



ISOLATION AND CHARACTERISATION OF FLAVONOIDS FROM OCIMUM SANCTUM LEAVES

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Article info

Received date:25-01-2026

Accepted date:03-05-2026

Published date:15-09-2026

ISSN;0975-8216

Keywords:

Ocimum sanctum, Flavonoids, Isolation, Structural characterisation, Tulsi

1. INTRODUCTION

Few medicinal plants carry as much cultural weight as holy basil. *Ocimum sanctum* Linn., known in most of India as tulsi, is grown in courtyards, offered in worship and brewed as a household remedy for coughs, fevers and stress. Reviews of its traditional and experimental uses list antimicrobial, anti-inflammatory, antidiabetic and adaptogenic effects, and much of that literature leans on the leaf [1, 2, 3]. The chemistry behind these claims is less settled than the folklore, because the leaf contains

Abstract

Holy basil (*Ocimum sanctum*, tulsi) is used widely in Indian medicine, and its leaves hold a mixed population of flavonoids that are often credited with its antioxidant and anti-inflammatory reputation. This review draws on published phytochemical and analytical literature to describe how those flavonoids are extracted, enriched, separated and identified. It covers solvent choice, chromatographic strategies, spectroscopic and mass spectrometric structure work, and the quantitation methods used for quality control. The review reports no new experimental data. The literature shows that several flavones and flavone glycosides have been characterised, but isolation reports are uneven, reference standards are scarce, and bioactivity is rarely tested on purified compounds.

volatile oil, triterpenoids, phenolic acids and flavonoids in the same extract. The leaf is also cheap and available through most of the

year, which partly explains why so many small laboratories choose it as test material. That convenience has produced a large volume of papers, though depth and methodological quality vary a great deal from one report to the next.

Flavonoids deserve separate attention. They are a large family of C₆–C₃–C₆ polyphenols that plants build in response to light, pathogens and oxidative stress, and they are the compounds most often invoked when an herbal extract is said to have antioxidant power [4, 5, 6]. In holy basil the flavone skeleton dominates, with apigenin and luteolin as the usual aglycones and a range of C- and O-glycosides attached to them [3, 7]. Knowing which of these are really present, and in what amounts, is the first step towards any standardised preparation. They also absorb ultraviolet light strongly, and that single property underlies most of the detection methods discussed below. Flavones such as apigenin and luteolin differ from flavonols and flavanones in the chemistry of ring C, which changes both their spectra and their behaviour on a column [8, 9].

Isolation and characterisation sound like routine laboratory tasks, yet the published record shows how much judgement they involve. A different solvent, a different drying method or a different column can change the apparent flavonoid profile of the same leaf. This review follows the path a flavonoid takes from fresh leaf to assigned structure, using published work on tulsi and on flavonoid methodology in general. It reports no new experiments. Its aim is to show what is established, where reports disagree, and what a laboratory would need to do to produce a reliable isolation. Where possible the sections below say which steps rest on tulsi-specific evidence and which are borrowed from work on other herbs, since the two are easy to confuse when a method is simply described as standard practice in the field.

2. PLANT MATERIAL AND PREPARATION

Everything downstream depends on the leaf. *Ocimum sanctum* is usually described as having green and purple forms, often called

Rama and Krishna tulsi, and some authors note differences in phenolic content between them, although taxonomic treatment of these forms is not uniform [1, 3]. Harvest season, altitude, soil and time of day all affect secondary metabolites in aromatic herbs. A voucher specimen lodged in a recognised herbarium is therefore the minimum record, since without it a later reader cannot tell which plant a given flavonoid profile describes [10]. Local names and market samples add further confusion, because several related basil species are sold under similar names in herbal markets, and substitution with *Ocimum basilicum* or other species is not unknown [3, 11]. Reporting the exact form studied is cheap and prevents years of later argument.

Drying is the next decision. Shade drying at moderate temperature is common in pharmacognosy practice, and oven drying above about 60 °C is generally avoided because heat-labile glycosides and ascorbic acid are lost and oxidative browning begins [12]. Freeze-drying preserves more of the native pattern but is costly for routine work. Leaves are then powdered and sieved to a defined particle size, because coarse powder extracts slowly and very fine powder clogs filters. Moisture content, ash values and foreign matter are checked against pharmacopoeial limits before extraction begins. Storage matters too. Powder kept in light and humid air loses phenolics steadily, so it is held in airtight containers away from light, and the date of grinding is recorded alongside the batch number so that any drift can be traced.

Defatting is often added before the main extraction. Light petroleum or hexane removes chlorophyll, waxes and much of the essential oil, which would otherwise smear across chromatographic columns and interfere with later spectra [12, 13]. The price is some loss of the least polar flavonoids, such as the methoxylated flavones, which dissolve partly in hexane. Several tulsi studies use this sequential extraction scheme, moving from non-polar to polar solvents, so that each fraction can be screened separately and the flavonoids concentrate in the ethyl acetate and alcohol fractions [3, 13]. Whether to

remove the oil by steam distillation first is a separate choice. The leftover marc then gives a cleaner polar extract, although it has by then been heated, and the effect of that heating on glycosides is seldom measured in tulsi studies.

Authentication is not only a matter of botany. Macroscopic and microscopic features, thin-layer chromatographic fingerprints and total phenolic assays together give a reasonable identity check for a batch of leaf [14, 15]. For flavonoid work, a quick fingerprint on silica plates sprayed with natural products reagent shows yellow, orange or green fluorescent bands under ultraviolet light that correspond to flavones and flavonols. Laboratories that record such a fingerprint for each batch can later explain why one extract behaved differently from another, which is more useful than a bare yield figure. Such fingerprints cost little and can be kept as a permanent record with each lot.

3. EXTRACTION AND ENRICHMENT

Flavonoid aglycones dissolve best in moderately polar solvents, whereas glycosides prefer aqueous alcohol. For that reason 60 to 80 per cent methanol or ethanol in water remains the workhorse solvent for tulsi leaf, and ethanol is favoured wherever the extract might later be used in a product [16, 17, 18]. Maceration at room temperature is gentle but slow. Soxhlet extraction is faster and exhaustive, though prolonged heating can hydrolyse or oxidise sensitive glycosides, so it suits aglycone-rich fractions better than glycoside work [13, 17]. Many authors keep some water in the solvent on purpose, since a water content of roughly a fifth to a half generally helps glycosides to dissolve and swells the plant tissue so the solvent can reach it. Fully absolute alcohol therefore tends to under-extract the more polar flavonoids.

Newer techniques try to shorten extraction time and reduce solvent use. Ultrasound-assisted extraction disrupts cell walls through cavitation, and microwave-assisted extraction heats the solvent and the plant water together, both giving comparable yields in minutes rather than hours for many polyphenols [16, 17].

These methods are described in general terms as greener alternatives. For tulsi specifically, direct comparisons of flavonoid yield are scarce, and the claimed advantages are mostly borrowed from other herbs, so a laboratory adopting them should confirm the benefit on its own material before relying on it. Solvent recovery is also part of the argument for these methods, because lower consumption cuts both cost and the amount of residual solvent that must later be removed from the extract [16]. Reproducibility depends on reporting power, frequency, temperature and vessel geometry, which many papers leave out.

A crude extract is too complex to separate directly, so an enrichment step usually comes first. Liquid-liquid partitioning of the aqueous alcohol extract against ethyl acetate and n-butanol pulls out flavone aglycones and glycosides respectively, leaving sugars, salts and proteins behind [12, 13]. Solid-phase extraction on reversed-phase or polyamide cartridges offers a more controlled alternative, since polyamide binds phenolic hydroxyl groups strongly and releases them with alcohol [9, 13]. Total flavonoid content is commonly tracked through these steps with the aluminium chloride colorimetric assay, which gives a useful relative number but not an absolute one [15, 19]. Recovery should be checked by spiking a representative aliquot with a known standard, so that losses during partitioning can be separated from a truly low content [20]. Without such a check, a missing compound may only mean that it was discarded in the wrong layer.

Choice of solvent also shapes what is lost. Acidified solvents help release bound phenolics but can cleave glycosidic bonds and create artefacts, while prolonged exposure to methanol may form methyl ethers or esters of other constituents [13, 18]. Reports that list a long series of flavonoids from a single hot methanolic extract therefore deserve a second look, since some of the compounds may arise during work-up rather than in the living leaf. Running a parallel extraction with cold solvent and comparing profiles is a simple control that few published studies describe. Reporting both profiles costs one extra extraction and sharply improves the credibility of any list of isolated compounds.

4. CHROMATOGRAPHIC ISOLATION

Open-column chromatography on silica gel remains the first separation tool in most natural product laboratories, with gradients of chloroform–methanol or ethyl acetate–methanol giving coarse fractions that are pooled on the basis of thin-layer behaviour [13, 14]. Flavone glycosides run poorly on silica because of tailing, so Sephadex LH-20 eluted with methanol is widely used as a second step. It separates by a mixture of size and adsorption, and flavonoids come off in an order that tracks the number of free hydroxyl groups [9, 12, 13]. Fractions are monitored on silica plates and visualised under ultraviolet light at 254 and 365 nm, then sprayed with natural products reagent, so that flavonoid-bearing fractions are recognisable by their fluorescence [14]. Column loading is kept low, commonly around one part of extract to thirty or more of adsorbent, because overloading ruins resolution.

Final purification is normally done by preparative or semi-preparative high-performance liquid chromatography on C18 columns, with water and acetonitrile or methanol modified by a little formic or acetic acid to keep phenols protonated and peaks sharp [13, 18, 21]. Photodiode array detection is particularly helpful for flavonoids because each class shows a characteristic two-band spectrum, with band I between about 300 and 380 nm and band II near 250 to 280 nm [9, 18]. Peak purity is judged from spectra across the peak, not from the chromatogram alone. Gradients are tuned so that the target peak elutes in a window free from the large caffeic and rosmarinic acid peaks that dominate many basil extracts [7, 22]. Fractions are collected by peak, evaporated under reduced pressure at moderate temperature, and stored cold under nitrogen where possible, since flavones with free catechol groups darken on standing.

Counter-current chromatography offers a different route. Because it uses two immiscible liquid phases and no solid support, there is no irreversible adsorption, which suits polyphenols that stick to silica [23, 24]. The difficulty lies in finding a solvent system with a suitable partition

coefficient, and empirical guides such as the GUESS approach were written to shorten that search [24]. Applications to tulsi flavonoids are few, so the technique is better described as promising than proven for this plant, although it has isolated flavonoids from many other leaves in good purity. Because partition and adsorption are decoupled, the same instrument can be run in a head-to-tail or tail-to-head mode, and sample loading is large compared with analytical columns. Those features make it attractive for the milligram quantities of rare glycosides needed for NMR, which is exactly the bottleneck described for tulsi.

Isolation is always judged by what comes out at the end. A flavonoid is generally regarded as sufficiently pure for spectral work when it appears as a single peak on two different chromatographic systems and shows no extra signals in the proton spectrum [13, 21]. Yields from a kilogram of dried leaf are typically a few milligrams to tens of milligrams for individual compounds, which explains why many publications stop at fractions or enriched extracts. Reporting the mass recovered at each stage would let readers judge whether a claimed isolation is realistic. A single compound in a figure caption is not proof of isolation unless the supporting data are shown. Authors who present chromatograms of the final material on at least two columns, with spectra and mass data in a supplement, give the reader a real chance to verify the claim.

5. STRUCTURAL CHARACTERISATION

Once a pure compound is in hand, identification proceeds in layers. Ultraviolet spectroscopy with diagnostic shift reagents, including sodium methoxide, aluminium chloride with and without hydrochloric acid, sodium acetate and boric acid, was the classic way to locate free hydroxyl groups on the flavone ring system [8, 9]. The method needs under a milligram and is still useful for a quick check, but it cannot place sugars or decide between isomers, which is why it is now treated as supporting evidence rather than proof. Rapid acquisition and modest sample needs made it popular in earlier decades, when mass spectral and NMR

facilities were rarer than they are today. Modern papers often retain it simply to show that the new compound behaves as expected.

Mass spectrometry supplies molecular mass and, through fragmentation, information on the sugar and aglycone parts. Electrospray ionisation in negative mode is typical for flavonoids, and tandem experiments can distinguish O-glycosides, which lose the sugar as a neutral fragment, from C-glycosides, which break within the sugar ring and show characteristic cross-ring losses [25, 26]. This distinction matters in holy basil, where orientin and vicenin-type C-glycosides have been reported alongside glucuronides of apigenin and luteolin [3, 27]. Mass data alone, however, rarely settle the linkage position. Sodium adducts and fragments from sugar chains can also complicate the interpretation of low-resolution spectra, so accurate mass measurement and an isotope pattern check are good practice before a formula is proposed [26].

Nuclear magnetic resonance is the decisive step. Proton and carbon spectra identify the substitution pattern of rings A and B, and two-dimensional experiments (COSY, HSQC, HMBC) connect protons to carbons across several bonds, locating the sugar attachment and its anomeric configuration [4, 5, 9]. Flavones show a distinctive one-proton singlet for H-3 near 6.5 to 6.8 ppm, and methoxyl signals mark compounds such as cirsimaritin and cirsilineol, which were isolated from tulsi leaf in work on cyclooxygenase inhibition [7]. Spectra are best acquired in deuterated dimethyl sulfoxide, which keeps hydroxyl protons visible. The anomeric proton signals and their coupling constants show whether a sugar is attached through an alpha or beta link, and long-range correlations to the aglycone carbons fix the point of attachment. For C-glycosides, the absence of a hydrolysable sugar and the shift of carbon 6 or 8 give the clue.

The reported chemistry of the plant is gathered in table 1, which sets published isolation and characterisation steps against what each step contributes to identification. The same information is tallied by category in

fig. 1. The pattern is clear enough: separation and enrichment steps are well documented in general, structural methods are mature, and the limiting factor for tulsi is the number of laboratories that have carried a single compound through the whole sequence and published the full spectral data. Readers should treat that table as a map of the evidence and not as an exhaustive inventory, because it condenses many studies into single lines. Entries without a tulsi-specific citation reflect borrowed methodology.

Table 1: Published steps in the isolation and characterisation of flavonoids from tulsi leaf

Step / method	Reported contribution	Ref.
Aqueous alcohol extraction 60–80% ethanol or methanol	Standard solvent for flavone aglycones and glycosides; heat can create artefacts.	[13, 17, 18]
Ultrasound or microwave assistance alternative to Soxhlet	Shorter extraction time with comparable polyphenol yield in other herbs; little tulsi-specific comparison.	[16, 17]
Liquid–liquid partition ethyl acetate, n-butanol	Concentrates aglycones and glycosides away from sugars and salts.	[12, 13]
Sephadex LH-20 column methanol elution	Separates flavonoids by hydroxyl content and size after silica fractionation.	[9, 13]
Preparative RP-HPLC C18, acidified mobile phase	Final purification with diode array confirmation of peak purity.	[18, 21]
Counter-current chromatography two-phase solvent system	No solid support loss; few tulsi applications reported so far.	[23, 24]
UV diagnostic shift reagents NaOMe, AlCl ₃ , NaOAc	Locates free hydroxyl groups on the flavone skeleton; supportive evidence only.	[8, 9]
ESI-MS/MS negative ion mode	Separates O- from C-glycosides and gives aglycone mass; linkage position remains uncertain.	[25, 26]
1D and 2D NMR DMSO-d ₆	Decisive for substitution pattern, sugar position and methoxyl groups, as in cirsimaritin.	[7, 9]
HPLC-UV quantitation ICH-validated	Gives content of marker flavones against authentic standards.	[20, 22]

6. QUANTITATION AND ACTIVITY

Quantitation turns an isolated compound into a quality marker. Reversed-phase HPLC with ultraviolet detection against an external standard curve is the usual method, and validation follows the ICH parameters of specificity, linearity, accuracy, precision, and limits of detection and quantitation [20]. Colorimetric assays for total flavonoids or total phenolics are cheaper and still common in tulsi papers, but they respond to non-flavonoid phenolics such as rosmarinic acid, so the number they give is an index and not a content [15, 19, 22]. Such methods also need a stable, pure standard, a defined extraction of the sample matrix and a stated stability period for the solutions, none of which is trivial for polyphenols that oxidise on standing [19, 20].

Biological claims should follow the chemistry, not precede it. Antioxidant activity of flavonoids is linked to the arrangement of hydroxyl groups, particularly the catechol group on ring B and the 2,3 double bond conjugated with the 4-keto group [28, 29, 30]. Tulsi leaf extracts show radical scavenging, anti-inflammatory and radioprotective effects in laboratory models [31, 32, 33], and phenolics from the leaf inhibit cyclooxygenase enzymes *in vitro* [7]. Most of those studies used crude extracts, so attributing the effect to flavonoids specifically is an inference, not a measurement. A rigorous design would pair the purified compound with the crude extract at matched content, so that any added or lost activity can be recognised, and would state the concentrations in molar terms to allow comparison between papers [28, 30, 33].

7. LIMITATIONS AND FUTURE DIRECTIONS

Several weaknesses run through the tulsi literature. Many papers name a plant but not the cultivar, collection site or voucher number, so results cannot be compared. Others report flavonoid identities from a single technique, often ultraviolet absorption or a colour reaction, and these assignments cannot be treated as firm [8, 10]. Mass balance is seldom given. And the term flavonoid is sometimes stretched to include

any phenolic, which inflates apparent diversity and clouds the link between a named compound and an observed effect [4, 6, 34]. Better reporting standards in the journals that publish this work would help as well, since a short checklist at submission could catch most of these omissions before they reach print [10, 21].

Practical improvements are not difficult to list. Authenticated, documented plant lots; cold and hot extraction compared side by side; orthogonal separation steps; full one- and two-dimensional NMR data deposited with each new report; and validated HPLC methods using purified marker compounds would together lift the standard considerably [18, 20, 21]. Biological testing on the purified flavonoids, with solvent and positive controls and with attention to bioavailability, would then show whether they account for the activities attributed to the whole leaf [30, 33, 35]. Collaboration between phytochemists, analysts and pharmacologists is the practical route, because no single group usually holds all the skills required to take one compound from leaf to validated marker. A shared repository of tulsi spectral data would make each new isolation easier to confirm and cheaper to carry out [9, 25].

8. CONCLUSION

Flavonoids from *Ocimum sanctum* leaves can be isolated and identified with methods that are mature and widely available: aqueous alcohol extraction, partitioning, gel and reversed-phase chromatography, and structure assignment by mass spectrometry and NMR. The published record shows apigenin, luteolin and several methoxylated and glycosylated relatives, though the number of compounds fully described is smaller than the number named [3, 7, 27]. Within a laboratory with ordinary equipment, a flavone can be taken from leaf to confirmed structure in a few weeks if the material is authenticated and the work is planned around the quantities each technique demands [9, 13]. The harder question is whether the compound is the one responsible for any given biological effect.

The most useful progress would come from better documentation instead of new techniques: authenticated material,

stage-by-stage yields, full spectra, validated quantitation against pure standards, and activity tests on purified compounds. Until those are routine, tulsi flavonoid claims should be read as plausible leads and not as settled chemistry [20, 21, 33]. For readers who work with tulsi products, the practical message is modest. Marker-based standardisation using two or three well-characterised flavones, quantified by a validated method, is feasible today and would be an improvement on total flavonoid figures [19, 20, 22].

9. ABBREVIATIONS

COSY-Correlation spectroscopy,
ESI-Electrospray ionisation,
HMBC-Heteronuclear multiple bond correlation,
HPLC-High-performance liquid chromatography,
HSQC-Heteronuclear single quantum coherence,
ICH-International Council for Harmonisation,
MS-Mass spectrometry, NMR-Nuclear magnetic resonance, UV-Ultraviolet, DMSO-Dimethyl sulfoxide, GUESS-Generally useful estimate of solvent systems

10. FUNDING

Nil

11. AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

12. CONFLICT OF INTERESTS

Declared none

13. REFERENCES

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