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Original Research

Development of a new highly standardized and granulated extract from *Monascus purpureus* with a high content of monacolin K and KA and free of inactive secondary monacolins and citrinin

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Abstract

Monacolins, well-known natural statins obtained from rice fermentation with *Monascus purpureus* (red yeast), are a class of fungal secondary metabolites able to inhibit HMG-CoA reductase. Interest in using fermented products as natural sources of monacolins, instead of chemically synthesized statins, has increased enormously in recent years mainly because these substances are perceived as 'natural'. This perception has also increased the demand for them. In this study we first analyzed the composition of several Chinese red yeast-fermented dried extracts commonly available on the market. Fifht percent of them (16 out of 32) did result to be likely adulterated products whilst the other 50% did not. Anyway, the not adulterated products did show 1) high variability in terms monacolin K and KA (considered to be the active monacolins), 2) relevant content in terms of inactive, or poorly active, secondary monacolins, 3) detectable content in terms of citrinin, a nephrotoxic compound and secondary metabolite yealded by fermentation process and 4) a total titre, expressed as monacolin K, correspondent instead to the sum of all monacolins present in the extract. We have therefore developed a method for purifying not adulterated red yeast fermented products in order to produce a highly purified, standardized extract (MonaKoPure-K20) characterized by a constant, high content of monacolin K+KA (≥20%), no detectable citrinin (<50 ppb) and very few secondary monacolins, where the global titre, expressed as monacolin K, exactly corresponds to the combined content of the two monacolins K and KA.

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Introduction

Cardiovascular disease, particularly coronary heart disease (CHD), is the main cause of mortality in developed countries. Atherosclerosis is the main risk factor for CHD and must be controlled in order to prevent cardiovascular and heart disease. Atherosclerosis is mainly due to foam cells which are former macrophage-type cells that have engulfed oxidized

low density lipoproteins (LDL) and are found in the intima of arteries. They gradually reduce artery and lumen diameter, putting the coronary and small arteries at risk. Consequently, LDL levels should be checked to avoid serious health problems [1-4]. Statins are a class of well-known synthetic substances commonly used because of their ability to reduce plasma LDL levels and cardiovascular risk. Considered worldwide the drugs of choice for treating hypercholesterolaemia, they act by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase. However, despite their positive pharmacological effects on plasma lipids, they have been reported to induce skeletal myopathy, which affects about 15% of patients [5-8].

Red yeast rice is fermented rice on which red yeast (*Monascus purpureus*) has been grown. It is a traditional food widely consumed in Asian countries, especially China, Japan and Korea, where it has been used for centuries. It has recently attracted attention because it contains a biologically active metabolite, called monacolin K and also known as mevinolin or lovastatin, which is able to reduce plasma levels of cholesterol (total and LDL). In addition to monacolin K, some other monacolins are produced during fermentation. Monacolins J, L, X, M, compactin and some other degradation products, such as dehydromonacolin K (DMK), have also been identified. Some of these are inactive or poorly active, while the hypocholesterolaemic effect of others has not yet been properly evaluated [9-11]. Most monacolins are present in an acidic and in a lactonic form (Fig. 1). As lactonic forms are more stable and are easily purified and crystallized, their structures have been better elucidated. The lactonic and acid forms of K-type monacolins are respectively indicated as MK and MKA and are normally the most prominent monacolins found in fermented red rice extracts. However their content, as well as that of one of the other monacolins, is not standardized and can vary considerably between *Monascus* extracts. Due to this variability, it is difficult to evaluate the biological effects of fermented red yeast extracts [12].

Although red yeast rice products have not been standardized, physicians often recommend *Monascus* extracts as an alternative treatment for hyperlipidaemia without considering that, beyond the issue of lack of standardization, they contain statins which, even if of natural origin, can cause adverse side effects [13-16]. Another concern is their citrinin content. Citrinin is a secondary toxic metabolite produced by several species of *Aspergillus*, *Penicillium* and *Monascus*. Originally isolated from *Penicillium citrinum*, citrinin is known to be nephrotoxic, hepatotoxic and possibly carcinogenic in humans and animals. It has been reported that, like ochratoxin A, citrinin is also a potential risk factor for human Balkan endemic nephropathy, originally described as a chronic tubulointerstitial kidney disease in south-eastern Europe. The mechanism of citrinin toxicity is not fully understood, particularly whether its toxicity and genotoxicity are the consequence of oxidative stress or the increased permeability of mitochondrial membranes. It has been suggested that citrinin requires complex cellular biotransformation to exert its damaging effects [17,18]. At present, the level of citrinin is regulated (EU No. 212/2014) and the limit in food supplements is set at 2 ppm [19].

The aim of our work was to first analyze the quality of some *Monascus* extracts available on the Italian market and then to develop a standardized *Monascus* dry extract only containing monacolin K and KA at a higher and standardized concentration, by removing most of the other monacolins produced during the fermentation process. We also sought to develop a product where the citrinin content was so low in the purified extract (<50 ppb) that human consumption did not result in toxicity.

Materials and methods

Materials

Monacolin standard, acetonitrile HPLC grade and ethanol were purchased from Sigma Aldrich Chemicals (USA).

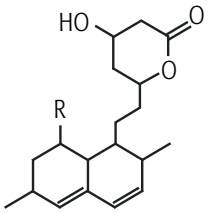
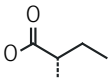
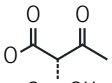
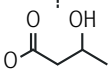
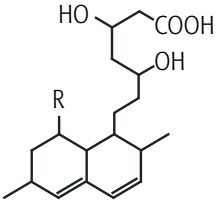
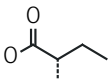
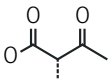
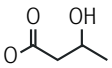
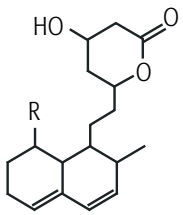
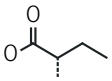
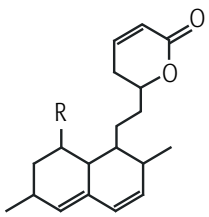
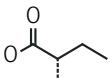
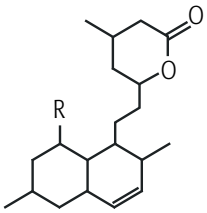
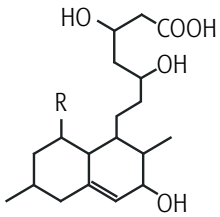
Structure	Name	R	MW	UV (λ_{min})	Ref.
	1. Monacolin K (MK)		404	230, 237, 246	10-12, 20
	2. Monacolin J (MJ)	OH	320	230, 237, 247	13
	3. Monacolin L (ML)	H	304	230, 237, 247	13
	4. Monacolin X (MX)		418	230, 237, 247	14
	5. Monacolin M (MM)		406		15
	1a. MK acid form (MKA)		422		25, 26
	2a. MJ acid form (MJA)	OH	338		
	3a. ML acid form (MLA)	H	322		
	4a. MX acid form (MXA)		436		
	5a. MM acid form (MMA)		424		
	6. Compactin (PI)		390	230, 237, 247	8,9
	7. Dihydroonacolin K (DMK)		386		6
	8. Dihydroonacolin L (DML)	H	306		14
	9. 3α-hydroxy-3,5-ihydroonacolin L (HDML)	H	340		23

Figure 1 Chemical structures of most of the investigated monacolins

Sample preparation

Samples of fermented red rice were placed in an ultrasound bath for 30 min at room temperature. In detail, 0.50 g samples were extracted using two 25 ml portions of ethanol 75%; the solution was placed in a 50 ml volumetric flask, and ethanol 75% was added to a final volume of 50 ml. The solution was then filtered with a 0.45 µm filter and injected for analysis into an HPLC analyzer.

Instrument conditions

An Agilent model 1260 Infinity instrument equipped with a DAD detector was used. Column: Spherisorb ODS-2 250×0.4 mm; 0.5 µm+precolumn Zorbax Reliance Cartridge. Detector: 237 nm. Flow: 1 ml/min. Eluent: A=0.2% phosphoric acid in water; B=acetonitrile. Gradient: A/B 65/35 to 25/75 in 20 min; A/B 25/75 to 25/75 in 28 min. Calibration: from 0.066 mg/ml to 0.530 mg/ml of monacolin K dissolved in ethanol.

Monascus extracts analyzed

All 32 samples of *Monascus* extracts for analysis were acquired on the European market and are available in Italy. All were manufactured by Chinese producers and were mostly imported into Europe by Chinese traders.

Method to evaluate adulteration of Monascus extracts

The 32 different samples of *Monascus* extracts produced by different manufacturers and imported by different traders were subjected to HPLC analysis to evaluate monacolins content. A *Monascus* extract is likely not adulterated if (a) the ratio between the sum of all secondary monacolins (monacolins J, L, X, M, in both the acidic and lactonic forms, and DMK), and the sum of monacolin K and KA is >0.02; and (b) the ratio between monacolin KA and the sum of monacolin K plus KA is >0.30. If other ratios are found, adulteration of the product is almost certain; if only the first of the two values differs, the product is likely adulterated; if only the second of the two values differs, adulteration is possible. Our method to evaluate the adulteration of *Monascus* extracts is original and the proposed cut-off values and criteria regarding monacolin subfractions have not been previously published.

Method to evaluate citrinin content

To evaluate citrinin content in the different *Monascus* extracts and in MonaKoPure-K20 (MP-K20; developed by Labiotre, Tavarnelle Val di Pesa, Florence, Italy), we coupled ultra-high performance liquid chromatography (HPLC) with fluorescence detection as described by Huertas-Pérez et al. [20].

Results and discussion

A total of 32 different fermented red rice extracts available on the European market and produced in China, and standardized as 1.5-3.0-5.0% of total monacolins, were analyzed. As shown in Table 1, most had an acceptable citrinin content, but 16 samples had some anomalies in monacolin content as measured by HPLC so that we assume they were likely adulterated by producers adding synthetic lactonic monacolin K (lovastatin) to red rice. According to our results, a single anomaly was found in 11 of the 32 samples: in four of them this concerned the value of the secondary monacolins and in the other seven concerned MKA content. Therefore these samples are considered as 'likely adulterated' and 'possibly adulterated', respectively. Five of the 32 are considered 'very likely adulterated' as they have two chemical anomalies regarding monacolin content. A pure *Monascus* extract has many peaks due to the presence of different monacolins. In particular, the ratio between the secondary and poorly active, or totally inactive, monacolins and the sum of the two peaks of monacolin K and KA should be >0.02. A lower value, for instance >0.01, is too close to the limit of detectability and to analytical sensitivity. Therefore, a ratio <0.02 indicates that secondary monacolins are absent, which is unusual since these monacolins are always produced during rice fermentation. A second parameter, easily analyzed by HPLC and

Sample number	Secondary monacolins/MKA+MK	MKA/MKA+MK	Citrinin content (ppm)	Manufacturer location	Trader location
LB3-001**	0.010	0.0251	<2	China	China
LB3-002	0.024	0.6788	<2	China	China
LB3-003	0.041	0.7840	<2	China	China
LB3-004	0.034	0.7968	<2	China	China
LB4-005*	0.058	0.0033	<2	China	China
LB3-006	0.058	0.3165	<2	China	China
LB3-007*	0.060	0.0057	<2	China	Europe
LB3-008**	0.000	0.0000	<2	China	China
LB3-009*	0.061	0.0032	<2	China	China
LB3-010	0.353	0.3524	<2	China	Europe
LB3-011	0.032	0.8521	<2	China	Europe
LB3-012*	0.023	0.0287	<2	China	Europe
LB3-013*	0.071	0.0050	<2	China	Europe
LB3-014*	0.012	0.9529	<2	China	China
LB3-015*	0.179	0.0448	>2	China	Europe
LB3-016*	0.014	0.7361	<2	China	Europe
LB3-017*	0.039	0.0251	<2	China	China
LB3-018**	0.002	0.1224	<2	China	China
LB3-019	0.067	0.4598	<2	China	China
LB3-020	0.132	0.4917	<2	China	China
LB3-021**	0.002	0.0571	<2	China	China
LB3-022	0.342	0.3584	<2	China	China
LB3-023*	0.012	0.3687	<2	China	China
LB3-024**	0.006	0.1165	>2	China	China
LB3-025	0.176	0.3821	<2	China	China
LB3-026	0.024	0.4532	<2	China	China
LB3-027	0.024	0.4532	>2	China	China
LB3-028	0.145	0.5432	<2	China	China
LB3-029	0.156	0.3567	<2	China	China
LB3-030*	0.016	0.3224	<2	China	China
LB3-031	0.121	0.3842	<2	China	China
LB3-032	0.056	0.4589	>2	China	China

Table 1 HPLC analysis performed on 32 different batches of *Monascus* extracts from different manufacturers and traders. A *Monascus* extract is likely not adulterated if (a) the ratio between the sum of all secondary monacolins and the sum of monacolin K+KA is >0.02, and (b) the ratio between monacolin KA and the sum of monacolin K+KA is >0.30. If other ratios are found, adulteration of the product is almost certain; if only the first of the two values differs, the product is likely adulterated; if only the second of the two values differs, adulteration is possible. A anomalous value in green is also indicated by an asterisk after the name of the analyzed batch.

useful for indicating possible adulteration, is the percentage of MKA over the sum of monacolin K plus KA. The two monacolins (K and KA) tend to be transformed into one another, with monacolin K normally being predominant with a percentage not above 70% of the total of the two monacolins combined. Therefore, if HPLC shows a higher monacolin K percentage, the product is likely adulterated. To illustrate this, Fig. 2 shows the HPLC chromatogram of sample LB3-008 (see Table 1) where a single peak of monacolin K in the lactonic form, without any of the other monacolins normally produced during the fermentation process, occurs. Since some conditions, for instance warming, are reported by the manufacturer to reduce the monacolin KA content so that only monacolin K is shown on HPLC, the sole presence of this anomaly implies, but does not confirm, adulteration. Of course, if both analytical anomalies are seen, adulteration is almost certain. As shown in Table 1, the other 16 samples had the correct composition of monacolins and did not seem to be adulterated. The HPLC chromatogram in Fig. 3 shows the main peaks of monacolin K and KA (lactonic and acidic forms) and numerous other inactive monacolins derived from the fermentation

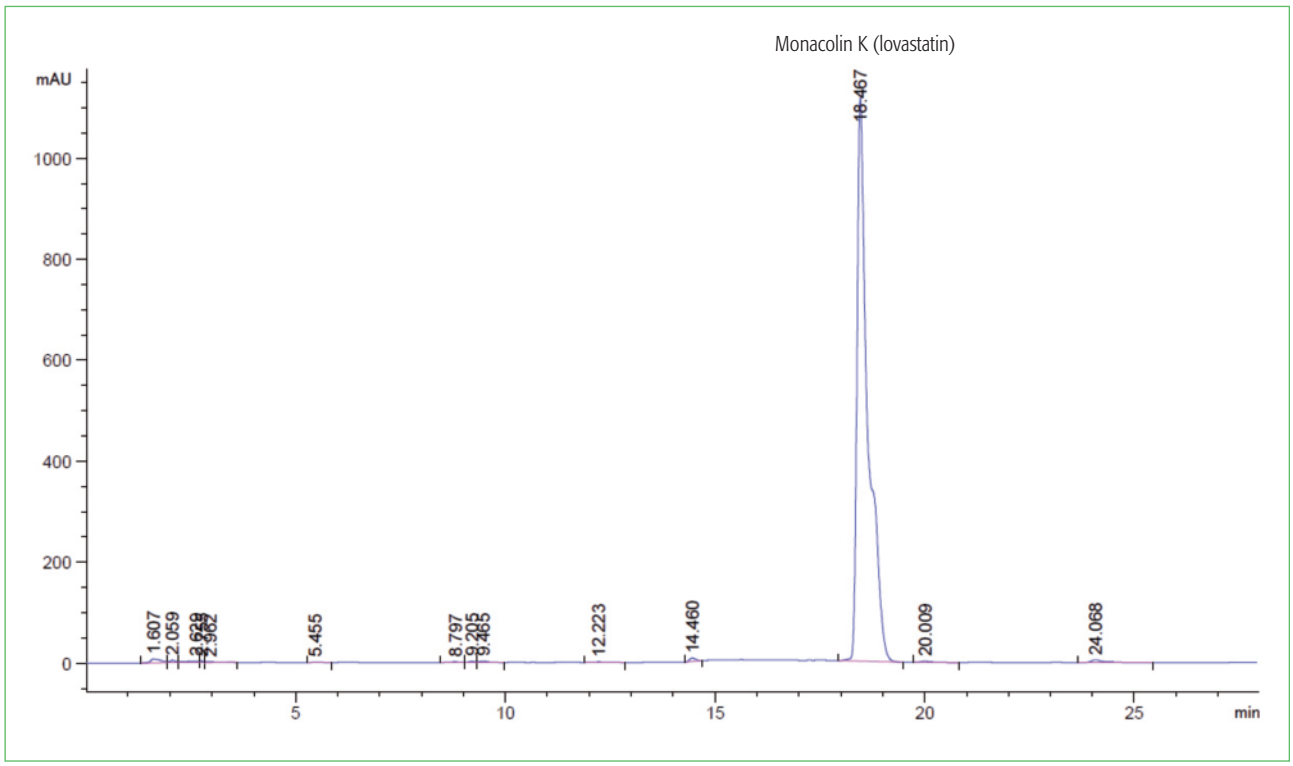


Figure 2 HPLC analysis of a surely adulterated *Monascus* extract shows that the only visible peak corresponds to lovastatin; no other peaks are produced

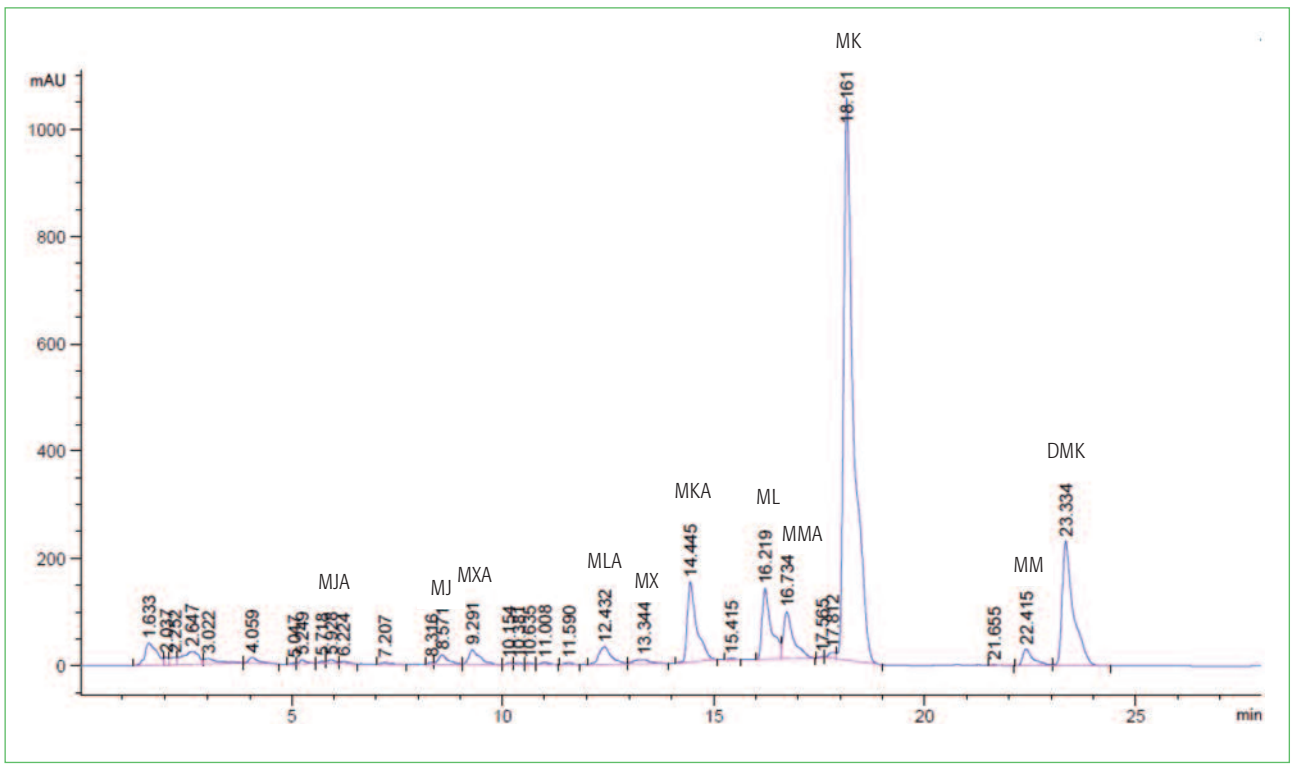


Figure 3 *Monascus* extract titred as 1.5% of monacolin K. HPLC analysis shows that monacolins K and KA combined constitute about 70% of the total monacolin content of the extract.

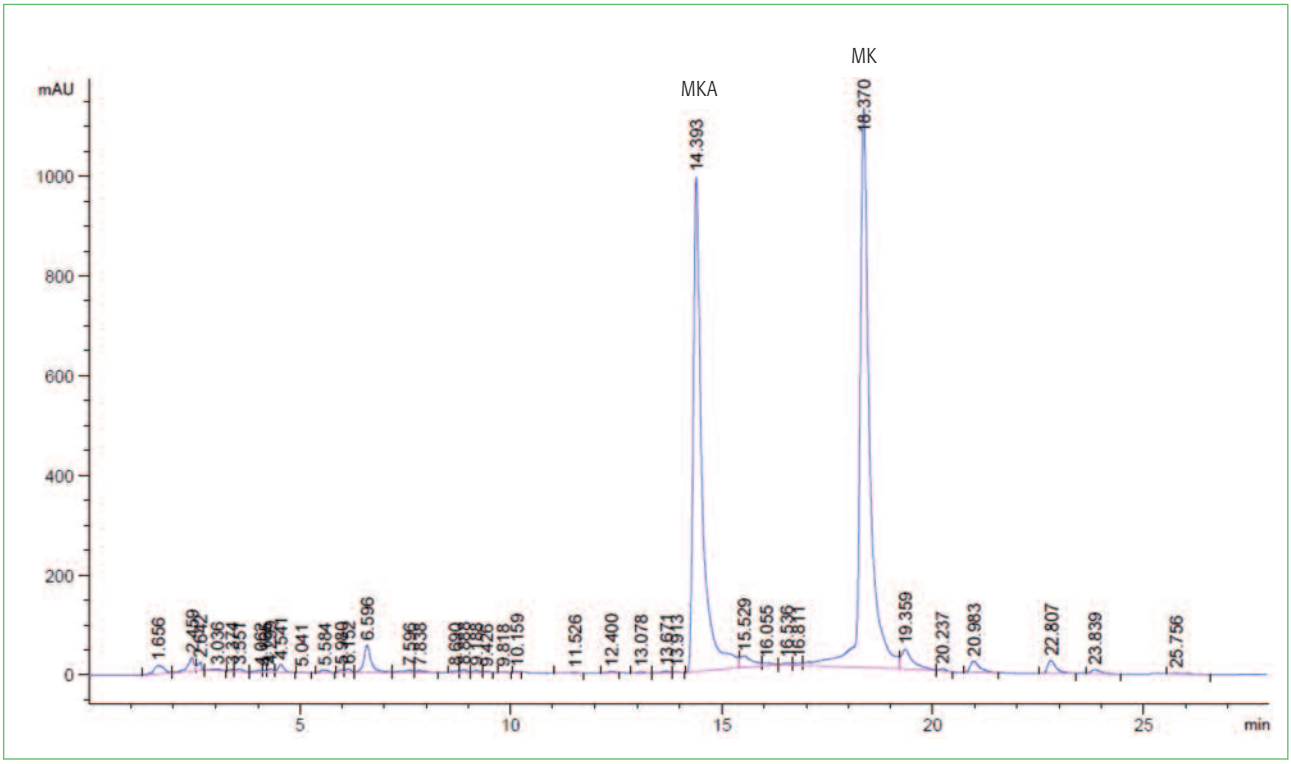


Figure 4 MonaKoPure-K20 standardized dry extract. HPLC only shows monacolins K and KA.

process. In terms of quality, the chromatogram in Fig. 3 (showing a titre of 1.5% calculated as monacolin K) is from a product (LB3-004 reported in Table 1) where monacolins K and KA combined constituted about 70% of the total monacolins in the extract. Analysis of the other 15 not adulterated products revealed combined monacolin K and KA percentages of 40–80%. Using an unadulterated *Monascus* extract containing about 70% monacolins K and KA and with a high level of MKA over the sum of K+KA (sample LB3-011), and only employing physical purification steps (such as extraction under specific conditions and adsorbent resin purification without the use of organic solvents and other chemicals), we produced a purified product (MP-K20) standardized as 20% monacolins K+KA and totally lacking of secondary monacolins (Fig. 4) and citrinin (less than 50 ppb). After analyzing several batches of MP-K20 produced from other samples, for instance batch LB3-003 or LB3-004, we observed that all chemical parameters were consistent. In particular, the ratio of monacolin K to monacolin KA was between 0.7:1.0 and 1.0:1.0. Micronization was applied to the obtained products to reduce particle size and granulometry. Fig. 5 shows the visible difference between MP-K20 and a conventional *Monascus* extract.

Monascus extracts are widely available dietary supplements used by millions of patients as an alternative treatment for hyperlipidaemia. They contain several active compounds, called monacolins, thought to inhibit hepatic cholesterol synthesis by acting on HMG-CoA reductase. Although some studies have suggested that some formulations of *Monascus* may be effective and safe for lipid lowering, monacolin levels are not standardized in products available in Europe and this possible variability is generally not noted on labels. Some monacolins are poorly effective, while others may be ineffective or even cytotoxic. It is also thought that at least 30% of the available *Monascus* extracts are heavily contaminated with citrinin, a nephrotoxic myco-

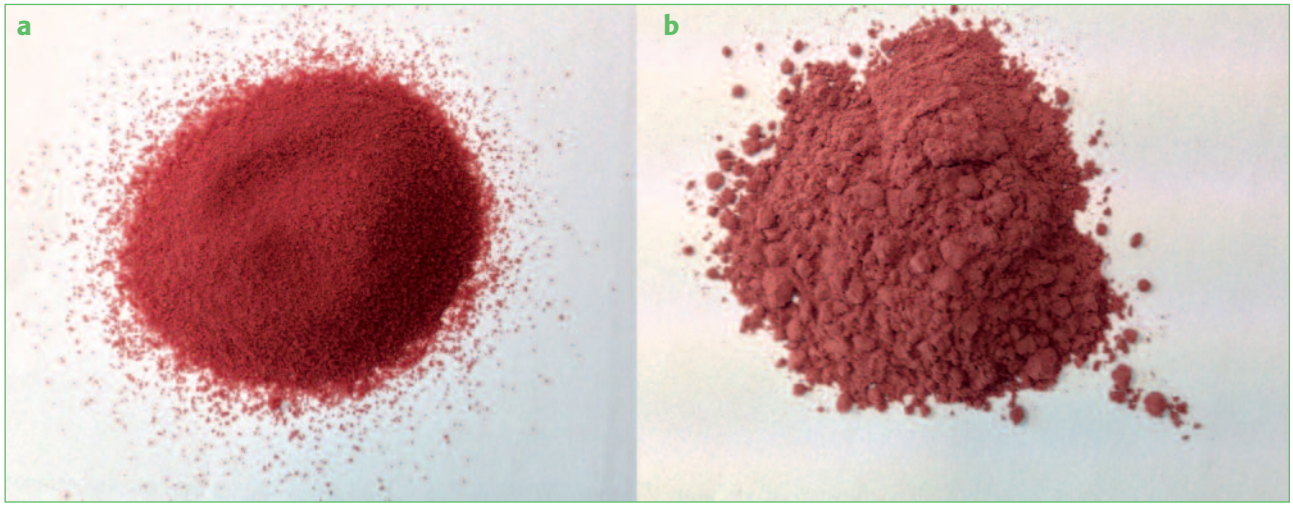


Figure 5 (**a**) MonaKoPure-K20 micronized extract containing 20% monacolins K+KA and citrinin <50 ppb. (**b**) Conventional fermented red rice extract containing 3% total monacolins and available on the EU market.

toxin [10]. We have therefore tried to improve the quality of *Monascus* extracts by physical purification to remove the useless secondary monacolins and the dangerous citrinin. We have developed a product standardized as 20% monacolins and characterized by the sole presence of monacolin K and KA. Last, by granulation and micronization processes applied to the purified extract, we have obtained a fine powder very different from that of conventional *Monascus* extracts and much easier to handle.

Our study has one limitation: the only way to definitely identify adulteration, isotopic analysis of C_{16}, was not employed.

In conclusion, although *Monascus* extracts may have potential as alternative lipid-lowering agents, we suggest caution in recommending them for the treatment of hyperlipidaemia and primary and secondary prevention of cardiovascular disease. These products are not standardized and may contain a toxic metabolite known as citrinin. However, a product with a standardized monacolin content of 20%, containing the only two monacolins considered active (K and KA), and free of other less effective, or toxic, secondary monacolins and, above all, with a very low citrinin content (<50 ppb), could be a safer alternative to all other *Monascus* extracts. Efficacy studies are currently ongoing to verify the clinical role of MP-K20 as an alternative or complementary treatment for hypercholesterolaemia.

Conflict of interest

Giulia Nannoni and Alessandro Alì are employed by Labiotre (the owner of MonaKoPure-K20). Francesco Di Pierro declares no conflict of interest.

Human and animal rights

This article does not contain any studies with human or animal subjects performed by any of the authors.

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