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In vivo antioxidant activities of the ethanolic extract of *Zehneria scabra* leaves on *Salmonella* Enteritidis infected quails

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ABSTRACT

Antioxidants are substances which are capable of protecting the body from damages caused by free radicals. A variety of free radical scavenging antioxidants exists in the body and many of them are derived from plant sources. The aim of the present study was to investigate the antioxidant activity of the absolute ethanolic extract of *Zehneria scabra* leaves on *Salmonella* Enteritidis infected quails. *Salmonella* Enteritidis infected quails were treated every day with different doses of plant extract (9, 18 and 37 mg/ kg or oxytetracyclin 20 mg/ kg). After 12 days of treatment, the results showed that the different doses of this extract contributed in curing the infected quails, healing marked by the absence of *Salmonella* in animal's cloacal swabs cultured on SS Agar. On the other hand, treatment with different doses of plant extract resulted in a relative and significant ($p < 0.05$) decrease in the level of tissues antioxidant parameters such as CAT, SOD, POD, MDA and NO as compared to the animals of the negative control group. These results showed that the absolute ethanolic extract of *Z. scabra* leaf possess antioxidant and antisalmonellal properties necessary to eradicate the free radical produced during avian salmonellosis infection.

Introduction

Avian salmonellosis is an infectious disease caused by the Gram-negative bacteria of the genus *Salmonella*. Salmonellosis caused by *Salmonella* Pullorum, *Salmonella* Gallinarum, *Salmonella* Enteritidis and *Salmonella* Typhimurium is a real limiting factor for the development of poultry farming because it is the cause of huge losses thus causing, the fall of egg laying, poor quality of eggs, low viability of chicks (Wigley et al., 2005; Awa and Achukwi, 2010). Since poultry is an important reservoir for this pathology, humans become contaminated by consuming contaminated meat, eggs and ovo products (Natasi et al., 1988). It is the cause of most zoonotic infections seen in humans. According to the World Health Organization, salmonellosis is one of the most important causes of foodborne enteric illness in humans and can cause sometimes severe illness and even death. Approximately 21 million cases of disease and approximately 161,000 typhoid fever-related deaths occur annually worldwide. In Cameroon, the number of cases increased from approximately 124,526 to 154,103 between 2015 and 2016 (WHO, 2016). This disease remains a challenge for health authorities in developing countries and especially in sub-Saharan Africa because of the precarious hygienic and sanitary conditions (Gatsing et al., 2006).

These *Salmonella* infections are characterized by the production of free radicals such as the superoxide anion and nitric oxide (O2, NO) by macrophages. These free radicals react with each other to form peroxynitrite which is a powerful biological oxidant. The biological consequence of this phenomenon is oxidative stress, whose level is proportional to the quantity of peroxynitrite produced (Madigan and Martinko, 2007). They have therefore become a public health problem justifying the involvement of whom. *Salmonella* are capable of by passing free radicals produced by macrophages against them by expressing the oxyR gene, responsible for the accumulation of free radicals in the body during their pathogenicity (Madigan and Martinko, 2007). An abnormally high level of free radicals in the body such as lipids and proteins, during the oxidation of lipids is the cause of damage of biological molecules, cytotoxic and mutagenic characters of the released metabolites (Delattre et al., 2005). To maintain the homeostasis of the organism it is therefore important to inhibit these radicals. In addition, given the accumulation of antibiotic residues in livestock products, the resistance developed by pathogenic microbes in livestock (Kabir, 2009) and the proven toxicity of

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certain synthetic products (Shukla et al., 2011) represent a major problem worldwide and constitute a handicap for the control of salmonellosis (Valnet et al., 2008). Therefore, Retnani et al. (2014) and Sokoudjou et al. (2019) showed respectively in their work that *Morinda citrifolia* and *Canarium schweinfurthii* are traditionally used for the treatment of human or avian salmonellosis. Plants are also recommended as an alternative to antibiotic growth promoters (Kana et al., 2017). Since plants are reservoirs of many bioactive compounds, their exploration could help in the development of new and more effective drugs. Previous study revealed that the ethanolic extract of *Zehneria scabra* exhibited a crucial antibacterial activity against *Salmonella* Typhimurium and *Salmonella* Enteritidis (Biekop et al., 2020). In this study, we therefore tested the antisalmonellal efficacy and antioxidant activities of this extracts on the *in vivo* model of quail orally infected by *Salmonella* Enteritidis.

Materials and Methods

Site of study

The trial was carried out at the experimental farm of the University of Dschang, located at 1420 m above sea level, between 5°26' North and 10°26'east. It has a moderate temperature ranging from 10°C to 25°C with average annual rainfall from 1500 to 2000 mm over a 9 months rainy season.

Collection and identification of plant material

Leaf of *Zehneria scabra* was collected in Foumbot, Noun Division (West Region-Cameroon) in April 2019. The identification of the plant was done by Mr. Tadjouteu Fulbert, a botanist at the National Herbarium, in Yaoundé-Cameroon, using a voucher specimen registered under the reference HNC N°66689.

Preparation of the plant

The leaves of *Z. scabra* were dried at room temperature until constant weight and powdered to coarse particles. One hundred gram (100g) of powder was soaked in 1 L of 95° ethanol. After 48h, it was filtered using Whatman paper N. 1. The filtrate was concentrated at 45°C in a rotatory evaporator under reduced pressure and the obtained volume was later dried at 40°C. The plant extract was stored in sterilized bottles at room temperature until usage Biekop et al. (2020).

Test bacterium and culture media

Salmonella Enteritidis (SE) was used in this study and was obtained from the medical bacteriology laboratory of "Centre Pasteur" of Yaoundé, Cameroon. Bacteria strain was maintained on agar slant at 4°C and sub-cultured on fresh appropriate agar plate 24h prior to antibacterial test. The culture media namely *Salmonella-Shigella* agar (SSA) was used for activation of *Salmonella* and during *in vivo* assay in quail for bacterial counts and identification.

Experimental animals

This test was carried out using *Salmonella* Enteritidis induced typhoid in quail model. Seventy two mature quails having weight between192-197gm (72 females, 5 weeks olds) were selected for this experiment. These animals were grouped randomly into six groups of twelve animals each with similar average body weight. After one week of acclimation all groups (2-6) were infected except group 1. They received orally 1 ml of suspension containing 1.5×10^5CFU of *S.* Enteritidis. During the experiment, animals of group 1 were not infected and were treated only with DMSO (5%), and thus serve as non-infected control. Animals of group 2 were infected and not treated (non treated control group) while those of group 3 were treated with oxytetracyclin (treated control). The remaining groups of animals were treated after infection with different doses of *Z. Scabra* @ 9 mg/ kg, 18 and 37 mg/ kg respectively for groups 4, 5 and 6. They were handled according to standard protocols for the use of laboratory animals. The studies were conducted according to the ethical guidelines of the Committee for Control and Supervision of Experiments on Animals as described in ''the European Community guidelines, EEC Directive 86/609/EEC'' (EEC, 1986), on the use of animals for scientific research.

Microbial confirmation after treatment

Cloacal swabs from the treated animals were collected every two days of treatment and inoculated on already prepared SS Agar on petri dishes. The inoculated plates were incubated at 37°C for 24 h. The counts of emerged colonies (black colour presentation) were used to evaluate the efficacy of treatment.

Dissection and sample collection

At the end of the experiment, animals were fasted overnight on the 12th day of treatment. Fifteen percent (15%) homogenate of organs (liver, kidney, heart and ovary) were prepared in normal saline solution, and then centrifuged at 3000 rpm for 15 minutes. The supernatant was used for the determination of biochemical parameters related to oxidative stress such as Catalase (CAT), Peroxidase (POD), Malondialdehyde (MDA), Nitric oxide (NO) and Superoxide Dismutase (SOD).

Enzymatic parameters

Three antioxidant enzymes were evaluated; Catalase (CAT), Peroxidase (POD), and Superoxide Dismutase (SOD).

Catalase assay

Catalase level was evaluated according to the method described by Dimo et al. (2006).Ten microliters of the serum or tissues homogenate were added into 150µL of phosphate buffer pH 7.4.Then, 40 µl of H_2O_2 (50 mM) were also introduced. After 1 minute, 400 µl of potassium dichromate (5%) prepared in 1% of acetic acid was introduced in the reaction solution. The mixture was heated in boiling water for 10 mins and cooled immediately. The absorbance was recorded at 570 nm using spectrophotometer "Schimadzu 1501, Japan". Enzymatic activity of catalase was inferred by the Beer-Lambert law (Chakroun et al., 2007) inmmol/ min per millilitre of serum or gram of tissue.

Peroxidase assay

Peroxidase level was determined in tissues as described by Habbu et al. (2008) with slight modifications. Two hundred and fifty microliters of organs homogenate were taken, and to this were added 500 µl of 10 mM KI solution and 500 µl of 40 mM sodium acetate. The absorbance of potassium per iodide was read at 353 nm, which indicates the amount of peroxidase. Then 10 µl of 15 mM H_2O_2 was added, and the change in the absorbance in 5 mins was recorded. Enzymatic activity of peroxidase activity was expressed in µmole/ min per milliliter of serum or gram of tissue by the Beer-Lambert law (Chakroun et al., 2007).

Superoxide dismutase (SOD) assay

SOD activity was determined in tissue by Mirsa et al. (1972) method with some modifications. To 150 µL of homogenates 1 650 µL of phosphate buffer (pH 7.2) and 200 µL of 0.3 mmol/ L epinephrine was also added. The self-oxidation of epinephrine was recorded at 480 nm in 30 seconds and then, one minute after its addition by spectrophotometer (Schimadzu 1501, Japan). The SOD activity expressed as percentage of inhibition was calculated taking into account that 50% inhibition correspond to one unit of activity.

Non enzymatic parameters

Estimation of lipid peroxidation

The extent of peroxidation in tissues was assessed by measuring the level of malondialdehyde (MDA) according to the method of Sujatha et al. (2013) with some modifications. A total of 0.5 mL of 1% orthophosphoric acid and 0.5 mL of precipitating mixture (1% thiobarbituric acid, 1% acetic acid) were added to 0.1 mL of tested sample. The mixture was homogenized and heated in boiling water for 15 min and cooled immediately. It was then centrifuged at 5000 rpm for 10 mins and the absorbance of the supernatant was recorded at 532 nm using Schimadzu 1501 spectrophotometer, Japan. The peroxidation in the tissues was calculated based on the molar extinction coefficient of malondialdehyde (MDA) (153 mM-1cm-1), and expressed in terms of micromoles of MDA/ g of tissue.

Determination of nitrite oxide concentration (NO)

This assay relies on a diazotization reaction that was described by Griess (Rukunga et al., 2007) with some modifications. The Griess Reagent is made up of a freshly prepared sulfanilamide and N-1-Napthyl Ethylenediamine Dihydrochloride (NED) under acidic conditions and protect from light. To 340µL of the experimental sample, 340 µL of freshly prepared 1% Sulfanilamide in 5% orthophosphoric acid were added and after 5 mins of incubation in the dark at room temperature (23 ± 2°C), 340µL of the NED solution (0.1% NED in water) were also added. The resulting solution was well mixed and then incubated away from light at room temperature for 5 mins, protected from light. The absorbance of the coloured azo compound formed was measured at 520 nm within 30 minutes. A standard curve was plotted using nitrite ($NaNO_2$) (100, 50, 25, and 12.5µM). The results were expressed as Micro molar of Nitrite Equivalents (µMNE) per gram (g) of tissue.

Statistical analysis

Data obtained were expressed as mean ± SEM (standard error on mean) and were statistically analyzed using one-way ANOVA with the Statistical Package for Social Sciences (SPSS) version 20.0 software. Post hoc analysis using Duncan test was used to compare means of different groups. A p-value of 0.05 was considered statistically significant.

Results

Effects of treatment on some biochemical parameters linked to oxidative stress

Effects of different doses of the treatment on catalase activity

The tissue catalase activity of infected and treated quails is presented in Table 1. It appears that the infection resulted in a significant ($p < 0.05$) increase in tissue catalase activity compared to the neutral control. However, the treatment generally induced a decrease in the activity of this enzyme compared to the negative control.

Table 1: Effect of treatment on catalase activity of tissue

Group	Liver (mmol/min/g)	kidney (mmol/min/g)	Heart (mmol/min/g)	Ovary (mmol/min/g)
Negative control	0.91±0.17^{a}	2.11±0.48^{a}	0.92±0.12^{a}	2.69±0.63^{a}
Oxytetracyclin20 mg/kg	0.50±0.09^{b}	1.58±0.43^{ab}	0.71±0.11^{bc}	1.04±0.42^{b}
Neutral control	0.47±0.07^{b}	1.70±0.50^{ab}	0.59±0.15^{c}	1.46±0.37^{b}
Extract 9mg/kg	0.81±0.19^{a}	1.73±0.53^{ab}	0.74±0.09^{b}	1.15±0.23^{b}
Extract 18mg/kg	0.53±0.11^{b}	1.73±0.52^{ab}	0.81±0.17^{ab}	1.42±0.45^{b}
Extract 37mg/kg	0.53±0.07^{b}	1.20±0.33^{b}	0.84±0.04^{ab}	1.54±0.52^{b}

In the same column, values bearing different letters are significantly different ($p < 0.05$).

Table 2: Tissue level of superoxide dismutase

Group	Liver (µmol/min/g)	Kidney (µmol/min/g)	Heart (µmol/min/g)	Ovary (µmol/min/g)
Negative control	0.26±0.06^{a}	0.29±0.06^{a}	0.30±0.08^{a}	0.41±0.09^{a}
Oxytetracyclin20 mg/kg	0.19±0.05^{b}	0.14±0.03^{c}	0.22±0.07^{a}	0.27±0.08^{b}
Neutral control	0.19±0.06^{b}	0.16±0.05^{bc}	0.22±0.03^{a}	0.25±0.05^{b}
Extract 9 mg/kg	0.18±0.03^{b}	0.21±0.05^{b}	0.22±0.07^{a}	0.21±0.05^{b}
Extract 18 mg/kg	0.15±0.04^{b}	0.17±0.04^{bc}	0.24±0.08^{a}	0.19±0.06^{b}
Extract 37 mg/kg	0.13±0.01^{b}	0.13±0.04^{c}	0.25±0.08^{a}	0.24±0.07^{b}

In the same column, values bearing different letters are significantly different ($p < 0.05$).

Table 3: Effect of treatment on tissue peroxidase activity

Group	Liver (µmol/min/g)	Kidney (µmol/min/g)	Heart (µmol/min/g)	Ovary (µmol/min/g)
Negative control	73.59±7.77^{a}	90.32±8.13^{a}	38.32±8.75^{a}	68.53±14.61^{a}
Oxytetracyclin 20 mg/kg	54.05±6.07^{b}	78.62±10.95^{ab}	33.41±8.09^{ab}	54.35±6.75^{b}
Neutral control	54.77±8.89^{b}	72.47±8.05^{b}	25.89±6.19^{b}	55.90±9.29^{b}
Extract 9mg/kg	56.89±7.77^{b}	88.21±14.51^{a}	33.02±9.89^{ab}	49.29±5.98^{b}
Extract 18mg/kg	57.84±6.01^{b}	75.01±5.91^{b}	34.40±7.19^{ab}	47.85±8.54^{b}
Extract 37mg/kg	54.28±7.40^{b}	69.89±9.13^{b}	29.14±4.38^{ab}	51.02±9.63^{b}

In the same column, values bearing different letters are significantly different ($p < 0.05$).

Effect of treatments on the level of superoxide dismutase

The evolution of the level of tissue superoxide dismutase after the treatment of the animals is presented in Table 2. It emerges from this table that, with the exception of the heart, the infection caused a significant ($p < 0.05$) increase in tissue superoxide dismutase activity compared to the neutral control. However, the treatment generally induced a decrease in the activity of this enzyme compared to the negative control.

Effects of different doses of treatment on peroxidase activity

The activity of tissue peroxidase in infected and treated quails is presented in Table 3. It appears from this table that the infection resulted in a significant ($p < 0.05$) increase in the activity of tissue peroxidase compared to neutral control. Nevertheless, the treatment induced a significant ($p < 0.05$) decrease in the activity of this enzyme in all organs compared to the negative control. The 37 mg /kg body weight dose also led to a normalization of the effects induced by the infection.

Effect of treatments on the level of malondialdehyde (MDA)

The evolution of the level of tissue malondialdehyde after treating the animals is presented in Table 4. It is seen that the infection resulted in a significant ($p <0.05$) increase in the level of tissue malondialdehyde in all the organs compared to the neutral control. Meanwhile, the treatment generally decrease the level of malondialdehyde in the treated animals compared to the negative control.

Table 4: Tissue concentration of malondialdehyde

Group	Liver (µmol/g)	Kidney (µmol/g)	Heart (µmol/g)	Ovary (µmol/g)
Negative control	1.44±0.29^{a}	1.88±0.39^{a}	1.05±0.23^{a}	1.10±0.19^{a}
Oxytetracyclin 20 mg/kg	1.23±0.16^{b}	1.41±0.16^{b}	0.67±0.09^{bc}	0.89±0.19^{b}
Neutral control	0.90±0.12^{c}	1.50±0.35^{b}	0.43±0.08^{d}	0.89±0.20^{b}
Extract 9 mg/kg	1.04±0.11^{bc}	1.54±0.15^{b}	0.56±0.09^{cd}	0.80±0.09^{b}
Extract 18 mg/kg	1.07±0.18^{bc}	1.51±0.19^{b}	0.56±0.09^{cd}	0.70±0.10^{b}
Extract 37 mg/kg	1.22±0.13^{b}	1.34±0.22^{b}	0.74±0.12^{b}	0.87±0.11^{b}

In the same column, values bearing different letters are significantly different ($p <0.05$).

Table 5: Tissue concentration of nitric oxide (NO)

Group	Liver (µmol/g)	Kidney (µmol/g)	Heart (µmol/g)	Ovary (µmol/g)
Negative control	37.16±8.55	40.34±8.88	34.35±8.12^{a}	43.03±9.02^{a}
Oxytetracyclin 20 mg/kg	34.17±4.89	35.52±4.09	31.39±8.61^{ab}	39.45±7.58^{ab}
Neutral control	32.02±3.86	34.76±6.12	25.71±7.05^{ab}	31.91±4.60^{b}
Extract 9 mg/kg	31.94±6.05	39.01±7.38	26.78±6.76^{ab}	35.05±5.51^{ab}
Extract 18 mg/kg	31.51±4.14	34.07±5.27	23.96±4.33^{b}	35.66±9.76^{ab}
Extract 37 mg/kg	33.07±3.51	33.91±5.03	23.62±6.47^{b}	33.59±2.89^{b}

In the same column, values bearing different letters are significantly different ($p <0.05$).

Effects of different doses of treatment on the level of nitric oxide (NO)

The evolution of the level of tissue NO after the treatment of the animals is presented in Table 5. It appears that the infection had a significant ($p < 0.05$) increase in the level of NO in the heart and the ovaries compared to the neutral control. However, in general, the treatment reduced the level of NO in the treated animals compared to the negative control and the dose 37 mg / kg body weight tends to normalize the effects induced by the infection.

Discussion

Quail has been used in this work simply because most of the salmonellosis seen in humans is due to food borne illness from poultry. Thus controlling this infection in poultry would greatly reduce the prevalence of *Salmonella* infections in humans. *Salmonella* Enteritidis being one of the most implicated serotypes in food borne illness in humans has been used. The antisalmonellal activity of *Z. scabra* extract was highlighted in this study. Indeed, several studies have demonstrated the effectiveness of certain plants (Retnani et al., 2014; Kamsu et al., 2019 and Sokoudjou et al., 2019) against certain serotypes of *Salmonella* using quails, rats and hens respectively as animal materials. The signs observed indicating the presence of infections in quails were: ruffled feathers, diarrhoea, and drowsiness with the eyes half closed thus the presence of blood in the faeces. These symptoms have been proven by the growth of *Salmonella* colonies on the petri dishes after culture. The administration of *Z. scabra* ethanolic extract to infected quails totally wiped off the negative effects caused by the infection. The results demonstrate that this plant possess compounds endowed with antibacterial activity. In fact, *Z. scabra* leaf was reported to contain phenols,

flavonoids, anthocyanin, triterpenes, tannins and saponins which had already shown several pharmacological properties including antibacterial properties (Ogutu et al., 2012; Biekop et al., 2020). The detection of these classes of secondary metabolites could explain the observed activity of this extract (Kengni et al., 2013).

Reactive oxygen species (ROS) are oxidants formed in the body due to exogenous and endogenous factors and are thought to be responsible for many diseases such as cancer, cardiovascular, neurodegenerative, inflammatory and aging diseases (Cohen et al., 2000). These free radicals are responsible of the accumulation of H_2O_2 in cells and of lipid peroxidation (Farombi et al., 2010). Antioxidant systems are normally implemented in living aerobic organisms to combat the effect of oxidative stress. In this context, the enzymatic antioxidants (catalase, superoxide dismutase and peroxidase) and two markers of oxidative stress (MDA and NO) were measured in the organs of quails infected with *Salmonella* Enteritidis and treated with ethanol 95% extract of *Z. scabra*.

Catalase, superoxide dismutase and peroxidase are markers of enzymes in peroxisomes. They function as destroyers of hydrogen peroxide. The increase in the activity of these enzymes (catalase, superoxide dismutase and peroxidase) in untreated quails could be due to the stimulation of cellular catalase activity by an excess of "its" substrate that is hydrogen peroxide. Indeed, the infection is accompanied by the stimulation of bacterial SOD, the hydrogen peroxide produced can only be accumulated, and inhibit the enzyme that ensures its conversion into water that is catalase. The decrease in catalase, superoxide dismutase and peroxidase activity in quails treated with different doses of the extract could be the result of the stimulation being lifted under the antioxidant effect of the extract which could have acted directly on the H_2O_2 on one hand or stimulate the catalase activity of the host cell on the other hand (Fodouop et al., 2014).

The increase in the level of lipid peroxidation products leads to an increase in the level of free oxygen radicals, which attack polyunsaturated fatty acids in cell membranes (Kehrer, 2000). The results of this work showed an increase in MDA in infected and untreated quails. This could be due to an imbalance in the cytosolic redox state in favour of pro-oxidants. Indeed, according to Fodouop et al. (2014), *Salmonella* infection leads to an imbalance in the cytosolic redox state in favour of pro-oxidants, by putting tissue cells in a state of oxidative stress, marked by an increase in MDA. The quails treated with the extract showed a decrease in the level of MDA in quail tissues compared to the negative control. This could suggest that the extract had a positive effect by reducing the level of MDA, thus preventing the destruction of the membrane bilayer (lipid peroxidation). These results corroborate those of Tchoua (2016); Atsafack et al. (2016); Kodjio et al. (2016) and Djimeli et al. (2017) who showed that the extracts of *Erica mannii*, *Albizia gummifera*, rhizomes of *Curcuma longa* and *Enantia chlorantha* had an elimination activity of superoxide ion and an inhibition activity of lipid peroxidation in liver microsomes of chicken and rat respectively. This finding corroborates those of Sokoudjou et al. (2019) in his previous works. This could be due to the increase in free radical formation and decrease of antioxidant enzymes. These results suggest that this extract exerts its antioxidant activity by preventing the oxidation of tissues by free radicals.

The decrease in the level of nitrogen monoxide in the tissues of infected and treated quails with the extract compared to the negative control, reveals that the extract regulated the production of nitrogen monoxide compared to the neutral control. This could further stimulate the immune system (macrophage) to fight against the germ *Salmonella* Enteritidis. Due to its ability to regulate the production of nitric oxide, the extract has both an antioxidant potential and an activity that can give it the potential to fight avian salmonellosis. The phenolic compounds present in the extract as shown by the dosage of total phenols may be responsible for the antioxidant effects observed in infected and treated quails. In fact, flavonoids inhibit the formation of free radicals and prevent the oxidation of macromolecules such as proteins, DNA (Van-Acker et al., 1996); they would form intermediate radical species which are not very reactive. They are secondary metabolites reputed to be the most antioxidants and very effective in the treatment of degenerative diseases (Montoro et al., 2005).

Conclusion

The *in vivo* antisalmonellal evaluation of the ethanolic extract of *Z. scabra* revealed that it can be successfully used in the treatment of avian salmonellosis. Infected animals treated with the *Z. scabra* extract at 18 and 37 mg/kg body weight recovered on day 9 after the beginning of the treatment. We also evaluated in the present

experiment the effect of ethanolic extract of *Z. scabra* on antioxidant profile of quails; the results showed that the antioxidant and anti-lipid peroxidation effects of the ethanolic extract of *Z. scabra* may be responsible for some of its pharmacological effects. These suggest that extracts from these plant species should be investigated in order to isolate bioactive components that might function as potential anti-salmonella agents, in order to minimize the damage caused by excessive oxidant production.

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Conflicts of interest/Competing interests

There is nothing to declare

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