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Rethinking the basic action modes of herbal medicine and pondering classical standardization

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ABSTRACT

Over the past two decades, the secondary metabolite platform has determined the scientific direction of herbal medicines, while plant sources have been assumed to be the object of lead discoveries through bioassay-guided fractionation efforts. Nonetheless, the majority of purification programs have resulted in fractions and pure compounds with much lower efficacy than their parent extracts. It is then assumed that co-working action modes among chemical constituents occur in the herbal preparations. Primary metabolites (polysaccharides, peptides, and fatty acids) and mineral groups, on the other hand, have been neglected in the herbal effect contributions. This review aims to understand the interplay of secondary metabolites in herbal preparations, particularly how they interact with primary metabolites and mineral groups. Thus, by adhering to classical methods, it is possible to address certain aspects that modern standardization lacks, thereby facilitating a more comprehensive approach to these issues.

Implication for health policy/practice/research/medical education:
The study demonstrates that multi-component herbal preparation paradigms should be revisited to prepare health and medical students for future multidisciplinary teamwork. In the industrial sector, integrating coworking action in herbal formulations is essential to establish a solid foundation for advancing herbal product development, particularly for comprehensive herbal component standardization.

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Introduction

Throughout the course of history, herbal medicines have garnered recognition for their esteemed status as substances that promote and maintain human health throughout many societies. Indeed, the initiation of the drug discovery process has been the isolation of natural chemicals obtained from plants. Notable examples include the extraction of morphine for its pain-relieving properties. Subsequently, this approach has led to the discovery of various drugs with anticancer properties such as taxol, camptothecin, vincristine, and vinblastine. Additionally, cardiotonic digoxin, anti-malarial agents like artemisinin and quinines, and anti-Alzheimer's medications such as galanthamine have also been derived from natural compounds (1,2). However, there has been a noticeable cessation in the development of novel pharmaceuticals derived from plants during the last twenty years. Subsequently, a significant proportion of natural drug discovery initiatives have transitioned towards using microbial or marine screening sources (3-5). Conversely, in the contemporary era of evidence-based medicine, herbal extract preparations have been categorized as complementary alternative medicines, specifically designed as preventative and promotional

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agents, owing to their uncertain outcomes. Some official authorities have provided herbal pharmacopoeias with comprehensive monograph outlines that include information on botanicals, secondary metabolite profiling, and contaminant threshold requirements, which align with the modern chemical-pharmacological paradigm. Since the uncertainty of outcomes has been mitigated through qualitative and quantitative analysis of chemical constituents, the main mission of these official documents is to focus on the safety of herbal preparations. The idea that certain isolated compounds from plants exhibit biological effects and have already been used in clinical therapy has led to the modernization of herbal medicine, especially in the use of the term specific standardization (6,7).

A number of major compounds in plant extracts could be the active principal constituents, such as hydroxycinnamic acid derivatives in *Sarcocaulon marlothii* stem (8), andrographolide in *Andrographis paniculata* leaf (9), 1'-acetoxychavicol acetate in *Alpinia galanga* rhizome (10), linalool in *Coriandrum sativum* fruit (11), xylaranic acid in *Xylaria primorskensis* (12), and hyperforin in *Hypericum perforatum* herb (13). The vast majority of perpetual bioassay-guided fractionation work has been aimed at discovering active compounds from crude materials. Technically, bioassay-guided fractionation is used to link drug discovery screening with biological responses mediated by an instrument analysis. It is done step by step from crude extract to purified materials (14). Unfortunately, numerous studies employing bioassay-guided fractionation have yielded bioactivity effects that are statistically insignificant for the isolated compounds when compared to their original extracts (15-17). In certain cases, these isolated compounds have even demonstrated lower effects (18-20), or no effects at all (21,22). Moreover, with regards to effectiveness, the primary active chemicals are frequently discovered in limited concentrations. There is not always a clear link between the amount of these compounds in the active parent extracts and their biological effects (23). The aforementioned concerns have been brought to attention through many findings on bioassay-guided fractionation, indicating the potential limitations of depending exclusively on certain chemicals as the main active metabolite (24-27). This review aimed to elucidate the current mainstream standardization of herbal medicinal preparations that may have deviated from the basic pharmacological theory. Further, it provides an evaluation of certain classical standardization methods as an alternative to address the limitations of some claimed comprehensive standardization methods.

Coworking mode of actions

Synergistic interaction

The first form of coworking mode of action is synergistic interaction, wherein the combination of two natural compounds yields effects greater than the cumulative responses elicited by each substance when administered individually (the general formula of 1+1 >2). The concept of pure synergism pertains to the phenomenon in which the presence of a non-active metabolite contributes to the augmentation or modulation of the effects of an active molecule. As recently revealed by Vidar et al (28), berberine antibacterial effect was enhanced by the presence of piperine. On the contrary, piperine itself did not have any antibacterial activity. Previously, Bilia et al (29) revealed that the antiplasmodial effect of artemisinin was enhanced by the presence of quercetin in certain ratios. Nevertheless, in vivo or clinical synergistic data should also be considered since Elfawal et al (30) and Cai et al (31) reported that some flavonoids inhibited P450 cytochromic metabolism of artemisinin and increased blood artemisinin concentration. Thus, in the context of non-pure synergy, the presence of a less active molecule enhances the effects of an active metabolite. The aforementioned phenomena have been elucidated by the utilization of in vitro antioxidant assays conducted on some polyphenols (32-34).

Additive interaction

The second proposed coworking modes is characterized by additive interaction, in which some compounds within mixtures have a shared action target. The observed quantitative effects arise from the aggregation of the individual effects of the compounds when they are given independently (follows the basic formula of 1 + 1 with approximate result of 2).

However, the additive mode is typically avoided in the clinical settings because of the possibility of unfavorable adverse outcomes (35,36). The published data on the interaction among natural compounds through pure additive mechanism, resulting from the combination among partial agonists, is quite scarce. Therefore, the explanation for additive mechanism could be approached by referring to the in vitro assay models such as antioxidant assays. As described by Heo et al in their endeavor to unveil the synergistic effects among phenolic catechin, chlorogenic acid, cyanidin, cyanidin 3-glucoside, cyanidin 3-rutinoside, epicatechin, peonidin, peonidin 3-glucoside, quercetin, quercetin 3-glucoside, quercetin 3-galactoside, and quercetin 3-rutinoside through ABTS (2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay method, they reported that the individual capacity of these phenolic compounds was similar to the summation of their combination effects (37). Hence, there was additive instead of synergistic effect among their combinations. Additionally, Palafox-Carlos et al examined the radical scavenge capacity (RSA) of polyphenol vanillic acid (A), protocatechuic acid (B), and chlorogenic acid (C) using 2,2-diphenyl-1-picrylhydrazy (DPPH) assay, resulting in RSAs of 5.6%, 17.30%, and 13.56%, respectively

(32). Meanwhile, the combination of the latter with the polyphenol showed 23.7% (B+A) and 20.4% (C+A) inhibition, respectively (32). Not only in those chemical constituents, but the additive mode has also been proven by the antioxidant assays in binary and ternary fruit extract combinations based on FRAP (ferric reducing *antioxidant* power), DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), and ORAC (oxygen radical absorbance capacity) assays (38). The addition of antioxidant capacities of individual phenolic compounds led to a total antioxidant capacity that exhibited an effect consistent with their summations from individual components, correlating with an additive action mode. Therefore, instead of relying on limited compounds as the principal active compound, it should be more logical to assume that there is probably a large orchestral mechanism among chemical constituents in plant material working on this target, which produces additive interactions (39). Hence, considering gradual effect as the main priority based on the bioassay-guided fractionation model must be reevaluated as the standard for finding pharmaceutical active materials. From an economic point of view, leveraging crude extracts for pharmaceutical dosage forms is more economical than the use of their fractions or purified compounds (40,41).

The role of aqueous extracts

In term of solvent choice, several investigations have shown that aqueous extracts from certain plant species display more pronounced activity compared to those obtained using organic solvents (42-47). A considerable amount of glycoside derivatives of secondary metabolites can be identified in aqueous extracts (48,49). However, these aqueous extracts mainly consist of primary metabolites, including peptides, carbohydrates, and certain inorganic compounds. In this regard, traditional preparation techniques for herbal medicine include decoction, boiling of dried plants, or expression of fresh material, which may attract predominantly primary instead of solely secondary metabolite groups (50,51). In fact, throughout history, long before the discovery of organic solvents for extraction, it has been widely recognized that traditional preparations involved extracting essential components from fresh herbs parts through methods such as juicing of fresh plant materials (52) or the utilization of plant materials in the form of decoctions or infusions with water (53,54). Therefore, it is plausible that the effectiveness of traditional infusion or decoction preparations arises from the interaction of primary and secondary metabolites, rather than from the effects of secondary metabolites alone.

Primary and secondary metabolites

Unfortunately, the majority of research papers predominantly have been focusing on the toxic aspects of metals and minerals in herbal products (55-57), leading to a scarcity of reports on the bioactivity of these minerals and trace elements in phytomedicine. However, the long-term consumption of these materials, particularly those present in herbal teas such as Al, Ba, Fe, Zn, Mn, Mg, K, Na, P, Cu, Sr, and Ca has been fundamentally acknowledged as playing a crucial role in human growth and overall health (58,59). Furthermore, some of these elements are vitally important for various preventive and promotive health states in human body (60,61). It is also widely recognized that minerals, when present in low concentrations, are soluble in water and may coexist with secondary or primary metabolites in traditional infusions or decoctions. Zhang et al reported that a synergistic effect occurs between Zn, Ca, Mn and flavonoids from *Epimedium koreanum* extract, which stimulates primary osteoblast by enhancing alkaline phosphatase activity (62). The combination of green tea extract containing rich polyphenols with Se, Cu, Mn and some amino acids demonstrated a significant inhibition of the growth of many cancer cell models (63,64). Trace elements such as Zn, Fe, and Cu, which are found in *Morus alba* extract (65), along with Cr and Mg in *Origanum grosii* leaf extract, are suggested to exhibit a synergistic antihyperglycemic effect when combined with the secondary metabolites present in these extracts (66). Biel et al reported that with varying content of Zn, Fe, Cr, and Mn in *Cynara scolymus* leaf extract, when interacting with a high polyphenol content and fixed carbohydrates, fats, and proteins, may contribute to the prevention of chronic non-communicable diseases, through protection from oxidative damage mechanism (67). Regarding the role of primary metabolites, particularly polysaccharide, Cho et al reported that a 3:1 combination of glucan polymer from *Aureobasidium pullulans* and *Textoria morbifera* in ovariectomized mice exhibit the strongest anti-osteoporotic effect (68). The combination of selenium with natural polyphenols, such as resveratrol (69,70) and chlorogenic acid (71) has been revealed to inhibit metal-induced Aβ aggregation involved in the development of Alzheimer's disease. Therefore, both secondary and primary metabolites may exert a potentially significant impact on various pathological conditions, particularly in the form of decoctions and infusions (68,72-74). Therefore, depending on the linked pharmacology class, it is urgent to provide the mineral and trace element profiles in herbal preparations, too. A summary of possible coworking action modes among secondary, primary, complex mixtures, and minerals is presented in Table 1.

Putative modeling of the pharmacological mechanisms of herbal preparations

Based upon those bioactivity mode overviews, relying on conventional pharmacological concepts, "one active compound toward one target" apparently is inappropriate to be applied to herbal preparations. To bridge the gap,

Table 1. Cooperative action mode among secondary metabolites, primary metabolites, and minerals

Component involved	Compound groups and bioactivity	Putative mechanism	Bioassay type	References
Secondary metabolites	Berberine and piperine: antibacterial	Synergist	*In vitro*	(28)
	Artemisinin and flavonoids: antimalaria	Synergist	*In vitro*	(29)
	Saponins and flavonoids: myocardial ischemia	Synergist	*In vivo*	(75)
	Prenylated xanthonoids (acetylshikonin, deoxyshikonin, β, β-dimethylacrylshikonin, β-hydroxyisovalerylshikonin, and trans-anethole): cell regenerating stimulation for wound healing	Additive	*In vitro*	(76)
	Berberine, hypaconitine and skimmianine: ulcerative colitis	Complementary	*In vivo*	(77)
	Tanshinone IIA terpenoid, salvianolic acid B and ginsenoside Rb1 saponin: myocardial ischemia	Synergist	*In vivo*	(78)
	Anthocyanidin and rosmarinic acid: anti colitis	Synergist	*In vivo*	(79)
Secondary metabolite with primary metabolite	Ginsenoside Rb1 and polysaccharides; Diabetic model and antistress	Synergist	*In vivo*	(80,81)
	Lignan and polysaccharide; hepatoprotective	Synergist	*In vivo*	(82)
	Puerarin and polysaccharide: diabetes type II	Synergist	*In vivo*	(83)
	Polysaccharide with arabinogalactan type II side chains of Piper nigrum fruits and its synergistic effect with piperine; antitussive	Synergist	*In vivo*	(84)
	Flavonoids and polysaccharides; dementia	Synergist	*In vivo*	(85)
	Polysaccharides of *Auricularia auricula* and tremella and flavonoid; anti-dyslipidemia	Synergist	*In vivo*	(86)
	Polyphenols and omega-3 fatty acid; antidepressant	Synergist	*In vivo*	(87)
	Procyanidin B3 and eicosapentaenoic acid Polyunsaturated fatty acids; anti-inflammatory	Synergist	*In vitro*	(88)
Among primary metabolites	Among polysaccharides; anti-inflammatory	Synergist	*In vitro*	(89)
	Among polysaccharides	Synergist	*In vitro*	(90)
	Glucagon peptide' glucose lowering effect	Synergist	*In vivo*	(91)
	Sulfated polysaccharides: anti-measles virus	Synergist	*In vitro*	(92)
	Secondary metabolites with polysaccharide with fatty acids or peptide with fatty acids	No report		
Secondary metabolite mineral/metal ions	Flavonoids and trace element ion of B, Cu, Co Mn, Zn: general health promotion	Possible complementary	-	(93)

more extended pharmacological mechanism theories may be proposed. There are four plausible mechanism concepts that could be approached. The first theory is based on the major compound effects present in herbal formulation. This concept follows conventional pharmacology, the pharmacological effects are produced by the binding of the major compound to a specific biomolecule such as an enzyme or receptor. The second idea is synergistic interactions. This is when one or more compounds, through specific actions, boost the activity of an active compound in an herbal preparation, as shown in many reports, including the most recent one by Vidar et al (28). The compound helpers have no effect or relatively do not have a significant effect on a specific target when each metabolite is examined separately. Meanwhile, when they are mixed in a dosage form, the active compound has a significant effect. The third form is additive interaction, as results of coworking of compounds which have similar working target, either enzymes, receptors, or other biomolecules. When each compound is tested at a certain dose individually against a specific target, the output becomes low because all targets are not occupied. However, it should be noted here that more detailed mechanisms, such as partial agonis, allosteric, and so on, are outside of this review's coverage. On the other hand, when all compounds are formulated in a dosage form, it will result in a maximum effect, which might be due to a mode of action combination such as agonist and allosteric or due to total occupancy of the target.

Reintroducing complementary effect

The last plausible coworking form is the complementary effect, as mentioned by Zhang et al (77). This coworking action mode involves multiple compounds with multiple targets, all of which are directed to produce the same main outcome of a certain pathological class improvement. As with the active drugs in hypertension therapy, there are different drug classes, e.g., angiotensin converting

enzyme (ACE) inhibitor, angiotensin receptor inhibitor, diuretic, and so on (77,94-96). Those agents have different specific targets, but all those sub-mechanisms are directed to lower blood pressure. Furthermore, because this complementary mode of action consists of proposed complex biological pathways, it has become the current pharmacology network paradigm (97,98). Nevertheless, those interactions must consider the concentration and ratio of the involving compounds.

Drawback of current metabolomic techniques

Over the last decade metabolomic techniques have emerged in response to the demand for a pattern-oriented approach that bridges the gap between holistic and comprehensive perspectives (99). However, a significant portion of reports has focused on small molecule groups and short-chain primary metabolites such as short sugars, amino acids, and short fatty acids (100,101), whilst their output data often exhibit limited direct correlations with bioactivity data. On the other hand, macromolecules (polysaccharides, peptides, and fatty acids), conjugates, and minerals in the herbal preparations might have bioactivity contributions that mainstream analytical methods have overlooked (102-105). Considering the secondary metabolite platform, therefore, a point to argue here is that there is some missing information from whole connectivity between the total metabolites and biological activities (106,107). Despite still being at the level of preclinical studies, the number of synergistic interaction reports between primary metabolite and other chemical constituent groups are relatively supportive of their existence (108).

Contribution of non-secondary metabolites

Shi et al (86) reported that polysaccharides of edible fungal *Auricularia* and Tremellan and flavonoid of *Crataegus* sp. demonstrated a synergistic effect as anti-dyslipidemia in vivo. Yan *et al* (82) reported that the combination of lignan from *Schisandrae chinensis* fruits and polysaccharide from Astragalus sp. showed a synergistic interaction as a hepatoprotective effect on a rat model. The combination of polysaccharide with arabinogalactan type II side chains and piperine was reported to have a synergistic effect as a cough suppressor (84). In vivo tests of the combination of puerarin and polysaccharide from Pumpkin (109) and polysaccharides and ginsenoside Rb1 from Panax ginseng (80) exhibited some synergistic effects as antidiabetic type 2. Fang et al (85) reported that ginkgo flavonoids and *Coriolus versicolor* polysaccharides showed synergistic effects as antidementia agents in a rat model. The coworking effect between secondary metabolites and fatty acids was demonstrated by the combinations of polyphenols and omega-3 fatty acids as synergistic antidepressants (87) and procyanidin B3 and eicosapentaenoic acid as anti-inflammatory agents (88). Meanwhile, the cooperative action mode between polysaccharides and peptides was reported on glucagon peptide as glucose lowering effect of *Ilex paraguariensis* (91) and conjugated polysaccharides with polypeptide, hypoglycemic, hypolipidemic-antiatherogenic, anticoagulant, and antithrombogenic agents (110). The report of possible cooperative action mode between secondary metabolite and minerals has been revealed on flavonoids and trace element ion of Cu, Co, Mn, or Zn in the composition of tea infusion (93). However, the current reports on potential coworking action modes predominantly are still at in vivo level. It is understandable since the "wet laboratory work" of natural product studies is mainly directed toward bioassay guided isolation for lead compounds. As a consequence, research reports on the macromolecules and mineral activity and their interaction toward secondary metabolites as well as their specific mechanism in herbal pharmacology are found quite rare. Nevertheless, from those ample of reported data, the contributions of polysaccharides, peptides, fatty acids or their conjugates, and minerals toward functional bioactivities to the crude drugs, in particular in aqueous and polar extracts, cannot be ignored. Therefore, once a defined pharmacology class of crude drugs has been established, the standardization requirements must be comprehensively fulfilled for all chemical groups. It is likely an unfair scenario if the standardization of herbal preparations sticks to relying on one type of secondary metabolite group or certain chemical markers as the specific standardization purpose. To address the gap, a comprehensive physical-chemical standardization, including the efforts to accommodate the classical quantification of polysaccharides, peptides, and mineral are indispensable with the initial guide of the biological activities (Figure 1).

Pondering classic herbal standardizations

Based upon the simplified pharmacological approaches, it implies that the current extract standardization might not adequately represent all contributing chemical constituents, particularly in terms of the terminology of specific parameters (111,112). Therefore, a more far-reaching paradigm should be considered. Once biologically active samples have been confirmed, the appropriate chemico-physical profiling methods of all metabolite types and all possible contributing minerals should also be conducted. For conventional extract batch uniformity tests, some investigators use certain chemical markers from secondary metabolite groups, which are called as active markers with HPTLC or HPLC quantification methods (113-115). Nevertheless, the efficacy of active markers is insufficient to show how all chemicals in certain plant species or even multiple components work together. This concept is only relevant to the active major compounds that have been identified so far. For this reason, the active markers very often fall into the category

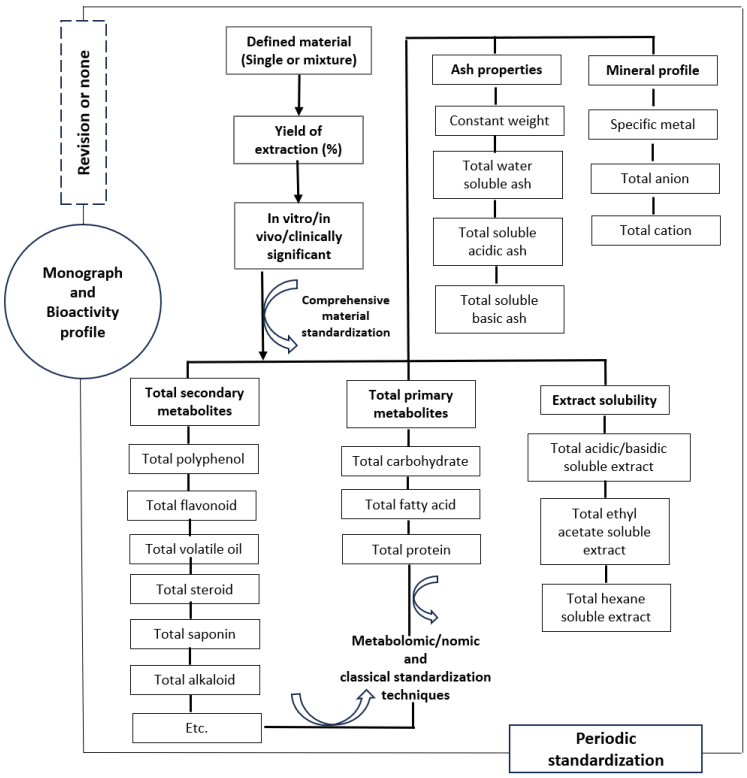

Figure 1. Flow chart of combination chemical finger printing and classical quantification of all chemical constituent groups.

of analytical markers without any correlation to quality, in particular efficacy (116). It is then clear that pattern-oriented approaches are more appropriate methodologies for possible synergistic, additive, and complementary interactions of compounds in herbal preparations. Current hyphenated instruments, in particular LC-MS and LC-NMR, in combination with chemometric platforms become choices as possibilities to provide "holistic or comprehensive views" in some senses (117,118). However, rather than a single system for all molecule groups, LC-MS instrumentations employ different solvents, stationary phases, and detection systems dependent upon the metabolite group in terms of molecular weight to accommodate a wide range of metabolites, including carbohydrates, peptides, fatty acids, and their conjugates. Therefore, for one sample, one might use several LC-MS types (79,119). Even though NMR spectrometry can cover all molecule groups with one spectrum, it is hard to imagine how many metabolite signals will overlap (120,121), and the sensitivity parameter and solvent choice solubility strength (122) become problems in the NMR method. Nonetheless, regarding the cost, in particular, LC-MS and NMR-based methods become issues for third-world countries. Considering these reasons, the scarcity, the high cost of the reference substances, and advanced instrumentation availability have been the three major bottlenecks in the implementation of multi-component routine quality control and pattern-oriented approach of herbal standardization. Thus, the extended standardization methods which align effectively with traditional practices, like the solubility of the total extract and ash in water, semi-polar solvents, and nonpolar solvents (Figure 1), could be reintroduced as the element of complete standardization approaches.

The traditional standardization methods based on colorimetry or gravimetry methods of total quantification of each metabolite group, such as flavonoid, polyphenol, alkaloid, tannin, coumarin, glucosinolate, protein, carbohydrate, fatty acid, and peptide groups, therefore, are still useful to fix the lack of thoroughness. Until recently, the vast majority of metal analysis in herbal medicines has been directed towards investigating their toxicity rather than recognizing their potential beneficial and fundamental contribution to human health. Further, assessing the total quantity of a specific mineral in an extract based on inductively coupled plasma-mass spectrometry (ICP-MS) could be applied since the interaction among any type of chemical constituent could be considered (123,124). ICP-MS is the current method of choice to quantify certain minerals or targeted mineral groups in very specific and sensitive quantification with a lower detection limit down to part per trillion (ppt) with efficient performance. Meanwhile, relatively uncovered matters by advanced analytical instruments in extract can be quantified by gravimetry methods with solubility division successively in water and ethanol prior

to the constant weight quantification. Ash characters representing whole acidic and basic minerals are dissolved in corresponding solvents before determining each constant weight according to USP (125). The chart of chemical group analysis and classical quantification of all chemical constituent groups is summarized in Table 2.

Periodic standardization and metabolite evolution

In addition to good agricultural practice implementations, considering the variability of regional resources and evolution's impact, official standards such as herbal pharmacopeia and monograph should be routinely evaluated every five to eight years. Several factors may trigger the secondary metabolite change due to hybridization (147), variability of herbivore-plant interaction (148) and insect resistance, and other environmental changes such as drought stress, salinity, higher-lower temperature, and soil microbial composition (149,150). These conditions will impact chemo-diversity on both metabolite composition and accumulation (151,152).

Conclusion

The bioassay guided fractionation program was only able to result in 0.01% clinically used drugs; besides, the purified matters have exhibited lower efficacy than that of the parent extracts. Thus, leveraging crude extracts will be more beneficial as the main raw material for health supporting products. Since the evidence based on herbal medicine is relatively lacking in data, a plethora of herbal medicine studies have been focused on secondary metabolites or macromolecules, which might not be so relevant to the traditional context, which is apparently based on complex constituents. As a consequence, once the bioactivity of an herbal extract is defined, conducting all aspects of chemical constituent groups is mandatory. Too much focus on the secondary metabolite group is apparently irrelevant to the basic pharmacological mechanism approach, which could result from multi-component interactions in the numerous biomolecular targets. Therefore, the quantification of primary metabolite groups such as polysaccharides, peptides, and fatty acids, as well as mineral content, will complete the data. Traditional methods such as gravimetry for constant weight determination and solubility in acidic or basic solvents of extract and ash are relevant to justify the basic character of material bulk and these methods have been unchangeable until now. Since ecological and climate changes could interfere with the chemical constituent in plant source, the periodic standardization may be conducted to provide official up dating on the official guides.

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Table 2. Chemical constituent groups and quantification methods

Constituent item	Method	References
Total polysaccharide	Phenol–sulfuric acid method, Colorimetry, Near-infrared hyperspectral imaging	(126-129)
Total fatty acids	One step extraction-esterification	(130,131)
Total peptides	Kjeldahl method, Spectrometry UV, Extraction with TCA-acetone	(132-134)
Specific mineral	ICP (inductively coupled plasma) Spectroscopy, AAS (atomic absorption spectrometry)	(135,136)
Total water-soluble extract	Gravimetry	(125)
Total alcohol-soluble extract	Gravimetry	(125)
Ash constant weight	Ignition method-gravimetry infrared spectrometry	(137)
Total ash soluble water	Fractionation-gravimetry	(125)
Total ash soluble in acidic water	Fractionation-gravimetry	(125)
Total ash soluble in basic water	Fractionation-gravimetry	(125)
Total polyphenol	Spectrophotometry	(138)
Total flavonoid	Spectrophotometry	(93,118,139)
Total coumarin	Spectrophotometry	(140)
Total steroid	Spectrophotometry	(141)
Total alkaloid	Gravimetry	(142-144)
Total glucosinolates	High performance liquid chromatography	(145,146)

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Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

The ethical clearance (number EC. No. 5116.2023) was approved by Health Research Committee of Universitas Muhammadiyah Surakarta according to the Helsinki Declaration 1975, Council for International Organizations of Medical Sciences (CIOMS), and World Health Organization (WHO) 2016.

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