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Mesoporous silica nanoparticles SBA-15 loaded with emodin upregulate the antioxidative defense of *Euproctis chrysorrhoea* (L.) larvae

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Abstract: The study presented here aims to elucidate the effects of emodin (EO = 1,3,8-trihydroxy-6-methylanthraquinone) in its free form and when loaded into a mesoporous silica nanocarrier SBA-15 (→ SBA-15|EO) on the activities of the main antioxidative enzymes, superoxide dismutase, catalase, glutathione S-transferase, and glutathione reductase, in larvae of a polyphagous insect pest, the browntail moth *Euproctis chrysorrhoea* (L.). The results show that only SBA-15|EO upregulates the activities of the tested antioxidative enzymes. These results point to significant differences in the effectiveness of the compound in the free versus the loaded form.

Key words: *Euproctis chrysorrhoea*, emodin, mesoporous nanosilica carrier SBA-15, antioxidative enzymes

1. Introduction

Emodin (EO) is a secondary plant metabolite identified in 17 plant families, which is mainly present in Rhamnaceae (*Rhamnus* spp.), Fabaceae (*Cassia* spp.), and Polygonaceae (*Rheum*, *Rumex*, and *Polygonum* spp.) (Izhaki, 2002). It is distributed in the leaves, fruits, flowers, bark, and roots, mostly in the form of different glycosides (sugar derivatives), but is also found in a free form as aglycone (Izhaki, 2002 and references therein). EO possesses numerous biological properties, including bactericidal, fungicidal, immunosuppressive, hepatoprotective, and anticancer activities (Srinivas et al., 2007; Martín-Cordero et al., 2012; Qu et al., 2013; Liu et al., 2015; Dong et al., 2016). EO is important in mediating plant–plant (Knight et al., 2007), plant–animal (Trial and Dimond, 1979; Sacerdote and King, 2014), plant–microorganism (Lee et al., 2013), and plant-abiotic environmental interactions (Izhaki, 2002). The principal activity of EO in mediating plant–animal interactions is feeding deterrence (Trial and Dimond, 1979; Georges et al., 2008; Akhtar et al., 2012). Commercially obtained emodin, similarly to the naturally occurring compound, reduces feeding at relatively low concentrations (0.0015–0.0003 mg/mL) and prolonged development. At high concentrations, EO produces elevated mortality in leaf-feeding gypsy moths (Trial and Dimond, 1979). Besides feeding deterrence, EO exhibits insecticidal (larvicidal) activity against mosquito species and aphids (Yang et al., 2003; Georges et al., 2008; Ateyyat and Abu-Darwish, 2009).

The molecular mechanisms of EO action depend on its chemical structure. The phenolic groups (at positions 1, 3, and 8) permit EO to interact with different proteins through hydrogen and ionic bonds (Wink and Schimmer, 1999). By interacting with enzymes, transporters, channels, and receptors, EO has access to multiple cellular targets and can interfere with many pathways, which is a common feature of many plant phenolics (Mueller et al., 1998; Srinivas et al., 2007 and references therein; Teng et al., 2012; Dong et al., 2016). As a quinone, EO has a marked oxidative impact on the cellular redox status and on the generation of reactive oxygen species (ROS) (Rahimipour et al., 2001; Srinivas et al., 2007; Mecklenburg et al., 2009; Martín-Cordero et al., 2012; Qu et al., 2013; Liu et al., 2015; Lennicke et al., 2016). EO can be reduced to its semiquinone, which in the presence of molecular oxygen generates the superoxide anion ($O_2^{\bullet-}$) (Rahimipour et al., 2001), from which a variety of ROS, such as H_2O_2, can be produced (Halliwell and Gutteridge, 2007; Mecklenburg et

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al., 2009; Lennicke et al., 2016). EO significantly increased ROS levels and oxidative stress in human T cells (Qu et al., 2013). The EO-induced disturbance of metabolic pathways in liver cells, including disruption of glutathione metabolism, has been considered as a mechanism of indirect toxicity of EO (Liu et al., 2015).

Euproctis chrysorrhoea (L.), the browntail moth, is a highly polyphagous insect pest that feeds on plants of 26 genera from 13 families (Forestry Compendium, 2005). It attacks deciduous plants of hardwoods, from oaks to wild roses, fruits, and ornamental trees, as well as shrubs and evergreens in Europe and the United States (Kniest and Hoffman, 1984). *E. chrysorrhoea* is characterized by a peculiar life cycle. It spends about 10 months in the larval stage and overwinters as young larvae inside communal nests (Frago et al., 2009). As some other Lepidoptera, it exhibits eruptive population dynamics and represents an economically important defoliator of forests (especially oaks), orchards, and parks (Kniest and Hoffman, 1984). Due to its appearance together with related gypsy moth species, it represents a special danger. This species is difficult to combat, especially during an outbreak, and requires considerable control by pesticides or biological agents (*Bacillus thuringiensis*, nucleopolyhedrovirus, or *Beauveria bassiana*) (Cory et al., 2000) and recently by the use of essential oils (Erler and Cetin, 2009). In addition, the larvae possess toxic, urticating hairs that are a public health problem (Cory et al., 2000).

Nanotechnology has rapidly developed into a promising field of application in diverse disciplines, from medicine to insect pest management (Rai and Ingle, 2012; de Oliveira et al., 2014). Mesoporous silica nanoparticles and other silica-based materials, notably SBA-15, are considered as exceptional particles due to their high pore volume, surface area, high loading capacities, controlled delivery, efficient cellular uptake (Vallet-Regí, 2012), and, importantly, their nontoxic behavior in cells, even at high concentrations of up to 1 mg mL^{-1} (Bensing et al., 2016).

Given the above information regarding the molecular mechanisms that underlie EO actions, we assumed that EO could act as a prooxidant when given with an artificial diet to 6th instar *E. chrysorrhoea* larvae. The aim of this study was to examine the effects of EO and mesoporous nanosilica carrier SBA-15 loaded with EO on the antioxidative defenses, specifically superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione reductase (GR), in *E. chrysorrhoea* larvae. Lepidopteran larvae rely on these antioxidative enzymes and ascorbate peroxidase (APOX), as well as on nonenzymatic cellular antioxidants, glutathione (GSH), ascorbic acid, and others to counteract the free-radical cascade of oxygen (Perić-Mataruga et al., 1997, 2014; Krishnan and Kodrík, 2006; Jena et al., 2013; Mirčić et al., 2013; Mrdaković et al., 2015; Renault et al., 2016). The superoxide radicals generated under oxidative stress are rapidly dismutated to hydrogen peroxide by SOD. Elevated levels of hydrogen peroxide induce the activity of CAT, the primary cellular scavenger of H_2O_2. SOD and CAT form a very efficient enzymatic pair that terminates the oxygen radical cascade in insects (Ahmad, 1992). The redox cycling of GSH is believed to be another important intracellular antioxidant component that prevents the toxic accumulation of hydroperoxide (Kalinina et al., 2014). We also assessed the activity of GR, which regenerates reduced GSH from its oxidized form (GSSG). We evaluated the activity of GST, which is involved in the second phase of detoxification of xenobiotics in insects, in the catalysis of GSH-dependent conjugation of xenobiotics, insecticides, and plant secondary metabolites (Yu, 2002; Freitas et al., 2007; Mirčić et al., 2013). Importantly, GST induction has been proposed to represent an evolutionarily conserved cellular response to oxidative stress (Hayes et al., 2005).

2. Materials and methods

2.1. Emodin and SBA-15|EO

Emodin was purchased from TCI Chemicals. SBA-15|EO was prepared as previously described (Krajnović et al., 2017).

2.2. Insect-rearing conditions and preparation of larval homogenates

Euproctis chrysorrhoea larvae were collected at the locality of Foča (43°30′N, 18°47′E) on 10 May 2016, usually in the 2nd and 3rd larval instar. During 1 to 2 days, the larvae were acclimatized to laboratory conditions at 23 °C with a 16-h light/8-h dark photoperiod. The larvae were kept on a synthetic high wheat-germ diet (O'Dell et al., 1985). The larvae (n = 6–10 per group) were reared on the synthetic diet ad libitum until the 6th instar, when they were randomly assigned to four experimental groups that were fed for 24 h with the same diet, supplemented as follows: the experimental larval group 1 was fed with an SBA-15 carrier-supplemented diet (15 μg/g of wet weight of the diet); group 2 was provided with the standard diet supplemented with EO in its free form at a concentration of 15 μg/g wet weight of the diet; group 3 was fed an SBA-15|EO-supplemented diet (15 μg/g of wet weight of the diet); and group 4 was the control group, provided with the standard diet (without any supplements).

After 24 h of receiving the different diets, *Euproctis chrysorrhoea* larvae were euthanized by freezing in liquid nitrogen on the third day of the 6th instar. Frozen larvae were kept at –24 °C until preparation of homogenates. The larvae were homogenized on ice in 0.25 M sucrose buffer (0.05 M Tris-HCl, 1 mM EDTA; pH 7.4) using an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany) for 3 × 10 s at 2000 rpm, followed by three 15-s sonication steps

with a 50-W sonifier (Bandelin SONOPULS HD2070, Berlin, Germany). The sonicated homogenates were centrifuged (Beckman L7-55 ultracentrifuge) at 37,000 × g for 100 min at 4 °C. The supernatants were collected and frozen at –24 °C until use.

2.3. Antioxidant enzyme assays

Total SOD (E.C. 1.15.1.1) activity was determined using the procedure of Misra and Fridovich (1972). This method is based on the ability of SOD to prevent adrenaline autoxidation in an alkaline medium. Adrenaline autoxidation was measured at 480 nm at 25 °C with a UV mc2 spectrophotometer SAFAS. SOD activity was expressed in enzyme units per milligram of protein.

The activity of CAT (E.C. 1.11.1.6) was determined according to Clairborne (1984) by spectrophotometric determination of the breakdown of the standard concentration of H_2O_2 (10 mM) at 230 nm (Shimadzu UV-1800 spectrophotometer). CAT activity was expressed in nanomoles of H_2O_2 reduced per minute per milligram of protein.

GR (E.C. 1.8.1.7) activity was determined according to the procedure of Glatzle et al. (1974), which is based on the change in the amount of NADPH consumed by the reduction of a standard amount of GSSG. The activity was expressed in nanomoles of NADPH per minute per milligram of protein.

GST (E.C. 2.5.1.18) activity was determined according to the method of Habig et al. (1974). The substrate, 1-chloro-2, 4-dinitrobenzene (CDNB), was used to determine the activity of GST. The amount of the CDNB-GSH complex was measured spectrophotometrically at 340 nm (Shimadzu UV-1800 spectrophotometer) and expressed in nanomoles GHS per minute per milligram of protein.

Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

The results were analyzed statistically with the program STATISTICA, version 10. Mean values of enzymatic activities and their standard errors were calculated for SOD, CAT, GST, and GR of *Euproctis chrysorrhoea* larvae from all experimental groups. The values were compared by one-way ANOVA applied on logarithmically transformed values and Fisher's least significant difference (LSD) test. P values less than 0.05 were considered as statistically significant.

3. Results

Synthetic EO supplemented in the artificial diet did not significantly affect the antioxidant enzyme activities of *E. chrysorrhoea* larvae. We did not observe significant differences in SOD, CAT, GST, and GR activities between control-diet fed larvae and larvae fed the mesoporous silica-carrier-supplemented diet, SBA-15 (Figures 1–4) in any of the experimental groups. In contrast, mesoporous silica nanoparticles loaded with EO, SBA-15|EO, affected larval antioxidative enzyme activities.

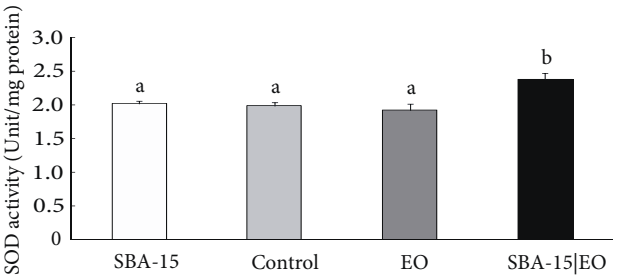

Figure 1. SOD activity in the *Euproctis chrysorrhoea* sixth instar larvae fed on an SBA-15-supplemented diet, control diet, EO-supplemented diet, and SBA-15|EO-supplemented diet. The bars represent the means ± SE. Different letters indicate significant differences between experimental groups (one-way ANOVA followed by post hoc LSD test, $P < 0.05$).

A marked increase in SOD activity was recorded in the SBA-15|EO group as compared to the SBA-15 ($P < 0.01$), control ($P < 0.001$), and EO group ($P < 0.001$) (Figure 1). No significant differences were recorded between the EO and control and the SBA-15 groups (Figure 1).

A statistically significant increase in CAT activity was observed only in the SBA-15|EO group when compared to the EO experimental group ($P < 0.05$) (Figure 2). Mean values for CAT activity in the EO and SBA-15|EO groups did not differ significantly from the values measured in the control and SBA-15 groups.

A significant increase in GST activity was observed in the SBA-15|EO group as compared to EO-treated larvae ($P < 0.01$) and control larvae ($P < 0.05$) (Figure 3).

The differences in GR activities between groups exposed to different diets were not statistically significant (Figure 4), although GR activity in whole larval homogenates was highest in the SBA-15|EO group (Figure 4).

These results show that the diet supplemented with SBA-15|EO induced increases in activities of the four tested antioxidant enzymes, whereas EO (in the free form) did not produce a significant effect on the antioxidative defenses in *E. chrysorrhoea* larvae.

4. Discussion

The naturally occurring and synthetic forms of EO exhibit many biological activities (Srinivas et al., 2007 and references therein), and its role in plant–animal interactions is well known (Izhaki, 2002 and references therein). The feeding-deterrent property of EO on many vertebrate and invertebrate species, including insects, is important (Trial and Dimond, 1979; Georges et al., 2008; Akhtar et al., 2012). Feeding deterrence probably depends

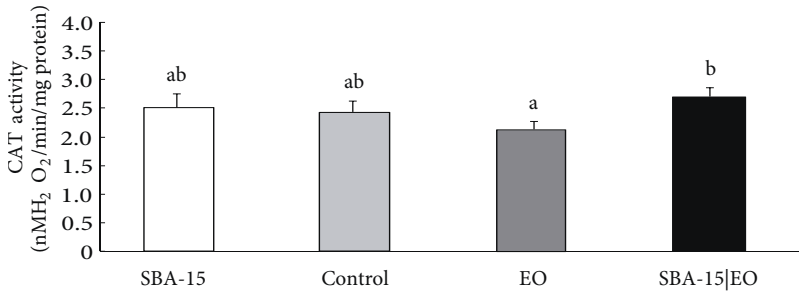

Figure 2. CAT activity in the *Euproctis chrysorrhoea* sixth instar larvae fed on an SBA-15-supplemented diet, control diet, EO-supplemented diet, and SBA-15|EO-supplemented diet. The bars represent the means ± SE. Different letters indicate significant differences between experimental groups (one-way ANOVA, followed by post hoc LSD test, $P < 0.05$).

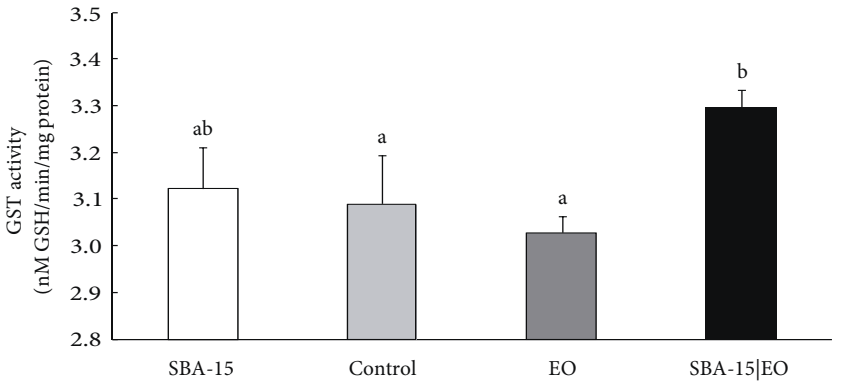

Figure 3. GST activity in the *Euproctis chrysorrhoea* sixth instar larvae fed on an SBA-15-supplemented diet, control diet, EO-supplemented diet, and SBA-15|EO-supplemented diet. Different letters indicate significant differences between experimental groups (one-way ANOVA, followed by post hoc LSD test, $P < 0.05$).

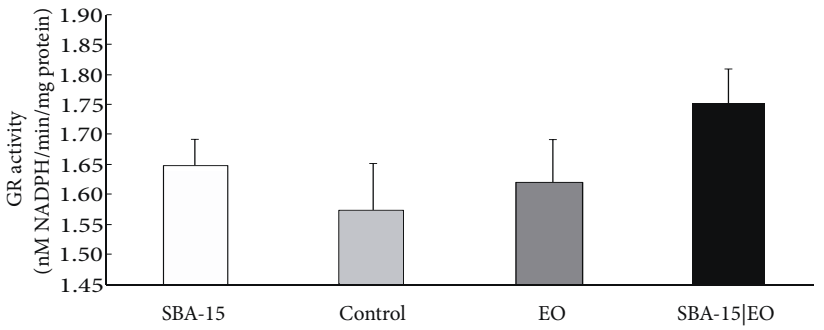

Figure 4. GR activity in the *Euproctis chrysorrhoea* sixth instar larvae fed on a SBA-15-supplemented diet, control diet, EO-supplemented diet, and SBA-15|EO-supplemented diet. Different letters indicate significant differences between experimental groups (One-way ANOVA, followed by post hoc LSD test, $P < 0.05$).

on both the number and positions of hydroxyl groups (Akhtar et al., 2012). The insecticidal (mosquitocidal, larvicidal) effects of EO and other anthraquinones have been demonstrated against a wide range of insects (Yang et al., 2003; Georges et al., 2008; Ateyyat and Abu-Darwish, 2009; Akhtar et al., 2012). Regarding the molecular mechanisms of EO action, it is clear that EO impacts the cellular redox status and the generation of oxidative

radicals (Huang et al., 1992; Srinivas et al., 2007; Qu et al., 2013; Liu et al., 2015; Zhao et al., 2017). However, the effects and molecular mechanisms of EO action on insect antioxidative defense are still unexplored.

In this study, we show that only SBA-15|EO significantly affected the antioxidant defense in *E. chrysorrhoea* larvae, in contrast to EO in its free form. This result suggests that EO and the SBA-15|EO after oral administration have different bioavailability, absorptive, and/or metabolic behaviors in the digestive tract or whole insect body. Pharmacokinetic studies in rat and human intestinal epithelium models revealed that EO, similarly to other dietary polyphenols, has low bioavailability in vivo because of poor intestinal absorption (Teng et al., 2012; Dong et al., 2016) and rapid elimination via extensive glucuronidation (Liu B et al., 2012; Wu et al., 2014). It was shown that the bioavailability of EO is dependent on both the number of free hydroxyl and methyl groups, because the methyl groups of EO can hamper the production of sulfated metabolites and increase the possibility of interaction between the hydroxyl groups and glucuronidation enzymes (Teng et al., 2012). Methylation also affects the redox potential and lipophilicity (Wessjohann et al., 2013). The formation of monoglucuronides in microsomes represents the second phase of biotransformation of anthraquinones (and EO) in vivo, and is the main metabolic pathway for its elimination (Wu et al., 2014). A likely reason for the greater efficiency of SBA-15|EO lies in the properties of mesoporous silica, a ceramic matrix that efficiently protects entrapped molecules against degradation or denaturation by enzymes, pH, temperature, or light (Vallet-Regí, 2012; Krajnović et al., 2017). Also, it is possible that substances loaded onto a mesoporous material such as the SBA-15 carrier enter the cells more easily, probably by macropinocytosis (unpublished data). On the other hand, the fate of orally ingested quinones also depends on the xenobiotic-metabolizing enzymes, transporters, and receptors found in intestinal epithelial cells (Teng et al., 2012), and in insects on the prevailing physicochemical conditions in the gut (Krishnan and Kodrík, 2006; Perić-Mataruga et al., 2014). The guts of leaf-feeding insects have an alkaline and in most cases a moderate to highly oxidizing environment. Thus, when EO reaches the cell it can undergo oxidation or reduction reactions. As mentioned previously, the reduction of EO initially yields its semiquinone, which can generate the superoxide anion ($O_2^{\cdot-}$) in the presence of oxygen due to electron transfer from the semiquinone to molecular oxygen (Rahimipour et al., 2001). Almost any oxidation of phenolics in the gut can generate $O_2^{\cdot-}$ because the reactive semiquinone can donate an electron to molecular oxygen. Also, EO is transformed by microsomal cytochrome P450 enzyme-dependent oxidation into hydroxymethyl-EO (ω-hydroxy-emodin) or 2-hydroxy-emodin, which can in turn produce active oxygen (Bachur et al., 1978; Kodama et al., 1987; Mueller et al., 1998). Finally, due to similarities with dimethyl naphthoquinone and mitochondrial ubiquinone, both of which have been reported to be capable of inducing ROS production (Teixeira et al., 1998), EO could induce ROS generation, as was shown in human T cells (Qu et al., 2013). The SBA-15 carrier loaded with EO can potentiate the EO prooxidant ability and increase ROS production. Indeed, EO exposure (at concentrations of 1–25 μg/mL in the peripheral blood leukocytes of fish larvae) was capable of inducing increased ROS generation, while EO at low concentrations increased the levels of Cu-Zn SOD and CAT mRNAs (Zhao et al., 2017). Increased SOD activity is consistent with previous works in which elevated activities of SOD and CAT in phytophagous insects ingesting prooxidant-rich food have been reported (Krishnan and Kodrík, 2006; Perić-Mataruga et al., 2014; Renault et al., 2016). Also, in larvae of *Megalobrama amblycephala*, EO treatment caused an increase in liver SOD activities that could enhance the antioxidative capacity and resistance to stress (Liu W et al., 2012). Treatment with EO that induced ROS generation and ER oxidative stress was shown to inhibit intracellular SOD activity and decrease the GSH/GSSG ratio in human T cells (Qu et al., 2013).

As EO has been shown to be capable of inducing $O_2^{\cdot-}$ production, SOD acts by eliminating it while producing H_2O_2, which could induce (elevate) catalase activity, among many other signaling effects (Lennicke et al., 2015). Additionally, it was shown that after treatment with anthraquinones (2-hydroxyemodin, one of the hydroxylated metabolites of EO), H_2O_2 can be generated both extracellularly (especially at alkaline pH levels) (Kodama et al., 1987), as well as intracellularly (Bachur et al., 1978). Since we recorded an increase in CAT activity in SBA-15|EO-treated larvae, we presumed that EO in the free form was not capable of inducing higher concentrations of H_2O_2, unlike nano-packed EO. It was shown that the toxic mechanisms of EO are partly mediated through the hydroperoxide generated from the semiquinone, because this effect was significantly inhibited by CAT (Huang et al., 1992). Ascorbate peroxidase could compete with H_2O_2 overproduction (Mirčić et al., 2013; Perić-Mataruga et al., 2014) due to its increased ability to lower H_2O_2 concentrations when compared to CAT. It should not be excluded that other mechanisms, such as the antioxidant defense in the midgut epithelium by the peritrophic envelope, which can function as a radical-scavenging antioxidant in caterpillars (Barbehenn and Stannard, 2004), or the increased levels of GSH, could be responsible for the increased resistance of late-instar larvae to phenolic compounds (Barbehenn and Kochmanski, 2013), and that they could compensate for stress.

The SBA-15|EO-supplemented diet significantly influenced GST activity in *E. chrysorrhoea* larvae; GST activity was elevated when compared to the control and the EO-supplemented diet experimental group (Figure 3). This is not surprising, because in insects, aside from detoxification of exogenous and endogenous toxins, GST activity plays an important role in protection against oxidative stress caused by ROS (Singh et al., 2001; Yu, 2002; Freitas et al., 2007; Yan et al., 2013). The main function of GST in mediating such a response is to reduce organic hydroperoxides to alcohols using GSH as the cosubstrate in the following reaction: ROOH + 2GSH (GST) → ROH + GSSG + H_2O (Hayes, 2005; Kalinina et al., 2014). The participation of GST in conjugation compounds containing a quinone structure could be a possible reason for the elevated GST activity under our experimental conditions.

Since GSH, inter alia, plays a role as a cosubstrate in the above-mentioned reactions, and more importantly, as it sustains the cellular redox status, the assessment of the contribution of GR is of particular importance. Only SBA-15|EO-treated larvae exhibited higher GR activity (Figure 4), which could indicate the involvement of this system in the prevention of the establishment of toxic conditions due to the accumulation of hydroxyperoxide or other ROS. It was shown that EO has the potential to disturb GSH metabolism in normal (human) liver cells (Liu et al., 2015), as judged by the decreased levels of both GSH and GSSG, and the increased level of its metabolite, glutamate (Liu et al., 2015).

It is obvious that EO impacts the cellular redox status and the generation of oxidative radicals (Bachur et al., 1978; Kodama et al., 1987; Huang et al., 1992; Srinivas et al., 2007; Qu et al., 2013; Liu et al., 2015; Zhao et al., 2017). In the present study, we demonstrated that only SBA-15|EO was capable of increasing the expression of the four antioxidative enzymes, SOD, CAT, GST, and GR. A possible reason for the better efficiency of EO in SBA-15 could be due to the ceramic matrix of SBA-15, which efficiently protects entrapped molecules against enzymatic degradation or denaturation (Vallet-Regí, 2012; Bensing et al., 2016). Nanopackaging appears to reduce the biotransformation of EO, thus increasing its bioavailability. As EO has been shown to have antifeedant and insecticidal properties, its packaging into a nontoxic nanocarrier could increase its effectiveness and facilitate its use for combating lepidopteran pests such as *E. chrysorrhoea* larvae in controlled environments.

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