Rational Design and Multi-Marker HPTLC Standardization of Polyherbal Antioxidant Formulations: A Review

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***Abstract*—Polyherbal antioxidant formulations are widely used in traditional and complementary medicine, yet their quality control is frequently limited to pharmacognostic and single-marker analysis, an approach insufficient for the complex, multi-herb matrices such products actually are. This review examines the rational, design-of-experiments-guided development and chemical standardization of a five-herb polyherbal antioxidant formulation comprising Phyllanthus emblica (Amla), Curcuma longa (Turmeric), Camellia sinensis (Green tea), Ginkgo biloba and Ocimum sanctum (Tulsi), optimised by a simplex-lattice mixture design for maximal phenolic and flavonoid content and free-radical-scavenging activity. The phytochemical basis, detection chemistry and structure-antioxidant-activity relationships of six representative markers, gallic acid, ascorbic acid, curcumin, catechin, quercetin and eugenol, are discussed alongside the validated high-performance thin-layer chromatography (HPTLC) fingerprinting methodology proposed for their simultaneous multi-marker quantification, the in-vitro antioxidant assay battery (DPPH, ABTS, FRAP, total phenolic and flavonoid content) against which the formulation is to be benchmarked, and the statistical framework proposed for correlating marker content with measured antioxidant activity. Precedent from twenty-nine directly relevant chromatographic, phytochemical and analytical-validation studies is synthesised to situate the proposed formulation within the wider marker-based standardization literature, and the regulatory basis for validating the resulting method is set out.**

***Index Terms*—Polyherbal formulation; HPTLC fingerprinting; multi-marker quantification; antioxidant activity; simplex-lattice mixture design; phytochemical standardization; DPPH; quercetin; curcumin; gallic acid**

I. INTRODUCTION

Oxidative stress, the imbalance between reactive oxygen species generation and endogenous antioxidant defence, is implicated in the pathogenesis of a wide range of chronic diseases including cardiovascular disorders, diabetes, neurodegeneration and cancer, and the free-radical chemistry underlying this imbalance is set out comprehensively in Halliwell and Gutteridge's standard reference on free radicals in biology and medicine [1]. Plant-derived polyphenols, flavonoids and other phytoconstituents are well documented as potent free-radical scavengers, and combinations of medicinal plants, polyherbal formulations, are widely used in Ayurveda and other traditional systems of medicine on the premise that multiple phytoconstituents acting synergistically confer superior therapeutic benefit compared with single-herb extracts.

Despite their widespread use, polyherbal formulations present a distinctive quality-control challenge: unlike single-molecule pharmaceuticals, they are complex, variable mixtures of dozens to hundreds of phytoconstituents, and pharmacopoeial standards for such products are frequently limited to organoleptic and pharmacognostic evaluation, insufficient to guarantee batch-to-batch chemical consistency, a limitation formally addressed in the World Health Organization's updated quality control methods for herbal materials [2] and in Mukherjee's systematic approach to the quality control and evaluation of botanicals [3]. High-performance thin-layer chromatography (HPTLC) has emerged as the

analytical technique of choice for herbal quality control owing to its low cost, high sample throughput, regulatory acceptance and ability to simultaneously resolve, visualise and quantify multiple marker compounds from a single chromatographic run. Marker-based standardization, selecting one or more chemically well-defined, pharmacologically relevant phytoconstituents from each constituent herb and validating an HPTLC method for their simultaneous quantification, is now the internationally recognised strategy for establishing the chemical identity and batch consistency of polyherbal products. This review synthesises the phytochemical, analytical and statistical literature supporting the rational development and multi-marker HPTLC standardization of a representative five-herb polyherbal antioxidant formulation, and situates the correlation of marker content with measured antioxidant activity as the specific methodological contribution that distinguishes this approach from conventional pharmacognostic standardization.

The literature synthesised here was drawn from primary phytochemical, chromatographic and analytical-validation studies together with the standard pharmacognosy and chromatography reference texts that establish the methodological baseline against which newer marker-based approaches are properly judged. Three bodies of evidence are brought together that are not always considered jointly in the polyherbal standardization literature: studies establishing the individual phytochemical and antioxidant credentials of each constituent herb, considered in Section 2; studies establishing HPTLC precedent for co-quantifying chemically diverse marker combinations, considered in Section 4; and studies, comparatively fewer in number, that go beyond marker quantification to statistically correlate chromatographic content with measured biological activity, considered in Section 7. The present formulation's proposed contribution lies specifically at the intersection of these three bodies of evidence, applying design-of-experiments rigour to the formulation step, ICH-compliant validation to the analytical step, and formal statistical correlation to the activity-linkage step, in a single coherent standardization package rather than treating any one of the three as sufficient on its own.

II. PHYTOCHEMICAL BASIS: THE FIVE CONSTITUENT HERBS AND THEIR MARKER PHYTOCONSTITUENTS

2.1 Phyllanthus emblica (Amla): gallic acid and ascorbic acid

Phyllanthus emblica fruit is among the richest known dietary sources of ascorbic acid and hydrolysable tannins, principally gallic and ellagic acid, and its antioxidant potency has been repeatedly confirmed by DPPH, FRAP and total phenolic content assays. Ridwan and colleagues' development and evaluation of a phytosome-based cream formulation from Phyllanthus emblica fruit extract confirms both the pharmaceutical formulability and the antioxidant relevance of Amla-derived polyphenols as a herbal marker source, directly supporting their inclusion as a chemically tractable standardization marker in the present formulation [4]. Gallic acid, a trihydroxybenzoic acid, and ascorbic acid, a water-soluble lactone-bearing sugar acid, together represent the two principal chemical classes, phenolic acid and ascorbate, through which Amla contributes to the formulation's overall antioxidant burden, and their markedly different polarities, gallic acid moderately polar and ascorbic acid highly polar, are a specific analytical consideration addressed in Section 4.

2.2 Curcuma longa (Turmeric): curcumin

Curcuma longa rhizome contributes curcumin, a symmetrical diarylheptanoid whose extended conjugation and phenolic hydroxyls confer potent radical-scavenging and metal-chelating activity, and which has been co-quantified with Amla-derived markers in prior HPTLC work. Manach and colleagues' review of polyphenol food sources and bioavailability situates curcumin within the wider dietary polyphenol landscape and documents the bioavailability limitations that, while a pharmacokinetic rather than a standardization concern, underline why chemical quantification of curcumin content in the finished formulation, rather than assumption of its presence from the rhizome input alone, is analytically necessary [5].

2.3 Camellia sinensis (Green tea): catechin

Camellia sinensis leaf is a well-established source of catechins, flavan-3-ols whose ortho-dihydroxy (catechol) B-ring substitution is mechanistically

linked to hydrogen-atom-transfer antioxidant activity. Nijveldt and colleagues' review of flavonoids, probable mechanisms of action and potential applications, catalogues this hydrogen-atom-donating capacity across the flavan-3-ol, flavonol and flavone subclasses and provides the mechanistic basis for treating catechin as a chemically representative, rather than merely conveniently available, marker of green tea's antioxidant contribution to the formulation [6].

2.4 Ginkgo biloba: quercetin

Ginkgo biloba contributes flavonol glycosides, principally quercetin and rutin, with well-documented free-radical-scavenging and metal-chelating activity. Amic and colleagues' structure-radical-scavenging-activity analysis of flavonoids identifies the C4-keto/C5-hydroxyl and catechol B-ring motifs of quercetin specifically as the structural determinants of both its radical-scavenging potency and its capacity for acid-stable metal chelation, the latter property exploited directly in the aluminium chloride-based total flavonoid content assay and HPTLC visualisation chemistry discussed in Section 5 [7].

2.5 Ocimum sanctum (Tulsi): eugenol

Ocimum sanctum contributes eugenol and related phenylpropanoids, valued in Ayurveda for combined antioxidant and anti-inflammatory activity. Unlike the polyphenolic markers contributed by the other four herbs, eugenol is a comparatively non-polar, monophenolic allylguaiacol, and Cheynier's account of the structural complexity of food polyphenols, together with Cai and colleagues' survey of antioxidant activity and phenolic compounds across a large panel of traditional Chinese medicinal plants, together establish that antioxidant capacity in plant-derived material is distributed across chemically heterogeneous classes rather than concentrated in a single compound type, reinforcing the rationale for a genuinely multi-marker, rather than single-marker, standardization strategy [8,9]. Selecting five herbs whose markers span phenolic acids, an ascorbate, a diarylheptanoid, a flavan-3-ol, a flavonol and a phenylpropanoid is intended to maximise the likelihood of synergistic, rather than merely additive, antioxidant activity when the extracts are combined in an optimised ratio, and to ensure that HPTLC standardization captures the chemical contribution of every constituent herb rather than only the most readily quantified one.

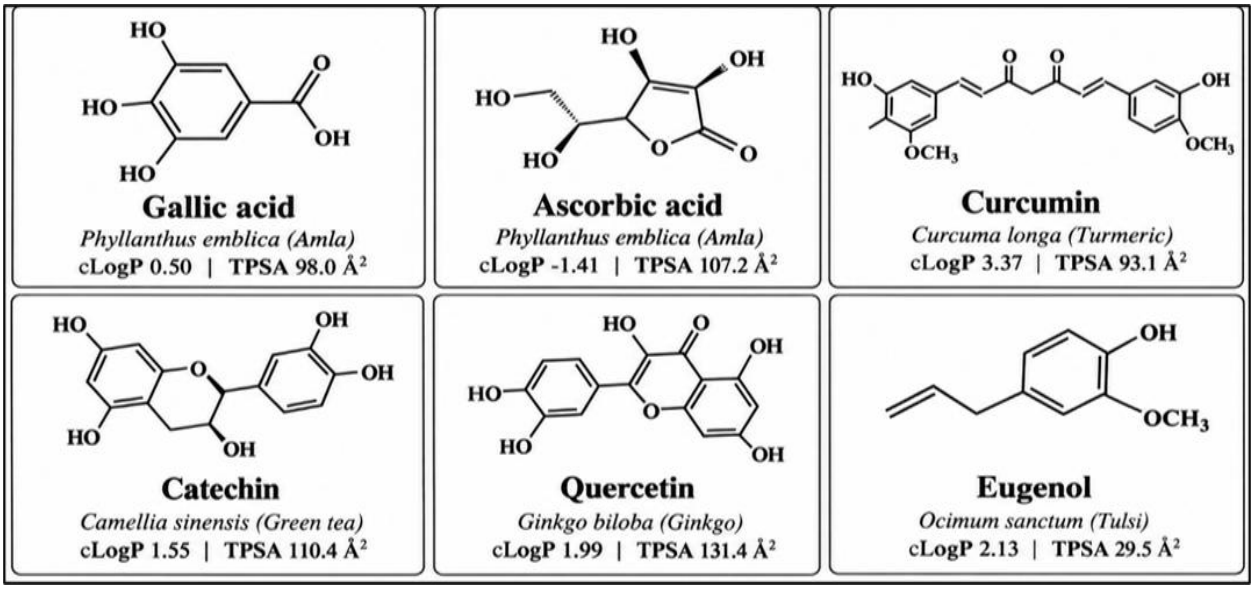


Figure 1. Structural panel of the six HPTLC marker phytoconstituents selected to represent the five constituent herbs, with cLogP and topological polar surface area (TPSA) computed in RDKit, illustrating the wide polarity range the multi-marker method must resolve [5,10].

III. RATIONAL OPTIMISATION OF THE POLYHERBAL RATIO: SIMPLEX-LATTICE MIXTURE DESIGN

Polyherbal formulations are traditionally combined in empirically fixed ratios rather than through a systematic optimisation process, a practice Mapeka and colleagues' design-of-experiments study of antioxidant synergy in polyherbal combinations of culinary herbs and spices directly addresses, using DPPH, ABTS and FRAP assays to identify synergistic extract pairs and providing methodological precedent for the mixture-design optimisation strategy adopted in the present formulation [11]. A simplex-lattice mixture design, in which each of the five herbal extract components is varied as a proportion of the total formulation subject to the constraint that all proportions sum to unity, is proposed to identify the extract ratio that maximises total phenolic content, total flavonoid content and DPPH radical-scavenging activity simultaneously. Cornell's definitive statistical reference on experiments with mixtures details the simplex-lattice and simplex-centroid design classes, the fitting of Scheffe polynomial models to mixture responses, and the specific constraint, distinct from a conventional factorial design, that a mixture design must respect, namely that the components are not independently variable but are instead constrained proportions of a fixed total, providing the statistical framework underpinning the rational optimisation of the herb ratio in the present study [12]. Design points comprising the five pure-component vertices, the binary edge midpoints and the overall centroid are proposed, augmented by interior check-points permitting a special-cubic model to be fitted should the quadratic model prove inadequate, with the model-predicted optimum ratio subsequently prepared and experimentally verified against the model's prediction rather than accepted on statistical grounds alone.

The five pure-component vertices serve a specific diagnostic purpose beyond simply anchoring the design space: each vertex batch, comprising a single herbal extract alone, provides the individual-extract antioxidant baseline against which any synergistic departure of a mixed-ratio batch can be judged, since a mixture response exceeding the linear combination of the constituent vertices' individual responses is the operational definition of synergy in a mixture-design context. The overall centroid, in which all five extracts are present in equal proportion, provides a further reference point representative of the empirically fixed, unoptimised ratio typical of conventional polyherbal practice, against which the model-predicted optimum can be compared to quantify the improvement, if any, that rational optimisation delivers over simple equal-weight combination. Fitting the measured total phenolic content, total flavonoid content and DPPH half-maximal inhibitory concentration at each design point to the corresponding Scheffe polynomial, and selecting between the linear, quadratic and special-cubic model forms on the basis of lack-of-fit testing rather than the coefficient of determination alone, is intended to ensure that the reported optimum reflects a genuinely well-fitted response surface rather than an artefact of an over-parameterised model.

IV. HPTLC AS THE ANALYTICAL PLATFORM FOR MULTI-MARKER STANDARDIZATION

4.1 Precedent for co-quantification of chemically diverse markers

A substantial prior literature has already applied HPTLC to the simultaneous quantification of structurally diverse phytoconstituent markers within polyherbal formulations, and the present proposal builds directly on this precedent. Khairnar and colleagues developed and validated an HPTLC method for the simultaneous estimation of quercetin, berberine, rutin and curcumin in a polyherbal anti-rheumatic formulation, achieving good resolution (Rf 0.093-0.663) and linearity (r2 greater than 0.997) across all four markers, providing a direct methodological precedent for multi-marker quantification of chemically diverse phytoconstituents of exactly the kind proposed here [13]. Kharat, Namdeo and Mehta developed and validated an HPTLC method for simultaneous estimation of curcumin and galangin in a polyherbal capsule formulation, achieving linear ranges of 80-450 and 200-1200 ng/spot respectively with correlation coefficients exceeding 0.9998, exemplifying the rigorous ICH-compliant validation standard the present method targets [14]. Ladva and colleagues standardized the patented polyherbal antidiabetic formulation SJT-DI-02 using four HPTLC markers, asarones, marmelosin, gallic acid and lupeol, demonstrating that marker-based HPTLC

fingerprinting can simultaneously authenticate and quantify chemically diverse constituents from an eight-herb formulation, a scale of chemical diversity comparable to the six-marker, five-herb system proposed here [15]. Jeganathan and colleagues standardized the Siddha polyherbal formulation Amukkara Curanam by HPTLC quantification of withaferine A and piperine, confirming that HPTLC-based multi-marker standardization is applicable across Ayurvedic, Siddha and other traditional polyherbal systems generally, not merely to a single formulation class [16]. Bidikar and colleagues developed a validated, regulatory-compliant USP-HPTLC protocol for quantification of polyphenols and antioxidants from polyherbal formulations containing Terminalia species, explicitly linking chromatographic marker quantification to antioxidant capacity assessment, the same conceptual link the present review's correlation framework, developed in Section 7, is designed to formalise statistically [17].

4.2 Method development across a wide polarity range

Because the six selected markers span a wide polarity range, from the highly polar ascorbic acid (TPSA approximately 107 A2) to the moderately polar gallic acid, catechin and quercetin, to the comparatively non-polar curcumin and eugenol, a single isocratic mobile phase may not resolve all markers simultaneously with adequate peak symmetry and resolution. Agatonovic-Kustrin and Morton's improved HPTLC-bioautographic method for direct visualisation and quantification of total free-radical-scavenging activity on the chromatographic plate itself offers a complementary bioautographic confirmation of antioxidant marker bands alongside the solution-phase assays discussed in Section 5, and demonstrates that HPTLC method development for chemically diverse marker sets can draw on precedent that goes beyond simple co-elution optimisation to include activity-guided plate visualisation [18]. Shah and colleagues' eco-friendly HPTLC method for the simultaneous estimation of gallic acid, ellagic acid and curcumin achieved a favourable AGREE greenness score while resolving all three markers with good linearity, directly validating the feasibility of co-quantifying Amla- and turmeric-derived markers of exactly the kind proposed in the present study, and Hazra and colleagues' rapid HPTLC method to estimate piperine across six Ayurvedic churna formulations further demonstrates the reproducibility of marker-based HPTLC standardization across diverse polyherbal matrices [19,20]. Reich and Schibli's practical reference on HPTLC analysis of medicinal plants, Sherma and Fried's standard handbook of thin-layer chromatography, and Wagner and Bladt's thin-layer chromatography atlas for plant drug analysis together provide the validated mobile-phase, derivatisation and densitometric precedent on which the present method's development is proposed to draw, so that method development proceeds from established practice for structurally similar marker combinations rather than requiring de novo discovery for every marker [21–23].

V. DETECTION CHEMISTRY OF THE SIX PHYTOCONSTITUENT MARKERS

Multi-marker HPTLC quantification and post-chromatographic visualisation rely on well-established detection chemistries that double, in several cases, as the same colorimetric reactions underlying the solution-phase antioxidant assays discussed in Section 7. Gallic acid, the Amla-derived phenolic acid marker, reduces the phosphomolybdic-phosphotungstic acid (Folin-Ciocalteu) reagent under mild alkaline conditions to a blue molybdenum-tungsten oxide chromophore read at 765 nm, the basis of the total phenolic content assay developed by Singleton and Rossi and benchmarked against gallic acid as reference standard [24]. Quercetin, the Ginkgo-derived flavonol marker, forms an acid-stable chelate with aluminium (III) at the C4-keto/C5-hydroxyl and catechol positions, the basis of both the total flavonoid content assay of Chang and colleagues, expressed as quercetin equivalents, and its independent use as an HPTLC chromogenic visualising reagent for flavonoid bands on the developed plate [25].

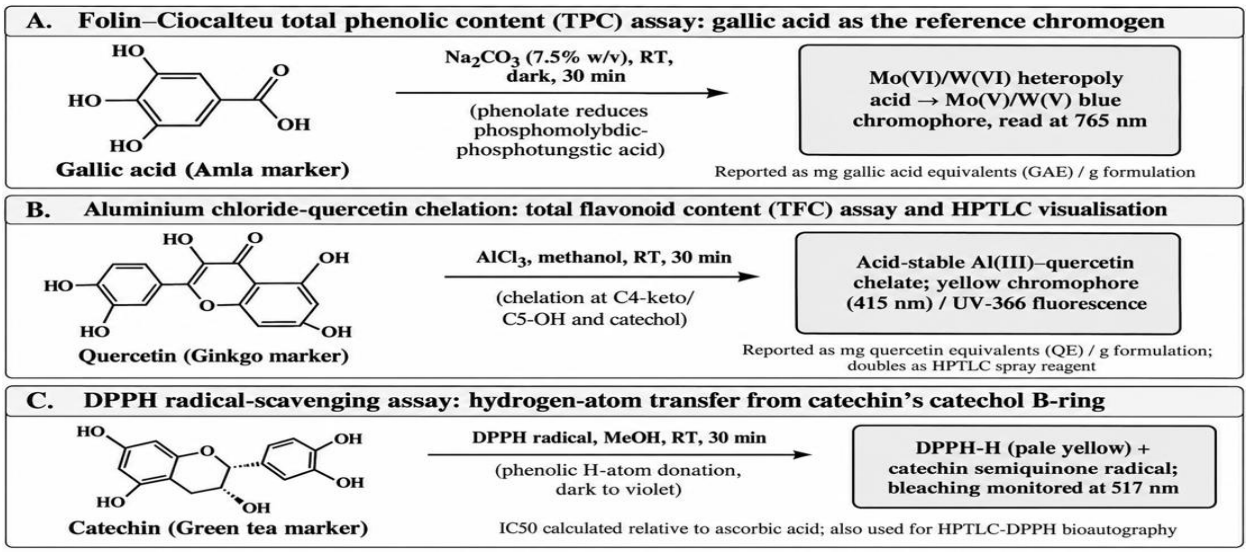


Figure 2. Detection chemistry linking three representative markers to their corresponding in-vitro antioxidant assay: (A) Folin-Ciocalteu total phenolic content assay of gallic acid; (B) aluminium chloride-quercetin chelation underlying the total flavonoid content assay and HPTLC visualisation; (C) DPPH hydrogen-atom-transfer assay of catechin [24–26].

Catechin, the green-tea-derived flavan-3-ol marker, donates a phenolic hydrogen atom from its catechol B-ring to the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical introduced by Blois, quenching its characteristic purple colour to the pale-yellow hydrazine DPPH-H, with the extent of bleaching at 517 nm used both in the solution-phase DPPH assay and in HPTLC-DPPH bioautography of the developed plate [26]. Ascorbic acid, the second Amla-derived marker, reduces the pre-formed blue-green 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation by single-electron transfer, itself being oxidised to dehydroascorbic acid, with the resulting decolorisation at 734 nm underpinning the improved ABTS radical-cation decolorisation assay of Re and colleagues [27]. Gallic acid additionally reduces the ferric-tripyridyltriazine (Fe(III)-TPTZ) complex to its intensely blue Fe(II) form at pH 3.6, the basis of the ferric reducing antioxidant power (FRAP) assay of Benzie and Strain, so that a single Amla-derived marker contributes chemically to three of the assay's five analytical readouts, total phenolic content, FRAP and, indirectly through its contribution to overall phenolic load, the DPPH and ABTS assays as well [28]. Curcumin and eugenol, the turmeric- and Tulsi-derived markers, are additionally distinguished on the developed plate by acid-catalysed condensation with vanillin and para-anisaldehyde respectively under charring conditions, generating characteristic-coloured bands used for densitometric scanning independent of the phenolic-specific derivatisation chemistries applied to the other four markers.

VI. STRUCTURE-ANTIOXIDANT ACTIVITY RELATIONSHIPS ACROSS THE MARKER CLASSES

The six selected markers are not chemically interchangeable proxies for a single generic antioxidant capacity; each represents a mechanistically distinct structural class whose radical-scavenging behaviour is governed by different structure-activity principles. Rice-Evans, Miller and Paganga's foundational structure-antioxidant-activity analysis of flavonoids and phenolic acids established that the number and arrangement of hydroxyl groups, particularly ortho-dihydroxy (catechol) substitution on an aromatic ring capable of delocalising the resulting phenoxyl radical, is the primary determinant of hydrogen-atom-transfer antioxidant potency across both flavonoid and simple phenolic-acid structural classes, directly rationalising why gallic acid's trihydroxy substitution and catechin's and quercetin's catechol B-rings are each independently effective radical scavengers despite their otherwise distinct core scaffolds [10]. Amic and colleagues extended this

structure-activity framework specifically to flavonoids, relating radical-scavenging activity quantitatively to bond dissociation enthalpy at the most reactive hydroxyl and to the degree of conjugation between the B-ring catechol and the chromone core, a relationship directly applicable to the comparison of catechin, a non-planar flavan-3-ol with a saturated C-ring, against quercetin, a fully conjugated flavonol, within the present six-marker set [7]. Ascorbic acid and curcumin depart from this phenolic hydrogen-atom-transfer paradigm in mechanistically distinct ways, ascorbic acid acting principally by single-electron transfer from its ene-diol moiety, and curcumin combining phenolic hydrogen-atom donation with the additional radical-stabilising and metal-chelating capacity of its extended, conjugated beta-diketone bridge, so that the formulation's overall antioxidant profile reflects the sum of at least three mechanistically distinct radical-scavenging strategies rather than one. Nijveldt and colleagues' review of flavonoid mechanisms of action further catalogues metal-chelation, principally of iron and copper, as a mechanistically separate antioxidant contribution to hydrogen-atom transfer, relevant to quercetin and, to a lesser extent, gallic acid and curcumin, and to the specific chelation chemistry exploited analytically in Section 5 [6]. Halliwell and Gutteridge's account of the broader free-radical biology within which these in-vitro assays are interpreted cautions that DPPH, ABTS and FRAP capture chemically distinct aspects of antioxidant behaviour, hydrogen-atom transfer, single-electron transfer and reducing power respectively, that need not correlate perfectly with one another or with biological antioxidant efficacy, a caveat directly relevant to the interpretation of the multi-assay correlation proposed in Section 7 [1].

VII. CORRELATING MULTI-MARKER HPTLC CONTENT WITH IN-VITRO ANTIOXIDANT ACTIVITY

A further limitation of conventional herbal standardization is that marker quantification is rarely linked back to the biological activity the formulation is intended to deliver. Hawryl, Hawryl and Waksmundzka-Hajnos combined liquid chromatography fingerprint analysis with chemometric calculations to correlate chromatographic profiles of lavender species with their antioxidant activity (DPPH, FRAP), establishing chemometric correlation as a validated approach for linking chromatographic fingerprints to bioactivity, the analytical strategy adopted for the present formulation [29]. Shalini and Ilango's preliminary phytochemical screening, GC-MS analysis and in-vitro antioxidant evaluation of selected medicinal plants and their polyherbal formulation confirmed that combined herbal extracts can exhibit enhanced antioxidant activity relative to single constituents, directly supporting the premise that the optimised five-herb blend, rather than any single constituent extract, is the appropriate unit of both chemical standardization and biological evaluation [30]. HPTLC-quantified marker content (percentage weight-by-weight of each of the six markers) is proposed to be correlated with DPPH, ABTS and FRAP activity using Pearson correlation coefficients and multiple linear regression, identifying the marker or markers contributing most significantly to overall antioxidant potency; because the assays differ mechanistically, as set out in Section 6, a marker correlating strongly with DPPH inhibition need not correlate equally strongly with FRAP reducing power, and the regression is accordingly proposed against each assay independently rather than against a single composite antioxidant score. This marker-activity correlation, rather than the mixture-design optimisation or the HPTLC method validation individually, is the specific element that converts a conventional pharmacognostic standardization exercise into a mechanistically interpretable quality-control package capable of informing future targeted enrichment of the formulation towards whichever marker the correlation identifies as principally responsible for its antioxidant potential.

VIII. COMPARATIVE PRECEDENT FOR HPTLC-BASED POLYHERBAL STANDARDIZATION

Table 1 summarises nine representative precedent studies spanning the marker-based HPTLC standardization literature, selected to illustrate the range of chemical diversity, validation rigour and activity-linkage already achieved across polyherbal formulations of varying composition and therapeutic intent. Shalini and Ilango's earlier macroscopic, microscopic and HPTLC densitometric analysis of

selected Indian medicinal plants and a polyherbal mixture, quantifying lupeol, rutin and shatavarin IV as chemical markers, establishes pharmacognostic authenticity as a prerequisite step preceding chemical standardization, a sequence the present formulation's plan of work also adopts by beginning with certified plant procurement and taxonomic authentication before extraction and marker analysis [31]. Salunkhe and colleagues determined the pharmacokinetic profile and oral bioavailability of diosgenin, charantin and hydroxychalcone from a polyherbal antidiabetic formulation using a validated bioanalytical HPTLC method, demonstrating that HPTLC utility extends beyond in-vitro quality control into pharmacokinetic marker quantification, a direction beyond the present formulation's immediate scope but indicative of the platform's broader analytical reach [32]. Kokate, Purohit and Gokhale's standard pharmacognosy reference and Trease and Evans's pharmacognosy text together detail the macroscopic, microscopic and chemical evaluation methods for crude drugs that underpin the pharmacognostic authentication step preceding chemical standardization in the present study, while Harborne's classic guide to modern techniques of plant analysis details the extraction, fractionation and chromatographic methods for the major phytoconstituent classes, phenolics, flavonoids and terpenoids, present in the five selected herbs [33–35].

Table 1. Representative HPTLC-based multi-marker standardization studies for polyherbal formulations

Study	Markers quantified	Formulation type	Ref.
Shah et al. (2024)	Gallic acid, ellagic acid, curcumin	Herbal formulation	[19]
Khairnar et al. (2023)	Quercetin, berberine, rutin, curcumin	Polyherbal anti-rheumatic	[13]
Bidikar et al. (2022)	Polyphenols/antioxidants (Terminalia)	Polyherbal (USP-compliant)	[17]
Shalini & Ilango (2022)	Lupeol, rutin, shatavarin IV	Polyherbal mixture	[31]
Kharat et al. (2017)	Curcumin, galangin	Polyherbal capsule	[14]
Hazra et al. (2019)	Piperine	Six Ayurvedic churna	[20]
Ladva et al. (2014)	Asarones, marmelosin, gallic acid, lupeol	SJT-DI-02 (8-herb, antidiabetic)	[15]
Jeganathan et al. (2008)	Withaferine A, piperine	Amukkara Curanam (Siddha)	[16]
Present formulation	Gallic acid, ascorbic acid, curcumin, catechin, quercetin, eugenol	5-herb polyherbal (mixture-design optimised)	--

IX. VALIDATION AND STABILITY: REGULATORY FRAMEWORK

The proposed HPTLC method is intended to be validated per the International Council for Harmonisation's Q2(R1) guideline on validation of analytical procedures, which specifies the linearity, accuracy, precision, specificity, detection and quantification limit, and robustness parameters against which the six-marker method is to be assessed [36]. Linearity is proposed across an appropriate nanogram-per-band calibration range for each marker individually, accuracy by recovery studies at multiple spiking levels, precision by intra-day and inter-day percentage relative standard deviation, specificity by spectral overlay comparison of sample and reference standard bands at the start, apex and end of each chromatographic peak, and robustness by deliberate small variation in mobile-phase ratio, chamber saturation time and plate batch. Because the optimised formulation is additionally proposed to be assessed for short-term stability under accelerated conditions, using the validated HPTLC method itself as a stability-indicating tool to monitor marker content over time, the International Council for Harmonisation's complementary Q1A(R2) guideline on stability testing of new drug substances and products provides the regulatory basis for that accelerated-stability design, specifying the storage conditions, testing frequency and acceptance criteria against which any decline in marker content, and by extension in antioxidant activity, would be assessed [37]. Read together, Q2(R1) and Q1A(R2) situate the present standardization package within the same regulatory framework applied to conventional pharmaceutical analytical methods, addressing directly the limitation, noted throughout the precedent literature reviewed in Section 4, that polyherbal quality control is too often confined to organoleptic and pharmacognostic

parameters that carry no equivalent validation standard.

9.1 Limitations of existing precedent and the present contribution

Several gaps recur across the precedent literature reviewed in this article. Most of the multi-marker HPTLC studies summarised in Table 1 validate a method for a formulation whose extract ratio was already fixed by the originating manufacturer or Ayurvedic recipe, rather than one arrived at through a documented optimisation process; the present formulation, by contrast, proposes to derive its extract ratio from a simplex-lattice mixture design before the HPTLC method is applied to the optimised product, so that standardization and formulation development are coupled rather than sequential, unrelated exercises. Second, only a minority of the precedent studies, principally those of Bidikar and colleagues and Hawryl and colleagues, explicitly link chromatographic marker content to antioxidant activity, and none of the nine studies summarised in Table 1 applies a mixture-design-optimised formulation, a six-marker HPTLC method and a formal Pearson/multiple-linear-regression correlation together within a single study, so that the present proposal's specific combination of all three elements, rather than any one of them individually, is the feature distinguishing it from existing precedent. Third, accelerated stability assessment using the validated HPTLC method as a stability-indicating tool, though methodologically straightforward given a validated method already in hand, is reported inconsistently across the polyherbal standardization literature, and its inclusion here is intended to ensure the resulting quality-control package addresses shelf-life as well as batch-release specification.

X. CONCLUSION

Polyherbal antioxidant formulations are widely used but rarely developed through a rational, evidence-based optimisation process, and their quality control is frequently limited to pharmacognostic evaluation rather than chemically validated, activity-linked multi-marker analysis. The literature reviewed here establishes four things with reasonable confidence: that HPTLC is the internationally accepted, regulator-compliant analytical platform for simultaneous multi-marker quantification of chemically diverse phytoconstituents in polyherbal formulations; that validated colorimetric assays with well-defined mechanistic bases exist for benchmarking the antioxidant potential of such formulations; that statistically robust precedent exists for correlating chromatographic marker content with antioxidant activity via Pearson correlation and chemometric or regression approaches; and that validated experimental-design methodology, specifically the simplex-lattice mixture design, exists for rationally optimising multi-herb extract ratios rather than relying on empirical formulation. Combining a mixture-design-optimised formulation, a validated six-marker HPTLC fingerprint, and a statistically formalised marker-activity correlation in a single coherent standardization package offers a scientifically rigorous route to a chemically standardized polyherbal antioxidant product suitable for regulatory documentation, and one whose specific contribution, treating pharmacognostic authentication as a necessary first step rather than a sufficient endpoint of standardization, addresses a gap that recurs across the precedent literature synthesised throughout this review.

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