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Review

Separation methods of quinonoid constituents of plants used in Oriental traditional medicines

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Abstract

Analysis of molecular constituents of traditional Oriental medicines has acquired a fresh perspective in view of a surge in interest in the consumption of herbal prescriptions all over the world. Several of them contain quinonoid compounds, and the long-standing therapeutic applications of these herbs have been vindicated, to some extent, through recent studies on the significant pharmacological properties of these compounds. In fact, the bioactive quinonoids and their analogues often serve as the 'marker' constituents of the respective plants of major commercial importance. Hence, shikonin, plumbagin, diospyrin, emodin analogues, sennosides, hypericin, tanshinone and related compounds have been discussed in this review which focuses on their extraction, separation and analysis from plant sources, cell cultures and biological fluids. As for the analysis of quinonoids, high-performance liquid chromatography connected with various detectors (ultraviolet, photodiode array, fluorescence, mass, nuclear magnetic resonance) has been the most useful technology succeeding the conventional methods such as thin layer and column chromatography. In some cases, high-performance thin layer chromatography and capillary electrophoresis are also used for this purpose.

Keywords: Reviews; Quinonoids separation; Medicinal plants

Contents

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Nomenclature

APCI	atmospheric pressure chemical ionization
ASE	accelerated solvent extraction
CC	column chromatography
CE	capillary electrophoresis
DCM	dichloromethane
DMSO	dimethyl sulphoxide
ESI-MS	electrospray ionization-mass spectroscopy
FL	fluorescence
HPTLC	high-performance thin layer chromatography
HSCCC	high-speed counter current chromatography
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
PLE	pressurised liquid extraction
PTLC	preparative thin layer chromatography
RP	reversed phase
r.t.	room temperature
SFE	supercritical fluid extraction
SPE	solid-phase extraction
SJW	St. John's wort (*H. perforatum*)
TLC	thin layer chromatography

1. Introduction

Traditional remedies based on natural products could be traced back over five millennia to written documents of the early civilizations, for example, Ayurveda in India, Pen Ts'ao in China, Kampo in Japan and Unani system of medicine in Near East [1]. Plant compositions continue to be the mainstay for health care even to this day in their respective countries of origin, mostly in the tropical and developing regions of the world. However, it is worthy to note that a tremendous surge of interest in herbal formulations has occurred all over the world in the past couple of decades; the consumption of medicinal plants has almost doubled in Western Europe [2], while 12.1% of the population in the USA spent roughly 5 billion dollars on non-prescription botanical drugs in 1997 [3]. In fact, according to WHO, 80% of the world's population still continue to rely on traditional medicines, and certain guidelines for assessment and standardization of such products have been recommended [4]. Thus, the role of phytochemical analysis has attained a new dimension in addition to its well-acknowledged input in finding pharmacologically active ingredients as 'leads' for drug development. Furthermore, a major impetus was generated towards improving the techniques of separation and analysis of plants of commercial importance for their standardization in terms of these recognized molecular entities [2,5]. It was also important to adapt these techniques to the requirements of pharmacokinetic estimation of biological fluids in order to establish the validity of the clinical usage of herbal products. Again, by this time, tissue culture techniques have started large-scale production of some useful plant metabolites, which have to be analyzed from the culture media.

In 1985, Farnsworth et al. [6] had reported the application of at least 119 compounds, obtained from 90 plant species, as constituent of medicines in one or more countries, and more than 70% of these were derived from plants used in traditional practices [1,7]. Thus, plants are the repositories for bioactive organic molecules among which quinones represent a class of ubiquitous secondary metabolites [8]. Some of them are components of the respiratory system of the plant cells playing important roles in the biochemistry of energy production. Quinones serve as vital links in the electron transport chains in the metabolic pathway by virtue of their capacity to undergo facile redox reactions [9]. This facility also leads to the inherent cytotoxicity of quinones, some of which might defend their hosts from invading pathogens, cf. bacteria, fungi or parasites. No wonder that quinones today form one of

Table 1
Separation of naphthoquinonoids (Figs. 3 and 5)

Compound	Sample	Extraction process	Separation technique	Reference
Phylloquinone	Food; human plasma	Extracted with hexane; washed with MeOH and H_2O; hexane layer evaporated; dissolved in MeOH and DCM	HPLC–FL	[66]
Shikonin	*L. erythrorhizon* root culture	Extracted with Et_2O; dissolved in $CHCl_3$	CC	[95]
Shikonin	*L. erythrorhizon* cell culture	Sonicated with MeOH (0 °C, 90 min); centrifuged (12,000 × g, 5 min); supernatant mixed with MeOH	HPLC	[11]
Shikonin	*L. erythrorhizon* root	Stirred with benzene (3 h); filtrate evaporated; dissolved in light petrol; hydrolysed (2% NaOH, stir 4 h); worked up (light petrol)	HSCCC–HPLC	[30]
Lawsone	*Impatiens glandulifera* leaf, stem, flower	Stirred with aqueous EtOH (35%, v/v; r.t., 30 min)	HPLC	[27]
Juglone	*J. regia* leaf	Macerated with $CHCl_3$ (r.t., 1 h); filtered; evaporated; dissolved in MeOH	HPLC	[117]
Plumbagin	*P. zeylanica* root	Extracted with aqueous EtOH (95%, v/v); partitioned between H_2O and *n*-hexane followed by EtOAc and *n*-BuOH; EtOAc extract collected	CC	[99]
Plumbagin	*P. zeylanica* root	Extracted with *n*-hexane–$CHCl_3$; diluted with $CHCl_3$	HPLC	[26]
Diospyrin	*Diospyros montana* stem bark	Refluxed with $CHCl_3$ (2 h)	HPTLC	[137]
Diospyrin	*D. montana* stem bark	Extracted with petrol ether–$CHCl_3$; refluxed successively with EtOH and $(CH_3)_2CO$; filtrate evaporated	HPLC	[25]

the largest classes of anti-cancer drugs, viz. the anthracycline antibiotics, for clinical use in the USA [10]. However, plant-derived quinonoids, despite their obvious usefulness as potential pharmacophores for drug design, have not so far been a much sought-after category as compared to alkaloids, flavones or terpenoids. Nevertheless, it transpires from a preliminary survey that a number of quinonoid compounds are present as the 'marker' constituents in some of the plants used in popular herbal preparations. Thus, in this review we would present the methods for separation of some of the predominant quinonoids present in these plants.

2. Quinonoids from Oriental medicinal plants

A variety of plant-derived quinonoids, most of them of the 1,4-type, will be discussed here, which comprise some benzo- and naphthoquinones (Sections 4.1 and 4.2), while the majority belongs to the anthraquinonoid class (Section 4.3). Two important types of dianthrones, viz. sennosides and hypericins, are also included in this review. Tanshinones, a group of diterpenes with an abietane-type skeleton containing a 1,2-quinone in the C-ring, have been considered as phenanthraquinonoids (Section 4.4).

3. Separation of quinonoids

3.1. Extraction methods

Extractions of quinonoids as summarized in Tables 1–5, were generally carried out using organic solvents like hexane, chloroform, acetone, acetonitrile, ethyl acetate, methanol, etc. either at ambient temperature or at refluxing condition. However, in some cases, homogenisation of the plant material in cold followed by sonication was performed to isolate sensitive compounds like shikonin [11] or aloe-emodin [12]. Ultrasonic bath was used to extract phylloquinone [13] and naphthodianthrones [14,15]. Samples of rhubarb, senna and herbal mixtures were also sonicated to extract the anthraquinonoid constituents [16–19]. Recently, microwave-assisted extraction was applied for isolation of tanshinones from *Salvia*

Table 2
Separation of alizarin and analogues (Fig. 6)

Compound	Sample	Extraction process	Separation technique	Reference
Alizarin, lucidin, ruberythric acid, lucidin primeveroside	Root and culture of *R. tinctorum*	Extracted with $CHCl_3$ and aqueous KOH at pH 5	HPLC	[21]
Alizarin	*R. tinctorum* cell culture	Sonicated in aqueous EtOH soaked (80 °C, 10 h); centrifuged; supernatant evaporated; dissolved in EtOH for SPE purification	HPLC	[153]
Alizarin, lucidin, ruberythric acid, lucidin primeveroside	*R. tinctorum* root	Refluxed with H_2O–EtOH (75:25, v/v; 6 h); filtrate diluted with H_2O–MeOH (50:50, v/v)	HPLC–PDA	[33]
Alizarin, lucidin, ruberythric acid, lucidin primeveroside	*R. tinctorum* root	Refluxed with H_2O–EtOH (50:50, v/v; 3 h); filtrate evaporated; suspended in H_2SO_4 (2%, v/v), refluxed; aliquots taken at intervals up to 48 h; diluted with H_2O–MeOH (50:50, v/v)	HPLC–PDA–MS	[44]

Table 3
Separation of emodin and its analogues, and sennosides (Figs. 7 and 8)

Compound	Sample	Extraction process	Separation technique	Reference
Emodin, physcion and their glycosides	*Polygonum hypolucum*	Extracted with MeOH (50 °C, 24 h); evaporated; residue partitioned with H_2O and *n*-BuOH (25:75, v/v); organic layer evaporated	Flash CC	[158]
Emodin, chrysophanol, rhein and their glycosides	Rhubarb root; senna leaf	Stirred with H_2O–MeOH (50:50, v/v; 15 min); filtered; extracted with $CHCl_3$; acidified worked up	HPLC	[22]
Sennosides A and B	*Cassia angustifolia* root, pod	Extracted with *n*-hexane; residue extracted with MeOH–H_2O (70:30, v/v)	HPLC–PDA	[176]
Rhein	*Rheum palmatum*	Sonicated with EtOAc (30 min); supernatant evaporated, dissolved in MeOH	HPLC	[17]
Rhein	Plasma, urine, CSF of rats fed with *R. palmatum*	Shaken with MeOH–EtOAc (12:88, v/v; 1 min); centrifuged (3000 rpm, 20 min); supernatant evaporated; dissolved in MeOH	HPLC	[17]
Emodin, aloe-emodin, rhein, physcion, chrysophanol, sennosides A and B	Rhubarb	Sonicated with aqueous MeOH (75%; 30 min); centrifuged; supernatant collected	HPLC	[18]
Aloe-emodin	Plasma of mice treated (i.p.) with aloe-emodin	Diluted with H_2O (50:50, v/v); shaken with DCM (30 min); centrifuged (4000 rpm, 10 min, 4 °C); organic phase dried under N_2; dissolved in MeOH; centrifuged (14000 rpm, 10 min); supernatant collected	HPLC–FL	[169]
Rhein	Human plasma from volunteers fed with *R. undulatum* root	Shaken with MeOH; centrifuged (3000 × *g*, 15 min); supernatant dried under N_2; reconstituted with MeOH; centrifuged (1400 × *g*, 15 min)	HPLC	[172]
Emodin, aloe-emodin, rhein, physcion, chrysophanol	Rhubarb root	Refluxed with 2N H_2SO_4 and $CHCl_3$; extracted with $CHCl_3$; dried; dissolved in 36 mM TEAP buffer (pH 2.5)–EtOH (65:35, v/v)	HPLC	[24]
Aloe-emodin, barbaloin	*A. barbadensis*, *A. natalensis*	Homogenised (MeOH, 0 °C); sonicated; centrifuged; supernatants collected	HPLC	[12]
Emodin, aloe-emodin, rhein, physcion, chrysophanol	*R. palmatum*	Sonicated with CH_3CN (30 min); centrifuged	CE–PDA	[16]
Sennosides A and B	*C. angustifolia* pod	Extracted with aqueous EtOH–H_2O; freeze dried	LC–MS	[42]
Rhein	*R. officinale*	Refluxed with EtOH; evaporated	HSCCC–HPLC	[170]
Emodin, aloe-emodin, rhein, physcion, chrysophanol	*R. officinale*	EtOH extract refluxed with EtOH–25% HCl (5:1, v/v; 4 h); worked up; extracted with diethyl ether	HSCCC–HPLC	[171]

Table 4
Separation of hypericins (Fig. 9)

Compound	Sample	Extraction process	Separation technique	Reference
Hypericin, pseudohypericin, protohypericin, protopseudohypericin	*H. perforatum* flower, leaf	Dissolved in MeOH; sonicated, centrifuged in absence of light	CE–electro-osmotic flow	[14]
Hypericin, pseudohypericin	*H. perforatum* extract	SFE with CO_2 (450 bar, 55 °C); residue macerated with aqueous EtOH (50 °C, 5 h); filtrate dried (100–140 mbar, 50–60 °C)	HPLC	[29]
Hypericin, pseudohypericin	*H. perforatum* extract	Sonicated with MeOH (−11 °C) in absence of light; centrifuged; supernatant collected	HPLC–PDA	[15]
Hypericin, pseudohypericin	*H. perforatum* extract	Sonicated with MeOH; diluted	HPLC–PDA	[38]
Hypericin, pseudohypericin	Plasma from human volunteers treated with *H. perforatum* extract	Extracted with a mixture of CH_3CN and (diluted H_3PO_4 + MeOH + THF) at 40 °C; supernatant collected; evaporated under N_2; reconstituted in the mixture (diluted H_3PO_4 + MeOH + THF) and 10% pyridine in MeOH	HPLC–UV–FL	[180]
Hypericin	*H. perforatum* culture	Extracted with $(CH_3)_2CO$–EtOH–MeOH (1:1:1, v/v/v); sonicated	LC–ESI-MS	[43]
Hypericin, pseudohypericin	*H. perforatum* plant	Extracted in hot MeOH	HPLC–PDA–ESI-MS	[40]
Hypericin, pseudohypericin, protohypericin, protopseudohypericin	*H. perforatum* extract	Extracted with MeOH (75%); for SPE purification	HPLC–UV–NMR–MS	[183]

Table 5
Separation of phenanthraquinonoids and tanshinones (Figs. 10 and 11)

Compound	Sample	Extraction process	Separation technique	Reference
Denbinobin, moniliformin	*D. moniliforme* stem	Sequentially extracted with *n*-hexane, EtOAc and MeOH (r.t.); residue of EtOAc extract collected for CC	CC–PTLC	[184]
Cryptotanshinone	*Perovskia abrotanoides* root	Sample extracted with EtOAc; evaporated	Vacuum LC	[196]
Tanshinone I, tanshinone IIA, cryptotanshinone	*S. miltiorrhiza* root	Mixed with aqueous EtOH; irradiated in microwave (80 °C, 25 s)	HPLC	[20]
Tanshinone IIA	*S. miltiorrhiza* root	SFE with CO_2, phytosol solvent extraction	HPLC	[28]
Tanshinone I, tanshinone IIA, cryptotanshinone	*S. miltiorrhiza* root	Extracted with MeOH; evaporated; worked up with H_2O and light petrol	HSCCC–HPLC	[31]
Tanshinone I, tanshinone IIA, cryptotanshinone	*S. miltiorrhiza* root	Extracted with light petrol	HSCCC–HPLC	[199]
Tanshinone I, tanshinone IIA, cryptotanshinone	*S. miltiorrhiza* root	Shaken with *n*-hexane–EtOH (50:50, v/v); centrifuged; supernatant diluted (1:2) with H_2O; organic layer washed with aqueous EtOH	HSCCC–HPLC	[200]

miltiorrhiza [20]. Many anthraquinonoid metabolites exist along with their glycosides in roots of madder and rhubarb, and leaves and fruits of senna. Thus, samples were first extracted with aqueous alcohol, followed by hydrolysis to separate the aglycon part [21–23]; the two steps could be performed simultaneously also [24]. Although quinonoids have been extracted by using various solvents, to our experience [25], chloroform or dichloromethane would be the solvent of choice for getting the maximum yield of the lipophilic constituents [26]; this has been established elegantly in the work of Liu et al. [24]. However, for an exception like lawsone, the tautomeric form helps it to be extracted preferentially with aqueous ethanol [27]. Other recent developments in extraction technology as applied to quinonoids are the use of a non-CFC solvent like phytosol [28], and liquid carbon dioxide for SFE of *Salvia* and *Hypericum* spp. [28,29].

3.2. Analytical techniques

Most of the naturally occurring quinonoids could be separated by conventional methods like TLC, PTLC and CC [8], which have now largely been supplemented by HPLC, one of the most useful techniques for the analysis of natural products in complex biological matrices (Tables 1–5). Comparison of chromatograms, used as fingerprints, between authentic samples and unknowns, leads to the identification of drugs and/or search for adulterants. However, hydroxyquinonoids often tend to remain strongly adsorbed in the silica gel column. This problem has been efficiently countered by HSCCC, which is a support-free liquid–liquid partition chromatography, and eliminates the irreversible binding of the sample onto the solid support. HSCCC is applied for analytical as well as preparative separation of a few quinonoids of scientific and commercial importance [30,31]. ASE and PLE are some other techniques for improving the yields obtained by conventional methods [32]. Again, droplet counter current chromatography was used to purify the quinonoids present in commercial ruberythric acid [33].

Hyphenated methods such as HPLC coupled to PDA, MS and NMR in combination with biological screening have lately been developed for rapid survey of natural products. However, as of now, these coupled techniques are expensive, and do not allow a full on-line identification, except for some well-known compounds, such as phylloquinone [13]. Other quinonoids reportedly isolated by such methods are 6-alkyl-substituted naphthoquinones from a Panamanian shrub *Cordia linnei* [34] and a ‘quinone methide’ from *Bobgunnia madagascariensis* from Zimbabwe [35], none of which are used in Oriental medicines. Detectors are based mostly on UV, and in some cases fluorescence or electrochemistry provided good detection of some quinonoids [36,37]. HPLC coupled with PDA detection has been used by many workers [38,39]. Some of the LC–MS interfaces are ESI and APCI [40,41]; such hyphenated techniques were used to analyze sennosides [42], hypericin [43] and alizarin analogues [44]. More reports on quinonoids in the near future are highly warranted, going by the recent reviews on the applications of such nascent technologies for separation of many bioactive phenolic compounds and flavonoids [45]. Recently, capillary electro-chromatography was applied for analysis of quinonoids [14,16]; this method when coupled to MS allows a rapid characterisation and quality control of quinonoid components in natural products [46]. Examples of some recent techniques employed for separation and detection of quinonoids are presented in Fig. 1a–e.

4. Overview of analysis of quinonoids

4.1. Benzoquinonoids

The first compound tested for anti-tumour activity in 1955 by the National Cancer Institute, USA, was a simple quinone, 2-methyl-*p*-benzoquinone [47]. Nevertheless, only a few of these compounds, e.g. irisquinone (Fig. 2) obtained from plant sources like *Iridaceaelatea pallasii* (Iridaceae) and used in antineoplastic Chinese medicines, has been found to be effective against transplantable rodent tumours [48,49]. It was also isolated from *Iris kumaoensis* in India [50], and used as a sensitiser for radiation therapy of cancer [51]. An

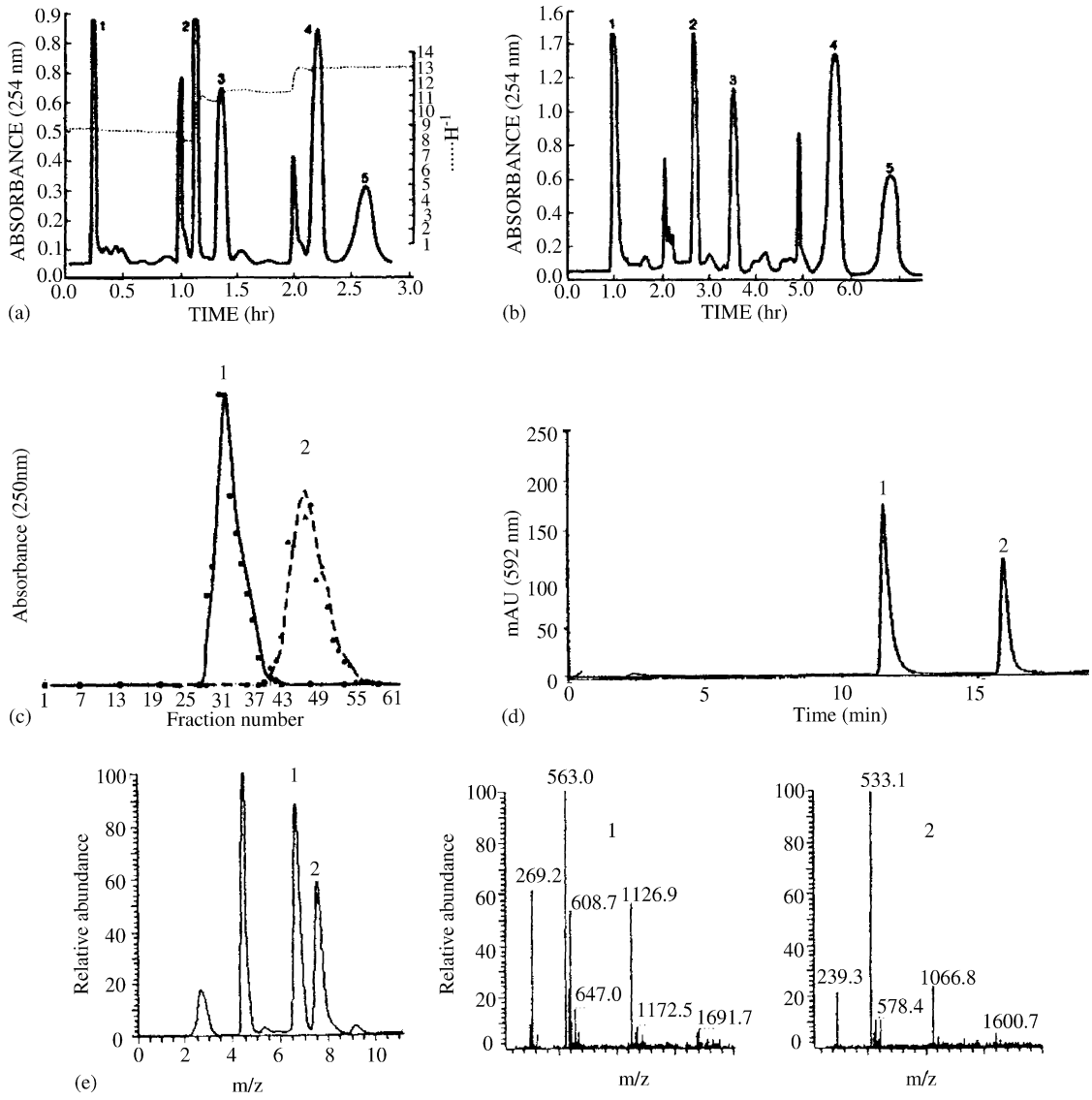
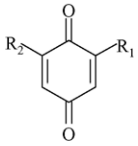


Fig. 1. Various analytical techniques for separation and detection of quinonoids. (a) Chromatogram of a crude sample of *R. officinale* Baill by analytical HSCCC. Solvent system: diethyl ether–basic water; stationary phase: upper organic phase; mobile phase: 35 ml of 4.0% $NaHCO_3$ and 55 ml of 0.7% Na_2CO_3 and 80 ml of 0.2% NaOH; flow rate: 1.0 ml/min; revolution speed: 1500 rpm; sample size: 10 mg dissolved in 1 ml stationary phase; retention of the stationary phase: 50%. Peaks: 1 = rhein; 2 = emodin; 3 = aloe-emodin; 4 = chrysophanol; 5 = physcion. Reproduced from Yang et al. [171] with permission from Elsevier. (b) Chromatogram of a crude sample of *R. officinale* Baill by preparative HSCCC. Solvent system: diethyl ether–aqueous base; stationary phase: upper organic phase; mobile phase: 120 ml of 4.0% $NaHCO_3$, 240 ml of 0.7% Na_2CO_3 and 480 ml of 0.2% NaOH; flow rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 300 mg dissolved in 20 ml stationary phase; retention of the stationary phase: 50%. Peaks: 1 = rhein; 2 = emodin; 3 = aloe-emodin; 4 = chrysophanol; 5 = physcion. Reproduced from Yang et al. [171] with permission from Elsevier. (c) Droplet counter current chromatography of commercially available crude ruberythric acid. Mobile phase: chloroform–methanol–water = 5:5:3 (v/v/v); flow rate: 0.48 ml/min; total separation time: 15 h and 30 min. Peaks: 1 = lucidin primeveroside; 2 = ruberythric acid. Reproduced from Derksen et al. [33] with permission from Elsevier. (d) Fluorescence detection of pseudohypericin (1) and hypericin (2) at 592 nm in HPLC chromatogram of a pharmaceutical preparation of St. John's wort. Mobile phase: solvent A (methanol–acetonitrile, 5:4, v/v) and solvent B (triethylammonium acetate buffer) in gradient mode; flow rate: 1.0 ml/min. Reproduced from Draves and Walker [181] with permission from Elsevier. (e) LC–UV–MS of a crude extract of *Rubia tinctorum* roots. Mobile phase: solvent A (ammonium formate–formic acid buffer, 0.2 M, pH 3, and EDTA, 30 mg/l) and solvent B (acetonitrile) in gradient mode; flow rate: 1.0 ml/min; UV detection at 254 nm. MS peaks for individual anthraquinones: 1 = lucidin primeveroside; 2 = ruberythric acid. Reproduced from Derksen et al. [44] with permission from Elsevier.

analytical method for its rapid determination has been reported in a Chinese journal [52]; a reduction wave of irisquinone with peak potential of −1.23 V (versus saturated calomel electrode) was recorded by single sweep oscillopolarography. The method was sensitive enough to detect at least 6×10^{-8} mol/l of irisquinone in raw medicine and capsule. Recently benzoquinone itself was found to show anti-bacterial activity against *Erwinia amylovora*, the causal agent of fire blight disease in pears. Benzoquinone was isolated from the aqueous extract of young shoots of Chinese pear (*Pyrus ussuriensis* Maxim.) by steam distillation in vacuum followed by charcoal powder chromatography [53].



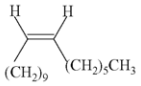
Compound	R_1	R_2
benzoquinone	H	H
irisquinone	OCH_3	

Fig. 2. Chemical structures of benzoquinonoids.

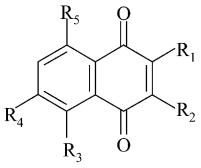
4.2. Naphthoquinonoids

4.2.1. Phylloquinone

The prenylquinones found in plant, animal and bacterial cells contain an isoprenoid side-chain, which is bound to a benzo- or naphthoquinone nucleus. Phylloquinone (Fig. 3 and Table 1), one of the Vitamin K group of naphthoquinonoids, occurs in green leafy vegetables and many higher plants. While the therapeutic efficacy of its synthetic analogue, i.e. menadione or Vitamin K_3, is well established for treatment of multi-drug resistant leukemia [54], a number of studies have demonstrated the anti-cancer effects of Vitamin K_1 (phylloquinone) also against several cell lines, e.g. liver, colon, lung, stomach, nasopharynx, breast, leukemia, etc. [55,56]. Moreover, Vitamin K, which is a critical co-factor in blood coagulation, has recently been found to play a role in bone metabolism to prevent osteoporosis [57,58]. Hence, for further understanding of the nutritional role of Vitamin K, there is a large demand for reliable analytical data on its content either in foodstuff or in blood and tissues of animals and human [41].

Classically, separation of such naturally occurring quinone mixtures was achieved by TLC on polyamide [59], Ag^+-impregnated silica gel plates, reversed-phase TLC on paraffin-impregnated Kieselguhr G with aqueous acetone [60], gel chromatography on Sephadex LH-20 and conversion of the quinones into 2,4-dinitrophenylhydrazones for separation by LC [61]. Subsequently, HPLC was used for separation of menaquinone and phylloquinone derivatives. Several solvent systems were adopted for resolution of various prenyllipids by both adsorption and RPLC [60,62,63].

More recently, gas chromatography was used to quantify Vitamin K_1 to a limit of 2 pg/ml of plasma [64]. HPLC coupled to fluorescence detection (after reduction to the hydroquinone form) also offered the requisite sensitivity and selectivity for its estimation in small amounts of analyte obtained from biological materials [13,65]. However, a sample clean-up step was required, e.g. a solid-phase extraction (SPE), prior to injection of the crude extract prepared from vegetable/herbal samples into the analytical column [13]. The post-column reactor consisted of a stainless steel column dry-packed with zinc powder. A gradient elution procedure was programmed with a mobile phase composed of methanol, to which 5.5 ml of aqueous solution (2 M zinc chloride, 1 M acetic acid, and 1 M sodium acetate) per litre was added (solvent A), and methylene chloride (solvent B) (90:10, v/v). In another method, the sample preparation was accomplished by using a liquid–liquid extraction purification step instead of SPE [66]. Phylloquinone in food was determined using triacontyl-bonded C_{30} column also [67].



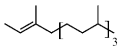
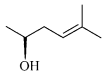
Compound	R_1	R_2	R_3	R_4	R_5
alkannin	H		OH	H	OH
juglone	H	H	H	H	OH
lawsone	H	OH	H	H	H
7-methyljuglone	H	H	H	CH_3	OH
phylloquinone		CH_3	H	H	OH
plumbagin	H	CH_3	H	H	OH
shikonin	H		OH	H	OH

Fig. 3. Chemical structures of naphthoquinonoids.

4.2.2. Shikonin

The naphthoquinone pigment, shikonin (Fig. 3 and Table 1), isolated from the roots of the Oriental herb *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae), and its derivatives, are the active components present in traditional medicines in the East [68]. Shikonin is the enantiomer of alkannin, which has historical references for treatment of ulcers as early as 4th century B.C. and was found in the roots of European dye-plant *Alkana tinctoria*. Readers are referred to an exhaustive review on the chemistry and biology of these naphthazarin natural products [69]. Traditionally, this has been used to treat macular eruption, measles, sore throat, carbuncles, wounds and burns. Extracts of *L. radix* roots in Japan were used for wound healing, and also taken internally as an antipyretic and anti-inflammatory agent. In recent times, multiple pharmacological actions have been attributed to shikonin, e.g. anti-bacterial [69], anti-fungal [70], anti-inflammatory [71], anti-tumour [72,73] and anti-HIV-1 [74,75]. Recent studies suggest that the anti-inflammatory and anti-cancer effects of shikonin derivatives may be attributable to several mechanisms of action, e.g. inhibition of leukotriene B4 biosynthesis [76], inhibition of phorbol 12-myristate 13-acetate-induced COX-2 expression [77], inhibition of TNF-α promoter activation [78], blockade of ligands binding to chemokine receptor [79], impairment of phosphatidylinositol signaling [80], DNA topoisomerase I inhibition [81], and inhibition of angiogenesis [82].

Shikonin is currently used in various medicinal preparations in China, Japan, and Korea, and also in cosmetics and dyestuffs in Japan, while alkannin is mainly used for food coloring and cosmetics in Europe and USA [69]. The ratio of the two enantiomers was found to vary with the species of the

respective plant sources, and could be determined by circular dichroism [83], and chiral stationary-phase HPLC [84].

Shikonin and the related compounds, 36 of them having been enlisted by Papageorgiou et al. [69], accumulate in the roots of *L. erythrorhizon*, *L. officinale* and many other Boraginaceae plants like *Arnebia hispidemia* and *A. tibetiana*. The *Lithospermum* root was extracted by application of SFE [85]. Conventional methods of CC and HPLC for preparative separation of shikonin derivatives from plant extracts are reportedly tedious, and only a few works describing their precise analytical estimation have been reported [84,86–90]. Fujita et al. [86] reported separation of seven shikonin derivatives by HPLC with mobile phase consisting of acetonitrile–water–triethylamine–acetic acid (70:30:0.3:0.3, v/v/v/v) at 40 °C. Nickel and Carrol achieved a similar separation at 70 °C in a MicroPak MCH-10 (Varian) column [87]. Based on these reports, a modified HPLC method was developed by Bozan et al. [91], and four derivatives of alkannin, found in relatively high amounts in the roots of Anatolian *Arnebia densiflora*, were determined quantitatively. The naphthoquinones were eluted within 12 min by RPLC using an Ultracarb ODS C_{20} column with UV–vis detection set at 520 nm. The mobile phase was methanol–water–formic acid (95:5:0.1, v/v/v) at ambient temperature.

Very recently, purification of shikonin from the crude extract of *L. erythrorhizon* root has been achieved by preparative HSCCC [30]. Prior to the analysis, *L. erythrorhizon* root extract, obtained by shaking with benzene at room temperature, was treated with 2% NaOH. The alkaline layer was separated and acidified with HCl. The resultant residue was processed to get crude shikonin for HPLC analysis which was done with a RP Symmetry C_{18} column using an isocratic mobile phase composed of methanol–water–acetic acid (70:28:2, v/v/v). Comparing the retention time and the UV–vis spectra from 220 to 620 nm by PDA against standard shikonin led to the identification of its constituents. The crude extract was contained several compounds among which shikonin represented 38.9% of the total and was subsequently isolated in 98.9% purity by application of HSCCC. A number of two-phase solvent systems were tested to select the ideal range of the partition coefficient for shikonin. Thus, preparative HSCCC was successfully performed with *n*-hexane–ethyl acetate–ethanol–water (16:14:14:5, v/v/v/v). A total amount of 19.6 mg of pure shikonin was obtained from 52 mg of the crude extract, the retention of the stationary phase was 34.5% and the total separation time was 200 min.

Presently, shikonin is produced in a large scale by liquid cultures of *L. erythrorhizon* root, originally established by Tabata et al. [92]. Research in this field was spurred by the increasing demand of shikonin and the acute shortage of the plant material in Japan, culminating into the world's first commercial production of a secondary metabolite by plant cell culture [69]. The cell suspension and hairy root cultures of *L. erythrorhizon* are known to produce more than 10 variable compounds including shikonin and furanobenzoquinone derivatives. Several methods for analyzing these quinonoids have been reported [86,93–95]. However, because of the wide differences in polarity and instability of the products under light, oxygen, and heating, it is difficult to analyze the dynamic accumulation patterns of these compounds. Recently, HPLC analysis based on a water–acetonitrile gradient program was established for the first time for simultaneous quantification of all these metabolites [11]. While doing so, Yamamoto et al. has also developed a new extraction procedure for such compounds, with methanol as the solvent, by use of ultrasonication in ice-water bath to prevent formation of artifacts. Although the ice chilling did weaken the efficiency of ultrasonication, but prolonged treatment for 90 min was sufficient to extract all these compounds from the plant cells and tissue materials. The HPLC analysis was done using an ODS column with acetonitrile, water and acetic acid in linear gradient condition. PDA detector was used for simultaneous identification and quantification of all the aromatic metabolites, water-soluble as well as the lipophilic shikonin derivatives, in one injection within 90 min.

4.2.3. Plumbagin and analogues

Plumbagin (Fig. 3 and Table 1) have been isolated from *Plumbago zeylanica* and other *Plumbago* spp. widely used in several Oriental systems of medicine in India, China and Far Eastern countries like Taiwan, Korea and Malaysia [8]. Traditionally, in India, *P. zeylanica* roots are used to treat diarrhoea, dyspepsia, piles, skin diseases including leprotic lesions, and for birth control [96,97]. In China, the whole plant and its root are used in rheumatic pain, menostatis, carbuncle and injury. Some of the activities of plumbagin have been studied on platelet aggregation and platelet–neutrophil interactions [98], anti-cancer, anti-leishmanial, anti-bacterial and anti-fungal properties [99–103], inhibitory effects on azoxymethane-induced intestinal carcinogenesis in rats [104], radiosensitising effects on experimental mouse tumours [105], potentiation of macrophage bactericidal activity in mice [106], and anti-fertility effects [107].

Classical separation methods, i.e. solvent extraction of plant sample followed by CC on silica gel, were adopted by various workers to isolate plumbagin and its derivatives [108–111]. The analytical separation of plumbagin analogues and few other non-isoprenoid naphthoquinonoid plant products by HPLC was first developed by Marston and Hostettmann [112]. A μBondapak CN column was used with an isocratic mobile phase of *n*-hexane with 1% acetic acid, and UV detection at 254 nm to separate the monomeric naphthoquinones, i.e. plumbagin, juglone, 7-methyl juglone, lawsone. Gupta et al. [26] described a mobile phase composition of *n*-hexane–chloroform–2-propanol (30:70:2, v/v/v) on a μSpherogel column with UV detection at 267 nm. Subsequently, other workers found it preferable to use RPLC for separation of plumbagin and its monomeric analogues [99,113].

Juglone and its 7-methyl derivative are close analogues of plumbagin found in many tropical plants alone, or in

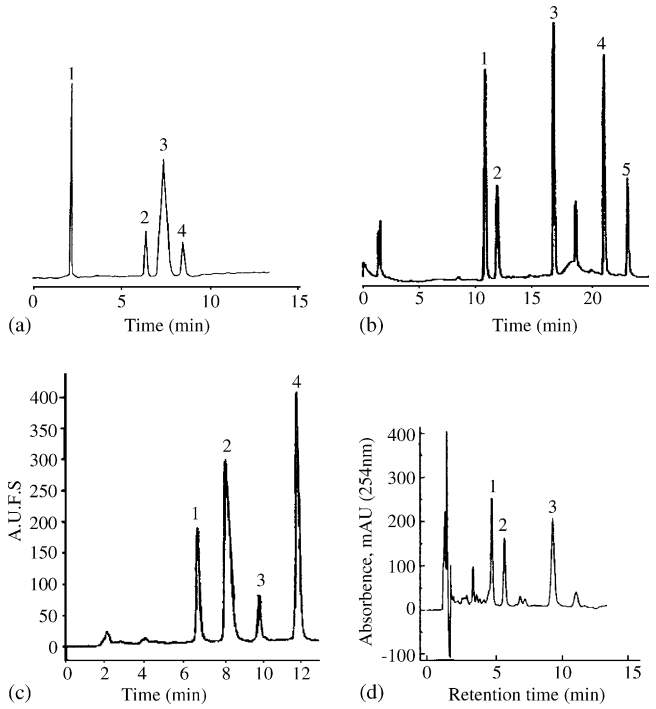


Fig. 4. Typical LC–UV chromatograms for separation of quinonoids. (a) Separation of a mixture of naphthoquinonoids. Mobile phase: acetonitrile–water (40:60, v/v) in isocratic mode; flow rate: 1.0 ml/min at ambient temperature; UV detection at 255 nm. Peaks: 1 = lawsone (1.9 min); 2 = juglone (6.4 min); 3 = menadione (7.4 min); 4 = plumbagin (8.7 min). (b) Chromatogram of a rhubarb sample by HPLC analysis. Mobile phase: solvent A (36 mM TEAP, pH 2.5) and solvent B (acetonitrile) in gradient mode; flow rate: 1.0 ml/min at ambient temperature; UV detection at 254 nm. Peaks: 1 = aloe-emodin; 2 = rhein; 3 = emodin; 4 = chrysophanol; 5 = physcion. Reproduced from Liu et al. [24] with permission from Elsevier. (c) Chromatogram of an extract of *H. perforatum*. Mobile phase: solvent A (methanol–acetonitrile, 5:4, v/v) and solvent B (0.1 M triethylammonium acetate buffer); flow rate: 0.6 ml/min; UV detection at 590 nm. Peaks: 1 = protopseudohypericin (6.7 min); 2 = pseduohypericin (8.2 min); 3 = protohypericin (9.9 min); 4 = hypericin (11.9 min). Reproduced from Piperopoulos et al. [182] with permission from Elsevier. (d) Chromatogram of *S. miltiorrhiza* Bunge roots by HPLC analysis. Mobile phase: methanol–tetrahydrofuran–glacial acetic acid–water (16:37.5:1:45.5, v/v/v/v); flow rate: 1.0 ml/min; UV detection at 254 nm. Peaks: 1= cryptotanshinone (4.79 min); 2 = tanshinone I (5.72 min); 3 = tanshinone IIA (9.31 min). Reproduced from Pan et al. [20] with permission from Elsevier.

association with other quinonoids in *Diospyros* and *Drosera* spp. [114–116]. HPLC method for determination of juglone in fresh leaves of walnut tree (*Juglans regia*) was described [117]. RPLC elution was done using a linear gradient program with the mobile phases of (A) water–phosphoric acid (95.5:0.5, v/v/v) and (B) acetonitrile–water (90:10, v/v). Juglone could be distinctly separated from the crude extract by detection at 420 nm. A more simple and efficient method was developed for separation of the monomeric naphthoquinone derivatives using isocratic elution with acetonitrile–water (40:60, v/v) on a μBondapak C_{18} RP column, followed by UV detection at 255 nm. [25]. Fig. 4a shows the distinct separation of lawsone, juglone, menadione and plumbagin from a mixture of the pure compounds by using this method.

4.2.4. Diospyrin and analogues

The *Diospyros* genus elaborates a large number of 1,4-naphthoquinone metabolites which include several monomers, dimers, a few trimers and even tetramers. In fact, such quinonoids are useful as taxonomic markers for this genus as proposed in an extensive review on this topic [116]. The leaf, wood, bark, fruit, seed and root of *Diospyros* plants have folkloric uses in many countries in Africa and Asia, mostly as astringents and chewing stick, for treatment of skin diseases, schistosomiasis, abdominal discomfort and female diseases; 12 of the species are documented in traditional medicinal systems in India for treatment of urinary disease, inflammation of the spleen, snake bite, and as astringent lotion for eyes. The leaves of *D. melanoxylon* have great commercial value for wrapping country cigarettes (bidi) in India. The pharmacological and biocidal studies on crude extracts and quinonoids from this genus are: piscicidal and molluscicidal [118–120], anti-inflammatory [121–124], antibacterial [125,126], anti-cancer [127–129], anti-tumour promoter [130] and anti-tubercular activities [131].

Diospyrin is one of the dimeric naphthoquinonoids abundantly present in *Diospyros* spp. (Fig. 5 and Table 1). The HPLC analysis of its isomers, viz. isodiospyrin and mamegakinone (Fig. 5) was first achieved by Marston and Hostettmann, using a normal phase column [112]. Recently, Lin et al. achieved the separation of four dimeric compounds, viz. chitranone, maritinone, elliptinone and isoshinanolone, through Sephadex LH-20 CC followed by preparative HPLC with methanol–water gradient [99]. Diospyrin was isolated by CC and TLC by several groups of workers in different countries [132–136]. A study on preparative TLC of diospyrin and isodiospyrin along with its monomer (7-methyl juglone) revealed the sensitivity of these compounds to exposure when adsorbed on silica gel [109]. The estimation of diospyrin in the stem bark of *D. montana* was first reported by Ravishankara et al. [137] using HPTLC technique. The analysis was performed on a pre-coated silica gel G60 HPTLC plate using a Linomat IV automatic sample spotter. The plate was developed in a solvent system of toluene–ethyl acetate–cyclohexane–glacial acetic acid (6:1:1:0.1, v/v/v/v) and scanned at 445 nm for detection of diospyrin (LOD = 30 ng). However, application of RPLC afforded a more sensitive analysis (LOD = 8 ng) of diospyrin in stem bark samples of *D. montana* collected from four different climatic regions of India [25]. The amount of diospyrin was quantitatively estimated within 15 min by using acetonitrile–water (50:50, v/v) as isocratic eluent.

4.3. Anthraquinonoids

4.3.1. Alizarin and analogues

The root and rhizomes of *Rubia cordifolia* and *R. tinctorum*, i.e. common madder, are rich sources of anthraquinonoids consisting mainly of alizarin and its analogues (Fig. 6 and Table 2). These are important components of traditional medicines in many Asian countries, particularly

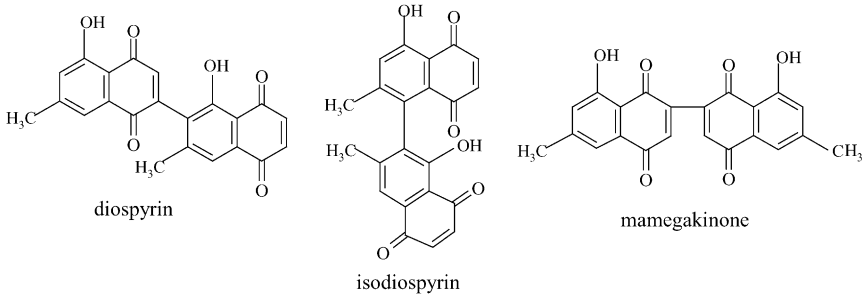


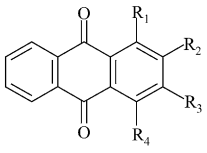
Fig. 5. Chemical structures of dimeric naphthoquinonoids.

India and China, for the treatment of arthritis, wound healing, dysmenorrhea, hemostatis and as a tonic. Various other uses of this plant have been reported such as remedy for ulcer, inflammation and swelling, poisoning due to snake and scorpion bite, leprosy, hepatic obstructions, indigestion, jaundice and paralysis [68,96,97].

Pharmacological studies on the crude extract and some of its purified constituents have shown anti-cancer [138–140], anti-oxidant [141], anti-inflammatory [142], anti-microbial [143], anti-viral [144], and hepatoprotective activities [145,146].

A search for novel chemopreventive agents in this plant led to the identification of mollugin, a naphthohydroquinone, as an active anti-proliferative compound [147]. Purpurin and lucidin have been found to possess mutagenic properties [148]. Nevertheless, the main interest in madder root is to produce the pigments, such as alizarin and lucidin, along with their respective glycosides (ruberythric acid and lucidin primeveroside) [21]. Screening and quantitative estimation of these compounds were mainly based on paper chromatography [149], TLC [150] and low pressure CC [151].

To separate the anthraquinone glycosides and their aglycons, a selective solvent extraction method was proposed by Masawaki et al. [21]. They performed a two-phase solvent extraction using chloroform and aqueous KOH by pH adjustment (Table 2). At pH 5, alizarin and lucidin were extracted from the organic phase whereas their primeverosides were obtained from the aqueous phase. Concentrations of the constituents were determined by HPLC. The aqueous KOH phase was subjected to enzymatic hydrolysis using β-glucosidase at 50 °C and at pH 5. Ruberythric acid was selectively and completely converted to alizarin within 6 h and it was separated from unreacted lucidin primeveroside by solvent extraction with hexane.



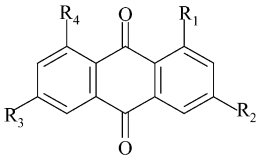
Compound	R_1	R_2	R_3	R_4
alizarin	OH	OH	H	H
lucidin	OH	CH_2OH	OH	H
lucidin primeveroside	OH	CH_2OH	O-β $glu^6 \rightarrow {}^1xyl$	H
munjistin	OH	COOH	OH	H
purpurin	OH	H	OH	OH
ruberythric acid	OH	O-β $glu^6 \rightarrow {}^1xyl$	H	H

Fig. 6. Chemical structures of alizarin and analogues.

Several other HPLC methods were also reported [152–154]. However, simultaneous analysis of both anthraquinone glycosides and aglycons present in the madder root was difficult because total baseline separation of glycosylated anthraquinones was not achieved by conventional HPLC method [152,155]. Hence, two glycosylated anthraquinones (lucidin primeveroside and ruberythric acid) along with five aglycons were quantitatively detected by Derksen et al. [33] in the extract of *R. tinctorum* in a single HPLC run. An end-capped C_{18} RP column was used for linear gradient elution with water–acetonitrile mixture and PDA detection at 250 nm. The anthraquinones were separated within 34 min with 55–95% purity. An improved LC–PDA–MS method was developed by same group of workers for the rapid characterisation of the anthraquinones in the *R. tinctorum* (Fig. 1e) [44]. HPLC separation was carried out on an end-capped C_{18} column and peaks were detected at 254 nm. For on-line mass detection APCI as well as ESI was used. The efficiency of the mass detection of the hydroxyanthraquinone aglycons was dependent on pk_a value of the component. Fifteen anthraquinonoids were identified using gradient elution with a mobile phase composed of (A) ammonium formate–formic acid buffer (0.2 M; pH 3) with EDTA (30 mg/l) and (B) acetonitrile.

4.3.2. *Emodin and analogues*

Some anthraquinonoid derivatives, particularly emodin, aloe-emodin, chrysophanol, physcion, rhein (Fig. 7 and Table 3) and their glycosides are the major constituents of *Aloe*, *Rheum* and *Polygonum* spp. used in many Oriental medicinal preparations. The leaves and fruits of all these plants have been widely used for laxative and purgative preparations since time immemorial. Incidentally, the leaves and pods of *Cassia* (syn. *Senna*) species spread all over the world have also been used for the same purpose, and found to contain bianthryls and their glycosides, represented by the sennosides. Two naphthalene glycosides, viz. tinnevellin glucoside



Compound	R_1	R_2	R_3	R_4
aloe-emodin	OH	CH_2OH	H	OH
chrysophanol	OH	CH_3	H	OH
danthron	OH	H	H	OH
emodin	OH	OH	CH_3	OH
physcion	OH	CH_3	OCH_3	OH
rhein	OH	COOH	H	OH

Fig. 7. Chemical structures of emodin and analogues.

and 6-hydroxymusicin, are the respective taxonomic markers for two types of senna, i.e. *C. angustifolia* or Tinnevelly senna, cultivated mainly in South India and Pakistan, and *C. acutifolia* (var. *senna*), known as Alexandrian senna from Africa [156].

In addition, many other medicinal applications of various parts of these plants are highly prevalent even to this day. For example, *Aloe barbadensis* (syn. *vera*) have been used in India and southeast Asian countries as abortifacient, emmenagogue, cathartic, cholerectic, uterine stimulant and anthelmintic, while its gel is applied for healing wounds and soothing damaged tissues [96,97]. The dried rhizome of *Rheum officinale* Baill, or *R. palmatum* L. commonly known as rhubarb, is a historically popular Chinese preparation (Da-Huang) known to possess stomachic, antifebrile and carminative properties and has been used for the treatment of bacterial dysentery, flatulence, congestion of pelvic organs, and menstrual disorders [68]. Japanese Kampo preparations like Dai-saiko-to, Choi-joki-to, San'o-shashin-to, etc. use it for cathartic, anti-inflammatory and anti-psychotic effects [18]. *R. undulatum* L. is traditionally cultivated in Korea for similar applications [157]. *Polygonum cuspidatum*, also known to contain these quinonoid constituents, has been traditionally used for menoxenia, skin burn, hepatitis, inflammation, and osteomyelitis in China [158].

It is presumed that the similarity in the traditional applications of these plants might be correlated to the ubiquitous presence of the danthron analogues in all of them. Furthermore, some recent studies carried out on pharmacological activity of these plant-derived quinonoids lend support to this contention [159] and might explain the popularity of such herbal preparations worldwide. Emodin and its analogues were found to possess anti-bacterial, anti-fungal, anti-inflammatory, immunosuppressive and estrogenic properties [160–164]. The tumour growth inhibitory activity [165] and related mechanisms [158,166] of emodin are also under investigation.

Since herbal preparations containing the aforesaid quinonoids are consumed indiscriminately as OTC laxatives all over the world, many analytical methods aim at their standardization by carrying out simultaneous separation of the anthraquinonoid constituents, particularly the ubiquitous quintet of emodin analogues (Fig. 7). On the other hand, some of these compounds have individually been found to possess significant pharmacological properties, as stated earlier. Thus, methods have also been developed for their independent isolation and analysis from plants as well as from biological samples with an eye to their pharmacokinetic assessment. Analytical approaches for separation of the antineoplastic constituents of Chinese medicines, and other anthraquinone-based anti-cancer drugs have been reviewed earlier [167,168]. Thus, mouse plasma was processed (Table 3), injected onto a RP column and eluted isocratically at 11.7 min with methanol–water–acetic acid (65:35:0.2, v/v/v) to estimate aloe-emodin using a fluorimetric detector [169]. The assay was linear from 10 to 1000 ng/ml with LOD of 4.5 ng/ml.

A similar RP column was used for analysis of rhein in a crude alcohol extract of *R. officinale* with a mobile phase composed of methanol–0.5% aqueous phosphoric acid (60:40, v/v). Rhein was eluted isocratically within 13 min, and was monitored at 254 nm by PDA detection [170]. Again, in another report, a distinct separation of all the five analogues, viz. aloe-emodin, rhein, emodin, chrysophanol and physcion, in this order, was achieved within 17 min. In this case, a sample of the same plant was processed differently prior to injecting onto the HPLC column (Table 3) and was eluted isocratically with a slightly modified mobile phase with a composition of methanol–0.1% aqueous phosphoric acid (80:20, v/v) [171]. Both these methods were adapted for HSCCC to carry out preparative separation of the anthraquinonoids in good yield. For purification of rhein from the crude ethanolic extract, a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v/v/v) was chosen. Thus, using two preparative units of the HSCCC, about 500 mg of crude extract of Chinese rhubarb yielded 6.7 mg of pure rhein at a purity of 97% [170]. The preparative isolation of five analogues of rhein was carried out by Yang et al. [171] using a pH-modulated stepwise elution in HSCCC. The two-phase solvent system was optimized by mixing diethyl ether and water, and a portion of the aqueous phase was basified by adding 4% $NaHCO_3$, 0.7% Na_2CO_3, and 0.2% NaOH. Thus, 300 mg of crude extract was well resolved into the constituent anthraquinonoids at a purity of 98%.

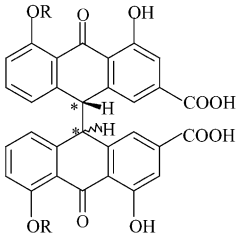
Recently, Ding et al. [17] has reported the simultaneous determination and distinct separation of the five anthraquinonoids from rhubarb samples, as well as from biological fluids (plasma, urine and CSF) collected from rats orally fed with rhubarb extract. In both the cases, HPLC analysis on a Zorbax SB-C_{18} column with a mobile phase composed of methanol–0.5% acetic acid (85:15, v/v) was performed within 20 min. The detection limit of 0.35–3.13 ng achieved in this method is sensitive enough for simultaneous determination of all the five compounds in the plant extract as well as in a biological sample.

Pharmacokinetic analysis of rhein in human plasma was conducted in Korea by feeding *R. undulatum* root extract to healthy volunteers [172]. RPLC was done by spiking the plasma sample with standard rhein solution using a mobile phase of acetonitrile–methanol–Mellvaine buffer (35:15:50, v/v/v) at pH 2.2. The presence of rhein in the plasma was also confirmed by a Quattro LC Triple Quadrupole Tandem MS and application of positive and negative electrospray conditions.

A recent work describes evaluation of 30 commercial rhubarb samples with respect to their anti-oxidative activity that was correlated with the concentration of its active components [18]. Thus, the specimens were analyzed on HPLC to quantify 18 constituents out of which 12 were quinonoids, anthrones and respective glucosides. HPLC of the crude ethanol extract was done on ODS column using gradient elution with acetonitrile and 20 mM phosphoric acid and monitored at 280 nm. The chromatogram showed the separation of 18 components within a time-span of 140 min. Multivariate regression analysis of the data was performed to correlate the concentration of each component vis-à-vis the anti-oxidative activity of the respective plant sample. Finally, five components, four of them quinonoids, were selected as markers for the pharmacological potency of the rhubarb sample.

Simultaneous determination of 17 anthraquinones and bianthryls (sennidin and sennosides) present in senna samples was standardized by Metzger and Reif [23]. HPLC was done on Nucleosil 5 RP-8 column, using a gradient elution with solvents (A) 0.02 M $KHPO_4$ in water adjusted to pH 2, and (B) acetonitrile. The anthraquinones and bianthryls were monitored at 435 and 270 nm, respectively, using a PDA detector. The gradient elution was optimized by means of a computer-assisted technique involving DryLab software, and the method was validated in accordance to the guidelines proposed by the European Community. The same software programme was also used for standardization of RP gradient elution of rhubarb by Liu et al. [24] to separate the quintet of emodin analogues. They have used a Zorbax RX-C_{18} column with a solvent gradient composed of (A) 36 mM triethylamine phosphate, pH 2.5, and (B) acetonitrile with UV detection set at 254 nm. All the five hydroxyanthraquinonoid ingredients of plant samples as well as medicinal preparations of rhubarb could be quantified in the concentration range of 0.5–50 μg/ml under the specified chromatographic conditions (Fig. 4b).

Thus, most of the analytical methods developed for separation of these use RPLC. Nevertheless, a simple normal phase method with a column of Spherisorb-CN was reportedly able to separate emodin, rhein and chrysophanol from rhubarb and senna samples in less than 10 min, using an isocratic mobile phase of chloroform–96% acetic acid (95:5, v/v). The absorption was monitored at 254 nm [22]. However, this method with LOD 0.005–0.02 mg/l was not sensitive enough as compared to the RP methods described earlier. Simultaneous determination of several anthraquinonoids from Chinese herbal preparations containing a mixture of plants like rhubarb and senna was achieved by HPLC [173] and capillary electrophoresis [174,175].



Compound	R
Sennidin A (10,10'-trans)	H
Sennidin B (10,10'-meso)	H
Sennoside A (10,10'-trans)	Glu
Sennoside B (10,10'-meso)	Glu

Fig. 8. Chemical structures of sennidins and sennosides.

4.3.3. Sennosides

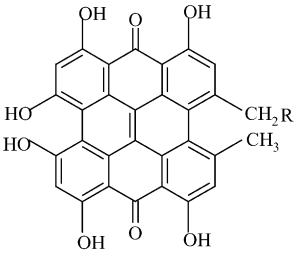
Sennosides A and B (Fig. 8 and Table 3) present in *C. angustifolia* was analyzed on a Symmetry C_{18} column using a mobile phase of (A) methanol–water–acetic acid (20:80:0.1, v/v/v; pH 4), and (B) methanol–water–acetic acid (80:20:0.1, v/v/v; pH 4) [176]. Elution was started with 80% A for 5 min, changing to 100% B over next 20 min, run isocratically at 100% B for 10 min, and again changing to initial condition over next 5 min. The flow rate was maintained at 0.6 ml/min for the first 20 min, and thereafter increased to 1 ml/min. The determination was linear in the range 2–50 μg, with LOD of 0.2 and 0.1 μg/ml, respectively, for the two bianthryl glucosides A and B. A PDA detector was used and the monitoring was done at 285 nm.

LC–MS analysis of *C. angustifolia* for detailed identification of its minor constituents, in addition to the major sennidins, has been done on a HP-1100 LC chromatograph coupled with a LCQ ion trap mass spectrometer [42]. A PDA detector was used, and UV detection was done at 270 nm. LC–MS analyses were performed with an electrospray interface in the negative ion mode without post-column buffer addition. In some cases, MS analyses of pure compounds were performed in the APCI mode.

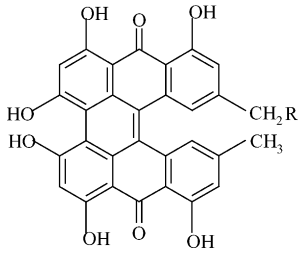
Barbaloin, an anthrone-*C*-glucoside, and its oxidation product aloe-emodin, occur in the yellow exudates from the inner epidermal cell layers which is used in purgative preparations of the *Aloe* species. Specimens of several *Aloe* were analyzed on a C_{18} RP column, using a mobile phase with a linear gradient of water–acetonitrile, 88:12 to 54:46 (v/v) over 39 min at a flow rate of 1 ml/min. A PDA detector was used and monitored at 290 nm. The assay was linear in the range 0.05–850 μg/ml and LOD was in the ng level [12].

4.3.4. Hypericin and analogues

Hypericin and its analogues (Fig. 9 and Table 4) are naphthodianthrones present in *Hypericum perforatum* L., popularly known as St. John's wort (SJW) in the West, where its traditional use as a powerful antidepressant continues to this day. The aerial part of the plant is used in Unani medicine popular in Arabic countries and also in India, where it is consumed as anthelmintic, diuretic and emmenagogue [177]. There has been a huge proliferation of studies on *Hypericum*



Compound	R
hypericin	H
pseudohypericin	OH



Compound	R
protohypericin	H
protopseudohypericin	OH

Fig. 9. Chemical structures of hypericins.

spp. during the last decade as summarized in a recent review by Verotta [178]. Currently, hypericin is under development for the photo-dynamic therapy of cancer, and as an anti-viral agent against HIV and hepatitis C [179]. It possesses a moderate anti-monoaminooxidase activity. However, the relevance of hypericins to the antidepressant activity has been questioned since this might also be due to some other compounds present in SJW [178].

Various methods of extraction of SJW were adopted for the analysis of hypericins and its cell culture (Table 4). In general, HPLC analysis of hypericin was carried out on RP-C_{18} columns of particle size not more than 5 μm using acidic aqueous phases (phosphate or acetate buffers) with gradients of methanol and acetonitrile with UV detection at 590 nm. A sensitive HPLC–electrospray tandem mass spectrometric method was developed by Tolonen et al. for fast and simultaneous determination of four major naphthodianthrones [43]. This method, based on multiple dissociation reaction monitoring, allowed the analysis of hypericin derivatives in less than 5 min in the concentration range of 2–500 ng/ml, with LOQ of 2 ng/ml for hypericin. The eluents were (A) aqueous 20 mM ammonium acetate and (B) acetonitrile in gradient mode. A different eluent composed of water–acetonitrile–methanol–trifluoroacetic acid was also used with a RP column having particle size of 5 μm [15]. The US Pharmacopoeia has introduced the method developed by Brolis et al. that uses a wide pore RP-18 column and elution with aqueous phosphoric acid–acetonitrile–methanol in a linear gradient program [40]. A combination of HPLC–PDA, HPLC–thermospray and electrospray MS was adopted for analysis of most of the important metabolites present in SJW. In another method, RPLC with a florescence detector was used for analysis of these compounds in human plasma samples [180], achieving a LOQ of 0.25 ng/ml. Fluorescence detection of hypericin and pseudohypericin is shown in Fig. 1d [181]. Recently, Schmidt [38] carried out the identification of naphthodianthrones from the plant extract by HPLC combined with on-line, pre-column photochemical conversion of the light-sensitive proto-forms of hypericin and pseudohypericin, followed by PDA detection. Separation was carried

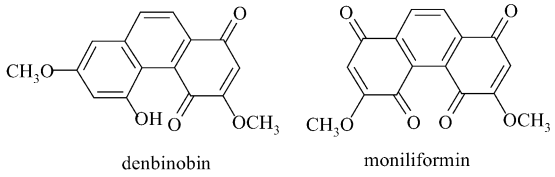


Fig. 10. Chemical structures of phenanthraquinonoids.

out on a C_{18} column under isocratic RP mode. The mobile phase consisted of methanol–ethyl acetate–0.1 M phosphate buffer at pH 2.1 (67:16:17, v/v/v). The compounds eluted in the order as protopseudohypericin, pseudohypericin, protohypericin and hypericin in less than 16 min. The method could accurately estimate the individual derivatives by recording the chromatograms before and after irradiation, and was successfully applied to the quality control of the plant extracts and its phytopharmaceutical preparations. A typical separation is presented in Fig. 4c [182].

In a hyphenated method, RPLC separation and structure elucidation of the components of SJW extract was carried out with ammonium acetate buffer using on-line UV–vis, NMR and MS [183]. The MS and MS/MS analyses were performed with negative ESI. The eluent from the HPLC column was split immediately after UV detection flow cell. Given the relative sensitivities of the two spectrometers, the major portion (95%) was directed towards the NMR detection with the rest (5%) to the MS. Using 'on-flow' and 'stopped flow' techniques, respectively, the MS and NMR spectra were obtained.

A fast technique to evaluate the composition of extracts of SJW within 8 min was achieved using non-aqueous capillary electrophoresis system with reversed electro-osmotic flow [14]. A solvent mixture of methanol, DMSO and *N*-methylformamide (3:2:1, v/v/v) was optimized with the additions of 50 mM ammonium acetate and 150 mM sodium acetate as electrolytes in electrophoresis media. The flow was reversed by the addition of the polycation hexadimethrine bromide (0.002%, w/v) and then negative voltage was applied. Use of a fluorescence detector lowered the LOD by almost 100 times of that obtained by UV detection (2.8 μg/ml).

4.4. Phenanthraquinonoids

4.4.1. Denbinobin

Denbinobin (Fig. 10 and Table 5), a phenanthraquinone derivative, was isolated from *Dendrobium moniliforme* Lindl. (Orchidaceae) and related species widely available in Japan, China, Korea, Indo-Malayan peninsula and Australia [184–187]. The plants were historically used for herbal preparations like Shih-hu in China. Traditional medicinal applications of such orchids have been reported in many other countries [188]. Denbinobin, also isolated from the stems of *Ephemerantha lonchophylla*, induced apoptosis in human colon cancer HCT-116 cells [189]. Anti-cancer activity was established in human lung carcinoma and HL-60 cell lines

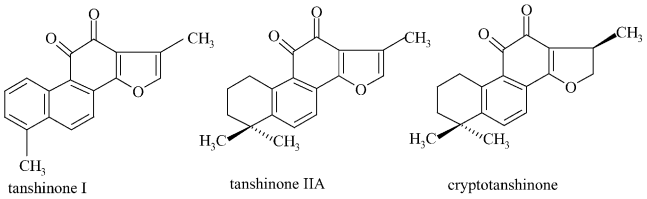


Fig. 11. Chemical structures of tanshinones.

[187]. Anti-inflammatory and anti-mutagenic activities of this compound have been reported recently [184,190]. To our knowledge, there is only one report on its HPLC analysis using a Cosmosil 5C-18 AR column and a mobile phase of water–acetonitrile (50:50, v/v) at a flow rate of 1 ml/min with UV detection at 236 nm [191].

4.4.2. Tanshinones

Tanshinones (Fig. 11 and Table 5), the major active constituents of Tan-shen, belong to a group of diterpenes with an abietane-type skeleton containing a 1,2-quinone in the C-ring. The compounds are present as a group of red pigments in 'Tan-shen' or 'Dan-shen', a well-known drug of Chinese traditional Pharmacopoeia prepared from the root and rhizomes of *S. miltiorrhiza* Bunge (Lamiaceae) [68]. Over 50 tanshinones have so far been identified from *Salvia* sp., and some other plants of Lamiaceae family, like *Perovskia abrotanoids*, a herb used to treat cutaneous leishmaniasis in Iranian folk medicine. Tan-shen has been widely used for traditional treatment of coronary and cerebrovascular diseases by promoting circulation to remove blood stasis, and also for relieving carbuncles, and to tranquilise the mind.

Pharmacological studies revealed a variety of activities of tanshinones, e.g. effects on cardiac functions [192,193], aldose reductase inhibitory activity [194], interaction with benzodiazepine receptor [195], leishmanicidal, antiplasmodial, cytotoxic [196] and induction of apoptosis [197].

Solvent extraction of tanshinones from the dried roots and rhizomes of the plants has been mostly done at room temperature [196] (Table 5). However, microwave-assisted method at 80 °C [20], and supercritical technique [28] for extraction using liquid carbon dioxide at 60 °C and 250 kg/cm^2 pressure have been described recently. Dean et al. compared the yields of tanshinone IIA from *S. miltiorrhiza* by using different extraction methods [28]. Recovery of tanshinone IIA was determined by HPLC on a C_{18} column using a mobile phase of acetonitrile–water–acetic acid (70:30:1, v/v/v), with UV–vis detection at 270 nm. The range of detection was 0.12–12 μg/ml by this method [28]. A Nova-Pak C_{18} column with methanol–water (85:15, v/v) as the mobile phase was used for the determination of cryptotanshinone and tanshinone IIA in pig plasma and urine [198]. A similar mobile phase of methanol–water (80:20, v/v) was used by Tian et al. [199]. However, separation of three tanshinones was done (Fig. 4d) using another mobile phase composed of methanol–tetrahydrofuran–glacial acetic acid–water (16:37.5:1:45.5, v/v/v/v) on a Zorbax SB guard column and Zorbax ODS by monitoring at 254 nm within a range of 0.16–1.4 μg of the compounds [20]. A HSCCC method for the preparative separation of six tanshinones, including three already discussed earlier, was developed recently, which was followed by HPLC in C_{18} RP column with a mobile phase composed of 0.075% aqueous trifluoroacetic acid and acetonitrile in gradient mode [31,200]. The effluent was monitored at 280 nm. HSCCC helped the extraction process and resulted in the separation of six tanshinones in a single run.

5. Conclusions

Studies in the molecular level carried out on the quinonoid constituents of herbal medicines are increasingly coming out in support of some of their traditional applications. Thus, recent analytical techniques are being implemented for separation and quantitation of plant-derived quinonoid compounds. Undoubtedly, reversed-phase HPLC coupled to PDA, fluorescence or electrochemical detection is the most favorable method for this purpose. A continuous development is under way to supplement it with modern extraction technology, such as HSCCC or SFE, and more efficient characterisation through MS, CE or NMR in tandem.

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