and placebo showed that green tea has thermogenic properties and promotes fat oxidation beyond that explained by its caffeine content per se [10584049]	During cold exposures noradrenaline is released to blood circulation and to BAT, where it acts via beta-adrenoceptors and cAMP to produce free fatty acids, which open the mitochondrial proton channel protein in BAT. Protons enter the mitochondria and inhibit ATP synthesis and energy is transformed into heat (adaptive thermogenesis) [16026418]. Hormone stimulated lipolysis of mouse and rabbit adipocytes as measured by both free fatty acid and glycerol release is proportionally elevated with increase in the adipocyte cAMP level [6164913]. Catechin-polyphenols inhibited catechol-*O*-methyl-transferase which degrades noradrenaline, caffeine inhibited trancellular phosphodiesterases which break down noradrenaline induced cAMP. They collectively stimulated thermogenesis by relieving different control points along the NA–cAMP axis [10702779]. Their synergism arises from complementary actions of maintaining levels of noradrenaline and cAMP for producing free fatty acids that lead to adaptive thermogenesis	Pharmacodynamic synergy because of complementary actions on different targets of the same pathway
Berberis (*Berberidaceae*) (berberine, 5′-methoxyhydnocarpin) [11801387, 12842327]	Antimicrobial action of berberine potentiated by 5′-methoxyhydnocarpin [10677479]		No report found	5′-methoxyhydnocarpin (1 μg/ml) inhibited the berberine effluxing multidrug pump and thus increased berberine bioavailability. When combined with subinhibitory amounts of berberine, 5′-methoxyhydnocarpin caused complete inhibition of growth at a concentration of 1 μg/ml. Berberine alone showed poor antimicrobial activity, and 5′-methoxyhydnocarpin alone had no antimicrobial activity at a concentration above 500 μg/ml [10677479]. Hence, 5′-methoxyhydnocarpin pharmacokinetically potentiated the effects of berberine by enhancing its bioavailability	Pharmacokinetic potentiation because of enhanced bioavailability

Kava (*Piper methysticum*) (kava lactones: kavain, dihydrokavain, yangonin, dimethoxyyangonin, methysticin and dihydromethysticin) [11991792]	Synergistic CNS depressive effects [11991792]	Six placebo-controlled, randomized trials of kava extract WS1490 in patients with nonpsychotic anxiety disorders, which showed that WS1490 and possibly other kava extracts are effective particularly in females and younger patients [15934028]. Three randomized, double-blind, placebo-controlled trials of kava, including one study with an active comparator (venlafaxine), in adult outpatients with DSM-IV generalized anxiety disorder, which showed that no effects were found for kava [16877894]	Kavalactones (300 μg/ml) and dihydrokavain (300 μM) enhanced agonist binding to GABA A receptor to promote its activation, leading to antidepression effects [12494336]. Kava lactones kavain, dihydrokavain and dihydromethysticin block voltage-gated Na^+ and Ca^{2+} channels in micromolar concentrations [11991792, 9690349]. Persistent exposure of brain neurons to GABA results in downregulation of GABA(A)R and uncoupling of GABA and benzodiazepine binding sites, and voltage-gated Ca^{2+} channel activation is required for GABA(A)R downregulation [11553685]. Thus, kavalactones and dihydrokavain's GABAergic enhancement activity is countered by voltage-gated Ca^{2+} channel mediated GABA(A)R reduction, which can be reduced by Kava lactones kavain, dihydrokavain and dihydromethysticin block voltage-gated Na^+ and Ca^{2+} channel, leading to pharmacodynamic synergism	Pharmacodynamic synergy because of anticounteractive actions on different targets of different pathways that regulate the same target

Cases of pharmacodynamic synergy

An example of pharmacodynamic synergism is Ginkgo (*ginkgo biloba*) extract (main active ingredients bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, flavonol glycosides and biflavones), which have been reported to show synergistic protection against ischemia-induced neuronal death *in vivo* and glutamate-induced neuronal death *in vitro* involving antiexcitotoxicity, inhibition of free radical generation, ROS scavenging and regulation of mitochondrial gene expression [39]. A double-blind placebo-controlled trial on neuropsychological functioning of 262 cognitively intact older adults has shown that those taking EGb761 daily for six weeks exhibited significantly more improvement on the selective remaining test (SRT) tasks involving delayed free recall and recognition of noncontextual, auditory-verbal material, compared with the placebo controls. The EGb761 group also demonstrated significantly greater improvement on the WMS-III FII subtest assessing delayed recognition of visual material (human faces), compared with the placebo group [40].

CNS inhibitory action is mediated by the activation of GABA(A) and glycine receptors (members of the same superfamily of ligand-gated ion channels with common transmembrane topology and structural features) [41]. Bilobalide antagonizes GABA(A) receptor with an IC_{50} of 3.7 μM [42] and rho(1) GABA(C) receptors with an IC_{50} of 5 μM [43], and increases mRNA levels of COXIII subunit of cytochrome *c* oxidase and subunit 1 of NADH dehydrogenase at 15–30 μM, thus reducing GABA(A)-mediated inhibitory neurotransmission and preserving brain energy metabolism [44]. Ginkgolide B antagonizes the glycine receptor with an IC_{50} of 0.27 μM [45], thereby reducing glycine-mediated inhibitory neurotransmission [46] and glycine-activated responses in pyramidal hippocampal neurons [45]. Glycine and GABA subunits can coassemble to form functional glycine receptors [47]. Thus, bilobalide antagonism of GABA(A) receptor complements ginkgolide B's antagonism of glycine receptor by hindering the formation of *trans*-assembled functional glycine receptors, leading to neuroprotective synergism.

Cases of pharmacokinetic potentiation

An example of pharmacokinetic potentiation is the Oolong tea extract (main active ingredients epigallocatechin gallate, gallocatechin gallate, catechin gallate, epicatechin and caffeine). A synergistic antibacterial effect of monomeric polyphenols epigallocatechin gallate and catechin gallate in the extract of Oolong tea has been observed in an *in vitro* test on *Staphylococcus mutans* cells. In a clinical trial of 110 patients receiving tea tree oil regimen versus 114 patients receiving a standard regimen for the treatment of methicillin-resistant *Staphylococcus aureus* colonization, tea tree has been found to be effective, safe and well tolerated [48]. A major ingredient in the extract, epigallocatechin gallate, produces its antibacterial effect partly by binding to and disrupting cell wall components [49] and by inhibiting bacterial DNA gyrase [50]. Another ingredient catechin gallate inhibits P-glycoprotein [51], which effluxes epigallocatechin gallate [52]. Thus, catechin gallate pharmacokinetically potentiates the antibacterial effect of epigallocatechin gallate by enhancing its bioavailability. Pharmacokinetic potentiation can also be achieved by combinations of herbal ingredients that improves bioavailability via such mechanisms as absorption (e.g. saponins increase

TABLE 2

Examples of pairs of herbs or herbal extracts reported to produce synergistic effects

Herb 1 (principal ingredients) [PMID]	Herb 2 (principal ingredients) [PMID]	Reported synergistic effect [PMID]	Pharmacodynamic synergism determination method and reliability of synergism detection result	Literature-reported clinical trial or *in vivo* study of pair of herbs or extracts [PMID]	Possible molecular mechanism of synergy [PMID]	Type of synergism
Onion (*Allium cepa* L) (quercetin, myricetin, kaempferol, dipropenyl sulfide) [12207471, 3802049]	Garlic (*Allium sativum*) (sulfurous substances: diallyl disulfide, diallyl disulfide, allicin, alliin, ajoene, *N*-acetyl cysteine, *S*-allyl cysteine, *S*-ethyl cysteine, *S*-methyl cysteine, *S*-propyl cysteine) [11238796]	Synergistic antioxidant activity in the inhibition of lipid peroxidation by lipoxygenase as tested in a lipid peroxidation assay [10780875]	Comparison of effects, effect of combination grater than individual alone combined. Synergism detection result reliable	Trial of ten healthy persons to test the effect of garlic and onion on alimentary hyperlipemia induced by feeding 100 g butter showed that garlic and onion have a significant protective action against fat-induced increases in serum cholesterol and plasma fibrinogen and decreases in coagulation time and fibrinolytic activity [1131298]	Quercetin inhibited 5-lipoxygenases (IC_{50} = 25 μM) [15120715, 15809148], myricetin inhibited lipoxygenase [3151014], kaempferol inhibited 15-lipoxygenase and phospholipase A2 [3938206], which reduced lipoxygenase-mediated lipoperoxidation. Diallyl sulfide inhibited superoxide production by xanthine oxidase [12784861]. 5-Lipoxygenase is activated by hydrogen peroxide or xanthine/xanthine oxidase in oxidative state [10691962]. Diallyl sulfide's inhibition of superoxide production by xanthine oxidase thus reduced the counteractive activation of 5-lipoxygenase against quercetin and myricetin's inhibition of 5-lipoxygenase, leading to pharmacodynamic synergism	Pharmacodynamic synergy because of anticounteractive actions on different targets of different pathways that regulate the same target
Hu-Zhang (*Rhizoma Polygoni Cuspidati*) (emodin, physcion, chrysophanol, anthraglycoside A, fallacinol, citreorosein, questin, questinol)	Huang-Qi (*Radix Astragali*) (Osthole, calycosin, astragaloside I, II, IV, soyasaponin I, formononetin, isomucronulatol, asparagine) [12166972,18272311, 18353340]	Synergistic antiviral effect as tested in HEp-2 cell system [12242802]		A trial of 60 chronic hepatitis C patients orally taking Bing Gan Ling liquid composed mainly of Shui-Niu-Jiao (*Cornu Bubali*), Hu-Zhang (*Rhizoma Polygoni Cuspidati*), Chi-Shao (*Radix Paeoniae Rubra*), Huang-Qi (*Radix Astragali*), etc. The total effective rate is 86.7%, which is considerably better than that of the control group [10453617]. This trial might implicate the synergistic antiviral effect of Hu-Zhang (*Rhizoma Polygoni Cuspidati*) and Huang-Qi (*Radix Astragali*)	Emodin binds to and disrupted membranes by inducing formation of hexagonal-H(II) phase which lead to antiviral and antimicrobial effects (MIC_{50} = 2.2 μM) [15242821], Osthole suppressed the secretion of HBV by increasing the glycosylation of surface antigen (20 μg/mL) [8781315]. HBV and other viral infections involve production of a viral envelope particle that contains membrane lipids, surface antigen and other proteins [10739944]. Emodin disruption of membranes facilitated osthole's activity by exposing surface antigen to osthole binding, leading to pharmacodynamic synergism	Pharmacodynamic synergy because of facilitating actions on different targets of the same pathway that regulate of the same target

Gou-Teng (*Uncaria rhynchophylla* (*Miq.*) *Jack*) (rhynchophylline, isorhynchophylline, corynoxeine, isocorynoxeine, corynantheine, dihydrocorynantheine, hirsutine, hirsuteine) [14668978, 2328929, 2792960]	Tian-Ma (*Gastrodia elata Bl*) (vanilline, vanillic acid, vanillyl alcohol, gastrodin) [10954052, 2618688]	Synergistic anticonvulsive effects as tested in male Sprague–Dawley rats	ANOVA + Scheffe's test. Synergism detection result reliable	*In vivo* test of male Sprague–Dawley rats (six in each group) that received intraperitoneal injection of kainic acid to induce epileptic seizures and generation of free radicals, with or without oral administration of Gou-Teng (*Uncaria rhynchophylla (Miq.) Jack*) alone or combination of Gou-Teng (*Uncaria rhynchophylla (Miq.) Jack*) and Tian-Ma (*Gastrodia elata Bl*). Herb combination exhibited greater inhibition on the onset time of wet dog shakes than UR alone. Testing results described in third column. A traditionally defined herb pair frequently used in formulating traditional Chinese medicine recipes for blocking wind and convulsion, and for soothing liver Yang [17267151]. It has been applied to patients without rigorous clinical test. It is thus classified into *in vivo* test category	Hirsuteine (300 nM to 10 μM) blocked ion permeation through nicotinic receptor channel complexes [8320880] which contribute to its anticonvulsive effect [10735809]. Vanilline inhibited acetylcholinesterase and butyrylcholinesterase [18810999], which reduces acetylcholine-induced seizure events to produce anticonvulsive effect [18333967]. Vanilline's inhibition of acetylcholinesterase produced a negative effect of reduced degradation of acetylcholine that enhances activation of nicotinic acetylcholine receptor leading to convulsive effect. This negative effect can be countered by hirsuteine's blocking of nicotinic receptor channel, leading to pharmacodynamic synergism	Pharmacodynamic synergy because of antinegative-effect actions on different targets of related pathways that regulate of the same target
Indigo bush (*Dalea versicolor Zucc*) (4′,6′-dihydroxy-3′,5′-dimethyl-2′-methoxychalcone; 3,5,4′-trimethoxy-*trans*-stilbene) [15043439]	Berberis (*Berberidaceae*) (Berberine) [11801387]	4′,6′-Dihydroxy-3′,5′-dimethyl-2′-methoxychalcone and 3,5,4′-trimethoxy-*trans*-stilbene potentiated the activity of berberine as shown by an *in vitro* test [15043439]		No report found	4′,6′-Dihydroxy-3′,5′-dimethyl-2′-methoxychalcone and 3,5,4′-trimethoxy-*trans*-stilbene inhibited berberine effluxing multidrug-resistant pumps with MICs of 250 and 500 μg/mL, respectively, but they caused complete growth inhibition at very low concentrations (~3.3 μg/mL) [15043439]. Thus the effects of berberine are potentiated by maintaining its concentration	Pharmacokinetic potentiation because of enhanced bioavailability

absorption of corticosteroids) [53] and enhanced solubility (e.g. in St. John's wort (*H. perforatum*) procyanidin B2 or hyperoside increases the solubility of hypericin (HI)) [54,55].

Pathway analysis of synergistic actions

Apart from its increasing applications in studying biological systems and disease processes, pathway analysis is highly useful for analyzing and illustrating the synergistic actions of herbal ingredients and drug combinations [27]. Figures 1–3 in supplementary information show the pathways regulated by the synergistic actions of the *Rosa damascene*, St. John's wort (*H. perforatum*) and tomato (*Lycopersicom esculentum*) fruit and leaf, respectively. *R. damascene* (main active ingredients kaempferol and 2-phenylethanol-*O*-(6-*O*-galloyl)-beta-D-glucopyranoside) shows anti-HIV synergy in cell assays. 2-Phenylethanol-*O*-(6-*O*-galloyl)-beta-D-glucopyranoside interacts with HIV gp120 to prevent its binding to CD4 (EC_{50} = 40 μg/mL), kaempferol inhibits HIV protease (EC_{50} = 2 μg/mL). Exposure of human T cells to soluble HIV-1 gp120 induces RANKL production; RANKL and TNF-α upregulate HIV replication and HIV gene transcription via NFκB enhancer elements in the HIV long-terminal repeat [56]. Thus, 2-phenylethanol-*O*-(6-*O*-galloyl)-beta-D-glucopyranoside complements kaempferol's inhibition of HIV protease by reducing HIV protease substrates via the reduction of RANKL-mediated HIV replication, leading to pharmacodynamic synergism. The corresponding paths (dashed lines in Supplementary Figure 1) involve soluble HIV-1 gp120 activation of CCR5, which, via MAPKs, activates NFκB/AP-1-regulated transcription processes that secrete RANKL, TNF-α and other cytokines. Secreted RNAKL and TNF-α via RANK–TRAF–IKK and TNFR–TRAF–IKK paths activate NFκB/Fos/Jun mediated transcription processes at sections covering HIV genes, which lead to the production of HIV polypeptides to be cleaved into maturing viral proteins by HIV-1 protease.

St. John's wort (*H. perforatum*) (main active ingredients HF, HI, pseudohypericin and flavonoids) produces a synergistic anticancer effect in leukemia K562 and U937 cells. HF disrupts mitochondrial transmembrane potential, downregulates Bcl-2 and Mcl-1, activates caspase3 and cleaves caspase substrate PARP-1, leading to proapoptotic action [57]. HI activates caspase8 and caspase3 to cause light-activated apoptosis in various tumor cells. Apoptotic signaling of the death receptor pathway is amplified via caspase8–Bid and c-myc-bak mediated activation of the mitochondria apoptotic pathway [58]. HFs downregulation of tBid inhibitor Bcl-2 therefore complements HIs activation of caspase8–Bid and c-myc-bak mediated activation of the mitochondria apoptotic pathway, leading to pharmacodynamic synergism. The corresponding paths are HI activation of the caspase8–caspase3 and caspase8–Bid–tBid–MMP–cytoC–caspase9–caspase3 paths that subsequently activates CAD-mediated DNA fragmentation and inhibits PARP-mediated DNA repair (dashed lines in Supplementary Figure 2), and HF downregulation of Bcl2 and MCL-1 that reduce these two proteins' inhibitory actions on tBid (doted lines in Supplementary Figure 2) to release the breaks against the signaling via caspase8–Bid–tBid–MMP–cytoC–caspase9–caspase3 path.

Low concentrations of tomato (*L. esculentum*) ingredients, lycopene and 1,25-dihydroxyvitamin D3 (VD), exhibit a synergistic effect on cell proliferation and differentiation in HL-60 promyelocytic leukemia cell line [59]. Lycopene at 2–3 μM inhibits cell cycle progression via the reduction of the cyclin D level and retention of p27 in cyclin E–cdk2 [60], VD at 0.1 μM regulates mineral homeostasis and exhibits potent antiproliferative, prodifferentiative and immunomodulatory activities by antagonizing vitamin D receptor [61]. The vitamin D receptor enhanced the expression of cyclins and cyclin-dependent kinases [62], possibly by interacting with HDAC complexes [63]. Therefore, vitamin D receptor's action in upregulating cyclin D counters lycopene's reduction of cyclin D. The antagonism of the vitamin D receptor by VD reduced this counteractive action, leading to pharmacodynamic synergism. The corresponding paths are displayed by dashed lines in Supplementary Figure 3. Lycopene's downregulation of cyclin D hinders the signaling via the cyclin D/CDK4/6–Rb/E2F/DP1 path in G1 phase. The network's counteractive action is via the path of myc expression – cyclin D and CDK4/6 upregulation. The action of VD is via VDR activation that facilitates the assembly of the VDR, RXR, 9cRA and HADC complex on vitamin D response elements to repress transcription of myc, SKP2, CYP27B and other genes. Myc downregulates cyclin D and CDK4/6 and, thus, represses myc expression and enhances cyclin D and CDK4/6 expression.

Coalistic actions of herbal ingredients

Some diseases or symptoms in specific cases may be redundantly regulated by multiple factors or involve dysfunction of multiple coregulators. In these cases, separately targeting each individual factor is ineffective, and coalistically targeting multiple factors may be effective for the treatment of these diseases or symptoms. An example of a coalistic combination of herbal products is the combination of glycyrrhizin (GL) and the saponin fraction of ginsenosides (GS), which has been reported to be individually ineffective and collectively useful for reducing ulcerative colitis in male Wistar/ST rats caused by 2,4,6-trinitrobenzene sulfonic acid [64]. Ulcerative colitis is associated with excessive production of inflammatory cytokines and repression of anti-inflammatory genes and involves defective Th1 (characterized by the production of IL-1, IL-2, IL-6, IL-12, IL-18, TNF-α and IFN-γ) and Th2 (characterized by the production of IL-4, IL-5 and IL-10) [65] responses. GS inhibits LPS-induced expression of TNF-α, IL-1β and IL-6 [66] and is, thus, partially effective against defective Th1 response. GL reduces levels of IL-4, IL-5 and IL-8 [67] and is, thus, partially effective against defective Th2 response. If the particular ulcerative colitis model involves both Th1 and Th2 responses, then GS and GL alone is insufficient, and GL–GS combination is effective in reducing defective responses. Other coalistic combinations may be found from such sources as immunostimulatory [68] and memory enhancing [69] herbal recipes for patients of multiple deficiencies.

Effects of ADME, complex formation, conformational change, concentration ratio, environmental condition and processing method

Apart from the illustrated examples of pharmacokinetic potentiation, there are other varieties of mechanisms for enhancing or reducing the bioavailability of active ingredients. In studying the synergistic actions and other molecular mechanisms of herbal ingredients, it is important to take into consideration the effects of ADME, complex formation, conformational change, ingredient ratios, environmental conditions (e.g. pre-

sence of salt) and processing method on the bioavailabilities and activities of the ingredients. For instance, some ingredients from two TCM formulae 'Xue-Fu-Zhu-Yu Tang' and 'Jing-Guan Tang' have been found to form colloid-like aggregates, which enable these ingredients to survive the gastro-intestinal environment, cross Caco-2 monolayers and inhibit cardiovascular targets [70]. An active ingredient trichosanthin of a TCM abortion formula 'Tian-Hua Fen' is a member of type-I ribosome-inactivating protein family, which is able to cross membrane at low pH via pH-dependent conformational change and subsequent membrane-insertion activities [71]. Despite low oral bioavailabilities of their parent ingredients, flavonoids and some of their bioactive phase II conjugates may accumulate adequate amount in human body to produce observable activities [72]. Glycyrrhizic acid is a herbal ingredient with broad antiviral activities, its activities can be reduced by the introduction of potassium, ammonium or sodium salt and its metabolic product 18β-glycyrrhetinic acid is more active against EBV [73]. A TCM decoction 'Dang-Gui Buxue Tang' produces a more potent cardioprotective effect than the mixture of its component herb extracts, primarily because of a much higher extraction yield of active ingredients by the decocting process [74] and the highest extraction yield of its active ingredients is reached at specific ratio of its two constituent herbs [75]. Some of these effects are promoted or regulated in traditional medicines by the addition of helper or effector herbs, for example 'Soldier' and 'Guide' herbs in TCM, into multiherb recipes [76].

Perspectives

The relevant literature reports suggest that herbal synergism may be exposed and investigated by clinical/*in vivo* studies using rigorous drug combination analysis methods and analysis of the MI profiles and pathway regulatory actions of active ingredients. A substantial number of reported cases of herbal synergism appear to be supported by indications from clinical trials, *in vivo* studies and literature-described MI profiles that show ingredient-mediated interactions and network regulations. Our analysis focused on the MI profiles of the active ingredients most relevant to the literature-reported synergistic effects of the herbal products studied. Apart from the reported synergistic effects, other effects were not considered. The active ingredients of these herbal products are known to interact with other molecules. A more complete analysis of the MI profiles of these herbal products is needed to provide a more complete picture about the overall effects of these products.

The discovered mechanisms of synergistic actions of herbal ingredients can be explored for designing new multitarget drugs and drug combinations and for discovering potent drug combinations that are individually subtherapeutic but efficacious in combination. The latter opens the possibility of using existing and new compounds of lower potencies that might otherwise be or have been abandoned. Synergistic actions involve interactions with multiple sites, targets and pathways that are sensitively influenced by genetic, environmental, behavioral and scheduling profiles [27]. Therefore, herbal ingredients or drugs of lower potencies need to be appropriately combined in accordance with these profiles and probably in personalized manner to achieve sufficient levels of efficacy. Biomarkers derived from molecules that regulate synergism-related interactions may be useful for determining treatment strategies for individual patients. Investigation of herbal synergism can be further facilitated by the rapid expansion of the knowledge of active ingredients of herbs and their MI profiles, molecular networks involved in human physiological and disease processes, and known mechanisms of actions of synergistic actions of clinical drugs, herbal ingredients and other compounds.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.drudis.2009.03.012.

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