RESEARCH ARTICLE

Effect of Annurca Apple Extract on Anti-hyper Cholesterol Action of a RYR based Formulation

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ABSTRACT

Recently, European Authorities imposed that any product for daily consumption shall provide less than 3 mg of monacolins from red yeast rice (RYR). This obliges to find new formulations able to guarantee an effect on cholesterol levels close to that performed by 10 mg of monacolin K. Two different formulations based on chitosan and xanthan gum containing 10 mg of lovastatin from RYR and a combination of lovastatin (3 mg) from RYR and annurca apple extract (AC), respectively, were compared to verify their anti-hyper cholesterol action in terms of inhibition of HMG-CoA reductase enzyme, protection of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) from oxidation and total free cholesterol depletion. Results highlighted that the combination of RYR and AC represents a valid alternative to the use of high dose of monacolin K. Indeed, formulations containing both nutraceuticals showed a significantly higher activity in terms of inhibition of HMG-CoA reductase, antioxidant activity towards both HDL and LDL and depletion of free cholesterol.

Keywords: Annurca apple, Red yeast rice, Cholesterol, HMG-CoA reductase, Chitosan, Xanthan gum.

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INTRODUCTION

Dyslipidemia, determining an abnormal amount of lipids in the blood, can be considered as one of the most relevant pathological conditions. Most of dyslipidemia cases are due to wrong diet and lifestyle and statins are at the forefront of strategy to manage them. Nevertheless, statins show several side effects and are not always well tolerated. Consequently, in the last decades, the interest in a nutraceutical approach has grown.[1] Red yeast rice (RYR) has been long studied for the cholesterol-lowering ability of its component, monacolin K (lovastatin). Lovastatin can reduce blood cholesterol levels acting as inhibitor of the cholesterol biosynthesis rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA). Lovastatin was the first anti-hypercholesterolemic drug approved by the United States Food and Drug Administration (FDA).[2] Starting from 2002 European authorities warned on the use of high doses of red yeast rice (RYR) and in 2011 determined in 10 mg of monacolins from fermented red yeast rice the amount to be consumed daily.[1] Our previous studies on the effect of different formulations on lovastatin release from RYR highlighted that 10 mg/day of monacolin k showed enhanced inhibitory activity against HMG-CoA reductase and lower cholesterol production compared with pravastatin and synthetic lovastatin.[3-6] Since the exact amount of monacolin K in RYR is highly variable, from 2022 June, the European authorities imposed that individual portions of any product for daily consumption shall provide less than 3 mg of monacolins from red yeast rice. This obliges finding new formulations able to guarantee an effect on cholesterol levels close to that performed by 10 mg of monacolin K. Several approaches have been tested, and the best one appears to be the combination of different nutraceuticals. Evidence of synergetic effects of substances, that act with different mechanisms on lipid metabolism, were found.[7] Supplement products containing RYR and other bioactive compounds have been developed and tested for their anti-hyper-cholesterol activity with very promising results.[8] A combination of RYR (providing 3 mg of monacolin K) and

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berberine (500 mg) have been developed (Armolipid Plus®) to join the inhibition of HMG-CoA reductase enzyme by RYR to the enhancement of the hepatic uptake of cholesterol by berberine.[7] Several other combinations have been tested, such as RYR and soybean, RYR and lupin protein or pea protein, RYR and artichoke.[9] Actually, high interest in polyphenolic compounds from apple extracts is growing thanks to their ability to play a key role in cholesterol metabolism.[10-12] Comparing different apple varieties (Red Delicious, Pink Lady, Fuji and Golden Delicious), polyphenolic extracts from Annurca apple appeared more effective in decreasing cellular cholesterol accumulation and protecting cells against oxidative stress.[13,14] The polyphenolic profile of the Annurca apple variety, a typical cultivar of the Campania region in Italy listed as a protected geographical indication (IGP) product, has been obtained and it resulted in one of the richest varieties.[15] In this paper, two different formulations containing 10 mg of lovastatin from RYR and a combination of lovastatin (3 mg) from RYR and annurca apple extract respectively, were compared to verify their anti-hypercholesterol action in terms of inhibition of HMG-CoA reductase enzyme, oxidation protection of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) and total free cholesterol depletion. The paper aims to evaluate if the combination of different nutraceuticals with different lipid-lowering activities, can provide an alternative to high dose of monacolin K.

MATERIALS AND METHODS

Materials and Samples

Xanthan gum (XG: Mw: 1 MDa), chitosan (C: Mw: 320 kDa, deacetylation degree ≥75%), cholesterol Quantification Kit (MAK043), HMG-CoA reductase assay kit (CS10909) and solvents were purchased from Sigma Aldrich (Milan, Italy). All solvents were analytical or HPLC grade. Red yeast rice (RYR, titrated at 5%) and Annurca apple extract (Annurca Complex: AC) were provided by MediBase s.r.l. (Prato, Italy). Cylindrical tablets (diameter: 0.5 cm; thickness: 1 cm) were prepared by mixing, by a vortex for 60 seconds (40 Hz), different amounts of components and pressing the powder using a hand press (5 tons pressure for 5 min). Commercial products (Lesstat® and Lesstat forte®) were used as reference. Rosuvastatin was purchased in a local pharmacy.

Methods

In-vitro gastrointestinal digestion

Tablets dissolution was performed following a procedure already reported and slightly modified.[3,14] Briefly, each tablet was immersed in 250 mL of gastric simulating fluid containing 0.5 g of pepsin dissolved in HCl 0.1 N, the pH was adjusted to 2.0 with HCl 6 N, and then incubated at 37°C for 2 hours. After the gastric digestion, the pH was raised to 6.5 with $NaHCO_3$ 0.5 N and then 5 mL of a mixture of pancreatin (8.0 mg/mL) and bile salts (50.0 mg/mL) (1:1; v/v), were added and incubated at 37°C for 24 hours. Samples were maintained under agitation (250 rpm). In 5 aliquots (10 mL) for each sample were collected after 3, 5 and 24 hours and lyophilized for the following analyses.

HMG-CoA reductase inhibition test and lovastatin quantification

Samples were prepared following the procedure previously reported.[6] Briefly, an aliquot of each sample was resuspended in 1-mL of PBS at pH 7.4 and 5 mL were added to the assay kit following the supplier protocol. The rate of NADPH oxidation by HMG-CoA was monitored every 15 seconds at 340 nm for a period of 5 minutes using an Ultraspec 200 UV (Biotech, USA).

To quantify lovastatin, samples (injection volume 20 µL) were analyzed using a HPLC-UV (Varian) equipped with a C18 column (250 × 4.6 mm, 5 µm, Varian). The mobile phase was a mixture of acetonitrile, water, and ethanol (5:3:1) and the flow rate was 1-mL/min. Lovastatin was quantified at 230 nm (LoD:0.05 mg/L; LoQ: 0.10 mg/L). Calibration curve (0.1–10 mg/L) was obtained with the internal standard method using simvastatin following a procedure already reported.[3]

Differential scanning calorimetry

Thermographs of different formulations were recorded using Q1000 DSC (TA Instruments). A tablet of each sample was pulverized, and 15 mg were sealed in aluminum pans, equilibrated at −90°C and then heated to 400°C, using a heating ramp rate of 10°C/min under nitrogen flow (50 mL/min).

Determination of DPPH· radical scavenging capacity

The determination of DPPH radical scavenging capacity was evaluated following the steady state method as described by Mishra *et al.*[16] and Cheng *et al.*[17] The method consists in monitoring the decrease in absorbance for different durations from 15 minutes to 6 hours depending upon the antioxidant and its concentrations to obtain a steady state of decrease in absorbance of DPPH·. The percentage of DPPH· remaining at steady state was determined.

$$\%\text{DPPH remaining} = (Af/A0) \times 100$$

where A0 and Af correspond to the absorbance at 515 nm of DPPH· at initial and steady-state, respectively. Af value was obtained at the steady state region where absorbance did not depict further observable decreases. The effective concentration (EC_{50}) value, defined as the concentration required to decrease the initial DPPH· concentration by 50%, was determined.

Inhibition of HDL and LDL oxidation

The antioxidant activity of formulations versus HDL and LDL was assessed following the procedure reported by Hillstrom *et al.*[18] with some modifications. Briefly, HDL (0.06 g of protein/L) and LDL (0.17 g of protein/L) were incubated at 37°C with Cu(II) ($CuCl_2$ 0.5 mmol/L) and 1 aliquot of each formulation or ascorbic acid (AA). Oxidation was assessed as the increase in absorbance at 245 nm during 3 hours of incubation.

Table 1: Composition of analyzed formulations

Sample	*Chitosan-Xanthan gum (%)*	*Monacolin K (mg)*	*AC (mg)*
C1	20–80	10.01	0.00
C2	20–80	0.00	200
C3	20–80	2.992	200
C4	80–20	10.01	0.00
C5	80–20	0.00	200
C6	80–20	2.992	200
Rosuvastatin 5 mg	/	/	/

[1]Corresponding to 200 mg of RYR; [2] corresponding to 59.8 mg of RYR

Free cholesterol depletion

An aliquot of each sample was redissolved in 1-mL of phosphate buffer (pH 6.8). Two mL of each sample were brought to a final volume of 50 µL with cholesterol assay buffer, and then, 50 µL of the provided reaction mix was added and incubated for 60 minutes at 37°C and then read at 570 nm by an Ultraspec 200 UV (Biotech, USA).[6]

RESULTS AND DISCUSSION

Changing the relative amount of chitosan and xanthan gum permits to modulation the release profile of monacolin K from RYR. Accordingly, two series of formulations were prepared (Table 1).

As reported in Table 1, formulations C1-C3 were obtained by combining chitosan and xanthan gum in a ratio 1:4. This ratio guarantees a prolonged release of lovastatin from RYR and it was the ratio used in the commercial product Lesstat® (Medibase s.r.l., Prato Italy).[6] Formulations C4-C6 were designed to guarantee a rapid release, obtainable by combining chitosan and xanthan gum in a ratio 4:1. This is the ratio that have been used in the realization of the commercial product Lesstat Forte® (Medibase s.r.l., Prato Italy). Each polymer ratio was loaded with a single active component, i.e. RYR or AC, or a mixture of them.

Anti HMG-CoA reductase activity

Monacolin K from RYR is able to reduce the blood cholesterol thanks to its ability to inhibit HMG-CoA reductase, enzyme that catalyzes the conversion of 3-hyroxyl-3-methylglutaryl CoA to mevalonate. When the enzyme is inhibited the production of cholesterol is inhibited. This activates a family of transcription factors that induce the expression levels of proteins involved in cholesterol biosynthesis and cholesterol uptake from the extracellular environment such as LDL receptor (LDLR). The elevation of hepatic LDLR expression levels facilitates the uptake of cholesterol from the blood circulation, which leads to the reduction of blood LDL cholesterol (LDL-C) level and disposal of cholesterol.[8] The effect of the presence of Aannurca complex (AC) on the RYR inhibiting action against HMG-CoA reductase was evaluated in both prolonged (C1 and C3) and rapid formulations (C4 and C6). After 3 hours of permanence in digestion fluid, formulations C3 and C6 resulted more active than the corresponding formulations C1 and C4. Indeed, we can see that either in formulations characterized by a prolonged release (C1–C3) or in formulations characterized by a delayed release (C4-C6) a significantly higher activity against HMG-CoA reductase enzyme was observed in the presence of AC, i.e., C6 resulted more active than C4 and C3 resulted more active than C1, despite both formulations C3 and C6 contained a lovastatin amount of 3 mg against 10 mg loaded in formulation C1 and C4. The different polymer ratio (C3 *vs*. C6 and C1 *vs*. C4) did not affect the inhibition activity versus HMG-CoA reductase. Interestingly, after 5h in the digestion fluid, some effect of the polymer ratio can be observed recording a slightly higher activity for formulation C6 than for formulation C3 (Figure 1). No significant difference was found after 24 hours. When only AC was present (formulations C2 and C5) no significant action was found against the enzyme.

The higher activity could be related to the capability of AC to enhance lovastatin release from RYR. The lovastatin profiles from tested formulations were superimposable but a significantly different amount of lovastatin was registered.

As shown in Figure 2a, either after 3 or 5 hours the polymer ratio affects the percentage of released lovastatin (C4>C1 and C6 >C3) accordingly with a more rapid release when xanthan gum and chitosan are combined in a ratio 1:4. The more rapid release can be related to the absence of interaction between RYR and xanthan gum when the polymer is present at a percentage lower than 70%.[6] Analyzing the absolute quantity of lovastatin released (Figure 2b) we can see that there is not a linear correlation (r = 0.53) between lovastatin and HMG-CoA reductase inhibition. This raises two main conclusions. First, it can be concluded that 3 mg of monacolin K are sufficient to significantly inhibit the enzyme, as also observed in other studies.[19] Second, the inhibition activity is related to different compounds or different monacolins from RYR.[3,19] Finally, as shown in Figure 2b in both series of formulations a higher release of lovastatin was recorded in the presence of AC at both after 3 and 5 hours. The capability of AC to affect lovastatin

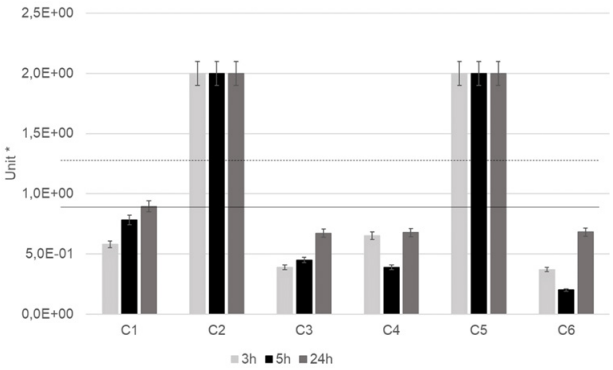

Figure 1: Inhibitory activity versus HMG-CoA reductase of all formulation as a function of kinetics release. One unit will convert 1.0 mmole of NADPH to NADPþ per 1 minute at 37°C. *The unit specific activity is defined as mmol/min/mg-protein (Units/mgP). The lower the value the higher the inhibiting action. Continuous line: aliquots obtained after digestion of two tablets of Rosuvastatin 5 mg; dotted line: aliquots obtained after digestion of one tablet of Rosuvastatin 5 mg).

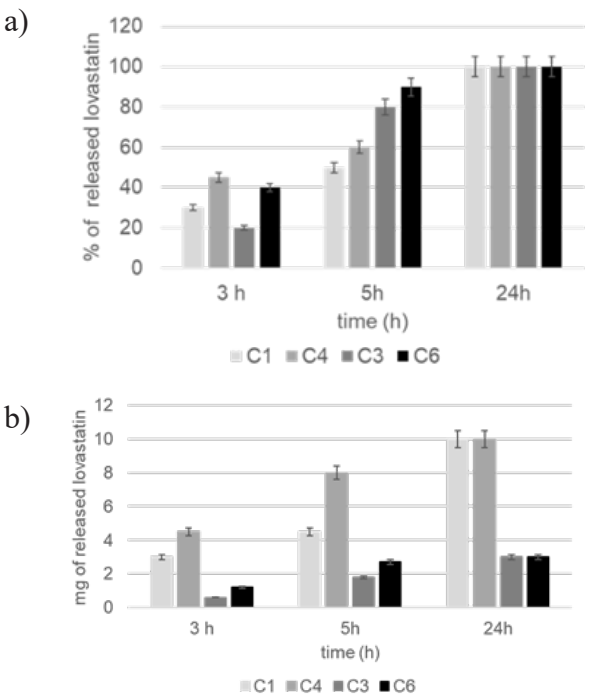

Figure 2: Release profile of lovastatin from formulations as a function of digestion time expressed as percentage of loaded lovastatin (a) and expressed as mg of loaded lovastatin (b)

release from RYR was evaluated by DSC. Thermographs of RYR, AC and a combination of RYR and AC were recorded and depicted in Figure 3.

No significant interactions can be derived between the two nutraceuticals even if the lovastatin melting point (171°C) is not evident in the thermograph of AC + RYR. AC decomposition, centered at 207°C, did not shift after combination with RYR. Thermographs of all the formulations were recorded and depicted in Figure 4.

Comparing the first series of formulations, C1–C3 (Figure 4a) no significant interaction can be observed among any components. Differently, from what was observed when only RYR in large amounts was present[6] the presence of AC destabilizes RYR xanthan gum interactions thus favoring lovastatin release. A superimposable trend was observed for formulations C4-C6 (Figure 4b). No interactions among components were found thus guaranteeing a complete release of free bioactive substances after complete digestion (24 hours).

Antioxidant Activity

The role of oxidative stress in the development and progression of dyslipidemia is well-recognized. HDL oxidation has huge consequences on its capability to perform the reverse transport of cholesterol and on protect LDL from oxidation.[20-22] Indeed, it is well known that cholesterol accumulation is not primarily due to native LDL but instead due to the uptake of the oxidized form of LDL.[23] HDL and LDL are susceptible to lipid oxidation with consequent loss of their protective properties. Several studies have demonstrated that vitamins E and C can

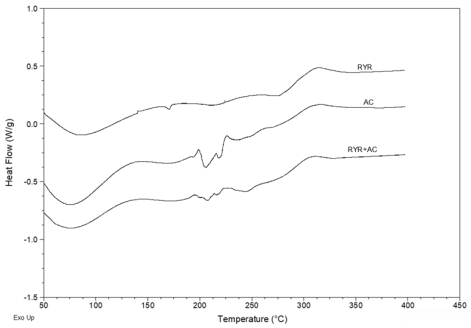

Figure 3: DSC thermographs of nutraceuticals, RYR, Ac and AC + RYR

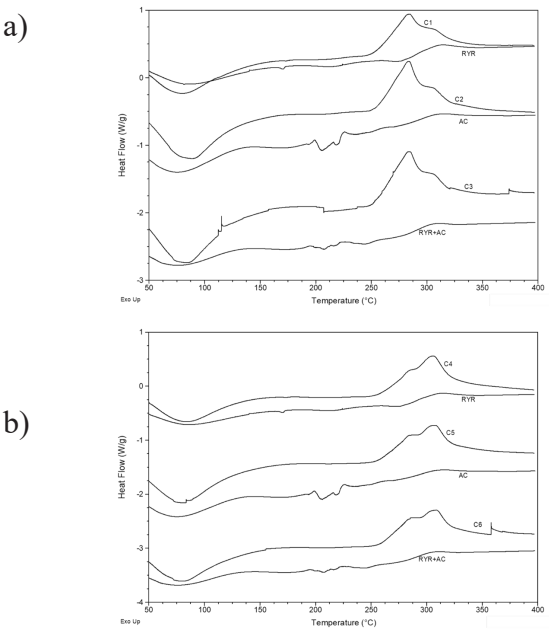

Figure 4: a) DSC thermographs of formulations C1–C3; b) DSC thermographs of formulations C4-C6.

protect HDL from lipid oxidation with the preservation of cardioprotective properties.[24] In this view, the antioxidant activity of all formulations was evaluated. The release kinetics were calculated and subsequently the antioxidant property was evaluated on the samples with respect to the DPPH˙ radical expressed in terms of EC_{50}, i.e., the quantity of antioxidant or extract (in weight) necessary to break down 50% of the radical. Therefore, the lower the value of EC_{50} the higher the antiradical power. For the analyses, ascorbic acid was used as a control and the readings were carried out at 45' for all the samples. Rosuvastatin was also tested but it had no antioxidant action. EC_{50} values for ascorbic acid was found to be 0.08 and it resulted significantly higher that the value obtained for all the tested formulations. Among the formulations, C3 and C6 showed significantly lower values in comparison with the others, thus highlighting a very strong antioxidant activity of both RYR and AC (Figure 5). Results highlighted a synergistic effect of the two active compounds whose antioxidant activity increases when they are in combination.

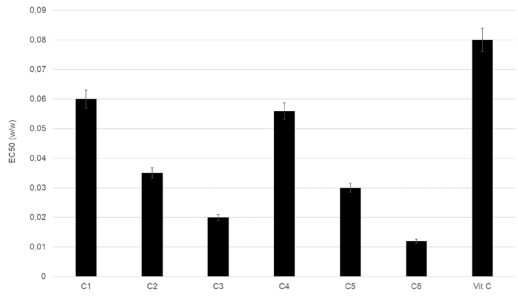

Figure 5: EC_{50} of all formulations

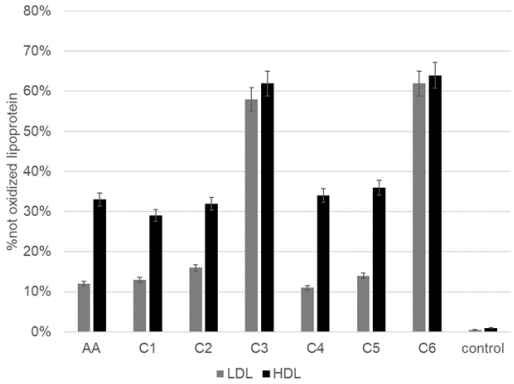

Figure 6: Percentage of not oxidized LDL (grey) and HDL (black). AA (ascorbic acid), control (absence of antioxidants)

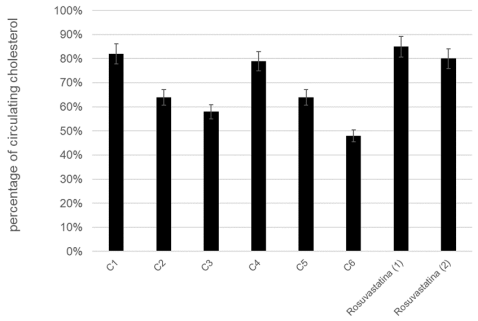

Figure 7: Percentage of free cholesterol after incubation with different formulations. Rosuvastatin (1): 1 tablet of rosuvastatin 5 mg; Rosuvastatin (2): 2 tablets of rosuvastatin 5 mg

Plasma lipoprotein oxidation is a key event in the development of atherosclerosis. It has been widely demonstrated that vitamins E and C reduce the oxidation of lipoproteins.[24] The ability of formulations to protect both HDL and LDL from the oxidizing action of copper ions (II) aliquots was evaluated. The results obtained, shown in Figure 6, highlighted that all formulations containing only RYR (C1 and C4), only AC (C2 and C5) and rosuvastatin showed superimposable antioxidant activity than ascorbic acid versus LDL and HDL with a higher protecting activity to HDL. Contrarily, formulations C3 and C6, containing both RYR and AC, showed the same excellent antioxidant capacity towards both lipoproteins.

Cholesterol depletion

The capability of formulations to deplete free cholesterol was evaluated (Figure 7). Rosuvastatin (both 1 and 2 tablets) and formulations containing only RYR, i.e., C1 and C4, showed a superimposable low capability to bind free cholesterol reducing its concentration of about 15%. Contrarily, formulations containing only AC, thanks to the high content of procyanidins, are able to favor the stable complexation of free cholesterol.14 Finally, formulations containing both RYR and AC showed the highest capability to deplete free circulating cholesterol, reducing its concentration of about 50%.

CONCLUSION

Two series of formulations were obtained combining chitosan and xanthan gum in different percentages. The obtained formulations were loaded with RYR or AC or a combination of them to evaluate their efficacy in lowering cholesterol. The combination of Annurca apple extract and RYR showed a synergistic effect in terms of inhibition of HMG-CoA reductase, antioxidant activity towards both HDL and LDL and depleting free cholesterol. Thus, a combination of phytochemical nutraceuticals with lipid-lowering activity represents a valid alternative to the use of high dose of monacolin K.

AUTHOR CONTRIBUTIONS

Conceptualization, G.L. and S.P; methodology, C.B., formal analysis, G.T. and M.C; .investigation, S.P., L.T. and F.B.; supervision, G.L. and A.M. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data available upon request to authors

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