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Two glucosinolates and their effects related to the prevention of cholesterol gallstones: a review

[Dos glucosinolatos y sus efectos asociados con la prevención de cálculos biliares de colesterol: una revisión]

Ibrahim Guillermo CASTRO-TORRES*, Minarda DE LA O-ARCINIEGA & Mariano MARTÍNEZ-VÁZQUEZ

[1]Instituto de Química, Universidad Nacional Autónoma de México, México DF, México.
[2]Área Académica de Farmacia, Instituto de Ciencias de la Salud, Universidad Autónoma del Estado de Hidalgo, Pachuca de Soto, México.
Contactos | Contacts: Ibrahim Guillermo CASTRO-TORRES - E-mail address: ibrahim1002@hotmail.com

Abstract*:* Two glucosinolates (glucoraphasatin and glucoraphanin) and their degradation products (raphasatin and sulforaphane) are secondary metabolites which have shown antioxidant properties and inhibitory properties against the hepatic cholesterol; these effects are very important for the prevention of cholesterol gallstones because in their pathophysiology there is an imbalance in the transport and secretion of cholesterol. These effects produce oxygen reactive species formation, which damages the hepatic and biliary tissues. Cholesterol gallstones are a public health problem; their pharmacological treatment is very limited and the invasive surgical treatment for symptomatic gallstones is the cholecystectomy. Current research focuses on the search for preventive treatments, as there are many risk factors associated with the development of gallstones; therefore, a natural therapeutic alternative may be the use of these glucosinolates and their degradation products.

Keywords: glucosinolates, gallstones, cholesterol, reactive oxygen species.

Resumen: Dos glucosinolatos (glucorafasatina y glucorafanina) y sus productos de degradación (rafasatina y sulforafano) son metabolitos secundarios que han demostrado propiedades antioxidantes y propiedades inhibidoras contra el colesterol hepático; estos efectos son muy importantes para la prevención de cálculos biliares de colesterol porque en su fisiopatología existe un desajuste en el transporte y secreción del colesterol. Estos efectos producen la formación de especies reactivas de oxígeno, que dañan los tejidos hepático y biliar. Los cálculos biliares de colesterol son un problema de salud pública, su terapia farmacológica es muy limitada y el tratamiento quirúrgico invasivo para cálculos biliares sintomáticos es la colecistectomía. Las investigaciones actuales están orientadas a la búsqueda de tratamientos preventivos, porque hay muchos factores de riesgo asociados al desarrollo de cálculos biliares; por lo tanto, una alternativa terapéutica natural podría ser el uso de estos glucosinolatos, así como sus productos de degradación.

Palabras Clave: Glucosinolatos, cálculos biliares, colesterol, especies reactivas de oxígeno.

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INTRODUCTION

Cholesterol gallstones are prevalent in Western countries such as the United States and in Latin-American countries such as Chile, Mexico and Argentina (Stinton and Shaffer, 2012). Due to the fact that this disease causes a considerable amount of medical appointments and hospitalizations, it is considered a public health problem for which there are is only a limited number of drugs that can be used for its treatment (Portincasa *et al*., 2012); the invasive prophylactic therapy for symptomatic gallstones is cholecystectomy, or the removal of the gallbladder (Maurer *et al*., 2009); therefore, investigations are focused on the search for preventive treatments, but the cost-benefit produced by the pharmacological and surgical treatment, coupled with the numerous risk factors for developing this disease (Portincasa and Wang, 2012). Various natural products that have preventive properties have been used in cholesterol gallstones research, some of them used in the traditional medicine. Glucosinolates are secondary metabolites present in the Brassicaceae family (Lelario *et al*., 2012). These natural products and their degradation products (isothiocyanates) have both been object of research due to their antioxidant properties, and it has recently been shown that these metabolites have the property of reducing hepatic cholesterol levels in *in vivo* models. (Hanlon *et al*., 2007; Rodríguez-Cantú *et al*., 2011). These effects might prevent the formation of cholesterol gallstones, as this gastrointestinal disorder is characterized by an imbalance in the concentration of hepatic cholesterol; the lipid is constantly secreted to the gallbladder, causing damage to the epithelium and the production of reactive oxygen species (Wang *et al*., 2009). Glucoraphasatin, raphasatin, glucoraphanin and sulforaphane are the glucosinolates which have shown to generate these important therapeutic effects. In their antioxidant activity have been reported trascendental action mechanisms in different enzymes, such as quinone reductase, glutathione s-transferases and thioredoxin reductase; the hypocholesterolaemic effects of glucosinolates are involved in the action mechanism of the sterol regulatory element-binding proteins (SREBP-1 and SRBP-2) and the fatty acid synthase enzyme (FAS) (Hanlon *et al*., 2007; Rodríguez-Cantú *et al*., 2011). In this review we discuss the reports that support these therapeutic effects and we present important information from databases such as PubMed, ScienceDirect and SciELO in order to discuss the effect of two glucosinolates regarding the prevention of gallstones. All prospective randomized controlled clinical studies and preclinical studies of cholesterol gallstones disease were considered. Articles written in English and Spanish were included in the search. The articles analyzed range from 2006 to 2012; we found them using keywords: glucosinolates, glucoraphasatin, glucoraphanin, Brassicaceae, reactive oxygen species, antioxidant, gallstones, cholesterol, gallbladder disease and biliary lipids. Most experimental studies analyzed were published in journals with a high impact factor.

RESULTS

We collected 80 papers, including review articles, clinical studies and basic experiments. Overall, 43 articles were eligible for the review. We selected two articles from the SciElO database and all remaining articles (41) were selected from the PubMed and ScienceDirect databases. Each manuscript was evaluated on the basis of scientific rigor of our article, considering the adequacy of the data, importance and originality of the studies, adequacy of the literature citations, clarity of the presentation, interest to the Journal's readership and our conclusion.

Cholesterol gallstones disease

This disease is the most common disorder of the gastrointestinal system, with a prevalence of 10%-15% in the Western population; Chile and Mexico are the countries with the highest prevalence of cholesterol gallstones in Latin-America, the genetic aspects being very important in these regions (Stokes *et al*., 2011; Kovacs *et al*., 2008). There are numerous risk factors associated with the development of cholesterol gallstones: female sex, obesity, sedentary lifestyle, high-fat diet, advanced age, hypercholesterolemia, among others (Stokes *et al*., 2011). The pathophysiology of cholesterol gallstones is complex and polygenic, many proteins and nuclear receptors are implicated in the hypersecretion of biliary cholesterol, crystallization/nucleation of cholesterol, biliary sludge formation, gallbladder stasis and intestinal hypomotiliy (Stokes and Lammert, 2012; Castro-Torres, 2012). Cholesterol homeostasis is very important within gallstones pathophysiology, because is the consequence of the complex regulatory mechanisms involving intestinal absorption and hepatic synthesis, as well as biliary secretion (Portincasa and Wang, 2012). These imbalances are

combined with all the pathophysiological process that takes place within the gallbladder, such as hypersecretion of mucin gel, inflammation reactions, alterations in immunological system and stasis (Maurer et al., 2009). Thus many proteins are implicated in biliary lipids secretion and are regulated it by several transcription factors, including nuclear receptors LXR and FXR (Vázquez et al., 2012). Bile salts, phospholipids and cholesterol are the three major lipids present in the bile and its transporters such as ABCB4, ABCB11, ABCG5, ABCG8 and NPC1L1 protein are involved in pathogenesis of gallstones (Stokes and Lammert, 2011).

Two cholesterol transport processes are important for the prevention of cholesterol gallstones: biliary secretion and intestinal absorption (Portincasa and Wang, 2012), and most of the research for a treatment is based on them. Currently, there is a drug called ezetimibe that inhibits the expression of the Niemann-Pick type C1 type 1 (NPC1L1) protein, thereby diminishing intestinal cholesterol absorption (de Bari *et al*, 2012). Ezetimibe inhibits the formation of cholesterol gallstones in mice (Wang et al, 2008; Zúñiga *et al*., 2008), but there are no specific clinical studies that evaluate NPC1L1 expression in gallstones prevention. Other prophylactic drugs are ursodeoxycholic acid and statins (Wang *et al*., 2008; Ahmed *et al*., 2011); it is important to consider that when gallstones are already formed, these therapeutic agents present a low capacity to dissolve them, ursodeoxycholic acid being the most used drug, but in order for it to work the gallstones must be radiolucent and the motility of gallbladder should not be altered (Tuncer *et al*., 2012). Although scientific research on a future therapeutic target is centered on cholesterol transport sites, it is important to consider the formation of reactive oxygen species as a result of the high concentration of hepatic cholesterol; these imbalances might be inhibited by the effect of glucosinolates and their hydrolysis products, but it is necessary to continue research on natural products, as they are considered important alternative treatments.

Glucosinolates

Glucosinolates constitute a well-defined group of plant secondary metabolites, with a characteristic structure and biochemistry. Structurally, they are anions composed of thiohydroximates that carry a sulphur-linked β-glucopyranosyl residue, an oxygen-linked sulfate residue, and an amino acid-derived, variable side chain (Figure 1). Occasionally, further substituents are attached to oxygen, sulfur or nitrogen atoms of the side chain or to the glucosyl moiety (Agerbirk and Olsen, 2012). Glucosinolates are structurally diverse and in recent years there has been major advances in understanding the molecular genetics and the biochemistry of their biosynthesis and accumulation. Glucosinolates are synthesized from a small number of primary amino acids such as tyrosine, phenylalanine and tryptophan (Sønderby *et al*., 2010). These secondary metabolites are found inside vacuoles, myrosinase being the enzyme responsible for hydrolyzing them into different products of degradation, such as nitriles, isothiocyanates and thiocyanates (Peñas *et al*, 2011). Most plants containing glucosinolates belong to the Brassicaceae family, also known as cruciferous; it includes radish, spinach, broccoli, cabbage, cauliflower, turnip, mustard, Brussels sprouts, capers, daikon, watercress and wasabi (Mithen *et al*., 2010). The pungent taste of these vegetables, and of the condiments and flavoring agents that are prepared with them, comes from the products of the myrosinase reaction that occurs soon after the tissue of the plant is damaged (Dinkova-Kostova and Kostov, 2012). The importance of this reaction for plant defense is emphasized by the extreme degree of metabolic specialization, including the degradation of cellular organelles, that takes place during the differentiation of the glucosinolate-rich sulphur cells (Tripathi and and Mishra, 2007; Mithöfer and Boland, 2012). The biology of glucosinolates is intrinsically connected to their characteristic chemistry and biochemistry; their degradation products are detrimental to a large number of organisms (Halkier and Gershenzon, 2006; Vig *et al*., 2009). In some cases, glucosinolates and/or their products are involved in herbivore insect defense against predators (Müller, 2009). Glucoraphasatin and glucoraphanin are the most studied glucosinolates and are found mainly in radishes and broccoli. These glucosinolates have shown significant antioxidant and hypocholesterolemic properties, which can be related to the prevention of cholesterol gallstones (Figure 2). No toxicity related to the different therapeutic effects of glucoraphasatin and glucoraphanin has been reported, but, with respect to cholesterol gallstones treatment, we have reported that the DL_{50} of black radish juice is over 5000 mg/kg and this plant has a high glucraphasatin concentration (Castro-Torres *et al.*, 2012). These results allow us to think that juice black radish is not toxic, that it is effective and that it is not necessary to look for a lethal dose at higher values than the one we analyzed because therapeutic effects produced with minimal doses are preferred.

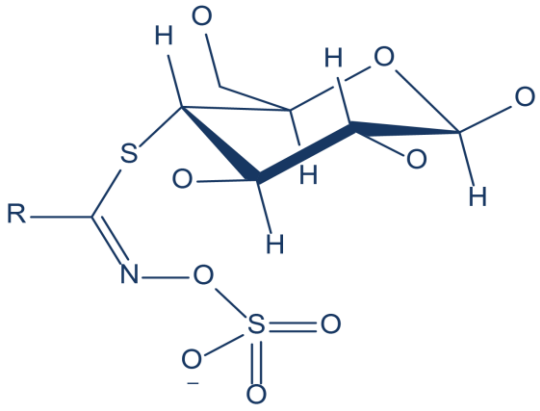

Figure 1
Chemical structure of glucosinolates.
The basic structure of glucosinolates comprises a glucose residue, a sulfate group, and a variable aglycone. Glucosinolate content varies with the species, the plant part and the cultivation and climatic conditions.

Glucoraphasatin. Glucoraphasatin is a glucosinolate mainly found in radish roots and sprouts (Montaut *et al*., 2010). This secondary metabolite is present in black radish (*Raphanus sativus* L. *var niger*) (Hanlon *et al*., 2009). Black radish has important ethnobotanical uses for the treatment of pigment and cholesterol gallstones in the Mexican traditional medicine. In mice fed with a lithogenic diet, the juice of black radish significantly decreased cholesterol and triglycerides levels and increased HDL levels (Castro-Torres and Naranjo-Rodríguez, 2012); the metabolism of cholesterol and triglycerides is very important in the pathophysiology of cholesterol gallstones (Smelt, 2010). Treatment with black radish juice at three concentrations (concentrated, diluted one hundredfold 1:100 and tenfold 1:10 in purified water) during 6 days, dissolved cholesterol gallstones in mice (Castro-Torres *et al*., 2012). The concentration of glucoraphasatin in an aqueous extract of the root of black radish is very high (approximately 30 μmol of glucoraphasatin per gram of radish dry weight) compared with other glucosinolates (Hanlon *et al*., 2007), and this glucosinolate is probably involved in the beneficial effect of decreasing cholesterol and triglycerides levels and dissolving cholesterol gallstones. Glucoraphasatin is metabolized into an isothiocyanate called raphasatin, which causes significant induction of detoxification enzymes (Scholl *et al*., 2011; Abdull-Razis *et al*., 2012). Raphasatin increases the activity of quinone reductase in HepG2 cells at concentrations of 1-30 μM (Hanlon *et al*., 2007), and also activates the antioxidant response element in cell lines (Scholl *et al*., 2011).

Glucoraphanin .Glucoraphanin is a secondary metabolite found naturally in black radish (in an aqueous extract of this plant, there is approximately 4 μmol of glucoraphanin per gram of radish dry weight). Broccoli sprouts are also very rich in glucoraphanin, which is enzymatically converted to sulforaphane (Hanlon *et al*., 2007; Rodríguez-Cantú *et al*., 2011). Sulforaphane is a potent chemopreventive agent, widely consumed in diets or as a diet supplement. This metabolite modulates phase II and III metabolic enzymes (Lubelska, *et al*., 2012). Glucoraphanin and sulforaphane affects cholesterol homeostasis by reducing hepatic cholesterol levels, an important effect for decreasing biliary cholesterol secretion and preventing the formation of cholesterol gallstones.

Antioxidant effects can prevent the formation of gallstones

Glucosinolates and isothiocyanates are known for their antioxidant properties, which are associated with cancer prevention (Fahey *et al*., 2012). However, the antioxidant effects of these compounds have not been studied in other pathologies. It is known that low levels of antioxidant nutrients and an imbalance in glutathione levels, the main antioxidant molecule in humans, are characteristic of gallstones disease (Khokonov, 2011; Vítek and Carey, 2012). A study was conducted in Hungary for evaluating the effect of the juice extracted from black radish root in male

Wistar rats fed with a hyperlipidaemic diet (cholesterol 2%, colic acid 0.5% and fat 20%). In this experiment, rats were given a 1:10 dilution of the extracted juice ad libitum for 9 days. The results showed a significant difference with respect to the products derived from lipid peroxidation between treated and untreated rats. In the same study, the untreated group showed a decrease in glutathione peroxidase enzyme levels compared to the treated group (Lugasi *et al*., 2005); it is known that diets rich in fats affect the efficacy of the blood antioxidant system. This study is important for the ongoing research about cholesterol gallstones prevention. The hyperlipidaemic diet administered to Wistar rats produced gallstones in mice because these organisms have a gallbladder, while rats cannot produce gallstones because they lack a gallbladder. The treatment with black radish juice produced significant antioxidant effects in rats, the same that can be expected in mice; these effects have the capacity to prevent the formation of gallstones. Black radish, in contrast to other cruciferous plants, is used in traditional medicine to prevent and dissolve cholesterol and pigmented gallstones, aspects that reinforce the scientifically demonstrated therapeutic effects associated to the prevention of gallstones.

Antioxidant enzymes prevent the formation of reactive oxygen species, which are known to reduce gallbladder motility, another important factor that predisposes people to the development of gallstones disease. Hence, it is important to consider the study of metabolites that are able to induce the expression of antioxidant enzymes that prevent the formation of gallstones, such as raphasatin, a potent inducer of enzymatic detoxification. Raphasatin (10 μM) induces the expression of relevant enzymes involved in enzymatic phase II detoxification such as quinone reductase, heme oxygenase and thioredoxin reductase in liver cell lines (HepG2) (Hanlon *et al*., 2007). Another study, which took place in the US, showed that the aerial parts (stem and leaves) of black radish were more effective for inducing the expression of antioxidant enzymes involved in enzymatic phase I and phase II detoxification (Hanlon *et al*., 2009). Altogether, these studies in cell lines have documented the significant antioxidant effect of black radish. In gallstones disease, reactive oxygen species act on both the liver and the gallbladder. In the gallbladder they induce cellular infiltration, edemas, granular hyperplasia and hypersecretion of mucin, resulting in the formation of biliary sludge and the accelerated formation of gallstones (Koppisetti, 2008); moreover, reactive oxygen species increase cholesterol crystallization. Therefore, antioxidant enzymes play a big role in preventing pathophysiological disorders associated to gallstones disease.

Inhibition of hepatic cholesterol levels

It has been observed that an increase in the secretion of cholesterol to the gallbladder is one of the necessary conditions for the formation of gallstones (Van Erpecum, 2011); it results from high cholesterol levels in hepatocytes; for this reason, the inhibition of high concentrations of hepatic cholesterol is a relevant target for the prevention of gallstones disease. In a study that used Syrian hamsters fed with a hypercholesterolaemic diet for 7 weeks, freeze-dried broccoli sprouts (*Brassica oleracea var italica*), high in glucoraphanin and sulforaphane, showed a significant decrease of hepatic cholesterol levels. These two metabolites decreased hepatic cholesterol levels, and this effect was associated to a decrease in the expression of SREBP-1, SREBP-2 and FAS (Rodríguez-Cantú *et al*., 2011); these proteins are known to play a relevant role in cholesterol and plant sterols homeostasis. The Syrian hamster has been widely accepted as a good animal model in gallstones formation studies; therefore, the results have shown that under a lithogenic diet, glucoraphanin and sulforaphane may play an important role in preventing the formation of gallstones by decreasing cholesterol levels. However, in the reported study, a hypercholesterolaemic diet was used, which was unable to induce the formation of gallstones. These results are inconclusive, as it is known that other proteins, such as those involved in the transport of cholesterol from the liver to the bile ducts, play a relevant role in gallstones formation; for example, proteins present in the hepatocyte canalicular membrane (ABCG5 and ABCG8). However, there are few studies on the transport and regulation mechanism of cholesterol inside hepatocytes and more studies are needed to fully understand SREB proteins, which are transcription factors involved in the homeostasis and biosynthesis of cholesterol in hepatocytes (Khesht and Hassanabadi *et al*., 2012). In C57L7J mice susceptible to gallstones formation, the abnormal expression of the SREBP-2 protein is associated with an increase in the secretion of cholesterol into the bile. Glucosinolates are known to inhibit the expression of this protein and, thereby, to induce a decrease in cholesterol secretion. SRBP-2 is also known to modulate the protein NPC1 L1, which is involved in the transport of cholesterol and inhibits its intestinal absorption (Pramfalk *et al*.,

2010). Further studies are required to fully understand the effects of glucosinolates and of their degradation products on the transport proteins of cholesterol, which could represent an alternative preventive therapy with few adverse effects.

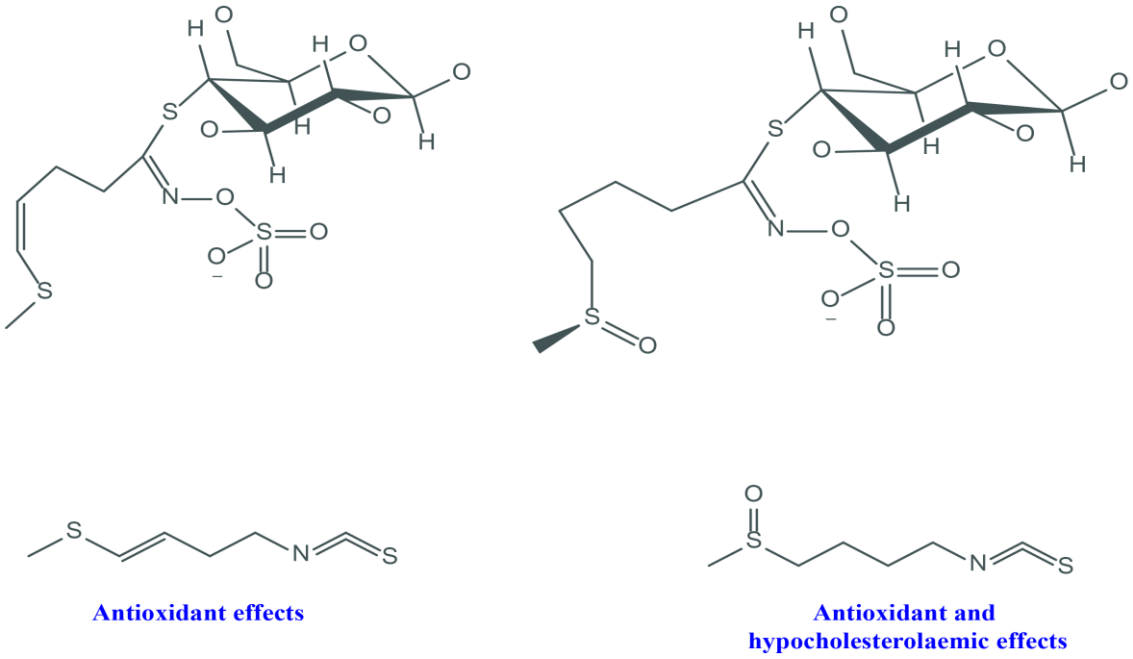

Figure 2
Antioxidant and hypocholesterolemic effects of glucoraphasatin, glucoraphanin and their degradation products. These effects can prevent the appearance of oxygen reactive species, which can appear in conditions of cholesterol gallstones, due to the high concentration of hepatic cholesterol. Glucoraphasatin exerts antioxidant activity by modulation in the expression of different hepatic antioxidant enzymes. Glucoraphanin inhibits hepatic cholesterol levels through the expression changes in enzymes and nuclear receptors of cholesterol metabolism.

CONCLUSION

The antioxidant and hypocholesterolaemic effects exerted by glucoraphasatin, glucoraphanin and their degradation products are very important for the prevention of cholesterol gallstones, a highly prevalent gastrointestinal disease; therefore, it is crucial to investigate the possible action mechanism that allows these secondary metabolites to prevent the development of this disease.

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