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Decontamination of Eggshell Contaminated with Salmonella Typhimurium Using Natural Plant Extracts

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ABSTRACT

Salmonella typhimurium is a major foodborne pathogen which causes foodborne infection related mainly to consumption of contaminated eggs. Eggshells become contaminated with S. typhimurium via different sources, emphasizing the need for effective methods for disinfection of egg surface (Background and objective). This study investigated the efficacy of four plant extracts (Rosemary, Licorice, Chicory and Neem) against S. Typhimurium, as natural and potent substitutes to artificial and chemical biocides (Purpose of study). These plants were purchased from local markets, the extracts were prepared in our lab in different concentrations, and then antibacterial activity and minimum inhibitory concentration were determined. Specific Pathogen Free (SPF) eggs were in vitro contaminated with reference Salmonella typhimurium strain and disinfected with the selected plant extract concentration for different times. The best plant extract and its concentration and time needed for complete contamination was determined using statistical analysis (One Way ANOVA) (Materials and Methods). Final results showed that, the use of licorice plant extract 1% concentration as immersion solution for washing eggshells for 5 minutes could completely decontaminate S. typhimurium infection (100%) to reduce the incidence of human salmonellosis and considered as effective bio preservative with no effect on the egg's physical quality. Results of licorice plant extract solution was significantly (P<0.05) higher than other plant extract solutions (Results). The presentation of different plant extracts, could be suggested as eco-friendly, safe and potent substitutes to other chemical sanitizers and disinfectants already used against the enteric Salmonella contamination in egg farms. The use of this biopreservation technique in table egg farms could increase the hygienic quality of finally produced table egg, with prolongation of its shelf life and decreasing the incidence of biological and chemical hazards threating the consumer (Conclusions).

Key words: *Antibacterial, S. Typhimurium, biopreservation, plant extracts, decontamination.*

INTRODUCTION

The quality of table eggs is reliant on of miscellaneous impacts before and after laying. After laying, the different stages involving egg manipulation, collecting, grading and packing processes and transportation considered as possible sources of possible hazards that may risk the sanitary conditions of the eggshells[1] . A recent shift in consumer preferences for consumption of unprocessed foods, potentially increasing the risk of salmonellosis[2].

Table eggs are watched as the most probable source of Salmonella contamination alongside the food chain. *Salmonella enteric* serovar *typhimurium* (*S. typhimurium*) and serovar *Enteritidis* (*S. enteritidis*) are the primary and predominant causes of foodborne salmonellosis, that routinely contaminate eggs[3].The Centers for Disease Control and Prevention (CDC) record about one million of Salmonella cases annually in the United States. Not only in US, 247 cases and 3 deaths in the UK, 130 cases in other European countries in 2014, were accountable for egg contamination in addition to 353 and 1895 cases in Australia in 2014 and 2015[4].

Trans-shell (horizontal transmission) of Salmonella to egg content is usually derived from faecal contamination or contamination through dust, crates, other contact surfaces and environmental vectors, such as farmers and rodents. That is smoothed by other factors as moist egg shells, storage temperature and shell damage[5].Therefore different biosecurity measures should be fulfilled in all table egg production steps starting at the farm level and ending by consumption, in order to control the risk points which, facilitate Salmonella contamination in the table egg processing chain[6].

As a preventative start point, vaccination of laying hens has high impact on reducing the incidence of human salmonellosis. The FAO organization had reported that, the laying hens vaccination against Salmonella; reduces egg contamination by about 75%. However, control measures should primary focus at farm level. Egg producers in US, Australia, Japan, and Canada have also strained to prevent Salmonella invasion from outside of the egg shell to inside egg content instead of vaccine treatment of laying hens through washing and rinsing of Grade A shell eggs which regarded as a safety procedure[7].

Preservation of egg is traditionally achieved by the use of chemical sanitizers for washing of whole egg shell in farm before casing such as: chlorine compound, quaternary ammonium compound and iodine compound. Contrariwise their repeated uses have resulted in the increase of chemical residues in food chain, acquirement of microbial resistance against the used chemicals in addition to the unpleasant adverse effects of these chemicals on the consumer health[8]. Because of such concern, the new strategies for controlling zoonosis focus on rising potentially effective, safe and natural food preservatives with verified antimicrobial activity[9].

The usage of medicinal plants has is not an emerging issue, it was recorded in the history of many cultures and they were treasured not only for their therapeutic and preservative potentialities but also as flavor enhancers. The Middle Eastern Mediterranean region is rich in plant species which are considered to have medicinal effects. The addiction on natural materials such as plant extracts, as potent substitutions to chemical and synthetic antimicrobial agents, is impressively anticipated from modern consumers[10]. Several plant extracts were positively functional as a control measure against enteric Salmonella in several food products[11].

In this regard, the area of concern of the current study was evaluation of some natural plant extracts as probable antibacterial compounds to be used against enteric Salmonella, i.e. *S. typhimurium* and their promising applications as biopreservative to decontaminate eggshells with reference to their effect on egg sensory quality.

MATERIAL AND METHODS

Microorganisms and test materials:

Salmonella enterica subsp. enterica serovar typhimurium (deposited as *Salmonella typhimurium*), i.e. *S. typhimurium* ATCC-13311 was used as a reference strain in this study. The bacterial strain was grown and maintained in Nutrient Broth (NB). Grade A freshly laid chicken eggs, were obtained from the National project for production of Specific Pathogen Free eggs, El fayoum, Egypt.

Preparation of plant extracts:

The examined plants were purchased from a local market of Medicinal Plants and Herbs, Cairo, Egypt. The whole dried plants were finely ground and kept in a tightly closed glass container at room temperature pending its extraction.

Extraction of the tested plants extracts:

The common, scientific names of plants, the used parts and solvents are mentioned in **Table (1):** To each (250 g/trials) of the dried plant powder, one liter of the solvent was added to a wide mouth plastic container and kept overnight. On the next morning, filtration of the container content was carried out using double layer gauze. The liquid extract was evaporated in a Rotatory evaporator at a temperature of 40 ˚C under reduced pressure using a vacuum pump. The used extract 1% from each plant was prepared in distilled water using few drops of Tween 80 as a suspending agent. The selected doses of each extract were calculated for the experiments.

In vitro determination of plants' antibacterial activity:

Antibacterial Activity was determined by the Gel Diffusion method according to the12. Petri plates containing Mueller Hinton agar medium were seeded with a 24 h culture of the bacterial strain (*S. typhimurium*). Wells (6 mm diameter) were cut into the agar and 50 µl of the plant extracts were tested in a concentration of 100 mg/ml. The inoculum size was adjusted so as to deliver a final inoculum of approximately 10^8colony-forming units (CFU)/ml. Incubation was performed at 37 °C for 24 h. The assessment of antibacterial activity was based on measurement of the diameter of the inhibition zone formed around the well. The sensitivity to the individual plant extract was classified by the diameter of the inhibition zones as follows[13, 14]:

• Not sensitive (–) for total diameter smaller than 8 mm.

• Sensitive (+) for total diameter 9–14 mm.

• Very sensitive (++) for total diameter 15–19 mm.

• Extremely sensitive (+++) for total diameter larger than 20 mm.

Each assay was performed in triplicates on three separate experimental runs.

Determination of Minimum Inhibitory Concentration (MIC) according to15:

The MIC was estimated by the broth dilution method in Brain-Heart infusion broth (Oxoid CM1135). Each plant extract was first diluted in dimethylsulfoxide; 80%. Serial dilutions of extracts were carried out with concentrations ranging from 0.1 % to 2%. One milliliter of a *S. typhimurium* (10^6 CFU/ml) and one-tenth ml of each extract were added to 2.9 ml of Brain Heart infusion broth. After 24 h at 37°C, MIC was determined as the lowest plant extract concentration inhibiting visible growth of bacteria.

Experimental contamination of eggs:

The experimental inoculation and disinfection of eggshells with Salmonella strain were carried out according to[16, 17]. *S. typhimurium* was grown in NB for 24 h at 37°C to obtain a cell density of approximately 10^8 CFU/ml. The eggshells were firstly sterilized by dipping eggs in 70% ethanol for 15s and air-dried for 1 h. Sterilized eggshells were experimentally contaminated by placing each egg in a sterile plastic bag along with 50 ml of Salmonella culture and keeping them for 1 min. Eggs were transferred to clean absorbent paper sheets and left for 30 min before the treatments were applied.

Disinfection of eggs with plant extracts:

Inoculated eggs were dipped in 2 liters of plant extract solutions, at their MIC levels, at various immersion periods, i.e. 1, 2, 3, 4 and 5 min, and air-dried.

Detection of S. typhimurium:

The decontaminated eggs, as well as control eggs (immersed for the same periods in sterile distilled water), were immersed in sterile buffered peptone water (BPW, 50 ml/each egg), shaken well and 100 µl from BPW spread on NA plates to count the viable colonies after incubation at 37°C for 24 h.

Egg content of the decontaminated and control eggs was also obtained and plated on NA plates to count the viable colonies after incubation at 37°C for 24 h. This step was applied to examine the possibility of migration of Salmonella organism to the egg content through the shell pores during immersion in the plant extract solution.

Sensory Evaluation of egg according to18:

Control eggs (SPF eggs) were immersed in 1% concentration of different plant extracts for 5 minutes (the highest concentration and immersion time used) and then evaluated for its sensory characteristics (odor, color, white consistency and yolk consistency, air sac and total acceptability) immediately after dryness according to Hedonic scale; 5 grade each. Panel test was done by experience 3 panelists (for each sample) from food hygiene department, faculty veterinary medicine, Cairo University.

Statistical analysis:

For the antibacterial activity of plant extracts, an egg per treatment (5 treatments) at every sampling point (each minute for 5 minutes) were included in all three replicated experiments (N = 75, n = 5). While for the sensory evaluation, three eggs for each treatment were tested (N = 15, n = 3). The analysis of variance (One Way ANOVA) test was conducted to analyze the possible significance ($P \leq 0.05$) between mean values of parameters using Fishers Least Significance Difference (LSD) using CoStat - Statistics Software Version 6.45.

This study was started in January 2017 and took about six months, starting from plant extraction till the end of research point.

RESULTS

The results presented in **table (1)** showed that licorice had higher antibacterial activity against *S. typhimurium* where zone of inhibition was 18.2 ± 0.12 then followed by Rosemary (17.8 ± 0.65), Chicory (16.0 ± 0.10) and finally Neem (12.3 ± 0.40).

MIC was estimated for each plant extract in different concentration (0.1%-2%) to determine the lowest plant extract concentration which can inhibit visible growth of bacteria. The data in **table (2)** presented that all plant extracts were effective at concentration 1% except chicory which can inhibit bacterial growth at 0.5%

The egg was contaminated experimentally with the bacteria then it has been added in the plant extracts solution at different time to detect the reduction percentage in the count of bacteria, the results in **table (3)** showed that licorice has the highest antibacterial effect as it has the ability to reduce the count of the bacteria from the first minute 93.96±0.80 then at 5 minute it reduced the count up to 100% and its effect was significantly different from the other plant extracts used at the first and second minutes. After two minutes Chicory showed high log reduction percent reached 70% to be the secondary extract after the licorice followed by Rosemary then Neem. By immersing the eggs for three minutes in 1% of the different plant extracts, there were no significant difference between licorice, Chicory and Rosemary with reduction log percent ranged from 97 to 99%, while Neem showed the lower inhibitory effect until the end of the 5 minutes of immersion.

The eggs have been immersed 5 minutes in the different plant extracts to show any changes in organoleptic characteristics or flavor threshold. The results represented in **table (4)** showing no significant changes in white consistency, yolk consistency and air sac , while the color of eggs immersed in rosemary extract solution was greatly affected . Also, the odor of eggs immersed in neem extract has been observed.

Table (1): Antibacterial activity of plant extracts against *S. typhimurium* measured as a zone of inhibition diameter (ZOI, mm).

Extracted plant	Part used	Scientific name	Solvent	ZOI (mm)
Rosemary	leaves	*Rosmarinus officinalis*	Alcohol 95%	17.8 ± 0.65
Licorice	roots	*Glycyrrhiza glabra*	Alcohol 70%	18.2 ± 0.12
Chicory	leaves	*Cichorium intybus*	water	16.0 ± 0.10
Neem	leaves	*Azadirachta indica*	Alcohol:hexan(60:40)	12.3 ± 0.40

*Zone of inhibition diameters are means of triplicates ± standard deviation.

Table (2): The Minimum Inhibitory Concentrations (MIC) of the plant extracts on *S. typhimurium*.

Plant Extract	***S. typhimurium* growth in different concentrations (%)**					
	Control*	0.1	0.3	0.5	1	2
Rosemary	++	+	+	+	_	_
Licorice	++	+	+	+	_	_
Chicory	++	+	+	_	_	_

Neem	++	+	+	+	−	−

*Plant extracts absence, + Growth present, - Growth absent.

Table (3): Reduction percentage in the count of *S. typhimurium* over eggshells at different immersion periods in plant extract solutions.

Plant Extract solutions	Reduction percentage (%) at different immersion time (min)				
	1 min	**2 min**	**3 min**	**4 min**	**5 min**
Rosemary	48.37 ± 2.94^{c}	50.09 ± 3.36^{c}	97.73 ± 0.17^{a}	99.19 ± 0.42^{a}	99.66 ± 0.15^{a}
Licorice	93.96 ± 0.80^{a}	99.59 ± 0.06^{a}	99.94 ± 0.06^{a}	99.97 ± 0.03^{a}	100 ± 0.00^{a}
Chicory	65.85 ± 6.08^{b}	70.98 ± 2.30^{b}	97.45 ± 2.05^{a}	98.72 ± 1.44^{a}	99.68 ± 0.14^{a}
Neem	28.24 ± 6.05^{d}	39.58 ± 5.50^{c}	87.45 ± 6.55^{b}	89.22 ± 3.30^{b}	92.71 ± 0.44^{b}
***Control**	35.20 ± 0.92^{cd}	35.55 ± 0.57^{c}	58.94 ± 0.47^{c}	73.17 ± 0.69^{c}	74.24 ± 1.56^{c}

Average values with different alphabetical superscripts within a column are significantly different at $P<0.05$.
* Control eggs, immersed in sterile distilled water instead of plant extract solutions for the same time.

Table (4): Sensory evaluation of Egg after immersion in the plant extract solutions for 5 minutes.

Sensory characteristics	Plant extracts				
	Rosemary	**Licorice**	**Chicory**	**Neem**	***Control**
Odor (5)	4.53 ± 0.06^{a}	4.62 ± 0.13^{a}	4.47 ± 0.06^{a}	4.07 ± 0.21^{b}	4.73 ± 0.12^{a}
Color (5)	3.00 ± 0.01^{b}	4.78 ± 0.07^{a}	4.67 ± 0.06^{a}	4.6 ± 0.20^{a}	4.7 ± 0.17^{a}
White consistency (5)	4.77 ± 0.06^{a}	4.70 ± 0.17^{a}	4.67 ± 0.15^{a}	4.63 ± 0.21^{a}	4.80 ± 0.10^{a}
Yolk consistency (5)	4.77 ± 0.15^{a}	4.80 ± 0.10^{a}	4.77 ± 0.12^{a}	4.63 ± 0.15^{a}	4.90 ± 0.00^{a}
Air sac (5)	4.60 ± 0.00^{a}	4.83 ± 0.06^{a}	4.73 ± 0.21^{a}	4.67 ± 0.15^{a}	4.77 ± 0.12^{a}
Average	4.33 ± 0.04^{c}	4.75 ± 0.10^{a}	4.66 ± 0.02^{a}	4.52 ± 0.09^{b}	4.78 ± 0.07^{a}

Average values with different alphabetical superscripts within raw are significantly different at $P<0.05$.
* Control eggs, immersed in sterile distilled water instead of plant extract solutions.

DISCUSSION

Human salmonellosis is a public health problem worldwide and hen's eggs are considered as the major source of these infections. Contamination of eggshells with Salmonella is considered a significant hazard threating the consumer health. The bacterium could penetrate the egg through the egg shell pores or egg contents could be contaminated during shell broking. Hence, decreasing or eliminating Salmonella on the egg shells would potentially outcome microbiologically safe egg products[19].

[20]Emphasized the importance of strict temperature control during table egg manufacturing process starting from egg collection till consumption, containing different process steps like; handling, packaging, storage, transportation and distribution; to reduce the incidence of Salmonella contamination. Salmonellosis caused by egg contamination remains the most common foodborne disease in the world, even though different management practices that had been already utilized during egg production as vaccination and egg shell washing and disinfection processes4. Controlling shell eggs contamination with Salmonella requires postharvest approaches which may include: 1- egg washing, either with water, electrolyzed oxidizing water, chlorine or ozone, 2- treating the eggs with ultrasound, UV, irradiation, pulsed light, or gas plasma. Egg washing is presently working and it includes either washing with water sustained at 32.2°C or slightly higher or using sanitization solutions containing some chemicals or sanitizers for decontamination[21].

In the current study, we investigated the efficacy of 4 plant extracts; rosemary, licorice, chicory, and neem, added to washing solutions for reducing S. typhimurium over shell eggs. The plants, evaluated for their antibacterial activity in current study, were carefully chosen based on their traditional practice as a treatment for several gastrointestinal disorders, especially in Arabic regions. The solicitations of these plants, for more than 1400 years, settle their biological safety for human.

Rosmarinus officinalis, commonly known as rosemary, is a woody, perennial herb, originally known Mediterranean region. It belongs to the mint family Lamiaceae, The herb has been hailed since ancient times for its medicinal properties[22]. Glycyrrhiza glabra also known as Licorice or sweet wood, it belongs to family Papilionaceae. It is a native of south-east Europe and south-west Asia, including Iran[22].

The trial plant Chicory (*Cichoriumintybus L.*) belongs to family Asteraceae. It is known as a small aromatic perennial herb. It grows on roadsides as a wild plant in its native Europe, North America and Australia. It may also be used as an appetite stimulant, and as a treatment for gastroenteritis and gallstones [23]. In tropical and semi-tropical regions, Neem products are believed to be anthelmintic, antifungal, antibacterial and antiviral[24].

An identified pure culture of *S. typhimurium* was used to settle the exactness and reliability of obtained results. It was informed by[25], that in vitro studies using pure cultures of identified microorganisms are recommended for developing scientific approaches.

By examination of the antibacterial effect of these plant extracts on *S. typhimurium* as seen in **Table (1)**, *S. typhimurium* was very sensitive to rosemary, licorice and chicory (ZOI between 15-19) while it was less sensitive to neem extracts. The most potent plant extracts inhibiting *S. typhimurium* growth, were licorice, rosemary, chicory and neem extract, respectively, as proved by the wider growth inhibition zones. These results are matching with[26], [27] who concluded that *E. coli* and *S. typhi* are less susceptible to neem extract. Also, [28] proved that Ethanolic neem leave extract has limited efficacy against Klebsiella and Salmonella at the highest concentration used (50 mg/mL). On the other side, [24] stated that neem leaf extract seems promising to combat *S. typhimurium*. Also, [29] proved that ethanol and methanol neem extract have a great antibacterial activity and[30] confirmed the antimicrobial potentiality of Chloroform leaf extract of neem against *S. typhimurium*.

Minimum inhibitory concentration (MIC) was done to determine the lowest plant extract concentration inhibiting visible growth of bacteria. The dilutions were set between 0.1-2% to be easily calculated and applied by the producers and farmers. Data illustrated in Table (2) showed that all plant extract solutions were effective at concentration 1% except Chicory which could inhibit the growth of *S. typhimurium* at lower concentration (0.5%). [31] suggested that the chicory root extracts contain the effective active constituents responsible for eliminating the bacterial pathogens. While [32] indicated that chicory extracts showed moderate activity as an antibacterial agent.

For rosemary, [33] pointed out that up to 1% of the ethanolic solution of rosemary had no activity on *Salmonella enteritidis*. While[34] found that rosemary leaves extract at a concentration of 400 mg/ml was active against *E.coli*.

[35] reported an in vitro inhibitory effect of licorice against the growth of *Salmonella typhi* at concentration 7.5%. It is well known that *S. enterica* should be present in high numbers (>10,000 cells) to produce gastroenteritis illness, but low numbers (<100 cells) nowadays found also to have the ability to produce sever illness[36]. Because of a relatively high count of *S. typhimurium* was identified over contaminated eggshells, in the current study (the initial bacterial load exceeds 10^8 CFU/eggshell), and that to verify the efficacy and ability of the used plant extracts to reduce or even eliminate Salmonella invasion and to be properly used as antibacterial agents.

Bacterial count was significantly reduced after immersion of egg in the used plant extracts and this reduction was continuous with increasing the immersion time. The maximum count reduction percent in *S. typhimurium* count, after immersion in sterile distilled water (control), was 74.24 ± 1.56 after 5 minutes. Complete reduction (100%) was achieved after egg immersion in licorice solution 1% for 5 minutes (Table 3). Licorice root extract has significant anti-bacterial and hydroxyl radical scavenging activities[37].

From data demonstrated in Table 3, it was obvious that, after the first minute the licorice solution showed the highest reduction percent (93.96 ± 0.80) with significant difference ($P<0.05$) than the other groups. By increasing the immersion time, the reduction percent increased with a significant difference between the plant extracts used; licorice showed the most powerful antibacterial activity followed by chicory then rosemary and finally neem extract. After three minutes of immersion there was no significant difference in the reduction percent between licorice, chicory, and rosemary, while neem still showing the lowest figure. By the end of immersion time (5 minutes); licorice, chicory, and rosemary were excellent as antibacterial solutions against *S. typhimurium* as the reduction percent reached nearly 100%. *S. typhimurium* was less sensitive to neem extract solution but it still more significant than only washing with water.

This result agreed with[37] who found that Licorice root extract has both radical scavenging and antibacterial activities, which could help in treating bacterial infection and scavenging hydroxyl radical generating during carcinogenesis. Also[38-40] supported the view that G. glabra root extract has powerful effects as antibacterial agents against Gram-positive and Gram-negative bacteria. Rosemary extract may be a promising therapeutic agent to control pathogenic microorganisms and biofilms, without using the cytotoxic or genotoxic[41].

For all examined eggs, the egg contents were examined for detection of *S. typhimurium* in the egg content but all samples were completely free from contamination. This step was conducted to deny the possibility of salmonella organism penetration through the pores on shell to the inside of egg content during immersion in the plant extract solution.

To make our results more applicable; the sensorial impact of the egg immersion in different plant extract solutions should be considered, as the use of plant extracts can alter the organoleptic characteristics or exceed acceptable flavor thresholds. **Table (4)** indicated that there was no significant difference (P<0.05) between all groups (including the control one) in the examined criteria; White consistency, Yolk consistency, and air sac. While the color of eggs immersed in rosemary extract solution was greatly affected by a significant difference from the other groups. Also, the odor of eggs immersed in neem extract significantly differed than other groups as the neem odor is highly characteristic and adhered to the eggshell. It should be pointed out that the overall acceptability of eggs after immersion in plant extract solutions was greatly accepted for all solutions including the control group except eggs treated with neem and rosemary were judged as slightly accepted according to Hedonic Scale.

The suggested antibacterial agents in our study, beside their great biological activity, they were obtained from cheap and available plants that have an economic importance on the both sides of producers and consumers. Data collected from different experiments in this study concluded that the use of licorice plant extract as immersion solution for washing of eggshells for 5 minutes could completely decontaminate *S. typhimurium* infection to reduce the incidence of Salmonella food poisoning and considered as effective biopreservative with has no effect on the egg's physical quality.

CONCLUSION

Food salmonellosis is not an emerging disease, but salmonella infection related to egg consumption is an ongoing global food safety issue. Reducing Salmonella flock occurrence is a direct resultant in a relational reduction of consumer health risk. Recently, interest has increased considerably in finding naturally occurring antimicrobials (Biopreservation) to substitute synthetic one, which has a well-known carcinogenicity. Data obtained from our study confirmed the hypothesis that these plants, which are traditionally used in various forms in popular medicine since early times, could be a potential be used as a good alternate to the already used chemical antimicrobial compounds which could completely prevent Salmonella contamination in egg production farms.

SIGNIFICANCE STATEMENTS

This study reveals the seriousness of the presence of biological and chemical hazard associated with table eggs consumption in Egypt with subsequent adverse health effect. The risk of these hazards found to be easily decreased at farm level by using natural plant extracts (especially licorice) as washing solution for short period of time before packaging of egg. These plant extracts are cheap in price and available in Egyptian markets, which make the application of this research on the farm level is easy and applicable with no extra financial resources. Also it was proved in the study that the use of these plant extracts doesn't affect the sensory and physical characters of eggs to satisfy the consumer needs.

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