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Research progress on separation and characterization of bioflavonoids from *Coreopsis tinctoria* Nutt

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Abstract

Coreopsis tinctoria Nutt (*C. tinctoria*) or a common name 'Snow chrysanthemum' because of its growing condition at high altitude,is one of the popular medicinal-food plants, mainly grows in the northwest area of China. Because of its rich nutrients, particularly polyphenols (majorly flavonoids), and volatile oils among others, *C. tinctoria* has become a research hotspot in recent years. In addition, the flavonoids of *C. tinctoria*, especially the flavanomarein, marein, okanin and other bioactive phytochemicals, have effective biological activity. They possess good antioxidant and anti-inflammatory effects, also have significant effects on lowering blood pressure, hypoglycemia and reducing levels of triglycerides and cholesterol. However, at present, studies on the bioactivity of *C. tinctoria* are mainly focused on crude extracts or total flavonoids. The study of monomer components is largely limited to the isolation and purification process, which is difficult for a systematic study because of the chemical-physical nature of marein, flavanomarein and other compounds in *C. tinctoria*. Therefore, to have an updated isolation of *C. tinctoria* bioactive components, this paper reviewed the current research status of the separation and purification methods of flavonoids from *C. tinctoria*, by analyzing and comparing the technical characteristics of these methods, and providing prospects on the development and application of the separation and purification methods of *C. tinctoria*.

Keywords: *Coreopsis tinctoria Nutt*; Marein; Flavanomarein; Flavonoid; Separation and purification.

1. Introduction

Coreopsis tinctoria Nutt, also known as plains coreopsis, or "Gulichar" in Uyghur, is an annual plant of the genus Chrysanthemum in the Asteraceae family. The native species of *Coreopsis tinctoria* (*C. tinctoria*) is originated from North America, and then it spread worldwide (Shen et al., 2021; Yao et al., 2017). *C. tinctoria*, also called 'snow chrysanthemum' in China, is mainly distributed in the area of the Kunlun Mountains in northwest region of China at an altitude of about 3 km. It is one of the alpine wild plants, unique to Xingjian province, China with traditionally claimed health promoting effects (Begmatoy et al., 2020; Guo et al., 2015; Du et al., 2018). It is very rich in flavonoids, as high as more than 20% in dry weight and also a rich source of polysaccharides, essential oils, amino acids and other nutrients, which has high nutritional and medicinal values (Jiang et al., 2014; Ma et al., 2023; Shen et al., 2021). It is often consumed as a flower tea in China and other countries, and also as a Uyghur folk medicine to prevent and treat cardiovascular and metabolic diseases. At present, the research on snow chrysanthemum worldwide is mainly focuses on flavonoids,

since it is found that flavonoids are the major bioactive components in *C. tinctoria*. For instance, one study has reported that 57 flavonoid components have been isolated and identified from *C. tinctoria*, among which include flavonoids, dihydroflavonoids, dihydroflavonols, chalcones, flavonols, flavones, and flavonols (Du et al., 2018; Li et al., 2015; Shen et al., 2021) and their main monomeric components are the flavanomarein, marein, and okanin among others (Lam et al., 2016).

However, due to the wide variety of bioactive ingredients in the flowers of snow chrysanthemum (Li et al., 2015), it is difficult to isolate a single substance individually, and the separation process is tedious and low in yield and purity. However, to have a more comprehensive understanding of the biological activities and associated mechanisms of targeting phytochemicals, an imperial need on single compounds with high purity becomes an urgent issue and the bottle-neck to concern in studying the biological activity of *C. tinctoria*. Strategies in optimizing the extraction conditions to improve the extraction efficiency and purity have become a key issue for current phytochemistry research of *C. tinctoria*. The aim of this review was to summarize the current extraction, isolation and purification methods of *C. tinctoria* flavonoids, compare the different methods and strategies and provide useful references for efficient separation of *C. tinctoria* flavonoids with high purity for the use and application in biological evaluation of *C. tinctoria* bioactive phytochemicals. Subsequently, an establishment of the structural activity relationship between the separated *C. tinctoria* flavonoids and their biological activities will lay the foundation for the principle targeted disease prevention with elucidated *C. tinctoria* flavonoids, which in turn will advance the application and product development of *C. tinctoria* in benefiting human health.

Overall, the extraction and separation of phytochemicals of *C. tinctoria*, in particular, flavonoids, have been studied and employed with various methods and technologies. Each application has its own theoretical basis and targeted extraction and or isolation. This review provided a systematic summary of the methods used associated with the intended biological application of extracts or single monomer of flavonoids. Thus, aspects of targeted extraction, tandem separation and other strategy are also suggested for consideration in a specific aimed or a combinatorial study.

2. Extraction of *C. tinctoria* flavonoids

There are various methods in the separation of natural products, but an appropriate extraction method is the first key step to efficiently extract targeted compounds. Hence, different extraction methods were employed by researchers to obtain crude *C. tinctoria* flavonoids. Extraction methods include solvent extraction, in which solvents used include water (cold or hot), aqueous ethanol with various ratios, ethyl acetate, chloroform and n-butanol and other organic solvents. The initial step for a stepwise extraction was to use 75% aqueous ethanol as the extracting solvent to treat ground *C. tinctoria* flower (Ma et al., 2016). According to a traditional fractionation method, the resulting extract was usually suspended in water and stepwise extracted with hexanes, ethyl acetate, chloroform and n-butanol, respectively, yielding five sub-extracts of *C. tinctoria.* i.e. sub-extracts of hexanes, ethyl acetate, chloroform and n-butanol (Lam et al., 2016; Ma et al., 2016). The hexanes portion extracted from the aqueous ethanol crude extract contained majorly essential oils of *C. tinctoria*. Major flavonoids of *C. tinctoria* were collected in the sub-extracts of ethyl acetate and n-butanol, including flavanomarein, marein, okanin and isookanin among others, detected by high performance liquid chromatography (HPLC) in the n-butanol fraction from the crude ethanol-water extract (Lam et al., 2016; Ma et al., 2016).

In extracting certain class of compounds, a specific appropriate method is usually chosen. The options of extracting method include but not limited to ultrasonic or ultrasonic-heating extraction, microwave assisted extraction, subcritical and or supercritical fluid extraction among others. More frequently, a combined extraction method is employed (Liu et al., 2015; Ma et al., 2016). For instance, supercritical fluid extraction is often used to extract lipophilic compounds, especially essential oils, but when the percentage of co-solvent methanol or ethanol increased, more polar compounds, e.g. marein and flavonmarein, could be extracted, similar to the extraction of other plants polyphenols. Thus different flavonoids in *C. tinctoria* could be selectively extracted as a group of mixture with similar polarity and or sharing same structure features (Lam et al., 2016; Ma et al., 2016).

As an example of common extracting method of phytochemicals, solvent extraction is chiefly used to prepare crude *C. tinctoria* flavonoids for animal experiments and also for further isolation of single flavonoid compounds (Liu et al., 2014). Solvent extraction from plant materials, also known as liquid-solid extraction, is a method that compounds transfer from solid to liquid. Due to different structures and dissolution physical properties among different flavonoids, the component and quantity of each *C. tinctoria* flavonoids in the extracts vary with different polarity of solvents (Liu et al., 2015), which is evidenced by a report that demonstrated a significant correlation between flavonoid content and antioxidant activity (Chen et al., 2016), The detection of antioxidant activity in different extracts correlated to the degree of flavonoid enrichment. In one study, authors examined the fractionated extracts with different solvents, i.e. petroleum ether, chloroform, ethyl acetate, 1-butanol and water, and revealed that the antioxidant capacity of the ethyl acetate fraction had the strongest antioxidant capacity, indicating that the ethyl acetate fraction had the richest components of flavonoids (Shao et al., 2018). When acetone, 50% and 95% aqueous ethanol were used the extraction solvents of *C. tinctoria*, the extract from 50% aqueous ethanol resulted in the strongest ability to scavenge DPPH free radicals (Liu et al., 2014), meaning that the 50% ethanol fraction had more flavonoids content than the other two extracts (that of acetone and 95% ethanol). Similar to some other plants, solvent extraction method is the most basic and often employed method for extracting *C. tinctoria* flavonoids (Liu et al., 2014). Despite the purity in initial extraction is low and the monomeric compounds were separated, it was the basis for further separation and purification.

3. Chromatographic separation and purification methods

Crude extracts of *C. tinctoria* yielded from the above mentioned extracting methods often consisted of a large amount of non-flavonoids such as pigments, polysaccharides, volatile components and others. Separation and purification is required to enrich the flavonoids content in an extract of *C. tinctoria*, in particular, flavonoids rich extract and also to improve the purity of the targeted compounds for definitive results of subsequent biological activity studies and associated structural-activity elucidation. At present, a variety of common separation and purification methods have been applied in the preparation and isolation process of snow chrysanthemum flavonoids (Table 1), and the commonly used methods mainly include macroporous sorbent resin method, polyamide column chromatography, silica gel column chromatography, dextran gel column chromatography and reversed-phase column chroma-

Table 1. Comparison of separation and purification methods currently used in separating *C. tinctoria* flavonoids

Purification methods	Advantages	Disadvantages
Macroporous adsorption resin method	Good yield and purity; simple equipment and easy operation; multiple types of adsorbents, wide range of applications; reusable/regenerable resin, long service life; overall low cost, suitable for industrial production	Bad desorption effect; time consuming; separation affected by adsorbent performance, eluent type, temperature, etc.
Polyamide column chromatography	Good separation effect and high product purity; good specificity; large sample capacity; reusable/regenerable resin, overall low cost, suitable for industrial production	Long separation period; large void adsorption volume; co-elution with low molecular weight impurities; adsorption process readily affected by solvents
Silica gel column chromatography	Large adsorption capacity and wide application range; easy separation effect; broad use; inexpensive material	Low separation efficiency; disposable material, organic solvent elution
Sephadex gel chromatography	Wide range of use; good separation efficiency; reusable/regenerable resin	Elution influenced by the number of phenolic hydroxyl groups and size and molecular weight of molecules; expensive materials
High-speed counter current chromatography or high-performance counter current chromatography	High purity of product; good reproducibility; separation of multiple components; automation	High cost; low productivity; low separation capacity; often time consuming
Reversed-phase high performance liquid chromatography	Good separation; multiple component separation capacity; automation	Very high cost equipment; low capacity; hard to meet animal study

tography separation, among which, the macroporous sorbent resin method is the most widely used purification method in obtaining *C. tinctoria* single compounds.

Table 1 listed several main chromatographic separation methods commonly used in isolating *C. tinctoria* flavonoids. In the separation of *C. tinctoria* flavonoids, the selection of a chromatographic method is dependent on separation factors of efficiency, specificity, readily availability, cost effectiveness and others. Moreover, to obtain an interested single compound, e.g. marein, particularly in large scale, a repeated separation with same or different chromatographic separation strategy is often practically employed, such as a normal phase silica gel chromatography coupled with a reverse-phase liquid chromatographic separation.

3.1. Normal phase silica gel column chromatography

Normal phase chromatography, or commonly silica gel column, is usually the first-line of chromatographic separation. Silica gel is a polar adsorbent with porous amorphous or spherical particles filled with silanol groups. The adsorption capacity of silica gel is related to the particle size, the number of effective silanol groups on the surface of silica gel and the shape of the silica gel. The higher the number of silanols the silica gel has, the stronger the adsorption capacity of silica gel (Xu et al., 2021) and the good the separation. As an example, the extract from 3.68 kg of dried *C. tinctoria* inflorescence with 70% aqueous ethanol at reflux conditions was fractionated with petroleum ether, ethyl acetate and n-butanol, respectfully. The n-butanol extract was separated by normal phase chromatography on a silica gel column, yielding white amorphous powder - dihydromarein and sub-fractions. Subsequent isolation with reversed-phase liquid chromatography generated pure marein in the form of orange powder. Flash silica gel chromatography has been popularly utilized in the separation of natural products and organic synthetic chemistry. Wang et al. partitioned the aqueous ethanol extract of *C. tinctoria* with ethyl acetate and hexanes, and then the ethyl acetate subfraction was separated by flash silica gel chromatography, yielding okanin with a purity of 97.4% after recrystallization, which was used in an *in vitro* anti-platelet aggregation assay, suggesting that the purified okanin effectively inhibited platelet aggregation induced by adenosine diphosphate and thrombin in healthy volunteers (Wang et al., 2018). The separation and purification of flavonoids from *C. tinctoria* using normal phase silica gel chromatography has been the first line of choice for its robustness and applicability, though the separation could be an effective fractionation and less pure components obtained.

3.2. Macroporous adsorption resin

Macroporous adsorption resin (MAR), an organic polymer adsorbent developed in the 1960s, belongs to polymer adsorption resin. MAR has good macroporous mesh structure, large area of adsorption surface, and strong capacity of adsorption (Wang et al., 2021). It also has the advantages of cost effectiveness, high performance of reusability and adsorption (Liu et al., 2014; Shao et al., 2018). MAR selectively adsorbs target components loaded with water and organic solvents based on intermolecular interactions between the polymer surface and the analytes (Liu et al., 2014). MAR is classified as nonpolar, weakly polar and polar macroporous resins depending on their polarity size. There are numerous types of resins with different polarity, which vary greatly for different phytochemical components in their adsorption properties. Therefore, it is important to select suitable MAR to achieve better separation and purification (Wang, et al., 2021; Zhang, Qi, and Feng, 2019). MAR is an effective method in the separation and purification of flavonoids. It has been used in isolating flavonoids from *Hippophae rhamnoides L.* (Zhang, Qi, and Feng, 2019), *Ginkgo biloba leaves* (Zain et al., 2020), *Lycium ruthenicum Murr.* and *Carthamus tinctorius L.* (Xie et al., 2016).

Upon extraction and fractionation, the crude extract of *C. tinctoria* flavonoids was often purified using macroporous resins such

as polystyrene-type non-polar adsorption resin, cross-styrene-divinylbenzene copolymer, spherical non-polar polymer adsorbent, and crossed-linked polystyrene with low polarity among others. For instance, Song et al (2016) subjected the crude extract of *C. tinctoria* to gradient elution with polystyrene-type macroporous resin (e.g. D101) and used 60% aqueous ethanol as eluent to yield a flavonoid-rich fraction that demonstrated very strong antioxidant activity. Many studies chose crossed-linked polystyrene (e.g. AB-8) macroporous resin to refine *C. tinctoria* flavonoids, because it has higher adsorption capacity, larger specific surface area and lower polarity than other similar type of MAR. Yao et al. (2016) compared 10 types of MAR and finally selected AB-8 resin for the purification of flavonoids of *C. tinctoria*. As an example, the sample was loaded to crossed-linked polystyrene type (AB-8) with 0.54 mg/mL of crude flavonoids with a loading volume of 2.1 bed volume (BV). After adsorption and equilibrium for 4 h, the water soluble polar components were washed with 2-3 BV distilled water. Then the resin bed was eluted with 2.2 BV of 50% aqueous ethanol and collected in sections. Under these conditions, a total of 29% *C. tinctoria* flavonoid content was separated (Yao et al., 2016). The total *C. tinctoria* flavonoids were greatly enriched after the purification with AB-8 macroporous resin, and also the monomeric substances such as flavanomarein,marein and okanin, were significantly increased. Although most of the macroporous resins could be used for purification, the separation efficiency is usually poor, thus cannot ensure that single components can be isolated. Therefore, more separation methods should be explored particularly when single compounds are needed to study the biological activity of monomers of *C. tinctoria* flavonoids.

3.3. Polyamide column chromatography

Polyamides are a class of polymers synthesized by the polymerization of amide groups, which could form hydrogen bonds with hydrogen donors and acceptors because of the multiple amide groups. Polyamide resin for column chromatography is white or off-white amorphous porous powder. The formation of hydrogen bonds between the surface amide groups and analytes, e.g. phenols, acids, quinones, flavonoids, and other phenolic hydroxyl-rich compounds for adsorption, differentiate theses analytes from compounds that cannot form hydrogen bonds. Then, the purpose of separation and purification is achieved (Xiao et al., 2013). Polyamide column chromatography is often used in the separation of flavonoids from natural plants. As an example, Nina et al. applied 60–100 mesh of polyamide resin to the separation of *C. tinctoria* flavonoids. The optimal elution parameters were 70% aqueous ethanol at an optimal pH value of 5, and an amount of 11 BV 70% ethanol. The content of total *C. tinctoria* flavonoids increased from 22.6% to 68.0% of the crude extract with this method (Mawulies et al., 2013). Although the single time separation of a polyamide column can enrich flavonoids of snow chrysanthemum, the separation and isolation of flavonoid monomers is less effective and relatively low purity. Therefore, many researchers have chosen to employ different column chromatography methods in a tandem manner to enhance the total flavonoid content and or to isolate monomeric flavonoid compounds. Zhang et al extracted buds of snow chrysanthemum with refluxing ethanol solution to obtained alcohol extracts, which was after concentration further extracted with ethyl acetate and 1-butanol. The yielded sub-extract was adsorbed by polyamide column chromatography for 12 h, and then eluted with different concentrations of aqueous ethanol at 3BV, respectively. The 10% ethanol fraction yielded flavanomarein with a purity of 99.6%, and the 40% ethanol collection yielded marein with a purity of 98.4% (Zhang et al., 2020). The purity of flavanomarein and marein prepared from this method was good. Polyamide column is cost-effective and reusable, and meantime, it has strong adsorption specificity and high loading capacity. Thus polyamide column plays an important role in the extraction and separation of natural product, such as *C. tinctoria* flavonoids.

3.4. High-speed counter-current chromatography

Counter-current chromatography (CCC) is a chromatographic technique and its working mechanism relies on gravity and centrifugal force to achieve liquid-liquid separation without the use of solid supports or carriers. There are advantages of CCC over traditional solid-state stationary-phase chromatography. CCC avoids the effects of irreversible adsorption, degradation and decomposition of the sample on the solid carrier, and enables continuous and efficient mixing and separation of targeted components in complex mixtures, thus improving the partition efficiency and shortening the separation time (Li et al., 2021; Qiu et al., 2022). In recent years, techniques such as high-speed counter current chromatography (HSCCC) and high performance counter current chromatography (HPCCC) have been explored further with modified techniques for fast and efficient separation, thus widely used in separation science, particularly in the separation and purification of natural products. Both HCSSS and HPCCC are based on technique of fast partition of analytes and dynamic equilibrium between two different phases (mobile and stationary phases) with unidirectional flow to achieve rapid separation of components (Li et al., 2021; Xu et al., 2019). Requiring less sample pretreatment than other chromatographic methods, the CCC technique provide fast separation, no sample adsorption and good reproducibility, thus can be used to refine the final product (Qiu et al., 2022; Sun et al., 2014).

Currently, the CCC technology of separation and purification of natural active substances is broadly used in China, but there are few studies on the isolation of *C. tinctoria* flavonoids that employed CCC. In an HSCCC separation of *C. tinctoria* flavonoids, Wang et al. (2017) used a mixture of four solvents, i.e. petroleum ether-ethyl acetate-methanol-water (v/v; 2: 8: 2: 8 for stationary phase, and 2: 8: 4: 6 for mobile phase, respectively) as the two-phase solvent system, with the upper phase as the stationary phase and the lower phase as the mobile phase. From the HSCCC separation, 3,5-dicaffeoyl quinic acid (11.2 mg, 92.8% purity) and okanin (10.7 mg, 97.5% purity) were isolated, respectively, from 200 mg of ethyl acetate extract of *C. tinctoria* (Wang et al., 2017). These isolating results have demonstrated that HSCCC separation of bioactive components in *C. tinctoria* was rapid and efficient with high purity, suggesting a new angle in isolating single and pure *C. tinctoria* flavonoids for their evaluation of biological activities.

3.5. Reversed-phase chromatography

Reversed-phase chromatography is commonly relative to normal phase, e.g. silica gel chromatography. After the silanol groups on the surface of silica gel (silica dioxide) are acetylated with C18, C8, C4 or other chain length of lipophilic hydrocarbons, the surface of the silica gel core becomes lipophilic, i.e. reversed relatively to initial hydrophilic silanols. Other polymers have also used to act as the silica core for acetylation of hydroxyl, amino groups on the surface of polymer core with hydrocarbon chains to yield 'reversed-phase' materials that have similar or better separation capacity than silica-core hydrocarbon derived materials in terms of separation efficiency. Solvent used in reversed-phase chromato-

graphic separation are water and several organic solvents, usually methanol and acetonitrile.

The reversed-phase chromatographic separation method, usually adapted in high performance liquid chromatography (HPLC), has been applied in the separation of *C. tinctoria* phytochemicals such as flavonoids (Chen et al., 2016). As an example, using reversed-phase C18 column chromatography, Song et al purified a fraction with D101 macroporous resin loaded with the crude extract of *C. tinctoria* after a gradient elution with 60% aqueous ethanol. A pure flavonoid, trans-3,4,2′,3′,4′-pentahydroxychalcone, was obtained, with a test DPPH IC50 value of 10.2 μg/mL, 6-fold lower than that of the positive control rutin, 60.5 μg/mL (Song et al., 2016).

3.6. Other method

There are other methods in the separation of phytochemicals including capillary zone electrophoresis, ion exchange chromatography, acid-base treatment and others. Using capillary zone electrophoresis, Deng at al. (2017) investigated the separation of *C. tinctoria* flavonoids and phenolic acid including flavanomarein, taxifolin-7-O-glucoside, quercetin-7-O-glucoside, okanin,okanin 4′-O-glucoside, and chlogenic acid in different parts of this plant and dieenernt origin as well as different species with hyperoside used as an internal standard. The sepataion was efficient and could be used as a quality control for pharmacological evaluation. However, this method is for analysis and detection, not applicable for large scale separation and isolation for the biological evaluation of *C. tinctoria* phytochemicals.

4. Conclusions

C. tinctoria flavonoids are the most important bioactive components discovered and evaluated up until the present. However, comparing to the phytochemicals from other plants, such as tea polyphenols, ginseng saponins and citrus flavonoids among others, bioactive compounds in *C. tinctoria* are relatively less explored and further separation and characterization particularly large scale isolation of bioactives including marein, flavanomarein, okanin, isookanin and others, for effective *in vivo* evaluation is needed to be performed. For example, systematic study in the methodology of efficient large scale separation of marein and flavanomarein remains a critical issue to be addressed sooner rather than later, to have a comprehensive understanding of their biological activity and associated mechanisms such as pathways to disease prevention and or treatment.

Methods of separation play critical roles in the research of natural products. There are multiple methods in the separation and purification of pure natural products. The advantages and disadvantages of different separation methods provide a combinatorial complement to enable the isolation of single phytochemicals in high purity and quantity. Therefore, the separation and purification methods summarized in this review and others such as supercritical fluid chromatography and chromatography coupled with mass spectrometry are worth of consideration and exploration in isolating *C. tinctoria* flavonoids and other natural products.

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