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Marjoram extract down-regulates the expression of *Pasteurella multocida* adhesion, colonization and toxin genes: A potential mechanism for its antimicrobial activity

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ABSTRACT

Due to the emergence of virulent and antibiotic-resistant microbes, natural antimicrobials from herbal origins have been given more attention as an alternative therapy. This study provides an *in vitro* research framework to investigate the antibacterial activities of 5 herbal (marjoram, garlic, onion, cinnamon and black seed) oil extracts against 16 multidrug-resistant (MDR) and virulent *P. multocida* serogroup A isolates recovered from dead and clinically diseased rabbits. Pathogenicity of the screened isolates was further proven experimentally and was verified by PCR analyses of 5 randomly selected virulence genes encoding attachment and colonization proteins (*ptf*A, *pfh*A, and *omp*87), sialidases (*nan*B) and dermonecrotoxin (*tox*A). A total of 12 *P. multocida* isolates were highly pathogenic with the possession of all examined virulence genes, while the other 4 isolates were of lower pathogenicity with expression of the target genes except *tox*A. *In vitro* anti-*P. multocida* activities of the 5 extracts and their synergism rates with 4 antibiotic drugs revealed that marjoram and cinnamon extracts had the highest antibacterial activities and the highest synergism rates against the screened isolates. *Pasteurella multocida* virulence gene expression profiles were assessed via real-time quantitative reverse transcription PCR (qRT-PCR) in response to marjoram extract. The quantitative analyses showed less than five-fold reduction in the targeted virulence genes expression in presence of marjoram extract compared with the control. The findings from this study document a novel molecular inhibitory activity of marjoram against *P. multocida* multiple virulence genes and provide a proof of concept for its implementation as an alternative candidate for the treatment of pasteurellosis in farm animals in future.

1. Introduction

Pasteurella multocida (*P. multocida*) is an important Gram-negative pathogen associated with a spectrum of animal diseases. Strains of *P. multocida* are designated into 5 capsular serogroups (A, B, D, E or F) and 16 lipopolysaccharides (LPS) somatic serotypes (1–16) [1]. Certain serotypes of *P. multocida* can cause pasteurellosis in rabbits (snuffles) resulting in considerable economic losses in rabbit production units [2]. The outcome of infections caused by *P. multocida* is influenced by the complex interactions of several hosts and pathogen-specific attributes [3]. The polysaccharide capsule and LPS are of major importance as virulence factors contributed in the pathogenesis of *P. multocida* in the host [4]. However, many other putative virulence determinants are related to pathogenicity including fimbriae, adherence and colonization factors, iron regulating and acquisition proteins, extracellular enzymes, exotoxins and a variety of outer membrane proteins [5].

Despite using the antimicrobial therapy as the most effective tool for controlling the infectious diseases caused by *P. multocida*, the used antibiotic agents are failing to bring an end to many infections due to the advent of MDR pathogens, which is recognized as an alarming threat to effective treatment and prevention of bacterial infections in humans and animals [6]. Increased resistance of *P. multocida* isolates to tetracyclines, erythromycin, trimethoprim/sulfamethoxazole, chloramphenicol, ciprofloxacin and cefotaxime antimicrobials was previously reported with the emergence of multidrug-resistant (MDR) strains [7–9]. In contrary, studies in France [10], Japan [11] and

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Hungry [12] demonstrated that cefotaxime, ciprofloxacin, tetracyclines and chloramphenicol were active drugs against *P. multocida* and recommended their use in pasteurellosis treatment. The undesirable effects and the high costs of antimicrobials administration still need to be considered in control of pasteurellosis. Thus, there is a dire need for developing alternative prophylactic strategies to combat the infections caused by these resistant strains. Currently, considerable researchers have evoked rekindled attentions towards the medicinal strength of some higher plants as a reasonable source for finding novel antimicrobial compounds [13], which have resulted in the development of alternative plant-based antimicrobial drugs with least side effects as compared to commercial antibiotics.

Indeed, few studies to date have focused on the antibacterial aspects of medicinal plants against *P. multocida* strains that target their cellular viability [14]. However, it is not yet known whether these anti-virulence potentials could be attributed to alterations of bacterial genes` expressions. This modulation of transcription can lead to subtle changes in the physiology of bacterial cell populations with consequences for their collective behaviors and thereby controlling pasteurellosis [15]. Considering the virulence and the MDR patterns within different strains of *P. multocida* [6], the informed selection of the virulence factors to be targeted for prevention of the struggle with infections caused by resistant *P. multocida* strains becomes vital. To the best of our knowledge, there is hardly any available report on the induced expression patterns of herbal oil extracts in *P. multocida*.

Over the last few decades, the use of natural compounds such as medicinal plants has gained attention due to increasing concerns about the safety of synthetic chemicals and emerging antibiotic resistance in bacteria. The current *in vitro* study is, therefore, the first to evaluate the antimicrobial activities of five common herbal oil extracts against MDR and virulent *P. multocida* isolates recovered from naturally infected rabbits suffering from respiratory diseases in Egypt giving a new safe approach for its treatment as a bio-control agent. In addition, the focus of this study was targeted to estimate the impact of the effective medicinal extract at the molecular level through the application of a wide scale genomic analysis using specific real-time quantitative reverse transcription PCR (real-time qRT-PCR) assay to investigate the expression levels of five critical virulence-associated genes putatively involved in modulating the pathogenesis of *P. multocida*.

2. Materials and methods

2.1. Bacterial isolates

Sixteen field bacterial isolates recovered from 200 rabbits were used in this study. They were previously isolated from clinical samples consisting of nasal swabs (3) from live rabbits with a clinical evidence of snuffles and lung tissue samples (13) those were collected from dead rabbits at different localities in Sharkia Governorate, Egypt. These isolates were definitively identified on the basis of standard laboratory findings [16]. For reliable and specific detection, the identity of *P. multocida* isolates was confirmed using a pair of specific primers which amplified a 460-bp fragment of the *kmt*1 gene according to the PCR protocol established by Townsend et al. [17]. The capsular serogrouping of the isolates was performed as was previously described [18].

2.2. In vivo pathogenicity test

All animals handling was performed in ordinance with the guidelines and permission established by the Committee on the Ethics of Animal Experiments of the Faculty of Veterinary Medicine, Zagazig University, Egypt and the animals general good health was observed regularly. For conducting the *in vivo* pathogenicity test, 20 Baladi pasteurella free rabbits with an average body weight of 0.750 kg and with an average of 8-10 weeks old were used in this experiment; one rabbit for each *P. multocida* isolate. All animals were accommodated and kept in separate cages under hygienic measures for one week. Water and pellets were offered ad libitum. The rabbits were challenged intranasally with 0.1 ml of pure *P. multocida* inoculums containing 2×10^5 CFU/ml in sterile PBS. Four rabbits were kept as negative controls and they were inoculated with 0.1 ml of sterile PBS alone [19]. Following a 7-day observation period for any clinical signs or mortalities, all surviving rabbits were euthanized. The dead rabbits were subjected to postmortem examinations and then heart blood, lungs, spleen, liver, and kidneys were subjected to re-isolation of *P. multocida*. Heart blood smears and impression smears prepared from the rabbits' liver, spleen and lung were stained with Giemsa's stain and examined microscopically to observe the bipolar characteristics of the bacterial cells.

2.3. Antimicrobials sensitivity assay

The listing of susceptibility of the 16 *P. multocida* isolates against a panel of 17 widely used and commercially available antimicrobial agents representing different antibiotic groups was conducted according to the standard operational procedures using Kirby-Bauer standardized disk diffusion method. The bacterial susceptibility was evaluated against erythromycin (15 μg), apramycin (15 μg), gentamicin (10 μg), neomycin (30 μg), tobramycin (10 μg), chloramphenicol (30 μg), colistin (10 μg), tetracycline (30 μg), doxycycline (30 μg), nitrofurantoin (300 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), amoxicillin (25 μg), amoxicillin /clavulanic acid (20/10 μg), ampicillin (10 μg), penicillin G (10 IU) and trimethoprim/ sulfamethoxazole (1.25/23.75 μg). The measured inhibition zones diameters were subsequently matched with the respective standard zone diameters outlined on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint table version 7.1 valid from 10 March 2017 [20]. Isolates that were resistant to at least three different antibiotic classes were classified as MDR.

2.4. Molecular screening of virulence-associated genes

In order to gather relevant information on the virulence of *P. multocida* strains demonstrating high levels of antimicrobial resistance, all MDR and phenotypically virulent isolates (confirmed with the pathogenicity test) were further analyzed for the presence of five randomly selected virulence determinants representing different categories. The virulence genes encoding colonization and adhesion-related proteins (*ptf*A, *pfh*A and *Omp*87), sialidases (*nan*B) and dermonecrotoxin (*tox*A) were tested via individual PCR protocols utilizing specific oligonucleotide primers and following the cycling conditions detailed previously [21–23]. All runs were repeated three times in parallel with the relevant positive and negative controls.

2.5. Herbal oil extracts antibacterial activity assays

To test the antimicrobial activities of herbal medicinal plant as a hope for developing alternative prophylactic strategies to combat *P. multocida* infections, an array of five herbal extracts including garlic (*Allium sativum*), onion (*Allium cepa*), black seed (*Nigella sativa*), marjoram (*Origanum majorana*) and cinnamon (*Cinnamomum zeylanicum*) (Star Chemical Pharmaceutical, Cairo, Egypt) were investigated for their antibacterial activities against all MDR and virulent isolates in accordance with the agar well diffusion assay explained previously [24]. The zone of inhibition above 7 mm in diameter was taken as a positive result. The minimal inhibitory concentration (MIC) values of the screened extracts were determined according to the standard broth microdilution technique as was previously stated [25]. The extracts diluent used was dimethylsulfoxide (DMSO).

2.6. Herbal oil extracts synergism with antibiotic agents

The sub-inhibitory concentrations of the plant extracts were used in the synergism assays with representative drugs of varying modes of actions. This assay was applied on the recovered *P. multocida* strains by disk diffusion method on MHA media. Two antibiogram sets were performed, in duplicate, for each *P. multocida* isolate in control plates, with plain MHA, and in plates containing MHA plus the sub-inhibitory concentrations of the respective extracts. The diameters (mm) of the each inhibitory zone were recorded after incubation at 37 °C/18 h [26].

2.7. RNA extraction and real-time qRT-PCR analysis of P. multocida virulence genes

Marjoram was prepared at sub-inhibitory concentrations and incubated with an overnight culture of the tested strains [27]. At the end of incubation, total RNA was extracted using the commercially available QIAamp RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturers` recommendations. The expression levels of mRNA transcripts were determined by one step real-time qRT-PCR using QuantiTect SYBR Green RT-PCR Kit (Qiagen GmbH, Hilden, Germany) in the MX3005 P real-time PCR thermal cycler (Stratagene, La Jolla, CA, USA). All reactions (25 µl) were performed using three technical replicates. The relative quantification of mRNA expression levels of genes of interest was normalized to the constitutive expression of the housekeeping gene (*Kmt*1) and it was calculated according to the $2^{-\Delta\Delta CT}$ comparative method [28]. The qPCR data were expressed as fold changes in mRNA expression levels of targeted genes in the candidate-treated *P. multocida* strains relative to their levels in the untreated control bacterial cells.

2.8. Statistical analysis

The data were described using means and standard errors (geometric mean ± SE). Descriptive statistics was analyzed by spss v.25

3. Results

3.1. Characteristics of P. multocida isolates

Standard conventional laboratory tests and PCR amplification of *kmt*1 gene were used for confirmation of the isolates as *P. multocida*. In capsular PCR assays, all the isolates amplified a product of approximately 1044 bp, which is a specific product for serogroup A.

3.2. Pasteurella multocida experimental infection

Pathogenicity tests were performed for the 16 *P. multocida* field isolates obtained from naturally infected rabbits. After being intranasally inoculated with *P. multocida* isolates, 12 rabbits died within 24 h postinfection with the appearance of snuffles symptoms. Therefore, these isolates were classified as highly virulent. The most significant macroscopic findings were the extensive cutaneous and subcutaneous hemorrhages and abscess formation (Supplement 1A–D). Postmortem examinations of laboratory rabbits revealed the pathognomonic characteristic lesions such as subcutaneous hemorrhages and severe congestion of the internal organs, especially trachea and lungs with severe hemorrhagic pneumonia. Spleen enlargement as a characteristic feature of pasteurellosis was observed in the infected rabbits in comparison with the control ones. On the other hand, the other 4 isolates displayed moderate rates of virulence as they caused rabbits death within 48 h post inoculation with mild or even no macroscopic cutaneous lesions. Postmortem lesions of these rabbits were of milder degree compared to those caused by the highly virulent isolates. The rabbits infected with either highly or moderately virulent isolates showed minimal excitation for a short time and then become depressed, anorexic and dull with the appearance of mucopurulent nasal discharge (Supplement 1E and F) along the experiment and finally death. All the rabbits belonging to the negative control group survived with no clinical signs of the disease or any macroscopic lesions or mortalities during the course of the experiment.

Pure *P. multocida* cultures were re-isolated onto 5% sheep blood agar and trypticase–soy agar (Oxoid, U.K) from heart blood and internal organs of the dead rabbits. The examined Giemsa stained smears of heart blood or internal organs showed the presence of the characteristic bipolar stained microorganism (safety pin appearance), and pleomorphic bacterial cells occurring singly, in pairs or in short chains (Supplement 2).

3.3. P. multocida sensitivity to antimicrobial agents

The susceptibility of 16 *P. multocida* clinical isolates was tested against 17 widely applied antimicrobials. The phenotypic antibiogram profiles of *P. multocida* isolates are illustrated in Supplement 3. Interestingly, MDR pattern was common among the 16 *P. multocida* isolates. All isolates from lung tissues and nasal swabs were resistant to at least 8 drugs. Moreover, most isolates were resistant to at least 13 antimicrobials from the different chemotherapeutic groups and only one isolate from lung tissues possessed resistance to the 17 used antimicrobials (Supplement 3).

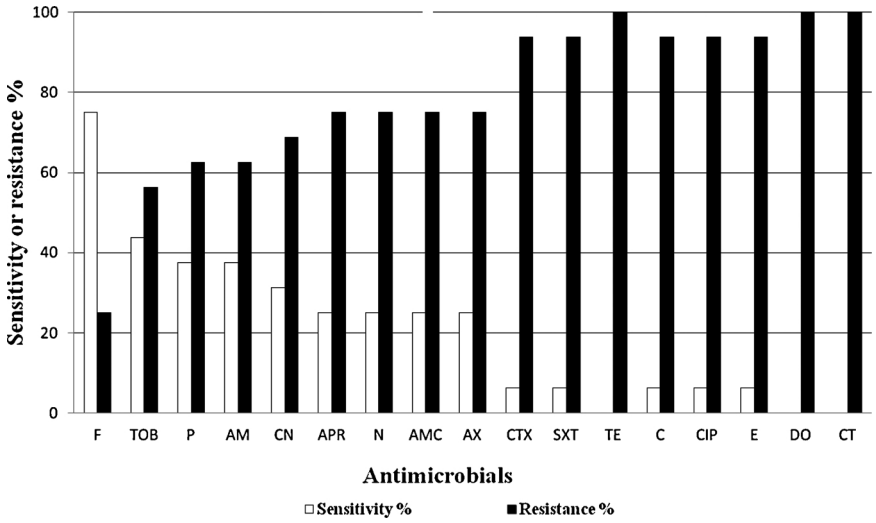

Fig. 1. Susceptibility percentages of 16 *P. multocida* clinical isolates against 17 common antimicrobial agents. F: Nitrofurantoin, TOB: Tobramycin, P: Penicillin G, AM: Ampicillin, CN: Gentamicin, APR: Apramycin, N: Neomycin, AMC: Amoxicillin/clavulanic acid, AX: Amoxicillin, CTX: Cefotaxime, SXT: Trimethoprim/sulfamethoxazole, TE: Tetracycline, C: Chloramphenicol, CIP: Ciprofloxacin, E: Erythromycin, Do: Doxycycline, CT: Colistin.

According to the *in vitro* antimicrobials susceptibility test (Fig. 1), the isolates showed different percentages of susceptibility with majority of the strains being resistant to most of the antibiotics tested. The resistance percentage to each of doxycycline, tetracycline, and colistin was 100% and this was a common feature among the tested *P. multocida* clinical isolates. It was also observed that 93.8% of *P. multocida* isolates were resistant to each of erythromycin, trimethoprim/ sulfamethoxazole, chloramphenicol, ciprofloxacin, and cefotaxime. On the other hand, the most prevalent antibiotic activities were reported against nitrofurantoin, followed by tobramycin with sensitivity rates of 75% and 43.8%, respectively.

3.4. Virulence genotyping of P. multocida isolates

All the tested MDR and phenotypically virulent isolates belonging to capsular type A were analyzed by PCR for the presence of the 5 randomly selected virulence associated genes previously confirmed to be indicators of *P. multocida* pathogenicity. PCR analysis identified the presence of these 5 chosen virulence genes in the 16 screened field isolates. Twelve of the isolates harbored all the examined genes (*nan*B, *omp*87, *ptf*A, *pfh*A, and *toxA*); meanwhile, the remaining 4 isolates possessed 4 of these virulence associated genes and did not express *toxA* gene.

3.5. Antibacterial activities of herbal extracts

The antibacterial potentials of 5 herbal extracts against the MDR and virulent *P. multocida* isolates were examined in terms of evaluating the inhibition zones of the bacterial growth, MICs and MBCs values. The collective analyses of antimicrobial activities of these extracts showed that they have different antibacterial activities against the tested isolates as was indicated by the average values of *P. multocida* isolates growth inhibition zone diameters, MICs and MBCs (Table 1). Additionally, DMSO (the extract diluent) was inactive against all investigated bacteria with no apparent inhibition zones. Among the five tested extracts, marjoram, followed by cinnamon were the most potent oil extracts forming the maximum inhibition zone diameters (up to 35 and 33 mm, respectively) and this efficacy was reflected with very low recorded MICs (up to 2 µg/mL each). Black seed and onion oil extracts exhibited moderate antibacterial activities against the tested *P multocida* isolates with inhibition zone diameters and MICs values up to 29 mm and 16 µg/mL, respectively. In contrast, garlic oil extract showed a limited antibacterial activity with the lowest inhibition zone diameters and highest MICs values (up to 15 mm and 64 µg/mL, respectively) (Data not shown).

3.6. Synergism assays between herbal extracts and antibiotic agents

The *in vitro* synergistic antibacterial activities of the 5 herbal extracts in the presence of 4 commonly used antibiotics (tobramycin, penicillin, cefotaxime, and nitrofurantoin) representing 4 chemotherapeutics groups and of varying modes of actions were verified. The overall results of plant-drug combinations documented oblivious synergism among the 5 used extracts and the 4 tested antibiotics. The tabulated results revealed that marjoram extract had the highest promising synergistic antibacterial activity against MDR *P. multocida* in combination with each of the above four mentioned antibiotics, followed by cinnamon that was synergistic with 3 of the tested antibiotics. Meanwhile, black seed and onion oil extracts exhibited synergistic activities in combination with only tobramycin and nitrofurantoin. Although the antimicrobial activity of garlic alone was limited, its synergistic activity was comparable with black seed and onion (Table 2). Synergistic rates of the drug-plant combination against MDR *P. multocida* isolates were variable. Protein synthesis inhibitor (tobramycin) together with ribosomal proteins, cell wall synthesis and DNA modifier (nitrofurantoin) presented the strongest synergistic rates (5 extracts/drug). Meanwhile, bacterial synthesis cell wall inhibitors recoded the weakest synergistic rates with the plant extracts as shown in Table 3.

Table 1
Multidrug resistance percentages of 16 *P. multocida* isolates from nasal and lung samples of diseased rabbits.

No. of antibiotics	No. of resistant isolates (%)		
	Nasal swabs (3)	Lung tissues (13)	Total (16)
8	0.0	1 (7.7)	1 (6.3)
12	0.0	2 (15.4)	2 (12.5)
13	2 (66.7)	5 (38.5)	7 (43.8)
14	0.0	1 (7.7)	1 (6.3)
15	1 (33.3)	2 (15.4)	3 (18.8)
16	0.0	1 (7.7)	1 (6.3)
17	0.0	1 (7.7)	1 (6.3)

Table 2
Antimicrobial activities of 5 herbal extracts against 16 *P. multocida* isolates from diseased rabbits.

Plant extract	Inhibition zones diameters (mm)	MIC (µg/ml)	MBC (µg/ml)
Marjoram	26.33 ± 2.72	8 ± 4.8	16 ± 9.6
Cinnamon	23.3 ± 2.91	36.5 ± 14.4	73 ± 28.8
Black seed	20.33 ± 2.36	51.2 ± 16.7	102.4 ± 33.4
Onion	19.50 ± 2.54	72 ± 37.4	144 ± 74.8
Garlic	13.33 ± 0.88	96 ± 14.3	192 ± 28.6

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; all values are geometric means ± SE.

3.7. Modulation of P. multocida virulence genes' expressions

To understand a possible molecular activity of the herbal extracts on *P. multocida* virulence-associated genes, the expression levels of the 5 randomly selected virulence genes were determined by real-time qRT-PCR after exposure to the sub-inhibitory concentration of marjoram extract that induced the most effective antimicrobial activity in this study. Data analysis indicated that all screened genes were down-regulated following exposure to marjoram purified extract (Fig. 2). Although different degrees of down-regulation were observed, the transcripts of *nan*B gene encoding neuraminidase were markedly repressed; all the down-regulated genes showed less than five-fold changes ranging from 0.214 to 0.4258-fold, except for the *nan*B gene that was markedly down-regulated in the range of 0.0526-fold when compared with the marjoram untreated control culture.

4. Discussion

The existence of antimicrobial resistance among *P. multocida* isolates considers a big problem in the veterinary medical field, specifically in rabbit industry. The implication of this problem can result in increasing the treatment cost, prolongation of illness due to treatment failure and it sometimes can lead to death. In this study, efforts were exerted to find an effective and safe antimicrobial(s) from natural sources such as herbal extracts with focusing on exploring their effects on the expression levels of critical virulence-associated genes putatively involved in the pathogenesis of *P. multocida*.

Pasteurella species use various virulence attachment and adherence elements to firmly bind the mucosal epithelium and to invade the mucosal surfaces, or evade the innate immunity and thereby cause the systemic diseases. The capsule, toxins, putative surface adhesion molecules, extracellular enzymes and iron acquisition proteins are among these effective virulence elements. Five common virulence-associated genes previously known as *P. multocida* pathogenicity indicators and

Table 3
Synergism rates between different antibiotic drugs and 5 herbal extracts against *P. multocida* isolates*.

Drug target	Drug	Marjoram	Cinnamon	Black seed	Onion	Garlic	Synergism rate (extract/drug)	Mean
Protein synthesis	TOB	x	x	x	x	x	5	5
Cell wall synthesis	P	x	–	–	–	–	1	1.5
	CTX	x	x	–	–	–	2	
Ribosomal proteins, cell wall synthesis, DNA	F	x	x	x	x	x	5	5
Total	4	4	3	2	2	2		

x: synergism; (-) no synergism; TOB: Tobramycin; P: Penicillin; CTX: Cefotaxime; F: Nitrofurantoin.
* The results were assessed by the Kirby and Bauer method.

proved to play a critical role in different *P. multocida* serotypes pathogenesis and disease outcomes were examined [20]. They encode adhesins [filamentous hemagglutinin (*pfh*A) and type 4 fimbriae (*ptf*A)], toxins (dermonecrotic toxin, *tox*A), sialidases (*nan*B) that may enhance bacterial virulence by unmasking key host receptors and/or reducing the effectiveness of host defenses with outer membrane proteins (*omp*87). The 16 used *P. multocida* field isolates were confirmed to be virulent by *in vivo* rabbit pathogenicity and PCR analysis of virulence genes. PCR analyses of these genes revealed that 12 isolates harbored 5 virulence genes (*nan*B, *omp*87, *ptf*A, *pfh*A and *tox*A) required for adherence, colonization and dermonecrotoxin production. Interestingly, those 12 isolates were highly virulent as was evidenced in the experimental *in vivo* pathogenicity study with the appearance of severe symptoms including external skin lesions and nasal discharges (Supplement 1A–F), followed by death of the challenged rabbits within 24 h, existence of marked postmortem internal lesions in addition to detections of the characteristic bipolar stained microorganisms (safety pin appearance) and pleomorphic bacterial cells occurring singly, in pairs or in short chains in the stained organs' smears and finally re-isolation of *P. multocida* from the laboratory rabbit's heart blood and internal organs (Supplement 2). Recent studies worldwide confirmed our findings and reported the predominance and occurrence of selected virulence genes encoding fimbriae and adhesions (*ptf*A and *pfh*A), outer membrane protein synthesis (*omp*87), extracellular enzymes (*nan*B) and dermonecrotoxin production (*tox*A) with other virulence-associated genes in *P. multocida* of all capsular serogroups (A, B, D, F and F) isolated from clinical cases in different animal species [6,29–34]. Four P. *multocida* clinical isolates did not express *tox*A virulence gene and possessed the rest of the 5 examined genes. Interestingly, these isolates were able to cause pasteurellosis, but with milder symptoms in comparison with the 12 isolates carrying the 5 examined virulence-associated genes. The *tox*A absence in these isolates may explain the milder or no cutaneous lesions in the laboratory rabbits and this was in agreement with a previous study in Brazil [29] Additionally, some authors described that the *tox*A gene encoding the toxin is not inserted into the bacterial chromosome, but in a lysogenic bacteriophage that infects the agent [35]. However, our study was based on the amplification of target genes using PCR and any false negative result cannot be ruled out.

Antimicrobial resistance among *P multocida* isolates was a common pattern in the tested isolates of the current study. The 16 isolates were resistant to at least 8 of the most widely used antimicrobials in Egypt and worldwide. Remarkable resistance (100%) was reported among all isolates against colistin, doxycycline, and tetracycline (Fig. 1). Food supplements containing tetracyclines have been widely used not only as therapeutic agents but also as growth promoters in animal husbandry. This is the most likely reason why an increased resistance to tetracyclines appeared in the examined isolates. Moreover, more than 93% of the strains were resistant to erythromycin, trimethoprim/ sulfamethoxazole, chloramphenicol, ciprofloxacin, and cefotaxime. These observations are in agreement with previous reports [7–9]. The MDR pattern may be attributed to the unregulated use of antibiotics in veterinary and human medicine in Egypt or due to the horizontal or vertical transfer of plasmid encoding antimicrobial resistance genes among different bacterial pathogens or from animals to humans as was reported elsewhere [36]. In contrary, some of the previous studies reported low frequencies of resistance among *P. multocida* isolates [10,37]. In a previous study in Ibadan, Nigeria reporting the antibiotic sensitivity patterns of 8 *P. multocida* isolates from some clinically ill New Zealand rabbits those were associated with snuffles against 12 commonly used antibiotics, *P. multocida* were highly sensitive, being sensitive to 11 of 12 tested antibiotics [38]. The authors also used ampicillin, chloramphenicol, erythromycin, gentamycin penicillin and tetracycline antibiotics as in our study. They revealed the following sensitivity percentages; 87.5, 100, 100, 100, 87.5 and 100%, respectively. However, our results were the contrary where the isolates showed high degrees of resistance to such antibiotics as shown in Fig. 1 and that confirms the aim of our research for the substantial need for alternative forms controlling *P. multocida* infection in rabbits. Other studies in Hungry [10], France [11] and Japan [12] recommended the

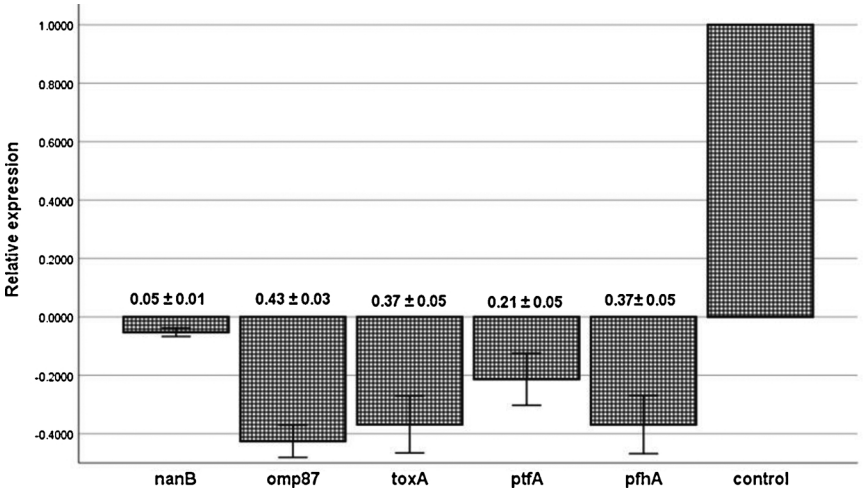

Fig. 2. Relative expressions of *P. multocida* virulence genes after treatment with sub-inhibitory concentrations of marjoram extract. Using the $2^{-\Delta\Delta CT}$ method, the data are presented as the fold changes in gene expressions normalized to an endogenous housekeeping gene and relative to the untreated control (value 1). Values are expressed as the mean values of the fold changes of all screened isolates for three independent experiments.

use of cefotaxime, ciprofloxacin, tetracyclines, and chloramphenicol drugs in treatment of *P. multocida* infection due to their effectiveness. Notably, the acceptable *in vitro* activities of aminoglycoside antibiotics against *P. multocida* isolates in our study were in agreement with the results reported previously [39]. As a simple solution for appropriate effective therapy and for the avoidance of MDR strains emergence, it is recommended to isolate the organism and to define its *in vitro* antibiotic susceptibility before treatment in addition to the need for restrictive rules regarding the use of antimicrobials in animals intended for human consumption.

Considering the present scenario, the development and introduction of new anti-pasteurella compounds are needed. The search for new anti-pasteurella drug(s) must consider drug(s) that are associated with low cost, low toxicity and high availability. Plant-derived products are gaining attention and are easily available and relatively inexpensive. So, one of the critical purposes of the present study was to evaluate the effects of 5 plant extracts from *Allium sativum*, *Allium cepa*, *Nigella sativa*, *Origanum majorana* and *Cinnamomum zeylanicum* against the virulent isolates of *P. multocida*. Critical analysis of the efficacy of these herbal-derived extracts against MDR and virulent *P. multocida* isolates indicated promising *in vitro* antibacterial activities. Marjoram and cinnamon were the most effective extracts, followed by black seed and onion those exhibited considerable antimicrobial effects against the screened isolates; meanwhile, garlic was recognized as the lowest effective extract against the isolates under the study. Studies have tested the antibacterial activities of onion, garlic, cinnamon, black seed and marjoram extracts and essential oils or their constituents against some Gram-negative pathogens [40–43]. Only one report in Pakistan evaluating the antibacterial activities of onion and garlic extracts against *P. multocida* strains is available [14]. The authors reported that the bacterial growth inhibition zones' diameters for each of onion and garlic extracts ranged from 9.3 to 14.4 mm, which are lower than the zone diameter means reported in this study for onion and garlic (19.50 and 13.33 mm, respectively). Clinical virulent and MDR *P. multocida* isolates tested against pure herbal extracts in our study compared with laboratory strains and whole plant extracts applied in their study could be among the possible causes for the inhibition zones` variations.

To our knowledge and until preparation of this paper for publication, there were no published data regarding the use of marjoram, cinnamon and black seed oil extracts as antimicrobials against *P. multocida*. The current report also appears to be the first to report synergistic antibacterial activities of the 5 tested herbal extracts and antibiotics against MDR *P. multocida* field isolates. Although the antimicrobial activities of these 5 medicinal plants have been previously reported against various bacterial species, little is known about their interaction with antimicrobial agents prescribed in controlling infections in domestic animals. Herein, marjoram and cinnamon showed significant synergistic activities with at least 3 antibiotic agents against MDR *P. multocida*; meanwhile, black seed, onion, and garlic represented considerable synergistic activities. Interestingly, protein synthesis inhibitor together with ribosomal proteins, cell wall synthesis and DNA modifier presented the strongest synergistic rates implicating their synergistic activities against *P. multocida*. Due to the unavailability of data dealt with these extracts as anti-pasteurella and the lack of information related to their mode of actions against *P. multocida*, we speculate that they exert their antibacterial and/or synergistic activities through different ways. The antimicrobial activities of these extracts may be through alteration of microbial cell permeability, thereby permitting the loss of macromolecules from the cell interior or through interference in the molecular machinery of cell wall synthesis and transportation of essential components through the cell wall [44]. Functional modification in bacterial DNA synthesis or generation of free toxic radicals by herbal effective ingredients could not be excluded. A reasonable explanation by which the herbal extracts could mediate their antimicrobial effects is through the interference with membrane functions and interaction with membrane proteins' components causing deformation in their structure and functionality [45]. Another proposed scenario explaining the antimicrobial activities of the used extracts may be related to the inactivation of bacterial extracellular enzymes as was documented elsewhere [46] or through inhibition of bacterial efflux pumps [47] those bacteria use as a self-defense mechanism to remove or inactivate antibiotics action from the cell to survive. From these results, it is reasonable to assume that a mixture of herbal compounds together with antimicrobials would have greater bioactivity than a single compound because a mixture of bioactive compounds has the ability to affect multiple targets in *P. multocida* bacterium [48]. Current guidelines for effective treatment of some clinical conditions in humans (e.g. septic shock) recommend the use of "bundles" of therapeutic interventions. These interventions have only been proven beneficial when used in association and failed to show benefits when used alone [49–50]. Therefore, the combination of several therapeutic agents or drugs, aiming at different therapeutic targets, is actually already being advocated and practiced by modern medicine and it is also the most claimed advantage of herbal medicines [51]. In addition, it may be cheaper. However, the exact mechanism(s) by which these herbal extracts perform their anti-*P. multocida* activities needed to be elucidated. Moreover, it is worthwhile to test these plant compounds for synergistic activity mechanisms with antibiotics both *in vitro* and *in vivo* as that may be a safe, helpful and unconventional tool in *P. multocida* infection control.

Identification of *P. multocida* virulence-associated factors are critical for evaluating the *P. multocida* molecular pathogenesis and it will also enhance the understanding of the survival mechanisms of the bacterium *in vivo*. Previous evidence indicated that the levels of capsule and other virulence elements expression in *P. multocida* responded to certain environmental conditions such as growth in the presence of antibiotics, low iron or specific iron sources as hemoglobin [52,53]. However, there is no information on the mechanism of virulence-associated genes expression in *P. multocida* after exposure to herbal extracts.

From the attachment/adhesion, colonization, immune evasion and toxin-virulence genes down-regulation data obtained from the virulence gene expression experiments and from the antimicrobials activities data obtained from using marjoram extract either alone or in combination with antibiotics (marjoram extract-antibiotic synergism) in this study, we can suggest the use of marjoram extract orally or intranasally either alone or in combination with antimicrobials as prophylactic and/or therapeutic regimens in pasteurellosis. Using marjoram extract as a safe antimicrobial could rapidly limit the snuffles symptoms and the *P. multocida* spread in the upper respiratory mucosa. This suggestion is based on its capacity as anti-virulence and its prominent down-regulation of genes required for adhesion/attachment, colonization, immune evasion, iron intake and toxin production those are effective tools for *P. multocida* pathogenicity. Previous studies used other herbal extracts orally in poultry industry to improve the body weight and food conversion and showed satisfactory results. Thus, there is a need for further immense potential for the research and development of plant-based antimicrobial therapies after knowing their exact modes of action. New investigations based on sound scientific bases and modern methodology, including using nationally recognized protocols and standards for microbial testing, the generation of minimum inhibitory concentrations, and standardization of the quality of plant materials used for testing should be considered.

A key and a novel finding in this study was the marjoram remarkable down-regulation of well-recognized virulence genes of *P. multocida* isolates those facilitate the bacterium colonization and invasion to the host, the avoidance or disruption of host defense mechanisms, injury to host tissues, and/or stimulation of a noxious host inflammatory response. These findings provide novel insights into the antimicrobial potentials of natural herbal products on *P. multocida* clinical-virulent isolates and suggest a promising therapeutic activity on such MDR and virulent bacteria. Thus, knowing the molecular pathway of marjoram that is apparent as a marked down-regulation of the 5 virulence-

associated genes might be useful for the prevention and control of *P. multocida* infections in rabbits and hopefully in other animal species. Further experiments to identify other *P. multocida* virulence genes expressed differentially after exposure to herbal extracts will help to elucidate the mechanisms of pathogenesis for this economically significant bacterium and may aid in designing protective vaccine candidates.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cimid.2018.11.007.

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